

Wydział Chemii Uniwersytetu Gdańskiego

mgr Klaudia Marta Kwidzińska

de domo Kropidłowska

Badania biochemicznych i fizjologicznych markerów wpływu farmaceutyków na sinice

Promotor:

dr hab. Magda Caban, prof. UG

Praca doktorska wykonana
w Katedrze Analizy Środowiska Wydziału Chemii

Gdańsk 2026

W szczególności pragnę podziękować

Pani dr hab. Magdzie Caban, prof. UG

za wprowadzenie w świat nauki, nieocenioną pomoc, motywację i opiekę naukową na najwyższym poziomie. Za wsparcie w dążeniu do celu i realizacji wszystkich kolejnych kroków do jego osiągnięcia, zawsze w milej atmosferze. Za radość ze wspólnych sukcesów i niesamowite pomysły na rozwiązywanie dosłownie każdego problemu.

Panu prof. Piotrowi Stepnowskiemu

za możliwość rozpoczęcia mojej drogi naukowej w Katedrze Analizy Środowiska i inspirację, jaką Pan Profesor stanowi jako naukowiec.

Pani dr hab. Annie Białk-Bielińskiej

za życzliwość, okazywaną pomoc i motywowanie do podejmowania nowych wyzwań.

Pracownikom i Doktorantom Katedry Analizy Środowiska

za przyjazną atmosferę i pomoc.

Mojemu mężowi, Damianowi Kwidzińskiemu

za rozbudzanie we mnie ambicji i nieustanne przekonywanie, że nie ma rzeczy niemożliwych do zrobienia. Za ogrom okazanego wsparcia, za szczerą radość z każdego mojego sukcesu i niezliczone słowa otuchy w trudniejszych chwilach.

Mamie i Tacie

*za fundament, na którym zbudowałam to, z czego mogę być dziś dumna.
Za to, że nauczyliście mnie wierzyć w siebie i mieć odwagę podążać własną drogą.*

Katarzynie Bethke

za ubarwienie tej wyboistej drogi, przyjaźń, niezliczone rozmowy i wspólną wiarę, że na wszystko potrzeba zdecydowanie mniej czasu, niż ostatecznie się okazywało.

Rodzeństwu i Przyjaciółom

za chwile wytchnienia, wspólne świętowanie i to, że zawsze trzymacie za mnie kciuki.

Rozprawa doktorska została oparta na cyklu pięciu publikacji [P1 – P6] oraz trzech manuskryptów będących w trakcie recenzji [M1 – M2].

Artykuły naukowe opublikowane w czasopismach znajdujących się w bazie Journal Citation Reports (JCR):

P1

Kropidłowska Klaudia, Caban Magda*, Effect of salinity on the toxicity of diclofenac, ibuprofen and naproxen toward cyanobacterium *Synechocystis salina*, Chemosphere, 2023, 338, 139521, DOI:10.1016/j.chemosphere.2023.139521 IF₂₀₂₃ 8,1 (Q1)

P2

Kwidzińska Klaudia, Hryniewicka Marta, Caban Magda*, Biogenic volatile organic compounds produced by two unicellular and two filamentous cyanobacteria after a high light shock, Atmospheric Environment, 2025, 353, 121252, DOI:10.1016/j.atmosenv.2025.121252. IF₂₀₂₅ 3,8 (Q2)

P3

Kwidzińska Klaudia, Caban Magda*, Extraction and separation of cyanobacterial photosynthetic and photoprotective pigments: comparison of practical and accessible methodologies, European Journal of Phycology, 2026, 1-17, DOI:10.1080/09670262.2026.2682186. IF₂₀₂₆ 2,2 (Q2)

P4

Lis Hanna*, Kwidzińska Klaudia, Bethke Katarzyna, Caban Magda, Cyanobacteria as generators of reactive oxygen species – A review of analysis methods and case studies, Talanta, 2026, 297, 128648, DOI:10.1016/j.talanta.2025.128648. IF₂₀₂₆ 6,7 (Q1)

P5

Kwidzińska Klaudia*, Zalewska Martyna, Aksmann Anna, Kobos Justyna, Mazur-Marzec Hanna, Caban Magda, Multi-biomarker response of cyanobacteria *Synechocystis salina* and *Microcystis aeruginosa* to diclofenac, Journal of Hazardous Materials, 2024, 471, 134373, DOI: 10.1016/j.jhazmat.2024.134373. IF₂₀₂₄ 11,3 (Q1)

P6

Kwidzińska Klaudia, Bethke Katarzyna, Caban Magda*, Growth Inhibition and Beyond: Hormetic and Environmentally Relevant Effects of Pharmaceuticals on Cosmopolitan Cyanobacteria, Aquatic Toxicology, 2026, 108035 <https://doi.org/10.1016/j.aquatox.2026.108035>. IF 4,6 (Q1)

Artykuły w trakcie procesu publikacyjnego:

M1

Kwidzińska Klaudia, Caban Magda*, Re-evaluation of dichlorodihydrofluorescein and dichlorofluorescein diacetates applicability for cyanobacteria cells staining, *Algal Research* (wysłano do recenzji). IF 5,0 (Q1)

M2

Kwidzińska Klaudia*, Zalewska Martyna, Aksmann Anna, Caban Magda, Nitrogen availability determines diclofenac toxicity in the diazotrophic cyanobacterium *Anabaena cylindrica* (wysłano odpowiedź na recenzję). IF 4,6 (Q1)

Sumaryczny IF¹ prac wchodzących w skład rozprawy doktorskiej – 36,5

Sumaryczna liczba cytowań² prac wchodzących w skład rozprawy doktorskiej – 18

¹ Wartości IF zgodnie z rokiem opublikowania

² Liczba cytowań publikacji według bazy Web of Science (WoS)

Spis treści

STRESZCZENIE.....	11
1. WSTĘP I AKTUALNY STAN WIEDZY	14
2. CEL I ZAKRES BADAŃ	20
3. OPIS KONCEPCJI EKSPERYMENTALNEJ I ZASTOSOWANYCH METOD BADAWCZYCH	21
3.1. BADANE SZCZEPY SINIC I SPOSÓB POMIARU WZROSTU	21
3.2. SPOSOBY POMIARU WYBRANYCH BIOMARKERÓW	22
3.2.1. Analiza chlorofilu i karotenoidów [P3, P5, M2]	23
3.2.2. Analiza fikobilin [P3, P5]	24
3.2.3. Analiza fluorescencji chlorofilu a i parametrów fotosyntetycznych [P5, M2]	24
3.2.4. Analiza cytometryczna [P5]	25
3.2.5. Analiza cyjanotoksyn [P5]	25
3.2.6. Analiza biogenicznych lotnych związków organicznych [P2]	26
3.2.7. Ocena przydatności dichlorodihydrofluoresceiny jako sondy produkcji ROS w komórkach sinic [M1]	27
3.3. STABILNOŚĆ FARMACEUTYKÓW I IDENTYFIKACJA PRODUKTÓW ICH PRZEMIAN	28
3.3.1. Analiza naproksenu, ibuprofenu oraz diklofenaku i jego metabolitów metodą HPLC- DAD [P1, P5, M2]	28
3.3.2. Identyfikacja lotnych produktów przemian diklofenaku metodą GC-MS [M2]	29
3.4. TESTY EKOTOKSYKOLOGICZNE	30
3.4.1. Wpływ zasolenia na toksyczność NLPZ względem <i>S. salina</i>	30
3.4.2. Wielomarkerowa odpowiedź sinic na diklofenak	30
3.4.3. Wpływ dostępności azotu na toksyczność diklofenaku względem <i>A. cylindrica</i>	31
4. WYNIKI BADAŃ WŁASNYCH I DYSKUSJA	33
4.1. OCENA METODY OZNACZANIA ROS PRODUKOWANYCH PRZEZ KOMÓRKI SINIC Z ZASTOSOWANIEM DICHLORODIHYDROFLUORESCYNY [P4, M1]	33
4.2. BIOGENICZNE LOTNE ZWIĄZKI ORGANICZNE PRODUKOWANE PRZEZ SINICE PO SZOKU WYSOKIEGO NATĘŻENIA ŚWIATŁA [P2]	36
4.3. EKSTRAKCYJA ORAZ ROZDZIAŁ FOTOSYNTETYCZNYCH I FOTOPROTEKCYJNYCH BARWNIKÓW SINIC [P3]	38
4.4. WPŁYW ZASOLENIA NA TOKSYCZNOŚĆ NLPZ [P1]	41
4.5. WIELOBIOMARKEROWA ODPOWIEDŹ SINIC <i>S. SALINA</i> I <i>M. AERUGINOSA</i> NA DCF [P5]	43
4.6. DOSTĘPNOŚĆ AZOTU A TOKSYCZNOŚĆ DIKLOFENAKU WOBEC DIAZOTROFICZNEJ SINICY <i>A.</i> <i>CYLINDRICA</i> [M2]	49
5. PODSUMOWANIE I WNIOSKI	56

6. BIBLIOGRAFIA	60
7. WYKAZ TABEL I RYSUNKÓW	67
8. DOROBIEK NAUKOWY	68
OŚWIADCZENIA WSPÓŁAUTORÓW PUBLIKACJI WCHODZĄCYCH W SKŁAD ROZPRAWY DOKTORSKIEJ.....	73
ORYGINALNE PRZEDRUKI OPUBLIKOWANYCH ARTYKUŁÓW NAUKOWYCH STANOWIĄCYCH CYKL ROZPRAWY DOKTORSKIEJ I MANUSKRYPTY PRZESŁANE DO RECENZJI.....	82

Wykaz skrótów

DCF – diklofenak

FCM – cytometria przepływowa

DChFH₂-DA - dwuocian 2',7'-dichlorodihydrofluoresceiny

DChF-DA - dwuocian 2',7'-dichlorofluoresceiny

DChF - 2',7'-dichlorofluoresceina

MV – metylowiologen

HPLC-DAD – wysokosprawna chromatografia cieczowa z detektorem fotodiodowym,
(ang. *High Performance Liquid Chromatography with Diode Array Detector*)

NLPZ - niesteroidowe leki przeciwzapalne

HS-SPME-GC-MS - mikroekstrakcji do fazy stałej z fazy nadpowierzchniowej ze chromatografią gazową sprzężoną ze spektrometrią mas

4-OH-DCF - 4'-hydroksydiklofenaku

Chl *a* – chlorofil *a*

5-OH-DCF - 5-hydroksydiklofenaku

IND-DCF - N-(2,6-dichlorofenylo)-2-indolinonu

2,6-DCA – 2,6-dichloroanilina

ECs - nowo pojawiające się zanieczyszczenia (ang. *Emerging Contaminants*)

OD - gęstość optyczna (ang. *Optical Density*)

IBF - ibuprofen

NPX - naproksen

OECD - Organizacja Współpracy Gospodarczej i Rozwoju (ang. *Organisation for Economic Co-operation and Development*)

TLC - chromatografia cienkowarstwowa

LPLC — niskociśnieniowa chromatografia cieczowa

ROS - reaktywne formy tlenu (z ang. *Reactive Oxygen Species*)

BVOC - biogeniczne lotne związki organiczne (z ang. *Biogenic Volatile Organic Compounds*)

MC - mikrocystyny

EC₅₀ – stężenie substancji powodujące 50% efektu w odniesieniu do wzrostu sinic (ang. *Half Maximal Effective Concentration*)

Streszczenie

Substancje aktywne farmakologicznie (dalej: farmaceutyki) należą do grupy nowo pojawiających się zanieczyszczeń środowiska, dla których ocena ryzyka środowiskowego jest przedmiotem coraz liczniejszych badań. Nadal jednak występują w tym zakresie luki, które wymagają uzupełnienia. Jedną z nich jest wpływ na sinice – fotosyntetyczne mikroorganizmy o ważnej roli ekologicznej. Podstawowe testy ekotoksykologiczne oparte na ocenie wzrostu sinic mogą nie odzwierciedlać ich pełnej odpowiedzi na farmaceutyki. Z tego względu celem rozprawy doktorskiej była ocena odpowiedzi sinic na poziomie biochemicznym i fizjologicznym na działanie wybranych farmaceutyków oraz przydatności wybranych parametrów do oceny ich subletalnego oddziaływania. Dodatkowo oceniono wpływ wybranych warunków środowiskowych na odpowiedź sinic ekspozowanych na farmaceutyki.

Badania przeprowadzono z wykorzystaniem pięciu szczepów sinic, oceniając przydatność biomarkerów obejmujących przyrost biomasy, funkcjonowanie aparatu fotosyntetycznego, skład barwników, produkcję cyjanotoksyn, reaktywnych form tlenu oraz biogenicznych lotnych związków organicznych (BVOC). Zanalizowano wpływ niesteroidowych leków przeciwzapalnych (NLPZ), które w odróżnieniu do antybiotyków, nie są związkami syntezowanymi z myślą o zapobieganiu chorobom bakteryjnym, stąd sinice są dla nich organizmami niecelowanymi. Wykazano, że brak wyraźnego wpływu NLPZ na wzrost sinic nie wyklucza występowania subletalnych zmian. W przypadku diklofenaku (DCF) obserwowano zmiany w funkcjonowaniu aparatu fotosyntetycznego oraz produkcji cyjanotoksyn pomimo niewielkiego wpływu na wzrost. Badania pochodnych fluoresceiny wskazały szereg ograniczeń tych sond do oceny produkcji aktywnych form tlenu w komórkach sinic i konieczność weryfikacji ich użycia przed zastosowaniem dla nowego typu komórek. Analiza BVOC wykazała, że stres świetlny zmienia profil lotnych metabolitów, wskazując na ich potencjał jako markerów stresu oksydacyjnego.

Ważnym elementem badań było określenie wpływu składu medium wzrostowego na toksyczność farmaceutyków. Nie wykazano wyraźnego wpływu zasolenia na toksyczność badanych NLPZ, natomiast ograniczenie dostępności azotanów wyraźnie zwiększyło toksyczność DCF wobec *A. cylindrica*. Wyniki wskazują zatem, że ocena wpływu farmaceutyków na sinice wymaga uwzględnienia nie tylko parametrów wzrostowych, ale również odpowiedzi fizjologicznej i biochemicznej oraz warunków środowiskowych, w jakich zachodzi ekspozycja. Uwzględnienie tych zależności może przyczynić się do bardziej realistycznej oceny zagrożeń związanych z obecnością farmaceutyków w środowisku wodnym.

Abstract

Pharmaceuticals belong to the group of emerging environmental contaminants and are frequently detected in aquatic environments, where they may affect organisms, including cyanobacteria, which play an important role in the functioning of aquatic ecosystems. Basic ecotoxicological tests based on the assessment of cyanobacterial growth may not fully reflect their responses to contaminants. The aim of this doctoral dissertation was to assess the biochemical and physiological responses of cyanobacteria to selected pharmaceuticals and the suitability of selected parameters for detecting their sublethal effects. Additionally, the influence of environmental conditions on the response of cyanobacteria exposed to pharmaceuticals was assessed.

The study was conducted using five cyanobacterial strains, evaluating the suitability of biomarkers including biomass increase, photosynthetic apparatus functioning, pigment composition, cyanotoxin production, reactive oxygen species, and biogenic volatile organic compounds (BVOCs). The toxicological studies examined the effects of non-steroidal anti-inflammatory drugs (NSAIDs), also considering the influence of salinity and the availability of biologically available forms of nitrogen. It was demonstrated that the absence of a clear effect of pharmaceuticals on cyanobacterial growth does not exclude the occurrence of sublethal changes. In the case of diclofenac (DCF), changes in photosynthetic apparatus functioning and cyanotoxin production were observed despite its limited effect on growth. It was demonstrated that the suitability of cyanobacterial response markers depends on the organism, stressor, and experimental conditions. Studies using DCFH₂-DA indicated limitations of this probe and the need for method verification before its application. Analysis of BVOCs demonstrated that light stress alters the profile of volatile metabolites, indicating their potential as stress markers.

An important part of the study was to determine the influence of environmental conditions on the toxicity of pharmaceuticals. No clear effect of salinity on the toxicity of the tested NSAIDs was demonstrated, whereas limited nitrate availability clearly increased the toxicity of DCF toward *A. cylindrica*. The results therefore indicate that the assessment of pharmaceutical effects on cyanobacteria requires consideration not only of growth parameters, but also of physiological and biochemical responses and the environmental conditions under which exposure occurs. Taking these relationships into account may contribute to a more realistic assessment of the risks associated with the presence of pharmaceuticals in the aquatic environment.

1. Wstęp i aktualny stan wiedzy

W ostatnich dekadach obserwuje się rosnące zainteresowanie grupą związków określanych jako nowo pojawiające się zanieczyszczenia (ang. *Emerging Contaminants* - ECs). Termin ten nie odnosi się tylko do substancji, które niedawno pojawiły się w środowisku. ECs obejmuje także związki chemiczne, których obecność przez wiele lat pozostawała poza rutynowym monitoringiem oraz oceną ryzyka środowiskowego, a dopiero rozwój metod analitycznych oraz wzrost zainteresowania ich oddziaływaniem pozwoliły wykazać, że substancje te występują powszechnie, mogą utrzymywać się w środowisku przez długi czas oraz wywoływać niepożądane efekty biologiczne u organizmów i wpływać na funkcjonowanie ekosystemów (Sauvé and Desrosiers, 2014). Do tej grupy zalicza się między innymi farmaceutyki, środki higieny osobistej, pestycydy, związki zaburzające funkcjonowanie układu hormonalnego, mikroplastiki, nanomateriały oraz PFAS (ang. *Per- and poly-Fluoroalkyl Substances*) (Das and Raj, 2025).

Spośród ECs szczególną uwagę zwracają farmaceutyki, ponieważ są one projektowane w celu wywoływania określonych efektów biologicznych, często już przy niskich stężeniach (Hejna et al., 2022). Choć aktywność ta ma charakter terapeutyczny u organizmów docelowych, obecność leków w środowisku może prowadzić do niezamierzonych oddziaływań na organizmy niecelowe, dla których skutki ich działania pozostają w dużej mierze nieznane.

Problem zanieczyszczenia środowiska pozostałościami farmaceutycznymi stale narasta, ze względu na duży rozwój przemysłu farmaceutycznego, medycyny i weterynarii. Drogi przedostawania się tego typu zanieczyszczeń są stosunkowo dobrze poznane. Szczególnie wysokie stężenia farmaceutyków obserwuje się w pobliżu zakładów produkujących leki, w rejonach zrzutu ścieków szpitalnych, intensywnej hodowli zwierząt i akwakultury oraz odprowadzania wód z oczyszczalni ścieków, które są najczęstszym źródłem zanieczyszczeń farmaceutycznych w środowisku wodnym. Substancje lecznicze stosowane przez ludzi są metabolizowane jedynie częściowo. Ich pozostała część jest wydalana z organizmu i wraz ze ściekami trafia do oczyszczalni. Konwencjonalne procesy oczyszczania nie zapewniają całkowitego usunięcia farmaceutyków, w związku z czym leki oraz ich metabolity wraz z oczyszczonymi ściekami przedostają się do rzek, jezior i innych zbiorników wodnych (Kazakova et al., 2021). Istotnym źródłem zanieczyszczeń farmaceutycznych są również ścieki

pochodzące z gospodarstw hodowlanych, ponieważ wybrane leki są stosowane jako dodatek do pasz między innymi w celu stymulacji wzrostu zwierząt (aus der Beek et al., 2016). Nie bez znaczenia pozostaje również niewłaściwa utylizacja leków, związana z brakiem świadomości konsumentów.

Środowisko wodne stanowi główny odbiornik zanieczyszczeń farmaceutycznych, dlatego to właśnie ekosystemy wodne są szczególnie narażone na ich obecność oraz potencjalne oddziaływanie na organizmy (Adedipe et al., 2024; Caban and Stepnowski, 2021). Dotychczas przeprowadzone badania wskazują, że farmaceutyki mogą wykazywać toksyczność wobec wielu organizmów wodnych już w stężeniach na poziomie środowiskowym (Świacka et al., 2021). Niekorzystny wpływ obserwowano na różnym poziomie troficznym od fitoplanktonu, przez bezkręgowce, aż po ryby i ssaki. Do najczęściej badanych leków należą antybiotyki, leki przeciwbólowe i przeciwzapalne, hormonalne, psychotropowe oraz regulujące ciśnienie krwi - β -blokery. Należy zaznaczyć, że antybiotyki budzą szczególne obawy, nie tylko, ze względu na częste wykrywanie w środowisku wodnym, ale także ze względu na poszerzającą się antybiotykoodporność (Arpin-Pont et al., 2016; Chaturvedi et al., 2021, [P6]). Ze względu na tak dużą skalę problemu konieczna jest niezwłoczna i kompletna ocena skutków obecności farmaceutyków w środowisku (Eapen et al., 2024). Obejmuje ona zarówno organizmy znajdujące się na wyższych poziomach troficznych, jak i niższych, takich jak fitoplankton. Ta druga grupa stanowi podstawę wodnych sieci troficznych i odgrywa kluczową rolę w produkcji pierwotnej, obiegu składników odżywczych i energii oraz wytwarzaniu tlenu, warunkując tym samym funkcjonowanie i stabilność całych ekosystemów wodnych. Wśród producentów pierwotnych wyróżnia się eukariotyczne mikroalgi oraz prokariotyczne sinice, które stanowią odrębne grupy mikroorganizmów fotosyntetyzujących.

Sinice są tlenowymi fotoautotrofami, klasyfikowanymi jako prokarioty, które są ważnymi organizmami zarówno w środowisku wód słodkich, jak i morskich (Hoiczyn and Hansel, 2000). Mikroorganizmy te pełnią istotne funkcje ekologiczne - mają znaczący udział w globalnej produkcji tlenu oraz stanowią ważny składnik fitoplanktonu, będącego podstawą łańcuchów pokarmowych w ekosystemach wodnych (Allaf and Peerhossaini, 2022a). Z drugiej strony, w sprzyjających warunkach środowiskowych mogą tworzyć masowe zakwity, które prowadzą do zaburzenia funkcjonowania całych ekosystemów, prowadząc do pogorszenia jakości wód, niedotlenienia zbiorników oraz śnięcia ryb. Ponadto wiele gatunków tworzących zakwity produkuje cyjanotoksyny,

które stanowią istotne zagrożenie dla organizmów wodnych, zwierząt lądowych, a także dla zdrowia ludzi (Catherine et al., 2013).

Długotrwała ewolucja w zróżnicowanych środowiskach sprawiła, że sinice wykształciły liczne mechanizmy adaptacyjne umożliwiające przetrwanie w warunkach silnie zmieniających się czynników środowiskowych (Srivastava, 2013). W zależności od gatunku obejmują one między innymi zdolność wiązania azotu atmosferycznego przez gatunki diazotroficzne, regulację położenia w słupie wody za pomocą pęcherzyków gazowych, tworzenie heterocytów i akinet, syntezę związków ochronnych oraz modyfikację aparatu fotosyntetycznego. Dzięki tym mechanizmom adaptacyjnym sinice występują w bardzo zróżnicowanym środowisku od wód słodkich po bardzo słone, a nawet środowiska ekstremalne, charakteryzujące się dużymi wahaniami temperatury, zasolenia, dostępności światła czy składników odżywczych (Mazur-Marzec et al., 2009). Ze względu na kluczową rolę sinic jako producentów pierwotnych nawet niewielkie zaburzenia ich funkcjonowania mogą wpływać na kolejne poziomy troficzne oraz funkcjonowanie całych ekosystemów wodnych. Z tego względu, a także dzięki możliwości prowadzenia kontrolowanych kultur laboratoryjnych, sinice są dobrymi organizmami modelowymi do badań ekotoksykologicznych (Allaf and Peerhossaini, 2022b; Hinners et al., 2015; Lu et al., 2021). Charakteryzują się stosunkowo krótkim czasem generacji, dobrze poznaną fizjologią oraz dostępnością licznych, dobrze scharakteryzowanych szczepów. Istotną zaletą sinic jako organizmów modelowych jest możliwość jednoczesnej oceny odpowiedzi na różnych poziomach organizacji biologicznej komórki. Oprócz klasycznych parametrów, takich jak wzrost czy biomasa, możliwa jest analiza zmian fizjologicznych i biochemicznych, obejmujących między innymi wydajność fotosyntezy, skład pigmentów, produkcję toksyn oraz odpowiedź na stres oksydacyjny. Zdolność sinic do uruchamiania licznych mechanizmów adaptacyjnych sprawia, że odpowiedź na czynniki stresowe nie zawsze prowadzi do zahamowania wzrostu, lecz często obejmuje również zmiany fizjologiczne i biochemiczne. W związku z powyższym istotne jest by dokonać oceny wpływu farmaceutyków na sinice nie tylko poprzez wyznaczenie ich toksyczności na podstawie wzrostu, ale wziąć pod uwagę również zmiany subletalne, które często zachodzą przy dużo niższych poziomach stężeń niż hamowanie wzrostu [P6].

Podstawę klasycznych badań ekotoksykologicznych prowadzonych na sinicach stanowi ocena ich wpływu na wzrost kultur. Ich celem jest wyznaczenie stężenia leku, który wywołuje założony efekt, w tym celu najczęściej wyznacza się parametr EC_{50} , czyli

takie stężenie leku, przy którym wzrost komórek sinic hamowany jest o 50 % w stosunku do kultur kontrolnych [P6]. W celu ujednolicenia badań i możliwości porównania toksyczności różnych substancji wobec sinic stosuje się ustandaryzowane zasady prowadzenia tych testów określone przez Organizację Współpracy Gospodarczej i Rozwoju (ang. *Organisation for Economic Co-operation and Development* – OECD) w wytycznych OECD Test Guideline No. 201 – „*Freshwater Alga and Cyanobacteria, Growth Inhibition Test*” (OECD, 2026). Wytyczne określają m.in. warunki prowadzenia testów, sposób obliczania wpływu toksycznych substancji na wzrostu, metody pomiaru, sposób wyznaczenia EC₅₀. Tego typu badania dostarczają ważnych informacji i umożliwiają porównanie toksyczności substancji wobec konkretnych organizmów. Niestety na ich podstawie nie można wyciągnąć wniosków dotyczących mechanizmu toksyczności. Ich zalety i ograniczenia zostały szerzej omówione w pracy P6 (Rozdział 3 i 6). Istotnym ograniczeniem oceny toksyczności wyłącznie na podstawie hamowania wzrostu jest również ryzyko pominięcia odpowiedzi występujących przy niskich stężeniach substancji. W przypadku niektórych farmaceutyków obserwowano efekty hormetyczne, polegające na stymulacji wybranych procesów fizjologicznych przy niskich stężeniach, które przy wyższych poziomach narażenia przechodzą w efekt inhibicyjny. Pomijanie takich odpowiedzi może prowadzić do niedoszacowania rzeczywistego wpływu farmaceutyków występujących w środowisku w stężeniach niższych niż te wywołujące wyraźne zahamowanie wzrostu (Rozdział 3 i Rozdział 5 [P6]). Dodatkowym wyzwaniem w ocenie ich wpływu na organizmy wodne jest fakt, że farmaceutyki występują w środowisku jako złożone mieszaniny, podczas gdy znaczna część badań ekotoksykologicznych koncentruje się na oddziaływaniu pojedynczych substancji. W konsekwencji rzeczywiste oddziaływanie środowiskowe może różnić się od efektów obserwowanych podczas ekspozycji na pojedyncze związki, ponieważ składniki mieszanin mogą wykazywać działanie addytywne, synergistyczne lub antagonistyczne. Problem ten został szczegółowo omówiony w pracy P6 - Rozdział 4.5.

Współczesna ekotoksykologia coraz częściej skupia się również na subletalnych efektach wpływu, które pozwalają na bardziej szczegółową analizę, a w konsekwencji określenie mechanizmu działania toksykanta. Takie zmiany obserwowane są zwykle przy dużo niższych stężeniach niż te przy którym obserwuje się hamowanie wzrostu.

W badaniach wpływu substancji chemicznych na sinice wykorzystuje się szeroki zakres biomarkerów. Generalnie, biomarker (inaczej wskaźnik biologiczny) to mierzalny parametr biologiczny, który wskazuje na stan fizjologiczny lub patologiczny organizmu,

obecność choroby albo reakcję na czynnik zewnętrzny (np. lek lub toksynę). W przypadku sinic badane biomarkery obejmują m.in. zmiany morfologiczne, zmiany w procesie fotosyntezy, pigmenty fotosyntetyczne, cyjanotoksyny, reaktywne formy tlenu (ROS, ang. *Reactive Oxygen Species*), lipidy oraz metabolity, takie jak biogeniczne lotne związki organiczne (z ang. *Biogenic Volatile Organic Compounds* – BVOC) (Miazek and Brozek-Pluska, 2019; Sharma et al., 2021). Coraz częściej stosuje się również zaawansowane analizy omiczne, pozwalające określić wpływ substancji chemicznych na ekspresję genów (transkryptomika), białka (proteomika) i lipidy (lipidomika). Badania omiczne dostarczają cennych danych pozwalających na określenie mechanizmów, ale ze względu na koszt i dostępność sprzętu, a także analizę ogromnych ilości danych są one jeszcze mniej rozwiniętą gałęzią.

Pomimo rosnącego zainteresowania obecnością farmaceutyków w środowisku wodnym wiedza na temat ich oddziaływania na sinice pozostaje ograniczona. Dotychczasowe badania ekotoksykologiczne koncentrowały się głównie na klasycznych parametrach, przede wszystkim wzroście, podczas gdy odpowiedź sinic na obecność farmaceutyków może obejmować zmiany biochemiczne i fizjologiczne niewidoczne na poziomie proliferacji komórek. Niewystarczająco poznana pozostaje zatem przydatność dodatkowych parametrów jako markerów tego oddziaływania oraz możliwość zastosowania podejścia wieloparametrowego do jego oceny. Istotną lukę stanowi również ograniczona wiedza na temat wpływu warunków środowiskowych na odpowiedź sinic eksponowane na farmaceutyki. Substancje aktywne leków to reaktywne związki chemiczne, ich cząsteczki zawierają wiele różnych grup funkcyjnych, w związku z czym są wrażliwe na fizykochemiczne zamiany warunków w miejscu ich występowania. Czynniki takie jak temperatura, pH, natężenie promieniowania słonecznego, czy stopień zasolenia mogą indukować zmiany w cząsteczce leku jak np. zmiana formy jonizacji zależna od pH wodnego medium, w której występuje dany lek (Patel et al., 2019). Zmieniające się warunki środowiska nie pozostają też obojętne dla komórek sinic, które często potrafią się dostosować, ale wiąże się to w m.in. z dużym wydatkiem energetycznym, zmianami morfologicznymi czy metabolicznymi jak na przykład w środowisku ubogim w azot, gdzie diazotroficzne sinice tworzą wyspecjalizowane komórki umożliwiające wiązanie azotu (Flores and Herrero, 2005). Wpływ zasolenia na toksyczność leków był wcześniej analizowany w odniesieniu do zielenic (Borecka et al., 2016), jednak brakowało danych dotyczących sinic, które mogą wykazywać odmienną wrażliwość i odpowiedź na obecność farmaceutyków. Niewystarczająco poznane

pozostaje również znaczenie dostępności składników odżywczych, w szczególności azotu, w kształtowaniu tej odpowiedzi. Wskazane braki w aktualnym stanie wiedzy uzasadniają potrzebę poszukiwania dodatkowych markerów umożliwiających pełniejszą ocenę oddziaływania farmaceutyków na sinice oraz określenia znaczenia wybranych czynników środowiskowych w kształtowaniu odpowiedzi tych organizmów na obecność farmaceutyków.

2. Cel i zakres badań

Celem rozprawy doktorskiej była ocena odpowiedzi biochemicznej i fizjologicznej sinic na działanie wybranych farmaceutyków oraz ocena przydatności wybranych parametrów biochemicznych i fizjologicznych do wykrywania ich subletalnego oddziaływania. Dodatkowym celem była ocena wpływu wybranych warunków środowiskowych na odpowiedź sinic eksponowanych na farmaceutyki.

Cele szczegółowe:

1. Ocena przydatności wybranych parametrów biochemicznych i fizjologicznych jako potencjalnych biomarkerów odpowiedzi sinic na czynniki stresowe, w tym możliwości wykorzystania pigmentów, reaktywnych form tlenu oraz biogenicznych lotnych związków organicznych. (P2, P3, P4, M1)
2. Ocena odpowiedzi sinic na wybrane farmaceutyki z wykorzystaniem klasycznych i dodatkowych markerów biochemicznych i fizjologicznych, obejmujących m.in. parametry wzrostu i funkcjonowania aparatu fotosyntetycznego, zawartość pigmentów oraz produkcję cyjanotoksyn, w celu identyfikacji efektów niewidocznych na poziomie wzrostu. (P1, P5, M2)
3. Ocena wpływu wybranych czynników środowiskowych – zasolenia oraz dostępności azotu – na odpowiedź sinic eksponowanych na wybrane farmaceutyki. (P1, M2)
4. Ocena przydatności podejścia wielobiomarkerowego do badania oddziaływania farmaceutyków na sinice oraz określenie jego ograniczeń. (P6, P5)

W pracy postawiono następujące hipotezy badawcze:

Wpływ farmaceutyków na sinice nie ogranicza się do zmian tempa ich wzrostu, lecz obejmuje również zmiany fizjologiczne i biochemiczne, których analiza z wykorzystaniem odpowiednio dobranych biomarkerów pozwala na pełniejszą ocenę odpowiedzi sinic na obecność farmaceutyków. Odpowiedź ta może być ponadto modyfikowana przez współwystępujące czynniki środowiskowe, takie jak zasolenie oraz dostępność składników odżywczych.

3. Opis koncepcji eksperymentalnej i zastosowanych metod badawczych

3.1. Badane szczepy sinic i sposób pomiaru wzrostu

W badaniach wykorzystano pięć szczepów bałtyckich sinic pochodzących z Kolekcji Kultur Sinic Północnej Polski (CCNP – ang. *Culture Collection of Northern Poland*, Pracownia Biotechnologii Morskiej, Uniwersytet Gdański). Wybrane gatunki reprezentowały trzy rzędy sinic oraz dwa typy budowy - formy jednokomórkowe i nitkowate. Numery kolekcji, miejsca izolacji oraz stosowane podłoża hodowlane do prowadzenia kultur sinic przedstawiono w **Tabeli 1**.

Microcystis aeruginosa jest jednokomórkową sinicą z rzędu Chroococcales, należąca do najlepiej poznanych i najczęściej wykorzystywanych organizmów modelowych w badaniach ekotoksykologicznych. Gatunek ten tworzy masowe zakwity a wybrane szczepy produkują mikrocystyny – hepatotoksyny stanowiące zagrożenie dla ludzi i zwierząt. *Synechocystis salina* to pikoplanktonowy gatunek sinic występujący w wodach słonawych, który jest ważnym składnikiem fitoplanktonu i charakteryzuje się wysoką tolerancją na zmiany zasolenia. *Anabaena cylindrica*, *Nodularia spumigena* oraz *Aphanizomenon flos-aquae* są nitkowatymi przedstawicielami rzędu Nostocales, zdolnymi do wiązania azotu atmosferycznego dzięki obecności heterocytów. Zdolność ta stanowi istotną adaptację do funkcjonowania w środowiskach o ograniczonej dostępności biologicznie przyswajalnych form azotu. *N. spumigena* dodatkowo produkuje nodularynę - hepatotoksynę charakterystyczną dla tego gatunku.

Wszystkie szczepy utrzymywano w kulturach laboratoryjnych w komorze fitotronowej w temperaturze 25°C przy ciągłym oświetleniu o natężeniu 35-70 $\mu\text{mol fotonów m}^{-2} \text{ s}^{-1}$, z wykorzystaniem odpowiednich pożywek (**Tabela 1**). Kultury były stale napowietrzane powietrzem przepuszczonym przez sterylne filtry oraz regularnie pasażowane poprzez przenoszenie do świeżego podłoża co 3-7 dni, w zależności od tempa wzrostu szczepu. Kultury prowadzono w warunkach sterylnych. Naczynia i narzędzia wykorzystywane w eksperymentach sterylizowano w autoklawie, a wszelkie manipulacje przy kulturach wykonywano w warunkach jałowych, z wykorzystaniem komory laminarnej. W przeprowadzonych badaniach wykorzystywano kultury znajdujące się w fazie wzrostu.

Wzrost sinic monitorowano za pomocą pomiarów spektrofotometrycznych UV-Vis, poprzez oznaczanie gęstości optycznej (OD, ang. Optical Density) przy długościach fali właściwych dla

poszczególnych szczepów i warunków doświadczenia (**Tabela 1**). W przypadku jednokomórkowych sinic *S. salina* i *M. aeruginosa* wzrost oceniano również na podstawie liczby komórek oznaczanej metodą cytometrii przepływowej.

Tabela 1. Ogólna charakterystyka badanych szczepów sinic

Rząd Gatunek i szczep	Budowa	Medium	Miejsce izolacji	Pomiar wzrostu	Informacje dodatkowe
Synechococcales <i>Synechocystis salina</i> CCNP1104	Jednokomórkowa (~2,2 µm, kuliste)	BG11/ BG11 (38 PSU)	Zatoka Gdańska	OD ₆₃₀ / Ilość komórek	Kosmopolityczne, pikocyano-bacteria
Chroococcales <i>Microcystis aeruginosa</i> CCNP1101	Jednokomórkowa (~5,6 µm, kuliste)	Z8	Zalew Wiślany	OD ₆₂₅ / Ilość komórek	Toksyny, Areotopy Kosmopolityczny Zakwity
Nostocales <i>Aphanizomenon flos-aqua</i> CL2019/05	Nitkowata	Z8	Zalew Kuroński	OD ₄₅₀	Diazotroficzne
Nostocales <i>Nodularia spumigena</i> CCNP1401	Nitkowata (dł. 5,0-6,2 µm; szer. 5,4-6,2 µm)	Z8	Zatoka Gdańska	OD ₄₅₀	Toksyny Diazotroficzna Zakwity
Nostocales <i>Anabaena cylindrica</i> CCNP1405	Nitkowata (komórki vegetatywne 1,8- 3,5 µm)	Z8 / Z8 _o	Zatoka Pucka	OD ₆₈₀	Diazotroficzna Akinety

3.2. Sposoby pomiaru wybranych biomarkerów

Farmaceutyki jako substancje biologicznie aktywne, mogą indukować stres oksydacyjny, zaburzać fotosyntezę, wpływać na metabolizm komórkowy oraz wywoływać zmiany morfologiczne często przy stężeniach, które nie powodują jeszcze zahamowania wzrostu. Mechanizmy takich odpowiedzi u sinic, stanowiących organizmy niedocelowe, pozostają w większości nieznane. W związku z tym w niniejszej pracy

oceniono przydatność wybranych biomarkerów biochemicznych i fizjologicznych w analizie reakcji sinic na obecność wybranych leków. W ramach badań opracowano nowe lub zoptymalizowano istniejące procedury oznaczania poszczególnych parametrów, adaptując je do specyfiki badanych mikroorganizmów.

3.2.1. Analiza chlorofili i karotenoidów [P3, P5, M2]

W ramach poszukiwania potencjalnych biochemicznych markerów odpowiedzi sinic na czynniki stresowe opracowano i porównano metody ekstrakcji oraz analizy pigmentów fotosyntetycznych i fotoprotekcyjnych. Analizę przeprowadzono z wykorzystaniem spektrofotometrii UV-Vis, chromatografii cienkowarstwowej (TLC), niskociśnieniowej chromatografii cieczowej (LPLC) oraz wysokosprawnej chromatografii cieczowej z detekcją diodową (HPLC-DAD). Badania obejmowały chlorofile oraz karotenoidy, których zmiany ilościowe i jakościowe mogą stanowić potencjalny marker odpowiedzi sinic na czynniki stresowe. Analizowano pigmenty pięciu szczepów sinic: *S. salina*, *M. aeruginosa*, *A. flos-aquae*, *N. spumigena* oraz *A. cylindrica*. Szczegółowy opis opracowania i optymalizacji zastosowanych metod analitycznych, w tym warunków ekstrakcji i rozdziału pigmentów, przedstawiono w publikacji **P3**. Poniżej przedstawiono ostatecznie zoptymalizowane warunki ekstrakcji i analizy HPLC-DAD, które następnie zastosowano w kolejnych badaniach cyklu doktorskiego.

Ekstrakcja

Do ekstrakcji pigmentów pobierano 1 ml kultur sinic o gęstości optycznej około 1,0. Próbkę wirowano przez 4 min przy $20\,000 \times g$, po czym supernatant odrzucano, a uzyskaną biomasę zamrażano. Do biomasy dodawano 1 ml mieszaniny acetonu z 50 mM octanem amonu w stosunku objętościowym 90:10. Próbkę homogenizowano za pomocą homogenizatora kulowego przez trzy cykle po 30 s, z chłodzeniem materiału pomiędzy kolejnymi cyklami. Następnie próbki inkubowano przez 48 godzin w temperaturze 4 °C. Po zakończeniu ekstrakcji uzyskane ekstrakty filtrowano i przeznaczano do dalszej analizy. Podczas procesu ekstrakcji próbki chroniono przed nadmiernym dostępem światła i podwyższoną temperaturą.

Analiza HPLC-DAD

Analizę poszczególnych pigmentów przeprowadzono z wykorzystaniem techniki HPLC-DAD. Rozdział prowadzono na kolumnie Gemini NX-C18 (150 × 4,6 mm; 5 µm) z przedkolumną, w temperaturze 30 °C. Składnikiem wodnym fazy ruchomej stanowiła mieszanina metanolu i 50 mM wodnego roztworu octanu amonu (80:20, v/v), natomiast

fazę organiczną stanowiła mieszanina metanolu i acetonu (80:20, v/v). Zastosowano gradientowy sposób wymywania. Przepływ fazy ruchomej wynosił 1 ml/min, a objętość dozowania 25 µl. Detekcję prowadzono z wybranymi długościami fal, zależnymi od barwnika. Identyfikację poszczególnych pigmentów przeprowadzano na podstawie porównania czasów retencji oraz widm UV-Vis z 17 wzorcami analitycznymi odpowiadającymi pigmentom potencjalnie występującym w badanych sinicach. Analizę ilościową barwników w ekstraktach przeprowadzono techniką wzorca zewnętrznego. Szczegółowy wykaz zastosowanych wzorców przedstawiono w publikacji **P3**.

3.2.2. Analiza fikobilin [P3, P5]

W ramach wyboru warunków ekstrakcji fikobilin zbadano warunki przygotowania biomasy, ekstrakcji oraz analizy spektrofotometrycznej. Pigmenty ekstrahowano z liofilizowanej biomasy sinic. W celu przygotowania materiału do ekstrakcji kulturę wirowano, supernatant odrzucano, a biomasę przepłukiwano wodą dejonizowaną i ponownie wirowano. Następnie biomasę liofilizowano w temperaturze -80 °C przez 24 h.

Ekstrakcję fikobilin prowadzono z wykorzystaniem 3 mg liofilizowanej biomasy zawieszonej w 0,9 ml 0,1 M buforu fosforanowego. Próbkę homogenizowano za pomocą homogenizatora kulkowego, a następnie dodawano 0,1 ml roztworu lizozymu o stężeniu 10 mg/ml. Próbkę inkubowano przez 2 godziny w temperaturze 37 °C, a następnie przechowywano w temperaturze 4 °C przez 48 h. Po zakończeniu ekstrakcji próbkę filtrowano i poddawano analizie spektrofotometrycznej UV-Vis.

Fikocyjaninę (główną fikobilinę badanych sinic) oznaczano półilościowo metodą spektrofotometryczną w zakresie stężeń 0,0016–2,0000 mg/l. Stężenie pigmentów normalizowano względem liczby komórek, określonej za pomocą cytometrii przepływowej. Dodatkowe informacje dotyczące zastosowanej metody przedstawiono w publikacji **P5** oraz jej materiale uzupełniającym.

3.2.3. Analiza fluorescencji chlorofilu *a* i parametrów fotosyntetycznych [P5, M2]

Pomiary wykonano w ramach współpracy z Katedrą Fizjologii Roślin Wydziału Biologii Uniwersytetu Gdańskiego. Analizę fluorescencji chlorofilu *a* (Chl *a*) przeprowadzono z wykorzystaniem fluorometru Handy PEA (Plant Efficiency Analyzer, Hansatech Instruments, Wielka Brytania). Rejestrowano krzywe fluorescencji typu O-J-I-P, na podstawie których, z wykorzystaniem oprogramowania PEA Plus w wersji 1.10,

wyznaczano parametry charakteryzujące funkcjonowanie aparatu fotosyntetycznego. Przed pomiarem próbki rozcieńczano do ujednoliconej gęstości optycznej $OD_{665} = 0,20 (\pm 0,02)$. Pomiary wykonywano w temperaturze pokojowej z zastosowaniem nasycającego impulsu światła o natężeniu $3000 \mu\text{mol fotonów m}^{-2} \text{ s}^{-1}$. Analizowano parametry fluorescencji związane z wydajnością i funkcjonowaniem fotosystemu II (PSII), w tym F_v/F_m , F_v/F_0 oraz RCM, a także parametry opisujące przepływ energii na aktywne centrum reakcji: ABS/RC, TR0/RC, ET0/RC, RE0/RC i DI0/RC. Uwzględniono również parametry wydajności kwantowej i efektywności transportu elektronów, takie jak $\phi E0$, $\phi R0$, $\psi 0$, $\phi D0$ oraz $\delta R0$. Charakterystykę analizowanych parametrów przedstawiono w manuskrypcie **M2** w **Tabeli 1**.

3.2.4. Analiza cytometryczna [P5]

Liczebność komórek sinic analizowano z wykorzystaniem cytometrii przepływowej. Metodę zastosowano w ramach wielomarkerowej oceny odpowiedzi jednokomórkowych sinic *S. salina* i *M. aeruginosa* na diklofenak (DCF) przedstawionej w publikacji **P5**. Pomiary wykonano z wykorzystaniem cytometru przepływowego CytoFLEX 2L6C (Beckman Coulter), wyposażonego w lasery o długości fali 488 i 638 nm. Oprócz pomiaru ilości komórek możliwa była analiza naturalnej fluorescencji barwników obecnych w komórkach sinic. Rejestrowano fluorescencję Chl *a* i fikocyjaniny oraz rozpraszanie światła w przód (FSC) i wstecz (SSC). Dla każdej próbki analizowano co najmniej 10 000 zdarzeń, a próbki o stężeniu przekraczającym 30 000 komórek/ μl rozcieńczano sterylną wodą. Na podstawie uzyskanych danych określano liczebność komórek oraz ich właściwości fluorescencyjne. Dane dotyczące liczebności komórek wykorzystano również do normalizacji wyników oznaczeń pigmentów.

3.2.5. Analiza cyjanotoksyn [P5]

Analizę cyjanotoksyn wykonano w ramach współpracy z Katedrą Biologii Morza i Biotechnologii Wydziału Oceanografii i Geografii Uniwersytetu Gdańskiego. Materiał do analizy stanowiła biomasa sinic, którą po zakończeniu testów ekotoksykologicznych oddzielano poprzez wirowanie, przepłukiwano wodą dejonizowaną, ponownie wirowano i liofilizowano. Z liofilizowanych komórek sinic przygotowywano ekstrakty z wykorzystaniem 75% wodnego metanolu. Cyjanotoksyny analizowano z wykorzystaniem chromatografii cieczowej sprzężonej z tandemową spektrometrią mas (LC-MS/MS). Detekcję prowadzono z wykorzystaniem spektrometru mas QTRAP 5500

w trybie MS/MS. Identyfikację poszczególnych wariantów mikrocystyn przeprowadzano na podstawie ich widm fragmentacyjnych oraz danych literaturowych. Ze względu na ograniczoną dostępność wzorców analitycznych oznaczenie miało charakter półilościowy. Szczegółowy opis zastosowanej procedury analitycznej przedstawiono w publikacji **P5** (Rozdział 2.8).

3.2.6. Analiza biogenicznych lotnych związków organicznych [P2]

W ramach badań nad potencjalnym wykorzystaniem BVOC jako markerów odpowiedzi sinic na stres zastosowano metodę mikroekstrakcji do fazy stałej z fazy nadpowierzchniowej z chromatografią gazową sprzężoną ze spektrometrią mas (HS-SPME-GC-MS). Analizę przeprowadzono dla czterech szczepów sinic: *S. salina*, *M. aeruginosa*, *N. spumigena* oraz *A. flos-aquae*. Jako czynnik stresowy zastosowano podwyższone natężenie światła. Lotne związki organiczne ekstrahowano bezpośrednio z fazy nadpowierzchniowej kultur sinic z wykorzystaniem włókna SPME typu mieszanego DVB/CAR/PDMS o grubości fazy sorpcyjnej 50/30 μm (Supelco). Przed pobraniem próbek napowietrzanie kultur wstrzymywano, aby ograniczyć wpływ zewnętrznego powietrza na skład analizowanej fazy gazowej. Czas ekspozycji włókna w fazie nadpowierzchniowej optymalizowano, porównując 1, 5 i 24 h; do dalszych analiz zastosowano czas 5 h. Jako próbę ślepą analizowano podłoże wzrostowe bez sinic.

Analizę chromatograficzną prowadzono z wykorzystaniem chromatografu gazowego sprzężonego ze spektrometrem mas Shimadzu QP2010-SE. Rozdział prowadzono na kolumnie polarnej DB-624 (20 m $\times$ 0,18 mm, 1,00 μm), stosując hel jako gaz nośny. Detekcję prowadzono w zakresie m/z 35-600 przy jonizacji elektronowej (70 eV). Identyfikację związków przeprowadzono na podstawie porównania widm masowych z biblioteką NIST, a w przypadku wybranych związków dodatkowo na podstawie czasów retencji odpowiednich wzorców analitycznych. W analizie wykorzystano m.in. wzorce limonenu, β -cyklocytralu, β -jononu, 4-oks-izoforonu i mentonu oraz mieszaninę wzorcową ISO 17943 Odor Mix, zawierającą geosminę, 2-metyloizoborneol (2-MIB), 2-etylo-4-metylo-1,3-dioksolan oraz 2-etylo-5,5-dimetylo-1,3-dioksan. Analiza miała charakter jakościowy i umożliwiła identyfikację lotnych związków organicznych uwalnianych przez badane sinice w odpowiedzi na stres świetlny. Szczegółowy opis zastosowanej procedury analitycznej przedstawiono w publikacji **P2**.

3.2.7. Ocena przydatności dichlorodihydrofluoresceiny jako sondy produkcji ROS w komórkach sinic [M1]

W ramach poszukiwania metod umożliwiających monitorowanie stresu oksydacyjnego u sinic przeprowadzono badania mające na celu ocenę przydatności octan 2',7'-dichlorodihydrofluoresceiny (DChFH₂-DA) jako sondy do wykrywania reaktywnych form tlenu (ROS). Badania prowadzono głównie z wykorzystaniem *S. salina*, natomiast w końcowym etapie wybrane obserwacje zweryfikowano również dla *M. aeruginosa*, *N. spumigena* oraz *A. cylindrica*. W celu zrozumienia działania sondy DChFH₂-DA dla porównania zastosowano inną sondę o podobnej strukturze – octan dichlorofluoresceiny - DChF-DA, która jest często wykorzystywana w badaniach określających niespecyficzne wykrywanie aktywności esteraz w komórkach mikroorganizmów, a w konsekwencji do określania żywotności komórek. Zastosowanie DChF-DA pozwoliło ocenić pobieranie i zatrzymywanie sondy przez komórki oraz prześledzić proces jej deacetylacji, co było istotne dla określenia czy powstawanie fluorescencyjnej dichlorofluoresceiny (DChF) może zachodzić wewnątrz komórek, poza nimi, czy w obu tych lokalizacjach. Trzecim analizowanym związkiem była DChF, będąca fluorescencyjnym produktem przemian DChF-DA oraz końcowym produktem przemian DChFH₂-DA. Jej fluorescencja stanowi podstawę odczytu stosowanego w obu typach oznaczeń, jednak nie może być bezpośrednio utożsamiana z ilością ROS w komórce.

Badania obejmowały kilka etapów:

I. Stabilność DChFH₂-DA i DChF-DA w medium bez komórek sinic

Stabilność i przemiany sond w podłożu DChFH₂-DA i DChF-DA badano w świeżym podłożu BG11 oraz w podłożu BG11 pochodzącym z standardowej kultury *S. salina* (odwirowanym i przefiltrowanym). Badanie polegało na umieszczeniu podłoży w kwarcowych kuwetach i dodawaniu każdego z badanych związków (DChF, DChF-DA i DChFH₂-DA) i monitorowaniu ich zmian w czasie 3h. Zawartość poszczególnych form śledzono zbierając widma UV-Vis oraz określając stężenia poszczególnych za pomocą techniki HPLC-DAD w określonych punktach czasowych.

II. Sorpcja DChF, DChF-DA i DChFH₂-DA przez komórki *S. salina*

W celu określenia zdolności sorpcji DChFH₂-DA, DChF-DA i DChF do komórek sinic *S. salina* do umieszczonej w fiolce kultury sinic (10 ml) dodawano roztwory badanych odczynników (ostateczne stężenie w próbce 24 µg/ml). Próbkę wytrząsano przez 60 min w ciemności, po czym zawartość badanych substancji w komórkach sinic analizowano za

pomocą cytometru z wykorzystaniem kanału FITC (filtr pasmowo-przepustowy 525/40 nm), po wzbudzeniu laserem o długości fali 488 nm. Natomiast stężenie odczynników pozostałych w roztworze analizowano za pomocą HPLC-DAD w supernatancie po odwirowaniu i odfiltrowaniu komórek. Jako kontrolę abiotyczną zastosowano przefiltrowany supernatant z wyjściowej kultury *S. salina*.

III. Ocena desorpcji DChF i DChF-DA z komórek *S. salina*

W celu zbadania desorpcji część sinic po wytrząsaniu przez 60 minut z dodatkiem badanych odczynników została odwirowana i umieszczona w świeżej pożywce BG11, a następnie mieszana przez 10 minut. Proces desorpcji monitorowano za pomocą cytometru wykonując pomiary po 1 i 10 minutach wytrząsania.

IV. Badanie przydatności DChFH₂ do monitorowania stresu oksydacyjnego

W celu oceny przydatności DChFH₂-DA do monitorowania stresu oksydacyjnego u *S. salina* zastosowano metyloviologen (MV) jako referencyjny czynnik indukujący stres oksydacyjny. Kultury eksponowano na MV w stężeniu 1 mg/l. Po 1 eksponowaniu na światło, sinice narażone na najwyższe stężenie MV poddano barwieniu DChFH₂-DA, przy czym DChF-DA zastosowano równolegle w celu porównania odpowiedzi uzyskiwanej z obu sond. Fluorescencję komórek analizowano metodą cytometrii przepływowej, uwzględniając kontrolę biologiczną bez barwnika.

W celu monitorowania stabilności, przemian oraz sorpcji badanych związków analizowano DChF, DChF-DA i DChFH₂-DA metodą HPLC-DAD. Analizy wykonano z wykorzystaniem systemu HPLC-DAD (Nexera XR, Shimadzu) wyposażonego w kolumnę Gemini NX-C18 (150 × 4,6 mm; 5 µm). Fazę ruchomą stanowiły 10 mM bufor octanu amonu (pH 3,5) oraz acetonitryl, stosowane w gradiencie, przy przepływie 1 mL min⁻¹. Detektor rejestrował widma w zakresie 190-800 nm, a identyfikację związków przeprowadzano na podstawie ich czasów retencji i charakterystycznych długości fal. Oznaczenia ilościowe wykonano metodą wzorca zewnętrznego, w zakresie 0,012–100 mg/l.

3.3. Stabilność farmaceutyków i identyfikacja produktów ich przemian

3.3.1. Analiza naproksenu, ibuprofenu oraz diklofenaku i jego metabolitów metodą HPLC-DAD [P1, P5, M2]

Stabilność ibuprofenu (IBF), naproksenu (NPX) i DCF monitorowano oznaczając ich stężenie każdego dnia w trakcie testów ekotoksykologicznych. Dodatkowo w przypadku DCF badano występowanie kilku najważniejszych produktów jego

przemiany w wodnej fazie, tj.: N-(2,6-dichlorofenylo)-2-indolinonu – IND-DCF, 4'-hydroksydiklofenaku – 4-OH-DCF, 5-hydroksydiklofenaku – 5-OH-DCF oraz acylo- β -D-glukuronidu diklofenaku – GLU-DCF. Analizę prowadzoną za pomocą HPLC-DAD. Rozdział chromatograficzny prowadzono z zastosowaniem kolumny Gemini NX-C18 (5 μ m, 110 Å, 150 $\times$ 4,6 mm, Phenomenex). Fazę ruchomą stanowiła mieszanina bufor octanowy (pH 4,0 – 4,1) oraz acetonitrylu. Detektor DAD rejestrował widma w zakresie 190–800 nm. Charakterystykę krzywych kalibracyjnych, obejmującą liniowość (R^2), zakres liniowości, granicę wykrywalności, dokładność oraz precyzję, przedstawiono w publikacjach **P4** i **P5** oraz ich materiałach uzupełniających.

3.3.2. Identyfikacja lotnych produktów przemian diklofenaku metodą GC-MS [M2]

Do identyfikacji lotnego produktu przemian DCF zastosowano HS-SPME-GC-MS. Analizę przeprowadzono po zakończeniu ekspozycji w próbkach kultur *A. cylindrica*, kontroli biotycznych oraz odpowiadających im kontroli abiotycznych zawierających DCF (**M2**). Do ekstrakcji wykorzystano włókno z mieszaną fazą sorpcyjną DVB/CAR/PDMS (50/30 μ m, 2 cm, 24 Ga, StableFlex; Supelco, Niemcy). Do fiolek o pojemności 20 ml wprowadzano 10 ml próbki oraz 2,5 g NaCl. Ekstrakcję z fazy nadpowierzchniowej prowadzono przez 30 min. Po zakończeniu ekstrakcji związki desorbowano z włókna w dozowniku chromatografu przez 3 min.

Analizę GC prowadzono na kolumnie Zebron ZB-SemiVolatiles (30 m $\times$ 0,25 mm, 0,25 μ m; Phenomenex). Temperatura dozownika wynosiła 200°C, zastosowano dozowanie bez podziału strumienia (splitless) przez 1 min, a jako gaz nośny stosowano hel. Program temperaturowy rozpoczynał się od 30°C przez 1 min, następnie temperaturę zwiększano z szybkością 16°C/min do 230°C i utrzymywano przez 1 min. Detekcję prowadzono z zastosowaniem spektrometru mas o pojedynczym kwadrupolu z wykorzystaniem jonizacji elektronowej (EI) przy energii 70 eV. Widma rejestrowano w trybie skanowania w zakresie 45–450 m/z oraz w trybie monitorowania wybranych jonów (SIM). Identyfikację 2,6-dichlorofluoresceiny (2,6-DCA) przeprowadzono na podstawie analizy wzorca analitycznego, wykorzystując jony o wartościach m/z 161, 163, 90, 63 i 52. Analiza miała charakter jakościowy i nie prowadzono ilościowego oznaczania 2,6-DCA. Szczegółowy opis procedury analitycznej przedstawiono w pracy **M2**.

3.4. Testy ekotoksykologiczne

3.4.1. Wpływ zasolenia na toksyczność NLPZ względem *S. salina*

Wpływ zasolenia na toksyczność niesteroidowych leków przeciwzapalnych (NLPZ) - IBF, NPX i DCF - oceniono względem sinic *S. salina*. Badania przeprowadzono w oparciu o wytyczne OECD 201 z modyfikacjami. Badania obejmowały testy wstępne, w których oceniono wpływ NLPZ na wzrost *S. salina* w stężeniach 0,1 - 5 mg/l. Ze względu na brak toksyczności w badanym zakresie stężeń w testach uwzględniających wpływ zasolenia sinice narażono na NLPZ w nominalnym stężeniu 100 mg/l. Stężenie to odpowiada najwyższemu stężeniu przewidzianemu w ramach tzw. testu granicznego (limit test) zgodnie z wytycznymi OECD 201, stosowanego w przypadku braku obserwowanych efektów toksycznych w badaniu wstępnym do stężenia 100 mg/l (OECD, 2026). Badania prowadzono w dwóch wariantach zasolenia: w standardowym podłożu BG11 (~3 PSU) oraz w BG11 uzupełnionym solą morską, uzyskując końcowe zasolenie 38 PSU. Uwzględniono odpowiednie kontrole biologiczne oraz abiotyczne dla obu poziomów zasolenia. Ekspozycję prowadzono przez 96 h, kontrolując wzrost do 24 h, pH i morfologię komórek. Wpływ zasolenia na wzrost *S. salina* oceniano na podstawie porównania wartości OD₆₃₀ kultur prowadzonych przy 38 PSU i w standardowym BG11, natomiast wpływ NLPZ określano poprzez odniesienie wzrostu kultur eksponowanych na badane związki do odpowiednich kontroli. Dodatkowo wyznaczano względne tempo wzrostu pomiędzy kolejnymi dnami ekspozycji. W przypadku IBF, ze względu na jego niższą rozpuszczalność oraz zakwaszenie podłoża po jego dodaniu, przeprowadzono również wariant testu z korektą pH do 7,5. Dokładny opis badań przedstawiono w publikacji **P1**.

3.4.2. Wielomarkerowa odpowiedź sinic na diklofenak

Przeprowadzono badania wpływu DCF na dwa gatunki jednokomórkowych sinic - *S. salina* i *M. aeruginosa*. Ocenę odpowiedzi sinic przeprowadzono z wykorzystaniem zestawu biomarkerów obejmujących parametry wzrostowe, fizjologiczne i biochemiczne. Szczegółowy opis badań przedstawiono w publikacji **P5**.

Testy prowadzono w warunkach identycznych z warunkami stosowanymi podczas prowadzenia kultur poszczególnych gatunków sinic (Rozdział 5.1). Kultury eksponowano na DCF w nominalnym stężeniu 100 mg/l przez 96 h. Dla każdej grupy zastosowano pięć powtórzeń biologicznych – pięć kultur eksponowanych na DCF oraz pięć kultur kontrolnych. Uwzględniono również odpowiednie kontrole abiotyczne,

obejmujące dwa rodzaje podłoża zawierające DCF, utrzymywane w warunkach testowych. Eksperyment przeprowadzono w trzech niezależnych powtórzeniach. Na początku oraz po zakończeniu ekspozycji mierzono pH kultur.

W trakcie ekspozycji, co 24 h, monitorowano wzrost kultur na podstawie pomiaru gęstości optycznej oraz liczebności komórek określonej za pomocą cytometrii przepływowej, zgodnie z metodą opisaną w Rozdziale 5.2.4. Jednocześnie obserwowano komórki pod mikroskopem optycznym w celu oceny dodatkowych zmian w ich morfologii.

Wpływ DCF na funkcjonowanie aparatu fotosyntetycznego oceniano na podstawie fluorescencji Chl *a* metodą OJIP, zgodnie z procedurą opisaną w Rozdziale 5.2.3. Pomiary wykonano pierwszego dnia eksperymentu, godzinę po dodaniu DCF oraz po 96-godzinnej ekspozycji.

Po zakończeniu ekspozycji przeprowadzono analizę barwników fotosyntetycznych. Określano zawartość Chl *a* i jego pochodnej – feofityny, wybranych karotenoidów oraz fikobilin, zgodnie z metodami opisanymi odpowiednio w Rozdziałach 5.2.1 i 5.2.2. Uzyskane wartości normalizowano względem liczby komórek, co umożliwiało porównanie zawartości barwników niezależnie od zagęszczenia kultur.

Ze względu na zdolność *M. aeruginosa* do produkcji cyjanotoksyn dodatkowym biomarkerem odpowiedzi na DCF była produkcja mikrocystyn. W ekstraktach biomasy oznaczano profile mikrocystyn metodą LC-MS/MS, zgodnie z procedurą opisaną w Rozdziale 5.2.5.

W trakcie eksperymentu monitorowano również stężenie DCF oraz obecność wybranych produktów jego przemian. Analizę DCF i jego metabolitów przeprowadzono metodą HPLC-DAD, zgodnie z procedurą opisaną w Rozdziale 5.3.1.

3.4.3. Wpływ dostępności azotu na toksyczność diklofenaku względem *A. cylindrica*

W badaniu oceniono wpływ DCF na diazotroficzną sinicę *A. cylindrica* hodowaną w warunkach różnej dostępności azotu – w standardowej pożywce Z8 oraz w pożywce Z8 bez azotanów (Z8₀). Oba warianty eksperymentalne prowadzono równolegle, wykorzystując sinice pochodzące z tej samej kultury wyjściowej. Przed rozpoczęciem eksperymentu kultury aklimatyzowano do warunków bez azotanów przez co najmniej 14 dni, a obecność heterocyst potwierdzano na podstawie obserwacji mikroskopowych.

Kultury *A. cylindrica* eksponowano na DCF w nominalnym stężeniu 100 mg/l przez 216 h. Każdy wariant eksperymentalny został przeprowadzony w trzech

niezależnych testach, a każdy z nich obejmował pięć powtórzeń biologicznych i odpowiednie kontrole abiotyczne. Testy prowadzono w warunkach takich jak prowadzono wyjściowe kultury (**Rozdział 5.1**). W trakcie ekspozycji codziennie monitorowano wzrost kultur na podstawie pomiaru OD₆₈₀. Na zakończenie ekspozycji mierzono również pH kultur.

Wpływ DCF oraz dostępności azotu oceniano na podstawie zmian wzrostu oraz wybranych parametrów fizjologicznych i biochemicznych. Analizowano efektywność procesu fotosyntetycznego na podstawie fluorescencji Chl *a* i parametrów testu OJIP, zgodnie z metodą opisaną w odpowiedniej sekcji części metodycznej. Oceniano również profil barwników fotosyntetycznych, obejmujący karotenoidy Chl *a* oraz jego pochodną feofitynę, zgodnie z procedurą opisaną w rozdziale **5.2.1**. Zmiany morfologiczne komórek monitorowano na podstawie obserwacji mikroskopowych.

Stabilność DCF oraz powstawanie wybranych produktów jego przemian monitorowano podczas całego okresu ekspozycji, pobierając codziennie próbki z kultur sinic oraz odpowiadających im kontroli abiotycznych. Analizy prowadzono zgodnie z metodą HPLC-DAD opisaną w rozdziale **5.3.1**.

Po zakończeniu ekspozycji przeprowadzono również analizę lotnych związków metodą HS-SPME-GC-MS. Analizę wykonano ze względu na obecność charakterystycznego zapachu obserwowanego po otwarciu naczyń zawierających DCF. Identyfikację 2,6-DCA jako produktu przemian DCF przeprowadzono zgodnie z metodą opisaną w Rozdziale 5.3.2. Szczegółowe informacje dotyczące przeprowadzenia badań oraz zastosowanych metod analitycznych przedstawiono w publikacji [M2].

4. Wyniki badań własnych i dyskusja

4.1. Ocena metody oznaczania ROS produkowanych przez komórki sinic z zastosowaniem dichlorodihydrofluoresceiny [P4, M1]

Produkcja ROS jest naturalnie związana z procesami metabolicznymi komórek, a u sinic jako organizmów fotosyntetyzujących, także transportem elektronów w aparacie fotosyntetycznym. Zwiększona produkcja ROS może towarzyszyć zaburzeniom równowagi oksydacyjno-antyoksydacyjnej wywołanym działaniem czynników stresowych, dlatego ich oznaczanie jest wykorzystywane do oceny odpowiedzi komórek na stres [P4].

Dioctan 2',7'-dichlorodihydrofluoresceiny (DChFH₂-DA) jest szeroko stosowany jako sonda fluorescencyjna do wykrywania wewnątrzkomórkowych ROS (Mondal and Singh, 2022; Rajneesh et al., 2017; Zhang et al., 2020). Ze względu na powszechne wykorzystanie tej sondy w badaniach nad sinicami, a jednocześnie niewystarczającą weryfikację jej zachowania w tych mikroorganizmach, przeprowadzono badania mające na celu ocenę jej przydatności na przykładzie komórek *S. salina*. Szczegółowe wyniki badań przedstawiono w manuskrypcie [M1].

Ocena obejmowała stabilność DChFH₂-DA w standardowym podłożu BG11, jej przemiany w warunkach występujących w kulturach sinic, oddziaływanie z komórkami oraz możliwość uzyskania i detekcji sygnału fluorescencyjnego. W badaniach dodatkowo wykorzystano DChF-DA oraz DChF jako związki odniesienia. DChF-DA pozwolił ocenić możliwość pobierania podobnej strukturalnie pochodnej przez komórki oraz zachodzącej w nich deacetylacji, natomiast DChF wykorzystano do określenia zachowania produktu fluorescencyjnego, w tym jego oddziaływania z powierzchnią komórek i podatności na desorpcję. Takie zestawienie pozwoliło określić, czy ewentualny brak sygnału uzyskiwanego po zastosowaniu DChFH₂-DA może być związany z ograniczonym pobieraniem sondy przez komórki lub z samą procedurą detekcji.

Badania stabilności wykazały, że DChFH₂-DA ulega przemianom już w standardowym medium BG11, gdzie obserwowano znaczny ubytek DChFH₂-DA, głównie związany z ograniczoną rozpuszczalnością sondy i jej częściowym wytrącaniem. W medium wzrostowym odwirowanym z kultur sinic (pH 9,7) stężenie sondy zmniejszyło się o około 66% już po 60 min, a po 180 min o około 91%. Jednocześnie obserwowano powstawanie produktów transformacji, m.in. DChF. Wyniki wskazują

zatem, że znaczny ubytek DChFH₂-DA może zachodzić niezależnie od obecności komórek, a w warunkach alkalicznych istotnym procesem jest jej szybka hydroliza. Obecność komórek powodowała dodatkowy ubytek DChFH₂-DA w stosunku do kontroli abiotycznej, wskazując na jej oddziaływanie z komórkami. Nie prowadziło to jednak do uzyskania mierzalnego sygnału fluorescencyjnego w komórkach sinic. W analizie cytometrycznej komórki *S. salina* po barwieniu DChFH₂-DA wykazywały intensywność fluorescencji w kanale FITC zbliżoną do kontroli biologicznej i kontroli rozpuszczalnikowej. Brak wzrostu sygnału potwierdzono w kolejnych doświadczeniach, z wykorzystaniem tego odczynnika od innego producenta oraz dla komórek *M. aeruginosa*. Dodatkowo, zastosowanie MV jako czynnika generującego ROS nie spowodowało wzrostu intensywności fluorescencji DChFH₂-DA w komórkach *S. salina*, która pozostawała na poziomie podobnym do kontroli. Wynik ten dodatkowo potwierdził brak możliwości wykorzystania DCFH₂-DA do wiarygodnego wykrywania odpowiedzi oksydacyjnej w zastosowanym układzie.

Porównanie z DChF-DA i DChF dostarczyło dodatkowych informacji istotnych dla interpretacji tego wyniku. Cząsteczki DChF-DA były efektywnie pobierane przez komórki *S. salina*, w których sonda ulegała deacetylacji. Wskazywał na to uzyskany w kanale FITC sygnał, który utrzymywał się po płukaniu komórek (**Rysunek 3 i Rysunek 5 [M2]**). DChF natomiast wykazywał niewielkie oddziaływanie z komórkami i był szybko usuwany z ich powierzchni podczas przepłukiwania komórek czystym medium. Wyniki te potwierdziły, że zastosowany układ umożliwia zarówno pobieranie odpowiedniej pochodnej fluoresceiny przez komórki, jak i detekcję powstałego produktu fluorescencyjnego. Tym samym brak sygnału po zastosowaniu DChFH₂-DA nie był jedynie konsekwencją nieskutecznego barwienia sinic lub ograniczeń zastosowanej detekcji.

Istotnym elementem oceny była również weryfikacja możliwości wykorzystania czytnika mikropłytek. W przeciwieństwie do cytometrii przepływowej metoda ta obejmuje pomiar całkowitej fluorescencji próbki, czyli zarówno sygnał związany z komórkami, jak i sam barwnik obecny w podłożu. W przypadku badanych sond wykazano, że część mierzonego sygnału może pochodzić z przemian zachodzących poza komórkami. Po płukaniu komórek obserwowano znaczne zmniejszenie fluorescencji (**Rysunek 5 [M2]**), co dodatkowo wskazało na znaczenie sposobu przygotowania próbki dla uzyskiwanego wyniku.

Jednocześnie wykazano, że etap wirowania stosowany w celu oddzielenia komórek od podłoża może stanowić dodatkowe źródło zakłóceń. W przypadku *S. salina* i *M. aeruginosa* obserwowano ubytek komórek po wirowaniu (**Rysunek S5**, suplement [M1]). Oznacza to, że zastosowanie płukania, a następnie wirowania i umieszczenia w świeżym medium, które mogłoby ograniczyć udział fluorescencji pochodzącej z barwnika obecnego w podłożu, może jednocześnie zaniżyć wyniki fluorescencji.

Na podstawie przeprowadzonych badań stwierdzono, że DChFH₂-DA nie zapewnia wiarygodnej oceny produkcji ROS w badanych komórkach sinic w zastosowanym układzie eksperymentalnym. Na uzyskiwany wynik wpływać może jednocześnie niestabilność sondy w alkalicznym podłożu, jej ograniczona rozpuszczalność, przemiany zachodzące poza komórkami oraz sposób przygotowania próbki do analizy. Szczególnie istotna jest szybka hydroliza DChFH₂-DA w podłożu o wysokim pH, ponieważ sinice silnie alkalizują środowisko podczas wzrostu. W takich warunkach podczas standardowej, 60-minutowej procedury barwienia może dojść do znacznego ubytku sondy jeszcze przed uzyskaniem sygnału pochodzącego z komórek.

Przeprowadzona analiza pokazuje, że samo powszechne zastosowanie danego markera w badaniach biologicznych nie jest wystarczające do uznania go za odpowiedni dla konkretnego organizmu i układu eksperymentalnego. W przypadku DChFH₂-DA konieczne okazało się uwzględnienie specyfiki prowadzenia kultur sinic, w szczególności zmian pH podłoża, a także potencjalnych artefaktów związanych ze stabilnością sondy i przygotowaniem próbek. W konsekwencji DChFH₂-DA została wykluczona jako wiarygodny marker produkcji ROS w dalszych badaniach prowadzonych w ramach niniejszej pracy. Badania wskazały, że ocena produkcji ROS u sinic z wykorzystaniem sond fluorescencyjnych może być obarczona istotnymi ograniczeniami wynikającymi zarówno z właściwości samej sondy, jak i ze specyfiki badanego układu.

W związku z tym postanowiono ocenić, czy problemy zidentyfikowane podczas badań DChFH₂-DA dotyczą również innych metod stosowanych do oznaczania ROS u sinic. Zagadnienie to podjęto w pracy przeglądowej **P4**, w której zestawiono i krytycznie przeanalizowano dostępne metody oznaczania wewnątrz- i zewnątrzkomórkowych ROS u sinic, ze szczególnym uwzględnieniem sond fluorescencyjnych. Przedstawiono ich możliwości w zakresie jakościowej i ilościowej oceny ROS oraz omówiono najważniejsze ograniczenia związane z ich selektywnością, czułością i sposobem detekcji. Przeprowadzona analiza prac eksperymentalnych wykazała, że większość stosowanych sond fluorescencyjnych nie pozwala na

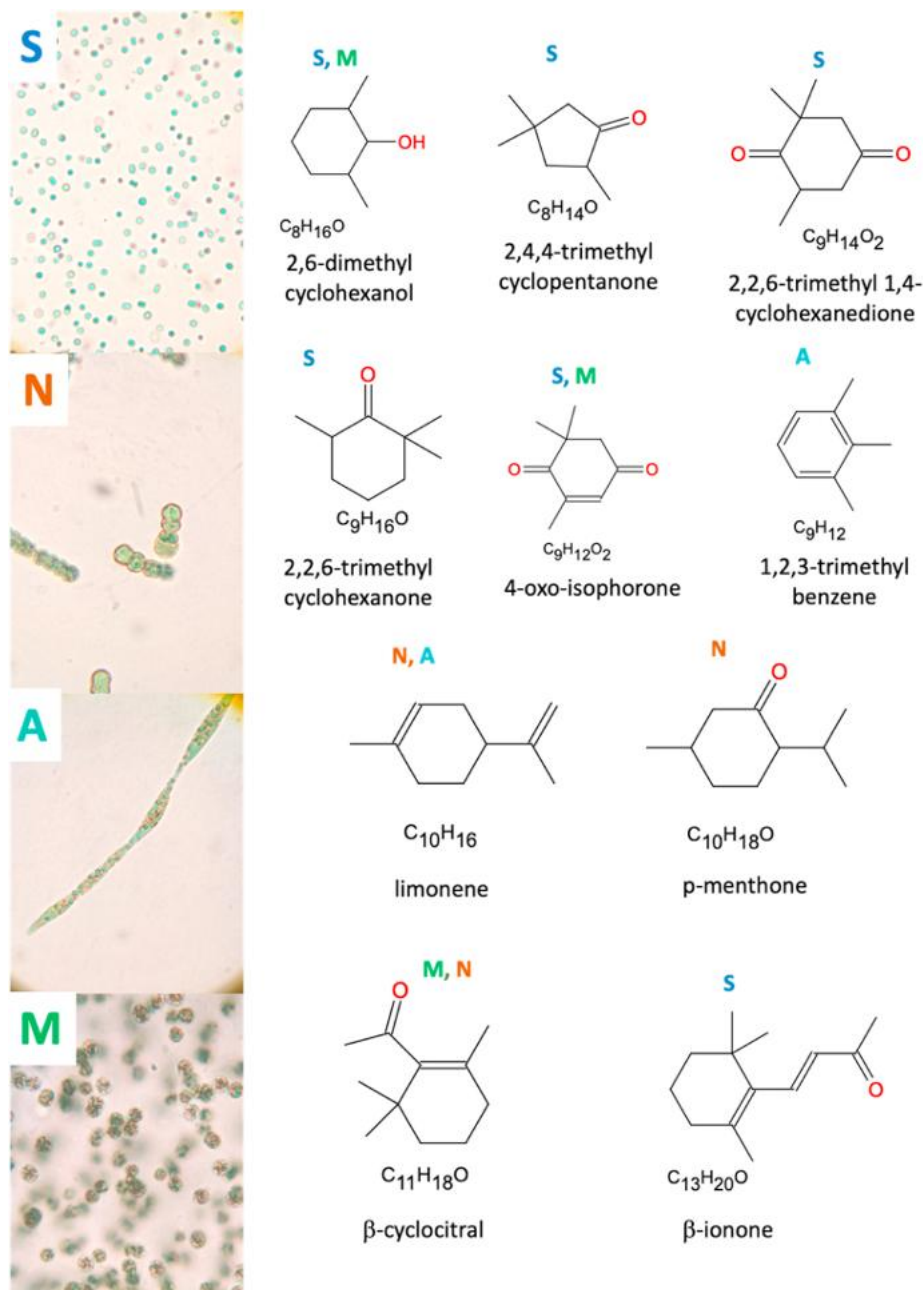
jednoznaczne wskazanie konkretnego rodzaju ROS, a uzyskiwany sygnał może zależeć od wielu czynników niezwiązanych bezpośrednio z produkcją ROS przez komórki. Wśród potencjalnych źródeł zakłóceń wskazano m.in. autooksydację sond pod wpływem światła, zależność ich właściwości od pH, obecność jonów metali i innych reaktywnych składników matrycy, a także możliwość powstawania produktów reakcji niezależnie od obecności komórek. Szczególnie istotne okazały się ograniczenia metod opartych na pochodnych dihydrofluoresceiny, w przypadku których wzrost fluorescencji nie może być utożsamiany bezpośrednio ze wzrostem stężenia określonego ROS. Analiza literatury w publikacji **P4** potwierdziła zatem, że ograniczenia zidentyfikowane eksperymentalnie dla DChFH₂-DA nie są wyłącznie problemem pojedynczej sondy, lecz wpisują się w szerszy problem wiarygodnego oznaczania ROS u sinic. Wskazują one na konieczność każdorazowej weryfikacji przydatności stosowanej metody w konkretnym układzie eksperymentalnym oraz ostrożnej interpretacji uzyskiwanego sygnału fluorescencyjnego.

4.2. Biogeniczne lotne związki organiczne produkowane przez sinice po szoku wysokiego natężenia światła [P2]

Biogeniczne związki lotne (BVOC, ang. *Biogenic Volatile Compounds*) zaliczane są do metabolitów wtórnych, a ich synteza może być aktywowana w odpowiedzi na stres wywołany przez czynniki biotyczne, jak i abiotyczne (Loreto and Schnitzler, 2010; Zuo, 2019). Chemicznie są to małowcząsteczkowe związki organiczne o dużej lotności, które mogą pełnić funkcje sygnałowe i obronne oraz uczestniczyć w ograniczaniu stresu oksydacyjnego poprzez wychwytywanie ROS (Mohanty et al., 2022; Zhang et al., 2015). Z tego względu zmiany w ich produkcji mogą stanowić potencjalny wskaźnik odpowiedzi komórki na stres. Sinice są jednym z głównych organizmów jednokomórkowych odpowiedzialnych za produkcję BVOC w wodach (Huang et al., 2018). W niniejszym badaniu jako czynnik stresowy zastosowano wysokie natężenie światła, które jest dobrze udokumentowanym czynnikiem stresowym dla sinic i może indukować produkcję BVOC (Muhetaer et al., 2020).

Badania wykazały, że w kulturach kontrolnych w optymalnych warunkach laboratoryjnych produkcja BVOC była poniżej granicy wykrywalności zastosowanej metody (SPME-GC/MS). Natomiast, we wszystkich kulturach sinic poddanych na stres świetlny wykryto związki z grupy BVOC. W zależności od gatunku zidentyfikowano od dwóch do siedmiu związków należących m.in.

do alkoholi, aldehydów lub terpenów. Struktury wykrytych BVOC przedstawiono na **Rysunku 1**.



Rysunek 1. Struktury biogenne lotnych związków organicznych (BVOC) wytwarzanych przez cyjanobakterie *Synechocystis salina* (S), *Microcystis aeruginosa* (M), *Aphanizomenon flos-aquae* (A) oraz *Nodularia spumigena* (N) w warunkach stresu wywołanego silnym światłem [P2].

Wśród wykrytych substancji znalazły się nor-karotenoidy, które mogą powstawać zarówno w wyniku biosyntezy, jak i degradacji pigmentów fotosyntetycznych. Powstają one w wyniku oksydacyjnego rozszczepienia karotenów i ksantofili, m.in. β -karotenu

i zeaksantyny (Watson et al., 2016). Obecność β -cyklocytralu, oznaczonego w kulturach *N. spumigena* i *M. aeruginosa*, najprawdopodobniej związana była z utlenianiem β -karotenu przez rodniki tlenowe, powstające w nadmiernej ilości w komórkach sinic pod wpływem szoku świetlnego. Związek ten wykrywano wcześniej u sinic po ekspozycji na wysokie natężenie światła. Natomiast nigdy wcześniej nie wykazano jego obecności u *N. spumigena*, co czyni niniejsze badanie pierwszym doniesieniem o jego występowaniu u tego gatunku (Havaux, 2020). W badanych kulturach nie wykryto żadnego ze związków wchodzących w skład mieszaniny referencyjnej 17943 Odor Mix. W *N. spumigena* wcześniejsze analizy wykazały możliwość produkcji geosminy przez szczepy brazylijskie, natomiast nie wykazano jej u szczepów pochodzących z Bałtyku i Japonii (Popin et al., 2020). Uzyskane wyniki wskazują, że profil BVOC zmieniał się w odpowiedzi na stres świetlny, a pojawienie się związków niewykrywanych w kontrolach, szczególnie produktów przemian karotenoidów, wskazuje na ich potencjalną przydatność jako markerów stresu, w szczególności oksydacyjnego.

W związku z tym postanowiono przetestować DCF jako potencjalny stresor powodujący stres oksydacyjny. W badaniach tych przeprowadzono analizę BVOC u *S. salina* eksponowanej na DCF w stężeniu 100 mg/l. W tym przypadku nie wykryto BVOC w kulturach kontrolnych oraz eksponowanych na DCF. Zastosowane stężenie DCF nie powodowało również zahamowania wzrostu *S. salina*. Wyniki tej analizy nie zostały dotychczas opublikowane. Odpowiedź w zakresie produkcji BVOC może zależeć od rodzaju i intensywności czynnika stresowego. Z tego względu zasadne jest dalsze badanie ich potencjału jako biomarkerów odpowiedzi sinic na działanie farmaceutyków, w tym oceny czy zmiany ich profilu mogą być obserwowane w odpowiedzi na związki o różnym mechanizmie i nasileniu działania.

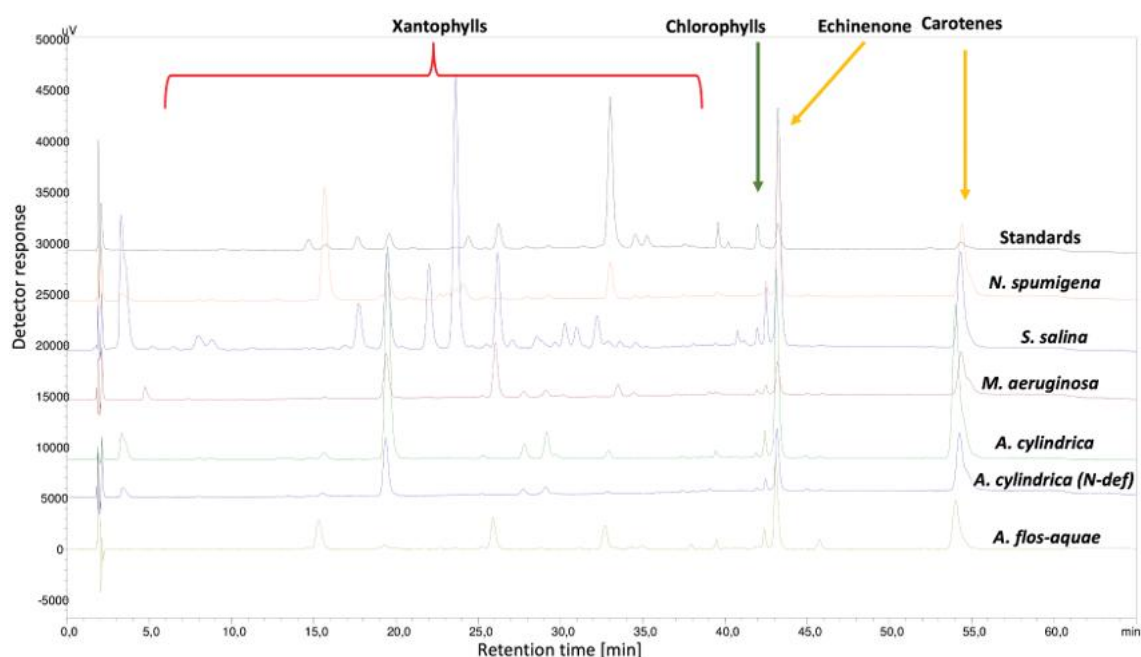
4.3. Ekstrakcja oraz rozdział fotosyntetycznych i fotoprotekcyjnych barwników sinic [P3]

Pigmenty fotosyntetyczne i fotoprotekcyjne, w szczególności chlorofile i karotenoidy, stanowią istotne wskaźniki funkcjonowania aparatu fotosyntetycznego i odpowiedzi sinic na zmieniające się warunki środowiskowe. Zmiany ich zawartości i składu mogą odzwierciedlać procesy aklimatyzacyjne oraz reakcję komórek na działanie czynników stresowych. Możliwość wykorzystania pigmentów jako potencjalnych biochemicznych wskaźników odpowiedzi sinic wymaga jednak opracowania

wiarygodnych i odpowiednio selektywnych metod ich ekstrakcji oraz oznaczania. W ramach poszukiwania potencjalnych biochemicznych markerów odpowiedzi sinic na czynniki stresowe opracowano metody ekstrakcji i analizy fotosyntetycznych chlorofili oraz fotoprotekcyjnych karotenoidów. Metody ekstrakcji były wcześniej dostępne, ale nie zostały wcześniej porównane w sposób systematyczny. Porównanie zastosowanych metod wykazało, że spektrofotometria UV-Vis pozwala na szybką ocenę całkowitej zawartości chlorofili i karotenoidów, natomiast TLC i LPLC mogą być wykorzystane do wstępnego rozdziłu i frakcjonowania ekstraktów. Metody te charakteryzowały się jednak ograniczoną selektywnością i nie pozwalały na jednoznaczną identyfikację poszczególnych pigmentów. Dodatkowo, wykazano, że badania zawartości głównych grup barwników bezpośrednio w kulturach sinic wiąże się z błędami ilościowymi związanymi ze zjawiskiem odbijania światła przez komórki (wyniki zawyżone). Ponadto, gdy barwniki są upakowane bardziej gęsto wewnątrz komórki, mogą pochłaniać więcej światła, jednak zmienia to również kształt widmowej krzywej absorpcji. Efekt upakowania (efekt pakietowy) wyjaśnia zmniejszoną absorpcję tej samej ilości barwnika znajdującego się wewnątrz komórek fitoplanktonu w porównaniu z barwnikiem wyekstrahowanym i rozpuszczonym w rozpuszczalniku. Ponadto, wykazano możliwą epimeryzację w cząsteczce Chl *a* podczas procedury ekstrakcji.

Największą selektywność uzyskano dla techniki HPLC-DAD, która umożliwiła identyfikację poszczególnych pigmentów (**Rysunek 2**) oraz ich ilościowe oznaczenie. Analiza ilościowa poszczególnych chlorofili i karotenoidów, przeprowadzona metodą wzorca zewnętrznego, charakteryzowała się precyzją oznaczeń poniżej 4,6% RSD oraz dokładnością w zakresie 85-116%. Opracowaną metodę HPLC-DAD zastosowano do charakterystyki profilu pigmentowego pięciu badanych gatunków sinic. We wszystkich analizowanych ekstraktach wykryto Chl *a*, echinenon oraz β -karoten, natomiast pozostałe składniki profilu pigmentowego różniły się między gatunkami. Pigmenty wykryte w poszczególnych gatunkach sinic przedstawiono w **Tabeli 1** publikacji **P3**. Uzyskane profile wskazują na wyraźne różnice w składzie karotenoidów między badanymi gatunkami. Porównanie z danymi literaturowymi wskazuje, że skład pigmentów może różnić się nawet między szczepami tego samego gatunku (**Tabela 3 [P3]**), a także zależeć od warunków prowadzenia hodowli (Assunção et al., 2023). W przypadku *S. salina* w tym samym szczepie we wcześniejszych badaniach wykazano obecność kantaksantyny (Wojtasiewicz and Stoń-Egiert, 2016) podczas gdy w przeprowadzonych analizach

pigment ten nie został zidentyfikowany. Podobne różnice obserwowano także dla pozostałych badanych gatunków.



Rysunek 2. Chromatogramy barwników fotosyntetycznych badanych sinic oraz mieszaniny wzorców barwników, zarejestrowane przy długości fali 452 nm - [P3]

Pomimo najwyższej selektywności spośród zastosowanych metod, HPLC-DAD miała pewne ograniczenia w jednoznacznej identyfikacji niektórych składników, związane m.in. z dostępnością odpowiednich wzorców analitycznych. W ekstrakcie *S. salina* zaobserwowano wyraźny dodatkowy sygnał, który na podstawie porównania widma, względnego czasu retencji oraz danych literaturowych (Graham i Bryant, 2008; Graham et al., 2008; Maresca et al., 2008) można wiązać z obecnością synechoksantyny. Jednoznaczne potwierdzenie jej obecności wymagałoby zastosowania bardziej zaawansowanych technik analitycznych, takich jak chromatografia cieczowa sprzężona ze spektrometrią mas (LC-MS). Ograniczenia te nie wpływają jednak istotnie na przydatność opracowanej metody do analizy profilu pigmentowego sinic, a w przypadku konieczności jednoznacznej identyfikacji wybranych składników możliwe jest zastosowanie dodatkowych technik analitycznych. Opracowana metoda ekstrakcji i analizy HPLC-DAD została następnie wykorzystana w dalszych badaniach niniejszej rozprawy doktorskiej w pracy **P5** i **M2**, w których barwniki potraktowane zostały jako ważny marker stresu oksydacyjnego.

4.4. Wpływ zasolenia na toksyczność NLPZ [P1]

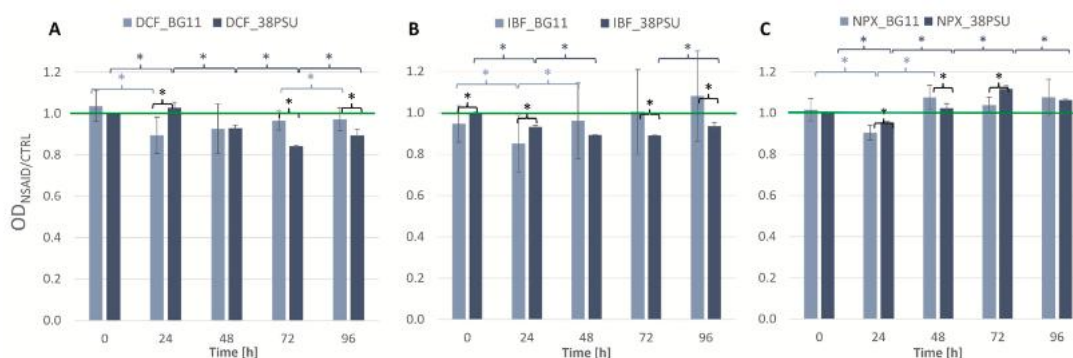
Zasolenie jest jednym z czynników fizykochemicznych, które mogą modyfikować toksyczność zanieczyszczeń obecnych w środowisku wodnym. Ma to szczególne znaczenie w przypadku substancji farmaceutycznych, ponieważ ich oddziaływanie na organizmy wodne mogą zależeć nie tylko od stężenia, ale również od właściwości fizykochemicznych środowiska, które może modyfikować biodostępność. Dotychczasowe badania wskazywały, że wzrost zasolenia może zmieniać toksyczność związków farmaceutycznych, m.in. prowadząc do obniżenia toksyczności sulfonamidów względem *Chlorella vulgaris* oraz toksyczności cieczy jonowych względem wybranych glonów i sinic (Borecka et al., 2016; Latała et al., 2010). Zależność ta może być związana zarówno ze zmianami biodostępności zanieczyszczeń, jak i z odpowiedzią fizjologiczną organizmu na stres osmotyczny. W związku z tym przeprowadzono badania oceny toksyczności DCF, NPX i IBF względem *S. salina* w warunkach standardowego zasolenia BG11 (3 PSU) oraz podwyższonego zasolenia (38 PSU).

Przed rozpoczęciem właściwych badań przeprowadzono testy wstępne oceniające wpływ DCF, NPX i IBF na wzrost *S. salina* w standardowym podłożu BG11. W zakresie stężeń 0,1–5 mg/L nie obserwowano istotnego wpływu badanych NLPZ na wzrost sinic. Właściwe doświadczenia przeprowadzono następnie przy stężeniu 100 mg/L, przy którym również nie stwierdzono wyraźnego zahamowania wzrostu *S. salina*.

W dalszej części oceniono zatem, czy podwyższone zasolenie może modyfikować odpowiedź *S. salina* na badane NLPZ. Ze względu na zdolność tej sinicy do dostosowania się do różnych warunków osmotycznych środowiska zewnętrznego, istotne było określenie również jej odpowiedzi na samo zwiększenie zasolenia. Wykazano, że samo zwiększenie zasolenia powodowało przejściowe zahamowanie wzrostu sinic. Po 24 godzinach ekspozycji wartość wskaźnika $OD_{38PSU/CTRL}$ wynosiła $0,87 \pm 0,04$, $0,87 \pm 0,04$, co wskazywało na przejściowe niewielkie zahamowanie wzrostu *S. salina* (**Rysunek 2 [P1]**). W kolejnych dniach różnice te ulegały jednak zmniejszeniu, wskazując na adaptację *S. salina* do podwyższonego zasolenia. Zaobserwowana adaptacja wynika z mechanizmów osmoregulacji opisanych dla sinic, obejmującymi m.in. aktywny transport jonów oraz akumulację kompatybilnych substancji osmotycznych, których synteza i transport wymagają dodatkowego nakładu energii (Pade and Hagemann, 2015).

Wpływ zasolenia na toksyczność badanych NLPZ okazał się niewielki. Wartości stosunku OD sinic narażonych na leki do OD kultur kontrolnych ($OD_{NSAID/CTRL}$) dla

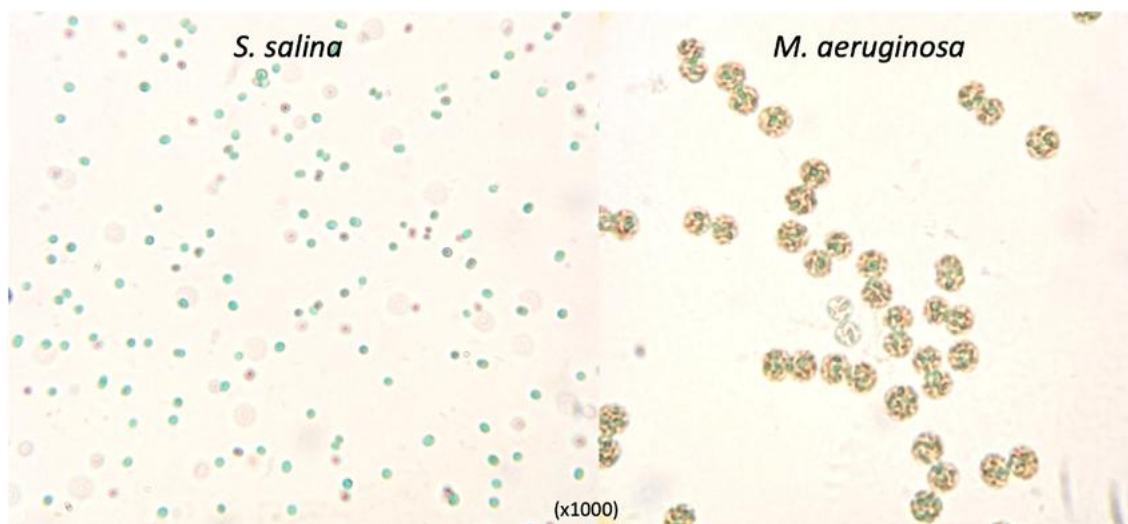
większości wariantów pozostawały zbliżone do 1, co wskazuje, że ich obecność nie powodowała wyraźnego zahamowania wzrostu *S. salina* zarówno przy zasoleniu 3, jak i 38 PSU (**Rysunek 3**). Najmniejszą zależność od zasolenia obserwowano w przypadku NPX, którego obecność praktycznie nie wpływała negatywnie na wzrost sinic niezależnie od warunków zasolenia. W przypadku DCF i IBF w 38 PSU obserwowano natomiast niewielkie wartości obniżenie tego wskaźnika w stosunku do kontroli, pojawiające się od 48 godziny ekspozycji i utrzymujące się do końca doświadczenia. Po 96 godzinach wartości OD_{NSAID/CTRL} były około 10% niższe względem odpowiednich kontroli. Efekt ten należy jednak uznać za niewielki. Dodatkowo, nie obserwowano jednokierunkowej zależności pomiędzy wzrostem zasolenia a toksycznością NLPZ, w poszczególnych punktach czasowych wartości wskaźnika były zarówno wyższe, jak i niższe w 38 PSU niż w BG11. Wyniki wskazują zatem, że podwyższone zasolenie nie stanowiło istotnego czynnika nasilającego toksyczność badanych NLPZ względem *S. salina*, a obserwowane niewielkie zmiany w odpowiedzi na DCF i IBF mogą być związane przede wszystkim z nakładaniem się efektu ekspozycji na farmaceutyki i fizjologicznej odpowiedzi sinic na stres osmotyczny. Jednym z możliwych wyjaśnień niewielkiego wpływu NLPZ na wzrost *S. salina* może być ujemny potencjał zeta powierzchni komórek. Jest on bowiem ujemny ($-43,1 \pm 2,4$ mV; dane literaturowe podane w publikacji [P1]), podobnie jak ładunek cząsteczek badanych NLPZ w warunkach doświadczenia, co może prowadzić do ich wzajemnego odpychania i ograniczać zbliżanie się cząsteczek do powierzchni komórek. W przypadku wielu związków organicznych nie jest znany mechanizm ich przenikania przez błony sinic (Stebegg et al., 2019). Wiadomo natomiast, że sinice posiadają szybki w odpowiedzi mechanizm usuwania jonów, który wymaga jednak nakładów energii (Mishra, Arun Kumar, D. N. Tiwari, and Amar N. Rai, 2020), co w konsekwencji prowadzić może, jak zauważono w niniejszej pracy, do zmniejszenia tempa wzrostu komórek w warunkach podwyższonego zasolenia.



Rysunek 3. Wartości OD_{NSAID/CTRL} uzyskane w ciągu 96 godzin ekspozycji *S. salina* na diklofenak (DCF), ibuprofen (IBF) i naproksen (NPX) w standardowym podłożu (BG11) oraz podłożu o podwyższonym zasoleniu (38 PSU) [P1].

4.5. Wielobiomarkerowa odpowiedź sinic *S. salina* i *M. aeruginosa* na DCF [P5]

W celu kompleksowej oceny odpowiedzi sinic na działanie DCF zastosowano podejście wielobiomarkerowe, obejmujące ocenę zmian na poziomie komórkowym, biochemicznym i fizjologicznym. DCF jest zanieczyszczeniem pojawiającym się w środowisku wodnym na całym świecie, którego obecność stwierdzano między innymi w Morzu Bałtyckim, Śródziemnym, Adriatyckim i Czerwonym oraz w Oceanie Spokojnym (Ali et al., 2019; Björlerius et al., 2018; Bonnefille et al., 2018; UNESCO and HELCOM, 2017). Jego znaczenie środowiskowe zostało również uwzględnione przez organizacje zajmujące się ochroną środowiska, w tym HELCOM (Baltic Marine Environment Protection Commission), która oznaczyła go jako priorytetowe zanieczyszczenie dla Morza Bałtyckiego (HELCOM, 2020). W 2013 r. DCF został włączony do pierwszej unijnej listy obserwacyjnej (*Watch List*) w ramach Ramowej Dyrektywy Wodnej, co zapoczątkowało jego monitoring w wodach powierzchniowych Europy. Z tych względów DCF wybrano jako modelowy farmaceutyk do oceny wielobiomarkerowej odpowiedzi dwóch jednokomórkowych gatunków sinic występujących w środowisku Morza Bałtyckiego – *M. aeruginosa* i *S. salina* (**Rysunek 4**). Sinice te różnią się pod względem ekologicznym, co opisano w publikacji (Mazur-Marzec et al., 2015). Ocenie poddano liczebność komórek, skład pigmentów fotosyntetycznych i fotoprotekcyjnych, zawartość cyjanotoksyn, funkcjonowanie aparatu fotosyntetycznego oraz zmiany stężenia DCF w obecności sinic. Wykorzystano wcześniej zwalidowane metody cytometryczne oraz chromatograficzne.



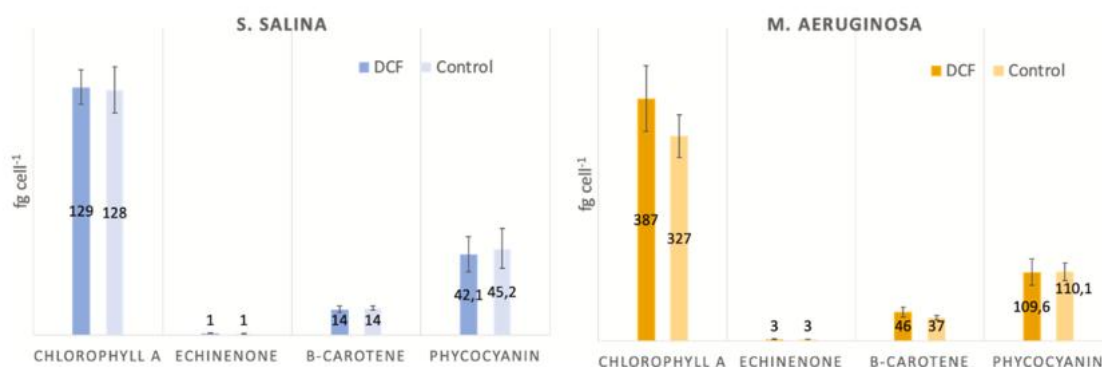
Rysunek 4. Obraz mikroskopowy (powiększenie 1000×) sinic: *Synechocystis salina* CCNP1104 (po lewej) i *Microcystis aeruginosa* CCNP1101 (po prawej) [P5]

Pomimo zastosowania wysokiego stężenia DCF (100 mg/l) i 96-godzinnej ekspozycji, nie stwierdzono istotnego wpływu leku na proliferację komórek badanych sinic. Kultury eksponowane na DCF rosły w tempie porównywalnym z kulturami kontrolnymi, a w przypadku obu gatunków nie wykazano statystycznie istotnych różnic zarówno w liczebności komórek, jak i wartościach OD (test t , $p = 0,05$; **Rysunek 4** [P5]). Odmienną odpowiedź *M. aeruginosa* obserwowano w badaniach Bácsi et al. (2016), w których ekspozycja na 100 mg/l DCF przez 10 godzin prowadziła do zahamowania tempa wzrostu (Bácsi et al., 2016). Sinice wykazywały większą odporność na DCF w porównaniu z innymi gatunkami fitoplanktonu (**Tabela 1** [P5]). Wysokie wartości EC_{50} wyznaczone dla NLPZ, a także LOEC i NOEC są nierealistyczne z punktu widzenia rzeczywistych warunków środowiskowych, niemniej jednak takie oceny mogą pomóc w określeniu mechanizmów odpowiedzialnych za zmiany w badanych organizmach.

Analiza pigmentów fotosyntetycznych

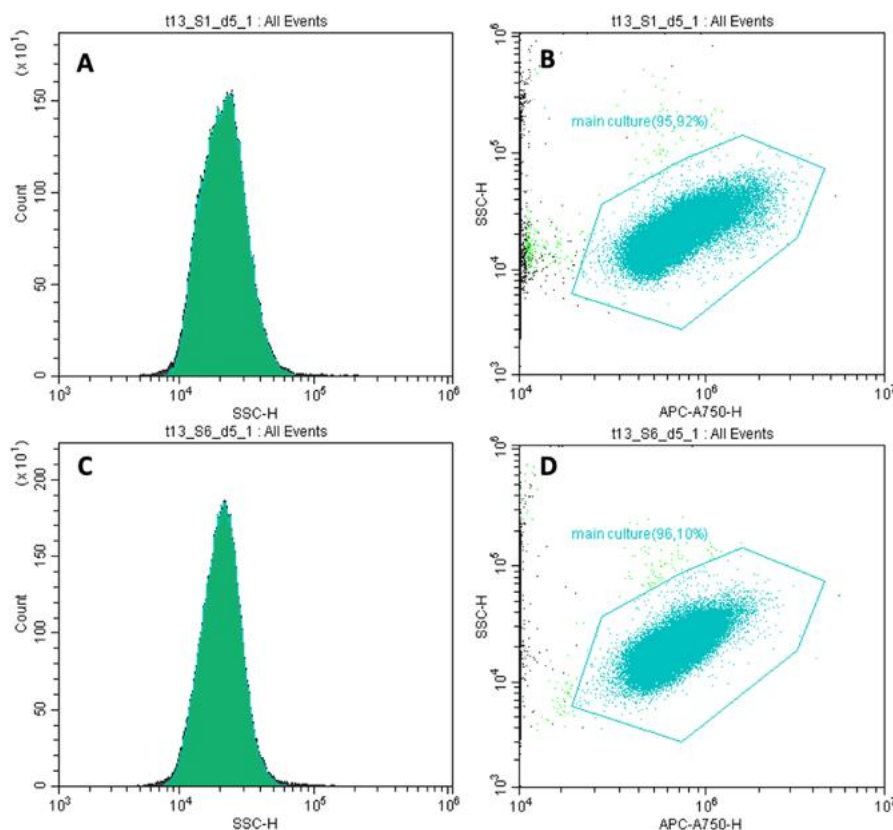
Analiza zawartości pigmentów fotosyntetycznych i fotoprotekcyjnych (w przeliczeniu na komórkę) nie wykazała istotnych zmian pod wpływem DCF. Po 96 godzinach ekspozycji oznaczono zawartość Chl *a*, feofityny, wybranych karotenoidów oraz fikobilin, a uzyskane wartości wyrażono w przeliczeniu na pojedynczą komórkę. Widma UV-Vis ekstraktów pigmentów obu badanych gatunków charakteryzowały się podobnym przebiegiem, wskazując na zbliżony ogólny skład analizowanych barwników (**Rysunek 6** [P5]). W przypadku ekstraktów fikobilin obserwowano dominujące

maksimum absorbancji przy 621-623 nm, odpowiadające fikocyjaninie. Ilościowa analiza zawartości pigmentów wykazała różnice pomiędzy *S. salina* i *M. aeruginosa*, dotyczące zarówno Chl *a*, karotenoidów, jak i fikobilin (**Rysunek 5**). Różnice te były związane przede wszystkim z odmienną fizjologią badanych sinic, ponieważ komórki *S. salina* były mniejsze, a ich liczebność była około pięciokrotnie większa niż w przypadku *M. aeruginosa* przy tej samej gęstości optycznej. Pomimo tych różnic wartości uzyskane dla obu gatunków wykazywały podobny ogólny profil pigmentowy, co pozostawało zgodne z podobieństwem ich widm UV-Vis (**Rysunek 6 [P5]**). Należy przy tym zaznaczyć, że oznaczenie fikocyjaniny miało charakter półilościowy, ponieważ zastosowany wzorzec nie był całkowicie czysty. Z tego względu wartości bezwzględne zawartości tego pigmentu należy interpretować z ostrożnością, natomiast porównanie kultur kontrolnych i eksponowanych na DCF pozostaje uzasadnione. Nie stwierdzono istotnych statystycznie różnic pomiędzy kulturami kontrolnymi i eksponowanymi na DCF w przypadku żadnego z analizowanych pigmentów (**Rysunek 5**). Dotyczyło to zarówno Chl *a* i fikobilin, związanych z funkcjonowaniem aparatu fotosyntetycznego, jak również analizowanych karotenoidów, które dodatkowo pełnią zarówno funkcje fotoprotekcyjne.



Rysunek 5. Stężenia [fg/komórka] wybranych pigmentów w ekstraktach sinic *S. salina* i *M. aeruginosa* w kulturach kontrolnych oraz kulturach eksponowanych na diklofenak (DCF) po 96 godzinach (stężenie fikocyjaniny oznaczono półilościowo) [P5].

Analiza cytometryczna wykazała, że ekspozycja na DCF nie powodowała istotnych zmian median fluorescencji Chl *a* i fikocyjaniny w komórkach obu badanych gatunków (**Rysunek 6**).



Rysunek 6. Histogramy i wykresy punktowe z analizy metodą cytometrii przepływowej dla hodowli *S. salina* ekspozowanych na diklofenak (A i B) oraz hodowli kontrolnych (C i D) po 96-godzinnej ekspozycji [P5].

Wydajność układu fotosyntetycznego

Brak zmian zawartości pigmentów nie oznaczał jednak braku odpowiedzi aparatu fotosyntetycznego. Bardziej szczegółowa ocena funkcjonowania aparatu fotosyntetycznego ujawniła wyraźne zmiany już po 1 godzinie ekspozycji u *M. aeruginosa* stwierdzono istotne zaburzenie funkcjonowania fotosystemu II (PSII), przejawiające się znacznym zmniejszeniem udziału aktywnych centrów reakcji PSII, przy jednoczesnym zachowaniu wysokiej wydajności funkcjonowania pozostałych aktywnych centrów. Taki układ może wskazywać na przekształcenie części centrów reakcji PSII w tzw. „heat sinks”, umożliwiające rozpraszanie energii wzbudzenia w postaci ciepła i ograniczenie jej nadmiaru w warunkach stresowych (Tsimilli-Michael and Strasser, 2008). Podobny mechanizm opisywano wcześniej u glonów zielonych (Harshkova et al., 2021; Majewska et al., 2018). *S. salina* wykazywała natomiast niewielkie zmiany w funkcjonowaniu PSII na początku ekspozycji, przy jednoczesnym zahamowaniu transportu elektronów poza PSII w kierunku końcowych akceptorów PSI. Po 96 godzinach ekspozycji u obu gatunków obserwowano poprawę parametrów

fotosyntetycznych, wskazującą na aklimatyzację aparatu fotosyntetycznego do warunków stresowych. U *M. aeruginosa* obejmowała ona przywrócenie optymalnych parametrów PSII i zwiększenie transportu elektronów, natomiast u *S. salina* obserwowano wzrost wydajności wykorzystania energii w PSII i PSI, który w przypadku części parametrów przekraczał poziom kontrolny, przy jednoczesnym zmniejszeniu niefotochemicznego wygaszania energii. Wyniki te wskazują zatem, że pomimo braku efektu na poziomie wzrostu, DCF wywoływał dynamiczną odpowiedź aparatu fotosyntetycznego, obejmującą zarówno początkowe zaburzenia procesów fotochemicznych, jak i późniejsze mechanizmy aklimatyzacyjne.

Cyjanotoksyny

Odpowiedź na DCF obserwowano również na poziomie metabolizmu - produkcji cyjanotoksyn. W ekstraktach *M. aeruginosa*, zarówno w kontrolach biotycznych, jak i w hodowlach eksponowanych na DCF, wykryto dziewięć wariantów mikrocystyn (MCs, ang. *Microcystins*) (**Tabela 2 [P5]**). Ekspozycja na DCF prowadziła do zwiększenia zawartości wszystkich oznaczonych wariantów MCs w komórkach w porównaniu z kontrolą, przy czym wzrost ten mieścił się w zakresie od 9% dla MC-LF do 46% dla MC-LW (warianty strukturalne mikrocystyn). Obserwowany wzrost zawartości MCs może wskazywać na ich udział w adaptacji *M. aeruginosa* do stresu wywołanego obecnością DCF, co jest zgodne z doniesieniami sugerującymi ich potencjalną rolę w ochronie komórek przed stresem oksydacyjnym (Jacinavicius et al., 2019). Wzrost produkcji MCs pod wpływem DCF nie był dotychczas opisywany, natomiast zmianę w ich wydzielaniu przez *M. aeruginosa* obserwowano po ekspozycji na antybiotyki, m.in. spiramycynę i amoksycylinę (Liu et al., 2012). Obserwowany wzrost zawartości MCs mógł zatem stanowić element odpowiedzi adaptacyjnej *M. aeruginosa* na stres wywołany obecnością DCF, jednak nie można jednoznacznie stwierdzić, czy był on bezpośrednim efektem działania DCF, czy też następstwem zmian fizjologicznych wywołanych ekspozycją.

Stabilność DCF

Pomimo obserwowanych zmian fizjologicznych i biochemicznych, stężenie DCF podczas czterodniowej ekspozycji spadło w niewielkim stopniu i nie różniło się istotnie pomiędzy układami biotycznymi i abiotycznymi. Wyniki oznaczeń stężenia DCF przedstawiono na **Rysunku 2** w publikacji **P5**. W próbkach pobranych w ostatnim dniu ekspozycji zaobserwowano dodatkowe sygnały na chromatogramach HPLC-DAD,

dlatego przeprowadzono celowaną analizę LC-MS wybranych metabolitów. Analiza ta wykazała, że dodatkowe sygnały nie odpowiadały metabolitowi 4-OH-DCF (najczęstszy hydroksylowany metabolit pierwszego etapu detoksykacji DCF), co sugerował zbliżony czas retencji tego metabolitu w analizie HPLC-DAD (**Rozdział S2 – materiał uzupełniający [P5]**). Brak wyraźnego ubytku DCF może wskazywać na jego ograniczone przenikanie do komórek sinic, a jednym z możliwych wyjaśnień jest odpychanie anionu DCF przez ujemnie naładowaną powierzchnię komórek sinic (Martinez et al., 2008; Mkaddem et al., 2022). DCF jest słabym kwasem, a jego właściwości lipofilowe zależą od pH, przy pH 8,5 wartość współczynnika dystrybucji ($\log D$) wynosi 0,75, podczas gdy przy pH 9 obliczona wartość wynosi -0,3. Ponieważ w kulturze pH było bliskie 9, zmniejszenie lipofilowości DCF mogło dodatkowo ograniczać jego zdolność do przenikania przez błony komórkowe. Dodatkowym czynnikiem może być obecność zewnętrznej bariery lipopolisacharydowej sinic, która może ograniczać dostęp związków chemicznych do wnętrza komórek (De Almeida Gonçalves and Figueredo, 2020; Lambert, 2002). Biorąc pod uwagę, że jedynie śladowe ilości DCF mogły przenikać do wnętrza komórek, a odpowiedź aparatu fotosyntetycznego na obecność farmaceutyku pojawiała się już na wczesnym etapie ekspozycji, nie można wykluczyć, że kaskada sygnałowa prowadząca do zmian w funkcjonowaniu komórek rozpoczynała się na poziomie błony komórkowej. Ponadto, możliwe, że już na początku ekspozycji komórki uruchomiły aktywne usuwanie DCF.

Wszystkie uzyskane wyniki wskazują, że brak wpływu DCF na wzrost sinic nie oznacza braku jego oddziaływania biologicznego. Badane sinice wykazywały większą odporność na DCF niż inne analizowane wcześniej grupy fitoplanktonu. Jednocześnie ekspozycja na ten związek prowadziła do zwiększenia zawartości mikrocystyn. Taka kombinacja cech może sprzyjać utrzymaniu lub zwiększeniu udziału sinic w społecznościach fitoplanktonowych poddanych presji farmaceutyków, podczas gdy toksyczność DCF wobec innych organizmów może dodatkowo modyfikować strukturę ekosystemu. W konsekwencji obecność DCF w środowisku może potencjalnie sprzyjać nasilaniu szkodliwych zakwitów sinic. Tym samym obecność farmaceutyków w środowisku może nie tylko bezpośrednio oddziaływać na poszczególne organizmy, ale również pośrednio modyfikować funkcjonowanie ekosystemów wodnych poprzez zmianę przewagi konkurencyjnej poszczególnych grup producentów oraz ich potencjalnych oddziaływań troficznych. Tego typu hipotezę należy rozstrzygnąć w przyszłych badaniach.

Podobną potrzebę kompleksowej oceny odpowiedzi sinic na działanie NLPZ wskazali Sha'aba et al. (2023), którzy wykazali, że IBF oddziaływał na *M. aeruginosa* nie tylko poprzez zmiany wzrostu, ale również poprzez modyfikację zawartości chlorofilu, odpowiedzi antyoksydacyjnej, składu biochemicznego komórek oraz produkcji i uwalniania cyjanotoksyn (Sha'aba et al., 2022). Co istotne, odpowiedź zależała od szczepu, a sinice wykazywały większą odporność na IBF niż badane zieleńce. Wyniki te wskazują, że ocena toksyczności NLPZ oparta wyłącznie na parametrach wzrostu może nie odzwierciedlać pełnego zakresu odpowiedzi fizjologicznej i biochemicznej sinic. Podobne zależności obserwowano również dla innych grup farmaceutyków, szczególnie antybiotyków. W przypadku fluorochinolonów, β -laktamów i makrolidów opisywano zarówno stymulację wzrostu przy niskich stężeniach, jak i zmiany procesów fotosyntetycznych, odpowiedzi antyoksydacyjnej czy produkcji cyjanotoksyn, nie zawsze odpowiadające zmianom wzrostu. Zagadnienia te, obejmujące różne grupy farmaceutyków i wielopoziomową odpowiedź sinic, zostały szerzej przeanalizowane w pracy P6.

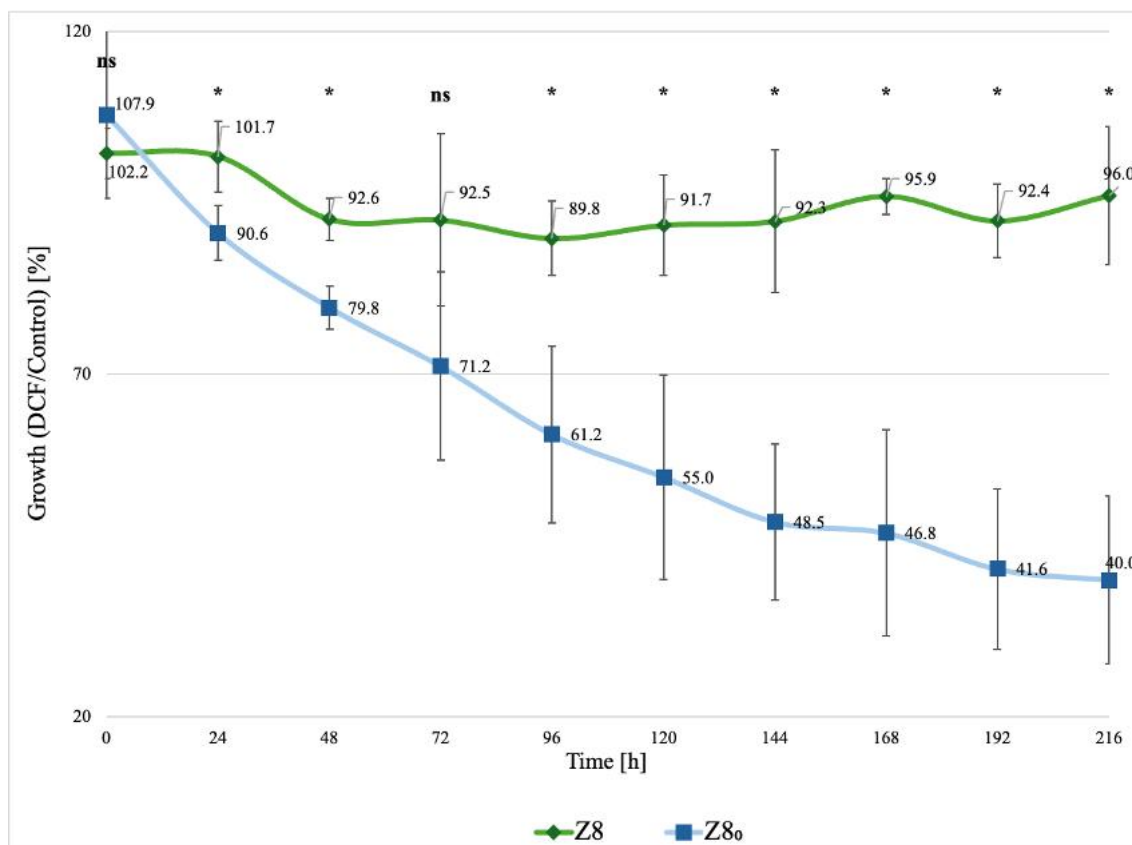
4.6. Dostępność azotu a toksyczność diklofenaku wobec diazotroficznej sinicy *A. cylindrica* [M2]

Obecność biodostępnego azotu jest jednym z kluczowych czynników determinujących wzrost i metabolizm sinic. W warunkach ograniczonej dostępności przyswajalnych form azotu sinice diazotroficzne, takie jak *A. cylindrica*, mogą wykorzystywać azot atmosferyczny dzięki procesowi wiązania azotu cząsteczkowego N₂. Proces ten wymaga jednak znacznych nakładów energetycznych oraz reorganizacji metabolizmu komórkowego, w tym różnicowania heterocyst i zmian w funkcjonowaniu aparatu fotosyntetycznego (Flores and Herrero, 2005; Herrero et al., 2001; Kumar et al., 2010). Z tego względu stan fizjologiczny komórek rosnących przy ograniczonej dostępności biologicznie przyswajalnych form azotu może różnić się od stanu komórek utrzymywanych w warunkach pełnej dostępności azotanów, co potencjalnie może wpływać również na ich odpowiedź na obecność zanieczyszczeń chemicznych. W związku z tym w badaniu oceniono odpowiedź *A. cylindrica* na DCF w dwóch odmiennych warunkach: w standardowej pożywce Z8 zawierającej azotany oraz w pożywce Z8₀ przygotowanej bez azotanów. Sinice ekspozowano na DCF w nominalnym stężeniu 100 mg/l przez 216 h. Oceniono wpływ ekspozycji na wzrost organizmów, zmiany pH, stabilność DCF w układzie eksperymentalnym, profil

barwników fotosyntetycznych oraz funkcjonowanie aparatu fotosyntetycznego na podstawie parametrów fluorescencji Chl *a*.

Kultury *A. cylindrica* inokulowane do podłoży Z8 i Z8₀ (kultury kontrolne) charakteryzowały się odmienną kinetyką wzrostu, co odzwierciedlają wartości OD₆₈₀ wyznaczone każdego dnia testów, przedstawione na **Rysunku S2** w materiale uzupełniającym publikacji **M2**. Faza intensywnego wzrostu sinic w podłożu Z8 trwała 96-144 h, a przyrost biomasy w tym okresie był znacznie większy niż w podłożu Z8₀, w którym faza intensywnego wzrostu trwała około 168 h, a wzrost był mniej intensywny. Średnie tempo wzrostu, obliczone zgodnie z wytycznymi OECD 201 dla całego okresu testu (216 h), wynosiło odpowiednio 0,22 i 0,18 na dobę dla kultur prowadzonych w Z8 i Z8₀.

Wpływ DCF na wzrost *A. cylindrica* był wyraźnie zależny od dostępności azotanów w podłożu. W standardowym podłożu Z8 nie zaobserwowano statystycznie istotnego wpływu DCF na przyrost biomasy *A. cylindrica*. Średnie tempo wzrostu sinic narażonych na DCF i kultur kontrolnych wynosiło odpowiednio 0,22 i 0,23 na dobę, a zahamowanie wzrostu w trakcie ekspozycji wynosiło jedynie 3,24%. Natomiast w warunkach ograniczonej dostępności azotanów *A. cylindrica* wykazywała znacznie większą wrażliwość na DCF. W podłożu Z8₀ średnie tempo wzrostu kultur eksponowanych na DCF było zdecydowanie mniejsze niż w kulturach kontrolnych i wynosiło odpowiednio 0,06 oraz 0,18 na dobę (**Rysunek 2 [M2]**). Zaobserwowano wyraźne zahamowanie wzrostu na poziomie 64,3%. Zależność tę potwierdzał również przebieg względnego wzrostu kultur przedstawiony na **Rysunku 7**. W Z8 stosunek OD₆₈₀ sinic narażonych na DCF do OD₆₈₀ kultur kontrolnych przez cały okres testu pozostawał zbliżony do 100%, natomiast w Z8₀ stosunek ten stopniowo się zmniejszał, aż do około 40% w ostatnim dniu testu. Uzyskane wyniki wskazują, że dostępność azotanów ma znaczący wpływ na toksyczność DCF wobec *A. cylindrica*. Należy jednak podkreślić, że zależność ta nie może zostać przypisana wyłącznie aktywacji procesu wiązania N₂, ponieważ utrzymywanie kultur w Z8₀ może wywoływać szereg różnych zmian w komórkach sinic, w związku z czym obserwowane zmiany mogą być wynikiem nakładania się stresu związanego z ograniczoną dostępnością przyswajalnych form azotu i ekspozycją na DCF.



Rysunek 7. Wzrost względny (stosunek DCF/kontrola [%]) kultur *A. cylindrica* eksponowanych na nominalne stężenie DCF 100 mg/l w standardowej pożywce Z8 oraz Z8_o. Dane przedstawiono jako średnią ± SD (n = 3, niezależne doświadczenia). Gwiazdki umieszczone nad poszczególnymi punktami czasowymi wskazują na statystycznie istotne różnice pomiędzy wariantami Z8 i Z8_o (*p < 0,05, test t-Studenta) [M2].

Zmiany pH

Różnicom we wzroście towarzyszyły wyraźne zmiany pH środowiska. Po 216 godzinach w kulturach prowadzonych w Z8 pH osiągnęło 9,68 w wariancie z DCF oraz 9,40 w kontroli, podczas gdy w kontroli abiotycznej wynosiło 7,66. W Z8_o pH końcowe było znacznie niższe i wynosiło odpowiednio 7,18 w obecności DCF oraz 6,12 w kontroli biologicznej (**Rysunek 4 [M2]**). Alkalinizacja środowiska podczas intensywnego wzrostu sinic jest związana m.in. z wykorzystaniem nieorganicznych form węgla oraz pobieraniem azotanów, któremu towarzyszą zmiany równowagi kwasowo-zasadowej środowiska (Brewer and Goldman, 1976; Nyholm and Källqvist, 1989). Brak azotanów w Z8_o ogranicza udział tego mechanizmu, a dodatkowe procesy metaboliczne zachodzące podczas wzrostu diazotroficznego mogą sprzyjać zakwaszeniu środowiska. Szczególnie istotna jest różnica pomiędzy kontrolą Z8_o a kulturą eksponowaną na DCF. Znacznie mniejszy spadek pH w obecności DCF jest zgodny z obserwowanym zahamowaniem

wzrostu i może wskazywać na ograniczenie aktywności metabolicznej kultur eksponowanych na farmaceutyk.

Stabilność DCF

Stężenie DCF stopniowo zmniejszało się podczas doświadczenia zarówno w kulturach sinic, jak i kontrolach abiotycznych. Po 216 godzinach jego stężenie zmniejszyło się o około 23% w Z8 i 24% w Z8₀, podczas gdy w kontrolach abiotycznych spadek wyniósł odpowiednio 12 i 13% (**Rysunek 5 [M2]**). Na chromatogramach HPLC-DAD zaobserwowano również dodatkowy sygnał o czasie retencji zbliżonym do czasu retencji produktu przemiany DCF – 4-OH-DCF (**Rysunek 6 [M2]**), który pojawił się wyłącznie w kulturach *A. cylindrica* eksponowanych na DCF.

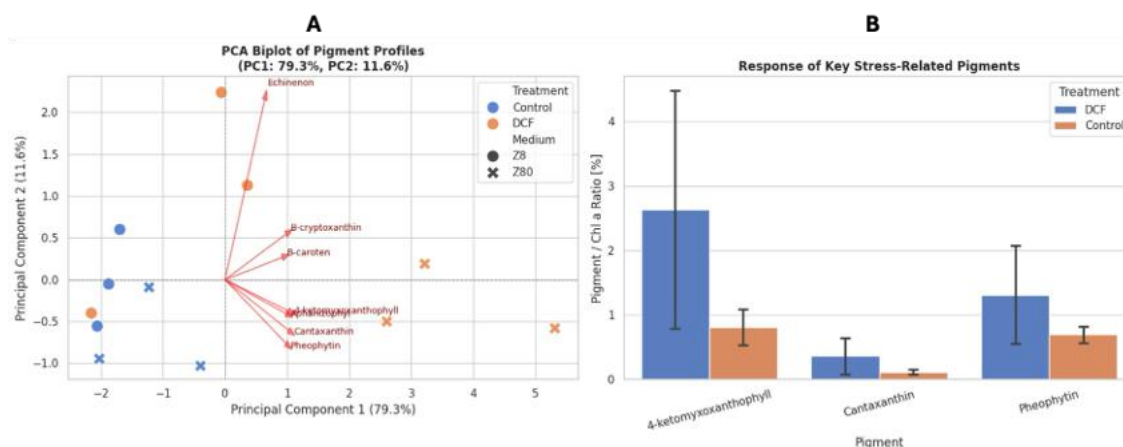
Dodatkowo przeprowadzono analizę lotnych związków metodą HS-GC-MS, w celu wykrycia powstawania ewentualnych produktów transformacji DCF. W analizowanych próbkach wykryto 2,6-DCA, będącą produktem rozpadu DCF. Związek ten zidentyfikowano zarówno w próbkach zawierających *A. cylindrica* i DCF, jak i w kontroli abiotycznej z DCF, natomiast nie stwierdzono jego obecności w kontroli biologicznej (**Rysunek 7 [M2]**). Obecność 2,6-DCA również w układzie abiotycznym wskazuje, że jej powstawanie nie wymagało obecności biomasy sinic i może być związane z fototransformacją DCF, opisywaną wcześniej w literaturze (Gackowska et al., 2025; Iovino et al., 2017). 2,6-DCA była także identyfikowana jako produkt transformacji DCF w innych procesach oksydacyjnych i degradacyjnych (Dadban Shahamat et al., 2019; Myint Sein et al., 2008; Najafinejad et al., 2026; Ziylan et al., 2014). W niniejszym badaniu po raz pierwszy wykazano jej obecność w układzie eksperymentalnym stosowanym do oceny toksyczności DCF wobec fototrofów. Ze względu na brak ilościowego oznaczenia 2,6-DCA nie można jednak określić jej udziału w obserwowanym efekcie toksycznym DCF.

Karotenoidy i chlorofile

Profil barwników fotosyntetycznych *A. cylindrica* różnił się w zależności od dostępności azotanów w pożywce. W warunkach Z8₀, w porównaniu z Z8, zawartość prawie wszystkich analizowanych barwników względem Chl *a* była większa. Wśród karotenoidów największy względny wzrost dotyczył 4-ketomyksozantofilu (69%) i kantaksantyny (64%), mniejsze zmiany obserwowano dla aphanizofilu/myksoksantofilu (30%), β-kryptoksantyny (27%) i β-karotenu (17%), natomiast zawartość echinenonu

zmniejszyła się o około 10% (**Rysunek 8 [M2]**). Zawartość pochodnej chlorofilu - feofityny wzrosła o około 39%. Wyniki te potwierdziła dwuczynnikowa analiza ANOVA, według której dostępność azotu istotnie wpływała na zawartość 4-ketomiksoksantofilu, kantaksantyny, feofityny, aphanizofilu/myksoksantofilu, β -karotenu i β -kryptoksantyny ($q < 0,05$), podczas gdy dla echinenonu nie stwierdzono istotnego wpływu podłoża ($q = 0,4101$) (**Tabela 2 [M2]**).

Wpływ DCF na profil pigmentów fotosyntetycznych u *A. cylindrica* różnił się wyraźnie w zależności od dostępności azotanów w pożywce. Analiza PERMANOVA wykazała istotną interakcję pomiędzy podłożem, a ekspozycją na DCF ($F = 8,705$; $p = 0,004$). Zróznicowanie profili barwnikowych sinic narażonych na DCF potwierdziła również analiza PCA, w której dwie pierwsze składowe wyjaśniały łącznie 90,9% zmienności danych (PC1 – 79,3%, PC2 – 11,6%) (**Rysunek 8A**). Próbkę eksponowaną na DCF w podłożu Z8₀ wyraźnie oddzielały się od pozostałych wariantów, a na ich położenie wpływały przede wszystkim 4-ketomiksoksantofil, kantaksantyna i feofityna. Dwuczynnikowa analiza ANOVA z korektą FDR wykazała istotną interakcję pomiędzy podłożem a ekspozycją na DCF dla 4-ketomiksoksantofilu ($q = 0,0047$), kantaksantyny ($q = 0,0155$) i feofityny ($q = 0,0295$) (**Tabela 2** w publikacji **M2**). Zmiany tych barwników w odpowiedzi na DCF były większe w warunkach Z8₀ niż w Z8 (**Rysunek 8B**). Podsumowując, niedobór azotu wiązał się z przeorganizowaniem profilu barwnikowego *A. cylindrica*, obejmującym zwiększenie względnej zawartości większości analizowanych karotenoidów oraz feofityny. DCF dodatkowo modyfikował ten profil, a charakter zmian zależał od dostępności azotu. Największe różnice związane z ekspozycją na DCF obserwowano dla 4-ketomiksoksantofilu, kantaksantyny i feofityny, których udział był wyraźnie większy w warunkach ograniczonego dostępu do azotu (Z8₀).



Rysunek 8. Analiza głównych składowych (PCA) oraz odpowiedź barwnikowa sinic na działanie diklofenaku (DCF) w podłożach wzrostowych Z8 i Z80. (A) Wykres PCA przedstawiający zróżnicowanie profili barwnikowych sinic w zależności od podłoża wzrostowego (Z8 vs. Z80) oraz ekspozycji na diklofenak (kontrola vs. DCF). Czerwone wektory przedstawiają ładunki poszczególnych barwników. (B) Względne zmiany zawartości wybranych barwników związanych z odpowiedzią na stres, wyrażone jako procentowy stosunek do Chl *a* [%]. Wartości przedstawiono jako średnią $\pm$ SD ($n = 3$, niezależne doświadczenia) [M2].

Wpływ na przebieg fotosyntezy

Analiza szybkiej indukcji fluorescencji Chl *a* metodą OJIP dostarcza informacji o funkcjonowaniu PSII, obejmującym m.in. wydajność fotosyntetyczną, transport elektronów oraz funkcjonowanie strony akceptorowej PSII (Stirbet and Govindjee, 2011). W niniejszym badaniu rejestrowany sygnał fluorescencji pochodził przede wszystkim z komórek wegetatywnych *A. cylindrica*, ponieważ heterocyst zawierają nieaktywny PSII i w niewielkim stopniu przyczyniają się do emisji fluorescencji Chl *a* (Malatinszky et al., 2017; Nozue et al., 2017). Pozwoliło to ocenić wpływ DCF na funkcjonowanie aparatu fotosyntetycznego *A. cylindrica* w warunkach różnej dostępności azotu, obejmujących podłoże zawierające azotany (Z8) oraz podłoże pozbawione azotanów (Z80).

W kulturach kontrolnych maksymalna wydajność fotochemiczna PSII (F_v/F_m) była zbliżona w obu warunkach i wynosiła 0,49 w Z8 oraz 0,48 w Z80. Różnica ta nie była statystycznie istotna, co wskazuje, że brak azotanów nie powodował istotnego obniżenia F_v/F_m .

Ekspozycja na DCF również nie powodowała wyraźnych zmian tego parametru, który zmniejszył się o około 2% w Z8, a w Z80 wzrósł o około 2%. Pozostałe parametry OJIP zmieniały się w zakresie 96–105% wartości kontrolnych w Z8 oraz 91–109% w Z80 (**Rysunek 10A i 10B** [M2]). Analiza poszczególnych wariantów wskazuje, że DCF miał niewielki wpływ na funkcjonowanie aparatu fotosyntetycznego *A. cylindrica* w pożywce

Z8, jest zgodna z wynikami wcześniejszych badań przeprowadzonymi na *Chlamydomonas reinhardtii* (Majewska et al., 2018) oraz na *S. salina* i *M. aeruginosa* [P5]. Z kolei w pożywce Z8₀ zmiany były bardziej wyraźne i były związane z zwiększaniem ilości aktywnych centrów reakcji i mniejszym przepływ energii i elektronów przypadający na pojedyncze centrum. Zmiany te mogą wskazywać na redystrybucję energii wzbudzenia między większą liczbę aktywnych centrów reakcji i mniejsze obciążenie pojedynczego centrum. Odmienne odpowiedzi *A. cylindrica* na DCF w zależności od dostępności azotanów może wynikać z różnic w stanie fizjologicznym komórek i dostępności zasobów potrzebnych do funkcjonowania aparatu fotosyntetycznego.

Podsumowując, brak dostępność azotanów zwiększył znacząco wrażliwość *A. cylindrica* na DCF, powodując silniejsze zahamowanie wzrostu oraz zmiany profilu barwników fotosyntetycznych. Różnice były również widoczne w analizie fluorescencji Chl *a*, gdzie zmiany dotyczyły wybranych parametrów OJIP, podczas gdy Fv/Fm pozostawał na zbliżonym poziomie. Wyniki wskazują, że forma azotu może istotnie modyfikować odpowiedź sinic na farmaceutyki, co wskazywano również we wcześniejszych badaniach na niediazotrficznej sinicy *M. aeruginosa* (Liu et al., 2015a, 2015b).

5. Podsumowanie i wnioski

Badania przeprowadzone w ramach niniejszej rozprawy doktorskiej miały na celu ocenę odpowiedzi fizjologicznej i biochemicznej sinic na działanie wybranych farmaceutyków oraz określenie przydatności różnych biomarkerów do wykrywania ich subletalnego wpływu. Przyjęto założenie, że oddziaływanie farmaceutyków może nie zawsze prowadzić do wyraźnych zmian wzrostu, a ich wpływ może być widoczny na poziomie procesów fizjologicznych i biochemicznych zachodzących w komórkach. Z tego względu, oprócz klasycznych parametrów wzrostowych wykorzystywanych do oceny wpływu farmaceutyków na biomasę, szczególną uwagę poświęcono parametrom związanym z zawartością barwników fotosyntetycznych, funkcjonowaniem aparatu fotosyntetycznego oraz zmianami w produkcji metabolitów wtórnych. Istotnym elementem pracy było również uwzględnienie warunków środowiskowych, takich jak zasolenie i dostępność biologicznie przyswajalnych form azotu.

Najważniejsze wnioski wynikające z przeprowadzonych badań są następujące:

- Farmaceutyki wywołują zmiany fizjologiczne i biochemiczne w komórkach sinic pomimo braku wyraźnego zahamowania ich wzrostu. Ocena ich wpływu wyłącznie na podstawie parametrów wzrostowych może zatem prowadzić do niedoszacowania ich oddziaływania na biologię sinic.
- Wyniki badań wskazują na zasadność stosowania podejścia wieloparametrowego w ocenie wpływu farmaceutyków na sinice, obejmującego parametry wzrostowe, fizjologiczne i biochemiczne. Szczególne znaczenie ma monitorowanie produkcji cyjanotoksyn, ponieważ obserwowane zmiany w ich produkcji i uwalnianiu mogą wpływać na funkcjonowanie ekosystemów wodnych.
- Poszczególne biomarkery mogą dostarczać informacji o różnych etapach odpowiedzi sinic na działanie farmaceutyków. Parametry związane z funkcjonowaniem aparatu fotosyntetycznego umożliwiają wykrycie szybkiej odpowiedzi już na początku ekspozycji, natomiast zmiany składu barwników mogą odzwierciedlać procesy adaptacyjne zachodzące podczas dłuższej ekspozycji.
- Analiza BVOC ma potencjał jako narzędzie oceny stresu u sinic, co potwierdziły zmiany ich profilu w warunkach silnego oświetlenia. Pomimo braku zmian po

ekspozycji na diklofenak, ich potencjał jako biomarkerów stresu uzasadnia dalsze badania.

- Przydatność biomarkerów i metod ich oznaczania wymaga wcześniejszej weryfikacji w odniesieniu do badanych organizmów i warunków ekspozycji. W przypadku DCFH₂-DA wykazano ograniczenia jego zastosowania do oceny produkcji ROS u sinic.
- Ocena wpływu farmaceutyków na sinice powinna uwzględniać zmienne warunki środowiskowe. Ograniczona dostępność azotu istotnie zwiększała toksyczność DCF wobec sinic diazotroficzných, wskazując na znaczenie dostępności składników biogennych w ocenie ich odpowiedzi na farmaceutyki. Według naszej najlepszej wiedzy wpływ dostępności azotu na toksyczność farmaceutyków wobec sinic diazotroficzných nie był dotychczas badany. W przypadku *S. salina* nie wykazano natomiast wpływu zasolenia na toksyczność NLPZ, co może wynikać z wysokiej halotolerancji tego gatunku i nie wyklucza znaczenia tego czynnika w przypadku innych sinic.

Jednym z ważniejszych osiągnięć rozprawy było wykazanie, że brak zahamowania wzrostu sinic nie musi oznaczać braku odpowiedzi biologicznej na działanie farmaceutyku. Wyniki uzyskane dla DCF wskazały, że zmiany w zawartości wewnątrzkomórkowych cyjanotoksyn oraz innych analizowanych parametrów mogą występować pomimo braku wyraźnego wpływu na wzrost. Oznacza to, że klasyczna ocena toksyczności oparta wyłącznie na zahamowaniu wzrostu sinic może nie uwzględniać istotnych efektów wywoływanych przez farmaceutyki. Uzyskane wyniki potwierdzają zasadność stosowania podejścia wieloparametrowego, w którym obok wzrostu analizowane są również biomarkery fizjologiczne i biochemiczne.

Ważną część rozprawy stanowiły również badania dotyczące możliwości wykorzystania wybranych parametrów jako markerów odpowiedzi sinic. Dużą uwagę skupiono na procesie fotosyntezy i zmianach ilości barwników jako kluczowych i czułych markerów odpowiedzi mikroorganizmów fotosyntezujących. Z kolei badania nad lotnymi związkami organicznymi wykazały, że stres środowiskowy może prowadzić do zmian w profilu BVOC sinic. Wykryto związki należące do różnych grup chemicznych, a część z nich po raz pierwszy opisano w odniesieniu do badanych gatunków lub warunków stresowych m.in. β -cyklocytral wydzielany przez *N. spumigena*. Wyniki te pokazały, że

BVOC mogą stanowić dodatkowe źródło informacji o odpowiedzi sinic na stres. W przypadku jednak wybranego do badań DCF nie potwierdzono, że indukuje produkcję BVOC.

Jednocześnie przeprowadzone badania wskazały, że nie każdy potencjalny marker może być stosowany bez wcześniejszej weryfikacji. Dotyczyło to między innymi metod wykorzystujących DCFH₂-DA do oceny produkcji reaktywnych form tlenu. Uzyskane wyniki wykazały ograniczenia stosowania DCFH₂-DA w badaniach nad sinicami. W związku z tym nie podjęto się badań nad jego wykorzystaniem do badania wpływu leków. Co należy podkreślić, w pracy przy testowaniu wybranych biomarkerów, dużą uwagę poświęcano walidacji metody w odniesieniu do sinic i warunków doświadczenia, zamiast bezpośredniego przenoszenia procedur opracowanych dla innych organizmów.

Ważną część rozprawy stanowiły również badania dotyczące możliwości wykorzystania wybranych parametrów jako markerów odpowiedzi sinic. Dużą uwagę skupiono na procesie fotosyntezy i zmianach w zawartości barwników jako kluczowych i czułych markerów odpowiedzi mikroorganizmów fotosyntetyzujących. Z kolei badania nad lotnymi związkami organicznymi wykazały, że stres środowiskowy może prowadzić do zmian w profilu BVOC sinic. Wykryto związki należące do różnych grup chemicznych, a część z nich po raz pierwszy opisano w odniesieniu do badanych gatunków lub warunków stresowych, m.in. β-cyklocytral wydzielany przez *N. spumigena*. Wyniki te pokazały, że BVOC mogą stanowić dodatkowe źródło informacji o odpowiedzi sinic na stres. W przypadku DCF nie potwierdzono jednak jego wpływu na indukcję produkcji BVOC.

Na podstawie całego cyklu badań można stwierdzić, że postawiona w rozprawie hipoteza została potwierdzona. Wykazano, że farmaceutyki mogą wywoływać zmiany w procesach fizjologicznych i biochemicznych zachodzących w komórkach sinic, które nie zawsze znajdują odzwierciedlenie w zmianach ich wzrostu. Jednocześnie wykazano, że odpowiedź ta jest zależna nie tylko od rodzaju badanego farmaceutyku, ale również od warunków prowadzenia ekspozycji. Oznacza to, że przy ocenie wpływu farmaceutyków na sinice nie można wskazać jednego uniwersalnego biomarkera, który byłby odpowiedni w każdych warunkach. Szczególnie przydatne mogą być jednak barwniki fotosyntetyczne, umożliwiające ocenę zmian związanych z adaptacją sinic oraz funkcjonowaniem aparatu fotosyntetycznego, ze względu na szybkość odpowiedzi i dostępność stosowanych metod.

Podsumowując, wyniki uzyskane w ramach rozprawy pokazują, że ocena oddziaływania farmaceutyków na sinice powinna opierać się na połączeniu klasycznych parametrów wzrostowych, uzupełnionych o analizę odpowiedzi fizjologicznej i biochemicznej oraz uwzględniać zmienność warunków środowiskowych.

Przeprowadzone badania pozwoliły nie tylko wskazać parametry przydatne w ocenie odpowiedzi sinic, ale również określić ograniczenia wybranych metod i wykazać konieczność ich weryfikacji przed zastosowaniem w badaniach toksykologicznych. Tym samym uzyskane wyniki przyczyniają się do lepszego rozpoznania mechanizmów odpowiedzi sinic na zanieczyszczenia farmaceutyczne, wskazują na zasadność rozszerzenia analizy o biomarkery, w szczególności związane z produkcją toksyn oraz na potrzebę prowadzenia badań toksykologicznych w warunkach bardziej zbliżonych do rzeczywistego środowiska. Sinice jako organizmy referencyjne są mniej poznane niż inne składniki fitoplanktonu. Jak wykazały przedstawione wyniki, mogą one wykazywać odmienną odpowiedź na zanieczyszczenia farmaceutyczne niż fotosyntetyczne organizmy eukariotyczne, co może wynikać z ich specyficznych cech biologicznych. W związku z tym oraz w obliczu rosnącego znaczenia zakwitów sinic, w przyszłości należy rozszerzyć analizę ryzyka środowiskowego farmaceutyków o badania ich wpływu na sinice.

6. Bibliografia

- Adedipe, D.T., Chen, C., Lai, R.W.S., Xu, S., Luo, Q., Zhou, G.J., Boxall, A., Brooks, B.W., Doblin, M.A., Wang, X., Wang, J., Leung, K.M.Y., 2024. Occurrence and potential risks of pharmaceutical contamination in global Estuaries: A critical review and analysis. *Environ. Int.* <https://doi.org/10.1016/j.envint.2024.109031>
- Ali, A.M., Sydnese, L.K., Alarif, W.M., Al-Lihaibi, S.S., Aly, M.M., Aanrud, S.G., Kallenborn, R., 2019. Diclofenac and two of its photooxidation products in the marine environment: Their toxicology and occurrence in Red Sea coastal waters. *Environmental Chemistry and Ecotoxicology* 1, 19–25. <https://doi.org/10.1016/j.enceco.2019.06.002>
- Allaf, M.M., Peerhossaini, H., 2022a. Cyanobacteria: Model microorganisms and beyond. *Microorganisms* 10, 696. <https://doi.org/10.3390/microorganisms10040696>
- Allaf, M.M., Peerhossaini, H., 2022b. Cyanobacteria: Model Microorganisms and Beyond. *Microorganisms*. <https://doi.org/10.3390/microorganisms10040696>
- Arpin-Pont, L., Martínez-Bueno, M.J., Gomez, E., Fenet, H., 2016. Occurrence of PPCPs in the marine environment: a review. *Environmental Science and Pollution Research* 23, 4978–4991. <https://doi.org/10.1007/s11356-014-3617-x>
- Assunção, J., Amaro, H.M., Tavares, T., Malcata, F.X., Guedes, A.C., 2023. Effects of Temperature, pH, and NaCl Concentration on Biomass and Bioactive Compound Production by *Synechocystis salina*. 13. <https://doi.org/10.3390/life13010187>
- aus der Beek, T., Weber, F.A., Bergmann, A., Hickmann, S., Ebert, I., Hein, A., Küster, A., 2016. Pharmaceuticals in the environment-Global occurrences and perspectives. *Environ. Toxicol. Chem.* 35, 823–835. <https://doi.org/10.1002/etc.3339>
- Bácsi, I., B-Béres, V., Kókai, Z., Gonda, S., Novák, Z., Nagy, S.A., Vasas, G., 2016. Effects of non-steroidal anti-inflammatory drugs on cyanobacteria and algae in laboratory strains and in natural algal assemblages. *Environmental Pollution* 212, 508–518. <https://doi.org/10.1016/j.envpol.2016.02.031>
- Björlenius, B., Ripszám, M., Haglund, P., Lindberg, R.H., Tysklind, M., Fick, J., 2018. Pharmaceutical residues are widespread in Baltic Sea coastal and offshore waters – screening for pharmaceuticals and modelling of environmental concentrations of carbamazepine. *Science of the Total Environment* 633, 1496–1509. <https://doi.org/10.1016/j.scitotenv.2018.03.276>
- Bonnefille, B., Gomez, E., Courant, F., Escande, A., Fenet, H., 2018. Diclofenac in the marine environment: A review of its occurrence and effects. *Mar. Pollut. Bull.* 131, 496–506. <https://doi.org/10.1016/j.marpolbul.2018.04.053>

- Borecka, M., Białk-Bielińska, A., Haliński, Ł.P., Pazdro, K., Stepnowski, P., Stolte, S., 2016. The influence of salinity on the toxicity of selected sulfonamides and trimethoprim towards the green algae *Chlorella vulgaris*. *J. Hazard. Mater.* 308, 179–186. <https://doi.org/10.1016/j.jhazmat.2016.01.041>
- Caban, M., Stepnowski, P., 2021. How to decrease pharmaceuticals in the environment? A review. *Environ. Chem. Lett.* 19, 3115–3138. <https://doi.org/10.1007/s10311-021-01194-y>
- Catherine, Q., Susanna, W., Isidora, E.S., Mark, H., Aurélie, V., Jean-François, H., 2013. A review of current knowledge on toxic benthic freshwater cyanobacteria - Ecology, toxin production and risk management. *Water Res.* 47, 5464–5479. <https://doi.org/10.1016/j.watres.2013.06.042>
- Chaturvedi, P., Shukla, P., Giri, B.S., Chowdhary, P., Chandra, R., Gupta, P., Pandey, A., 2021. Prevalence and hazardous impact of pharmaceutical and personal care products and antibiotics in environment: A review on emerging contaminants. *Environ. Res.* <https://doi.org/10.1016/j.envres.2020.110664>
- Dadban Shahamat, Y., Zazouli, M.A., Zare, M.R., Mengelizadeh, N., 2019. Catalytic degradation of diclofenac from aqueous solutions using peroxymonosulfate activated by magnetic MWCNTs-CoFe₃O₄ nanoparticles. *RSC Adv.* 9, 16496–16508. <https://doi.org/10.1039/c9ra02757b>
- Das, R., Raj, D., 2025. Sources, distribution, and impacts of emerging contaminants – a critical review on contamination of landfill leachate. *Journal of Hazardous Materials Advances* 17. <https://doi.org/10.1016/j.hazadv.2025.100602>
- De Almeida Gonçalves, C., Figueredo, C.C., 2020. What we really know about the composition and function of microalgae cell coverings? - An overview. *Acta Bot. Brasilica* 34, 599–614. <https://doi.org/10.1590/0102-33062020abb0309>
- Eapen, J.V., Thomas, S., Antony, S., George, P., Antony, J., 2024. A review of the effects of pharmaceutical pollutants on humans and aquatic ecosystem. *Exploration of Drug Science* 484–507. <https://doi.org/10.37349/eds.2024.00058>
- Flores, E., Herrero, A., 2005. Nitrogen assimilation and nitrogen control in cyanobacteria. *Biochem. Soc. Trans.* 33, 164–167. <https://doi.org/10.1042/BST0330164>
- Gackowska, A., Studziński, W., Kudlek, E., Przybyłek, M., 2025. Environmental fate and ecotoxicity of diclofenac degradation products generated by photo-assisted advanced oxidation processes. *J. Hazard. Mater.* 489, 137708. <https://doi.org/10.1016/j.jhazmat.2025.137708>
- Harshkova, D., Liakh, I., Bialevich, V., Ondrejmišková, K., Aksmann, A., Bišová, K., 2021. Diclofenac alters the cell cycle progression of the green alga *Chlamydomonas reinhardtii*. *Cells* 10, 1936. <https://doi.org/10.3390/cells10081936>

- Havaux, M., 2020. Review β -Cyclocitral and derivatives: Emerging molecular signals serving multiple biological functions.
- Hejna, M., Kapuścińska, D., Aksmann, A., 2022. Pharmaceuticals in the Aquatic Environment: A Review on Eco-Toxicology and the Remediation Potential of Algae. *Int. J. Environ. Res. Public Health*. <https://doi.org/10.3390/ijerph19137717>
- HELCOM, 2020. Diclofenac in the Baltic Sea.
- Herrero, A., Muro-Pastor, A.M., Flores, E., 2001. Nitrogen control in cyanobacteria. *J. Bacteriol.* 183, 411–425. <https://doi.org/10.1128/JB.183.2.411-425.2001>
- Hinners, J., Hofmeister, R., Hense, I., 2015. Modeling the role of pH on baltic sea cyanobacteria. . 5, 1204–1217. <https://doi.org/10.3390/life5021204>
- Hoiczuk, E., Hansel, A., 2000. Cyanobacterial cell walls: News from an unusual prokaryotic envelope. *J. Bacteriol.* 182, 1191–1199. <https://doi.org/10.1128/JB.182.5.1191-1199.2000>
- Huang, H., Xu, X., Shi, C., Liu, X., Wang, G., 2018. Response of Taste and Odor Compounds to Elevated Cyanobacteria Biomass and Temperature. *Bull. Environ. Contam. Toxicol.* 101, 272–278. <https://doi.org/10.1007/s00128-018-2386-5>
- Iovino, P., Chianese, S., Canzano, S., Prisciandaro, M., Musmarra, D., 2017. Photodegradation of diclofenac in wastewaters. *Desalination Water Treat.* 61, 293–297. <https://doi.org/10.5004/dwt.2016.11063>
- Jacinavicius, F.R., Pacheco, A.B.F., Chow, F., Verissimo da Costa, G.C., Kalume, D.E., Rigonato, J., Schmidt, E.C., Sant’Anna, C.L., 2019. Different ecophysiological and structural strategies of toxic and non-toxic *Microcystis aeruginosa* (cyanobacteria) strains assessed under culture conditions. *Algal Res.* 41, 101548. <https://doi.org/10.1016/j.algal.2019.101548>
- Kazakova, J., Villar-Navarro, M., Ramos-Payán, M., Aranda-Merino, N., Román-Hidalgo, C., Bello-López, M.Á., Fernández-Torres, R., 2021. Monitoring of pharmaceuticals in aquatic biota (*Procambarus clarkii*) of the Doñana National Park (Spain). *J. Environ. Manage.* 297. <https://doi.org/10.1016/j.jenvman.2021.113314>
- Kumar, K., Mella-Herrera, R.A., Golden, J.W., 2010. Cyanobacterial heterocysts. *Cold Spring Harb. Perspect. Biol.* 2. <https://doi.org/10.1101/cshperspect.a000315>
- Lambert, P.A., 2002. Cellular impermeability and uptake of biocides and antibiotics in Gram-positive bacteria and mycobacteria. *Journal of Applied Microbiology Symposium Supplement* 92, 35–45. <https://doi.org/10.1046/j.1365-2672.92.5s1.7.x>
- Latała, A., Nędzi, M., Stepnowski, P., 2010. Toxicity of imidazolium ionic liquids towards algae. Influence of salinity variations. *Green Chemistry* 12, 60–64. <https://doi.org/10.1039/b918355h>

- Liu, Y., Gao, B., Yue, Q., Guan, Y., Wang, Y., Huang, L., 2012. Influences of two antibiotic contaminants on the production, release and toxicity of microcystins. *Ecotoxicol. Environ. Saf.* 77, 79–87. <https://doi.org/10.1016/j.ecoenv.2011.10.027>
- Liu, Y., Wang, F., Chen, X., Zhang, J., Gao, B., 2015a. Influence of coexisting spiramycin contaminant on the harm of *Microcystis aeruginosa* at different nitrogen levels. *J. Hazard. Mater.* 285, 517–524. <https://doi.org/10.1016/j.jhazmat.2014.11.027>
- Liu, Y., Wang, F., Chen, X., Zhang, J., Gao, B., 2015b. Cellular responses and biodegradation of amoxicillin in *Microcystis aeruginosa* at different nitrogen levels. *Ecotoxicol. Environ. Saf.* 111, 138–145. <https://doi.org/10.1016/j.ecoenv.2014.10.011>
- Loreto, F., Schnitzler, J.P., 2010. Abiotic stresses and induced BVOCs. *Trends Plant Sci.* <https://doi.org/10.1016/j.tplants.2009.12.006>
- Lu, T., Zhang, Q., Zhang, Z., Hu, B., Chen, Jianmeng, Chen, Jun, Qian, H., 2021. Pollutant toxicology with respect to microalgae and cyanobacteria. *J. Environ. Sci. (China)* 99, 175–186. <https://doi.org/10.1016/j.jes.2020.06.033>
- Majewska, M., Harshkova, D., Guściora, M., Aksmann, A., 2018. Phytotoxic activity of diclofenac: Evaluation using a model green alga *Chlamydomonas reinhardtii* with atrazine as a reference substance. *Chemosphere* 209, 989–997. <https://doi.org/10.1016/j.chemosphere.2018.06.156>
- Malatinszky, D., Steuer, R., Jones, P.R., 2017. A comprehensively curated genome-scale two-cell model for the heterocystous cyanobacterium *Anabaena* sp. PCC 7120. *Plant Physiol.* 173, 509–523. <https://doi.org/10.1104/pp.16.01487>
- Martinez, R.E., Pokrovsky, O.S., Schott, J., Oelkers, E.H., 2008. Surface charge and zeta-potential of metabolically active and dead cyanobacteria. *J. Colloid Interface Sci.* 323, 317–325. <https://doi.org/10.1016/j.jcis.2008.04.041>
- Mazur-Marzec, H., Błaszczuk, A., Felczykowska, A., Hohlfeld, N., Kobos, J., Toruńska-Sitarz, A., Devi, P., Montalvão, S., D'souza, L., Tammela, P., Mikosik, A., Bloch, S., Nejman-Faleńczyk, B., Węgrzyn, G., 2015. Baltic cyanobacteria – a source of biologically active compounds. *Eur. J. Phycol.* 50, 343–360. <https://doi.org/10.1080/09670262.2015.1062563>
- Mazur-Marzec, Hanna, Pliński, Marcin, Mazur-Marzec, H, Pliński, M, 2009. Do toxic cyanobacteria blooms pose a threat to the Baltic ecosystem? *Oceanologia* 51, 293–319.
- Miazeck, K., Brozek-Pluska, B., 2019. Effect of PHRS and PCPS on microalgal growth, metabolism and microalgae-based bioremediation processes: A review. *Int. J. Mol. Sci.* 20. <https://doi.org/10.3390/ijms20102492>
- Mishra, Arun Kumar, D. N. Tiwari, and Amar N. Rai, (Eds.), 2020. *Cyanobacteria: From basic science to applications*. Academic Press.

- Mkaddem, H., Rosales, E., Pazos, M., Ben Amor, H., Sanromán, M.A., Mejjide, J., 2022. Anti-inflammatory drug diclofenac removal by a synthesized MgAl layered double hydroxide. *J. Mol. Liq.* 359, 119207. <https://doi.org/10.1016/j.molliq.2022.119207>
- Mohanty, B., Majedi, S.M., Pavagadhi, S., Te, S.H., Boo, C.Y., Gin, K.Y.H., Swarup, S., 2022. Effects of Light and Temperature on the Metabolic Profiling of Two Habitat-Dependent Bloom-Forming Cyanobacteria. *Metabolites* 12. <https://doi.org/10.3390/metabo12050406>
- Mondal, S., Singh, S.P., 2022. Flow Cytometry-based Measurement of Reactive Oxygen Species in Cyanobacteria. *Bio. Protoc.* 12, 1–11. <https://doi.org/10.21769/BioProtoc.4417>
- Muhetaer, G., Asaeda, T., Jayasanka, S.M.D.H., Baniya, M.B., Abeynayaka, H.D.L., Rashid, M.H., Yan, H.Y., 2020. Effects of light intensity and exposure period on the growth and stress responses of two cyanobacteria species: *Pseudanabaena galeata* and *Microcystis aeruginosa*. *Water (Switzerland)* 12. <https://doi.org/10.3390/w12020407>
- Myint Sein, M., Zedda, M., Tuerk, J., Schmidt, T.C., Golloch, A., Von Sonntag, C., 2008. Oxidation of Diclofenac with Ozone in Aqueous Solution 42, 6656–6662. <https://doi.org/https://doi.org/10.1021/es8008612>
- Najafinejad, M.S., Fenti, A., Sokhansanj, A., Falco, G., Iovino, P., Chianese, S., Musmarra, D., 2026. Diclofenac degradation through electro-oxidation using a continuous reactor equipped with meshed electrodes: removal efficiency, degradation pathways and reactor modelling. *J. Environ. Chem. Eng.* 14, 121637. <https://doi.org/10.1016/j.jece.2026.121637>
- Nozue, S., Katayama, M., Terazima, M., Kumazaki, S., 2017. Comparative study of thylakoid membranes in terminal heterocysts and vegetative cells from two cyanobacteria, *Rivularia* M-261 and *Anabaena variabilis*, by fluorescence and absorption spectral microscopy. *Biochim. Biophys. Acta Bioenerg.* 1858, 742–749. <https://doi.org/10.1016/j.bbabi.2017.05.007>
- OECD, 2026. Test Guideline No. 201 - Freshwater Alga and Cyanobacteria, Growth Inhibition Test, OECD Guidelines for the Testing of Chemicals, Section 2. OECD Publishing. <https://doi.org/10.1787/9789264069923-en>
- Pade, N., Hagemann, M., 2015. Salt acclimation of cyanobacteria and their application in biotechnology. . 5, 25–49. <https://doi.org/10.3390/life5010025>
- Patel, M., Kumar, R., Kishor, K., Mlsna, T., Pittman, C.U., Mohan, D., 2019. Pharmaceuticals of Emerging Concern in Aquatic Systems: Chemistry, Occurrence, Effects, and Removal Methods. *Chem. Rev.* 119, 3510–3673. <https://doi.org/10.1021/acs.chemrev.8b00299>

- Popin, R.V., Delbaje, E., De Abreu, V.A.C., Rigonato, J., Dörr, F.A., Pinto, E., Sivonen, K., Fiore, M.F., 2020. Genomic and metabolomic analyses of natural products in *Nodularia spumigena* isolated from a shrimp culture pond. *Toxins (Basel)*. 12. <https://doi.org/10.3390/toxins12030141>
- Rajneesh, ., Pathak, J., Chatterjee, A., Singh, S., Sinha, R., 2017. Detection of Reactive Oxygen Species (ROS) in Cyanobacteria Using the Oxidant-sensing Probe 2',7'-Dichlorodihydrofluorescein Diacetate (DCFH-DA). *Bio. Protoc.* 7, 1–8. <https://doi.org/10.21769/bioprotoc.2545>
- Sauvé, S., Desrosiers, M., 2014. A review of what is an emerging contaminant, *Chemistry Central Journal*.
- Sha'aba, R.I., Chia, M.A., Gana, Y.A., Alhassan, A.B., Gadzama, I.M.K., 2022. The growth, biochemical composition, and antioxidant response of *Microcystis* and *Chlorella* are influenced by Ibuprofen. *Environmental Science and Pollution Research* 30, 13118–13131. <https://doi.org/10.1007/s11356-022-22837-9>
- Sharma, L., Siedlewicz, G., Pazdro, K., 2021. The toxic effects of antibiotics on freshwater and marine photosynthetic microorganisms: State of the art. *Plants* 10, 1–15. <https://doi.org/10.3390/plants10030591>
- Srivastava, A.K.R.A.N.N.B.A. (Ed.), 2013. *Stress Biology of Cyanobacteria Molecular Mechanisms to Cellular Responses*. CRC Press, Boca Raton.
- Stebegg, R., Schmetterer, G., Rompel, A., 2019. Transport of organic substances through the cytoplasmic membrane of cyanobacteria. *Phytochemistry*. <https://doi.org/10.1016/j.phytochem.2018.08.013>
- Stirbet, A., Govindjee, 2011. On the relation between the Kautsky effect (chlorophyll a fluorescence induction) and Photosystem II: Basics and applications of the OJIP fluorescence transient. *J. Photochem. Photobiol. B* 104, 236–257. <https://doi.org/10.1016/j.jphotobiol.2010.12.010>
- Świacka, K., Michnowska, A., Maculewicz, J., Caban, M., Smolarz, K., 2021. Toxic effects of NSAIDs in non-target species: A review from the perspective of the aquatic environment. *Environmental Pollution* 273, 115891. <https://doi.org/10.1016/j.envpol.2020.115891>
- Tsimilli-Michael, M., Strasser, R.J., 2008. In vivo Assessment of Stress Impact on Plant's Vitality: Applications in Detecting and Evaluating the Beneficial Role of Mycorrhization on Host Plants, in: *Mycorrhiza: State of the Art, Genetics and Molecular Biology, Eco-Function, Biotechnology, Eco-Physiology, Structure and Systematics*. Berlin, pp. 679–703.

- UNESCO and HELCOM, 2017. Pharmaceuticals in the aquatic environment of the Baltic Sea region in the aquatic environment of the Baltic Sea region - A status report. UNESCO Emerging Pollutants in Water Series – No. 1, UNESCO Publishing, Paris.
- Watson, S.B., Monis, P., Baker, P., Giglio, S., 2016. Biochemistry and genetics of taste- and odor-producing cyanobacteria. *Harmful Algae*. <https://doi.org/10.1016/j.hal.2015.11.008>
- Wojtasiewicz, B., Stoń-Egiert, J., 2016. Bio-optical characterization of selected cyanobacteria strains present in marine and freshwater ecosystems. *J. Appl. Phycol.* 28, 2299–2314. <https://doi.org/10.1007/s10811-015-0774-3>
- Zhang, D., Dechatiwongse, P., del Rio-Chanona, E.A., Maitland, G.C., Hellgardt, K., Vassiliadis, V.S., 2015. Modelling of light and temperature influences on cyanobacterial growth and biohydrogen production. *Algal Res.* 9, 263–274. <https://doi.org/10.1016/j.algal.2015.03.015>
- Zhang, M., Steinman, A.D., Xue, Q., Zhao, Y., Xu, Y., Xie, L., 2020. Effects of erythromycin and sulfamethoxazole on *Microcystis aeruginosa*: Cytotoxic endpoints, production and release of microcystin-LR. *J. Hazard. Mater.* 399. <https://doi.org/10.1016/j.jhazmat.2020.123021>
- Ziylan, A., Dogan, S., Agopcan, S., Kidak, R., Aviyente, V., Ince, N.H., 2014. Sonochemical degradation of diclofenac: Byproduct assessment, reaction mechanisms and environmental considerations. *Environmental Science and Pollution Research* 21, 5929–5939. <https://doi.org/10.1007/s11356-014-2514-7>
- Zuo, Z., 2019. Why Algae Release Volatile Organic Compounds—The Emission and Roles. *Front. Microbiol.* 10. <https://doi.org/10.3389/fmicb.2019.00491>

7. Wykaz tabel i rysunków

Tabela:

Tabela 1. Ogólna charakterystyka badanych szczepów sinic

Rysunki:

Rysunek 1. Struktury biogennych lotnych związków organicznych (BVOC) wytwarzanych przez cyjanobakterie *Synechocystis salina* (S), *Microcystis aeruginosa* (M), *Aphanizomenon flos-aquae* (A) oraz *Nodularia spumigena* (N) w warunkach stresu wywołanego silnym światłem [P2].

Rysunek 2. Chromatogramy barwników fotosyntetycznych badanych sinic oraz mieszaniny wzorców barwników, zarejestrowane przy długości fali 452 nm - [P3].

Rysunek 3. Wartości tempa wzrostu (R_G) dla *S. salina* traktowanej NLPZ (diklofenakiem – DCF, ibuprofenem – IBF, naproksenem – NPX) w podłożu BG11 oraz w podłożu BG11 z dodatkiem soli morskiej (38 PSU) [P1].

Rysunek 4. Obraz mikroskopowy (powiększenie 1000×) sinic: *Synechocystis salina* CCNP1104 (po lewej) i *Microcystis aeruginosa* CCNP1101 (po prawej) [P5].

Rysunek 5. Stężenia [fg/komórka] wybranych pigmentów w ekstraktach sinic *S. salina* i *M. aeruginosa* w kulturach kontrolnych oraz kulturach ekspozycyjnych na diklofenak (DCF) po 96 godzinach (stężenie fikocyjaniny oznaczono półilościowo) [P5].

Rysunek 6. Histogramy i wykresy punktowe z analizy metodą cytometrii przepływowej dla hodowli *S. salina* ekspozycyjnych na diklofenak (A i B) oraz hodowli kontrolnych (C i D) po 96-godzinnej ekspozycji [P5].

Rysunek 7. Wzrost względny (stosunek DCF/kontrola [%]) kultur *A. cylindrica* ekspozycyjnych na nominalne stężenie DCF 100 mg/l w standardowej pożywce Z8 oraz Z8_o. Dane przedstawiono jako średnią ± SD (n = 3, niezależne doświadczenia). Gwiazdki umieszczone nad poszczególnymi punktami czasowymi wskazują na statystycznie istotne różnice pomiędzy wariantami Z8 i Z8_o (*p < 0,05, test t-Studenta) [M2].

Rysunek 8. Analiza głównych składowych (PCA) oraz odpowiedź barwnikowa sinic na działanie diklofenaku (DCF) w podłożach wzrostowych Z8 i Z8_o. (A) Wykres PCA przedstawiający zróżnicowanie profili barwnikowych sinic w zależności od podłoża wzrostowego (Z8 vs. Z8_o) oraz ekspozycji na diklofenak (kontrola vs. DCF). Czerwone wektory przedstawiają ładunki poszczególnych barwników. (B) Względne zmiany zawartości wybranych barwników związanych z odpowiedzią na stres, wyrażone jako procentowy stosunek do Chl *a* [%]. Wartości przedstawiono jako średnią ± SD (n = 3, niezależne doświadczenia) [M2].

8. Dorobek naukowy

DANE LICZBOWE

Sumaryczny IF¹ wszystkich publikacji	61,0
Sumaryczna liczba cytowań^{2/2*} wszystkich publikacji	13/58
Sumaryczna punktacja ministerialna MEiN	1290
Indeks Hirscha^{3/4}	2/5

Wartości IF według Journal Citation Reports, prezentowane w Bazie Wiedzy Uniwersytetu Gdańskiego, właściwe dla roku opublikowania poszczególnych prac.

²Sumaryczna liczba cytowań (bez autocytowań) według Web of Science Core Collection.

^{2*}Sumaryczna liczba cytowań (bez autocytowań) według Web of Science Core Collection z uwzględnieniem pracy oczekującej na weryfikację panińskiego nazwiska

³Indeks Hirscha według Web of Science Core Collection

⁴Indeks Hirscha według Google Scholar

*Dane bibliometryczne według stanu na 26.09.2026 r., z wyłączeniem publikacji w trakcie recenzji.

Artykuły z czasopism

2023

- Kropidłowska, K., Caban, M.*, 2023. Effect of salinity on the toxicity of diclofenac, ibuprofen and naproxen toward cyanobacterium *Synechocystis salina*. Chemosphere 338, 139521. <https://doi.org/doi.org/10.1016/j.chemosphere.2023.139521>
- Bethke, K., Kropidłowska, K., Stepnowski, P., Caban, M.*, 2023. Review of warming and acidification effects to the ecotoxicity of pharmaceuticals on aquatic organisms in the era of climate change. Science of The Total Environment 877, 162829. <https://doi.org/10.1016/j.scitotenv.2023.162829>
- Łukaszewicz, P., Siudak, P., Kropidłowska, K., Caban, M., Haliński, Ł.P.*, 2023. Unicellular cyanobacteria degrade sulfoxaflor to its amide metabolite of potentially higher aquatic toxicity. Chemosphere 337, 139440. <https://doi.org/10.1016/j.chemosphere.2023.139440>

2024

- Bethke, K., Kwidzińska, K., Caban, M.*, 2024. Investigation of pharmaceutical bioaccumulation in *Daphnia* sp. living in a wastewater treatment plant. Science of the Total Environment 950. <https://doi.org/10.1016/j.scitotenv.2024.174915>

- Kwidzińska, K., Zalewska, M., Aksmann, A., Kobos, J., Mazur-Marzec, H., Caban, M., 2024. Multi-biomarker response of cyanobacteria *Synechocystis salina* and *Microcystis aeruginosa* to diclofenac. J. Hazard. Mater. 471. <https://doi.org/10.1016/j.jhazmat.2024.134373>

2025

- Kwidzińska, K., Hryniewicka, M., Caban, M.*, 2025. Biogenic volatile organic compounds produced by two unicellular and two filamentous cyanobacteria after a high light shock. Atmos. Environ. 353. <https://doi.org/10.1016/j.atmosenv.2025.121252>

2026

- Kwidzińska, K., Caban, M.*, 2026. Extraction and separation of cyanobacterial photosynthetic and photoprotective pigments: comparison of practical and accessible methodologies. Eur. J. Phycol. 61, 322–338. <https://doi.org/10.1080/09670262.2026.2682186>
- Lis, H., Kwidzińska, K., Bethke, K., Caban, M.*, 2026. Cyanobacteria as generators of reactive oxygen species – A review of analysis methods and case studies. Talanta 297. <https://doi.org/10.1016/j.talanta.2025.128648>
- Kwidzińska, K., Bethke, K., Caban, M.*, 2026. Growth Inhibition and Beyond: Hormetic and Environmentally Relevant Effects of Pharmaceuticals on Cosmopolitan Cyanobacteria. Aquatic Toxicology, 108035. <https://doi.org/10.1016/j.aquatox.2026.108035>

Staż naukowy

Uniwersytet Techniczny w Dreźnie, Instytut Chemii Wody; 07.11.2023 - 01.12.2023

Tytuł stażu: „Ecotoxicity tests on water organisms”

- badania bioakumulacji w organizmach wodnych (*Daphnia magna* i mikroglony)
- analiza farmaceutyków i bisfenoli metodą LC-MS/MS
- ekstrakcja związków chemicznych z materiału biologicznego
- prowadzenie testów ekotoksykologicznych z wykorzystaniem *Daphnia magna*

Wygłoszone referaty

Konferencje międzynarodowe

- Klaudia Kropidłowska, Katarzyna Bethke, Magda Caban "Effect of naproxen, ibuprofen and diclofenac on cyanobacterium *Synechocystis Salina* with relation

to salinity of medium", SETAC Europe 33rd Annual Meeting, Dublin, Ireland, 2023

- Katarzyna Bethke, Klaudia Kropidłowska, Weronika Koczur, Magda Caban, Piotr Stepnowski "Effect of lowering pH and increasing temperature on toxicity of sulfametoxazole to *Daphnia magna*", SETAC Europe 33rd Annual Meeting, Dublin, Ireland, 2023
- Magda Caban, Martyna Zalewska, Anna Aksmann, Justyna Kobos, Hanna Mazur-Marzec, Katarzyna Bethke and Klaudia Kropidłowska "Resistance of cyanobacterium *Microcystis aeruginosa* to diclofenac measured by various marker", SETAC Europe 33rd Annual Meeting, Dublin, Ireland, 2023
- Katarzyna Bethke, Klaudia Kwidzińska, Weronika Koczur, Anna Białk-Bielińska, Magda Caban „Acidification increases the toxicity of diclofenac to aquatic organisms” SETAC Europe 34th Annual Meeting, Sevilla, Spain, 2024

Konferencje krajowe

- Klaudia Kropidłowska, Katarzyna Bethke, Piotr Stepnowski, Magda Caban „Wykorzystanie metod chromatograficznych i spektrofotometrii do analiz pigmentów fotosyntetycznych sinic *Synechocystis salina*”, XI Polska Konferencja Chemii Analitycznej (PoKoChA), Łódź, 2022
- Katarzyna Bethke, Klaudia Kropidłowska, Magda Caban, Piotr Stepnowski „Analiza bioakumulacji wybranych farmaceutyków w zooplanktonie żyjącym w osadniku wtórnym oczyszczalni ścieków z wykorzystaniem techniki LC-MS”, XI Polska Konferencja Chemii Analitycznej (PoKoChA), Łódź, 2022
- Magda Caban, Klaudia Kropidłowska, Katarzyna Bethke, Piotr Stepnowski „Analiza lotnych związków produkowanych przez sinice techniką mikroekstrakcji do fazy stałej wspomaganą próżniowo”, XI Polska Konferencja Chemii Analitycznej (PoKoChA), Łódź, 2022
- Klaudia Kropidłowska, Katarzyna Bethke, Piotr Stepnowski, Magda Caban „Wykorzystanie metod chromatograficznych i spektrofotometrii do analiz pigmentów fotosyntetycznych sinic *Synechocystis salina*”, XI Polish Conference on Analytical Chemistry in Łódź, 2022

- Klaudia Kropidłowska, Magda Caban „Siła jonowa a toksyczność leków względem sinic” XV Konferencję pt. „Chemia, Geochemia I Ochrona Środowiska Morskiego”. Gdynia, 2023
- Klaudia Kwidzińska, Hanna Mazur-Marzec, Martyna Zalewska, Magda Caban „Odpowiedź sinic na obecność diklofenaku” XX Ogólnopolskie Seminarium Doktorantów i Studentów „Na pograniczu chemii, biologii i farmacji” Jarnołówek, 2024
- Katarzyna Bethke, Klaudia Kwidzińska, Magda Caban „Zakwaszenie wód zwiększa toksyczność diklofenaku wobec organizmów wodnych”, XX National Seminar of Doctoral Students and Graduate Students "Na pograniczu chemii, biologii i farmacji". Jarnołówek, 2024

Postery

Konferencje międzynarodowe

- Klaudia Kropidłowska, Katarzyna Bethke, Magda Caban "Effect of naproxen, ibuprofen and diclofenac on cyanobacterium *Synechocystis Salina* with relation to salinity of medium" - SETAC Europe 33rd Annual Meeting, Dublin, Ireland, 2023
- Klaudia Kwidzińska, Katarzyna Bethke, Magda Caban „Miniaturization of ecotoxicological tests and their application to assess the toxicity of pharmaceuticals towards Baltic cyanobacteria” BEING SEA-EU, Valetta, Malta, 2024
- Katarzyna Bethke, Klaudia Kwidzińska, Weronika Koczur, Magda Caban „Effect of pH reduction on chronic toxicity of diclofenac to *Daphnia magna*”. BEING SEA-EU, Valetta, Malta, 2024
- Klaudia Kwidzińska, Hanna Mazur-Marzec, Martyna Zalewska, Magda Caban “Toxicity of diclofenac towards Baltic cyanobacteria and their biochemical response” SETAC Europe 34th Annual Meeting, Sevilla, Spain, 2024
- Klaudia Kropidłowska, Katarzyna Bethke, Magda Caban, tytuł: „Application of flow cytometry for testing of pharmaceuticals toxicity toward cyanobacteria” SETAC Europe 34th Annual Meeting, Sevilla, Spain, 2024
- Klaudia Kwidzińska, Magda Caban, Ewa Mulkiewicz, „Does DCF influence antioxidant enzyme system in Baltic cyanobacteria? Biochemical response of the

cells to environmental emergent pollutant” SETAC Europe 34th Annual Meeting, Sevilla, Spain, 2024

Konferencje krajowe

- Klaudia Kropidłowska, Magda Caban „Porównanie wpływu farmaceutyków na sinice i zielenice” Konferencja Naukowa e-Factory od Science, 2022
- Klaudia Kropidłowska, Magda Caban „Wpływ antybiotyków na sinice *Microcystis aeruginosa*” III Pomorskie Studenckie Sympozjum Chemiczne, 2021
- Magda Caban, Klaudia Kropidłowska, Piotr Stepnowski „Application of chromatographic techniques for screening of diclofenac effect into pigments production by cyanobacteria *Synechocystis salina* CCNP1104” - XII Polska Konferencja Chromatograficzna (PKChrom), 2021
- Magda Caban, Klaudia Kropidłowska, Piotr Stepnowski “The application of spme-gc/ms for study of volatile compounds produced by cyanobacteria *Synechocystis salina* CCNP110” – XII Polska Konferencja Chromatograficzna (PKChrom), 2021
- Klaudia Kwidzińska, Hanna Mazur-Marzec, Martyna Zalewska, Magda Caban „Czy diklofenak indukuje odpowiedź biochemiczną u bałtyckich sinic?”, I Polskiego Sympozjum „Leki-kontra-Środowisko” (LEKOŚ’2024), Gdańsk, 2024

Projekty

Kierownik projektów BMN (Badania Młodych Naukowców), finansowanych przez Uniwersytet Gdański:

- „Wpływ zasolenia wód na stabilność wybranych niesteroidowych leków przeciwzapalnych oraz możliwość degradacji tych związków przez sinice z grupy *Synechocystis*”, 2021
- „Sprawdzenie możliwości miniaturyzacji testów ekotoksykologicznych z wykorzystaniem sinic”, 2023

Główny wykonawca w projekcie OPUS (NCN)

- „Badania biochemicznych i fizjologicznych markerów wpływu diklofenaku na bałtyckie sinice”, 2020 – 2024

**Oświadczenia współautorów publikacji wchodzących w skład
rozprawy doktorskiej**

dr hab. Magda Caban, prof. UG
Katedra Analizy Środowiska
Wydział Chemii

27.07.2026r., Gdańsk

Oświadczenie

Oświadczam, iż w poniżej wymienionych pracach:

P1 Bethke Katarzyna, Kropidłowska Klaudia, Stepnowski Piotr, Caban Magda*, Review of warming and acidification effects to the ecotoxicity of pharmaceuticals on aquatic organisms in the era of climate change, Science of the Total Environment, 2023, vol. 877, s.1-24, Numer artykułu:162829. DOI:10.1016/j.scitotenv.2023.162829, 200 punktów, IF₂₀₂₃ 8,2 (Q1)

P2 Bethke Katarzyna, Kwidzińska Klaudia, Caban Magda*, Investigation of pharmaceutical bioaccumulation in *Daphnia* sp. living in a wastewater treatment plant, Science of the Total Environment, 2024. vol. 950, s.1-8, Numer artykułu:174915. DOI:10.1016/j.scitotenv.2024.174915, 200 punktów, IF₂₀₂₄ 8,0 (Q1)

P3 Bethke Katarzyna*, Caban Magda, Effect of acidification on the chronic toxicity of diclofenac to *Daphnia magna*, Aquatic Toxicology, 2025, vol. 287, s.1-7, Numer artykułu:107497. DOI:10.1016/j.aquatox.2025.107497, 140 punktów, IF₂₀₂₅ 4,6 (Q1)

P4 Bethke Katarzyna*, Caban Magda, Exploring the impact of pH on the toxicological response of *Lemna minor* to pharmaceuticals - insights from sulfamethoxazole exposure, Environmental Toxicology and Chemistry, 2026, vol.45, s.2610-2621, DOI: 10.1093/etoxjnl/vgag136, 100 punktów, IF₂₀₂₅ 3,6 (Q2)

P5 Bethke Katarzyna, Caban Magda*, Context-Dependent Toxicity of Diclofenac in *Daphnia magna*: Roles of Water Chemistry, Neonate Source and Preparation Procedure, Aquatic Toxicology, 2026, Numer artykułu: 108029. DOI: 10.1016/j.aquatox.2026.108029, 140 punktów, IF₂₀₂₅ 4,6 (Q1)

mój udział polegał na konceptualizacji, opiece merytorycznej, nadzorowaniu badań oraz współtuczestniczeniu nad pisanem manuskryptów.


dr hab. Magda Caban, prof. UG



Katedra Analizy Środowiska

ul. Wita Stwosza 63
80-308 Gdańsk

mgr Martyna Zalewska
Katedra Biologii Eksperymentalnej i Biotechnologii Roślin
Wydział Biologii
Uniwersytet Gdański

Oświadczenie

Oświadczam, iż w poniżej wymienionych publikacjach:

Kwidzińska K., Zalewska M., A. Anna, K. Justyna, Mazur-Marzec H., Caban M., Multi-biomarker response of cyanobacteria *Synechocystis salina* and *Microcystis aeruginosa* to diclofenac, *Journal of Hazardous Materials*, 2024, 471, 134373, DOI:10.1016/j.jhazmat.2024.134373

Kwidzińska K., Zalewska M., A. Anna, Caban M., Nitrogen fixation induces diclofenac toxicity in the diazotrophic cyanobacterium *Anabaena cylindrica*, *Aquatic toxicology* (w recenzji)

mój udział obejmował przeprowadzenie badań dotyczących procesów fotosyntetycznych oraz współpracę przy przygotowaniu manuskryptów.

.....Zalewska.....
.....
podpis

Gdańsk, 16.09.2026

dr hab. Anna Aksmann, prof. UG
Katedra Biologii Eksperymentalnej i Biotechnologii Roślin
Wydział Biologii
Uniwersytet Gdański

Oświadczenie

Oświadczam, iż w poniżej wymienionych publikacjach:

Kwidzińska K., Zalewska M., Aksmann A., Kobos J., Mazur-Marzec H., Caban M., Multi-biomarker response of cyanobacteria *Synechocystis salina* and *Microcystis aeruginosa* to diclofenac, *Journal of Hazardous Materials*, 2024, 471, 134373, DOI:10.1016/j.jhazmat.2024.134373

Kwidzińska K., Zalewska M., Aksmann A., Caban M., Nitrogen fixation induces diclofenac toxicity in the diazotrophic cyanobacterium *Anabaena cylindrica*, *Aquatic Toxicology* (w recenzji)

mój udział obejmował konsultacje merytoryczne podczas analizowania wyników eksperymentów oraz współtworzenie manuskryptów w wersji pierwotnej i zrewidowanej.


podpis

Gdynia 16.08.2026
miejsce i data

dr Justyna Kobos
Katedra Biologii Morza i Biotechnologii
Wydział Oceanografii i Geografii
Uniwersytet Gdański

Oświadczenie

Oświadczam, iż w poniżej wymienionych publikacjach:

Kwidzińska K., Zalewska M., A. Anna, K. Justyna, Mazur-Marzec H., Caban M., Multi-biomarker response of cyanobacteria *Synechocystis salina* and *Microcystis aeruginosa* to diclofenac, *Journal of Hazardous Materials*, 2024, 471, 134373, DOI:10.1016/j.jhazmat.2024.134373

mój udział obejmował współpracę w przygotowaniu manuskryptu.


podpis

dr Hanna Lis
Katedra Analizy Środowiska
Wydział Chemii
Uniwersytet Gdański

Oświadczenie

Oświadczam, iż w poniżej wymienionych publikacjach:

Lis H., Kwidzińska K., Bethke K., Caban M., Cyanobacteria as generators of reactive oxygen species – A review of analysis methods and case studies, Talanta, 2026, 297, 128648, DOI:10.1016/j.talanta.2025.128648

mój udział obejmował analizę i opracowanie treści literaturowych, współtworzenie i przygotowanie manuskryptu, jego redakcję oraz opracowanie odpowiedzi na uwagi recenzentów.


.....
podpis

Białystok, 14.09.2026
.....
miejsce i data

dr Marta Hryniewicka
Zakład Analiz Farmaceutycznych i Żywności
Wydział Chemii
Uniwersytet w Białymstoku

Oświadczenie

Oświadczam, iż w poniżej wymienionej pracy:

Kwidzińska K., Hryniewicka M., Caban M., Biogenic volatile organic compounds produced by two unicellular and two filamentous cyanobacteria after a high light shock, Atmospheric Environment, 2025, 353, 121252, DOI:10.1016/j.atmosenv.2025.121252

mój udział obejmował współudział w tworzeniu manuskryptu, wizualizacji wyników oraz przeprowadzeniu badań.

Hryniewicka
.....
podpis

Prof. dr hab. Hanna Mazur-Marzec
Katedra Biologii Morza i Biotechnologii
Wydział Oceanografii i Geografii
Uniwersytet Gdański

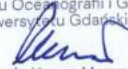
Oświadczenie

Oświadczam, iż w poniżej wymienionych publikacjach:

Kwidzińska K., Zalewska M., A. Anna, K. Justyna, Mazur-Marzec H., Caban M., Multi-biomarker response of cyanobacteria *Synechocystis salina* and *Microcystis aeruginosa* to diclofenac, *Journal of Hazardous Materials*, 2024, 471, 134373, DOI:10.1016/j.jhazmat.2024.134373

mój udział obejmował przeprowadzenie badań dotyczących cyjanotoksyn oraz współpracę przy tworzeniu manuskryptu.

Kierownik
KATEDRY BIOLOGII MORZA I BIOTECHNOLOGII
Wydziału Oceanografii i Geografii
Uniwersytetu Gdańskiego


prof. dr hab. Hanna Mazur-Marzec

.....
podpis

Gdańsk 23.09.2026
miejsce i data

Mgr Katarzyna Bethke
Katedra Analizy Środowiska
Wydział Chemii
Uniwersytet Gdański

Oświadczenie

Oświadczam, iż w poniżej wymienionych publikacjach:

Lis H., Kwidzińska K., Bethke K., Caban M., Cyanobacteria as generators of reactive oxygen species – A review of analysis methods and case studies, Talanta, 2026, 297, 128648, DOI:10.1016/j.talanta.2025.128648

Kwidzińska K., Bethke K., Caban M., Growth Inhibition and Beyond: Hormetic and Environmentally Relevant Effects of Pharmaceuticals on Cosmopolitan Cyanobacteria, Aquatic toxicology (w recenzji)

mój udział obejmował współtworzenie manuskryptów i udział w przygotowaniu odpowiedzi na uwagi recenzentów.

Katarzyna Bethke
podpis

**Oryginalne przedruki opublikowanych artykułów naukowych
stanowiących cykl rozprawy doktorskiej i manuskrypty przesłane
do recenzji**

Publikacja – P1

K Kropidłowska Klaudia, Caban Magda*, Effect of salinity on the toxicity of diclofenac, ibuprofen and naproxen toward cyanobacterium *Synechocystis salina*, Chemosphere, 2023, 338, 139521, DOI:10.1016/j.chemosphere.2023.139521



Effect of salinity on the toxicity of diclofenac, ibuprofen and naproxen toward cyanobacterium *Synechocystis salina*

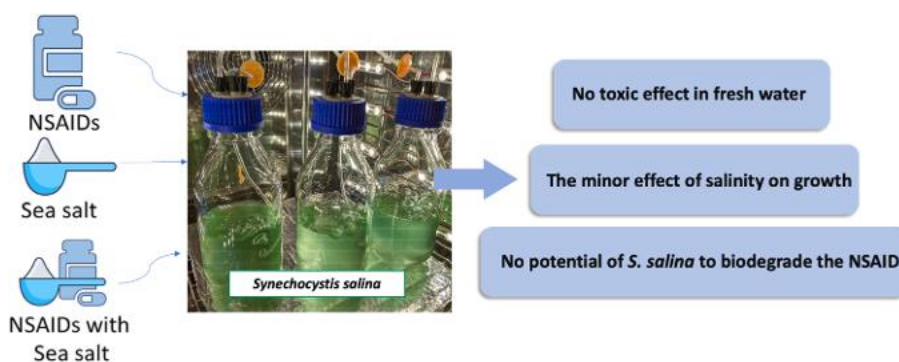
Klaudia Kropidłowska, Magda Caban*

University of Gdansk, Department of Environmental Analysis, Faculty of Chemistry, ul. Wita Stwosza 63, 80-308, Gdańsk, Poland

HIGHLIGHTS

- *S. salina* is opportunistic to high concentrations of NSAIDs.
- The high salinity (38 PSU) caused salt shock, but cyanobacteria cells adapted.
- The NSAIDs were stable in test conditions, and they did not bioaccumulate.
- The 38 PSU had a minor effect on the toxicity of NSAIDs toward *S. salina*.

GRAPHICAL ABSTRACT



ARTICLE INFO

Handling Editor: Magali Houde

Keywords:

Pharmaceuticals
Ecotoxicology of climate change
Synechocystis sp.
Non-steroidal anti-inflammatory drugs
Effect of salinity

ABSTRACT

Aquatic species are continuously exposed to pharmaceuticals and changeable water conditions simultaneously, which can induce changes in the toxicity of pollutants. Cyanobacteria are an organism for which less ecotoxicological tests have been performed compared to green algae. In this study, we decided to check how selected non-steroidal anti-inflammatory drugs (NSAID) affect the growth of *Synechocystis salina*, picocyanobacterium isolated from the Baltic Sea, with salinity as potential modulator of toxicity. *S. salina* was exposed to diclofenac (DCF), ibuprofen (IBF) and naproxen (NPX) (nominal 100 mg L⁻¹) in BG11 medium and sea salt supplemented BG11 medium (38 PSU) over 96 h in continuous light at 23 °C. No acute toxicity was found in both tested salinity levels. The comparable growth rate in exposed culture compared to control culture over 4 days indicate lack of stress for several generations which need to be overcome with substantial energy consumption. *S. salina* was found to be halotolerant and can be species for ecotoxicology test where salinity is an additional stressor. Furthermore, resistance of *S. salina* to target NSAIDs provides a competitive advantage over other phytoplankton species.

* Corresponding author.

E-mail address: magda.caban@ug.edu.pl (M. Caban).

<https://doi.org/10.1016/j.chemosphere.2023.139521>

Received 18 April 2023; Received in revised form 13 July 2023; Accepted 14 July 2023

Available online 21 July 2023

0045-6535/© 2023 Elsevier Ltd. All rights reserved.

1. Introduction

Pharmaceuticals are increasingly used both in medicine as well as in aquaculture and animal husbandry. Several pharmaceuticals are not completely metabolized in treated organisms, so they end up in the sewage system and then in wastewater treatment plants (WWTPs), where they are not effectively removed (UNESCO and HELCOM, 2017). The pathways of entering the environment varied between pharmaceuticals classes (Caban and Stepnowski, 2021). Depending on the pharmaceuticals, they are introduced into the environment in metabolized and non-metabolized forms, with both possibly active biologically (Mulkiwicz et al., 2021). Aquatic organisms are exposed chronically to pharmaceuticals in all types of waters around the world (aus der Beek et al., 2016; Caban and Stepnowski, 2021; Łukaszewicz et al., 2018; Patel et al., 2019).

One of the most used and widely available pharmaceuticals are non-steroidal anti-inflammatory drugs (NSAIDs) (Izadi et al., 2020). Unfortunately, most NSAIDs are inefficiently treated in WWTPs (Almeida et al., 2020; Izadi et al., 2020; Kolečka et al., 2019). Furthermore, it has been shown that NSAIDs can affect both eukaryotic and prokaryotic organisms, with high distribution of effects. For example, in a study conducted with three species of cyanobacteria (*Synechococcus elongatus*, *Microcystis aeruginosa*, *Cylindrospermopsis raciborskii*) and three species of green algae (*Desmodesmus communis*, *Haematococcus pluvialis*, *Cryptomonas ovata*), the toxicity of several NSAIDs (ibuprofen, diclofenac, mefenamic acid, diflunisal, piroxicam) at concentrations of 100 mg L⁻¹ was performed (Bácsi et al., 2016). None of the NSAIDs tested had a significant effect on the growth of single-celled *S. elongatus*. In the case of *M. aeruginosa*, diclofenac had the greatest effect on growth inhibition (38% relative to the tenth-day control). The filamentous of *C. raciborskii* was more sensitive, with the extent of growth inhibition by NSAIDs ranging from 73.5 to 84.5% relative to the control. In contrast, the NSAIDs tested significantly inhibited the growth of all eukaryotic algae, and growth inhibition ranged from 21 to 97%. Studies by Bácsi et al. show that prokaryotic cyanobacteria are more resistant to NSAIDs than eukaryotic algae. Other studies, in turn, have shown that pharmaceuticals can also have a stimulating effect on cyanobacteria (Pomati et al., 2004). It is generally stated that the presence of NSAIDs has a toxic effect on aquatic organisms, and their accumulation is a potential risk to public health (dos Santos et al., 2021; Lapworth et al., 2019). This statement comes from laboratory studies, where standard conditions are preferred (Świacka et al., 2022a). However, the fate (stability, distribution, bioavailability, among others) of pollutants is influenced by many factors, such as temperature, pH, light exposure and the salinity (Bethke et al., 2023; Pinheiro et al., 2021).

The last factor seems to be unexplored for pharmaceuticals. The bioavailability and toxicity of pollutants in saline water can vary greatly compared to that in freshwater (Borecka et al., 2016; Klok et al., 2017). Literature provides information on the effect of salinity on the ecotoxicity of selected classes of organic and inorganic compounds, such as petroleum hydrocarbons, polycyclic aromatic hydrocarbons, pesticides and metals (Hall and Anderson, 1995). However, with the exception of metals, whose toxicity decreases with increasing salinity, these compounds do not show consistent trends in the effect of salinity on their toxicity (Xiong et al., 2018). In the case of new emerging pollutants, data is limited, and general trends are unavailable. Nevertheless, an exemplary report shows the substantial effect of salinity. For example, the toxicity of sulfonamide antibiotics in *Chlorella vulgaris* decreased with increasing salinity (Borecka et al., 2016). The toxicity of ionic liquids in cyanobacterium *Geitlerinema amphibium* and algae *Oocystis submarina*, *C. vulgaris* was also lowered by increased salinity (Latala et al., 2010). Pharmaceuticals are mostly ionizable (more than 80% of pharmaceutical representatives have this attribute (Manallack, 2007)), and they get into environments with different salinities. Furthermore, the salinity of freshwater can be accelerated as the effect of failures in WWTPs or through the intended introduction of wastewater. The most dramatic

noted malfunction of a WWTP took place in Poland, when more than 3.6 million m³ untreated wastewater was introduced into the Vistula, the longest Polish river, in 2019 from a single point (Stepnowski et al., 2020). An accelerated salinity, as well as NSAIDs concentration was noted. Unfortunately, in 2022, the second longest Polish river, Warta, was subjected to an ecological disaster, as the cumulative effect of increased temperature and salinity caused a bloom of toxic algae and the massive death of fish and other organisms (Nazaruk and Korzeniowska, 2022).

Aquatic organisms are therefore exposed to variable salinity and a constant supply of pharmaceuticals. Among aquatic organisms, the most ecotoxicologically tested are green algae. For cyanobacteria, the number of data is much lower, even though they are found in every kind of water and can cause massive blooms more often. The cosmopolitan cyanobacteria can live in waters with varying salinities. The Baltic Sea is a body of water with a large fluctuation of salinity between regions. Due to its geographical location, the Baltic Sea is particularly vulnerable to pollution, including pharmaceutical. In a study by Björnlén et al. in the Baltic Sea, 39 of the 93 pharmaceuticals studied were detected at concentrations between 0.01 and 80 ng L⁻¹ (Björnlén et al., 2018), and, among other classes, NSAIDs were detected (UNESCO and HELCOM, 2017). In coastal areas, concentrations may even reach several hundred ng L⁻¹ for diclofenac (DCF) (Szymczycha et al., 2020). This means that the toxicity of the same pharmaceutical can be different depending on location and obtained salinity level.

The aim of this work was to evaluate the toxicity of selected NSAIDs – naproxen (NPX), ibuprofen (IBF) and DCF (Table 1) – toward *Synechocystis salina* cyanobacterium (order of Chroococcales, isolated from the Baltic Sea in 2006) at two salinity. Tested picocyanobacterium does not form massive blooms but is an important component of biocenosis. Target NSAIDs are one of the most sold (Kolečka et al., 2022). DCF deserves special attention due to its stability, relatively low reduction in WWTPs, high frequency of detection in the environment and proven toxic effect on wild-living organism (dos Santos et al., 2021; Helcom, 2023). Salinity effect on pharmaceuticals toxicity was already observed with green algae (Borecka et al., 2016), but generally it is unexplored stressor factor in pharmaceutical ecotoxicology. For example, it was never tested for cosmopolitan cyanobacteria knowing to habit in different salinity level. We hypothesized that salinity can affect also toxicity of NSAIDs toward cosmopolitan cyanobacteria, similarly as it was observed with green algae. The salinity of natural fresh water is more often increased, and, as in the case of the Polish Warta River, it can lead to dramatic consequences. The constantly increasing consumption of pharmaceuticals without parallel investments into wastewater treatment technology, makes highly probable that pharmaceuticals concentration in environment will increase. All these circumstances confirm the need to study the effect of salinity on the ecotoxicity of pharmaceuticals.

2. Materials and methods

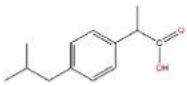
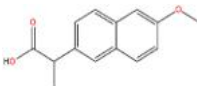
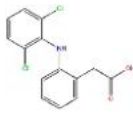
2.1. Chemicals

The analytical standards: diclofenac sodium (DCF, CAS 15307-79-6), ibuprofen (IBF, CAS 15687-27-1), naproxen (NPX, CAS 22204-53-1), *N*-(2,6-dichlorophenyl)-2-indolinone (IND, CAS 15362-40-4), 4'-hydroxydiclofenac (4'-OH DCF, CAS V64118-84-9), 5-hydroxydiclofenac (5-OH DCF, CAS 69002-84-2), diclofenac acyl-β-D-glucuronide (GLU-DCF, CAS 64118-81-6), were purchased from Sigma-Aldrich (Germany). HPLC grade acetonitrile (ACN) was purchased from POCH S.A. (Poland), while ammonium acetate and glacial acetic acid from VWR International (USA). Sea salt for reconstituting sea water was purchased from Aquaforest (Poland).

2.2. Batch culture

The research used a culture of *Synechocystis salina* cyanobacterium

Table 1
Selected properties of the tested NSAIDs.

NSAIDs	Ibuprofen (IBF)	Naproxen (NPX)	Diclofenac (DCF)	Ref.
CAS	15687-27-1	22204-53-1	15307-79-6	
Structure				
Solubility in water [mg L ⁻¹]	21.0 ¹	29.9 ¹	14180 ²	¹ (“DrugBank,” 2023) ² (Kincl et al., 2004)
pKa (strongest acidic)	5.2	4.19	4	(“DrugBank,” 2023)
Max. conc. of NASIDs detected in aquatic ecosystems worldwide [ug L ⁻¹]	17.40	11.40	15.09	(Świacka et al., 2021)

(strain CCNP1104) isolated from the Baltic Sea in the Gulf of Gdańsk (Poland, salinity approx. 7 PSU). The batch culture of cyanobacterium was obtained from the Culture Collection of Northern Poland (CCNP, Institute of Oceanography, University of Gdańsk, Poland). Cultures were carried out in borosilicate glass bottles, in a BG11 medium prepared from a BG11 medium concentrate purchased from Sigma-Aldrich (Germany). The pH of the medium was 7.2–7.5. The culture was constantly aerated and agitated by an air stream. The air was filtered through a FW600 (Wanza) carbon filter and a sterile polyethylene terephthalate (PET) syringe filter with a pore size of 0.2 µm (CHROMAFIL®). A picture of bottles with cyanobacterium cultures and their cells under a microscope is presented in Fig. S1 (Supplementary Information). The cyanobacterium was acclimated to the experimental conditions at least 10 days before the test was performed. Cultures were performed under sterile conditions. The cyanobacterium inoculation and sampling were performed under a laminar chamber UVC/T-M-AR (SIA Biosan, Latvia). The water for the preparation of the medium was sterilized with autoclave at 121 °C for 20 min. The cyanobacterium culture bottles were sterilized by heating for 2 h at > 120 °C. Aeration hoses and bottle tops were sterilized with methanol. The culture morphology was checked microscopically before the test. Optical density OD₆₃₀ (absorbance measured at 630 nm) was used to track the density of culture. The wavelength of 630 nm corresponds to the absorption maximum of phycocyanin, a characteristic pigment found in the cyanobacterial photosynthesis apparatus. The starting value of OD₆₃₀ was approx. 0.2–0.5 and allowed us to maintain a linear growth of cyanobacteria over the 96 h-day experiment.

2.3. Monitoring of NSAIDs by HPLC/DAD

To determine the stability of the tested NSAIDs under the test conditions and to determine the potential of cyanobacteria to eliminate (degrade or bioaccumulate) pharmaceuticals, changes in pharmaceutical concentrations and the presence of selected degradation products (metabolites of diclofenac: 4'-OH DCF, 5-OH DCF, IND, GLU-DCF) in the medium was monitored by HPLC-DAD. The concentration of the respective NSAIDs was evaluated in each sample on each test day. The abiotic controls (NSAID in medium, without cyanobacterium) were performed in each test. The stability of NSAID (S_{NSAID}), both in biotic and abiotic samples, was calculated as:

$$S_{NSAID} = \frac{C_{day\ x}}{C_{day\ (x-1)}} \quad (\text{eq. 1})$$

where C_{day x} is the concentration in day x, and C_{day (x-1)} is the concentration [mg L⁻¹] on the previous day to day x. The S_{NSAID} was calculated starting from the 24 h after exposition (first day).

Samples of NSAID-exposed and control cultures were filtered using 0.2 µm PET syringe filters prior to analysis. The Nexera XR HPLC/DAD system with the SPD-M20A detector, SIL-20AC autosampler, LC-20AD pump and CTO-20AC column oven (Schimadzu) with the Gemini-NX column (type C18 110A, particle size 5 µm, dimensions 150 × 4.6 mm, Phenomenex) with precolumn (Phenomenex) was used for

quantification. The injection volume was 25 µL. The mobile phase component A consisted of acetonitrile (ACN), while phase component B consisted of a 5 mM ammonium acetate buffer (pH 4.1) with 5% ACN (volume ratio). The parameters of mobile phase flow, isocratic elution composition, retention times of analytes, analytical wavelength with the chromatograms of standards analysis are given in Fig. S2 (Supplementary Information). The description of calibration curves (linearity (R²), linearity range, detection limit, accuracy and precision) are presented in Table S1 (Supplementary Information).

2.4. Exposure of *S. salina* on NSAIDs and/or salinity

The basis of the ecotoxicological study was the OECD 201 protocol (OECD, 2011). The modification introduced by us is the extended duration of the experiment to 96 h, due to the lower growth rate of the cyanobacterium compared to that given in OECD 201 and the use of BG11 as a test and growth medium adapted to *S. salina*.

A series of single tests was performed in which the cyanobacterium of *S. salina* were exposed to DCF, IBF and NPX at concentrations of 100 mg L⁻¹. A summary of the tests performed is presented in Table 2. The main study was preceded by preliminary studies in which *S. salina* was treated with DCF, IBF and NPX at concentrations ranging from 0.1 to 5 mg L⁻¹ in a standard salinity of BG11 medium. Their results are presented in Fig. S3 (Supplementary Information) and show no effect on the cyanobacterium growth at lower concentration levels of NSAIDs.

Exposure experiments were performed at the given concentration in at least 3 replicates. The biotic controls were untreated cyanobacterium. The abiotic controls were a BG11 medium or BG11 medium supplied with sea salt, both with the addition of a given pharmaceutical in the tested concentration (100 mg L⁻¹). The effect of salinity (nominal 35 PSU) was tested by the addition of sea salt before exposure to NSAIDs. With respect to the fact that BG11 has a salinity of approx. 3 (measured by conductometer), the accurate final salinity was 38 PSU.

The pharmaceuticals were added directly as a powder to the BG11 medium. Due to the lower solubility of IBF in the BG11 medium (and generally water, Table 1), the solutions containing this pharmaceutical were mixed for a few minutes and then filtered. Therefore, the actual concentrations of IBF differ significantly from the nominal concentrations (actual concentrations given in Table 2). Furthermore, the concentration of IBF varies between repetitions due to the problems with solubility. DCF and NPX were used as the sodium salt, while IBF was added in an acid form. The acidic nature of IBF lowered the pH of the BG11 medium to approx. 5.5. To exclude the effect of the lowered pH on the toxicity of IBF to the cyanobacterium, additional tests were conducted for IBF that was alkalinized with a solution of NaOH (1%) to pH 7.5.

The tests were carried out in the IL 65-W incubator from Wamed (Poland) for 96 h at 23 °C with constant white light illumination (LED) at an intensity of 70 ± 10 µmol m⁻² s⁻¹. The experiments were carried out in 250 mL borosilicate glass bottles, in which 200 mL volumes of cyanobacterial cultures were placed. All samples were constantly mixed and aerated with sterile air, which caused the evaporation of a few mL

Table 2

Description of set of samples prepared (IBF – ibuprofen, NPX – naproxen, DCF – diclofenac, abio – abiotic, bio – biotic, ctrl – control). n.d. = no data.

Test mark	NSAID	pH at the start to (→) the end of test	Presence of <i>S. salina</i> inoculum	Mean actual concentrations during the test [mg L ⁻¹]	Salinity
IBF_BG11	Ibuprofen	7.5 → 9.3 (±0.3)	yes	38.42 (±7.92)	3 PSU (BG11)
IBF_pH 5.5		5.5 → 9.1 (±0.4)	yes	37.67 (±8.93)	3 PSU (BG11)
IBF_38PSU		7.8 → 9.9 (±0.2)	yes	39.00 (±2.65)	38 PSU
IBF_abio-ctrl_BG11		7.5 → 7.51 (±0.3)	no	38.66 (±0.69)	3 PSU (BG11)
IBF_abio-crt_38PSU		7.8 → 8.2 (±0.1)	no	42.49 (±1.74)	38 PSU
NPX_BG11	Naproxen	7.5 → n.d.	yes	81.84 (±3.61)	3 PSU (BG11)
NPX_38PSU		7.8 → 9.3 (±0.3)	yes	76.20 (±3.40)	38 PSU
NPX_abio-ctrl_BG11		7.5 → n.d.	no	80.79 (±3.18)	3 PSU (BG11)
NPX_abio-crt_38PSU		7.8 → n.d.	no	78.51 (±5.00)	38 PSU
DCF_BG11	Diclofenac	7.5 → 9.9 (±0.5)	yes	112.96 (±5.20)	3 PSU (BG11)
DCF_38PSU		7.8 → n.d.	yes	92.18 (±6.33)	38 PSU
DCF_abio-ctrl_BG11		7.5 → 7.5 (±0.2)	no	108.53 (±8.08)	3 PSU (BG11)
DCF_abio-crt_38PSU		7.8 → n.d.	no	100.38 (±7.34)	38 PSU
Bio-ctrl_BG11	not supplied	7.8 → 10.3 (±0.6)	yes	not applicable	3 PSU (BG11)
Bio-ctrl_38PSU	not supplied	7.8 → 9.9 (±0.1)	yes	not applicable	3 PSU (BG11)

per day. In order to maintain a constant level of NSAIDs during the tests, an appropriate amount of sterile distilled water was added each day before the sampling.

During the tests, the pH was controlled, and the cultures were observed under a microscope. Cyanobacterial cell growth was evaluated spectrophotometrically by measuring absorbance at 630 nm (OD₆₃₀) every 24 h. During the tests, the entire UV–Vis spectrum in the range of 350–800 nm was recorded. The selected wavelength for the OD measurement represented the maximum absorption of the characteristic signal in the obtained spectrum derived from phycocyanin, a photosynthetic pigment characteristic for cyanobacteria. The absorption spectrum of *S. salina* is included in the supplement in Fig. S7 in the Supplementary Information. The relative standard deviation of the OD₆₃₀ values measured for one culture of *S. salina* (ten replicates) was 0.61%. The OD₆₃₀ measurements have been correlated with the number of *S. salina* cells, and this relationship is shown in Fig. S8 in the Supplementary Information. The relationship was determined for the culture of blue-green algae with the OD₆₃₀ value from 0.27 to 2.38. In the tested range, this relationship was linear, the value of R² was 0.9903. This means that the OD₆₃₀ directly correspond to cell number and it is applicable to determine the multiplication of culture.

The effect of salt addition on the growth of *S. salina* was presented using a ratio of the OD₆₃₀ for the cyanobacterium impacted by 38 PSU (OD_{38PSU}) to the OD₆₃₀ of the corresponding control culture (OD_{CTRL}) according to the equation:

$$OD_{38PSU/CTRL} = \frac{OD_{38PSU}}{OD_{CTRL}} \quad (\text{eq.2})$$

The effect of the tested NSAIDs on *S. salina* was presented as the ratio of the growth of the NSAID-treated culture to that of the corresponding control cultures according to the equation:

$$OD_{NSAID/CTRL} = \frac{OD_{NSAID}}{OD_{CTRL}} \quad (\text{eq.3})$$

where OD_{NSAID} is the OD₆₃₀ of the cyanobacterial culture treated with the selected pharmaceutical, and OD_{CTRL} is the OD₆₃₀ of the corresponding control culture.

For each of the samples with cyanobacterium culture, the rate of growth (R_G) values - ratio of the OD₆₃₀ of the cultures on each test day to the OD₆₃₀ of the previous day - were also determined according to the following equation:

$$R_G = \frac{OD_{day\ x}}{OD_{day\ (x-1)}} \quad (\text{eq.4})$$

where OD_{day x} is the OD₆₃₀ of the sample on test day x, and OD_{day (x-1)} is the OD₆₃₀ of the same sample on the previous day. R_G was calculated

both for the cyanobacterium cultivated in BG11 and 38 PSU.

The statistical importance of differences was calculated using two sample t-tests (for independent samples with unequal variance, two-sides) using Microsoft® Excel software. The distribution (Shapiro-Wil test) and homogeneity of variance (F-test) were checked before statistical comparison. The significance level was set at p < 0.05.

3. Results and discussion

3.1. Stability of the NSAIDs

In all sample sets (Table 2), the nominal concentration of NSAIDs was 100 mg L⁻¹. The actual concentration differs (Table 2) due to limited solubility. To normalize the values, the stability of NSAIDs was calculated using equation (1). The values of S_{NSAID} are presented in Fig. 1 (for day 1, 2, 3 and 4). The concentration ratios in the abiotic controls were between 1.02 and 1.06 for DCF (mean 1.04 ± 0.07), 0.91–1.09 (mean 1.01 ± 0.07) for NPX and 0.91 to 1.00 (mean 0.99 ± 0.05) for IBF. Mean values of S_{NSAID} close to 1 indicate that DCF, IBF and NPX were stable (no change in concentrations between days) under the tested conditions for the entire duration of the tests (96 h). Furthermore, the concentrations of NSAIDs did not change significantly during the tests, neither in a pure culture medium (BG11) nor in a sea salt-supplied medium (38 PSU). The presence of *S. salina* cyanobacteria did not affect the stability of the pharmaceuticals tested. No additional signals were observed in the chromatograms obtained from the HPLC/DAD analysis from all studied NSAIDs that would indicate the formation of metabolites or transformation products.

The tested NSAIDs were shown to be stable under the obtained conditions, and none of the biotic and abiotic factors affected its concentration. Similarly, studies conducted by Toński et al. have shown that NPX and IBF are hydrolytically stable over a wide range of temperatures (20–70 °C) and pH (4–9) (Toński et al., 2019), which shows that their half-lives are more than 1 year according to guidelines (OECD, 2004). A study conducted by Koumaki et al. to evaluate the biotic and abiotic degradation of NSAIDs in water and river sediment found that DCF, NPX and IBF were stable in sterile experiments (Koumaki et al., 2017). Their concentrations remained constant or changed slightly, ruling out abiotic removal processes such as hydrolysis and sorption. In contrast, the study showed that the compounds were biodegradable in non-sterile samples, and their removal rate depended on the amount of dissolved oxygen in the water. The study performed by Świacka et al. showed a high stability of DCF and 4-OH DCF in seawater in the absence of light (Świacka et al., 2022b). Another study showed that DCF dissolved in water were rapidly degraded when exposed to sunlight (Packer et al., 2003). However, in that study, the drug solutions were placed in quartz bottles, which may

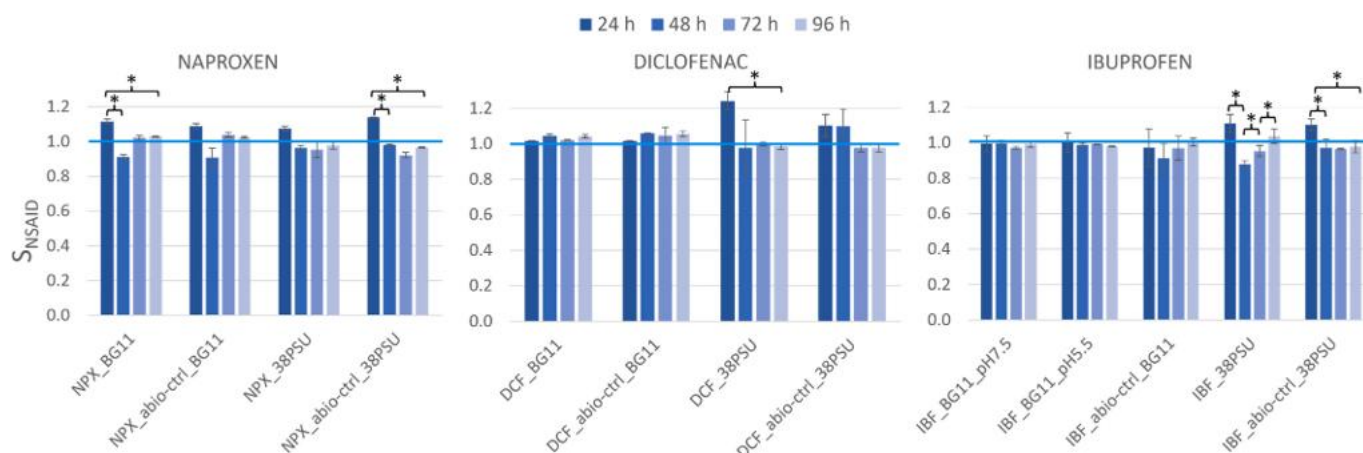


Fig. 1. Stability (S_{NSAID}) of ibuprofen – IBF, naproxen – NPX, diclofenac – DCF (each 100 mg L⁻¹) in pure culture medium (abio-ctrl) and in the presence of *S. salina* cyanobacteria with (38 PSU) or without (BG11) the addition of sea salt. Stability was expressed as the ratio of the drug concentration on day x to that of the previous day (day x-1), presented for days 1–4 of exposure.

have had a significant effect on the rate of degradation, due to the higher transmission of UV. The bottles applied in our test prevent UV transfer to the medium. The high stability at 23 °C for 96 h (4 days) and light exposure ($70 \pm 10 \mu\text{mol m}^{-2} \text{s}^{-1}$) suggest that removal from the environment solely by the abiotic factor is limited, which was also noted by mentioned reports.

3.2. Effect of salinity on the growth of *S. salina*

Part A of Fig. 2 presents the values of the ratio $OD_{38PSU}/CTRL$ on each day of the experiment between controls (without NSAID addition), which grow in sea salt supplemented medium (38 PSU), and a standard medium (BG11). At the start of the experiment, this parameter was equal to 1.00, while on the first day, it was 0.87 (± 0.04), suggesting a lower growth rate of the culture with the presence of sea salt. There was a statistically significant difference in the $OD_{38PSU}/CTRL$ parameter between the start of the tests (day 0) and first day (after 24 h of exposure) (*t*-test, $p < 0.05$; $p = 0.019$). There was also a statistical difference between the start of tests (day 0) and last day of the experiment (*t*-test, $p < 0.05$; $p = 0.00027$). This suggests that the cyanobacterium grow less in the sea salt supplemented BG11 medium compared to the BG11 medium itself. The mean growth rate (R_G , part B of Fig. 2) between the start of test and first day was lower in the 38 PSU medium compared to the 3 PSU medium (BG11) ($p < 0.05$), but there were no statistically significant differences between these parameters on the last day of the

experiment.

The reason for the reduced growth of the cyanobacterium in the sea salt supplemented medium may be due to salt shock, which was however quickly overcome by the cells (the grow rate was lower at the start of the experiment but then stabilized). Generally, cyanobacteria are hyperosmotic compared to the surrounding water, and to maintain a stable water level via the osmotic process, the level of osmotically active substances must be higher inside the cell. These cells apply a “salt-out” strategy in a salt-rich environment to maintain turgor at a stable level. The basis of this strategy is to keep the internal ion concentration low, and the necessary osmotic acclimation is achieved via the accumulation of small organic molecules called compatible solutes. This strategy consists of two parts. First, the cells accumulate compatible solutes that do not interfere with metabolism at high concentrations, and second, they actively export inorganic ions that steadily diffuse along their electrochemical gradients into the cytoplasm (Pade and Hagemann, 2015).

It appeared that the *S. salina* is halotolerant, similarly to the model organism *Synechocystis* sp. PCC 6803 (Marin et al., 2006), which accumulates glucosyl glycerol as the major compatible solute (Klähn and Hagemann, 2011). *Synechocystis* PCC 6803 is a model organism for experimental work and first cyanobacterial strain for which the total genome was sequenced (Korelusova et al., 2009), with known biology and preferable growing condition. *S. salina* is marine species, and was isolated from the Gulf of Gdańsk (Baltic Sea), where the average salinity

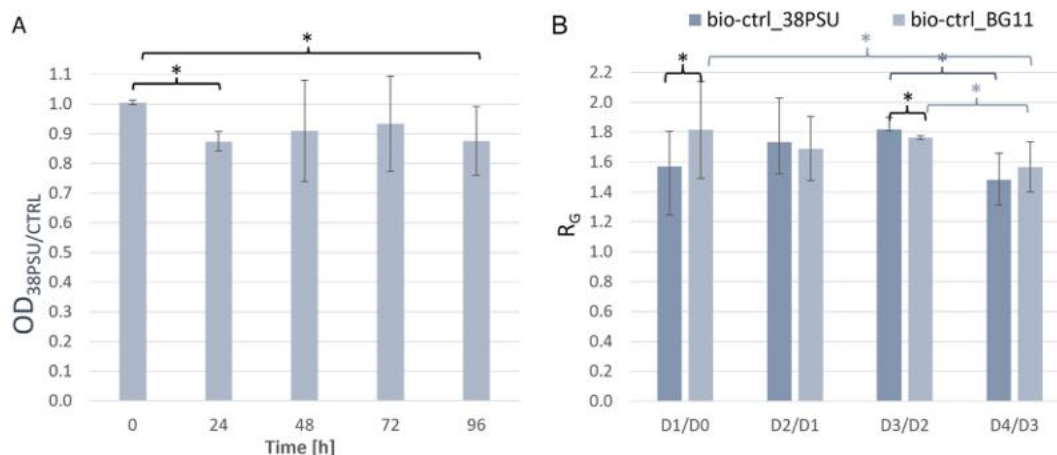


Fig. 2. Values of A. $OD_{38PSU}/CTRL$ ratio and B. rate of growth (R_G) for biotic controls of *S. salina* cultivated in the BG11 medium (CTRL) and BG11 supplied with sea salt (38_PSU) (* denotes the statistically significant difference between marked bars).

is approx. 7 PSU. Despite the initial reduction in growth in 38 PSU, the cyanobacterium adapted to the increased salt concentration. Nevertheless, lower salinity can affect positively to this species. For example, the biomass production of *S. salina* LEGE 06,155 was found to be optimal at 10 PSU (Assunç et al., 2023). In another study, the maximum production of polyhydroxybutyrate in *Synechocystis* sp. was observed with 12 PSU (Rueda et al., 2022). The tolerance to salinity can be different between strains. *S. salina* in our lab cultivated in BG11 with salinity 3, and the maximum grow rate observed was close to 2, meaning duplication of culture density twice per day. This means that *S. salina* is halotolerant within a high range of salinity. Most probably this adaptation was caused by the rapid response of active ion transport to an increase in the intracellular concentration of Na^+ ions (Mishra et al., 2020). In the cyanobacteria cell, the intracellular Na^+ concentration increases rapidly after exposure to high salinity then decreases at a lower rate (minutes to hours), as active ion transport eliminates excess Na^+ ions to maintain the intracellular ion concentration at 10–30 mM. The uptake of K^+ allows for immediate regulation. Exposure to high salinity also induces water loss from the cell. This process in cyanobacterium of the species

Synechocystis sp. can occur simultaneously with ion transport. The mentioned change in ion transport and the intensive production of compatible solutes cause energy consumption, which was responsible for the lower growth rate at the start of the experiment. The halotolerant species are desired in large scale production systems, because they can be cultivated in sea water, much more abundant than fresh water. The *S. salina* CCNP1104 can be also the species for testing of toxicity in different salinity level, what was missed up to day.

3.3. Effect of NSAIDs on the growth of the cyanobacterium *S. salina*

The maximum solubility of IBF and NPX in the growth medium was reached (Table 2). The solubility of pharmaceuticals was not significantly affected by the addition of salt. It was not possible to determine the value of EC_{50} according to OECD 201 (72 h; the concentration causing 50% growth inhibition) for IBF and NPX, as the saturated solution of pharmaceuticals had no toxic effect on growth. Only in the case of a DCF concentration higher than 100 mg L^{-1} can this be obtained. We did not perform such additional experiments, as testing such a high

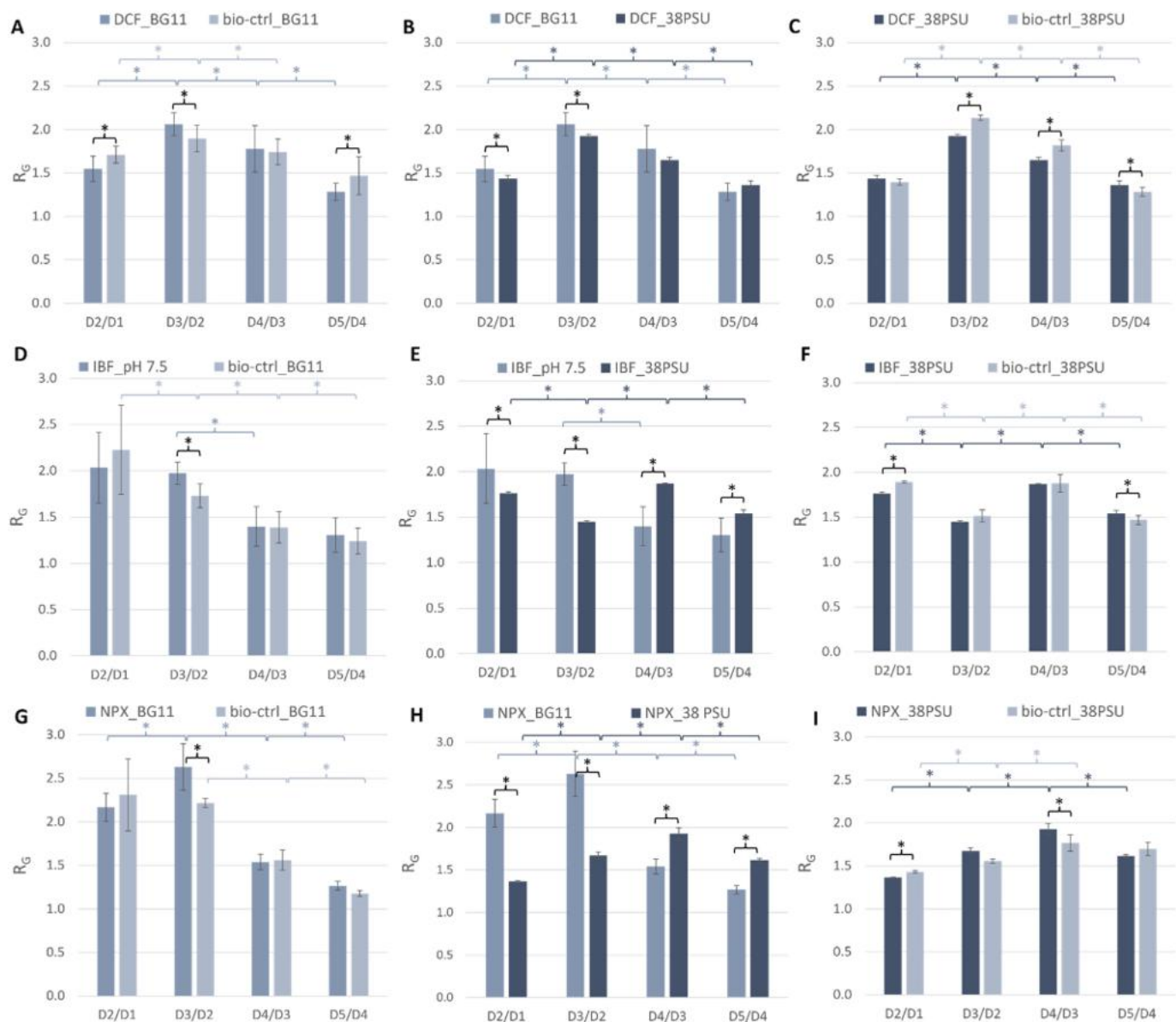


Fig. 3. Values of the rate of growth (R_g) for *S. salina* treated with NSAIDs (diclofenac – DCF, ibuprofen – IBF, naproxen – NPX) in a BG11 medium and BG11 supplied with sea salt (38 PSU).

concentration would not be reasonable keeping in mind that the environmental concentrations reached a dozen $\mu\text{g L}^{-1}$ (Table 1). Thereby, the effect on the fitness of the culture was determined as changes in the ratio of growth (R_G) (eq. (4)), which is presented in Fig. 3. The raw values of OD_{630} measured in the culture with and without NSAIDs are presented in Figs. S4–6 (Supplementary Information) for all tests and days of exposure. Table S2 (Supplementary Information) presents the specific description of growth of *S. salina* in the control and NSAID supplemented medium. The growth, as already presented, can be described by the growth rate (R_G) between days and the comparison of OD between the NSAID impacted culture and appropriate control culture ($\text{OD}_{\text{NSAID}/\text{CTRL}}$) on each day of exposure. Fig. 3 present any statistically important differences (t -test, $p < 0.05$), if obtained, between bars.

The changes in R_G in cultures with and without DCF have a similar pattern, independent from salinity (Fig. 3A and C). The differences obtained were rather minor. The DCF had no impact on the growth rate of *S. salina* in the performed test, independent of salinity tested (Fig. 3C).

There was no difference between R_G values of cultures growing in BG11 and BG11 + IBF (Fig. 3D), what suggest no toxic/stimulative effect of this NSAID. Similarly, IBF had no effect on R_G values for the cyanobacterium cultivated in BG11 supplemented with sea salts (Fig. 3F). However, differences can be observed when comparing the exact values of R_G between cultures with IBF in the standard medium and the sea salt supplemented medium (Fig. 3E). Specifically, at the start of the test, higher growth was noted for cultures in BG11, while after 48 h (2 days), higher growth was seen for the sea salt supplemented cultures. This is in agreement with the previous statement that the sea salt supplemented cultures start to grow later due to the effect of salt shock and not the effect of exposure to IBF.

The effect of NPX on growth rate was similar to DCF and IBF. The minor differences obtained were between the growth rate of the biotic control and the NPX-exposed cultures (Fig. 3G and I). Growth rate trends over 96 h (4 days) of NPX-exposed cultures were similar to those in the corresponding control cultures. As with the results noted for IBF exposure, the cultures with the sea salts started to grow later (Fig. 3H), and this was not due to the toxic effect of NSAID but the salts themselves.

Comparison of the $\text{OD}_{\text{NSAID}/\text{CTRL}}$ values of cultures grown in BG11 medium and in BG11 medium with the addition of sea salt allows the determination of the effect of salinity on NSAID toxicity. In general, $\text{OD}_{\text{NSAID}/\text{CTRL}}$ values in all tests did not differ significantly from unity (green line in Fig. 4). This indicates little effect of NSAIDs on the culture of *S. salina* within 96 h of exposure as cultures exposed to NSAIDs grew at a similar rate to control cultures (0.84 ± 0.002 – 1.17 ± 0.002) (Fig. 4). On most days during testing, there were statistical differences between the $\text{OD}_{\text{NSAID}/\text{CTRL}}$ values of the NSAID_BG11 culture and the NSAID_38PSU culture. However, the NSAID_BG11 culture had a higher

$\text{OD}_{\text{NSAID}/\text{CTRL}}$ value on some days, and on other days, a higher $\text{OD}_{\text{NSAID}/\text{CTRL}}$ value was seen in the NSAID_38PSU culture, and this lack of uniform trend.

NPX shows almost no negative effects on the growth of *S. salina*, independent of salinity level (Fig. 4C). DCF and IBF have an effect on the cyanobacterium growth in 38 PSU (Fig. 4A and B, respectively), while the values of $\text{OD}_{\text{NSAID}/\text{CTRL}}$ were lower than the optimal value of 1. What is interesting is that this negative effect started to be observable on day 2 and prolonged to day 4. On the last day of exposure, DCF and IBF reduced the OD ratio by approx. 10% when dissolved in 38 PSU. However, this negative effect is rather minor.

The lack of a significant effect of the tested pharmaceuticals on the growth of the cyanobacterium may have been due to the inability of these compounds to penetrate the cell. The value of the Zeta potential, among other things, provides information about the charge on the cell surface. The Zeta potential value for *S. salina* is $-43.1 (\pm 2.4)$ mV (Gonçalves et al., 2015), which means that the outer cell surface of these cyanobacteria have a negative charge just like the molecules of the tested NSAIDs, what cause mutual repulsion. The negative charge on the surface of cyanobacterial cells prevents them from coagulating and may also prevent negatively charged pharmaceutical molecules from approaching the cyanobacterial cell. In the work of Bácsi et al. (2016), the negative effect of IBF on growth of *C. raciborskii* was observed, despite the constant concentration of IBF in medium per 10 day exposure. The stability of NSAIDs was also noted in cultures of cyanobacteria for which pharmaceuticals had no toxic effect. The lack of sorption of NSAIDs by cyanobacteria need additional study, because negative charge of molecule is most probably not the sole factor which modulates transport. Selected organic substances can penetrate cell membrane of cyanobacteria by passive or active way (Stebeegg et al., 2019). They belong to saccharides, aminoacidic, peptides, carbonic acids, alcohols, organic dyes, and antibiotics. For most of them, mechanism of uptake is unknown.

In literature, there is little information about the effects of NSAIDs on cyanobacteria. In studies conducted by Pomati et al., a stimulating effect of IBF on the growth of *Synechocystis* sp. In all tested concentrations (0.001 – 1 mg L^{-1}) was observed (Pomati et al., 2004). The highest growth stimulation was observed at a concentration of 0.01 mg L^{-1} , where the increase in the average density of the culture exposed to IBF, expressed as a percentage in relation to the control cultures, was as high as 72%. At concentrations of 0.001 , 0.1 and 1 mg L^{-1} , this was 12%, 42% and 31%, respectively. Our research does not indicate the stimulating effect of IBF on *S. salina*, and this discrepancy may result from morphological differences between the studied cyanobacterium or from the conditions under which the tests were conducted. In this case, the aeration of the cyanobacterium with air and stronger lighting in the tests carried out as part of this study could have been of key importance.

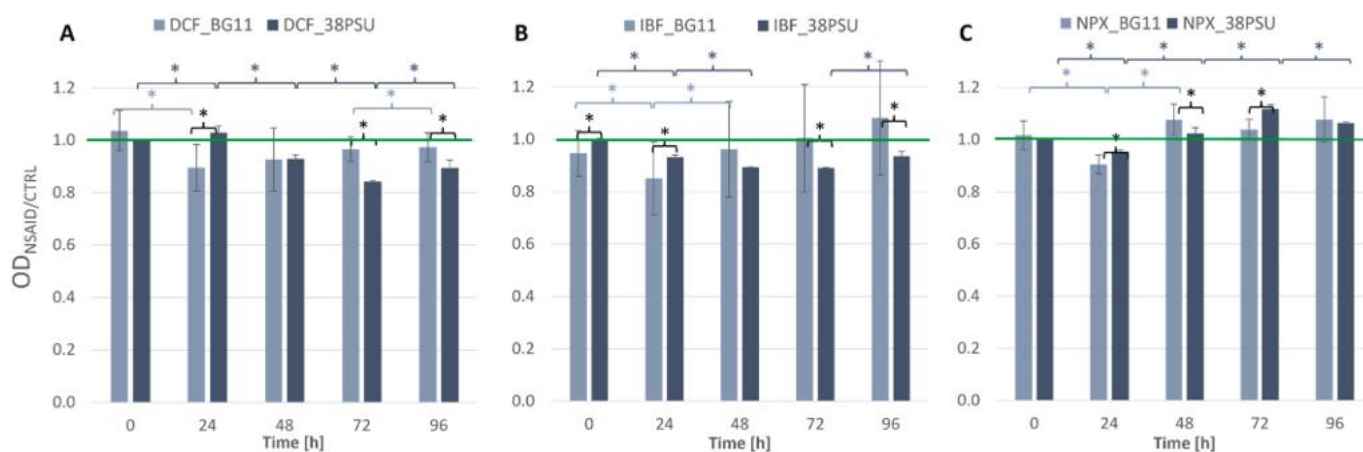


Fig. 4. Values of $\text{OD}_{\text{NSAID}/\text{CTRL}}$ obtained over 96 h of exposure of *S. salina* to diclofenac (DCF), ibuprofen (IBF) and naproxen (NPX) in a standard (BG11) and increased salinity (38 PSU) medium.

Blue-green algae of the *Synechocystis* species are mixotrophs, i.e. they can feed through photosynthesis and take food from the environment, e.g. in the form of organic carbon (Trentin et al., 2019). For this reason, the presence of CO₂ and strong irradiation causes cyanobacteria gain energy via photosynthesis. Lower light intensity (25 μmol s⁻¹ m⁻²) and the lower amount of supplied CO₂ in tests conducted by Pomati et al. (2004) may have contributed to the fact that cyanobacteria used organic forms of carbon available in water and perhaps even ibuprofen as carbon source.

IBF in concentrations of 100 mg L⁻¹ did not show a significant effect on the growth of another species of the cyanobacterium - *Synechococcus elongatus* and *Microcystis aeruginosa* (Bácsi et al., 2016). On the other hand, it strongly inhibited the growth of the cyanobacterium *Cylindrospermopsis raciborskii*, where the inhibition rate was 74.5% compared to control cultures after 10 days (Bácsi et al., 2016). DCF slightly inhibited the growth of *M. aeruginosa* and *S. elongatus* cyanobacterium (Bácsi et al., 2016), and for the *Synechococcus leopoliensis*, the EC₅₀ for DCF was 14.5 mg L⁻¹ (Ferrari et al., 2004). There is no information in literature concerning the effect of NPX on cyanobacteria. More information can be found on the effects of these drugs on green algae. One of the frequently tested species is *D. subspicatus*, with an EC₅₀ determined by Cleuvers of 625.5 mg L⁻¹ (Cleuvers, 2004), whereas in the studies conducted by Isidori et al., the EC₅₀ 31.82 mg L⁻¹ was reported (Isidori et al., 2005). The *S. salina* found to be resistant to toxic effect of NSAIDs manifested as reduction of grow rate. This mean that it have competitive advantage over other phytoplankton species. It should be aware that despite reduction of grow rate, the other endpoint of stress should be included in modern ecotoxicological studies. In a case of study performed by us, was assumed that the main biology of cells stay intact during 4 day-long exposure of several cyanobacteria generations, mainly due to lack of absorption of NSAIDs, and testing molecular response was not reasonable. The reasons of such assumption was fact that OD₆₃₀ was comparable in exposed and control cultures, and this parameter reflect the chlorophyll content, which in turn show the photosynthesis stability. The cyanobacterium grow constantly with no need to spend energy to overcome stress.

The effect of pH should be investigated, as pharmaceuticals are ionic. The tested NSAIDs have a pKa between 4 and 5.2 (Table 1). This means that in the pH range obtained in all tests performed in our study, all molecules of the target NSAIDs are anions, and an increase in pH during the test had no effect on charge distribution. Only in the case of acid IBF was the starting pH 5.5. There was no effect of the acidic IBF solution compared to the IBF solution with corrected pH in a standard BG11 on the growth of *S. salina* (Fig. S5 Supplementary Information). The reason is the increase of pH during culture cultivation in each flask. The initial pH of the BG11 medium was 7.5. In flasks with *S. salina* (both impacted and not impacted by NSAIDs), the pH increased to 9.34 (±0.13) (Table 2). The pH of the abiotic control remained constant. The increase of pH originate from OH⁻ efflux from the cells in a process designed to balance the HCO₃⁻ and nutrient uptake by the cells (Chaves et al., 2015). The optimal pH for the cyanobacteria growth varies between species. The alkaline pH is optimal for grow of *Synechocystis*. The pH of natural water is 6.5–8.5, and in seawater, around 8.2, though this can range between 7.5 and 8.5. The pH of the Baltic Sea is around 8, with a seasonal fluctuation of 0.5 (Wesslander et al., 2010). The blooms of cyanobacteria increase pH (Hinnert et al., 2015). This was noted, for example, for *Microcystis* spp (Zepernick et al., 2021), which caused a lower abundance of other species in the phytoplankton community, e.g., diatoms. This means that testing of the pH effect on NSAID toxicity would be problematic due to high modification of pH by growing cyanobacteria. Nevertheless, it could be realized by automatic pH regulation by CO₂ stream into culture of buffering (Assunç et al., 2023; Chaves et al., 2015).

4. Conclusion

The study shows that IBF, NPX and DCF do not affect the growth of *S. salina* cyanobacterium under pharmaceuticals at relatively high concentrations (100 mg L⁻¹), long exposure (4 days) and two salinity level (3 and 38 PSU). In contrast, high salinity (38 PSU) had an inhibitory effect on the growth of *S. salina*, but the culture had adapted and continued to grow. The tested cyanobacterium did not show a potential for removal/degradation of the target pharmaceuticals. The resistance of the cyanobacterium to selected pharmaceuticals, as well as their lack of ability to degrade them, may be due to the mutual repulsion of NSAID molecules with *S. salina* cells, which also carry a negative charge, but exact reason need to be tested in separate study. The *S. salina* was seen to be halotolerant and insensitive to high concentrations of target NSAIDs. This means that it can be opportunistic in the actual environment and can survive a high level of pollution. Study support also the previous observations that the cyanobacteria are tolerant to NSAIDs in mg L⁻¹ level. Despite the lack of salinity impact into toxicity of NSAIDs presented in this study, testing of salinity as an ecotoxicity-modulated factor needs to be explored further using phytoplankton as target organisms, as the environment is subjected to increase salinity regionally.

CRediT authorship contribution statement

Klaudia Kropidłowska: Conceptualization, Visualization, Writing – original draft, Writing – review & editing. **Magda Caban:** Conceptualization, Writing – original draft, Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Magda Caban reports financial support was provided by National Science Center (Poland).

Data availability

Data will be made available on request.

Acknowledgements

Financial support was provided by the National Science Center (Poland) under grants UMO-2019/35/B/NZ9/00253 and the research program of young scientists at the University of Gdańsk 539-T010-B927-21. The authors would like to thank the Culture Collection of Northern Poland (CCNP, Division of Marine Biotechnology, University of Gdańsk, Poland) for providing the cyanobacterium monocultures.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.chemosphere.2023.139521>.

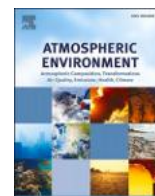
References

- Almeida, Â., Solé, M., Soares, A.M.V.M., Freitas, R., 2020. Anti-inflammatory drugs in the marine environment: bioconcentration, metabolism and sub-lethal effects in marine bivalves. *Environ. Pollut.* 263 <https://doi.org/10.1016/j.envpol.2020.114442>.
- Assunç, J., Amaro, H.M., Tavares, T., Malcata, F.X., Guedes, A.C., 2023. Effects of Temperature, pH, and NaCl Concentration on Biomass and Bioactive Compound Production by *Synechocystis Salina*.
- aus der Beek, T., Weber, F.A.A., Bergmann, A., Hickmann, S., Ebert, I., Hein, A., Küster, A., 2016. Pharmaceuticals in the environment-Global occurrences and perspectives. *Environ. Toxicol. Chem.* 35, 823–835. <https://doi.org/10.1002/etc.3339>.
- Bácsi, I., B-Béres, V., Kókai, Z., Gonda, S., Novák, Z., Nagy, S.A., Vasas, G., 2016. Effects of non-steroidal anti-inflammatory drugs on cyanobacteria and algae in laboratory

- strains and in natural algal assemblages. *Environ. Pollut.* 212, 508–518. <https://doi.org/10.1016/j.envpol.2016.02.031>.
- Bethke, K., Kropidłowska, K., Stepnowski, P., Caban, M., 2023. Review of warming and acidification effects to the ecotoxicity of pharmaceuticals on aquatic organisms in the era of climate change. *Sci. Total Environ.* 877, 162829 <https://doi.org/10.1016/j.scitotenv.2023.162829>.
- Björklén, B., Ripszám, M., Haglund, P., Lindberg, R.H., Tysklind, M., Fick, J., 2018. Pharmaceutical residues are widespread in Baltic Sea coastal and offshore waters – screening for pharmaceuticals and modelling of environmental concentrations of carbamazepine. *Sci. Total Environ.* 633, 1496–1509. <https://doi.org/10.1016/j.scitotenv.2018.03.276>.
- Borecka, M., Białk-Bielińska, A., Haliński, Ł.P., Pazdro, K., Stepnowski, P., Stolte, S., 2016. The influence of salinity on the toxicity of selected sulfonamides and trimethoprim towards the green algae *Chlorella vulgaris*. *J. Hazard Mater.* 308, 179–186. <https://doi.org/10.1016/j.jhazmat.2016.01.041>.
- Caban, M., Stepnowski, P., 2021. How to decrease pharmaceuticals in the environment? A review. *Environ. Chem. Lett.* <https://doi.org/10.1007/s10311-021-01194-y>.
- Chaves, J.E., Kirst, H., Melis, A., 2015. Isoprene production in *Synechocystis* under alkaline and saline growth conditions. *J. Appl. Phycol.* 27, 1089–1097. <https://doi.org/10.1007/s10811-014-0395-2>.
- Cleuvers, M., 2004. Mixture toxicity of the anti-inflammatory drugs diclofenac, ibuprofen, naproxen, and acetylsalicylic acid. *Ecotoxicol. Environ. Saf.* 59, 309–315. [https://doi.org/10.1016/S0147-6513\(03\)00141-6](https://doi.org/10.1016/S0147-6513(03)00141-6).
- dos Santos, C.R., Arcanjo, G.S., de Souza Santos, L.V., Koch, K., Amaral, M.C.S., 2021. Aquatic concentration and risk assessment of pharmaceutically active compounds in the environment. *Environ. Pollut.* 290. <https://doi.org/10.1016/j.envpol.2021.118049>.
- DrugBank, 2023 [WWW Document]. <https://go.drugbank.com/salts/DBSALT000949>.
- Ferrari, B., Mons, R., Vollat, B., Frayssé, B., Paxéus, N., Lo Giudice, R., Pollio, A., Garric, J., 2004. Environmental risk assessment of six human pharmaceuticals: are the current environmental risk assessment procedures sufficient for the protection of the aquatic environment? *Environ. Toxicol. Chem.* 23, 1344–1354. <https://doi.org/10.1897/03-246>.
- Gonçalves, A.L., Ferreira, C., Loureiro, J.A., Pires, J.C.M., Simões, M., 2015. Surface physicochemical properties of selected single and mixed cultures of microalgae and cyanobacteria and their relationship with sedimentation kinetics. *Bioresour. Bioprocess.* 2 <https://doi.org/10.1186/s40643-015-0051-y>.
- Hall, L.W., Anderson, R.D., 1995. The influence of salinity on the toxicity of various classes of chemicals to aquatic biota. *Crit. Rev. Toxicol.* 25, 281–346. <https://doi.org/10.3109/10408449509021613>.
- HELCOM, 2023. Diclofenac. HELCOM Pre-Core indicator report. Online. <https://indicators.helcom.fi/indicator/diclofenac/>.
- Hinners, J., Hofmeister, R., Hense, I., 2015. Modeling the role of pH on Baltic Sea cyanobacteria. *Life* 5, 1204–1217. <https://doi.org/10.3390/life5021204>.
- Isidori, M., Lavorgna, M., Nardelli, A., Parrrella, A., Previtera, L., Rubino, M., 2005. Ecotoxicity of naproxen and its phototransformation products. *Sci. Total Environ.* 348, 93–101. <https://doi.org/10.1016/j.scitotenv.2004.12.068>.
- Izadi, Parnian, Izadi, Parin, Salem, R., Papry, S.A., Magdouli, S., Pulicharla, R., Brar, S.K., 2020. Non-steroidal anti-inflammatory drugs in the environment: where were we and how far we have come? *Environ. Pollut.* 267, 115370 <https://doi.org/10.1016/j.envpol.2020.115370>.
- Kinčl, M., Meleh, M., Veber, M., Vrečer, F., 2004. Study of physicochemical parameters affecting the release of diclofenac sodium from lipophilic matrix tablets. *Acta Chim. Slov.* 51, 409–425.
- Klähn, S., Hagemann, M., 2011. Compatible solute biosynthesis in cyanobacteria. *Environ. Microbiol.* 13, 551–562. <https://doi.org/10.1111/j.1462-2920.2010.02366.x>.
- Klok, C., de Vries, P., Jongbloed, R., Tamis, J., 2017. Literature review on the sensitivity and exposure of marine and estuarine organisms to pesticides in comparison to corresponding fresh water species. *EFSA Support. Publ.* vol. 9 <https://doi.org/10.2903/sp.efsa.2012.en-357>.
- Kolecka, K., Gajewska, M., Caban, M., 2022. From the pills to environment – prediction and tracking of non-steroidal anti-inflammatory drug concentrations in wastewater. *Sci. Total Environ.* 825 <https://doi.org/10.1016/j.scitotenv.2022.153611>.
- Kolecka, K., Gajewska, M., Stepnowski, P., Caban, M., 2019. Spatial distribution of pharmaceuticals in conventional wastewater treatment plant with Sludge Treatment Reed Beds technology. *Sci. Total Environ.* 647, 149–157. <https://doi.org/10.1016/j.scitotenv.2018.07.439>.
- Korelusova, J., Kastovský, J., Jirř, K., 2009. Heterogeneity of the cyanobacterial genus *Synechocystis* and description of a new genus, *Geminocystis* 1. *J. Phycol.* 45, 928–937. <https://doi.org/10.1111/j.1529-8817.2009.00701.x>.
- Koumaki, E., Mamais, D., Noutsopoulos, C., 2017. Environmental fate of non-steroidal anti-inflammatory drugs in river water/sediment systems. *J. Hazard Mater.* 323, 233–241. <https://doi.org/10.1016/j.jhazmat.2016.03.026>.
- Lapworth, D.J., Lopez, B., Laabs, V., Kozel, R., Wolter, R., Ward, R., Vargas Amelin, E., Besien, T., Claessens, J., Delloye, F., Ferretti, E., Grath, J., 2019. Developing a groundwater watch list for substances of emerging concern: a European perspective. *Environ. Res. Lett.* 14 <https://doi.org/10.1088/1748-9326/aaf4d7>.
- Latala, A., Nędzi, M., Stepnowski, P., 2010. Toxicity of imidazolium ionic liquids towards algae. Influence of salinity variations. *Green Chem.* 12, 60–64. <https://doi.org/10.1039/b918355h>.
- Łukaszewicz, P., Białk-Bielińska, A., Dołzonek, J., Kumirska, J., Caban, M., Stepnowski, P., 2018. A new approach for the extraction of tetracyclines from soil matrices: application of the microwave-extraction technique. *Anal. Bioanal. Chem.* 410, 1697–1707. <https://doi.org/10.1007/s00216-017-0815-7>.
- Manallack, D.T., 2007. The pKa distribution of drugs: application to drug discovery. *Perspect. Med. Chem.* 1 <https://doi.org/10.1177/1177391x0700100003>, 1177391x07001000.
- Marin, K., Stirnberg, M., Eisenhut, M., Krämer, R., Hagemann, M., 2006. Osmotic stress in *Synechocystis* sp. PCC 6803: Low tolerance towards nonionic osmotic stress results from lacking activation of glucosylglycerol accumulation. *Microbiology* 152, 2023–2030. <https://doi.org/10.1099/mic.0.28771-0>.
- Mishra, A.K., Tiwari, D.N., Rai, A.N. (Eds.), 2020. *Cyanobacteria: from Basic Science to Applications*. Academic Press.
- Mulkiewicz, E., Wolecki, D., Świacka, K., Kumirska, J., Stepnowski, P., Caban, M., 2021. Metabolism of non-steroidal anti-inflammatory drugs by non-target wild-living organisms. *Sci. Total Environ.* 791 <https://doi.org/10.1016/j.scitotenv.2021.148251>.
- Nazaruk, Z., Korzeniowska, K., 2022. Oder River Environmental Disaster [WWW Document]. URL. <https://wyborcza.pl/7,73236,28796675,oder-river-environmental-disaster-timeline-map.html?disableRedirects=true>.
- OECD, 2011. Test No. 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test, OECD Guidelines for the Testing of Chemicals. <https://doi.org/10.1787/9789264069923-en>. Section 2.
- OECD, 2004. OECD guidelines for the testing of chemicals. 111. Hydrolysis as a function of pH. *Analysis* 1–16.
- Packer, J.L., Werner, J.J., Latch, D.E., McNeill, K., Arnold, W.A., 2003. Photochemical fate of pharmaceuticals in the environment: naproxen, diclofenac, clofibric acid, and ibuprofen. *Aquat. Sci.* 65, 342–351. <https://doi.org/10.1007/s00027-003-0671-8>.
- Pade, N., Hagemann, M., 2015. Salt acclimation of cyanobacteria and their application in biotechnology. *Life* 5, 25–49. <https://doi.org/10.3390/life5010025>.
- Patel, M., Kumar, R., Kishor, K., Mlsna, T., Pittman, C.U., Mohan, D., 2019. Pharmaceuticals of emerging concern in aquatic systems: chemistry, occurrence, effects, and removal methods. *Chem. Rev.* 119, 3510–3673. <https://doi.org/10.1021/acs.chemrev.8b00299>.
- Pinheiro, J.P.S., Windsor, F.M., Wilson, R.W., Tyler, C.R., 2021. Global variation in freshwater physico-chemistry and its influence on chemical toxicity in aquatic wildlife. *Biol. Rev.* 96, 1528–1546. <https://doi.org/10.1111/brv.12711>.
- Pomati, F., Netting, A.G., Calamari, D., Neilan, B.A., 2004. Effects of erythromycin, tetracycline and ibuprofen on the growth of *Synechocystis* sp. and *Lemna minor* 67, 387–396. <https://doi.org/10.1016/j.aquatox.2004.02.001>.
- Rueda, E., Altamira-Algarra, B., García, J., 2022. Process optimization of the polyhydroxybutyrate production in the cyanobacteria *Synechocystis* sp. and *Synechococcus* sp. *Bioresour. Technol.* 356, 127330. <https://doi.org/10.1016/j.biortech.2022.127330>.
- Stebegg, R., Schmetterer, G., Rempel, A., 2019. Transport of organic substances through the cytoplasmic membrane of cyanobacteria. *Phytochemistry* 157, 206–218. <https://doi.org/10.1016/j.phytochem.2018.08.013>.
- Stepnowski, P., Wolecki, D., Puckowski, A., Paszkiewicz, M., Caban, M., 2020. Anti-inflammatory drugs in the Vistula River following the failure of the Warsaw sewage collection system in 2019. *Sci. Total Environ.* 745, 140848 <https://doi.org/10.1016/j.scitotenv.2020.140848>.
- Świacka, K., Maculewicz, J., Kowalska, D., Caban, M., Smolarz, K., Świeżak, J., 2022a. Presence of pharmaceuticals and their metabolites in wild-living aquatic organisms – current state of knowledge. *J. Hazard Mater.* 424, 127350 <https://doi.org/10.1016/j.jhazmat.2021.127350>.
- Świacka, K., Maculewicz, J., Smolarz, K., Caban, M., 2022b. Long-term stability of diclofenac and 4-hydroxydiclofenac in the seawater and sediment microenvironments: evaluation of biotic and abiotic factors. *Environ. Pollut.* 304 <https://doi.org/10.1016/j.envpol.2022.119243>.
- Świacka, K., Michnowska, A., Maculewicz, J., Caban, M., Smolarz, K., 2021. Toxic Effects of NSAIDs in Non-target Species: A Review from the Perspective of the Aquatic Environment * 273. <https://doi.org/10.1016/j.envpol.2020.115891>.
- Szymczycha, B., Borecka, M., Białk-Bielińska, A., Siedlewicz, G., Pazdro, K., 2020. Submarine groundwater discharge as a source of pharmaceutical and caffeine residues in coastal ecosystem: bay of Puck, southern Baltic Sea case study. *Sci. Total Environ.* 713 <https://doi.org/10.1016/j.scitotenv.2020.136522>.
- Toński, M., Dołzonek, J., Stepnowski, P., Białk-Bielińska, A., 2019. Hydrolytic stability of selected pharmaceuticals and their transformation products. *Chemosphere* 236. <https://doi.org/10.1016/j.chemosphere.2019.06.206>.
- Trentin, G., Bertucco, A., Sforza, E., 2019. Mixotrophy in *Synechocystis* sp. for the treatment of wastewater with high nutrient content: effect of CO₂ and light. *Bioproc. Biosyst. Eng.* 42, 1661–1669. <https://doi.org/10.1007/s00449-019-02162-1>.
- UNESCO and HELCOM, 2017. UNESCO Emerg. Pollut. Water Ser. – No. 1. In: *Pharmaceuticals in the Aquatic Environment of the Baltic Sea Region in the Aquatic Environment of the Baltic Sea Region - A Status Report*. UNESCO Publ, Paris.
- Wesslander, K., Omstedt, A., Schneider, B., 2010. Inter-annual and seasonal variations in the air-sea CO₂ balance in the central Baltic Sea and the Kattegat. *Cont. Shelf Res.* 30, 1511–1521. <https://doi.org/10.1016/j.csr.2010.05.014>.
- Xiong, J.Q., Kurade, M.B., Jeon, B.H., 2018. Can microalgae remove pharmaceutical contaminants from water? *Trends Biotechnol.* 36, 30–44. <https://doi.org/10.1016/j.tibtech.2017.09.003>.
- Zepernick, B.N., Gann, E.R., Martin, R.M., Pound, H.L., Krausfeldt, L.E., Chaffin, J.D., Wilhelm, S.W., 2021. Elevated pH Conditions Associated With Microcystis spp. Blooms Decrease Viability of the Cultured Diatom *Fragilaria crotonensis* and Natural Diatoms in Lake Erie. *Front. Microbiol.* 12, 1–12. <https://doi.org/10.3389/fmicb.2021.598736>.

Publikacja – P2

Kwidzińska Klaudia, Hryniewicka Marta, Caban Magda*, Biogenic volatile organic compounds produced by two unicellular and two filamentous cyanobacteria after a high light shock, Atmospheric Environment, 2025, 353, 121252, DOI:10.1016/j.atmosenv.2025.121252.



Biogenic volatile organic compounds produced by two unicellular and two filamentous cyanobacteria after a high light shock

Klaudia Kwidzińska^a, Marta Hryniewicka^b, Magda Caban^{a,*}

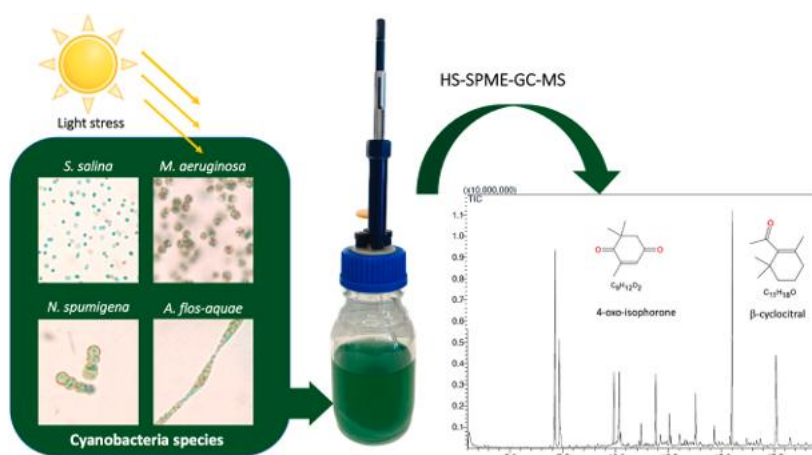
^a University of Gdansk, Faculty of Chemistry, Department of Environmental Analysis, ul. Wita Stwosza 63, 80-308, Gdańsk, Poland

^b University of Białystok, Faculty of Chemistry, Department of Analytical and Inorganic Chemistry, ul. K. Ciołkowskiego 1K, 15-245, Białystok, Poland

HIGHLIGHTS

- Analysis of biogenic volatile compounds (BVOCs) was performed by HS-SPME-GC/MS.
- Four tested cyanobacteria produce volatile compounds as an effect of light stress.
- Detected chemicals were 6C–13C molecules (alcohol, aldehyde and terpenes).
- Unknown substance noted in three cyanobacteria cultures was Sulfur-based molecule.
- 4-oxo-isophore was detected for the first time as BVOC of cyanobacteria.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:

Biogenic volatile organic compounds
Cyanobacteria
Volatilome
Nor-carotenoids
4-Oxo-isophore

ABSTRACT

The emission of biogenic volatile organic compounds (BVOCs) is increasing due to the effects of climate change, and the related increases in biomass production and phytoplankton blooms. The Baltic Sea is subject to increased pollution and average temperature rises, with cyanobacterial blooms happening more often. For the majority of Baltic cyanobacteria, there is no information about BVOCs production. Thereby, in our study, we decided to qualitatively analyze these chemicals in four species common for the Baltic Sea and its lagoons: *Synechocystis salina*, *Microcystis aeruginosa*, *Nodularia spumigena*, and *Aphanizomenon flos-aquae*. A high light stressor was used as a trigger for the production of BVOCs in monocultures. Depending on the species, two to seven compounds were identified from groups of cyclic and aliphatic alkanes, alcohols, aldehydes, and terpenes. β -cyclocitral (representative of nor-carotenoids) was obtained in *M. aeruginosa*, and for the first time, it was detected as being produced by *N. spumigena*. Two unidentified compounds were also found. For one of them, the analysis of mass spectra showed that it could be a sulfur-based compound. The study presents for the first time BVOCs that are produced by *S. salina*, *N. spumigena*, and *A. flos-aquae* after light stress.

* Corresponding author.

E-mail address: magda.caban@ug.edu.pl (M. Caban).

<https://doi.org/10.1016/j.atmosenv.2025.121252>

Received 3 June 2024; Received in revised form 14 October 2024; Accepted 16 April 2025

Available online 17 April 2025

1352-2310/© 2025 Elsevier Ltd. All rights are reserved, including those for text and data mining, AI training, and similar technologies.

1. Introduction

Cyanobacteria are the oldest oxygen-evolving photoautotrophic Gram-negative bacteria. They are also the major biomass producers in both aquatic and terrestrial ecosystems, together with plants (Bar-On et al., 2018; Häder et al., 2007). The versatility of cyanobacterial life forms and productivity makes them especially useful for biotechnology (Zinicovscaia and Cepoi, 2016). Phytoplankton is a source of various active molecules and biogenic volatile organic compounds (BVOCs), among others. BVOCs are treated as secondary metabolites, whose syntheses are activated through biotic or abiotic stresses (Loreto and Schnitzler, 2010; Zuo, 2019), as well as constitutive chemicals (Niederbacher et al., 2015). Generally, in plants, BVOCs function as signaling molecules or as a biochemical defense method (Leflaive and Ten-Hage, 2007; Saha, 2022; Zuo et al., 2017). BVOCs also play the role of lowering oxidative stress through the scavenging of reactive oxygen species. Furthermore, the BVOCs released from the cyanobacteria, especially the ruptured cells, exhibit a repelling effect on herbivores (Zuo, 2023). The nutrient source was found to be an important factor for the modulation of BVOCs production by selected cyanobacteria (Zuo et al., 2017). Furthermore, BVOC, similarly to several other metabolites production is regulated by the temperature and light (Mohanty et al., 2022). The light is generally one of the main trigger of blooms, but after the threshold level of light the cyanobacteria cells are experiencing stress, which reduce its functions and growth (Muhetaer et al., 2020; Zhang et al., 2015).

Volatile organic chemicals (VOCs) emissions have continuously increased, and their flux on a global scale has been dominated by natural VOCs (Duan et al., 2023). BVOCs constitute 80–90 % of total VOCs emissions, with isoprene and monoterpenes providing the highest contributions (Duan et al., 2023; Peñuelas and Llusà, 2003). Monoterpene production has increased, especially over the last few years (Boy et al., 2022; Dayan et al., 2020). Lea-Smith et al. (2015) attempted to quantify the production of C15 and C17 aliphatic alkanes from *Prochlorococcus* and *Synechococcus* over the open ocean, and they extrapolated that the production of these two hydrocarbons alone may range from 308 to 771 million tons every year (Lea-Smith et al., 2015). These numbers are significant, thereby the chemistry of BVOCs in the troposphere (Atkinson and Arey, 2003) and its contributions to air quality and global warming (Dicke and Loreto, 2010) are under investigation. The BVOCs are removed from the air through oxidation (Mahilang et al., 2021), which can lead to the formation of aldehydes and radicals (Rossell, 2019). Furthermore, BVOCs contribute to the production of secondary organic aerosols (SOAs), which significantly affect Earth's radiation balance (Bianchi et al., 2019; Dayan et al., 2020; Duan et al., 2023; Mahilang et al., 2021). BVOCs presence can also affect to a localized increase in photochemical ozone pollution (Curci et al., 2009) or a decrease in ozone due to its fast oxidation via these molecules (Calfapietra et al., 2013). The presence of BVOCs contributes to increasing the lifetime of methane (an important greenhouse gas) because the BVOCs rapidly consume hydroxyl radicals (Peñuelas and Llusà, 2003). It has been estimated that BVOCs can have both negative and positive feedback on climate change (Peñuelas and Llusà, 2003; Peñuelas and Staudt, 2010; Yáñez-Serrano et al., 2020). The emission of BVOCs from marine sources is influenced by observable increase of seas temperatures, which dramatically changes the phytoplankton community in some regions (Dayan et al., 2020). Production of BVOC during cyanobacterial blooms need to be noted and quantified as an additional source of organic chemicals in atmosphere (Huang et al., 2018; Lea-Smith et al., 2015; Liu et al., 2021; Zuo, 2023). Unfortunately, knowledge of BVOC produced by cyanobacteria is limited to selected species and compounds. Furthermore, performing only targeted chemical analyses make it impossible to expand knowledge about new volatile compounds produced by cyanobacteria. Thereby, carrying out “volatilome” type of chemical analysis is urgently needed (Meredith and Tfaily, 2022; Saha, 2022; Steinke et al., 2002).

In this study, we decided to investigate the BVOCs produced by common species of cyanobacteria. The trigger for BVOCs production was light shock, which is a known stressor for cyanobacteria (Hudnell, 2008) and can promote BVOCs production (Zuo, 2019, 2023). We decided to test four cyanobacterial species living in or close to the Baltic Sea, because this sea is highly impacted by blooms each summer period (Munkes et al., 2021; Olofsson et al., 2020), when the light flux is high. Furthermore, to our best knowledge, there is data about BVOCs production for only one species. Expanding knowledge about compounds produced by cyanobacteria will allow us to examine their presence during actual environmental blooms in the future and then assess the impact on changes in atmospheric chemistry and role in ecology (Saha, 2022; Steinke et al., 2002).

2. Experiment

2.1. Chemicals

ISO17943 Odor Mix (geosmin, 2-methylisoborneol, 2-ethyl-4-methyl-1,3-dioxolane, and 2-ethyl-5,5-dimethyl-1,3-dioxane), and analytical grade standards of limonene, β -cyclocitral, β -ionone, 4-oxoisophorone, and menthone were purchased from Sigma Aldrich (Germany). Organic solvents (methanol and acetone for standard dissolutions) were supplied by POCH (Poland). The medium BG11 was prepared from a concentrate purchased from Sigma-Aldrich (Germany). The water for the medium was autoclaved (20 min at 121 °C, HMC-Europe HV-50, Germany).

2.2. Sources of cyanobacterial strains

Four cyanobacterial monocultures came from the Culture Collection of Northern Poland (CCNP, Division of Marine Biotechnology, University of Gdańsk, Poland). *Synechocystis salina*, CCNP 1104 (order of Synechococcales), was isolated from the Gulf of Gdańsk in 2006 (Mazur-Marzec et al., 2015). *Microcystis aeruginosa*, CCNP 1101 (order of Chroococcales), was isolated from Vistula Lagoon in 2005. It contains freshwater and brackish water, with cosmopolitan species being observed in the coastal waters and lagoons. *Aphanizomenon flos-aquae*, CL2019/03 (order of Nostocales), was isolated from Curonian Lagoon in 2018. *Nodularia spumigena*, CCNP1401 (order of Nostocales), was isolated from the Gulf of Gdańsk in 1997, causes massive blooms in the Baltic Sea and produces the toxin nodularin. *S. salina* and *M. aeruginosa* are unicellular, while *N. spumigena* and *A. flos-aquae* are filamentous.

2.3. Cyanobacterial growing conditions and exposure to high light

Cyanobacteria were grown in a photo-chamber with an LED source of white light at a PAR (photosynthetically active radiation) intensity of $35 \pm 5 \mu\text{mol m}^{-2} \text{s}^{-1}$ (or $\text{Watts m}^{-2} \text{h}^{-1}$) (Biogenet, model Fito700, Poland). This light provides optimal growth under our laboratory conditions (a duplication of culture density every 24 h). An experimental study has shown that at about $200 \mu\text{mol m}^{-2} \text{s}^{-1}$, Baltic *Synechococcus* show growth saturation (Śliwińska-Wilczewska et al., 2017); thereby, we decided to test high light for our species. Generally, cyanobacteria can grow even at a PAR that is lower than $5 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Śliwińska-Wilczewska et al., 2017), and they can even adapt to $2000 \mu\text{mol m}^{-2} \text{s}^{-1}$ when preceded by acclimation using several intermediate stages (Kana and Glibert, 1987). The studies on the cyanobacteria show that they can change the structural features in their photosynthetic system over a relatively short term as an effect of high light (Dann et al., 2021; Dobson et al., 2021). We decided to increase the light by about 5-fold to observe the effect of increased light, but with no acute negative impact on the cells. The control of eight light channels was possible in the LED panel (Table S1, Supplementary Information). Cultures were kept in glass bottles of 100 mL volume (the volume of the culture was 60 mL) with continuous aeration and air purification using an activated

carbon filter (FW600, Wanza, Poland), and with continuous irradiation in an incubator at 23 °C. All of the studied cyanobacteria were cultured in BG11 medium (pH 7.5). To maintain sterile conditions, a UVC/T-M-AR laminar chamber (SIA Biosan, Latvia) was used for any manipulation of the culture. For high light stress, the culture was exposed to $195 \pm 5 \mu\text{mol m}^{-2} \text{s}^{-1}$ for 4 h by increasing the intensity of the light channels (Table S1, Supplementary Information). Other conditions of cultivation were the same as for the stock culture. The light stress was tested in duplicate, using fresh cultures each time.

The growth of each culture was measured using its optical density (OD) at wavelengths of 630 nm and 680 nm (Genesys150, UV-Visible Spectrophotometer, Thermo Scientific, USA) before light stress, directly after exposure to high light (4 h), and after the regeneration of the culture under normal light (24 h).

2.4. Identification of volatile chemicals via HS-SPME-GC-MS

Volatile organic compounds were extracted using head-space solid phase microextraction (HS-SPME). The SPME fiber adsorbent phase was a mixed mode (DVB/CAR/PDMS, 50/30 μm , 2 cm on stableflex, 24 Ga) supplied by Supelco (Germany). The fibers were cleaned via pre-incubation in a hot injector before sampling in HS. The Odor Mix added to deionized water was applied as a reference substance to check the SPME system performance, and all the standards were detected. The instrument detection limits (IDLs) for analytes in the Odor Mix were determined via the injection of standards at known amounts in the organic solvent, and these were 0.5–2.0 ng per injection to the GC-MS.

HS-SPME was performed in duplicate directly in the culture bottle with the growing cyanobacteria via the immersion of the fiber into the air above the liquid. The three rounds of exposition in HS were tested – at 1 h, 5 h, and 24 h, for comparisons of the sampling time. The fiber needle was introduced through a silicon septum of 4-port cap bottles. After exposure, the fiber was returned to the needle. The needle was transported in a nitrogen-filled vial between the biological and instrumental laboratories. For the exposition, aeration with air was stopped to be sure that any peaks found in the chromatograms were products only of the culture's metabolism. In addition, this improves the detection of organics (Johnson et al., 2016). A blank sample (BG11 medium, no cyanobacteria) was also performed. It was found that the heating and acidification of water for the SPME extraction was responsible for the production of β -cyclocitral in cyanobacteria cultured (Yamashita et al., 2020). Therefore, in our study, we have not used an increase in temperature for SPME. Furthermore, we have not added NaCl, which is common during SPME extraction, to avoid provoking additional stress to the culture. The ambient temperature of the HS extraction caused the need for a prolonged extraction of at least 5 h.

The GC-MS analysis was performed on the Shimadzu QP2010-SE instrument. The Merlin septum designed for SPME was used in an injector. The capillary column was DB-624 (20 m, 0.18 mm, 1.00 μm , Agilent). Helium at 100 kPa was used as a carrier gas. A splitless injection (a sampling time of 1 min, then at a ratio of 40:1) was used. The injector temperature, and interface and EI (70 eV) source temperatures were 200 °C, 235 °C, and 200 °C, respectively. The temperature gradient was as follows: a starting temperature of 40 °C, a rate of 10 °C min^{-1} up to 90 °C, a rate of 16 °C up to 230 °C, and finally a 5 min hold at 230 °C (a total run time of 19.75 min). The solvent cut time was 3 min. Mass spectra were recorded at an m/z range of 35–600, with a speed of three scans per second. The NIST library of mass spectra version 2.0 was used. For compound qualification, the average mass spectrum of each peak was extracted and cleaned of noise. The match between the found spectra and library spectra was presented in the results. For the Odor Mix compounds, limonene, β -cyclocitral, β -ionone, 4-oxoisophorone, and menthone, the retention times were checked for further identification in the real samples.

The free software Mass Spec Calculator Professional 5.1 (Quadtech Associates, Inc.) was used for interpretation of unknown GC/MS spectra

of one of found substance. This program is designed to assist in the interpretation of mass spectra from small molecules (~1000 Da or less). The proposed structure was drawn in software and fragmented by an automatic algorithm using generally known fragmentation principles. Next, the simulated spectrum was compared to the real one. This program was also used for isotope distribution calculation.

3. Results and discussion

3.1. The effect of high light on cyanobacterial growth

The ODs at wavelengths 630 nm and 680 nm corresponded to carotenoid and phycobilin peaks on the UV-Vis spectrum, which were checked by recording the spectra within 300–800 nm. Table 1 presents the mean ($n = 2$) percentage changes in the optical density on these two specific wavelengths before stress, just after the end of stress, and after 24 h regeneration under normal light. For all cyanobacteria, the OD values increased slightly (1–14 %) after 4 h stress. After a 24 h regeneration period under normal light, the *S. salina* density was lower compared to the end of the stress phase. The same was observed for *N. spumigena*. The density of the *M. aeruginosa* culture increased, but this extent was lower compared to the standard culture growth rate of about 1.5–2.0 (a 150–200 % increase in OD within 24 h). This observation suggests that after 4 h light stress, a negative impact on growth was not observed, but after the 24 h period, it was measurable for both the carotenoid and phycobilin concentrations. Probably, after the light stress, the cultures needed the regeneration time, and the energy went not into cell division, but into the treatment of broken cell compartments. Nevertheless, Table 1 presents the mean from only two repetitions of the experiment. Notably, the intensity of the light applied had a stressor value, which was the intention of this experiment. For comparison, the full sunlight is about $2200 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Ritchie, 2010), but only 34 % can be used for photosynthesis, and even lower part in the water. For cyanobacteria the optimal light intensity depends on temperature and species (Muhetaer et al., 2020; Sabour et al., 2009). The tested cyanobacteria were cultivated for more than two years in $35 \pm 5 \mu\text{mol m}^{-2} \text{s}^{-1}$, and the increase of light to about $200 \mu\text{mol m}^{-2} \text{s}^{-1}$ is for sure light stress. The 4 h long stress was performed, by somehow close the study to a real environmental condition in the midday period. Can be suspected that the compound eventually detected after applied light stress can be produced by wild-living cyanobacteria. Nevertheless, in environment cyanobacteria have possibilities to escape from an unfavorable high light by changing buoyancy (Bormans et al., 1999).

In one of the previous works, the control *M. aeruginosa* was growing at $50 \mu\text{mol m}^{-2} \text{s}^{-1}$, and the inhibition of growth was measured at 500 and $1000 \mu\text{mol m}^{-2} \text{s}^{-1}$, or a 10-fold higher light intensity under stress conditions (Zheng et al., 2020). The inhibition of growth in the aforementioned report was connected with increased cyclocitral production, and thereby, the proposed function was for quenching the reactive oxygen species. In our study, cyanobacteria were subjected to 5.6 times higher light compared to control.

Table 1

The percentage change in optical densities at two wavelengths (630 and 680 nm) for four cyanobacteria species' cultures measured before light stress, after stress and after a 24 h regeneration period (mean from two repetition, 100% for control).

Measurement set	After 4 h-long light stress compared to start value		24 h after light stress compared to end of stress	
Wavelength [nm]	630	680	630	680
<i>S. salina</i>	114 %	113 %	94 %	93 %
<i>M. aeruginosa</i>	103 %	103 %	114 %	115 %
<i>N. spumigena</i>	102 %	103 %	91 %	91 %
<i>A. flos-aquae</i>	108 %	101 %	105 %	98 %

3.2. The volatile organics produced by cyanobacteria

In the control cultures not subjected to high light, no BVOCs were noted. This means that under optimal growing conditions, the production of BVOCs was limited to an amount that could not be detected when using the applied technique. Similarly, no BVOC was detected in algal control culture subjected to grazing stress (Reese et al., 2019). The chromatogram of the blank sample was reached in silicone-type compounds, which came from the septums of the growing bottles and the PDMS sorbent, but there were no compounds other than silicones. The high number of chromatographic peaks from experimental setup was presented also by other researchers who track volatile production by phytoplankton (Koteska et al., 2022; Reese et al., 2019). Figs. S1–S5 (SI) present the fragments of overlapping TICs (total ion chromatograms) of the analytical grade standards of β -ionone (Fig. S1), menthone (Fig. S2),

β -cyclocitral (Fig. S3), 4-oxo-isophorone (Fig. S4), and limonene (Fig. S6), and TICs from the sampled HS of cyanobacterial cultures. The retention times and matches of the mass spectra to the library (Table S2) were the same for the standards and the detected compounds.

Fig. 1 presents the chemical structures of substances found after high light exposure and 5 h sampling in HS. Table S2 (Supplementary Material) presents the retention time and library match (%) for the detected substances, and the same parameters for the analytical standards, if obtained. In each bottle with a cyanobacterial culture after light stress, BVOCs were detected, and samples of *S. salina* gave the highest numbers of compounds (seven). It was noted that not all substances were found in two repetitions of the light shock test. The reason for this could be the lower density of the starting culture in the first set. Furthermore, the signal to noise of chromatographic peak was set to 10, and some of the peaks do not reached this requirement. Similarly, low reproducibility of

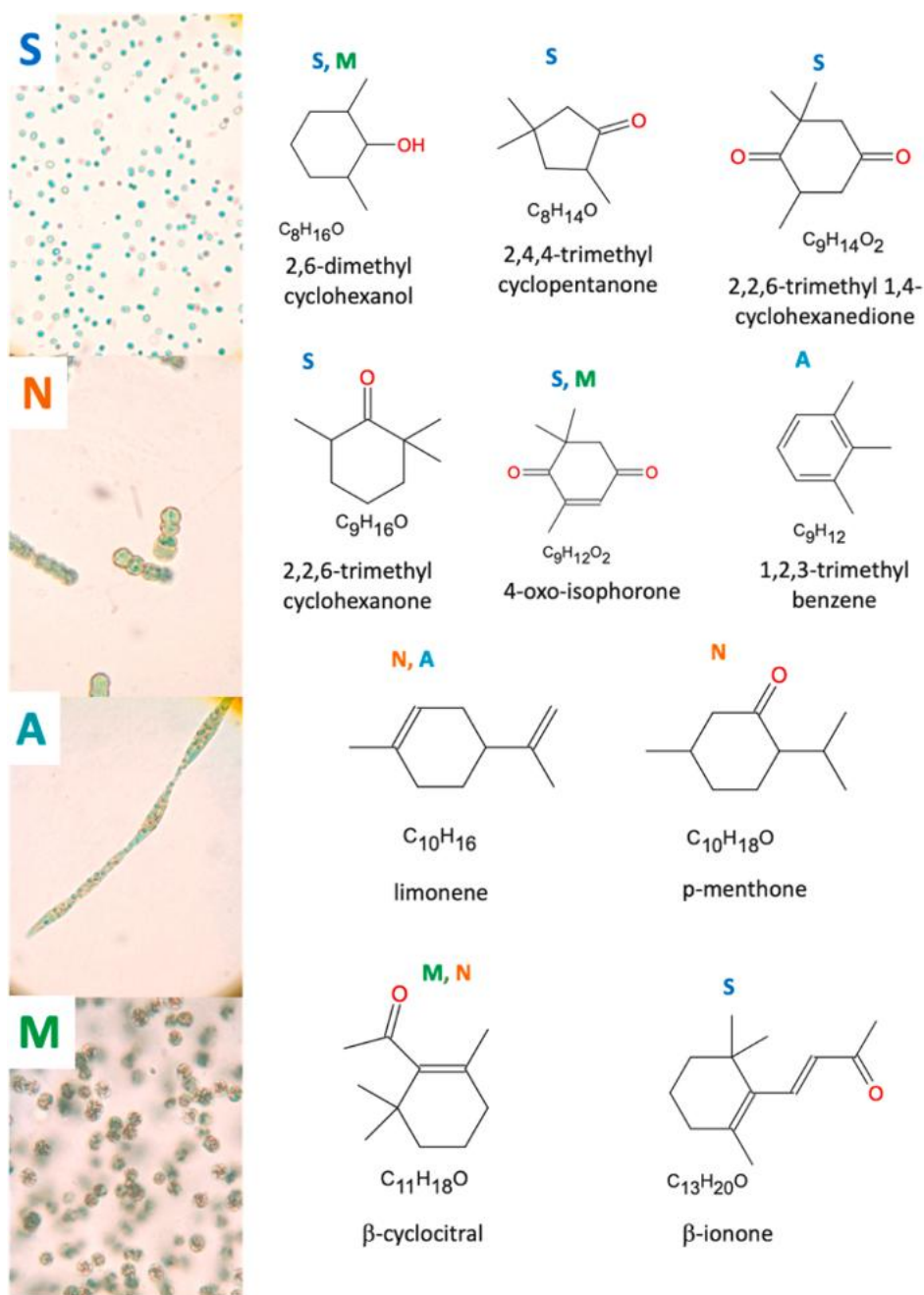


Fig. 1. The structures of biogenic volatile organic compounds (BVOCs) produced by cyanobacteria *Synechocystis salina* (S), *Microcystis aeruginosa* (M), *Aphanizomenon flos-aquae* (A), and *Nodularia spumigena* (N) after high light stress.

SPME-GC/MS analysis was found by (Reese et al., 2019), who analyze the algal BVOC stressed by grazing, and (Koteska et al., 2022) who analyze volatiles produced by dinoflagellate. Several unidentified compounds were noted in our experiments. The components of the Odor Mix were not detected in the cyanobacterial cultures. For comparison, geosmin and 2-methylisoborneol were detected in the cyanobacterial species *Phormidium retzii*, *Microcoleus vaginatus* (Alghanmi et al., 2018), *Anabaena circinalis*, and *Phormidium amoenum* (Li et al., 2012).

The selected detected substances are nor-carotenoids that can be both the products of synthesis and/or the degradation of pigments. There are several points of scientific evidence supporting this statement. Nor-carotenoids are derived from the oxidative cleavage of carotenes and xanthophylls (e.g., β -carotene and zeaxanthin) (Watson et al., 2016). The degradation of carotenoids contributes to odor-like substances in water, such as β -cyclocitral, β -ionone, and geranylacetone (Ikawa et al., 2001; Ozaki et al., 2008; Zuo, 2019) (the two first noted in our study). It has been reported that β -cyclocitral can be produced by cyanobacteria under high irradiation and that it is the product of β -carotene cleavage (Zuo, 2023). The tobacco-odor metabolite β -cyclocitral, 6-methylhept-5-en-2-one, and β -ionone are the most commonly reported nor-carotenoids, and they are often associated with cyanobacterial blooms. The reactions of nor-carotenoids syntheses are catalyzed by carotenoid cleavage dioxygenases (CCDs), and they are important in the photoadaptive regulation of pigments (Ozaki et al., 2006). Carotenoids are also vulnerable to non-specific oxidation by other catalytic enzymes (lipoxygenases and peroxidases), reactive oxygen species, UV, and other stressors, but these processes typically do not generate significant extracellular levels of nor-carotenoids during vigorous growth (Peter et al., 2009).

β -cyclocitral was detected in *N. spumigena* and *M. aeruginosa*. Previously, this compound was stated to be characteristic for *M. aeruginosa*, and it was found only after cell disturbance, density stress, or in aged cultures (Arii et al., 2021; Jüttner et al., 2010; Ozaki et al., 2008). Little of this aldehyde is produced externally by intact cells (Zuo, 2023). The presence of β -cyclocitral suggests that the integrity of some cells was broken (Fujise et al., 2010). This chemical can be generated by enzymatic or non-enzymatic oxidation by reactive oxygen species of β -carotene (Havaux, 2020). β -cyclocitral biochemistry and gene regulation pathways have been investigated (Watson et al., 2016). It regulates nuclear gene expression through several signaling pathways, leading to stress acclimation. Although gene-encoded CCDs were found in other cyanobacteria, until 2016, β -cyclocitral was noted only for *M. aeruginosa*. Thus, this work presents for the first time the presence of β -cyclocitral in *N. spumigena*. Interestingly, a genome analysis shows that geosmin can be produced by Brazilian *N. spumigena*, but not by the Baltic and Japanese strains (Popin et al., 2020).

In cyanobacteria, β -cyclocitral functions as an inhibitor of competing microalgae and as a repellent against grazers, but it can be found in various organisms, plants, lichens, and mosses. An increase in light intensity was already found as a factor that stimulated the production of β -cyclocitral by *Microcystis* (Aparecida et al., 2022).

In addition to β -cyclocitral, several other small cyclic or aliphatic compounds were detected (Fig. 1). They were most probably products of the carotenoids' degradation, as well as β -cyclocitral. Cyanobacteria have several other carotenoids alongside β -carotene (Jaiswal et al., 2018), and most of them play a photoprotection role. The compound 2-methylbutanol was found to be produced as a β -cyclocitral oxidation product (Fujise et al., 2010; Tomita et al., 2016). The compound 4-oxo-isophorone (2,6,6-trimethyl-2-cyclohexene-1,4-dione) is a key intermediate in the synthesis of carotenoids and flavoring agents, which suggests that cyanobacteria start a healing process after exposure to high light. It was found as a degradation product of paprika (Krammer et al., 2001), and is used as a product of *M. aeruginosa* and *S. salina* metabolism. β -ionone was detected in *S. salina* culture. This compound is a commonly detected product of carotenoids degradation in plants (Zuo, 2019). The β -cyclocitral, 2,6-trimethyl-cyclohexanone, and β -ionone,

among other similar chemicals, were detected as the marker of algal culture crash (Reese et al., 2019), and produced in low amount by selected dinoflagellates (Koteska et al., 2022).

The terpenes menthone and limonene that were detected in *N. spumigena* were previously found in fungi and various plants (Grabarczyk et al., 2020). Limonene has also been previously found in *Microcystis flos-aquae* (Xu et al., 2017).

To our best knowledge, this is the first article presenting the production of volatiles by *N. spumigena*, *S. salina*, and *A. flos-aquae* after light shock. The nitrogen-based volatile compounds were detected in *A. flos-aquae*, but from lyophilized cells (Dembitsky et al., 2000). In one of the old reports (1988), geosmine and phytol were detected in *A. flos-aquae* after distillation of the medium (Tsuchiya and Matsumoto, 1988).

The mass spectrum of the first unidentified molecule (M^+ m/z 166) is presented in Fig. 2A. The reconstruction of chemical structure from the mass spectra shows that it could be a sulfur-based compound, the structure of which is proposed in Fig. 2C. The characteristic isotopic ion for sulfur-containing molecules at $M+2$ was present. The real and simulated isotope distribution for $C_4H_6O_3S_2$ is presented in Fig. S6 (SI). The ratios of $M+1$ and $M+2$ for the registered spectrum (7 % and 12 %, respectively) are close to the ideal distribution (6 % and 10 %) for a simulated isotopic distribution. In Fig. 2B, the simulated mass spectrum (Mass Spec Calculator Professional 5.1, Quadtech Associates, Inc.) was presented, with good agreement between the obtained peaks and relative intensities compared with the real mass spectrum of an unknown compound. The chromatographic peak of this molecule was found in the *S. salina*, *M. aeruginosa*, and *A. flos-aquae* cultures. Cyanobacteria are known to produce volatile organic sulfur compounds, and DMS is one of the most commonly detected compounds (Watson et al., 2016). In our study, DMS was not found. Marine phytoplankton are capable of synthesizing more complex volatile organic sulfur compounds (VOSCs) (Bentley and Chasteen, 2004); for example, dimethyl-sulfoniopropionaldehyde and organo-sulfur acid. The dimethylsulfoniopropionate (MDSP) was found to be produced by marine bacteria and phytoplankton as osmoprotectant (Vila-Costa et al., 2006; Yoch, 2002). A database using SMILE for unknown compounds ($O=CSSCCC(=O)O$) showed no results. The closest compound found was 3,3'-dithiodipropionic acid (CAS 1119-62-6), which is found in magnolia-type trees (Natural Product ID: NPC16947 in the database NPASS), suggesting that similar compounds can be found as an effect of biological activity. What interesting, unidentified compound with mass 166 m/z was detected in two laboratory cultivated dinoflagellates species (Koteska et al., 2022).

The mass spectrum of the second unknown compound is presented in Fig. S7 (SI). This spectrum shows a fragmentation pattern that is close to limonene, the spectrum of which is also presented in Fig. S7. With the information from low-resolution MS, we are unable to confirm that this compound can be categorized as a terpene-like molecule.

4. Conclusions

An inventory of the BVOCs produced by four common cyanobacterial species in the Baltic Sea was performed. High light was found to be a trigger, while control cultures (normal light) showed no BVOCs production. The selected substances are nor-carotenoids, which means that the light shock hurt the photosynthetic apparatus, and a biochemical response was activated. The growth of each culture was stopped by light stress, which means that the cyanobacteria changed the direction of their metabolism to heal themselves from shock. The compounds identified by SPME-GCMS had between 6 and 13 carbon atoms. The β -cyclocitral characteristic for *M. aeruginosa* was also detected in *N. spumigena*. Several of the found BVOCs have never previously been reported. One of the unknown compounds was most likely to be a sulfur-based molecule.

The study reports on the variety of BVOCs produced, with most of

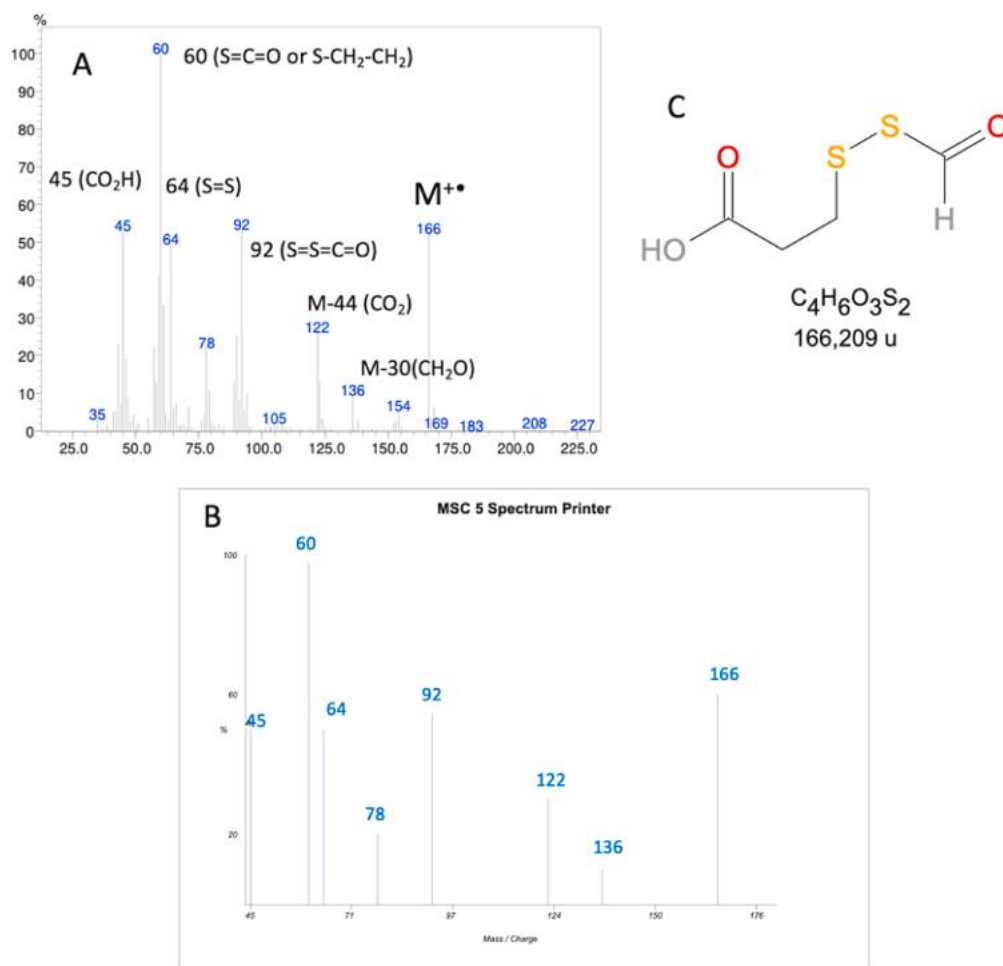


Fig. 2. The mass spectra of unknown compounds found using HS-SPME-GC-MS after high light stress in tested cyanobacteria (A), the simulated mass spectrum (Mass Spec Calculator Professional 5.1, Quadtech Associates, Inc.) (B), and the proposed chemical structure (C).

them being related to photosynthetic system stress. Such compounds can be released in high amounts during or after cyanobacterial blooms and are potential stress signals. The Baltic Sea is subjected to an increased number of cyanobacteria blooms, which holistic impact can be only determined knowing the volatiles compound emission in addition to water soluble metabolites, such as toxins. The quantification and probable effects of BVOC on air quality (the production of radicals, aerosols, and odors) and cyanobacteria ecology need to be investigated in the future.

CRediT authorship contribution statement

Klaudia Kwidzińska: Writing – review & editing, Writing – original draft, Visualization, Investigation. **Marta Hryniewicka:** Writing – original draft, Visualization, Investigation. **Magda Caban:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Magda Caban reports financial support was provided by National Science Centre Poland.

Acknowledgments

Financial support was provided by the National Science Center (Poland) under grants UMO-2019/35/B/NZ9/00253. The authors would like to thank the Culture Collection of Northern Poland (CCNP, Institute of Oceanography, University of Gdańsk, Poland) for providing cyanobacterial monocultures.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.atmosenv.2025.121252>.

Data availability

Data will be made available on request.

References

- Alghanmi, H.A., Alkam, F.M., Al-Tae, M.M., 2018. Effect of light and temperature on new cyanobacteria producers for geosmin and 2-methylisoborneol. *J. Appl. Phycol.* 30, 319–328. <https://doi.org/10.1007/s10811-017-1233-0>.
- Aparecida, J., Moretto, S., Nathane, P., Freitas, N. De, Almeida, É.C. De, Altarugio, L.M., Vieira, S., Fiore, M.D.F., Pinto, E., 2022. Effects of different cultivation conditions on the production of β -cyclocitral and β -ionone in *Microcystis aeruginosa*. *BMC Microbiol.* 22, 78. <https://doi.org/10.1186/s12866-022-02473-6>.
- Arii, S., Yamashita, R., Tsuji, K., Tomita, K., Hasegawa, M., Bober, B., Harada, K., 2021. Differences in susceptibility of cyanobacteria species to lytic volatile organic compounds and influence on seasonal succession. *Chemosphere* 284, 131378. <https://doi.org/10.1016/j.chemosphere.2021.131378>.

- Atkinson, R., Arey, J., 2003. Gas-phase tropospheric chemistry of biogenic volatile organic compounds: a review. *Atmos. Environ.* 37, 197–219. [https://doi.org/10.1016/S1352-2310\(03\)00391-1](https://doi.org/10.1016/S1352-2310(03)00391-1).
- Bar-On, Y.M., Phillips, R., Milo, R., 2018. The biomass distribution on Earth. *Proc. Natl. Acad. Sci. USA* 115, 6506–6511. <https://doi.org/10.1073/pnas.1711842115>.
- Bentley, R., Chasteen, T.G., 2004. Environmental VOCs – formation and degradation of dimethyl sulfide, methanethiol and related materials. *Chemosphere* 55, 291–317. <https://doi.org/10.1016/j.chemosphere.2003.12.017>.
- Bianchi, F., Kurtén, T., Riva, M., Mohr, C., Rissanen, M.P., Roldin, P., Berndt, T., Crounse, J.D., Wennberg, P.O., Mentel, T.F., Wildt, J., Junninen, H., Jokinen, T., Kulmala, M., Worsnop, D.R., Thornton, J.A., Donahue, N., Kjaergaard, H.G., Ehn, M., 2019. Highly oxygenated organic molecules (hom) from gas-phase autoxidation involving peroxy radicals: a key contributor to atmospheric aerosol. *Chem. Rev.* 119, 3472–3509. <https://doi.org/10.1021/acs.chemrev.8b00395>.
- Bormans, M., Sherman, B.S., Webster, I.T., 1999. Is buoyancy regulation in cyanobacteria an adaptation to exploit separation of light and nutrients? *Mar. Freshw. Res.* 50, 897–906. <https://doi.org/10.1071/MF99105>.
- Boy, M., Zhou, P., Kurtén, T., Chen, D., Xavier, C., Clusius, P., Roldin, P., Baykara, M., Pichelstorfer, L., Foreback, B., Bäck, J., Petäjä, T., Makkonen, R., Kerminen, V.M., Pihlatie, M., Aalto, J., Kulmala, M., 2022. Positive feedback mechanism between biogenic volatile organic compounds and the methane lifetime in future climates. *npj Clim. Atmos. Sci.* 5. <https://doi.org/10.1038/s41612-022-00292-0>.
- Calfapietra, C., Fares, S., Manes, F., Morani, A., Sgrigna, G., Loreto, F., 2013. Role of Biogenic Volatile Organic Compounds (BVOC) emitted by urban trees on ozone concentration in cities: a review. *Environ. Pollut.* 183, 71–80. <https://doi.org/10.1016/j.envpol.2013.03.012>.
- Curci, G., Beekmann, M., Vautard, R., Smiatek, G., Steinbrecher, R., Theloke, J., Friedrich, R., 2009. Modelling study of the impact of isoprene and terpene biogenic emissions on European ozone levels. *Atmos. Environ.* 43, 1444–1455. <https://doi.org/10.1016/j.atmosenv.2008.02.070>.
- Dann, M., Ortiz, E.M., Thomas, M., Guljamow, A., Lehmann, M., Schaefer, H., Leister, D., 2021. Enhancing photosynthesis at high light levels by adaptive laboratory evolution. *Nat. Plants* 7, 681–695. <https://doi.org/10.1038/s41477-021-00904-2>.
- Dayan, C., Fredj, E., Misztal, P.K., Gabay, M., Guenther, A.B., Tas, E., 2020. Emission of biogenic volatile organic compounds from warm and oligotrophic seawater in the Eastern Mediterranean. *Atmos. Chem. Phys.* 20, 12741–12759. <https://doi.org/10.5194/acp-20-12741-2020>.
- Dembitsky, V.M., Shkrob, I., Lev, O., 2000. Occurrence of volatile nitrogen-containing compounds in nitrogen-fixing cyanobacterium. *J. Chem. Ecol.* 26, 1359–1366.
- Dicke, M., Loreto, F., 2010. Induced plant volatiles: from genes to climate change. *Trends Plant Sci.* 15, 115–117. <https://doi.org/10.1016/j.tplants.2010.01.007>.
- Dobson, Z., Ahad, S., Vanlandingham, J., Toporik, H., Vaughn, N., Vaughn, M., Williams, D., Reppert, M., Fromme, P., Mazon, Y., 2021. The structure of photosystem I from a high-light tolerant cyanobacteria. *Elife* 10, 1–23. <https://doi.org/10.7554/eLife.67518>.
- Duan, C., Liao, H., Wang, K., Ren, Y., 2023. The research hotspots and trends of volatile organic compound emissions from anthropogenic and natural sources: a systematic quantitative review. *Environ. Res.* 216, 114386. <https://doi.org/10.1016/j.envres.2022.114386>.
- Fujise, D., Tsuji, K., Fukushima, N., Kawai, K., Harada, K.I., 2010. Analytical aspects of cyanobacterial volatile organic compounds for investigation of their production behavior. *J. Chromatogr. A* 1217, 6122–6125. <https://doi.org/10.1016/j.chroma.2010.07.008>.
- Grabarczyk, M., Mączka, W., Żolnierczyk, A.K., Wińska, K., 2020. Transformations of monoterpenes with the p-menthane skeleton in the enzymatic system of bacteria, fungi and insects. *Molecules* 25, 4840. <https://doi.org/10.3390/molecules25204840>.
- Häder, D.P., Kumar, H.D., Smith, R.C., Worrest, R.C., 2007. Effects of solar UV radiation on aquatic ecosystems and interactions with climate change. *Photochem. Photobiol. Sci.* 6, 267–285. <https://doi.org/10.1039/b700020k>.
- Havaux, M., 2020. β -Cyclocitral and derivatives: emerging molecular signals serving multiple biological functions. *Plant Physiol. Biochem.* 155, 35–41. <https://doi.org/10.1016/j.plaphy.2020.07.032>.
- Huang, H., Xu, X., Shi, C., Liu, X., Wang, G., 2018. Response of taste and odor compounds to elevated cyanobacteria biomass and temperature. *Bull. Environ. Contam. Toxicol.* 101, 272–278. <https://doi.org/10.1007/s00128-018-2386-5>.
- Hudnell, H.K., 2008. Cyanobacterial harmful algal blooms: state of the science and research needs, cyanobacterial harmful algal blooms: state of the science and research needs. <https://doi.org/10.1007/978-0-387-75865-7>.
- Ikawa, M., Sasner, J.J., Haney, J.F., 2001. Activity of cyanobacterial and algal odor compounds found in lake waters on green alga *Chlorella pyrenoidosa* growth. *Hydrobiologia* 443, 19–22. <https://doi.org/10.1023/A:1017535801766>.
- Jaiswal, A., Koli, D.K., Kumar, A., Kumar, S., Sagar, S., 2018. Pigments analysis of cyanobacterial strains. *Int. J. Chem. Stud.* 6, 1248–1251.
- Johnson, T.J., Zahler, J.D., Baldwin, E.L., Zhou, R., Gibbons, W.R., 2016. Optimizing cyanobacteria growth conditions in a sealed environment to enable chemical inhibition tests with volatile chemicals. *J. Microbiol. Methods* 126, 54–59. <https://doi.org/10.1016/j.mimet.2016.05.011>.
- Jüttner, F., Watson, S.B., Elert, V.E., 2010. β -Cyclocitral, a grazer defence signal unique to the cyanobacterium *Microcystis*. *J. Chem. Ecol.* 36, 1387–1397. <https://doi.org/10.1007/s10886-010-9877-0>.
- Kana, T.M., Glibert, P.M., 1987. Effect of irradiances up to 2000 $\mu\text{E m}^{-2}\text{s}^{-1}$ on marine *Synechococcus* WH7803—I. Growth, pigmentation, and cell composition. *Deep-Sea Res., Part A* 34, 479–495. [https://doi.org/10.1016/0198-0149\(87\)90001-X](https://doi.org/10.1016/0198-0149(87)90001-X).
- Koteska, D., Sanchez Garcia, S., Wagner-Döbler, I., Schulz, S., 2022. Identification of volatiles of the dinoflagellate *Prorocentrum cordatum*. *Mar. Drugs* 20, 1–17. <https://doi.org/10.3390/md20060371>.
- Krammer, G.E., Werkhoff, P., Sommer, H., Schmidt, C.O., Gatfield, I., Bertram, H.-J., 2001. Carotenoid degradation products in paprika powder. In: *ACS Symposium Series*; American Chemical Society, pp. 206–219. <https://doi.org/10.1021/bk-2002-0802.ch015>.
- Lea-Smith, D.J., Biller, S.J., Davey, M.P., Cotton, C.A.R., Perez Sepulveda, B.M., Turchyn, A.V., Scanlan, D.J., Smith, A.G., Chisholm, S.W., Howe, C.J., 2015. Contribution of Cyanobacterial Alkane Production to the Ocean Hydrocarbon Cycle. *Proceedings of the National Academy of Sciences*, pp. 13591–13596. <https://doi.org/10.1073/pnas.1507274112>.
- Leflaive, J., Ten-Hage, L., 2007. Algal and cyanobacterial secondary metabolites in freshwaters: a comparison of allelopathic compounds and toxins. *Freshw. Biol.* 52, 199–214. <https://doi.org/10.1111/j.1365-2427.2006.01689.x>.
- Li, Z., Hobson, P., An, W., Burch, M.D., House, J., Yang, M., 2012. Earthy odor compounds production and loss in three cyanobacterial cultures. *Water Res.* 46, 5165–5173. <https://doi.org/10.1016/j.watres.2012.06.008>.
- Liu, M., Wu, T., Zhao, X., Zan, F., Yang, G., Miao, Y., 2021. Cyanobacteria blooms potentially enhance volatile organic compound (VOC) emissions from a eutrophic lake: field and experimental evidence. *Environ. Res.* 202. <https://doi.org/10.1016/j.envres.2021.111664>.
- Loreto, F., Schnitzler, J.P., 2010. Abiotic stresses and induced BVOCs. *Trends Plant Sci.* 15, 154–166. <https://doi.org/10.1016/j.tplants.2009.12.006>.
- Mahilang, M., Deb, M.K., Pervez, S., 2021. Biogenic secondary organic aerosols: a review on formation mechanism, analytical challenges and environmental impacts. *Chemosphere* 262, 127771. <https://doi.org/10.1016/j.chemosphere.2020.127771>.
- Mazur-Marzec, H., Felczykowska, A., Hohlfield, N., Kobos, J., Toruńska-Sitarz, A., Devi, P., Montalvão, S., D'souza, L., Tammela, P., Mikosik, A., Bloch, S., Nejman-Faleńczyk, B., Węgrzyn, G., 2015. Baltic cyanobacteria – a source of biologically active compounds. *Eur. J. Phycol.* 50, 343–360. <https://doi.org/10.1080/09670262.2015.1062563>.
- Meredith, L.K., Tfaily, M.M., 2022. Capturing the microbial volatilome: an oft overlooked “ome”. *Trends Microbiol.* 30, 622–631. <https://doi.org/10.1016/j.tim.2021.12.004>.
- Mohanty, B., Majedi, S.M., Pavagadhi, S., Te, S.H., Boo, C.Y., Gin, K.Y.H., Swarup, S., 2022. Effects of light and temperature on the metabolic profiling of two habitat-dependent bloom-forming cyanobacteria. *Metabolites* 12, 1–16. <https://doi.org/10.3390/metabo12050406>.
- Muheta, G., Asaeda, T., Jayasanka, S.M.D.H., Baniya, M.B., Abeynayaka, H.D.L., Rashid, M.H., Yan, H.Y., 2020. Effects of light intensity and exposure period on the growth and stress responses of two cyanobacteria species: *pseudanabaena galeata* and *Microcystis aeruginosa*. *Water (Switzerland)* 12. <https://doi.org/10.3390/w12020407>.
- Munkes, B., Löptien, U., Dietze, H., 2021. Cyanobacteria blooms in the Baltic Sea: a review of models and facts. *Biogeosciences* 18, 2347–2378. <https://doi.org/10.5194/bg-18-2347-2021>.
- Niederbacher, B., Winkler, J.B., Schnitzler, J.P., 2015. Volatile organic compounds as non-invasive markers for plant phenotyping. *J. Exp. Bot.* 66, 5403–5416. <https://doi.org/10.1093/jxb/erv219>.
- Olofsson, M., Suikkanen, S., Kobos, J., Wasmund, N., Karlson, B., 2020. Basin-specific changes in filamentous cyanobacteria community composition across four decades in the Baltic Sea. *Harmful Algae* 91, 101685. <https://doi.org/10.1016/j.hal.2019.101685>.
- Ozaki, K., Ohta, A., Iwata, C., Horikawa, A., Tsuji, K., Ito, E., Ikai, Y., Harada, K., Marasco, E.K., Vay, K., Schmidt-dannert, C., 2006. Identification of carotenoid cleavage dioxygenases from *Nostoc* sp. PCC 7120 with different cleavage activities. *J. Biol. Chem.* 281, 31583–31593. [https://doi.org/10.1016/S0021-9258\(19\)84072-3](https://doi.org/10.1016/S0021-9258(19)84072-3).
- Ozaki, K., Ohta, A., Iwata, C., Horikawa, A., Tsuji, K., Ito, E., Ikai, Y., Harada, K.I., 2008. Lysis of cyanobacteria with volatile organic compounds. *Chemosphere* 71, 1531–1538. <https://doi.org/10.1016/j.chemosphere.2007.11.052>.
- Peñuelas, J., Llusà, J., 2003. BVOCs: plant defense against climate warming? *Trends Plant Sci.* 8, 105–109. [https://doi.org/10.1016/S1360-1385\(03\)00008-6](https://doi.org/10.1016/S1360-1385(03)00008-6).
- Peñuelas, J., Staudt, M., 2010. BVOCs and global change. *Trends Plant Sci.* 15, 133–144. <https://doi.org/10.1016/j.tplants.2009.12.005>.
- Peter, A., Köster, O., Schildknecht, A., von Gunten, U., 2009. Occurrence of dissolved and particle-bound taste and odor compounds in Swiss lake waters. *Water Res.* 43, 2191–2200. <https://doi.org/10.1016/j.watres.2009.02.016>.
- Popin, R.V., Delbaje, E., Augusto, V., Abreu, C. De, Rigonato, J., Dörr, F.A., Pinto, E., Sivonen, K., Fiore, M.F., 2020. Genomic and metabolomic analyses of natural products in *Nodularia spumigena* isolated from a shrimp culture pond. *Toxins* 12. <https://doi.org/10.3390/toxins12030141>.
- Reese, K.L., Fisher, C.L., Lane, P.D., Jaryenneh, J.D., Moorman, M.W., Jones, A.D., Frank, M., Lane, T.W., 2019. Chemical profiling of volatile organic compounds in the headspace of algal cultures as early biomarkers of algal pond crashes. *Sci. Rep.* 9, 1–10. <https://doi.org/10.1038/s41598-019-50125-z>.
- Ritchie, R.J., 2010. Modelling photosynthetic photon flux density and maximum potential gross photosynthesis. *Photosynthetica* 48, 596–609. <https://doi.org/10.1007/s11099-010-0077-5>.
- Rossell, R.K., 2019. Cyanobacterial emissions of biogenic volatile organic compounds: impacts on the remote marine atmosphere. *Bachelor Sci.* 49. <https://doi.org/10.1017/CBO9781107415324.004>.
- Sabour, B., Sbiyyaa, B., Loudiki, M., Oudra, B., Belkoura, M., Vasconcelos, V., 2009. Effect of light and temperature on the population dynamics of two toxic bloom forming Cyanobacteria - *microcystis ichthyoblabe* and *Anabaena aphanizomenoides*. *Chem. Ecol.* 25, 277–284. <https://doi.org/10.1080/02757540903062525>.

- Saha, M., 2022. Algal volatiles – the overlooked chemical language of aquatic primary producers. *Biol. Rev.* 97, 2162–2173. <https://doi.org/10.1111/brv.12887>.
- Śliwińska-Wilczewska, S., Cieszyńska, A., Latała, A., 2017. The impact of temperature and photosynthetically active radiation on the growth and pigments concentration in Baltic picocyanobacterium *Synechococcus* sp. *Ann. Univ. Paedagog. Cracoviensis Stud. Naturae* 1, 59–68. <https://doi.org/10.24917/25438832.2.4>.
- Steinke, M., Malin, G., Liss, P.S., 2002. Trophic interactions in the sea: an ecological role for climate relevant volatiles? *J. Phycol.* 38, 630–638. <https://doi.org/10.1046/j.1529-8817.2002.02057.x>.
- Tomita, K., Hasegawa, M., Arai, S., Tsuji, K., Bober, B., 2016. Characteristic oxidation behavior of β -cyclocitral from the cyanobacterium *Microcystis*. *Environ. Sci. Pollut. Res.* 23, 11998–12006. <https://doi.org/10.1007/s11356-016-6369-y>.
- Tsuchiya, Y., Matsumoto, A., 1988. Identification of volatile metabolites produced by blue-green algae. *Water Sci. Technol.* 20, 149–155. <https://doi.org/10.2166/wst.1988.0236>.
- Vila-Costa, M., Simó, R., Harada, H., Gasol, J.M., Slezak, D., Kiene, R.P., 2006. Dimethylsulfoniopropionate uptake by marine phytoplankton. *Science* 314, 652–654. <https://doi.org/10.1126/science.1131043>.
- Watson, S.B., Monis, P., Baker, P., Giglio, S., 2016. Biochemistry and genetics of taste- and odor-producing cyanobacteria. *Harmful Algae* 54, 112–127. <https://doi.org/10.1016/j.hal.2015.11.008>.
- Xu, Q., Yang, L., Yang, W., Bai, Y., Hou, P., Zhao, J., Zhou, L., Zuo, Z., 2017. Volatile organic compounds released from *Microcystis flos-aquae* under nitrogen sources and their toxic effects on *Chlorella vulgaris*. *Ecotoxicol. Environ. Saf.* 135, 191–200. <https://doi.org/10.1016/j.ecoenv.2016.09.027>.
- Yamashita, R., Bober, B., Kanei, K., Arai, S., Tsuji, K., Harada, K. ichi, 2020. Analytical technique optimization on the detection of β -cyclocitral in microcystis species. *Molecules* 25. <https://doi.org/10.3390/molecules25040832>.
- Yáñez-Serrano, A.M., Bourtsoukidis, E., Alves, E.G., Bauwens, M., Stavrakou, T., Llusà, J., Filella, I., Guenther, A., Williams, J., Artaxo, P., Sindelarova, K., Doubalova, J., Kesselmeier, J., Peñuelas, J., 2020. Amazonian biogenic volatile organic compounds under global change. *Glob. Change Biol.* 26, 4722–4751. <https://doi.org/10.1111/gcb.15185>.
- Yoch, D.C., 2002. Dimethylsulfoniopropionate: its sources, role in the marine food web, and biological degradation to dimethylsulfide. *Appl. Environ. Microbiol.* 68, 5804–5815. <https://doi.org/10.1128/AEM.68.12.5804-5815.2002>.
- Zhang, D., Dechatiwongse, P., del Rio-Chanona, E.A., Maitland, G.C., Hellgardt, K., Vassiliadis, V.S., 2015. Modelling of light and temperature influences on cyanobacterial growth and biohydrogen production. *Algal Res.* 9, 263–274. <https://doi.org/10.1016/j.algal.2015.03.015>.
- Zheng, T., Zhou, M., Yang, L., Wang, Yan, Wang, Yaya, Meng, Y., Liu, J., Zuo, Z., 2020. Effects of high light and temperature on *Microcystis aeruginosa* cell growth and β -cyclocitral emission. *Ecotoxicol. Environ. Saf.* 192. <https://doi.org/10.1016/j.ecoenv.2020.110313>.
- Zinicovscaia, I., Cepoi, L. (Eds.), 2016. *Cyanobacteria for Bioremediation of Wastewaters*. Springer International Publishing, Cham. <https://doi.org/10.1007/978-3-319-26751-7>.
- Zuo, Z., 2023. Emission of cyanobacterial volatile organic compounds and their roles in blooms. *Front. Microbiol.* 14. <https://doi.org/10.3389/fmicb.2023.1097712>.
- Zuo, Z., 2019. Why algae release volatile organic compounds - the emission and roles. *Front. Microbiol.* 10, 1–7. <https://doi.org/10.3389/fmicb.2019.00491>.
- Zuo, Z., Yang, Y., Xu, Q., Yang, W., Zhao, J., Zhou, L., 2017. Effects of phosphorus sources on volatile organic compound emissions from microcystis flos-aquae and their toxic effects on *Chlamydomonas reinhardtii*. *Environ. Geochem. Health* 40, 1283–1298. <https://doi.org/10.1007/s10653-017-0055-y>.

Publikacja – P3

Kwidzińska Klaudia, Caban Magda*, Extraction and separation of cyanobacterial photosynthetic and photoprotective pigments: comparison of practical and accessible methodologies, European Journal of Phycology, 2026, 1-17, DOI:10.1080/09670262.2026.2682186.



Extraction and separation of cyanobacterial photosynthetic and photoprotective pigments: comparison of practical and accessible methodologies

Klaudia Kwidzińska & Magda Caban

To cite this article: Klaudia Kwidzińska & Magda Caban (17 Jun 2026): Extraction and separation of cyanobacterial photosynthetic and photoprotective pigments: comparison of practical and accessible methodologies, European Journal of Phycology, DOI: [10.1080/09670262.2026.2682186](https://doi.org/10.1080/09670262.2026.2682186)

To link to this article: <https://doi.org/10.1080/09670262.2026.2682186>



© 2026 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group.



[View supplementary material](#)



Published online: 17 Jun 2026.



[Submit your article to this journal](#)



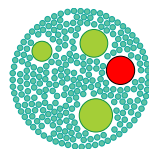
Article views: 64



[View related articles](#)



[View Crossmark data](#)



Extraction and separation of cyanobacterial photosynthetic and photoprotective pigments: comparison of practical and accessible methodologies

Klaudia Kwidzińska and Magda Caban

Faculty of Chemistry, Department of Environmental Analysis, University of Gdansk, ul. Wita Stwosza 63, Gdansk 80-308, Poland

ABSTRACT

Cyanobacterial pigments are complex mixtures of water-soluble and lipophilic compounds with diverse properties and biological roles. There is a growing interest in investigating these pigments due to their potential applications in biotechnology, food production and various industrial sectors. Additionally, they serve as important markers in ecological studies, phylogenetic analyses and ecotoxicology research. Low-pressure liquid chromatography (LPLC), thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) are commonly used laboratory techniques for the fractionation and separation of pigments, with spectrophotometry being the preferred detection method. However, to date, no single study has combined all these techniques for the comprehensive analysis of cyanobacterial photosynthetic pigments. This paper presents the application of these accessible and straightforward techniques for the separation and characterization of cyanobacterial lipophilic pigments. The extraction was performed on two unicellular species, *Synechocystis salina* and *Microcystis aeruginosa*, and three filamentous cyanobacteria, *Aphanizomenon flos-aquae*, *Nodularia spumigena* and *Anabaena cylindrica*. The study highlights the capabilities and limitations of spectrophotometric detection of carotenoids and chlorophylls in *in vivo* cultures and extracts, as well as the challenges related to chlorophyll stability. Experimental work is supplemented with relevant literature and provides practical guidance on the use and interpretation of results from widely available techniques, making it a valuable resource for researchers across various fields.

HIGHLIGHTS

- This study integrates LPLC, TLC, and HPLC for cyanobacterial pigment analysis.
- This study assesses spectrophotometric detection and chlorophyll stability.
- This study provides practical guidance for pigment separation using five cyanobacterial species.

ARTICLE HISTORY Received 11 March 2025; Revised 26 January 2026; Accepted 25 May 2026

KEYWORDS Allomerization; Baltic Sea cyanobacteria; chlorophyll stability; cyanobacterial pigments; fractioning of pigments; liquid chromatography

Introduction

The cyanobacteria are the oldest oxygen-evolving photoautotrophic prokaryotes and the most primitive Gram-negative bacteria. They are also the major biomass producers in aquatic and terrestrial ecosystems (Häder *et al.*, 2007), and play a vital role in the carbon and nitrogen cycle, thereby exerting an important control on greenhouse gas concentrations (Prashant Singh *et al.*, 2020). Furthermore, they are versatile life forms, with high potential to be used in biotechnology for bioremediation (Zinicovscaia & Cepoi, 2016; Badiale-Furlong *et al.*, 2023), and as a source of active compounds (Mazur-Marzec *et al.*, 2015; Bouyahya *et al.*, 2024). Several cyanobacteria-derived substances are already used at an industrial scale (Saini *et al.*, 2018; Santos-Merino *et al.*, 2019). Pigments are among the most widely used cyanobacterial biochemicals, with economic, ecological and nutritional value. They are mostly used as antioxidants in food, cosmetics, pharmacology, nutraceuticals and animal-feed supplements (Saini *et al.*, 2018; Deepika

et al., 2022; Castro *et al.*, 2023). However, their applications are much broader (Sandybayeva *et al.*, 2022).

The photosystems of cyanobacteria have several differences from plants. Firstly, they are located within the internal membranes, while in plants they are in the thylakoid membranes of the chloroplast. Furthermore, the cyanobacteria have a different configuration of the photosynthetic apparatus. They have light-harvesting complexes (LHC) that include chlorophylls (Chls) and carotenoids (CRs) as in plants, but also have phycobiliproteins (PBPs) located externally to the membrane. Chls are crucial for light harvesting and selectively absorb red and blue regions of light. Chl *a* is a primary type Chl found in cyanobacteria, but there are some species with other Chl types (Averina *et al.*, 2019). CRs (divided into carotenes and xanthophylls) absorb light at wavelengths different from that of Chls (450–570 nm), and have photosynthetic and photoprotective functions (Hirschberg & Chamovitz, 1994; Prashant Singh *et al.*, 2020). The

CONTACT Magda Caban magda.caban@ug.edu.pl

© 2026 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group.

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/4.0/>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited. The terms on which this article has been published allow the posting of the Accepted Manuscript in a repository by the author(s) or with their consent.

protective role of CRs is due to their ability to absorb energy from photosensitized chlorophyll and quench single oxygen (Prashant Singh *et al.*, 2020). Xanthophylls are oxygenated forms of carotenes, often present as glycosylated forms, or as esters. Ketocarotenoids (for example echinenone), 2-hydroxy-derivatives and glycosides are unique to cyanobacteria. The genes that regulate carotenoid biosynthesis in cyanobacteria are relatively well known, and there are differences in regulation between unicellular and filamentous types (Liang *et al.*, 2006). There are databases for CRs (for example, <http://carotenoiddb.jp/>), where the common and unique ones can be found. Knowledge of pigment composition can help taxonomic identification and determine the usefulness of an algal strain in biotechnology. Furthermore, changes in compositions of pigments, e.g. overproduction of one of them, can be a stress biomarker.

There are several strategies for lipophilic pigment extraction and qualification (Pagels *et al.*, 2021a, 2021b; Deepika *et al.*, 2022). In most cases, the CRs and Chls are extracted from cells by solid-liquid extraction (SLE) and then analysed by UV-Vis spectrophotometry (Schagerl & Künzl, 2007; Ilavarasi *et al.*, 2012; Hu *et al.*, 2013). Extracted pigments mixtures in a solvent are analysed in bulk, and collectively, and there is no precise specification of individual pigments, which may limit new knowledge and ecological or biochemical insights. Pagels *et al.* (2021a) provided a comprehensive overview of extraction techniques of cyanobacterial pigments for bioprocessing, with special attention given to extraction acceleration. Essentially, most of the disadvantages of separation techniques are related to high cost and energy consumption, and their application for routine analysis in the laboratory with small sample sizes is deemed not to be economic. Standardizing a protocol for extracting biomass to obtain the desired pigments is still a challenge (Sandybayeva *et al.*, 2022). Currently, low-pressure liquid chromatography (LPLC) using silica gel, and thin-layer chromatography (TLC), despite their suitability for screening assays, are little used in analytics of cyanobacterial pigments. These techniques are easy to implement in any laboratory, and their applicability for lipophilic pigments analysis is the subject of this paper. In addition, there is a lack of standardized comparative evaluations of pigment separation and analysis methods. Supplementing spectrophotometric analysis with other techniques can provide qualitative and quantitative information. To date, there have been few studies on the applicability of LC techniques for analysis of photosynthetic and photoprotective pigments.

This study examines the applications of and limitations of spectrophotometry, LPLC, TLC and high-performance liquid chromatography (HPLC) to the specification of photosynthetic and photoprotective

lipophilic pigments (CRs and Chls) produced by cyanobacteria (two unicellular, and three filamentous) isolated from the Baltic Sea. These pigments are a wide mixture of compounds with different properties, and understanding the composition of this mixture is often crucial in the biological, ecological and chemical analysis of cyanobacteria.

Materials and methods

Chemicals

Standards of chlorophylls and carotenoids: chlorophyllide *a* (CAS 14897-06-4), violaxanthin (CAS 126-29-4), 4-ketomyxoxanthophyll (CAS n.d.), astaxanthin (CAS 472-61-7), aphanizophyll (CAS 35930-16-6), myxoxanthophyll (CAS 11004-68-5), diatoxanthin (CAS 31063-73-7), zeaxanthin (CAS 144-68-3), lutein (CAS 127-40-2), canthaxanthin (CAS 514-78-3), chlorophyll *b* (CAS 519-62-0), beta-cryptoxanthin (CAS 472-70-8), chlorophyll *a* (CAS 479-61-8), echinenone (CAS 432-68-8), lycopene (CAS 502-65-8), pheophytin (CAS 603-17-8) and β -carotene (CAS 7240-7) were purchased from DHI Lab Products (Denmark) in the form of ethanol or acetone solutions and stored at -20°C . Dichloromethane (HPLC plus) was purchased from Sigma-Aldrich (USA), cyclohexane from Chempure (Poland), acetone (HPLC grade) was purchased from J.T. Baker (Germany), while diethyl ether (HPLC grade) was from POCH (Poland).

Cyanobacterial strains and growing conditions

The five cyanobacterial monocultures came from the Culture Collection of Northern Poland (CCNP, Division of Marine Biotechnology, University of Gdansk, Poland). The place of isolation and growth medium are presented in Supplementary table S1. *Synechocystis salina* and *Microcystis aeruginosa* are unicellular, while *Aphanizomenon flos-aquae*, *Nodularia spumigena* and *Anabaena cylindrica* are filamentous. The species tested represent the orders Synechococcales, Chroococcales and Nostocales. *A. cylindrica* grew in Z8 growth medium with and without the nitrates, which was further marked as N-def (nitrogen deficient). Additional information on the species tested can be found in the literature (Mazur-Marzec *et al.*, 2015).

Cyanobacteria were grown in a photo-chamber with an LED source of white light (Biogenet, model Fito700, Poland) with continuous photosynthetically active radiation (PAR) of $35 \pm 5 \mu\text{mol m}^{-2} \text{s}^{-1}$ as measured by a photoradiometer (Delta OHM HD2302.0 with probe LP 471 PAR) close to the bottle walls. Details of the spectral composition of the LEDs are presented in Supplementary table S2. The temperature of cyanobacterial cultivation was set to 23°C . Cultures (~ 60 ml) were kept in glass bottles (100 ml) with continuous aeration by air passing through an

activated carbon filter (FW600, Wanza, Poland) and syringe filter (0.22 µm, PET, Chromafil, Germany). To maintain sterile conditions, a laminar chamber UVC/T-M-AR (SIA Biosan, Latvia) was used for any manipulation of the cultures. Once a week, cultures were inoculated in fresh medium to obtain a starting optical density (OD) at 663 nm of about 0.1. Cells were harvested in their late exponential growth phase (96 h after inoculation).

Pigment extraction procedure

Extraction of Chls and CRs was performed as follows: 1 ml of sample was taken from stock culture (OD ≈ 1.0), centrifuged (4 min, 20 000 rpm), and the supernatant was discarded. It was noted that *M. aeruginosa* cells were problematic during centrifugation as some of the cells accumulated on the surface of the liquid (Supplementary fig. S1), related to their gas-filled vesicles. The cyanobacterial cells were then rinsed with deionized water and centrifuged again, and the water was decanted. Then biomass lyophilization was performed using a freeze dryer (FreeZone 2.5 l, Labconco, USA) at −80°C for 24 h. The preliminary study with acetone, 90% acetone (aq.) and a mixture of acetone with 50 mM ammonium acetate buffer (90:10 v/v) (described in Supplementary methods S1, spectra Supplementary fig. S2), showed that the 100% acetone was the least efficient for extraction, while the two other extraction mixtures had the same efficiency. Finally, the mixture with acetate buffer was chosen, because of its compatibility with HPLC mobile phases. The volume of 1 ml of cold acetone solution with 50 mM ammonium acetate (90:10 v/v) was added to the dry cells. Subsequently, the cells were homogenized using a Bead Ruptor Elite homogenizer (Omni International, USA) for three cycles of 30 s shaking (4 m s^{−1}) with ice cooling between cycles. The samples were then placed in a refrigerator (4°C) for 48 hours. After this, the extracts (~ 800 µl) were filtered and placed into chromatographic vials for HPLC-DAD and UV-Vis analysis. During the extraction of pigments, the samples were protected from excess light and temperature.

Thin layer chromatography

TLC analysis was performed for the acetone-based extracts (acetone, 90% acetone (aq.), and a mixture of acetone with 50 mM ammonium acetate buffer (90:10 v/v)). Silica Gel 60 TLC plates (Merck, Germany) were used, activated by heat (120°C for 1 h), and conditioned by washing with the mobile phase. The mobile phase was a mixture of cyclohexane, diethyl ether and acetone in a volume ratio of 50:25:25. The Chls and CRs extract for TLC analysis was concentrated to approximately 0.1 ml with a stream of nitrogen and then applied to silica

gel plates using a glass capillary tube. The development of a chromatogram was performed in a beaker covered by parafilm with head-space saturated by mobile phase vapours. After completion of development, the plate was left in the dark until the organic solvent evaporated, and a photo was taken. Selected colour dots were scratched and hollowed out, and the resulting material was placed in 1 ml of acetone to extract the pigments. Extraction was performed by vortexing for 1 minute at room temperature, then the liquid extract was separated from the sorbent by centrifugation at 20 000 rpm (21°C, 2 min). The resulting sample was then concentrated with a stream of nitrogen and subjected to HPLC-DAD analysis to identify the pigment.

UV-Vis spectrophotometry

A Genesys 150 (Thermo Scientific™, USA) spectrophotometer was used for UV-Vis spectrum measurement of cultures and extracts. Before the measurement, the extracts were centrifuged. Quartz microcuvettes (1 cm optical length, total volume 400 µl) were used. The absorbance spectrum was measured in the range 300–800 nm with a measurement frequency of 0.2 or 1 nm. The blank was an extraction medium or growth medium.

High-performance liquid chromatography

The parameters of HPLC-DAD analysis were those previously applied for tracking the change in three pigments (Chl *a*, echinenone, β-carotene) in unicellular cyanobacteria (Kwidzińska *et al.*, 2024). Here, we expand it to other cyanobacterial pigments. Chromatographic analysis of Chls + CRs was performed using the HPLC/DAD Nexera XR system (Shimadzu) with an autosampler SIL-20AC, a pump LC-20AD, a column oven CTO-20AC and a detector SPD-M20A. The autosampler temperature was set to 10°C, while column oven temperature was set to 30°C. The separation of the pigments was performed on an analytical column ReproSil Gold (C18, 5 µm, 120 Å, 20% carbon load, double bond encamping, surface 300 m² g^{−1}, Dr. Maisch, Germany) with a pre-column. The base of separation was the method presented in Freiberg *et al.* (2011). The mobile phase component A was MeOH with aq. ammonium acetate 50 mM (80:20, v/v), while component B was MeOH with acetone (80:20, v/v). A gradient elution was applied for separation. Initially, the B-phase concentration was maintained at 30% (0–2.5 min), then increased to 70% (from 2.5–30 min), then to 100% (30–35 min) and was maintained for 20 min (35–55 min), after which the concentration of phase B decreased to 30% (55–60 min), and finally remained at that level for 3 min (60–63 min). The flow rate of the mobile phase was 1 ml min^{−1}. The injection volume was 25 µl. The DAD detector scan wavelengths were in the range 200–1000

nm. Calibration curves with pigment standards from DHI were prepared in a concentration range from 0.0023–0.0210 (LOQ) to 0.0657–0.3208 mg l⁻¹, depending on the analyte. The precision within this concentration range was <4.6% (relative standard deviation). The accuracy was between 85 and 116%. The validation values for each analyte are listed in Supplementary table S3.

Diatoxanthin was applied as the control analyte, even though this pigment is specific to diatoms. The absence of diatoxanthin in cyanobacterial extracts proved that our protocol of analysis applied could distinguish between cyanobacterial pigments and other microalgal organisms, as no co-elution was obtained.

Low-pressure liquid chromatography

This test was performed using a pure acetone-based extract of *S. salina*. LPLC was performed to separate the pigments according to lipophilicity. 20 g silica (high purity grade, pore size 60 Å, 230–400 mesh, particle size 40–63 µm, Sigma-Aldrich) was sufficient to create a 10 cm high layer in the glass column (internal diameter 18 mm). The residual water from the silica was removed by drying for 1 h at 150°C. The bottom of the glass column was protected by glass wool and a small cellulose filter. Before packing, the silica was preconditioned in starting elution solvent for 1 h with mixing (orbital shaker, 150 rpm). Such preconditioning was needed to prevent bubble formation in the silica, caused by the heat emitted during wetting by the solvent. The solvent from the 5 ml of acetone extract of *S. salina* was exchanged into 250 µl of starting elution solvent to get

a dense solution. Pretreated silica was added in a low amount to the sample, which improved the resolution at the start of separation. This solution was introduced into the top of the wet column. Two tests of different elution solvent were done. Test I was with solvents in order: diethyl ether, diethyl ether:acetone (1:1, v/v), and acetone. Test II started with methyl chloride, then methyl chloride:diethyl ether (1:1, v/v), diethyl ether, diethyl ether:acetone (1:1, v/v), and acetone. The volumes of fractions collected depended on the bands visible on the column and are presented in the Results section. The flow of eluent was quite low, about 5–10 ml min⁻¹, and depended on the viscosity of the applied eluent. The fractions were collected in glass headspace-type vials. They were further analysed by spectrophotometry and HPLC-DAD.

Results

General description of UV-Vis spectrum of cyanobacterial cultures and extracts

Figure 1A presents the UV-Vis spectra (350–750 nm) of native cultures of the four species tested. The general characteristics of the spectra in order of maxima were similar. Absorbance peaks in region 350–450 nm correspond to the overlapping absorbance of each analysed group of pigments so this region cannot be used to quantify a specific type of pigments. Peaks observed near 635 nm were attributed to phycobilins, while the peaks at 440 nm and 680 nm originated from Chls (Malhotra & Örmeci, 2023).

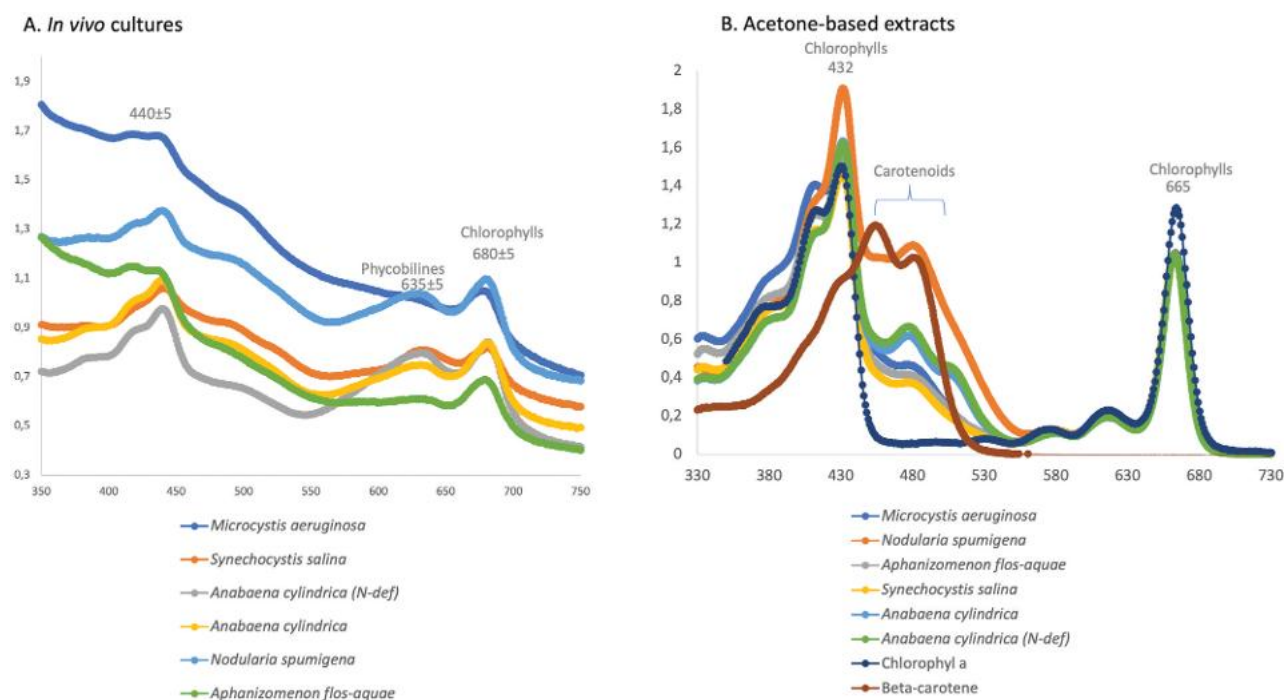


Fig. 1. UV-Vis spectra of all tested cyanobacterial species in (A) raw cultures and (B) organic extracts (ChLs + CRs).

Fig. 1B presents spectra of organic solvent extracts (Chls + CRs) from tested species, in addition to the spectrum of Chl *a* and β -carotene standards. There are similarities in the order of the spectrum characteristic of the cellular extracts. The peaks at 431 nm and 665 nm correspond to Chl *a*, while bands between 450 and 530 nm mainly correspond to CRs.

From the spectrum in Fig. 1A, it can be observed that N-deficient *A. cylindrica* has fewer carotenoids and more phycobilins compared to nutrient-replete *A. cylindrica* at the same chlorophyll concentration. This observation is consistent with results from previous work with *Pseudanabaena* sp. and *Oscillatoria splendida* (de Loura *et al.*, 1987). The differences in carotenoid production in *Anabaena* sp. have previously been reported by Kosourov *et al.* (2016), who studied H_2 photoproduction. Under severe N deficiency, *Anabaena* sp. have lower echinenone and β -carotene production but accumulate glycosylated and hydroxylated carotenoids, such as myxol (or ketomyxol) 20-methylpentosides, 30-hydroxyechinenone and zeaxanthin (Kosourov *et al.*, 2016). The pigment composition and abundance shift is aimed at optimizing light harvesting and energy production (Murton *et al.*, 2017).

TLC for pigments screening

TLC plate pictures for the extract of the tested cyanobacterial species showed that it was not possible to separate all pigments with the eluent compositions

applied, and there was an observable dominance of chlorophyll concentrations (Fig. 2). The selected dots were further analysed by HPLC-DAD for identification of the exact pigment and identified dots on plates were named. *N. spumigena* had a substantial amount of 4-ketomyxoxanthophyll and echinenone and *S. salina* had a pigment that was most likely synechoxanthin (investigated later in the text). Echinenone and carotene were shared by all species.

In the TLC chromatograms (Fig. 2), there was a problem with the most polar pigments, which remained on the start line. This issue may be related to the elution solvent applied, which can be modified by a change in composition. We also observed that the solvent in which the extract was transferred onto the plate could change the resolution and dot shapes as shown in Supplementary fig. S3 for *S. salina* extracts prepared in three solvents. The transfer solvents were evaporated before development, but the viscosity of the applied drop affected the starting and final dot shapes. The application of extracts in pure organic solvents made the starting dots wider, but after separation, the pigments dots were better focused into lines. The application of more aqueous extracts made the dots more focused at the start, and gave more rounded dot shapes at the end of development.

Another issue was the stretching of chlorophylls across the plates, caused by their high content compared to other pigments. Reducing the sample volume at the start avoids overloading, but some

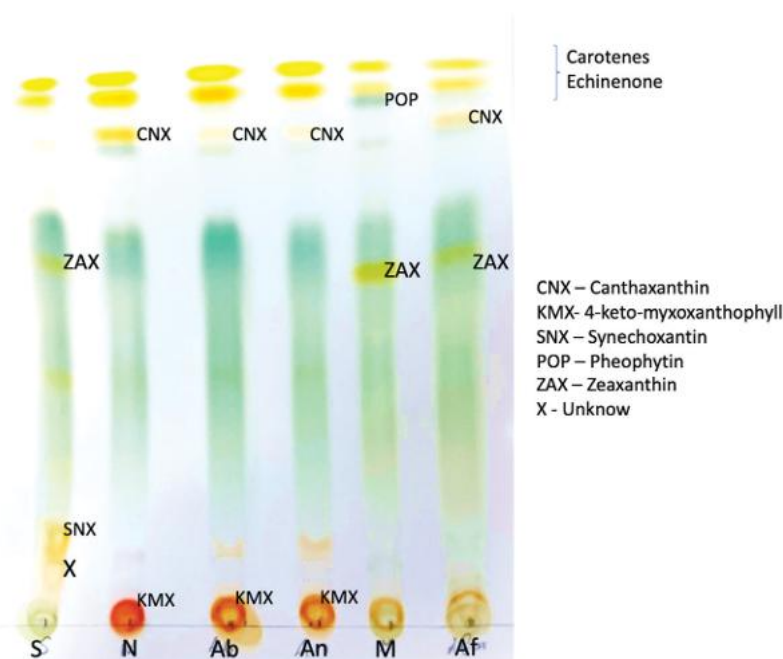


Fig. 2. TLC chromatograms of organic extract from tested cyanobacteria: S – *Synechocystis salina*, N – *Nodularia spumigena*, Ab – *Anabaena cylindrica* without nitrogen in the growth medium, An – *Anabaena cylindrica* with nitrogen in the growth medium, M – *Microcystis aeruginosa*, Af – *Aphanizomenon flos-aquae*. Pigments: CNX – canthaxanthin; KMX – 4-ketomyxoxanthophyll; SNX – synechoxanthin; POP – pheophytin; ZAX – zeaxanthin; CR – carotene; ECN – echinenone.

low concentration pigments could then be lost for observation. We developed a technique named “fast-start”, where a plate with applied extracts was firstly developed for 5–10 seconds in pure acetone (the highest elutropic power solvent from the elution mixture), to move all components from their starting points, then, after drying, separation was continued with normal elution mixture. With this, dots of polar xanthophylls were more focused and separated, as presented in Supplementary fig. S3. The automation of sample applications can help the TLC separation too (Charegaonkar, 2011; Morlock, 2021; Xu *et al.*, 2022).

Hydrophobic pigment quality in cultures

Several peaks were found in each HPLC-DAD chromatogram (Fig. 3). The signal intensities were not proportional to concentration, because each pigment had its specific absorbance maximum, while extraction of the chromatogram was performed only at 452 nm absorption. For the same reason, the peaks of phaeophytin and chlorophyll were relatively low in these chromatograms, despite their high concentration in extracts. The most polar xanthophylls elute first, then chlorophylls, and then the most lipophilic carotenes (Fig. 3). Table 1 lists the identified pigments in the different organic extracts of cyanobacteria. The pigments with bold names were those shared by several species and those marked “+/-” indicate that there was a peak at the analytical standard retention time, but the absorbance was too low to compare the spectrum with the standard spectrum.

In the extract from *S. salina*, six pigments were identified, but at least two more were present in the

chromatogram for which analytical standards were not obtained. We hypothesized that the most abundant unknown signal (retention time about 23 min) was synechoxanthin (chi,chi-caroten-18,18'-dioic acid), because of the very similar spectrum of synechoxanthin detected previously in *Synechococcus* sp. PCC 7002 (Graham & Bryant, 2008). Its spectrum was nearly identical to a spectrum of peaks number 1 to 4, indicating that it is xanthophyll (oxidized carotenoid). Chemically, synechoxanthin (PubChem CID 44237248) is different from other xanthophylls, because it possesses a carboxyl group on the outer rings (aromatic carotenoid diacid). Future studies will identify it by high-resolution mass spectrometry (HRMS). This compound gave an orange colour on the TLC chromatogram (Fig. 2, SNX dot).

In the extract of *M. aeruginosa*, seven pigments were identified, while in the extract of *N. spumigena* and *A. flos-aquae* there were eight (Table 1). In *A. cylindrica* the 11 peaks have the same retention time to standards, and six have been confirmed by comparison of UV-Vis spectra with standards.

Phaeophytin was found in each extract with a low area of chromatographic peak. This pigment is the product of chlorophyll losing its central Mg cation, which can be an artefact during extraction steps. It was present despite the special attention to extraction conditions – reduced light and low temperature of sample and special extract storage.

Violaxanthin was not identified in the tested species. A peak with the same retention time as violaxanthin was only detected in *Anabaena* growing in

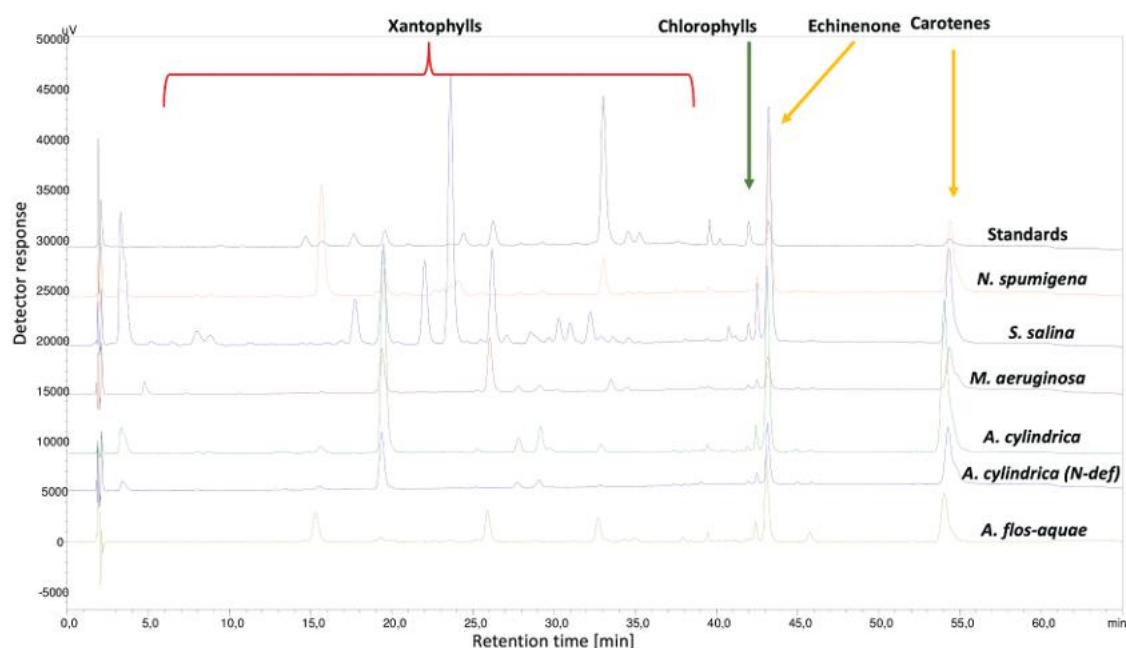


Fig. 3. Chromatograms (extracted for 452 nm) of organic extract of tested cyanobacteria and mixture of pigments (listed in section 2.1).

Table 1. Identified lipophilic pigments in five tested cyanobacterial species (*Anabaena cylindrica* in full growth medium and N-deficient growth medium); +/- means that pigment cannot be confirmed, because of low area of signal and problematic comparison with standard spectrum.

R _t [min]	λ max [nm]	Pigment	<i>Synechocystis salina</i>	<i>Microcystis aeruginosa</i>	<i>Nodularia spumigena</i>	<i>Aphanizomenon flos-aqua</i>	<i>Anabaena cylindrica</i> (N-deficient)	<i>Anabaena cylindrica</i>
3.98	420, 653	chlorophyllide <i>a</i>	-	-	+	+	+/-	+/-
14.88	415, 440, 469	violaxanthin	-	-	-	-	+/-	-
16.13	480, 503	4-ketomyxoxanthophyll	-	-	+	-	+	+
18.11	478	astaxanthin	-	-	-	-	-	-
20.13	474, 505/ 474, 505	aphanizophyll/myxoxanthophyll	+	+	+	+	+	+
23.00	416, 440, 468	unknown	+	-	-	-	-	-
		possible synechoxanthin						
24.93	451, 478	diatoxanthin	-	-	-	-	-	-
26.78	451, 476/444, 472	zeaxanthin/lutein	+	+	-	+	+/-	+/-
32.26	476	canthaxanthin	-	+/-	+	+	+/-	+
39.41	467, 650	chlorophyll <i>b</i>	-	-	-	-	-	-
41.77	451, 476	β-cryptoxanthin	-	-	-	-	+/-	+/-
43.01	431, 665	chlorophyll <i>a</i>	+	+	+	+	+	+
43.87	460	echinenone	+	+	+	+	+	+
50.42	471, 501	lycopene	-	-	-	-	-	-
52.37	408, 666	phaeophytin <i>a</i>	+	+	+	+	+	+
54.41	452, 475	β-carotene	+	+	+	+	+	+

N-deficiency condition, but the low abundance prevented a definite identification.

The aphanizophyll/myxoxanthophyll pair was detected in all the tested species, in a manner analogous to echinenone, β-carotene and Chl *a*. This pair of pigments, which shared the same UV/Vis spectrum, was not separated in our HPLC setup. Therefore it is not certain which one was detected in extracts. Chromatographic analysis of carotenoid standards revealed that zeaxanthin and lutein exhibit identical retention times in applied HPLD-DAD system.

The semi-quantitative analysis was conducted on four cyanobacterial species to determine their pigment concentrations (Supplementary table S4, which reports the pigment concentrations in mg per ml of extract and illustrates the relationship between the concentrations of selected pigments and Chl *a* in acetone extracts from these species). It is important to note that the extraction and analysis were performed twice independently, which limits the comparison to a semi-quantitative level rather than precise quantification. Preliminary results indicate that *N. spumigena* exhibits the highest proportion of echinenone, with a ratio of 0.1, whereas *M. aeruginosa* shows the highest levels of zeaxanthin, aphanizophyll/myxoxanthophyll and β-carotene. Overall, Chl *a* remains the most abundant pigment in the extracts, with other pigments reaching a maximum of approximately 25% of the Chl *a* concentration. These findings provide insight into the pigment composition of cyanobacteria and highlight the dominance of Chl *a* in their pigment profile, which is consistent with their photosynthetic activity and ecological roles.

Low-pressure liquid chromatography performance

The main aim of applying low-pressure liquid chromatography (LPLC) was to separate fractions of pigment type from cyanobacterial organic extracts with a simpler and lower cost method than HPLC. The fractions were analysed by HPLC-DAD to verify the composition. The performance of two tested elution systems (test I and test II) is presented in Table 2. Pictures of the column during test II development, and the fractions collected in vials are shown in Supplementary fig. S5. In test I we applied quick elution starting with the diethyl ether. In the first fraction carotene and echinenone were identified, while in the second one chlorophyll *a* was found. The next fractions (3–7) included xanthophylls. Synechoxanthin (suspected) and myxoxanthophyll eluted together in the last fraction. The elution solvent order applied in the test I was quite inefficient in separating pigments. In response, we improved the elution order in test II, in which separation was

Table 2. Performance of low-pressure liquid chromatography of *Synechocystis salina* organic extract using two elution procedures.

Test	Fraction	Volume and eluent	Pigments (*HPLC confirmed)
I	1	9 ml diethyl ether	Carotene* and echinenone*
	2	18 ml diethyl ether	Chlorophylls (mainly Chl <i>a</i> *)
	3	25 ml diethyl ether	Xanthophylls
	4	15 ml mixture diethyl ether + acetone (1:1, v/v)	Traces of xanthophylls
	5	9 ml acetone	Xanthophylls
	6	15 ml acetone	Xanthophylls
	7	40 ml acetone	Synechoxanthin (?) and myxo-xanthophyll*
II	1	20 ml dichloromethane	Beta-carotene* (95% purity)
	2	40 ml dichloromethane	Echinenone* (100% purity)
	3	40 ml dichloromethane	Pheophytin* and carotene traces
	4	40 ml dichloromethane + diethyl ether (3:1, v/v)	Chlorophylls
	5	55 ml dichloromethane + diethyl ether (3:1, v/v)	Chlorophylls
	5'	50 ml diethyl ether	Conditioning, no peaks
	6	25 ml diethyl ether + acetone (2:1, v/v)	Chlorophylls and xanthophylls
	7	45 ml diethyl ether + acetone (2:1, v/v)	Chlorophylls and xanthophylls
	8	55 ml diethyl ether + acetone (2:1, v/v)	Synechoxanthin (?) + xanthophylls
	9	50 ml acetone	Myxoxanthophyll* + xanthophylls
	10	10 ml MeOH	Low-intensity unidentified peaks

prolonged about three times, because of the application of nine solvent mixtures. The efficiency of separation was improved. The first fraction was mainly carotene (β -carotene was confirmed, but the peak shape suggests the presence also of another form of carotene). The second fraction contained echinenone (with 100% purity). In fraction 3 there were pheophytin and carotene traces. The chromatograms of fractions 1–3 together with overlaid chromatograms of standards are shown in Supplementary fig. S6. Fractions 4 and 5 were enriched in chlorophylls. Fractions 8 and 9 were suspected synechoxanthin and myxoxanthophyll, respectively, with residual content of unidentified xanthophylls of low abundance (Supplementary fig. S7).

Fig. 4 presents overlapping chromatograms of selected fractions from LPLC of extract from *S. salina*, standards of Chl *a* and Chl *b*, and extract before fractioning. Chl *b* was absent in the extract, as expected (Palenik & Haselkorn, 1992; Averina *et al.*, 2019). The chlorophylls were obtained in fractions 4–6 (Table 2). However, there is a question about the nature of these chlorophylls, as not all peaks in Fig. 4 have the same retention time as the Chl *a* standard. Chl *a* was detected in the native extract of *S. salina* and fraction 2 from test I. In test II, surprisingly, there was no peak with the same retention time as the Chl *a* standard. The peaks in fractions 4–6 had the same UV-Vis spectrum as Chl *a*.

Discussion

Biochemistry of pigments

Violaxanthin, which was not detected, is the product of the epoxidation of zeaxanthin as a product of

reactive oxygen species (ROS) attack (Michel Havaux, 1999). Such ROSs arise when a cell is subject to excess irradiance, greatly exceeding the dissipation of energy in photosynthetic electron transport. Violaxanthin can be formed from zeaxanthin through a reversible activity of epoxidase and de-epoxidase in a process called the violaxanthin cycle (Pagels *et al.*, 2021b). It is not likely that violaxanthin will be detected in *Anabaena*, as Paliwal *et al.* (2016) reported this pigment only in Chlorophyta among microalgae.

Either aphanizophyll or myxoxanthophyll was detected in all the tested species, but our HPLC setup could not distinguish them. However, myxoxanthophyll was confirmed previously in *N. spumigena* (Schlüter *et al.*, 2008), and this pigment is a precursor for 4-ketomyxoxanthophyll, also detected in *N. spumigena* (Schlüter *et al.*, 2008). In our study, 4-ketomyxoxanthophyll was identified in the extract of *A. cylindrica*. The detection of myxoxanthophyll was reported by the majority of studies presented in Table 3. Myxoxanthophyll, together with echinenone, was proposed to be a chemotaxonomic marker for Cyanophyta (Paliwal *et al.*, 2016). Finally, in our study, in the analysis of aphanizophyll/myxoxanthophyll, the detection of myxoxanthophyll was more probable. However, due to the limitations of the chromatographic separation, definitive identification remains challenging. Further refinement of the separation process or supplementary analytical techniques are recommended to enhance certainty in future analyses.

Lutein has not been detected in cyanobacterial samples, as reported (Takaichi, 2011; Lehmann *et al.*, 2021). Consequently, the peak observed at a retention time of

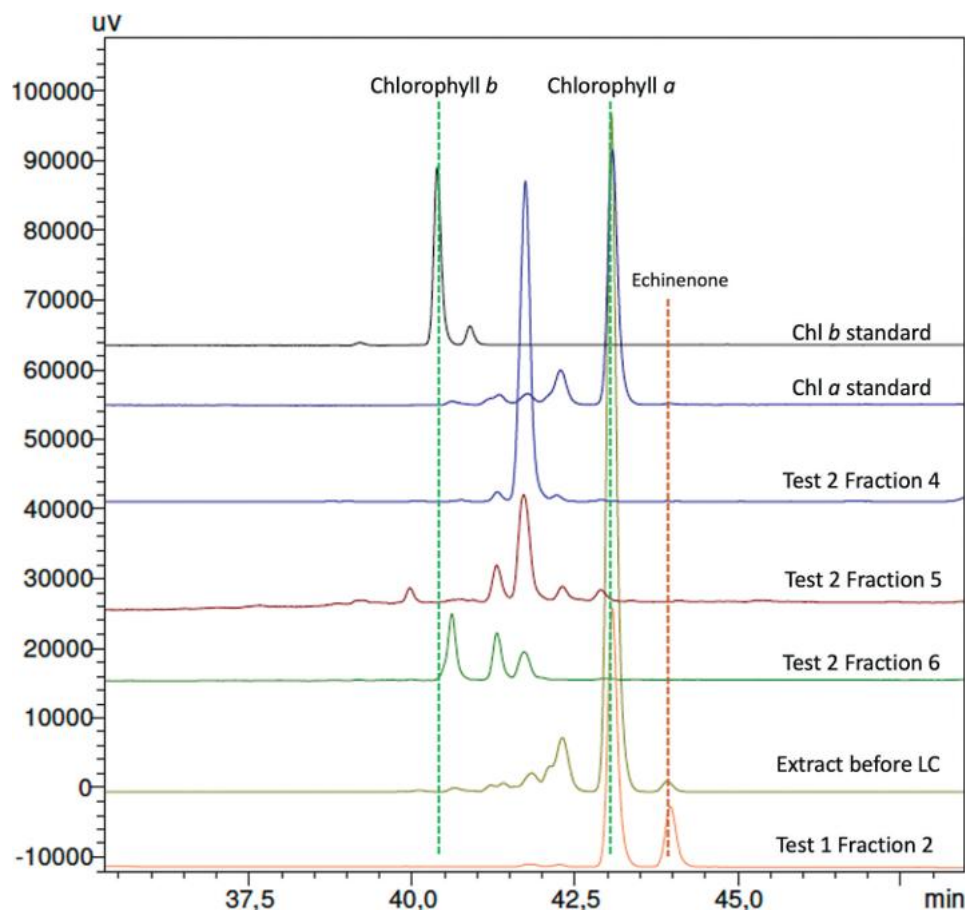


Fig. 4. HPLC-DAD chromatograms of selected fractions from low-pressure liquid chromatography of organic extract from *Synechocystis salina*, standards of Chl *a* and Chl *b*, and extract before fractioning.

27 minutes was attributed to zeaxanthin. Assunção *et al.* (2021) reported the detection of lutein in *S. salina*, indicating variability in carotenoid composition among different species or under varying experimental conditions. These findings underscore the importance of precise chromatographic identification and separation of close-structural pigments.

Table 3 synthesizes reports on the presence of xanthophyll pigments in the cyanobacteria tested. Chl *a* and β -carotene were omitted from the table because they were universal. In the case of *S. salina*, we found four reports. Wojtasiewicz & Stoń-Egiert (2016) tested the same strain and found canthaxanthin in addition to zeaxanthin and echinenone, while we have not identified canthaxanthin. Myxoxanthophyll was identified in *S. salina* by us and in one other report (Nováková *et al.*, 2020). In addition to this identification, we tentatively identified synechoxanthin.

Various *A. flos-aqua* strains were analysed in order of xanthophyll composition (Table 3). Canthaxanthin and echinenone were obtained in all strains, and zeaxanthin in three out of four. In the case of *M. aeruginosa*, we found a report for the same strain (Wojtasiewicz & Stoń-Egiert, 2016), and in addition to the reported zeaxanthin and echinenone, we identified

myxoxanthophyll. For *N. spumigena* we found four reports for the same strain. Only echinenone was identified in each strain, while canthaxanthin was present in *N. spumigena* CHS1, *N. spumigena* CS-584 and *N. spumigena* CCNP 1401 (our study). Schlüter *et al.* (2008) identified aphanizophyll (4-hydroxymyxol, PubChem CID16061296) in this species. The pigments 4-keto-myxoxanthophyll, echinenone and canthaxanthin were identified in each reported study with *Anabaena* spp. and in our study. In addition to these pigments, *Anabaena* PCC 7120 4-ketomyxol 2'-fucoside and myxol 2'-fucoside, belonging to glycosylated xanthophylls were identified (Kosourov *et al.*, 2016).

A 4-keto-myxoxanthophyll-like pigment was also found in samples taken during intense blooms of *N. spumigena* and correlated with the concentration of the algal toxin nodularin ($r = 0.97$) (Schlüter *et al.*, 2004). This pigment was identified further as 4-keto-myxol-20-fucoside (Schlüter *et al.*, 2008). The lack of zeaxanthin, the marker of cyanobacteria, was noted in *N. spumigena* in our study, and the same was reported previously for this strain (Wojtasiewicz & Stoń-Egiert, 2016).

The identity of the chlorophyll *a* like peaks can be ascertained from the literature. There are two

Table 3. Xanthophylls reported in tested cyanobacterial species.

Cyanobacterial strain	Xanthophylls	Reference
<i>Synechocystis salina</i>		
<i>S. salina</i> LEGE 06,155	lycopene, lutein, echinenone, zeaxanthin	(Assunção <i>et al.</i> , 2021)
<i>S. salina</i> CCNP 1104	zeaxanthin, canthaxanthin, echinenone	(Wojtasiewicz & Stoń-Egiert, 2016a)
<i>S. salina</i> PCC6906	myxoxanthophyll	(Nováková <i>et al.</i> , 2020)
<i>S. salina</i> CCNP 1104	myxoxanthophyll, zeaxanthin, echinenone, synechoxanthin (presumably)	Our study
<i>Aphanizomenon flos-aqua</i>		
<i>A. flos-aqua</i> KAC15	zeaxanthin, canthaxanthin, echinenone	(Wojtasiewicz & Stoń-Egiert, 2016a)
<i>A. flos-aqua</i> BS15	astaxanthin, myxoxanthophyll, canthaxanthin, echinenone	(Staal <i>et al.</i> , 2003a)
<i>Aphanizomenon</i> sp.	oscillaxanthin, aphanizophyll, myxoxanthophyll, canthaxanthin, zeaxanthin, echinenone, beta-cryptoxanthin	(Schlüter <i>et al.</i> , 2004c)
<i>A. flos-aqua</i> CL2019/03	chlorophyllide a, zeaxanthin, canthaxanthin, echinenone	Our study
<i>Microcystis aeruginosa</i>		
<i>M. aeruginosa</i> CCNP 1101	zeaxanthin, echinenone	(Wojtasiewicz and Stoń-Egiert 2016a)
<i>M. aeruginosa</i> (not given)	zeaxanthin, echinenone	(Walsh <i>et al.</i> , 1997)
<i>M. aeruginosa</i> CCNP 1101	myxo-xanthophyll, zeaxanthin, echinenone	Our study
<i>Nodularia spumigena</i>		
<i>N. spumigena</i> CCNP 1401	astaxanthin, echinenone	(Wojtasiewicz & Stoń-Egiert 2016a)
<i>N. spumigena</i> CHS1	canthaxanthin, echinenone	(Hassan <i>et al.</i> , 2020)
<i>N. spumigena</i> BS19	myxoxanthophyll, echinenone	(Staal <i>et al.</i> 2003b)
<i>N. spumigena</i> CS-584	4-ketomyxoxanthophyll, myxoxanthophyll, canthaxanthin, cis-canthaxanthin, echinenone	(Schlüter <i>et al.</i> , 2008a)
<i>N. spumigena</i> CCNP 1401	4-ketomyxoxanthophyll, canthaxanthin, echinenone	Our study
<i>Anabaena</i> spp.		
<i>Anabaena</i> sp. PCC 7120	4-ketomyxol 2'-fucoside, myxol 2'-fucoside, zeaxanthin, 3'-hydroxyechinenone, canthaxanthin, echinenone	(Kosourov <i>et al.</i> , 2016)
<i>Anabaena lemmermannii</i> KAC 16	4-ketomyxoxanthophyll-like pigment, echinenone, canthaxanthin4-keto-myxo-xanthophyll-like pigment, echinenone, canthaxanthin	(Schlüter <i>et al.</i> , 2004c)
<i>Anabaena cylindrica</i> CCNP1405	4-ketomyxoxanthophyll, zeaxanthin, myxoxanthophyll, echinenone, canthaxanthin	Our study

stereoisomeric forms (epimers) of Chl *a*, caused by the enolization at position C13² (Senge *et al.*, 2014). The epimer 13²S-stereoisomer is termed Chl *a'*. The reaction of epimerization occurs easily in an aqueous environment (Schaber *et al.*, 1984). The epimer Chl *a'* has a higher retention time in the reverse phase compared to Chl *a* (Schaber *et al.*, 1984; Nakamura *et al.*, 2003). Therefore, the product on the retention time of 41 min is most probably not a Chl *a'* because of lower retention time compared to Chl *a*. The low-intensity peaks close to the true Chl *a* could be chlorophyllide *a* esterified with geranylgeranyol, dihydrogeranylgeranyol and tetrahydrogeranylgeranyol, biosynthetic intermediates of Chl *a* (Nakamura & Watanabe, 1998; Nakamura *et al.*, 2003), but without the analytical standard we were unable to investigate this hypothesis.

Spectrophotometry technique

Spectrophotometry is often used as a quick technique for estimating the density of cultures by OD, for

example for growth rate determination in ecotoxicology studies (Breuer *et al.*, 2016). The *in vivo* absorption spectrum after mathematical operations (such as log-transformation and derivative spectrum calculation) can be applied to identification of phytoplankton classes (Johnsen *et al.*, 1994). However, this approach is rarely applied to the determination of CRs and Chls concentrations for reasons explained below.

Even though cell pigments have no potential for absorption in regions of wavelengths longer than 750 nm, absorbance with an intensity close to 0.5 was noted in UV-Vis spectra of cultures (Fig. 1A). Such a situation is caused by the presence of unicellular or filamentous structures and the deflection of light (scattering), which reduces the intensity of light reaching the detector perpendicular to the light beam. Light decreases rapidly during its passage through the cuvette due to absorption by pigments and scattering by cells in suspensions (Ma *et al.*, 2022), but in wavelength longer than 750 nm the apparent absorbance value is entirely due to

scattering (Yeh *et al.*, 2023a). Multiple scattering limits the application of the Bouguer–Lambert–Beer (BLB) law in pigment determination in culture (Yeh *et al.*, 2023b). Use of an integrating sphere type of detector together with dilution of the sample can reduce scattering and improve the absorbance measurement, and allow the distinction between molecular absorption and scattering to be made (Gaigalas *et al.*, 2009). For example, Ritchie & Sma-Air (2020) observed that real absorbance was at least two times lower for various microalgal species when comparing spectra between a standard dual-beam detector and an integrating sphere detector (Ritchie & Sma-Air, 2020). The scattering coefficient of the microalga *Chlorella sorokiniana* was approximately 50 times larger than the absorption coefficient, which indicates that scattering plays a main role in light attenuation in culture (Ma *et al.*, 2022). Therefore, with the standard configuration of a spectrophotometer, only a relative estimation of pigment concentrations can be given from UV-Vis spectrum of native culture. However, for the plants, the reflectance and the transmittance spectrum were applied for Chls and CRs content (Pérez-Patricio *et al.*, 2018). Moreover, a corrected version of the BLB law equations has been proposed for determination of pigments in *in vivo* cultures (Yeh *et al.*, 2023b).

The pigment package effect also should be taken into account when analysing *in vivo* spectra. This refers to the situation where the arrangement and concentration of pigments within a phytoplankton cell influence how much light energy they absorb (Huan *et al.*, 2022). When pigments are packed more densely within a cell, they can absorb more light, but this also alters the spectral shape of the absorption curve. The package effect explains the reduced absorption of the same amount of pigment contained within phytoplankton cells comparing to the extracted pigment dissolved in the solvent (Soja-Woźniak *et al.*, 2022). The package effect can also increase the error estimation of the Chl *a* concentration from space by remote sensing methods (Soja-Woźniak *et al.*, 2020) and is more pronounced for larger cells (Huan *et al.*, 2022).

Scattering was not an issue in a case of solvent extracts (Fig. 1B), because the solutions were homogeneous liquids. If a high baseline at 750 nm remained, this meant that there was residual debris in the solution, and the sample needed to be additionally filtered or centrifuged. The quantification of pigment groups using absorbance in solvent extract is carried out very widely. Similar to *in vivo* spectra of cultures, the UV-Vis spectrum of extracts (Fig. 1B, 1C) cannot give precise information on specific pigment composition. However, by the comparison of the peak intensity (preferably normalized to a mass-related factor, or, as in our situation, to the

chlorophyll peak), the relations between pigment types or rough differences in pigment group concentrations can be estimated. It is standard procedure to calculate the Chls and CRs concentrations using previously established equations ('trichromatic equations' (Aminot & Rey, 2000)). The coefficients in equations depend on the solvent applied (Lichtenthaler, 1987; Ritchie, 2008). The application of a trichromatic equation for cyanobacterial extracts can be problematic because this technique was developed for extracting Chl *a*, Chl *b* and Chl *c*, while cyanobacteria have only Chl *a*. The best option is to perform in-house calibration curves using Chl *a* standards prepared in the same solvent as cell extracts. However, it should be noted that the result of these equations is expressed as pigment content per ml of extract, not native culture, and appropriate re-calculation for volume of culture (or biomass) should be performed.

The observation that N-deficient *A. cylindrica* has fewer carotenoids and more phycobilins compared to nutrient-replete *A. cylindrica* at the same chlorophyll concentration is consistent with results from previous work with *Pseudanabaena* sp. and *Oscillatoria splendida* (de Loura *et al.*, 1987). The differences in carotenoid production in *Anabaena* sp. have previously been reported by Kosourov *et al.* (2016), who studied H₂ photoproduction. Under severe N deficiency, *Anabaena* sp. have lower echinenone and β-carotene production but accumulate glycosylated and hydroxylated carotenoids, such as myxol (or ketomyxol) 20-methylpentosides, 30-hydroxyechinenone and zeaxanthin (Kosourov *et al.*, 2016). The pigment composition and abundance shift are aimed at optimizing light harvesting and energy production (Murton *et al.*, 2017).

TLC technique

The visualization of pigments on a TLC plate can give initial valuable information about the lipophilic-solvent extractable pigments. From the literature, we know that the CRs have various colours and the maximum wavelength of absorption increases with the number of conjugated double bonds (Hirschberg & Chamovitz, 1994). The xanthophylls have the following colours: astaxanthin – red, zeaxanthin – yellow, canthaxanthin – orange, and beta-carotene – orange. This matches our observations. An increase in the number of double bonds increases the ability to scavenge radical species and improves photoprotective properties. Knowing the colour and spectra, predictions about the antioxidant properties can be hypothesized. The carotenes are chemically tetraterpenoids with no oxygen atoms and two rings at the chain ends. Xanthophylls are the oxygenized derivatives of carotenes. Selected xanthophylls, such as

myxoxanthophyll, are glycosylated carotenoids. Myxoxanthophyll is a yellow glycoside carotenoid exclusive to cyanobacteria, and its production is required for cell wall structure and thylakoid organization (Mohamed *et al.*, 2005). It exhibited a colour that was more orange than yellow, with the intensity of this colour derived from the concentration of the substance. Aphanizophyll had the same retardation factor (Rf) as myxoxanthophyll, another glycoside carotenoid, and it was not possible to resolve them on the TLC plate.

Generally, TLC is a quick method for screening of CRs and Chls presence, and at-a-glance comparison of extracts composition. TLC was used for screening of pigments composition in thermal mats, rich in cyanobacterial consortia (Portillo *et al.*, 2009), and the green algae *Coelastrella* sp. (Pushpalatha *et al.*, 2021). Another application was an investigation of chemotypes, for example for specimens from section Peltidea (lichen-forming fungi) (Miadlikowska *et al.*, 2018). A detailed protocol for analysis of secondary carotenoids in microalgae by TLC and UV-Vis spectroscopy includes more methods for TLC (Minyuk & Solovchenko, 2018). The micro-thin layer chromatography (MTLC) has been used for the separation of *Spirulina* pigments from various food products (Zarzycki *et al.*, 2011). Because of the low-resolution properties of the applied TLC chromatographic system, the exact composition of lipophilic pigments cannot be determined. However, there is room for TLC separation improvement. High-performance thin layer chromatography (HPTLC) can be applied for the identification and quantification of CRs and Chls, for example, in dietary supplements with *Chlorella vulgaris* and *Spirulina platensis* (Hynstova *et al.*, 2018).

HPLC-DAD technique

The identification of pigments in HPLC-DAD chromatograms was performed by comparing peak retention times and UV-Vis spectra with those of standards. There are databases containing pigment spectra (Clementson & Wojtasiewicz, 2019), which can support pigment identification. However, the solvent effect on spectrum performance needs to be considered. In addition, some pigments have identical spectra, and without comparing retention time with the standard, the identification can be incorrect. Thereby, purchasing and analysis of pigment standards is the most reliable method for accurate identification.

The inference of synechoxanthin in *S. salina* is based on the similarity of the spectrum reported in other studies (Graham & Bryant, 2008). In addition, this pigment was found in *Synechococcus* sp. PCC 7002 (Graham *et al.*, 2008), and several other cyanobacteria,

including the freshwater and soil *Synechocystis* sp. PCC 6803, *Nostoc* sp. PCC 7120 (Maresca *et al.*, 2008) and *Synechococcus* sp. BD154 strain (Ten *et al.*, 2016). Synechoxanthin, together with other xanthophylls such as zeaxanthin and echinenone, prevents the bioaccumulation of reactive oxygen species (ROS) in cyanobacterial cells and protects them from photo-oxidative stress (Zhu *et al.*, 2010). *Synechococcus* sp. synthesizes synechoxanthin from β -carotene with the help of the gene *cruE* (SYNPCC7002_A1248). Synechoxanthin can be found in the carotenes database (<http://carotenoiddb.jp/Entries/CA00779.html>).

The present study primarily focuses on evaluating the quality of pigments extracted from cyanobacteria. A semi-quantitative comparison was conducted to assess pigment concentrations within the extracts: Supplementary table S4 presents the concentration data, and Supplementary fig. S4 illustrates the ratio of selected pigment concentrations relative to Chl *a*. The analysis emphasizes the importance of accurate quantification methods, with HPLC-DAD identified as the most reliable technique for pigment analysis in biological extracts. Normalization of pigment concentrations to the biomass of native cell material enables meaningful comparisons across different cyanobacterial strains, facilitating a comprehensive understanding of pigment variability and biosynthesis efficiency among the strains studied. This approach ensures that the data are standardized, allowing for precise inter-strain comparisons and supporting the development of targeted strategies for pigment extraction and application in various biotechnological fields. Such a concept was presented in our previous study, where we analysed pigments as the marker of the stress of *S. salina* and *M. aeruginosa* caused by diclofenac and described the concentration of selected pigments in fg per cell (Kwidzińska *et al.*, 2024). Other studies with the same concept can be found (Jaiswal *et al.*, 2018; Lee *et al.*, 2021), for example for extremophile cyanobacteria (Silva *et al.*, 2024). There are techniques (mostly based on light moderation) to increase pigment production in microalgae for further high-scale applications (Elisabeth *et al.*, 2021; Pagels *et al.*, 2021a; Aizpuru & González-Sánchez, 2024), and in such cases, quantitative analysis by HPLC-DAD is needed.

The comparison of identified pigments with existing literature data (Table 3) serves as an indicative reference point. It is important to recognize that each cyanobacterial strain may produce a unique pigment profile, and intra-strain variations can occur due to differences in environmental growth conditions. For example, Schlüter *et al.* (2004) analysed differences in pigment production in low, medium and high light for *N. spumigena*, *Aphanizomenon* sp. and *Anabaena lemmermannii*, and results indicated an overproduction of carotenoids under high light (554 μ mol

photons $\text{m}^{-2} \text{s}^{-1}$). Similarly, *M. aeruginosa* demonstrated doubling of the level of zeaxanthin, echinone and β -carotene relative to Chl *a* under high light ($70 \mu\text{mol m}^{-2} \text{s}^{-1}$) (Walsh *et al.*, 1997). Several studies and strategies for the optimization of a cyanobacteria-based bio-production, mostly using light, but also pH, temperature and growth medium composition have been presented (Pagels *et al.*, 2021a, 2021b). These strategies aim to maximize the accumulation of specific pigments, which are often targeted for commercial or biotechnological applications.

Chlorophylls stability

Chlorophyll can be altered in many ways during the procedure of extraction and analysis (Schaber *et al.*, 1984), such as loss of magnesium (phaeophytin as a product) and photochemical degradation. The degradation (hydrolysis) of Chl *a* to chlorophyllide *a* can occur during the extraction (Hu *et al.*, 2013). The most problematic is the process of allomerization – the replacement of a hydrogen atom at the C-10 position with an oxygen-containing moiety (Hynninen & Hyvärinen, 2002), first demonstrated in 1918 (Willstätter & Stoll, 1913). The oxidation can be unnoticed, because of the same spectroscopic spectra of unaltered and oxidized Chl *a*. Brereton *et al.* (1994) observed progressive allomerization of Chl *a* on a chromatogram with time of degradation experiments. The allomerization can occur in polar (nucleophilic) organic solvents, and the mechanism depends on the solvent applied (Hynninen, 1981). For example, methanol solution exposed to atmosphere and light (Schaber *et al.*, 1984) completely changed Chl *a* to 10-hydroxychlorophyll *a* (10-OH Chl *a*) and the 10-methoxylactone of chlorophyll *a* (10-Me lactone Chl *a*). Both these products showed a lower retention time compared to Chl *a* on the C18 type column. The Chl *a* was degraded in methanol both in the dark and in the light, and under O_2 and N_2 (Brereton *et al.*, 1994). The carotenes were found to prevent the process of Chl *a* allomerization in the dark (Hynninen, 1981), because they are free radical scavengers, which is also the mechanism of photo-protection during photosynthesis. Schagerl & Künzl (2007) reported that extraction with *N,N'*-dimethylformamide, and methanol seems to promote the formation of degradation products (chlorophyllide *a*, allomer, epimer and phaeophytin *a*). Acetone was not only superior in Chl extraction, but it also caused fewer artefacts than the other solvents.

In the case of our extract, it is uncertain whether the same extract gave pure Chl *a* in test I, while probably an allomerization product was formed in test II of LPLC. However, the application of the HPLC allowed us to detect that the transformation of chlorophyll occurred (Fig. 4). The UV-Vis spectra

alone would be unable to detect this alteration, because of the similar spectrum of Chl *a* and its transformation products (Supplementary fig. S8). Therefore, it would be beneficial to confirm that intact Chl *a* is extracted by performing HPLC-DAD analysis.

Comparison of detection techniques

Five species of cyanobacteria living in the Baltic Sea were tested for photosynthetic and photoprotective pigments by three liquid chromatography techniques and/or spectrophotometry. Comparison of pigments for the same growing conditions but different species was presented. Differences in pigment composition between tested species and the literature data were noted, which are attributed to variations in strains and growing conditions. *S. salina* probably contains synechoxanthin, like other *Synechococcus* and *Synechocystis* species.

The HPLC analysis was needed to prove pigment identity in dots of TLC and fractions from LPLC. Furthermore, the HPLC helps to investigate the degradation of Chl *a*. The UV-Vis spectra of raw cultures and extracts differed in peaks of pigments, because of specific chromophore-protein interactions in cells, disturbed by extraction, but also due to the solvent effect on absorption. Scattering at 750 nm in UV-Vis *in vivo* spectra of cultures was also noted.

Each of the tested techniques shows applicability for pigment characterization, with the lowest selectivity being that of spectrophotometry, and the highest HPLC-DAD. Although spectrophotometry can give information about total CRs and Chls, only HPLC-DAD can differentiate the pigments. The TLC and LPLC can be applied for fractioning the organic extract with quite a good separation with proper eluent choice. These cheap and easy-to-perform techniques can support preliminary characterization of extracts. For both techniques, we make recommendations for improvement of separation. The full scan of lipophilic pigments by spectrophotometry, TLC, LPLC and HPLC is beneficial for comparison of cyanobacteria, screening for new valuable pigments, and purity of pigments. The SLE extraction and UV-Vis detection could be the technique of choice for total quantitative analysis, but the exact identification requires more effort and application of advance detection and additional sample processing. Extraction, separation and specification of cyanobacterial photosynthetic and photoprotective lipophilic pigments by easily available techniques for analytical purposes is possible. If the total content of the CRs and Chls is desired, this can be achieved by the spectrophotometry of *in vivo* cultures or extracts, with the recommendations presented in this study.

However, the identification of specific carotenoids and chlorophylls can be performed only with HPLC.

Acknowledgements

The authors would like to thank the Culture Collection of Northern Poland (CCNP, Institute of Oceanography, University of Gdansk, Poland) for providing cyanobacterial monocultures. An AI-based language model (ChatGPT, OpenAI) was used exclusively to assist with English language refinement. The AI tool did not contribute to the study design, data analysis, interpretation of results or writing of the scientific content. The authors reviewed and take full responsibility for the final manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

Financial support was provided by the National Science Center (Poland) under grants UMO-2019/35/B/NZ9/00253.

Supplementary material

The following supplementary material is accessible via the Supplementary Content tab on the article's online page at <https://doi.org/10.1080/09670262.2026.2682186>

Supplementary methods S1. Test of extraction solvent for lipophilic pigments.

Supplementary table S1. The strains applied in the study with basic information.

Supplementary table S2. Specification of power of light panel under the phytotron (Biogenet, model Fito700, Poland).

Supplementary table S3. Parameters of retention, detection, and calibration lines by HPLC-DAD for tested cyanobacterial pigments.

Supplementary table S4. Concentration of pigment determined for tested cyanobacteria species in mg of pigment per 1 ml of extract (two repetitions of extraction, nd – not detected).

Supplementary fig. S1. *Microcystis aeruginosa* and *Synechocystis salina* after centrifugation in Eppendorf type vials (4 min, 20,000 rpm); accumulation of *M. aeruginosa* on the top of supernatant marked.

Supplementary fig. S2. The UV-Vis spectra of extracts of *Synechocystis salina* pellet using acetone, 90% aq. acetone, or a mixture of acetone with 50 mM ammonium acetate buffer (90:10 v/v).

Supplementary fig. S3. The TLC plates of separation of *Synechocystis salina* extracts: part A. normal application of extracts using following media I. acetone, II. 90% aq. acetone, III. acetone + ammonium acetate buffer (9:1, v/v), and IV. 90% aq. acetone, III. acetone + ammonium acetate buffer (9:1, v/v) with the total removal of phase and resuspension of extract in acetone, part B. 90% aq. acetone extract developed with the “fast-start” technique.

Supplementary fig. S4. The ratio of concentrations of selected pigments toward chlorophyll a concentration obtained in extracts of acetone extract of four tested cyanobacterial species (n = 2).

Supplementary fig. S5. LPLC of organic extract from *Synechocystis salina*. Upper pictures – the column during the development of separation, lower pictures – collected fractions.

Supplementary fig. S6. The chromatograms of fractions 1 to 3 from test II of low-pressure liquid chromatography of extract from *Synechocystis salina*.

Supplementary fig. S7. The chromatograms of fractions 8 to 10 from test II of low-pressure liquid chromatography of extract from *Synechocystis salina*.

Supplementary fig. S8. Comparison of UV-Vis spectra of GLC fraction 2 and 4 from test 1 and test 2, respectively – the region 550-750 overlaps, despite the difference in compounds: in fraction 2 there is Chl *a*, while in fraction 4 there is an allomerization product of Chl *a*.

Author contributions

K. Kwidzińska: HPLC and TLC analysis, validation of chlorophylls and carotenes quantification by HPLC-DAD, preparation of extracts, editing manuscript; M. Caban: instrumental analysis, LPLC, HPLC and TLC analysis, conceptualization, funding acquisition, supervision, visualization of results, drafting and editing manuscript.

Data availability statement

The data that support the findings of this study are openly available in MostWiedzy repository at <http://doi.org/10.34808/50x0-we31>.

ORCID

Klaudia Kwidzińska  <http://orcid.org/0000-0002-5927-5813>

Magda Caban  <http://orcid.org/0000-0002-8040-5862>

References

- Aizpuru, A. & González-Sánchez, A. (2024). Traditional and new trend strategies to enhance pigment contents in microalgae. *World Journal of Microbiology & Biotechnology*, **40**: 1–26.
- Aminot, A. & Rey, F. (2000). Standard procedure for the determination of chlorophyll *a* by spectroscopic methods. *ICES Techniques in Marine Environmental Sciences*, **30**: 1–17.
- Assunção, J., Amaro, H.M., Lopes, G., Tavares, T., Malcata, F.X. & Guedes, A.C. (2021). *Synechocystis salina*: potential bioactivity and combined extraction of added-value metabolites. *Journal of Applied Phycology*, **33**: 3731–3746.
- Averina, S.G., Velichko, N.V., Pinevich, A.A., Senatskaya, E. V. & Pinevich, A.V. (2019). Non-a chlorophylls in cyanobacteria. *Photosynthetica*, **57**: 1109–1118.
- Badiale-Furlong, E., Pagnussatt, F.A. & Scaglioni, P.T. (2023). Antioxidant and antifungal compounds of microalgae. In *Handbook of Food and Feed from Microalgae* (Jacob-Lopes, E., Queiroz, M.I., Maroneze, M.M. & Zepka, L.Q. eds.), 253–266. Academic Press, New York.
- Bouyahya, A., Bakrim, S., Chamkhi, I., Taha, D., El Omari, N., El Mneyiy, N., El Hachlafi, N., El-Shazly, M., Khalid, A., Abdalla, A.N., Goh, K.W., Ming, L.C., Goh, B.H. & Aanniz, T. (2024). Bioactive substances of

- cyanobacteria and microalgae: Sources, metabolism, and anticancer mechanism insights. *Biomedicine & Pharmacotherapy*, **170**: 115989.
- Breterton, R.G., Rahmani, A., Liang, Y. & Kvalheim, O.M. (1994). Investigation of the allomerization reaction of chlorophyll a: use of diode array HPLC, mass spectrometry and chemometric factor analysis for the detection of early products. *Photochemistry and Photobiology*, **59**: 99–110.
- Breuer, F., Dören, L. & Ebke, K.-P. (2016). Comparison of four measuring techniques to assess growth inhibition in standardized tests with seven freshwater algae and cyanobacteria. *Toxicological & Environmental Chemistry*, **98**: 848–859.
- Castro, V., Oliveira, R. & Dias, A.C.P. (2023). Microalgae and cyanobacteria as sources of bioactive compounds for cosmetic applications: A systematic review. *Algal Research*, **76**: 103287.
- Charegaonkar, D. (2011). High-performance thin-layer chromatography: excellent automation. In *High-Performance Thin-Layer Chromatography (HPTLC)* (Srivastava, M.M., ed.), 55–65. Springer, Berlin, Heidelberg.
- Clementson, L.A. & Wojtasiewicz, B. (2019). Dataset on the absorption characteristics of extracted phytoplankton pigments. *Data in Brief*, **24**: 103875.
- Deepika, C., Wolf, J., Roles, J., Ross, I. & Hankamer, B. (2022). Sustainable production of pigments from Cyanobacteria. *Advances in Biochemical Engineering/Biotechnology*, **183**: 171–251.
- de Loura, I.C., Dubacq, J.P., Thomas, J.C., Loura, I.C.D.E., Dubacq, J.P. & Thomas, J.C. (1987). The effects of nitrogen deficiency on pigments and lipids of cyanobacteria. *Plant Physiology*, **83**: 838–843.
- Elisabeth, B., Rayen, F. & Behnam, T. (2021). Microalgae culture quality indicators: a review. *Critical Reviews in Biotechnology*, **41**: 457–473.
- Freiberg, R., Nömm, M., Tönno, I., Alliksaar, T., Nöges, T. & Kisand, A. (2011). Dynamics of phytoplankton pigments in water and surface sediments of a large shallow lake. *Estonian Journal of Earth Sciences*, **60**: 91–101.
- Gaigalas, A.K., He, H.-J. & Wang, L. (2009). Measurement of Absorption and Scattering With an Integrating Sphere Detector: Application to Microalgae. *Journal of Research of the National Institute of Standards and Technology*, **114**: 69–81.
- Graham, J.E. & Bryant, D.A. (2008). The biosynthetic pathway for synechoxanthin, an aromatic carotenoid synthesized by the euryhaline, unicellular cyanobacterium *Synechococcus* sp. strain PCC 7002. *Journal of Bacteriology*, **190**: 7966–7974.
- Graham, J.E.E., Lecomte, J.T.J.T.J., Bryant, D.A.A., Park, U. V. & Pennsylv, V. (2008). Synechoxanthin, an aromatic C40 xanthophyll that is a major carotenoid in the cyanobacterium *Synechococcus* sp. PCC 7002. *Journal of Natural Products*, **71**: 1647–1650.
- Häder, D.P., Kumar, H.D., Smith, R.C. & Worrest, R.C. (2007). Effects of solar UV radiation on aquatic ecosystems and interactions with climate change. *Photochemical & Photobiological Sciences*, **6**: 267–285.
- Hirschberg, J. & Chamovitz, C. 1994. Carotenoids in Cyanobacteria. In *The Molecular Biology of Cyanobacteria* (Bryant, D.A., ed.), 361–390. Springer, Dordrecht.
- Hu, X., Tanaka, A. & Tanaka, R. (2013). Simple extraction methods that prevent the artifactual conversion of chlorophyll to chlorophyllide during pigment isolation from leaf samples. *Plant Methods*, **9**: 1.
- Huan, Y., Sun, D., Wang, S., Zhang, H., Li, Z., Zhang, Y. & He, Y. (2022). Phytoplankton package effect in oceanic waters: Influence of chlorophyll-a and cell size. *Science of the Total Environment*, **838**: 155876.
- Hynninen, P.H. (1981). Mechanism of the allomerization of chlorophyll: inhibition of the allomerization by carotenoid pigments. *Zeitschrift für Naturforschung - Section B Journal of Chemical Sciences*, **36**: 1010–1016.
- Hynninen, P.H. & Hyvärinen, K. (2002). Tracing the allomerization pathways of chlorophylls by ¹⁸O-labeling and mass spectrometry. *Journal of Organic Chemistry*, **67**: 4055–4061.
- Hynstova, V., Sterbova, D., Klejdus, B., Hedbavny, J., Huska, D. & Adam, V. (2018). Separation, identification and quantification of carotenoids and chlorophylls in dietary supplements containing *Chlorella vulgaris* and *Spirulina platensis* using High Performance Thin Layer Chromatography. *Journal of Pharmaceutical & Biomedical Analysis*, **148**: 108–118.
- Ilavarasi, A., Pandiaraj, D., Mubarak Ali, D., Mohammed Ilyas, M.H. & Thajuddin, N. (2012). Evaluation of efficient extraction methods for recovery of photosynthetic pigments from microalgae. *Pakistan Journal of Biological Sciences*, **15**: 883–888.
- Jaiswal, A., Koli, D.K., Kumar, A., Kumar, S. & Sagar, S. (2018). Pigments analysis of cyanobacterial strains. *International Journal of Chemical Studies*, **6**: 1248–1251.
- Johnsen, G., Samset, O., Granskog, L. & Sakshaug, E. (1994). In vivo absorption characteristics in 10 classes of bloom-forming phytoplankton - Taxonomic characteristics and responses to photoadaptation by means of discriminant and HPLC analysis. *Marine Ecology Progress Series*, **105**: 149–158.
- Kosourov, S., Murukesan, G., Jokela, J. & Allahverdiyeva, Y. (2016). Carotenoid biosynthesis in *Calothrix* sp. 336/3: Composition of carotenoids on full medium, during diazotrophic growth and after long-term H₂ photoproduction. *Plant and Cell Physiology*, **57**: 2269–2282.
- Kwidzińska, K., Zalewska, M., Aksmann, A., Kobos, J., Mazur-Marzec, H. & Caban, M. (2024). Multi-biomarker response of cyanobacteria *Synechocystis salina* and *Microcystis aeruginosa* to diclofenac. *Journal of Hazardous Materials*, **471**: 134373.
- Lee, H., Noh, Y.J., Hong, S.J., Lee, H., Kim, D.M., Cho, B. K., Lee, C.G. & Choi, H.K. (2021). Photosynthetic pigment production and metabolic and lipidomic alterations in the marine cyanobacteria *Synechocystis* sp. PCC 7338 under various salinity conditions. *Journal of Applied Phycology*, **33**: 197–209.
- Lehmann, M., Vamvaka, E., Torrado, A., Jahns, P., Dann, M., Rosenhammer, L., Aziba, A., Leister, D. & Rühle, T. (2021). Introduction of the carotenoid biosynthesis α -branch into *Synechocystis* sp. PCC 6803 for lutein production. *Frontiers in Plant Science*, **12**: 1–13.
- Liang, C., Zhao, F., Wei, W., Wen, Z. & Qin, S. (2006). Carotenoid biosynthesis in cyanobacteria: Structural and evolutionary scenarios based on comparative genomics. *International Journal of Biological Sciences*, **2**: 197–207.
- Lichtenthaler, H.K. (1987). Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. *Methods in Enzymology*, **148**: 350–382.
- Ma, S., Zeng, W., Huang, Y., Zhu, X., Xia, A., Zhu, X. & Liao, Q. (2022). Revealing the synergistic effects of cells, pigments, and light spectra on light transfer during

- microalgae growth: A comprehensive light attenuation model. *Bioresource Technology*, **348**: 126777.
- Malhotra, A. & Örmeci, B. (2023). Detection and identification of a mixed cyanobacteria and microalgae culture using derivative spectrophotometry. *Journal of Photochemistry & Photobiology, B: Biology*, **238**: 112616.
- Maresca, J.A., Graham, J.E. & Bryant, D.A. (2008). The biochemical basis for structural diversity in the carotenoids of chlorophototrophic bacteria. *Photosynthesis Research*, **97**: 121–140.
- Mazur-Marzec, H., Błaszczyk, A., Felczykowska, A., Hohlfeld, N., Kobos, J., Toruńska-Sitarz, A., Devi, P., Montalvão, S., D'souza, L., Tammela, P., Mikosik, A., Bloch, S., Nejman-Faleńczyk, B. & Węgrzyn, G. (2015). Baltic cyanobacteria – a source of biologically active compounds. *European Journal of Phycology*, **50**: 343–360.
- Miadlikowska, J., Magain, N., Pardo-De La Hoz, C.J., Niu, D., Goward, T., Sérusiaux, E. & Lutzoni, F. (2018). Species in section Peltidea (aphthosa group) of the genus *Peltigera* remain cryptic after molecular phylogenetic revision. *Plant and Fungal Systematics*, **63**: 45–64.
- Michel Havaux, K.N. (1999). The violaxanthin cycle protects plants from photooxidative damage by more than one mechanism. *Proceedings of the National Academy of Sciences USA*, **96**: 8762–8767. <https://www.pnas.org/doi/10.1073/pnas.96.15.8762>
- Minyuk, G.S. & Solovchenko, A.E. (2018). Express analysis of microalgal secondary carotenoids by TLC and UV-Vis spectroscopy. *Methods in Molecular Biology*, **1852**: 73–95.
- Mohamed, H.E., Van De Meene, A.M.L., Roberson, R.W. & Vermaas, W.F.J. (2005). Myxoxanthophyll is required for normal cell wall structure and thylakoid organization in the cyanobacterium *Synechocystis* sp. strain PCC 6803. *Journal of Bacteriology*, **187**: 6883–6892.
- Morlock, G.E. (2021). High-performance thin-layer chromatography combined with effect-directed assays and high-resolution mass spectrometry as an emerging hyphenated technology: A tutorial review. *Analytica Chimica Acta*, **1180**: 338644.
- Murton, J., Nagarajan, A., Nguyen, A.Y., Liberton, M., Hancock, H.A., Pakrasi, H.B. & Timlin, J.A. (2017). Population-level coordination of pigment response in individual cyanobacterial cells under altered nitrogen levels. *Photosynthesis Research*, **134**: 165–174.
- Nakamura, A., Akai, M., Yoshida, E., Taki, T. & Watanabe, T. (2003). Reversed-phase HPLC determination of chlorophyll a' and phylloquinone in Photosystem I of oxygenic photosynthetic organisms: Universal existence of one chlorophyll a' molecule in Photosystem I. *European Journal of Biochemistry*, **270**: 2446–2458.
- Nakamura, A. & Watanabe, T. (1998). HPLC determination of photosynthetic pigments during greening of etiolated barley leaves: Evidence for the biosynthesis of chlorophyll a'. *FEBS Letters*, **426**: 201–204.
- Nováková, M., Fábryová, T., Vokurková, D., Dolečková, I., Kopecký, J., Hrouzek, P., Tůmová, L. & Cheel, J. (2020). Separation of the glycosylated carotenoid myxoxanthophyll from *Synechocystis salina* by HPLC and evaluation of its antioxidant, tyrosinase inhibitory and immune-stimulating properties. *Separations*, **7**: 1–15.
- Pagels, F., Pereira, R.N. & Vicente, A. (2021a). Extraction of pigments from microalgae and cyanobacteria - a review on current methodologies. *Applied Sciences*, **11**: 5187.
- Pagels, F., Vasconcelos, V. & Guedes, A.C. (2021b). Carotenoids from cyanobacteria: Biotechnological potential and optimization strategies. *Biomolecules*, **11**: 753.
- Palenik, B. & Haselkorn, R. (1992). Multiple evolutionary origins of prochlorophytes, the chlorophyll b-containing prokaryotes. *Nature*, **355**: 265–267.
- Paliwal, C., Ghosh, T., George, B., Pancha, I., Maurya, R., Chokshi, K., Ghosh, A. & Mishra, S. (2016). Microalgal carotenoids: Potential nutraceutical compounds with chemotaxonomic importance. *Algal Research*, **15**: 24–31.
- Pérez-Patricio, M., Camas-Anzueto, J.L., Sanchez-Alegria, A., Aguilar-González, A., Gutiérrez-Miceli, F., Escobar-Gómez, E., Voisin, Y., Rios-Rojas, C. & Grajales-Coutiño, R. (2018). Optical method for estimating the chlorophyll contents in plant leaves. *Sensors (Switzerland)*, **18**: 650.
- Portillo, M.C., Sririni, V., Kanoksilapatham, W. & Gonzalez, J.M. (2009). Pigment profiles and bacterial communities from Thailand thermal mats. *Antonie Van Leeuwenhoek International Journal of General & Molecular Microbiology*, **96**: 559–567.
- Pushpalatha, S., Sangeetha, R., Ariraman, S., Ashokkumar, B. & Varalakshmi, P. (2021). Photocatalyst (TiO₂) as an enhancer: an attempt to enhance the production of carotenoids and lipids with the combined oxidative stresses in *Coelastrella* sp. M60. *Clean Technologies and Environmental Policy*, **23**: 41–53.
- Ritchie, R.J. (2008). Universal chlorophyll equations for estimating chlorophylls a, b, c, and d and total chlorophylls in natural assemblages of photosynthetic organisms using acetone, methanol, or ethanol solvents. *Photosynthetica*, **46**: 115–126.
- Ritchie, R.J. & Sma-Air, S. (2020). Using integrating sphere spectrophotometry in unicellular algal research. *Journal of Applied Phycology*, **32**: 2947–2958.
- Saini, D.K., Pabbi, S. & Shukla, P. (2018). Cyanobacterial pigments: Perspectives and biotechnological approaches. *Food and Chemical Toxicology*, **120**: 616–624.
- Sandybayeva, S.K., Kossalbayev, B.D., Zayadan, B.K., Sadvakasova, A.K., Bolatkhan, K., Zadneprovskaya, E. V., Kakimov, A.B., Alwasel, S., Leong, Y.K., Allakhverdiev, S.I. & Chang, J.S. (2022). Prospects of cyanobacterial pigment production: Biotechnological potential and optimization strategies. *Biochemical Engineering Journal*, **187**: 108640.
- Santos-Merino, M., Singh, A.K. & Ducat, D.C. (2019). New applications of synthetic biology tools for cyanobacterial metabolic engineering. *Frontiers in Bioengineering and Biotechnology*, **7**: 1–24.
- Schaber, P.M., Hunt, J.E., Fries, R. & Katz, J.J. (1984). High-performance liquid chromatographic study of the chlorophyll allomerization reaction. *Journal of Chromatography A*, **316**: 25–41.
- Schagerl, M. & Künzl, G. (2007). Chlorophyll a extraction from freshwater algae - A reevaluation. *Biologia*, **62**: 270–275.
- Schlüter, L., Garde, K. & Kaas, H. (2004). Detection of the toxic cyanobacteria *Nodularia spumigena* by means of a 4-keto-myxoxanthophyll-like pigment in the Baltic Sea. *Marine Ecology Progress Series*, **275**: 69–78.
- Schlüter, L., Lutnæs, B.F., Liaaen-jensen, S., Garde, K., Kaas, H., Jameson, I. & Blackburn, S. (2008). Correlation of the content of hepatotoxin nodularin and glycosidic carotenoids, 4-ketomyxol-20-fucoside and novel 10-O-methyl-4-ketomyxol-20-fucoside, in 20 strains of the cyanobacterium *Nodularia spumigena*. *Biochemical Systematics and Ecology*, **36**: 749–757.
- Senge, M.O., Ryan, A.A., Letchford, K.A., MacGowan, S.A. & Mielke, T. (2014). Chlorophylls, symmetry, chirality, and photosynthesis. *Symmetry*, **6**: 781–843.

- Silva, R., Gonçalves, T., Morone, J., Moreira, G.A., Morais, J., Hentschke, G.S., Álvarez-Gutiérrez, P.E., Batista-García, R.A., Vasconcelos, V. & Lopes, G. (2024). Pigments profile and antioxidant potential of extremophile cyanobacteria isolated from the Mexican Volcanic Lake Chichonal. *Algal Research*, **8**: 103578.
- Singh, P., Kumar, A., Singh, V.K. & Shrivistava, A., eds. (2020). *Advance in cyanobacterial biology*. 1st ed. Elsevier, London.
- Soja-Woźniak, M., Clementson, L., Wojtasiewicz, B. & Baird, M. (2022). Estimation of the global distribution of phytoplankton light absorption from pigment concentrations. *Journal of Geophysical Research: Oceans*, **127**: 1–16.
- Soja-Woźniak, M., Laiolo, L., Baird, M.E., Matear, R., Clementson, L., Schroeder, T., Doblin, M.A. & Suthers, I.M. (2020). Effect of phytoplankton community size structure on remote-sensing reflectance and chlorophyll a products. *Journal of Marine Systems*, **211**: 103400.
- Takaichi, S. (2011). Carotenoids in algae: Distributions, biosyntheses and functions. *Marine Drugs*, **9**: 1101–1118.
- Ten, L.N., Yoon, G.J. & Chae, S.M. (2016). Fatty-acid and carotenoid compositions of cyanobacterium *Synechococcus* sp. BD154. *Chemistry of Natural Compounds*, **52**: 689–691.
- Walsh, K., Jones, G.J. & Dunstan, R.H. (1997). Effect of irradiance on fatty acid, carotenoid, total protein composition and growth of *Microcystis aeruginosa*. *Phytochemistry*, **44**: 817–824.
- Willstätter, R. & Stoll, A. (1913). *Untersuchungen über Chlorophyll*. Sustainability (Switzerland). Springer Berlin, Heidelberg, Berlin Heidelberg.
- Wojtasiewicz, B. & Stoń-Egiert, J. (2016). Bio-optical characterization of selected cyanobacteria strains present in marine and freshwater ecosystems. *Journal of Applied Phycology*, **28**: 2299–2314.
- Xu, H., Zhang, D. & Mo, F. (2022). High-throughput automated platform for thin layer chromatography analysis. *STAR Protocols*, **3**: 101893.
- Yeh, Y.C., Ebbing, T., Frick, K., Schmid-Staiger, U., Haasdonk, B. & Tovar, G.E.M. (2023a). Improving determination of pigment contents in microalgae suspension with absorption spectroscopy: light scattering effect and Bouguer–Lambert–Beer Law. *Marine Drugs*, **21**: 619.
- Yeh, Y.C., Haasdonk, B., Schmid-Staiger, U., Stier, M. & Tovar, G.E.M. (2023b). A novel model extended from the Bouguer–Lambert–Beer law can describe the non-linear absorbance of potassium dichromate solutions and microalgae suspensions. *Frontiers in Bioengineering and Biotechnology*, **11**: 1–11.
- Zarzycki, P.K., Zarzycka, M.B., Clifton, V.L., Adamski, J. & Głód, B.K. (2011). Low-parachor solvents extraction and thermostated micro-thin-layer chromatography separation for fast screening and classification of spirulina from pharmaceutical formulations and food samples. *Journal of Chromatography A*, **1218**: 5694–5704.
- Zhu, Y., Graham, J.E., Ludwig, M., Xiong, W., Alvey, R.M., Shen, G. & Bryant, D.A. (2010). Roles of xanthophyll carotenoids in protection against photoinhibition and oxidative stress in the cyanobacterium *Synechococcus* sp. strain PCC 7002. *Archives of Biochemistry and Biophysics*, **504**: 86–99.
- Zinicovscaia, I. & Cepoi, L., eds. (2016). *Cyanobacteria for Bioremediation of Wastewaters*. Springer International Publishing, Cham.

Supplementary Information

to

Extraction and separation of cyanobacterial photosynthetic and photoprotective pigments: comparison of practical and accessible methodologies

Klaudia Kwidzińska¹, Magda Caban^{1*}

¹*University of Gdansk, Faculty of Chemistry, Department of Environmental Analysis, ul. Wita Stwosza 63, 80-308 Gdańsk, Poland*

***Corresponding author:** Phone (+48 58) 523 5208; E-mail address: magda.caban@ug.edu.pl

Supplementary methods S1. Test of extraction solvent for lipophilic pigments

The lyophilized mass of *S. salina* in amount 2.0 mg was added to a plastic vial with 3 2 ceramic homogenization beads (2.8 mm), β10 mg of glass homogenization microspheres (0.1 mm) (Ceramic Bead Media, OMNI, USA). Then, 1 mL of cold solvents: acetone (HPLC grade), 90% aq. acetone or a mixture of acetone with 50 mM ammonium acetate buffer (90:10 v/v) was added to the vial. The cells were then homogenized using a Bead Ruptor Elite homogenizer (Omni International, USA) for three cycles of 30 s shaking (4 m s⁻¹) with ice cooling between cycles. The extracts were collected and the UV-Vis spectrum was measured (Figure SX). The acetone without water has a lower potential for penetration of cell pellets. The addition of water (pure or buffered), increases the extraction efficiency. At the second round of extraction, for the water-based solvents, there was about 5% more extracted, while with acetone there was still not a negligible amount of pigments. The 90% aq. acetone is one of the most applied solvents, well established for photosynthetic pigments extraction (Schagerl & Künzl, 2007)

Supplementary table S1. The strains applied in the study with basic information.

Strain	Isolation place and year	Growth medium	Selected ecology facts
<i>Synechocystis salina</i> CCNP 1104 (order of Synechococcales) unicellular	Gulf of Gdansk, 2006	BG11	Marine pico-cyanobacteria, do not produce blooms and toxins
<i>Microcystis aeruginosa</i> CCNP 1101 (order of Chroococcales) unicellular	Vistula Lagoon, 2005	Z8	Freshwater and brackish water, cosmopolitan species
<i>Aphanizomenon flos-aquae</i> CL2019/03 (order of Nostocales) filamentous	isolated from Curonian Lagoon, 2018	Z8	Colony forming causes massive blooms in brackish water
<i>Nodularia spumigena</i> CCNP1401 (order of Nostocales) filamentous	Gulf of Gdansk, 1997	Z8	Because of the massive blooms in the Baltic Sea and the toxins nodularin, The Gulf of Gdansk was first observed in 1994 [26]
<i>Anabaena cylindrica</i> CCNP1405 (order of Nostocales) filamentous	Puck Bay, 2005	Z8 or Z8 without nitrates (N-def)	Can assimilate nitrogen from the air, create massive blooms

Supplementary table S2. Specification of power of light panel under the phytotron (Biogenet, model Fito700, Poland).

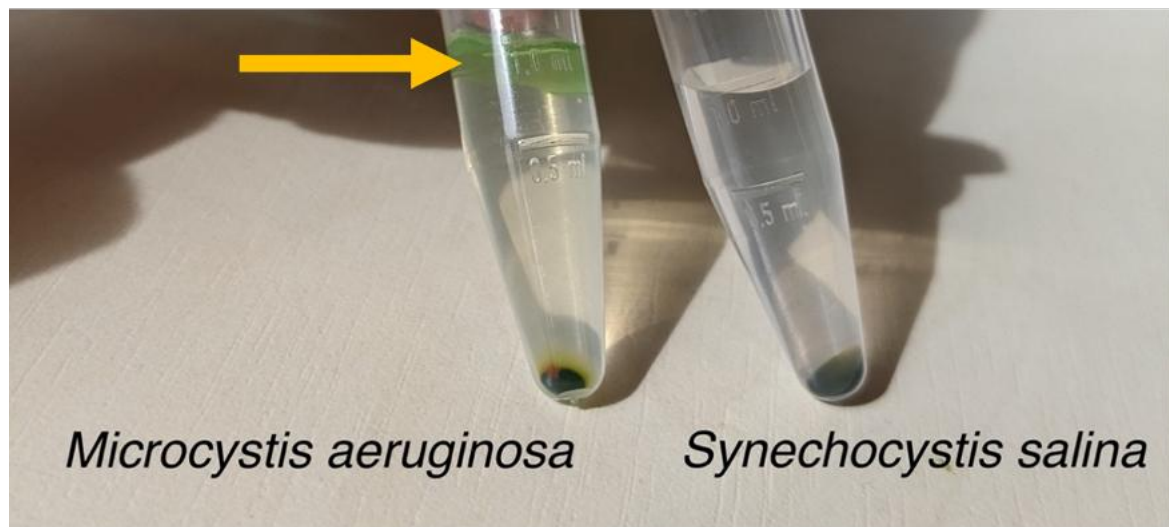
Canal	Percentage of intensity of light [%]
CW (cool white)	1
WW (warm white)	1
B (blue)	1
DB (deep blue)	1
R (red)	2
DR (deep red)	4
FR (far red)	1
UV (ultraviolet)	0
Total PAR analyzed by luminometer close to bottles with cultures	$35 \pm 5 \mu\text{mol m}^{-2} \text{s}^{-1}$

Supplementary table S3. Parameters of retention, detection, and calibration lines by HPLC-DAD for tested cyanobacterial pigments.

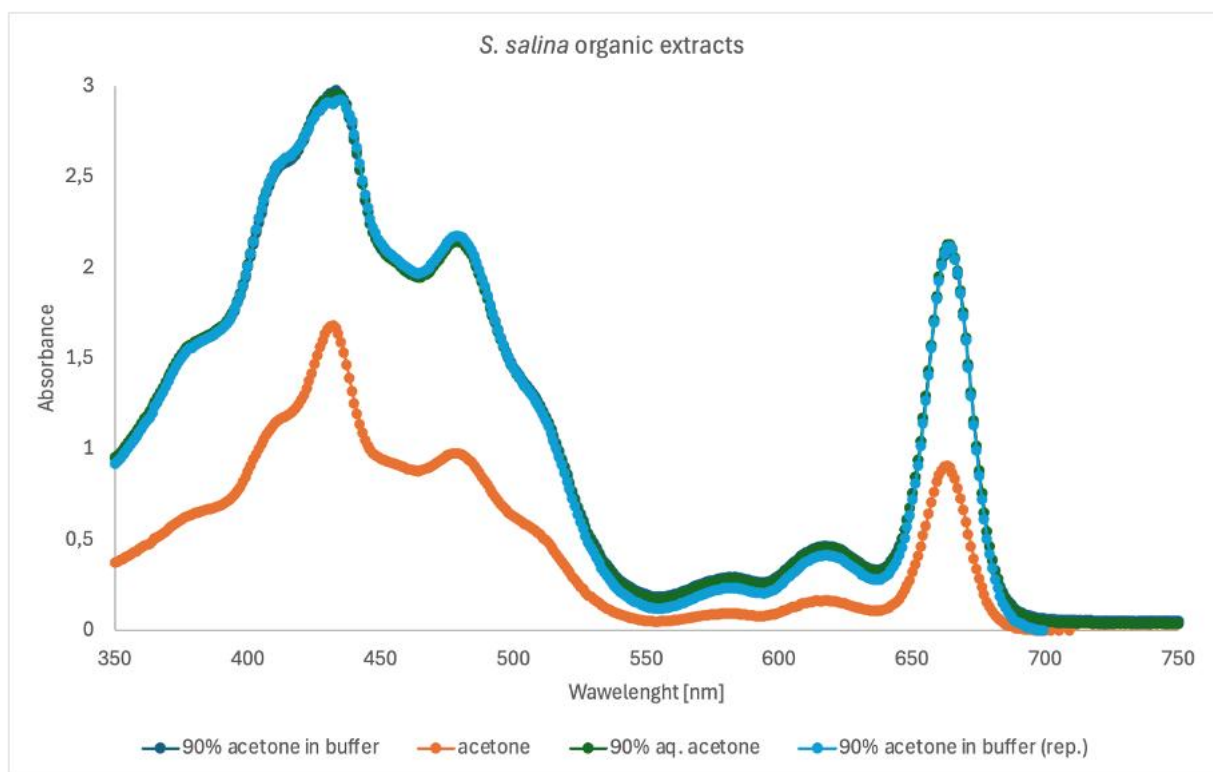
No.	Name	RT [min]	Wavelength [nm]	LOQ [mg L ⁻¹]	R ²	RSD [%]	Accuracy [%]
1	Chlorophyllide a	3.98	402	0.0201	0.9999	<4.6	98.05-101.52
2	Violaxanthin	14.88	480	0.0035	0.9996	<1.4	98.07-116.67
3	4-ketomyxoxanthophyll	16.13	480	0.0021	0.9994	<2.7	93.71-105.00
4	Astaxanthin	18.11	480	0.006	0.9995	<3.7	88.89-100.63
5	Aphanizophyll/Myxoxanthophyll	20.13	452	0.0028	0.9998	<2.0	93.33-102.73
6	Diatoxanthin	24.93	452	0.0023	0.9995	<3.8	90.00-115.00
7	Zeaxanthin/Lutein	26.78	445	0.0053	0.9998	<2.5	95.00-113.00
8	Canthaxanthin	32.26	480	0.0052	0.9999	<1.1	98.10-104.00
9	Chlorophyll b	39.41	655	0.0074	0.9993	<3.4	92.5-114.62
10	B-cryptoxanthin	41.77	452	0.0035	0.9998	<0.9	98.25-116.67
11	Chlorophyll a	43.01	655	0.011	0.9997	<2.4	97.05-106.50
12	Echinenone	43.87	460	0.003	0.9995	<2.0	84.29-113.33
13	Lycopene	50.42	475	0.003	0.9994	<1.9	85.71-104.35
14	Pheophytin	52.37	402	0.01	0.9995	<2.5	96.05-111.11
15	β-carotene	54.41	452	0.0052	0.9994	<2.2	96.74-104.00

Supplementary table S4. Concentration of pigment determined for tested cyanobacteria species in mg of pigment per 1 ml of extract (two repetition of extraction, nd – not detected).

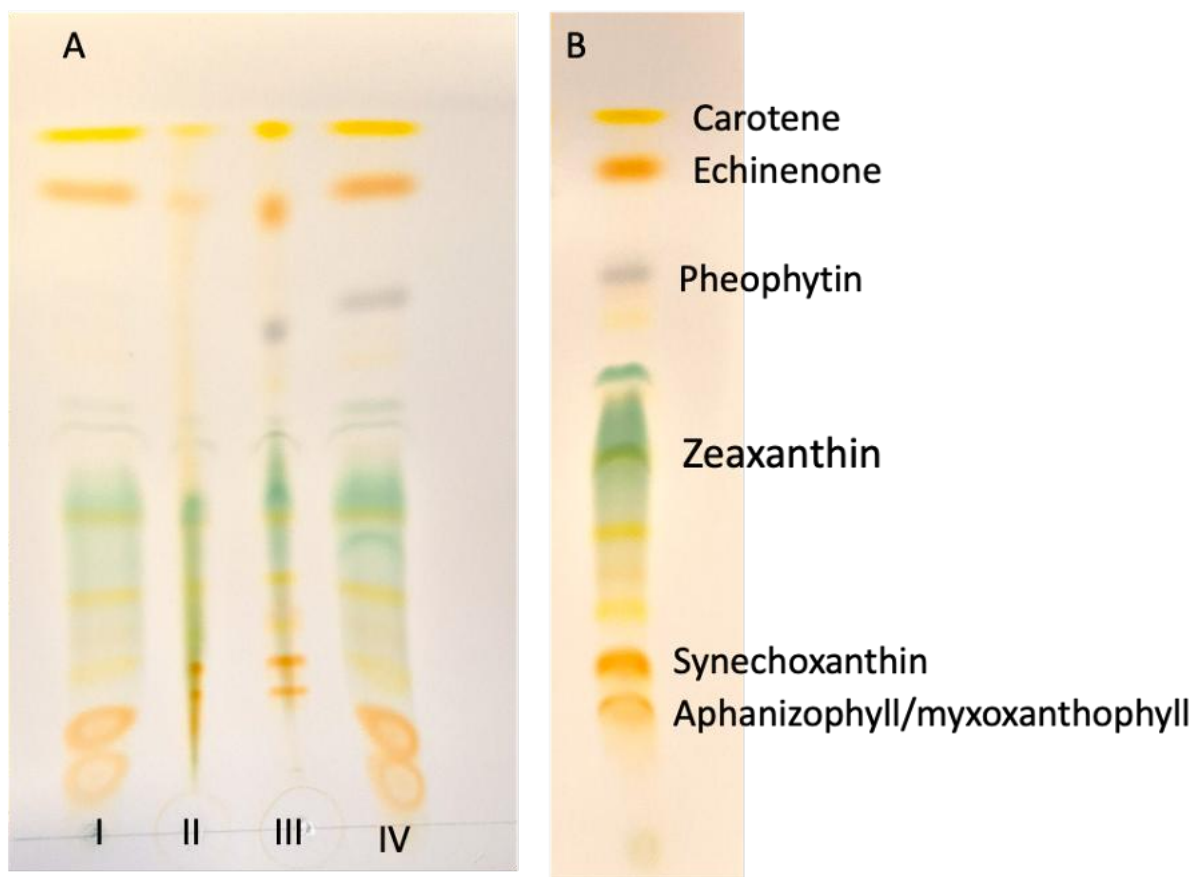
Pigment	<i>Synechocystis salina</i>		<i>Microcystis aeruginosa</i>		<i>Nodularia spumigena</i>		<i>Aphanizomenon flos-aqua</i>	
	Test 1	Test 2	Test 1	Test 2	Test 1	Test 2	Test 1	Test 2
Chlorophyllide <i>a</i>	nd	nd	nd	nd	0.104	0.190	0.141	0.126
Violaxanthin	nd	nd	nd	nd	nd	nd	nd	nd
4-ketomyxoxanthophyll	nd	nd	nd	nd	3,174	4,171	nd	nd
Astaxanthin	nd	nd	nd	nd	nd	nd	nd	nd
Aphanizophyll/ myxoxanthophyll	0.310	0.307	0.501	0.629	0.233	0.268	0.066	0.074
Diatoxanthin	nd	nd	nd	nd	nd	nd	nd	nd
Zeaxanthin/Lutein	0.458	0.590	1.292	1.618	nd	nd	0.076	0.072
Canthaxanthin	nd	nd	nd	nd	0.081	0.107	0.015	0.010
Chlorophyll b	nd	nd	nd	nd	nd	nd	nd	nd
B-cryptoxanthin	nd	nd	nd	nd	nd	nd	nd	nd
Chlorophyll a	27.784	25.229	12.303	19.284	16.579	15.587	7.681	7.227
Echinenone	0.910	1.160	0.493	0.835	1.421	1.432	0.262	0.261
Lycopene	nd	nd	nd	nd	nd	nd	nd	nd
Pheophytin	0.543	0.164	0.104	0.145	0.116	0.107	0.035	0.016
β -carotene	3.156	5.402	3.160	4.865	3.090	3.208	0.411	0.427



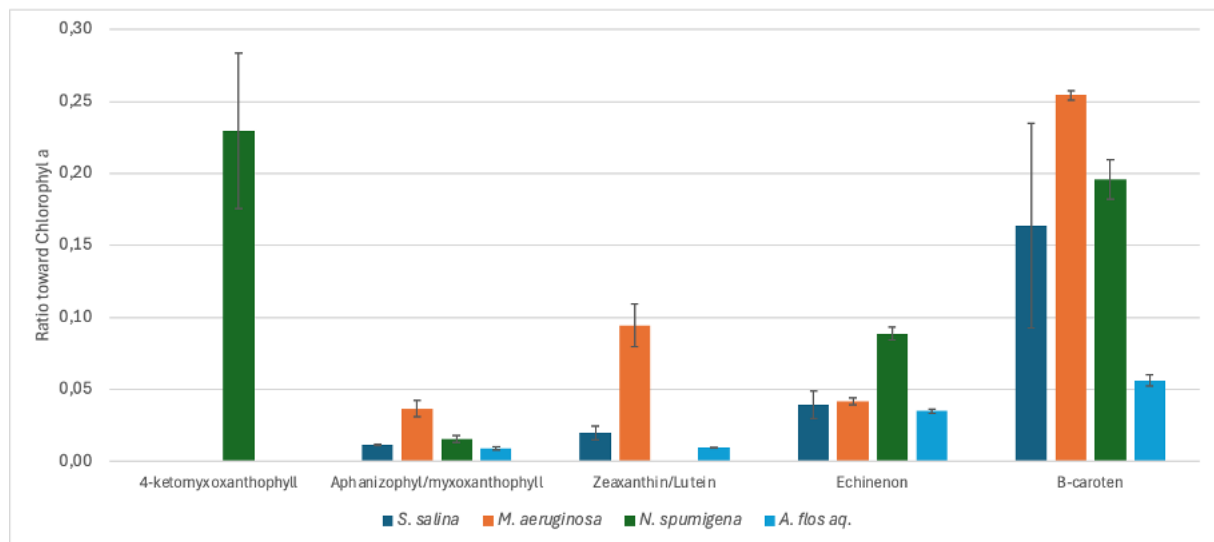
Supplementary fig. S1. The picture of *Microcystis aeruginosa* and *Synechocystis salina* after centrifugation in Eppendorf type vials (4 min, 20,000 rpm); accumulation of *M. aeruginosa* on the top of supernatant marked.



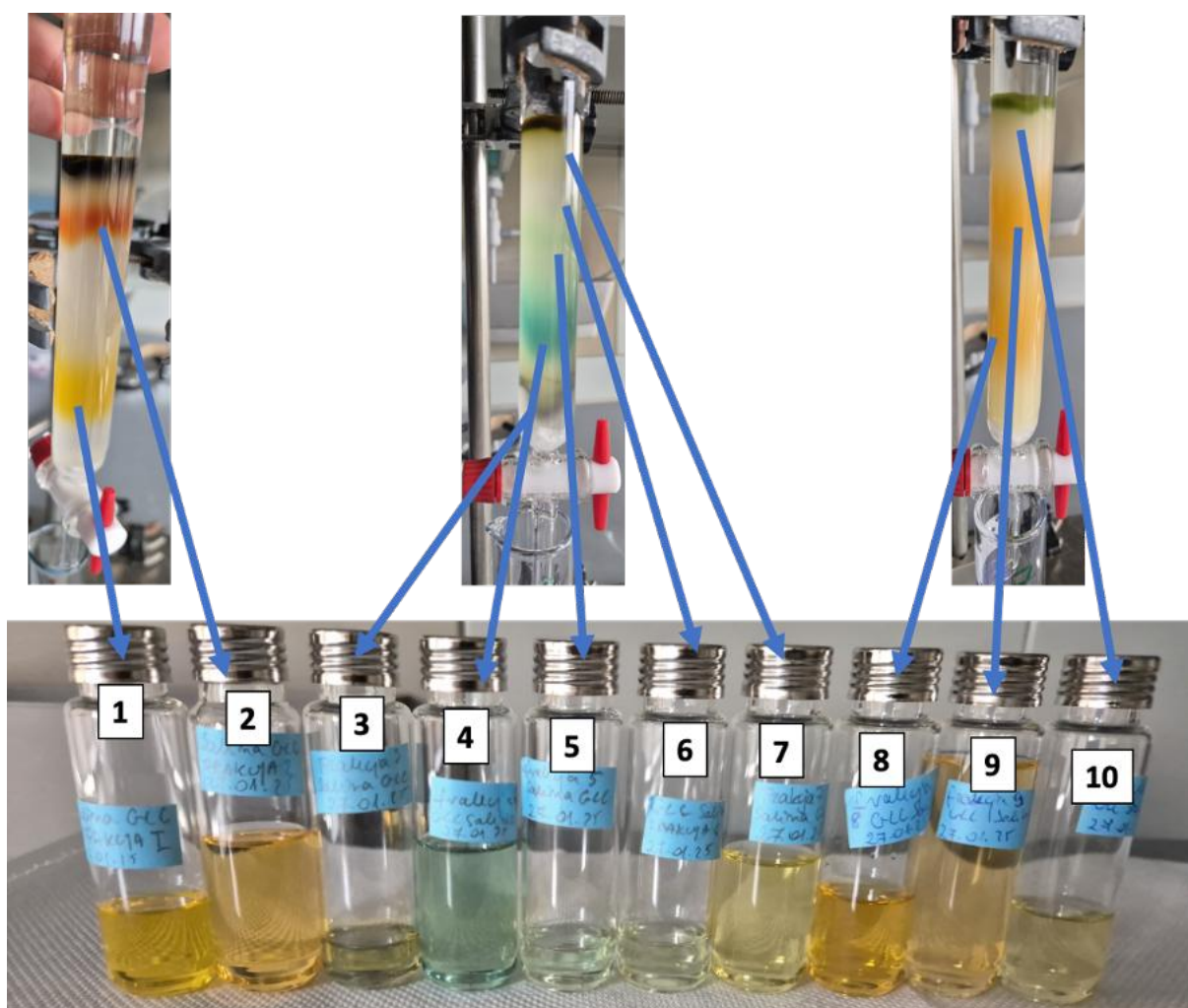
Supplementary fig. S2. The UV-Vis spectrums of extracts of *Synechocystis salina* pellet using acetone, 90% aq. acetone, or a mixture of acetone with 50 mM ammonium acetate buffer (90:10 v/v).



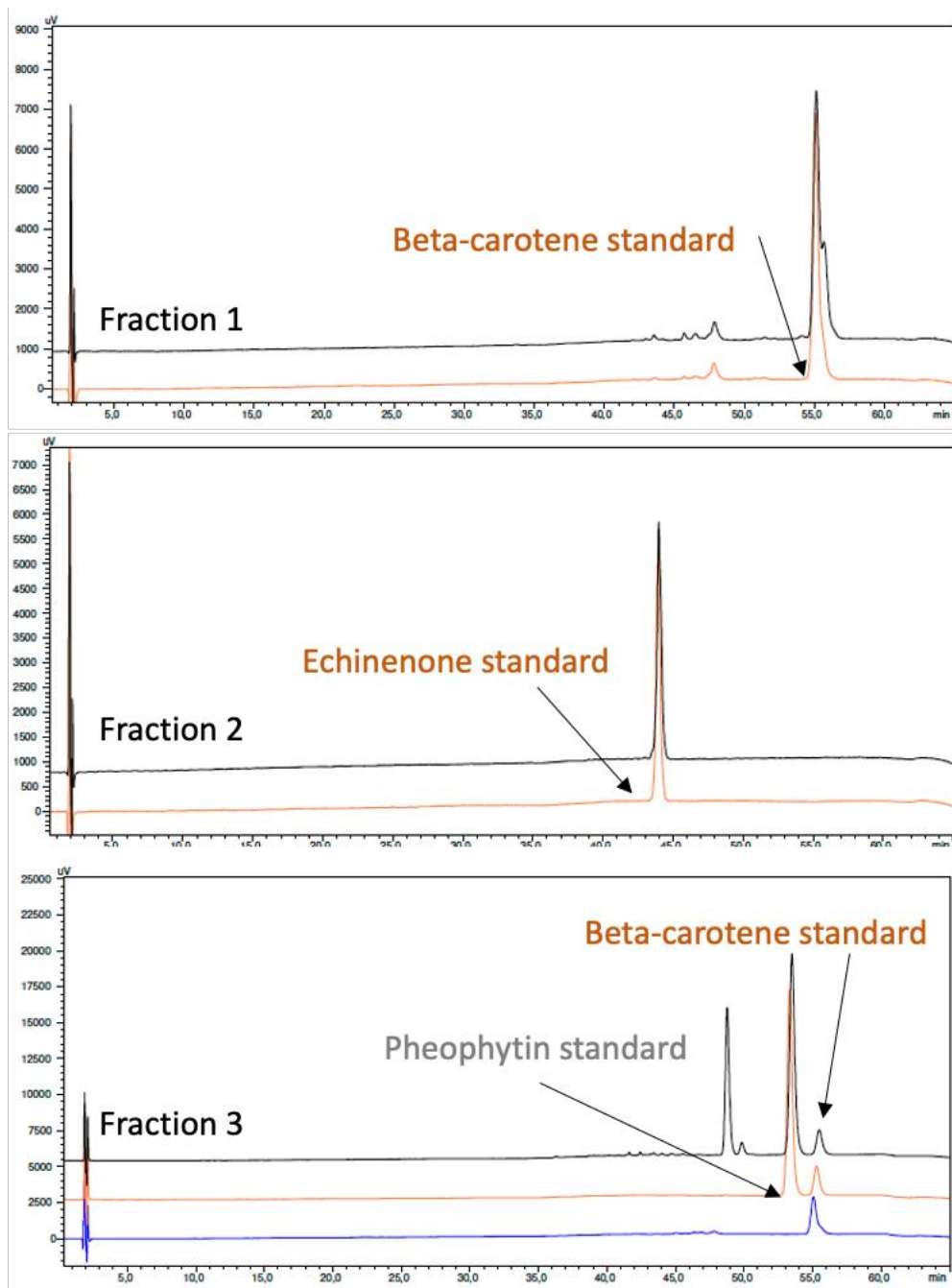
Supplementary fig. S3. The TLC plates of separation of *Synechocystis salina* extracts: part A. normal application of extracts using following mediums I. acetone, II. 90% aq. acetone, III. acetone + ammonium acetate buffer (9:1, v/v), and IV. 90% aq. acetone, III. acetone + ammonium acetate buffer (9:1, v/v) with the total removal of phase and resuspension of extract in acetone, part B. 90% aq. acetone extract developed with the “fast-start” technique.



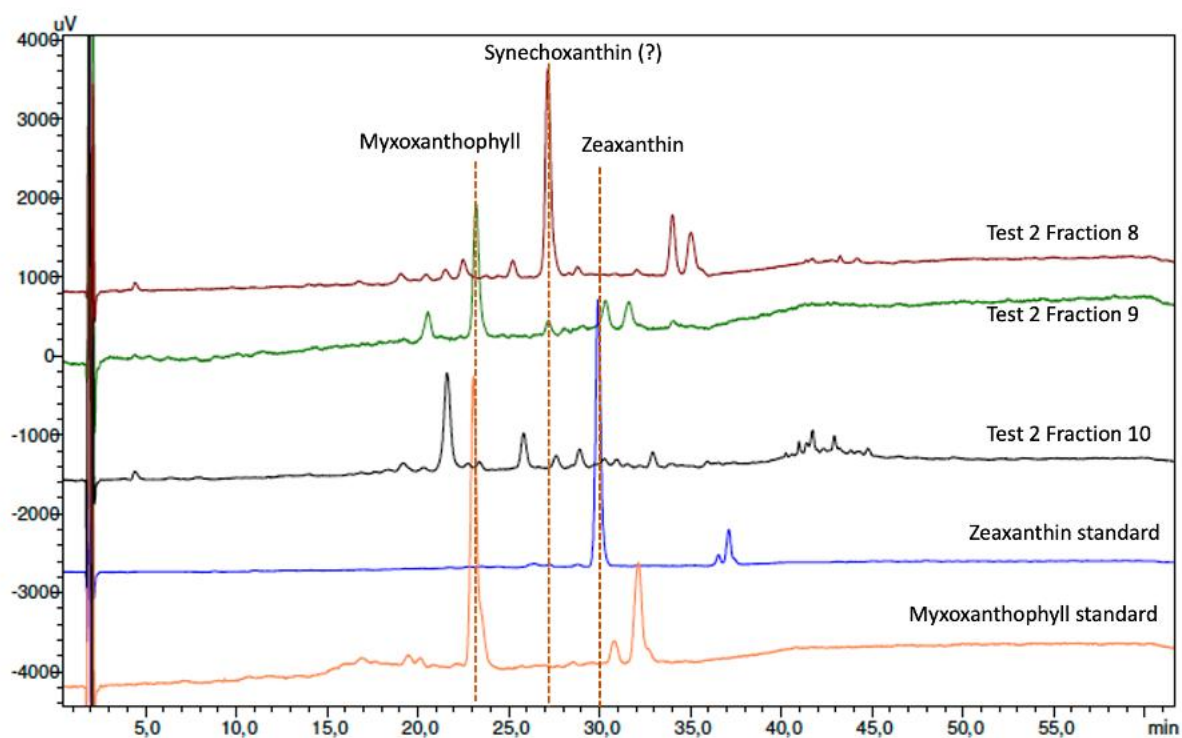
Supplementary fig. S4. The ratio of concentrations of selected pigments toward chlorophyll a concentration obtained in acetone extract of four tested cyanobacteria species (n = 2).



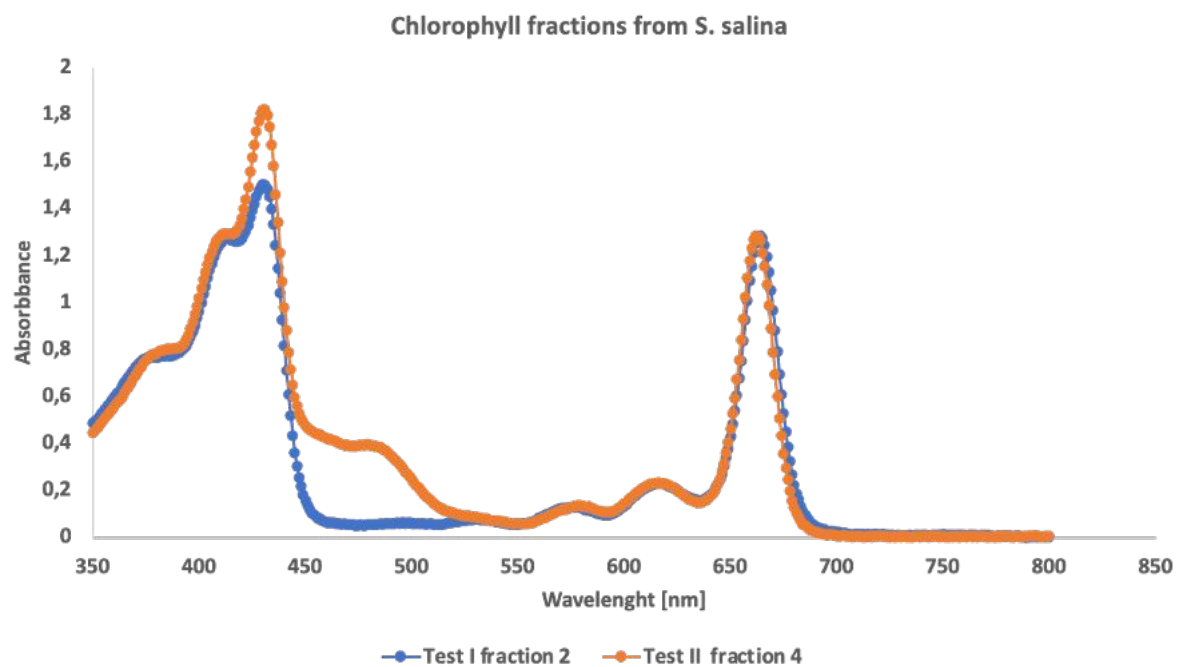
Supplementary fig. S5. The pictures form the LPLC of organic extract from cyanobacterium *S. salina*. Upper pictures – the column during the development of separation, lower pictures - collected fractions.



Supplementary fig. S6. The chromatograms of fractions 1 to 3 from test II of low-pressure liquid chromatography of extract from *Synechocystis salina*..



Supplementary fig. S7. The chromatograms of fractions 8 to 10 from test II of low-pressure liquid chromatography of extract from *Synechocystis salina*.



Supplementary fig. S8. Comparison of UV-Vis spectrums of GLC fraction 2 and 4 from test 1 and test 2, respectively – the region 550-750 overlap, despite the difference in compounds: in fraction 2 there is Chl *a*, while in fraction 4 there is allomerization product of Chl *a*.

Publikacja – P4

Lis Hanna*, Kwidzińska Klaudia, Bethke Katarzyna, Caban Magda, Cyanobacteria as generators of reactive oxygen species – A review of analysis methods and case studies, Talanta, 2026, 297, 128648, DOI:10.1016/j.talanta.2025.128648.



Cyanobacteria as generators of reactive oxygen species – A review of analysis methods and case studies

Hanna Lis, Klaudia Kwidzińska, Katarzyna Bethke, Magda Caban^{*}

University of Gdansk, Department of Environmental Analysis, Faculty of Chemistry, ul. Wita Stwosza 63, 80-308, Gdańsk, Poland

ARTICLE INFO

Keywords:

Cyanobacteria
Reactive oxygen species
Fluorescence probes
Dichlorodihydrofluorescein
Oxidative stress measurement

ABSTRACT

Reactive oxygen species (ROS) are omnipresent in the environment and serve as important modulators of geochemical cycles. Phytoplankton is the major producer of ROS in water. Cyanobacteria, on one hand, are the most sensitive component of phytoplankton to ROS; on the other, they are producers of ROS with ecological roles that are still not fully understood. The correlation of blooms with increased ROS levels necessitates a deep understanding of the mechanisms responsible for ROS production by cyanobacteria. Reliable analytical methods are thus needed. This review begins with the presentation of ROS as a factor in water biochemistry and the interactions between ROS and cyanobacteria. It then presents reports on ROS analysis following cyanobacteria exposure to physical and chemical stressors using fluorescence probes. The possibilities for selective and qualitative analysis of ROS were critically discussed. In the conclusion section, the main drawbacks of the methods based on fluoresceine-based fluorescence probes, challenges to overcome and future needs are highlighted.

1. Introduction

Reactive oxygen species (ROS) can be defined as short-lived molecules containing oxygen atoms with redox state between O_2 and H_2O , following reduction reactions [1,2].

- (1) $O_2 + e^- \rightarrow O_2^-$ (superoxide – e.g. via photoreduction)
- (2) $O_2^- + e^- + 2H^+ \rightarrow H_2O_2$ (hydrogen peroxide - e.g. via photochemical reaction with dissolved organic matter - DOM)
- (3) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^\bullet$ (hydroxyl radical via Fenton or Haber-Weiss Reaction)
- (4) $HO^\bullet + H^+ + e^- \rightarrow H_2O$

ROS have half-lives ranging from nanoseconds to hours in aqueous environments [1–3]. In a normal metabolic process in aerobic cells, ROS arises mainly from oxygen reduction during respiration. In cyanobacteria and other photosynthetic organisms, photosynthetic electron transport serves as an additional ROS source [4]. This process also generates reactive nitrogen species (RNS), and there is significant crosstalk between ROS and RNS [5]. Certain ROS levels play essential roles in metabolic regulation, while elevated ROS levels can damage cellular components. Since most ROS, aside from H_2O_2 , cannot easily diffuse or be actively transported across membranes, cells have evolved

both enzymatic and non-enzymatic defense mechanisms to manage oxidative stress [6]. Enzymatic defenses include catalases, superoxide dismutase, and peroxidases, while non-enzymatic defenses involve glutathione, vitamins A, C, and E, as well as carotenoids. The commonly known state of oxidative stress occurs when the balance between oxidants and antioxidants is disrupted. The sources and regulation of oxidative stress in cyanobacteria are comprehensively reviewed by Lafiti et al. [4]. Environmental stressors, such as high light, increased temperature, salt stress, nutrient limitations, and chemical toxicants, are known inducers of oxidative stress in cyanobacteria [7,8]. The impact of pollution from anthropogenic chemicals on oxidant-antioxidant balance in cyanobacteria remains one of the least explored areas of research.

Oxidative stress is probably a factor in cyanobacteria bloom limitation and cell apoptosis, which is further connected with the presence of cyanotoxins in water [4]. Another issue is ROS released outside the cells, as such molecules can act as antibiotics or signaling agents with a potentially underappreciated role in ecosystems. For example, the production of extracellular ROS was proven for filamentous dinitrogen-fixing cyanobacteria in the genus *Trichodesmium* [9]. The cyanobacteria are found to be especially sensitive toward ROS in comparison to other phytoplanktonic taxa [9,10], which has already been used in natural water reservoirs for controlling blooms [2]. On the other hand, cyanobacteria can produce external ROS to improve iron

^{*} Corresponding author.

E-mail address: magda.caban@ug.edu.pl (M. Caban).

<https://doi.org/10.1016/j.talanta.2025.128648>

Received 25 April 2025; Received in revised form 19 July 2025; Accepted 27 July 2025

Available online 28 July 2025

0039-9140/© 2025 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

absorption. This suggests that ROS could influence the structure and function of cyanobacterial communities, and the increased number of cyanobacterial blooms can accelerate ROS concentration in water. ROS plays a key role in the aquatic oxygen cycle and actively interacts with other geochemical cycles [2,11]. Therefore, tracking ROS production by cyanobacteria is crucial for understanding environmental ecology and assessing the broader implications of oxidative processes in aquatic ecosystems. Comprehensive studies on oxidative stress in cyanobacteria are needed to understand, among other things, the bloom cycle and the effects of climate change and anthropogenic pollution on primary producers.

The invention of fluorescence methods in 1990's enable tracking nanomoles of H_2O_2 in surface water [2]. Currently, the primary technique for intracellular and extracellular ROS detection is based on fluorescence probes activated by oxidation. However, recent research has shown that many of the methods used so far are not selective enough to distinguish between ROS types [12]. Furthermore, fluorescence probes' performance was never verified for cyanobacterial cells. To date, no comprehensive review of the application of fluorescence probes for cyanobacterial ROS tracking has been conducted. Therefore, the aim of this review is to present a possibility in order to analyze intracellular and extracellular ROS produced by cyanobacteria and critically discuss the qualitative and quantitative powers of obtained methods. We summarize the methods for ROS analysis under laboratory conditions focused on cyanobacteria as emerging microorganisms. The critical discussion aims to highlight the limitations and advantages of fluorescence probes for ROS detection. Furthermore, case studies on the impact of physical and chemical stressors, with special attention to anthropogenic pollutants, are presented.

2. Comparison of selected species of ROS produced by cyanobacteria

Reactive oxygen species (ROS) produced by cyanobacteria differ in both their intracellular functions and environmental roles.

Singlet oxygen ($^1\text{O}_2$) is primarily generated in photosystem II (PSII) due to the excitation of chlorophyll by light. Upon light absorption, chlorophyll reaches a triplet excited state ($^3\text{Chl}^*$), which can transfer energy to molecular oxygen, forming $^1\text{O}_2$ - a highly reactive and damaging form of oxygen. Within cyanobacterial cells, $^1\text{O}_2$ can oxidize chlorophyll molecules, damage PSII proteins, and attack membrane lipids, resulting in the degradation of the photosynthetic machinery.

Despite its toxicity, $^1\text{O}_2$ also plays a role in cellular signaling - a phenomenon referred to as the dual role of ROS. It triggers repair mechanisms in PSII, especially by promoting the replacement of the D1 protein, a key component of the PSII reaction center [13]. It also activates stress-responsive genes such as *psbA* (encoding D1) and *ocp*, which encodes the Orange Carotenoid Protein (OCP). OCP mitigates ROS generation by reducing energy transfer from the light-harvesting phycobilisomes to photosystems, thus limiting the formation of $^3\text{Chl}^*$ and subsequently $^1\text{O}_2$ [14].

Superoxide anion (O_2^-), though less reactive than other ROS, is a major precursor for more harmful species. It is mainly produced in photosystem I under high light stress [4]. Due to its charge, O_2^- cannot diffuse across lipid cell membranes and acts near its production site, where it damages thylakoid membranes and releases Fe^{2+} from iron-sulfur clusters, disrupting key enzymatic processes (Fig. 1). Importantly, O_2^- is also part of a redox-dependent signaling system that responds to light stress. It is sensed by the PrqR-CalA regulatory system, which activates the sigma factors SigB and SigD. These sigma factors promote transcriptional responses that enhance the expression of protective genes and contribute to the oxidative stress defense network [4, 15].

The released Fe^{2+} from O_2^- damaged proteins can participate in the Fenton reaction, leading to the formation of the highly reactive hydroxyl radical ($\text{OH}^\bullet$). This reaction is preceded by the conversion of O_2^- to

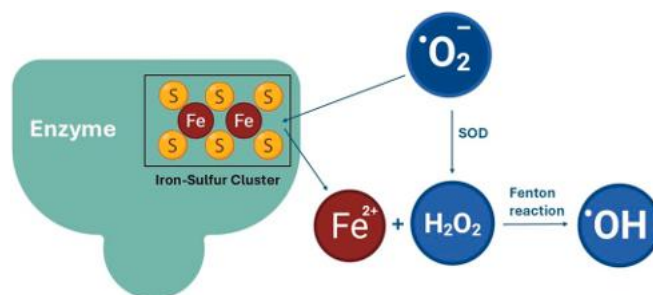


Fig. 1. Formation of the hydroxyl radical from superoxide anion in cyanobacterial cells.

hydrogen peroxide (H_2O_2) via superoxide dismutase (SOD). H_2O_2 , though less reactive than $\text{OH}^\bullet$, is membrane-permeable and can exert oxidative effects in both the thylakoid and cytoplasmic compartments.

Cyanobacteria counteract H_2O_2 through specialized defense systems, including PerR and the histidine kinase Hik33, which differ from regulatory mechanisms in heterotrophic bacteria like *E. coli* [4,16]. However, the complete network of ROS defense in cyanobacteria remains incompletely understood, and additional regulatory components are likely involved.

ROS, especially $\text{OH}^\bullet$, pose significant internal threats to nucleic acids, proteins, and lipids. Radical $\text{OH}^\bullet$ is extremely short-lived and reacts immediately at its site of formation. In DNA, it causes strand breaks and base modifications, leading to mutations [17]. Oxidative damage to mRNA can impair translation and protein synthesis [18]. Lipid peroxidation, particularly targeting unsaturated fatty acids, compromises membrane integrity and can generate further ROS, including $^1\text{O}_2$. Proteins are similarly vulnerable: $\text{OH}^\bullet$ indiscriminately modifies side chains and backbones, while $\text{O}_2^{\bullet -}$ and H_2O_2 selectively damage iron-containing proteins like ferritin [17]. Even essential enzymes, such as the β -subunit of ATP synthase, can suffer oxidative damage, impairing core cellular functions.

In addition to their intracellular effects, ROS produced by cyanobacteria also play roles in ecological interactions, including allelopathy, ichthyotoxicity, and iron mobilization. In some cases, they may even promote growth in microbial communities [19].

3. The interdependence between ROS and phytoplankton dynamics

ROS are the components of the oxygen cycle in the environment. Nevertheless, full knowledge of spatial and temporal occurrence of ROS in water is lacking. The primary sources of ROS in water are phytoplankton and chromophoric dissolved organic matter (CDOM) [2]. Only a few studies have been conducted to determine the natural temporal and seasonal levels of ROS in water. Morris et al. [2] provide a comprehensive review of H_2O_2 concentrations across diverse aquatic environments globally, reporting values ranging from 2 nM to 4.5 μM . Notably, H_2O_2 concentrations in rainwater are substantially higher than those in surface waters, ranging from 1 to 85 μM , primarily due to photochemical reactions occurring in the atmosphere. Seasonal variations significantly influence H_2O_2 dynamics. Yoon et al. [20] demonstrated that summer H_2O_2 concentrations can be up to three times higher than winter levels, highlighting the role of temperature and irradiance in oxidative processes. Also, Cory et al. [21] reported that temporal fluctuations in H_2O_2 concentrations were strongly associated with seasonal bloom dynamics in lacustrine ecosystems. In natural aquatic systems, ROS are often undetectable due to their rapid reactions with dissolved organic matter and metals. Consequently, most reported ROS concentrations are derived from controlled laboratory experiments, which may not fully capture their dynamics in complex environmental matrices.

Phytoplankton presence is intricately linked to ROS dynamics in

natural waters, as these organisms both generate and respond to ROS. Review of reactive species production by photosystem II (PSII) as a response to high light and high temperature can be found in literature [7]. The primary phytoplankton-derived ROS are H_2O_2 and O_2^- . H_2O_2 is generated both intracellularly and through cell surface reactions, while O_2^- is produced during intracellular metabolic processes, as mentioned in previous section. However, O_2^- has an extremely short half-life (seconds to minutes) and does not diffuse outside the cell, as it is rapidly converted into H_2O_2 by superoxide dismutase (SOD). The extracellular O_2^- is a process of surface activity of enzymes. The concentrations ($\text{fmol cell}^{-1} \text{ hr}^{-1}$) of ROS produced by selected species across phytoplankton can be found in review [19].

Despite its ecological significance, the role of extracellular ROS in phytoplankton physiology remains incompletely understood. Hypothesized ecotoxicological functions include ichthyotoxicity, allelopathy, growth regulation, and iron speciation, but these effects have only been documented in selected species and cannot be generalized across all phytoplankton. It's important to note that ROS can have beneficial effects on the environment [22]. They serve as important cell signaling molecules [23] that participate in organismal protective responses and may regulate resistance to pathogens. Additionally, ROS participate in redox reactions with trace metals (Fe, Cu, Mn), enhancing nutrient bioavailability, which supports phytoplankton growth and influences carbon and oxygen cycles [2,22]. Extracellular ROS production has been implicated as a toxic mechanism in harmful algal species, such as *Chattonella marina* [24], and various others (review in [25]). However, the rate of extracellular ROS production varies among species, as highlighted in a review by Diaz and Plummer [19]. In addition to species-specific differences, factors such as growth stage, cell density, cell lysis, light intensity, temperature, salinity, and iron availability further regulate extracellular ROS production.

Exposure to ROS in the environment can induce oxidative stress in phytoplankton, influencing their physiological responses [2]. Cyanobacteria, which are particularly sensitive to ROS, can experience oxidative damage from light-irradiated CDOM [10,20]. Additionally, environmental stressors such as temperature fluctuations, acidification, and contaminants (e.g., pesticides, pharmaceuticals, and other toxic substances) can further exacerbate oxidative stress in phytoplankton [2, 26]. ROS can interfere with redox reactions involving trace metals, thereby altering the bioavailability of essential nutrients crucial for phytoplankton growth [2]. Studies have shown that H_2O_2 can affect the speciation of iron, rendering it unavailable to phytoplankton and consequently inhibiting their growth [27]. The extracellular reduction of Fe^{3+} has been observed in *Microcystis aeruginosa*, enhancing iron uptake. This process is mediated by organic exudates, which, upon irradiation, induce the generation of O_2^- , facilitating Fe^{3+} reduction [28]. One of the first cyanobacteria to be subjected to experiments testing growth inhibition under the influence of H_2O_2 was *Prochlorococcus*, a cyanobacterium that inhabits temperate waters and tropical oligotrophic oceans. It has been found that *Prochlorococcus* relies on heterotrophic bacteria for increased tolerance to H_2O_2 [29,30]. In the absence of these bacteria, oceanic ROS levels can rise, rapidly inhibiting cyanobacterial growth. *Prochlorococcus* is a particularly sensitive cyanobacterial species to ROS, possibly due to the lack of enzymes responsible for defense against H_2O_2 in its genome. While other cyanobacterial species are generally less sensitive than *Prochlorococcus*, they are still more susceptible to ROS compared to many other aquatic organisms, as reviewed by Morris et al. [2]. Unicellular cyanobacteria of the genus *Synechocystis* [31], *M. aeruginosa* [32] and the filamentous cyanobacteria *Trichormus variabilis* [32] showed higher sensitivity (mortality already at exposure to $10 \mu\text{M}$ H_2O_2 [2]) compared to green algae such as *Pseudokirchneriella subcapitata* and diatoms like *Navicula semimulum* [32], which survived exposure up to 1 mM H_2O_2 [2].

Researchers suggest that environmental ROS concentrations are generally lower than the toxicity thresholds observed in laboratory studies of aquatic organisms [33]. However, this assumption requires

careful verification, as culture media used in laboratory experiments may contain unrecognized ROS sources, potentially leading to artificially elevated resistance levels in cultivated microorganisms [1,34]. This discrepancy complicates assessments of wild aquatic organisms' true sensitivity to ROS in their natural habitats. Furthermore, accurately quantifying ROS levels in environmental samples remains challenging due to technical limitations in current analytical methods, further hindering direct comparisons between laboratory and field conditions.

4. Methods of extracellular and intercellular ROS analysis

There are direct and indirect methods for quantification of ROS in natural waters [35], and in water in general [3,36]. Various techniques can be applied for ROS and RNS analysis, including fluorescence, luminescence, spectrophotometry, high-performance liquid chromatography (HPLC) with UV-Vis detection, gas chromatography, electron spin resonance, nuclear magnetic resonance, iodine titration, and capillary electrophoresis [5]. According to the latest literature, the fluorescent methods has been used for cyanobacteria (Tables 1 and 2). In general, probes react with various forms of ROS to produce a product that exhibits fluorescence and can provide spatial and temporal information about cellular systems [36] (Fig. 2).

Some probes penetrate directly into cells, while others detect ROS in the environment surrounding the cells. Like other ROS detection methods, fluorescent probes vary in selectivity. However, due to the short-lived and highly reactive nature of ROS, most fluorescent probes are considered non-selective, meaning they primarily indicate overall ROS levels rather than distinguishing between specific ROS types.

To improve accuracy, combining multiple detection methods can provide a more reliable assessment of both intercellular and intracellular ROS distribution. In addition, the use of appropriate, controlled, and reproducible procedures, such as conducting positive controls (with ROS donors), negative controls (with ROS scavengers), proper sample washing, maintaining constant staining/scanning times within a set of experiments, and using optimal pH and turgor pressure, can contribute to accurate results. It is also critical to find the optimal experimental conditions: incubation time, temperature, or probe concentration, as these values vary significantly between different model phototrophic organisms (higher plants, algae, and cyanobacteria), fungi, or "fungus-like" organisms (oomycetes) [57].

In general, unicellular structures, like pollen, green algae, and thin-walled spores can be stained easily [57]. However, in the case of cyanobacteria, effective staining requires a higher probe concentration and an extended incubation time to achieve optimal results. For example, in the case of the unicellular cyanobacterium *Synechocystis*, a widely used photosynthetic model, the Singlet Oxygen Sensor Green (SOSG) concentration is usually increased from the commonly used $50 \mu\text{M}$ – $250 \mu\text{M}$, along with an increase in incubation temperature from room temperature to 37°C [58]. This adjustment is necessary due to the cyanobacterial cell wall's higher cross-linking of polysaccharides and the production of an external mucoid sheath. The average cell wall thickness is 10 nm in unicellular species and 15 – 35 nm in filamentous species. An extreme case is *Oscillatoria princeps*, where the cell wall is 700 nm wide [57]. Cyanobacteria are, therefore, challenging species for intracellular ROS analysis. In the paper of Cho et al. [25] there is review of methods for analysis of the ROS in harmful algal bloom (HAB)-forming phytoplankton and it seems that the topic of ROS analysis for algae is more known compared to cyanobacteria.

The *in-situ* detection of extracellular ROS in water environment can be performed by fluorescence-based assays and electrochemical methods [59]. For example, H_2O_2 can be performed by flow injection analysis acridinium ester chemiluminescence. The base is reaction of H_2O_2 with an acridinium ester 10-methyl-9-(*p*-formylphenyl)-acridinium carboxylate trifluoromethanesulfonate. The system was used to determine H_2O_2 concentrations in natural waters, including rain, freshwater, and seawater with detection limit 352

Table 1

The overview of reports on DCFH₂-DA application for reactive oxygen species (ROS) detection in cyanobacteria after exposure to physical and chemical stressors (PAR- photosynthetically active radiation, FCM – flow cytometry, FM - fluorescence microscopy, FS - fluorescence spectrophotometry, F – green fluorescence).

Cyanobacteria Species	Stressor Exposure Time	Stressing Conditions	Cell Density	Probe (final conc.)	Incubation with Stain	Detection Technique	ROS Quantification	Excitation/ Emission [nm]	Ref.
Physical stressors									
<i>Anabaena variabilis</i>	UV-A, UV-B, PAR 12 h	23 °C, shaken regularly	–	DCFH ₂ - DA (5 μM)	1 h (after exposure, shaken)	FM, FS	No	Green F 485/500-600	[37]
<i>Anabaena variabilis</i>	UV-A, UV-B, PAR 24 h	23 °C, shaken regularly	–	DCFH ₂ - DA (5 μM)	1 h (after exposure, shaken)	FM	No	Green F and red F	[38]
<i>Rivularia</i> sp.	UV-A, UV-B, PAR 24 h	23 °C, shaken regularly	–	DCFH ₂ - DA (5 μM)	1 h (after exposure, shaken)	FS	No	Green F 485/520	[39]
<i>Synechococcus elongatus</i> PCC <i>Fremyella diplosiphon</i>	–	–	1 × 10 ⁵ cells mL ⁻¹	DCFH ₂ - DA	1 h (after exposure, shaken, in dark)	FMC	Yes	Green F (emission 515–545 nm) and red F (emission range >670 nm)	[40]
<i>Nostoc</i> sp. strain <i>HKAR-2</i>	UV-A, UV-B, PAR 24 h	25 ± 2 °C, stirred 15–20 rpm	–	DCFH ₂ - DA (5 μM)	1 h (after exposure, shaken, in dark)	FM, FS	No	Green F 488/500-600	[41]
<i>Nostoc</i> sp. strain <i>HKAR-2</i> <i>Scytonema</i> sp.	UV-A, UV-B, PAR 72 h	25 ± 2 °C, stirred 15–20 rpm	optical density of 0.5 at 750 nm	DCFH ₂ - DA (5 μM)	1 h (after exposure, shaken, in dark)	FS	No	Green F 485/500-600	[42]
<i>Fischerella</i> sp. strain <i>HKAR-14</i>	UV-A, UV-B, PAR 72 h	20 ± 2 °C, photoperiod of 14/ 10 h light/dark cycle	–	DCFH ₂ - DA (5 μM)	1 h (after exposure, shaken, in dark)	FM, FS	No	Green F 485/525	[43]
<i>Synechococcus</i> sp.,	Temperature (20, 23 and 26 °C) 96 h	Two-factor design: 20, 23 and 26 °C and salinity 33, 36 and 39 PSU in different configurations	10 ⁴ cells mL ⁻¹	DCFH ₂ - DA	30 min (after exposure, in dark) 10 ⁴ cells·mL ⁻¹	FMC	No	Green F 488/530	[44]
<i>Synechocystis</i> sp.	UV-A, UV-B, PAR (rhythmic light variations) 24 h	photoperiod of 12/ 12 h light/dark cycle	optical density of 0.7 at 750 nm	DCFH ₂ - DA	1 h (after exposure, shaken, in dark)	FS	No	Green F 485/500-600	[45]
<i>Phormidium tenue</i>	UV-B	UV-B and PAR photoperiods	2–5 × 10 ⁴ cells/ mL	DCFH ₂ - DA (5 μM)	1 h (after exposure, shaken, in dark)	FS	No	Green F 485/500-600	[46]
Chemical stressors									
<i>Microcystis aeruginosa</i> <i>Aphanizomenon flos- aquae</i> <i>Anabaena flos-aquae</i> <i>Microcystis aeruginosa</i>	Natural water	–	–	DCFH ₂ - DA	6/12 h	FS	No	Green F 480/530	
<i>Microcystis aeruginosa</i>	Polyphenolic allelochemical pyrogalllic acid (2, 7 and 14 mg L ⁻¹) 72 h	25 ± 2 °C light/dark cycle of 12 h/12 h	–	DCFH ₂ - DA (5 μM)	1 h (after exposure, in dark)	FCM	No	Green F 485/535	[47]
<i>Fischerella</i> sp.	Methyl parathion 96 h	–	–	DCFH ₂ - DA (5 μM)	1 h (after exposure, shaken, in dark)	FM	No	Not specified	[48]
<i>Anabaena sphaerica</i>	Malathion (pesticide) 5, 10, 20 and 30 μg mL ⁻¹ 8 days	28 ± 2 °C, photoperiod of 14/ 10 h light/dark cycle	–	DCFH ₂ - DA (5 μM)	1 h (after exposure, in dark)	FM	No	Not specified	[49]
<i>Synechocystis</i> sp	CNT, CAP, CT 96 h	–	1 × 10 ⁶ cells mL ⁻¹	DCFH ₂ - DA	in the dark at 25 °C for 30 min	FS	No	Green F 485/530	[46]
<i>Microcystis aeruginosa</i> <i>Pseudokirchneriella subcapitata</i>	Allelochemicals 4 h	Room temperature, light/dark conditions, static	5 × 10 ⁶ cells mL ⁻¹ 2 × 10 ⁶ cells mL ⁻¹	DCFH ₂ - DA (5 μM)	1 h (before exposure)	FS	No	Green F 485/530	[50]
<i>Anabaena fertilissima</i>	250 mM NaCl, NaCl + Na ₂ SO ₄ (125 mM) 12 h	28 °C	–	DCFH ₂ - DA (5 μM)	45 min (after exposure)	FM	No	Red F and green F	[51]

(continued on next page)

Table 1 (continued)

Cyanobacteria Species	Stressor Exposure Time	Stressing Conditions	Cell Density	Probe (final conc.)	Incubation with Stain	Detection Technique	ROS Quantification	Excitation/Emission [nm]	Ref.
<i>Rivularia halophila</i>	NaAsO ₂ Na ₃ AsO ₄ ·12H ₂ O 15 days	Stirred	–	DCFH ₂ -DA (5 µM)	1 h (after exposure, shaken, in dark)	FM	No	Green F 492/527	[52]
<i>Synechococcus</i> sp.,	salinity (33, 36 and 39 PSU) 96 h	Two-factor design: 20, 23 and 26 °C and salinity 33, 36 and 39 PSU in different configurations	10 ⁴ cells mL ⁻¹	DCFH ₂ -DA	30 min (after exposure, in dark)	FCM	No	Green F 488/530	[44]

Table 2

The methods based on other than DCFH₂-DCA probe for ROS analysis in cyanobacteria.

Probe	ROS type	Cyanobacteria Stressor	Observed Effect	Ref.
Intracellular Singlet Oxygen Sensor Green (SOSG)	Singlet oxygen	<i>Synechocystis</i> sp. No stressor	Prove that small CAB-like proteins prevented formation of singlet oxygen in the damaged photosystem II complex of the cyanobacterium.	[53]
Extracellular AmplexTM Red	Total ROS	<i>Microcystis aeruginosa</i> , <i>Aphanizomenon flos-aquae</i> , <i>Anabaena flos-aquae</i> and green algae <i>Chlorella</i> sp. heavy metals, antibiotics tetracycline and sulphamethoxazole (1 µg/L)	The higher H ₂ O ₂ level was measured in cyanobacteria culture after antibiotics exposure compared to heavy metals.	[20, 54]
AmplexTM Red	Total ROS	<i>Microcystis aeruginosa</i> <i>Aphanizomenon flos-aqua</i> UV-irradiated natural organic matter	Cyanobacteria are 2 order of magnitude more sensitive to H ₂ O ₂ compared to green algae.	[55]
6-(4-methoxyphenyl)-2-methyl-imidazo [1,2-a] pyrazine-3(7H)-one (MCLA)	Superoxide	<i>Trichodesmium</i> sp. No stressor, natural level measured	Superoxide production rate per cell determined.	[43]
6-(4-methoxyphenyl)-2-methyl-imidazo [1,2-a] pyrazine-3(7H)-one (MCLA) [2-[4-[3,7-dihydro-2-methyl-3-oxoimi-dazo [1,2-a]pyrazin-6-yl] phenoxy] butyramido] ethylamino] sulforhodamine10 (red-CLA)	Superoxide	<i>Trichodesmium erythraeum</i>	Superoxide production rate per cell determined.	[9]
Titanium chloride method	H ₂ O ₂	<i>Phormidium ambiguum</i> combined PAR and nutrient depletion (phosphorus concentrations)	Both PAR regimes and P concentration shortages are shown to enhance H ₂ O ₂ concentrations in cyanobacterial cultures. Protein-related quantification was performed.	[56]

pM in ocean seawater to 58.1 nM in rain [60,61]. The advantages of this method is negligible background fluorescence matrix effects in organic-rich environment. The emitted luminescence at 470 nm can be detected by flow injection analysis using a FeLume system (Waterville Analytical, Waterville, ME), as performer for groundwater [62]. This system was also applied for laboratory analysis of extracellular superoxide of several species of ocean microbes with application of chemiluminescent probe MCLA [63]. MCLA was also used with success for tracking the superoxide in the water column in the eastern equatorial Pacific Ocean [64]. What interesting, the same flow system, but with both probes (acridinium ester, and MCLA) was applied for detection of the extracellular H₂O₂ and O₂⁻ produced by diatoms [65]. The ROS in-situ can be also analyzed by electrochemical methods, for example, scanning electrochemical microscopy (SECM) [66]. For more information we advise to check review of methods for ROS detection in aqueous environments [19,67]. Despite quite well established methods for extracellular ROS analysis, we were unable to found the *in-situ* analysis of ROS produced by cyanobacteria blooms. Such data are obtained for laboratory cultivated phytoplanktonic species [19], with predominance of harmful green algae species.

4.1. Non-selective fluorescence probes for intracellular ROS detection

There are many dihydrocompounds, such as 2',7'-dichlorodihydrofluorescein (DCHF), dihydrorhodamine 123, dihydrorhodamine 6G,

dihydrocalcein, dihydroethidium, 10-acetyl-3,7-dihydroxyphenoxazine (AmplexTM Red) and 2,3,4,5,6-pentafluorodihydrodihydro-4-methylamine, which can be used as fluorescent probes for ROS detection [36]. However, all dihydrocompounds have two major drawbacks. First, it is known that they are originally highly photosensitive compounds, to a greater or lesser degree, and as a result, tend to auto-oxidize, producing high background fluorescence in the absence of ROS during fluorescence measurements that involve excitation light irradiation by laser. Therefore, experimental results obtained with these compounds should be interpreted with caution since the increase in fluorescence intensity is derived from both the presence of ROS and autooxidation by excitation light. Another issue is that these dihydrocompounds have long been widely used as fluorescent probes for the selective detection of H₂O₂. However, as is now known, these probes can react with different types of ROS. Therefore, dihydrocompounds are not suitable for the selective detection of individual ROS; however, they are effective for measuring overall oxidative activity in living cells or tissues.

The derivatives of fluorescein are routinely applied for ROS detection. Among them, 2',7'-dichlorodihydrofluorescein diacetate (DCFH₂-DA) is the most widely used, and due to its high permeability across membranes and cell walls, is used to detect intracellular ROS. Dihydrofluorescein diacetate (DHF-DA) was found to be superior for detecting intracellular oxidants compared with DCFH₂-DA among other fluorescence probes [68]. At the same time, all such probes show no selectivity, and provide an overall response toward oxidative reaction

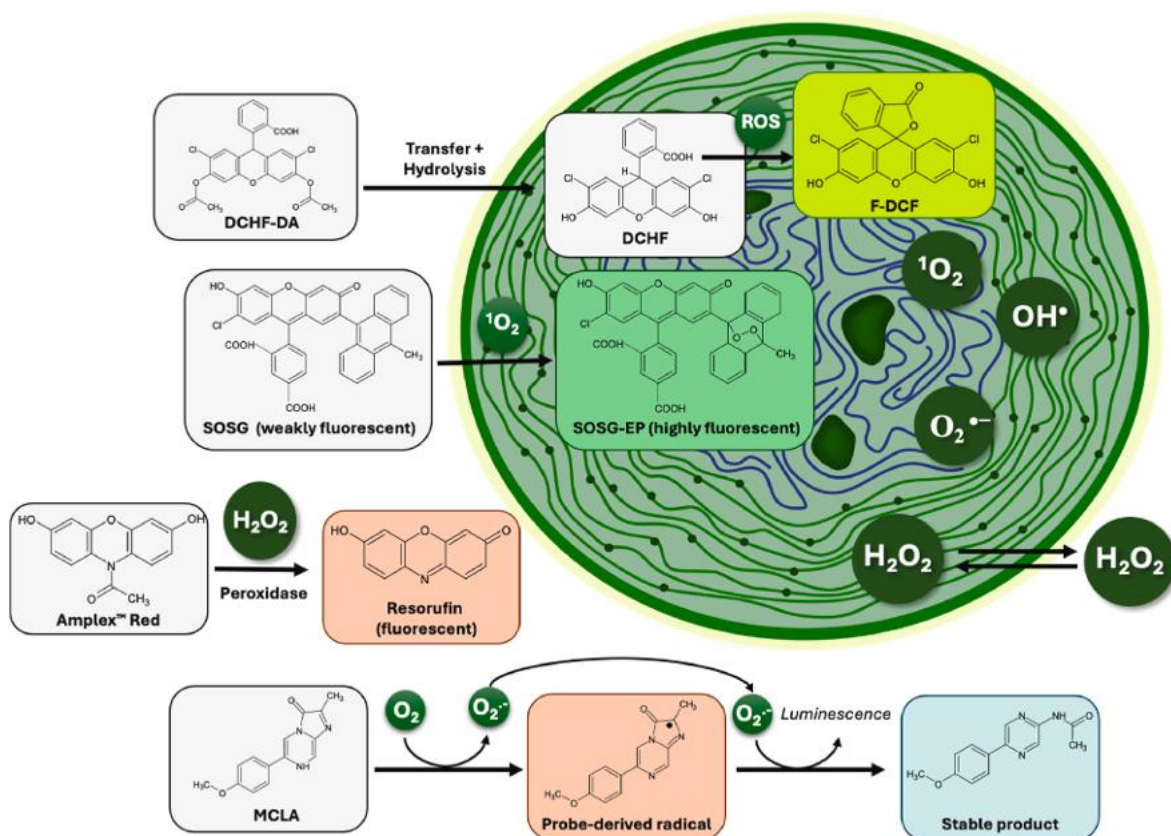


Fig. 2. The fluorescence probes applies for tracking of intracellular and extracellular ROS produced by cyanobacteria.

rather than selective response to single factor. In laboratory tests, the fluorescence was found to constantly grow with incubation time [68, 69], which is also our observation (data not published). DCFH₂-DA assay for analysis the intracellular ROS based on two reaction: first, uptake by cell and deacetylation by esterase to oxidant sensitive species (conversion of DCFH₂-DA to DCHF), and second, oxidation by ROS (DCHF to fluorescent DCF (dichlorofluorescein), named as f-DCF in further text) (Fig. 2) [69]. Besides ROS, reactive nitrogen species (RNS) may also directly or indirectly cause f-DCF formation [37].

Table 1 presents reported studies of cyanobacteria derived intracellular ROS analysis using DCFH₂-DA. Fluorescence spectrophotometry (FS) and fluorescence microscopy (FM) are primarily used as detection techniques, often in combination. In three reports, flow cytometry (FCM) was applied [44,70,71]. Each of these techniques relies on fluorescence detection, and the choice of method in the studies presented in Tables 1 and 2 appears to depend primarily on instrument availability and the specific research objectives. In general, fluorescence-based methods are approximately 1000 times more sensitive than spectrophotometric approaches [72]. FCM, in addition to fluorescence detection, also quantifies cell numbers, while FM provides spatial information about probe distribution on or within cells. Notably, only FS allows for the detection of probe fluorescence in solution, enabling the monitoring of extracellular ROS. To date, no study has directly compared FS, FM, and FCM in the context of ROS analysis. A common feature shared by these techniques is the excitation and emission behavior of the fluorescent probes, as DCF-based probes operate via the same physicochemical mechanisms. Excitation of f-DCF is carried out at wavelengths from 480 nm to 488 nm, while emission is measured at values from 500 nm to 600 nm. Most of the work did not attempt to validate or calibrate the methods used and only determined the overall level of ROS produced by various species of cyanobacteria (and not only) under natural conditions and because of various stressors in controlled laboratory studies.

DCFH₂-DA is particularly applicable to cyanobacteria, as the

structure of their cell wall prevents most probes from penetrating the cell interior. In real-case studies, DCFH₂-DA has been used most frequently (Table 1). Typically, incubation of the test solution with the probe is performed after exposing the cells to a factor that induces oxidative stress, such as UV-A, UV-B, or high photosynthetically active radiation (PAR). The effects of salinity, allelochemicals, pesticides, or carbon nanotubes on oxidative stress in cyanobacterial cells have also been evaluated. DCFH₂-DA is usually used at a final concentration of 5 μM, with a common incubation time of 30–60 min. Since DCFH₂-DA is a light-sensitive fluorescence probe, experiments should be conducted in minimal light. Therefore, incubation is carried out in the dark while shaking the cyanobacteria solution mixed with the probe. The following paragraphs present case study data on the use of DCFH₂-DA in detecting ROS produced by cyanobacterial species, sorted by the factor causing oxidative stress.

A model study type involves determining ROS induced by radiation mimicking solar UV ranges (UV-B, UV-A). For example, in the widely cited work of Rastogi et al. [37], studies were conducted on the cyanobacterial species *Anabaena variabilis*. Both FM and FS were used, obtaining compatible results. The excitation/emission values used were 485/500–600 nm, which are common for the DCFH₂-DA probe. In testing UV-R radiation effects on biochemical changes in cyanobacterial cells (and their surroundings), cut-off filters as monochromators are necessary to determine the accuracy of the wavelength affecting the test organisms. During exposure, the temperature was 23 °C, and the solution was regularly shaken. These conditions are like those used in most studies on this subject. Temperatures typically range from 20 °C [43] to 25 °C [71,41,42], with the solution being stirred or shaken. According to Table 1, UV light exposure time vary from 12 to 72 h [43]. It was observed that DCFH₂-DA was characterized by extensive green background fluorescence due to autooxidation and noticeably increased fluorescence in ROS-generating cells. Post-exposure results revealed that UV-B radiation induced a significant increase in ROS levels, most visible

when a 295 nm cut-off was applied. Additionally, Singh et al. [73] published results on the correlation of rhythmic changes in time and radiation components on ROS production by *Synechocystis* species. The highest ROS concentrations occurred with continuous radiation across the full range of natural light, including UV-B. In 2014, the same team conducted similar research on temporal dynamics of ROS biogenesis using *Anabaena* species and FM [74]. This study used seven different cut-off filters ranging from 280 nm to 400 nm. Images showed no ROS induction during the first 8 h of exposure to simulated solar radiation with different cut-off filters. After 24 h, ROS generation signals were observed in all samples except those kept in the dark. The signals were more pronounced in samples with 320, 305, 295, or 280 nm cut-off filters, indicating the highest ROS generation.

In the same year, Rastogi et al. [37] published a paper that included information on the effects of UV radiation on oxidative stress in *Rivularia* sp. The procedure is similar to the one presented in papers [37], fluorescence spectrophotometry with 485/520 nm emission and excitation, respectively, was used and the cut-off filters were 295 nm, 320 nm and 395 nm. Also in 2017, a full protocol was published for a method of determining ROS in cyanobacteria *Nostoc* sp. strain HKAR-2, where UV-R was the stressor [41]. They showed that solar UV radiation harms cyanobacteria directly by acting on DNA/proteins or indirectly through ROS-induced oxidative damage. As in most tests of this type, samples were taken after 12 h and after 24 h of exposure of cyanobacteria to radiation. A marked increase was observed after 12 h, doubling after 24 h. Similar conclusions were presented for cyanobacteria isolated from the environment *Scytonema* sp. and lab-cultured species *Nostoc* sp. strain HKAR-2 [42], hot spring cyanobacterium *Fischerella* sp. strain HKAR-14 [43].

In 2023, Kholssi et al. [44], investigated temperature and salinity effects on ROS production by *Synechococcus* sp. A temperature range of 20, 23, and 26 °C was investigated. Elevated concentrations of ROS were consistently observed across all tested temperatures, with a slight augmentation noted at the highest temperature, likely due to cell mortality correlating with increased ROS production.

Several papers have used DCFH₂-DA to assess the effects of structurally diverse chemicals on oxidative stress in cyanobacteria, such as allelochemicals [70,50] and pesticides like malathion [49], methyl parathion and arsenic salts [52].

Wang et al. [50] applied DCFH₂-DA to measure the oxidative stress caused by allelochemicals using FS (excitation/emission 485/530 nm). Other analytical parameters also do not differ from those standardly used for DCFH₂-DA (Table 1). However, this work deserves special attention for two reasons. First, it compares the concentration of ROS produced by two different species: cyanobacteria and green algae. Second, an attempt was made to validate the method, which is not usually done, due to the low selectivity of the DCFH₂-DA probe. Validation of the method was performed as a positive control using H₂O₂ to test the reliability of DCFH₂-DA as a probe for detecting intracellular ROS in *M. aeruginosa* and *P. subcapitata*. H₂O₂ significantly increased f-DCF fluorescence generation compared to the respective controls in both cultures. However, the generation of f-DCF fluorescence in *P. subcapitata* cells induced by H₂O₂ was about twice as high as in *M. aeruginosa* at all exposure concentrations over 4 h. The results showed that of all the stressors tested, only (+)-catechin and pyruvic acid could induce ROS formation both in cyanobacteria *M. aeruginosa* and green algae *P. subcapitata* cells. In all of the tested experimental conditions, ROS levels were higher when green algae were used as the test organism. In another work on the influence of allelochemicals on cyanobacteria *M. aeruginosa* [47], FCM technique was used for the intracellular ROS levels measurement with excitation/emission wavelengths of 485/535 nm. Also in this work, a positive correlation was proven between allelochemical pyrogallol acid concentration and intracellular ROS levels, suggesting the autooxidation of pyrogallol acid.

Two studies investigated insecticides' impact on ROS levels in cyanobacteria: Chakraborty et al. [49] tested stressor malathion (192 h of

exposure), and Tiwari et al. [48] tested methyl parathion (MP, 96 h of exposure). In both cases the detection technique applied was FM (Table 1). The cyanobacterial species used were *Anabaena sphearica* [49] and *Fischerella* sp. [48]. It was stated that high dose of malathion (30 mg L⁻¹) induced the generation of ROS. What interesting, the goal of the work of Tiwari et al. [48] was not only to correlate ROS concentration with insecticide concentration, but also to study the MP removal and degradation capacity of this cyanobacterium along with the mechanism it adopted to combat pesticide toxicity at different doses. It was found that at 20 mg L⁻¹ MP, the cyanobacterium effectively modulated its antioxidant defense system, fatty acid and hydrocarbon profiles to promote growth. However, the results obtained after exposing cyanobacterial cells to MP found that the pesticide caused a significant increase in ROS levels in a dose-dependent manner.

Rueda et al. [52] investigated the impact of the presence of NaAsO₂ and Na₃AsO₄·12H₂O across concentrations ranging from 0 to 15,000 mg L⁻¹ on ROS production by *Rivularia halophila* cyanobacterial cells. The measurement of ROS was conducted under conditions typical for DCFH₂-DA (Table 1), apart from the cyanobacteria exposure duration to the stressor, which extended up to 15 days. Initially, an escalation in arsenic salt concentration was accompanied by a gradual rise in ROS concentration, as anticipated. Furthermore, their findings elucidated that the bioreduction of As(V) to As(III) emerged as the principal transformation process, simultaneously diminishing levels of antioxidants and protective mechanisms aimed at ROS mitigation.

In the paper by Swapnil et al. [51], the influence of salinity (250 mM) on ROS production by *Anabaena fertilissima* was determined using DCFH₂-DA. Salt-treated cells showed a distinct biphasic accumulation of ROS. The first phase in ROS accumulation lasted 2 h, the second generation, less intense, was observed for from the 4th hour of salt stress to 12th hour of exposure. The biphasic timing of ROS accumulation corresponded to the response of cyanobacterial cells to salt stress and may serve as a potential trigger for an early defense response. Also, Pathak et al. [42] presented results on the effect of salinity (up to 250 mM) on oxidative stress in cyanobacteria from the species *Scytonema* sp. and *Nostoc* sp. strain HKAR-2. ROS level peaked after 60 h of exposure to salinity and then began to decrease. Possible causes were cell death caused by the extremely high salinity or early defense response. The mentioned works differ in the detection technique used: in the case of Swapnil et al. [51] is FM, while Pathak et al. [42] used FS. The study conducted by Kholssi et al. [44] also aimed to elucidate the impact of salinity on ROS production by cyanobacteria, employing FCM for analysis. However, no definitive correlations were discerned across the spectrum of salinity investigated (33–39 PSU), except for the consistent increase in ROS generation in the presence of salt. Various exposure durations were also employed, spanning 12, 72, and 96 h, alongside a range of temperatures from 20 to 28 °C.

There is also an interesting paper published in 2021 on measuring oxidative stress in different species: cyanobacteria *M. aeruginosa*, *Aphanizomenon flos-aquae*, *Anabaena flos-aquae* and green algae *Chlorella* sp. in water coming directly from the natural environment [20]. The detection was performed using FS under standard conditions (excitation/emission: 480/530 nm). Controls were performed for all selected species under commonly used conditions: under fluorescent light (4000 lx) with a cycle consisting of 14 h of light and 10 h of darkness at 20 ± 1 °C, stirred every 8 h. The composition of the medium was adjusted to reflect the composition of the environmental water under study. In addition to DCFH₂-DA, the AmplexTM Red was applied for extracellular ROS detection. Unfortunately, it is not clear if both reagents were added to same samples or measurement was performed in separate samples and how the samples were treated.

There is also data in the literature on the effect of carbon nanotubes (CNTs) on the toxic effects of chloramphenicol and tetracycline on the cyanobacterium *Synechocystis* sp. [75]. Both ROS quenching experiments and proteomic analysis were performed. FS was employed to detect ROS after staining by DCFH₂-DA. The results showed that

ROS-induced oxidative stress contributed to the toxicity of CNTs.

As was presented, the staining with DCFH₂-DA can be detected by FM, FS of FCM. The FCM is an alternative tool for ROS analysis in cyanobacteria analysis and possess several advantages. In a study of Mondal et al. [71] the FCM protocol utilized single-celled cyanobacteria, *Synechococcus elongatus*, and the filamentous *Fremyella diplosiphon*. They employed an FCM-based method to measure ROS in a large population of cyanobacterial cells efficiently. The authors rightly highlight the limitations of FM, which is restricted to small sample sizes due to time-consuming processes and background noise from dye photooxidation. Additionally, FM struggles with visualizing ROS in small, spherical cells. The study proposes FCM-based ROS monitoring as a solution, offering high-throughput capabilities and the simultaneous recording of various cellular parameters. However, unlike other detection methods, the FCM-based method lacks complete validation. The protocol includes optimization of the DCFH₂-DA dye concentration to ensure maximal ROS detection while minimizing cell death. Practical advice for using FCM with cyanobacterial cells is provided, emphasizing the need for careful sonification to disperse filamentous cyanobacteria or cell aggregates. Nonetheless, not all species may be suitable for this treatment.

Technically, the staining with the DCFH₂-DA is easy to perform and the price of reagent relatively low. The DCFH₂-DA can be prepared in DMSO [50] or ethanol [37,43,42,74,51]. In most cases, ROS are measured by DCFH₂-DA by *in vivo* techniques, what mean that appropriate mass of staining reagent is added directly to treated cultures (sometimes to washed pellet). The experiment can also be performed in reverse way, by firstly loading cells with stain, then performing treatment. As example, Wang et al. [50] performed test by pre-incubation of cells with DCFH₂-DA in PBS buffer, then cells were washed and resuspended in respective culture medium, and after adjustment of cell density (2-5 x 10⁶ cells mL⁻¹) the allelochemical was added. The control was the cells with only DCFH₂-DA; solvent control (DMSO) was also performed. The background fluorescence formation was determined with the mixture of BG-11 + growth medium, cells without loading the DCFH₂-DA and different concentrations of the allelochemicals. Obviously, in a case of PAR or UV as stressor only *in vivo* studies can be performed, because the DCFH₂-DA is light-unstable.

What was noticed after reports analysis, that the majority of works did not present a way of negative or positive controls performance. The presence of stress was measured by comparison of response of treated and not-treated cultures. DCFH₂-DA is a probe widely used for ROS detection in various cell types over several decades. However, its use comes with certain limitations: A. the oxidized fluorescent product of DCHF is membrane-permeable and can leak out of cells over time; B. incomplete DCF trapping may complicate data interpretation and hinder precise evaluation of intracellular oxidation; C. some cells have low esterase activity; D. the exact species responsible for oxidation is unknown [69].

4.2. Non-selective AmplexTM red for extracellular ROS determination

AmplexTM Red (10-acetyl-3,7-dihydroxyphenoxazine) reagent is used for non-selective determination of ROS. However, due to its inability to penetrate cell membranes/walls, it only allows the determination of extracellular ROS. The advantages of its use are simple procedures, fast reaction, and commercial availability of full experimental setups. Additionally, this reagent offers stability, generates less background noise, and produces a red-fluorescent product that is more easily detected than similar reduced methylene blue derivatives. In the presence of horseradish peroxidase (HRP), AmplexTM Red reacts with H₂O₂ in a 1:1 stoichiometry to form highly fluorescent resorufin (Fig. 2). However, it should be remembered that this represents a simplification, and AmplexTM Red is also capable of reacting with other ROS. The reference to DCFH₂-DA or AmplexTM Red for the determination of H₂O₂ content is since H₂O₂ is the most stable form of ROS. However, the ability of these probes to interact not only with various forms of ROS, but

also with RNS, cannot be neglected.

This simplification was still widely used until 2015. A paper from 2014 by Leunert et al. (2014) indicated the possibility of AmplexTM Red reagent application determining H₂O₂ produced by cyanobacteria, but the H₂O₂ concentrations were very low compared to those obtained using green algae. In fact, AmplexTM Red is more commonly used for green microalgae [76,77]. FS as detection technique for AmplexTM Red is used with the following excitation and emission values used: 530/590 nm [20], and 572/585 nm [10] (Table 2). In the work of Leunert et al. [10], the Amplex Red was used to quantify the ROS produced by UV-irradiated natural organic matter (NOM), and calibration was performed using six H₂O₂ levels ranging between 10⁻⁹ and 10⁻⁶ M. Unfortunately, in the work [20] no specific parameters of AmplexTM method were given. The authors stated that the analysis of ROS was performed both by AmplexTM and DCFH₂-DA, and test heavy metals, antibiotics tetracycline and sulphamethoxazole (1 g L⁻¹) as stressor. The higher H₂O₂ level was measured in cyanobacteria culture after antibiotics exposure compared to heavy metals.

Despite its advantages, AmplexTM Red has not been widely used for the determination of extracellular ROS, and the reasons for this remain unclear. One possible explanation is that its use has been largely overshadowed by other probes, such as DCFH₂-DA. In addition, while Amplex offers excellent sensitivity and stability, its reliance on horseradish peroxidase (HRP) to react with H₂O₂ can introduce variability under different experimental conditions. This may limit its wider application.

4.3. Selective Singlet Oxygen Sensor Green for intracellular singlet oxygen determination

Singlet Oxygen Sensor Green (SOSG) reagent was used to visualize singlet oxygen (¹O₂) production inside cells [78]. This reagent is also based on fluorescence, like DCFH₂-DA or AmplexTM Red. However, unlike other available fluorescent and chemiluminescent singlet oxygen detection reagents, SOSG does not show any appreciable response to other ROS, including hydroxyl radical, superoxide and nitric oxide [79]. Its limitation is unequal penetration between cell types (especially limited transport through mucoid sheath of cyanobacteria) and photo-sensitization [78]. This probe shows weak blue fluorescence with excitation peaks at 372 and 393 nm and emission peaks at 395 and 416 nm. In contrast, in the presence of ¹O₂ it produces SOSG endoperoxide (SOSG-EP), which emits green fluorescence like fluorescein (excitation/emission maxima ~504/525 nm) (Fig. 2). The SOSG reagent can degrade over time in some solutions and can become fluorescent at alkaline pH in the absence of singlet oxygen. Nevertheless, with proper control, the intensity of the green-fluorescent signal can be correlated with the concentration of singlet oxygen, without significant interference from other ROS. However, quantitative analysis of ¹O₂ formation performed exclusively based on SOSG endoperoxide fluorescence images is misleading [78].

Sinha et al. [58] used SOSG to determine ¹O₂ in cells of cyanobacteria of the *Synechocystis* species (Table 2). However, in this case, the stressor was not used to induce oxidative stress in cyanobacteria, and the goal was to see if small CAB-like proteins prevented formation of ¹O₂ in the damaged PSII complex. Cells were stained with SOSG and kept at 37 °C for 15 min to increase the efficiency of fluorochrome uptake by the mucous envelope surrounding the cyanobacterial cell wall. The samples were then incubated for 3 h at room temperature in the dark or with photosensitizing exposure. Suspensions of cells in deuterium oxide and histidine served as positive and negative controls, respectively. SOSG may be easily photosensitized during incubation and microscopy [57]. Therefore, to minimize artifacts, it is recommended that the sample be stained in the dark and visualized by a confocal laser scanning microscope. Based on the results obtained, the presence of ¹O₂ was indicated, and the role of small CAB-like proteins in stress reduction proven (most probably by the binding of free chlorophyll released from the damaged

PSII complexes) [58]. No other current publications on the use of SOSG against cyanobacteria were found, but it was used for diatoms [79].

4.4. Chemiluminescence probe for extracellular superoxide determination

Hansel et al. proposed a method based on the absorption of radiation produced by chemiluminescence [40] by adapting the method of [9]. This method was used to measure extracellular O_2^- production by the cyanobacterium *Trichodesmium*. The chemiluminescent probe used was 6-(4-methoxyphenyl)-2-methyl-imidazo [1,2-a]pyrazine-3(7H)-one (MCLA), which is a Cypridina luciferin analog [80] (Fig. 2). In the absence of a natural bioluminescent protein, MCLA generates chemiluminescence in response to superoxide production by the cells [40]. A possible mechanism for the chemiluminescence of MCLA was described in work by Zielonka et al. [81].

The advantage of this probe is that it does not sensitize to O_2^- production and therefore does not lead to false-positive results. Its pH optimum for luminescence generation is closer to the physiological near-neutral range [82]. However, MCLA-dependent chemiluminescence is enhanced by naturally occurring NADH in the cell, and since this electron donor is present in most cells, the true release of O_2^- can be masked [83]. In the research by Hansel et al. [40] (Table 2) extracellular O_2^- was measured by passing filtered seawater (AFSW) through filter-supported *Trichodesmium* colonies directly into the FeLume Mini system (two separate fluid lines, one for the analyte solution and the other for the MCLA reagent), where it was mixed with MCLA. This method, unlike most ROS detection methods, was fully calibrated, allowing the quantification of O_2^- in the test solutions. For calibration, standard solutions of potassium dioxide (KO_2) in NaOH (pH 12.5) were prepared with the addition of 90 μ M diethylene triamine pentaacetic acid (DTPA) to capture trace impurities that could otherwise significantly shorten the peroxide lifetime. Superoxide concentrations in the primary standards were quantified by measuring the difference in absorbance at 240 nm before and after the addition of superoxide dismutase. The secondary standard was the aged, filtered solutions amended with DTPA. The limit of detection for these measurements was 100 ± 7 pM of O_2^- . The rate of O_2^- production by populations sampled in the Sargasso Sea was normalized to the total number of *Trichodesmium* colonies or *Coscinodiscus* cells added to provide normalized rates (final pmol units of colonies per hour). As the conclusion, was stated that *Trichodesmium* colonies produce O_2^- independent to light presence and are a key ROS source in the surface of oligotrophic ocean. MCLA assay was applied to track O_2^- in various species of harmful algal blooms [25]. Considering the lack of calibration or validation of the methods used in most already presented work, the validation performed in the described work is impressive.

5. Findings

This review highlights several key points associated with current detection methods for extracellular and intracellular ROS in cyanobacteria, summarized in Fig. 4, but they can be extended to other cell types.

5.1. The non-selectivity of ROS detection methods

At the outset, it is necessary to emphasize the already known facts of using DCFH₂-DA as a fluorescence probe for ROS. Unfortunately, in contradiction with the generally repeated opinion, DCFH₂-DA does not react directly with H_2O_2 but requires a peroxidase or transition metal catalyst [84,85] and can produce H_2O_2 itself in intermediate reactions ('self-fulfilling prophesy') [86]. Furthermore, the relationship between the intensity of f-DCF fluorescence and the concentration of the ROS is often nonlinear [87]. It can be an effect of artifactual amplification of the fluorescence intensity via a redox-cycling mechanism involving an intermediate radical, DCF[•], because of several one-electron-oxidizing species oxidize DCHF to f-DCF, as presented in work by Bonini et al.

[88] and by Wardman et al. [85]. In addition, cytochrome c, a heme protein that is released from mitochondria to the cytosol during apoptosis, is capable of oxidizing DCHF directly or indirectly via peroxidase-type mechanism, forming f-DCF [89]. In the work of Chen et al. [90] more than 20 more interferences for f-DCF formation are reviewed. Therefore, even if differences in DCF fluorescence between control and exposed/stressed cells are observed, one must consider the various strictures listed above for proper observation interpretation. As a conclusion, DCFH₂-DA probe cannot be reliably used to measure intracellular H_2O_2 and other ROS [91]. The only useful information from the DCHF-DA probe is the exploration of redox signaling mechanisms in cells. This probe, despite its widespread use, primarily provides qualitative data (relative differences) rather than precise quantitative measurements. This fact is often overlooked in microalgae studies. The other weaknesses of the fluorescent probes, mainly DCFH₂-DA, were the topic of several previous reviews [90,91,91–93].

5.2. False-positive and false-negative results

There are more reactive species in cells than ROS and RNS [92,93] for which we researchers do not have tools for measuring in cyanobacteria. What is essential is that most available methods for ROS analysis in aqueous solutions lack specificity. They do not find specific ROS but rather show a general response to oxidative stressors. Many fluorescence probes work based on non-specific oxidation and therefore do not distinguish between O_2^- , H_2O_2 and OH^- , even though these species have different biological effects. For example, presumably DCFH₂-DA is used to detect H_2O_2 , but it also reacts with peroxynitrite ($ONOO^-$) [91], and other cellular ROS [94], leading to false-positives results. The non-selectivity of ROS detection methods threatens understanding of ROS dynamics in natural environments, potentially leading to misinterpretation of their ecological roles. Non-selectivity also affects experimental design, data interpretation, and reproducibility. One key issue may be overestimating or underestimating the impact of ROS on phytoplankton communities. For example, if DCFH₂-DA is used without controlling selectivity, leading researchers to falsely conclude that cyanobacteria are under oxidative stress, when in fact other factors (e.g., nutrient deficiency) may be at work. AmplexTM Red is used to quantitatively measure H_2O_2 , but its signal is affected by metal ions or organic matter, which can lead to an underestimation of extracellular ROS.

Interpretation of ROS measurements is often inadequate, especially with chemical stressors that can interfere with the detection process. Chemical stressors can interfere with ROS detection assays, leading to false-negative results. For example, some chemicals can inhibit deacetylation of the DCFH₂-DA probe, leading to underestimation of oxidative stress [95]. Cellular antioxidants present in biological samples can scavenge ROS, reducing their availability to react with detection probes and leading to underestimation of ROS levels, as highlighted by review [96].

Furthermore, natural components such as DOM, salinity and pH fluctuations also affect the performance of ROS probes, leading to false-negative or false-positive results. For example, SOSG is widely used to detect singlet oxygen, but high DOM concentrations in freshwater ecosystems quench fluorescence, leading to underestimation of singlet oxygen levels. Therefore, a conclusion could be that photochemical ROS production is negligible in some lakes, when in fact singlet oxygen is present but undetectable due to DOM interference [97]. Many ROS-sensitive probes show pH-dependent fluorescence [98]. Deviations from the optimal pH can alter the behavior of the probe, leading to inaccurate detection of ROS [55]. discussed that changes in environmental pH can interfere with ROS detection, causing misleading results. What can be done is to use proper buffering agents to support a constant pH level during experiments, ensuring stable probe performance.

Two other factors that can lead to false-negative results are the chemical reactivity of the chemical components present in the test solution and nanoparticle interference. Both factors can cause quenching

or enhancement of fluorescence signals (so both can also cause false positives) [56,99]. In addition, some compounds can generate ROS when exposed to light, which can lead to artifactual results in ROS assays, as indicated by ref. [100] in the case of pharmaceuticals, e.g. quinine, tamoxifen, ketoprofen, diclofenac and many other. In addition, in buffered phytoplankton culture media there is continuous H_2O_2 production, causing sublethal chronic exposure to cyanobacteria [1]. Similarly - redox-active metals, e.g. Fe^{2+} can induce oxidation of the probe. False-positive results can be also obtained by artifact generation. For example, in the case of DCFH₂-DA the intermediate radical form during oxidation can lead to superoxide production and finally more H_2O_2 [95]. In the case of AmplexTM Red enzymatic interference can be a big obstacle regarding getting correct results [101,102]. Because the probe functioning depends on horseradish peroxidase (HRP) presence, endogenous reducing agents like NADH and glutathione (GSH) can interfere. Therefore, they can go auto-oxidation or HRP-mediated oxidation and generate H_2O_2 .

5.3. Lacks in ROS detection studies

Current studies (Tables 1 and 2) predominantly focus on unicellular cyanobacteria, while filamentous types remain underrepresented. This gap is significant, as filamentous cyanobacteria contribute to ROS production differently than their unicellular counterparts. Additionally, a cell type in general plays a crucial role in the consistency and reliability of ROS measurements. Research has shown that ROS probe behavior can vary between different cell types, and in some cases, extracellular probes may be taken into the cell interior, potentially altering the accuracy of ROS detection (e.g. in case of SOSG) [103].

There is a notable absence of simultaneous quantification of extracellular and intracellular ROS in the studies reviewed. Such studies can be performed, for example, for mouse cells [104]. This dual quantification is essential for a comprehensive understanding of the role of cyanobacteria in the total ROS in the environment. Fig. 3 summarizes the fluorescence channels used to detect intracellular and extracellular ROS in cyanobacterial cultures. The most common approach involves using stains such as DCFH₂-DA and AmplexTM Red, whose fluorescence

differs from the natural autofluorescence of chlorophylls and phycobilins found in cyanobacteria. Only a few studies utilizing fluorescence microscopy have specifically analyzed the natural red fluorescence of cyanobacteria [105]. None of the work track autofluorescence of phycobilins. Tracking the natural autofluorescence of cyanobacterial cells (Fig. 3) together with synthetic probes can provide deeper insights into the stress responses of cyanobacteria, and ROS evolving. The development of methods for the simultaneous quantification of extracellular and intracellular ROS is essential to obtain a complete assessment of the oxidative environment. This is particularly important for cyanobacteria, whose mechanisms of ROS production remain insufficiently understood.

5.4. Validation improvements and limits of detections

There are technical needs for ROS detection performance. Rigorous validation of ROS detection methods is essential to ensure reliable and reproducible results under different experimental conditions. The use of positive and negative controls, such as H_2O_2 or other ROS donors and scavengers, is crucial for the standardization of these methods. A reference protocol using DCFH₂-DA could facilitate the comparison of stressors, strains, and experimental factors. This protocol should specify reference cell concentrations for unicellular cyanobacteria and biomass measurements for filamentous forms. In addition, quantification and normalization of ROS should be performed in a more standardized way, such as reporting median fluorescence per cell or per biomass unit. The establishment of universal standardized measurement protocols adapted to aquatic environments would enhance the comparability, reproducibility, and transparency of data.

Some of the publications presented in Tables 1 and 2 attempted to quantify the ROS content of water produced by cyanobacteria. In principle, DCFH, AmplexTM Red and other ROS probes (e.g. titanium chloride – Table 2) allow some degree of quantification of ROS in surface water samples. However, there are important limitations to be aware of. Firstly, DCFH₂-DA and AmplexTM Red react not only with H_2O_2 , but also with $\cdot\text{OH}$, $\text{ONOO}\cdot$, $\text{NO}\cdot$ and other ROS. None of the samplers presented in this paper provide information about which specific ROS were involved in the reaction; instead, they only indicate the total amount of

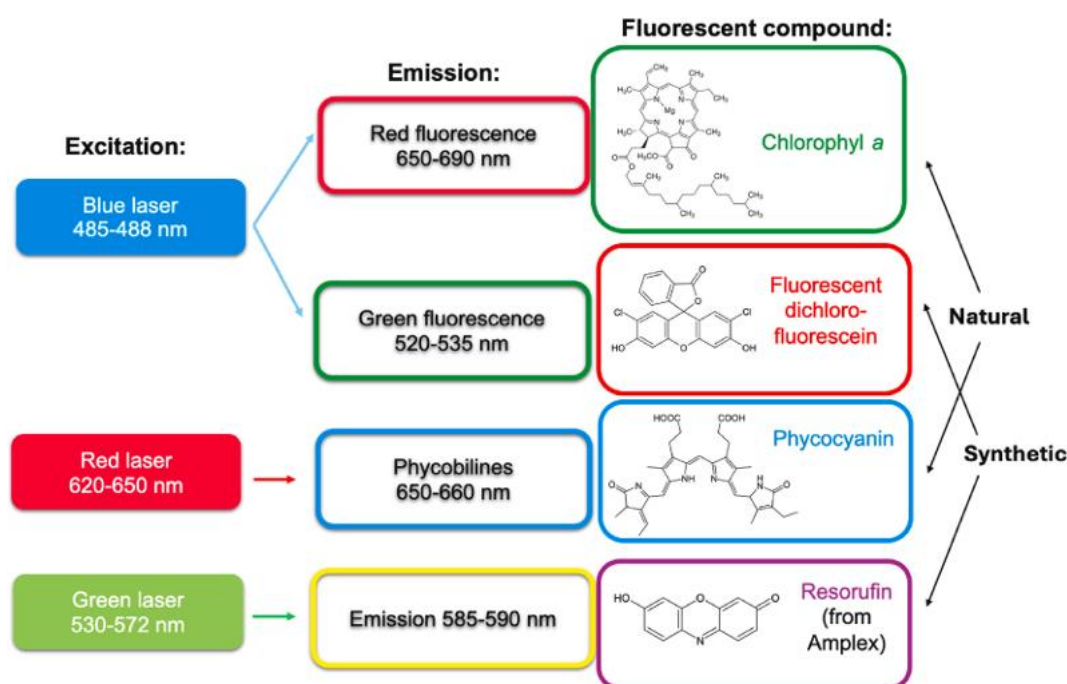


Fig. 3. Canals of fluorescence of naturally occurred substances in cyanobacteria (chlorophyll *a*, phycobilines) and stains - dichlorofluorescein for intracellular ROS detection and resorufin (product of AmplexTM Red) for extracellular ROS detection.

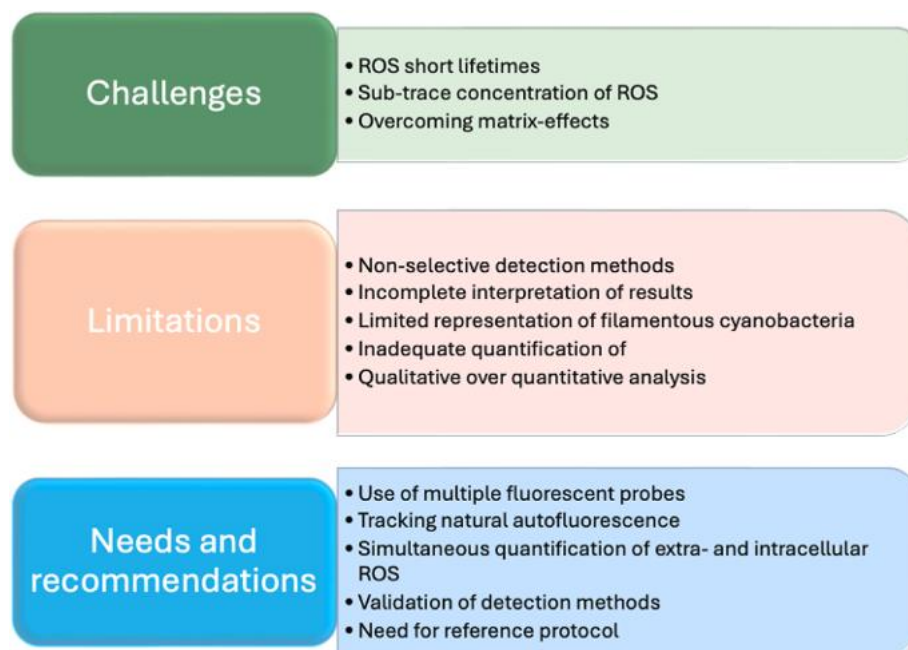


Fig. 4. The summary of challenges of ROS detection, the limitations of available methods and the recommendation for future studies for tracking of ROS production by cyanobacteria.

reactive species that oxidized the probe. In other words, the measurement is quantitative but non-selective, representing the aggregate reactivity of all ROS (i.e., total ROS) (Table 3).

AmplexTM Red also requires catalysts like peroxidase (HRP), which complicates conversions. Most samplers also auto-oxidize easily (especially with light/heat), which increases the fluorescence background, and the effect of pH, oxygen levels and metal ions on the efficiency of the probes - can interfere with the linearity and repeatability of measurements. Thus, the matrix of natural waters (e.g. DOM, metals, organic pollutants) can mask, generate or absorb ROS, requiring standardization of methods and daily calibration (e.g. H₂O₂), background control and avoidance of light exposure. Several research papers have proposed possible solutions to this situation (Table 2), such as: H₂O₂ per unit of protein (μmol/g) [106], fluorescent RFU/MFI, converted by calibration to ROS [71] (Table 1), comparing fluorescence intensity between treatments [46]. These methods use titanium chloride [106], DCFH₂-DA + cytometry, with calibration against H₂O₂ [71,46] and DCFH₂-DA + spectrofluorometry, with calibration by relative quantification [46] allowing results to be presented in quantitative units Table 1.

Studies of reactive oxygen species (ROS) produced by cyanobacteria - whether qualitative assessments, observations of concentration

changes, or the limited number of quantitative analyses - have all been conducted under laboratory conditions, including those using natural water samples (Table 1). This is due to the extreme instability of ROS, which typically persist for only seconds to minutes in aqueous environments. Accurate quantification therefore requires that samples be processed immediately after collection. The available literature reports only laboratory-based measurements of ROS in cyanobacteria, most often employing fluorescent probes such as DCFH-DA or AmplexTM Red [71,41]. To date, no validated methodologies exist for the direct, quantitative measurement of ROS released by cyanobacteria in field conditions. As a result, rapid sample collection and transport under controlled conditions - low temperature and protection from light - are essential to minimize ROS degradation and ensure reliable analytical results.

The graphical relative comparison of probes in order of sensitivity, selectivity, quantification potential and applicability for intra- and extracellular ROS detection is presented on Fig. 5.

6. Conclusions and perspectives

The ROS plays a critical role in both biogeochemical cycles and the

Table 3
Comparison of ROS samplers used in measuring oxidative stress of cyanobacteria.

Probe	ROS Detected	Selectivity	Sensitivity	Detection Method	Quantification	Intracellular Use	Extracellular Use	Used In
DCFH ₂ -DA (2',7'-Dichlorodihydrofluorescein diacetate)	General ROS (e.g., H ₂ O ₂ , •OH, ROO•, ONOO ⁻)	Non-selective	Moderate (~μM range)	Fluorescence (~485/530 nm)	Semi-quantitative	Yes (esterase-dependent)	Possible fluorescent leakage	[37, 41]
Amplex Red + HRP	General ROS (dedicated to H ₂ O ₂)	Low-moderate (HRP-aided, not specific)	High (1–10 nM)	Fluorescence (~560/590 nm)	Semi-quantitative	No	Yes	[40]
SOSG (Singlet Oxygen Sensor Green)	Singlet oxygen (¹ O ₂)	Moderate-high (selective in vitro)	Moderate (~nM, but qualitative)	Fluorescence (~504/525 nm)	Qualitative	Weak penetration in cyanobacteria	No	[37, 53]
MCLA (Methoxy-luminol analog)	Superoxide (O ₂ ⁻ , extracellular only)	Good (with SOD control)	High (100–500 pM)	Chemiluminescence	Quantitative (with KO ₂ calibration)	No	Yes	[40]

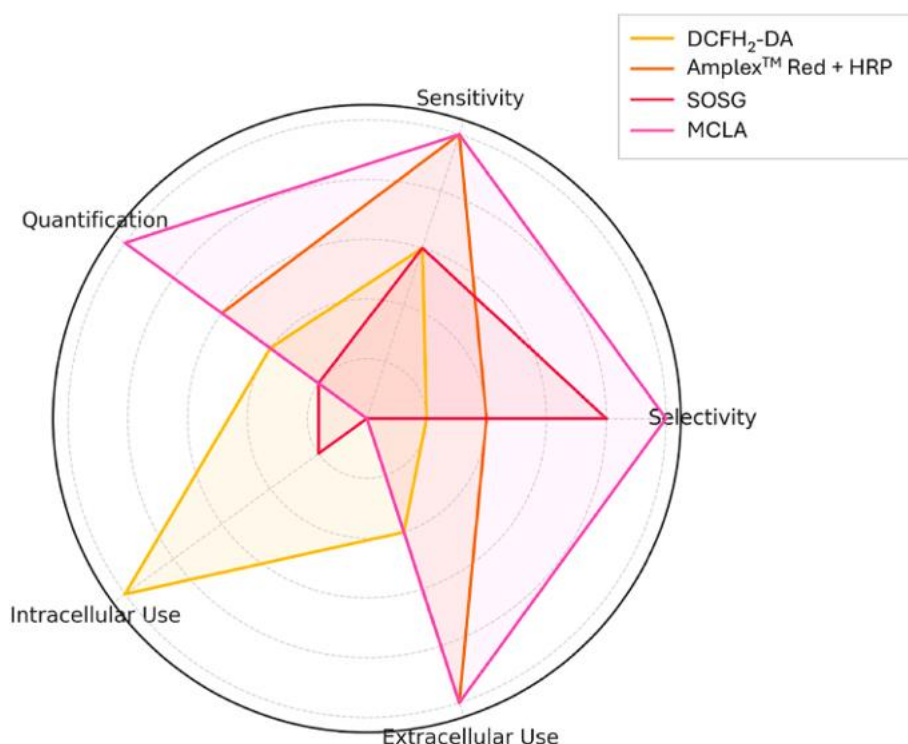


Fig. 5. Relative comparison of probes in order of sensitivity, selectivity, quantification potential and applicability for intra- and extracellular ROS detection for cyanobacterial cells.

modulation of phytoplankton communities within aquatic environments. However, the comprehensive understanding of factors influencing ROS concentrations in natural water bodies remains incomplete. Monitoring of cyanobacterial-derived ROS in natural waters is infrequent, with most data derived from controlled laboratory studies. One of the limitations is the lack of verified methods for tracking intracellular and extracellular ROS, as presented in this review. In practice, the methods track changes in ROS level after stress with comparison to control what is not quantitative information.

As the conclusion of this review, we present the following advice, and perspectives. Improving methodologies for ROS detection is crucial due to the key role of ROS in geochemical cycles. Current methods, while valuable, have significant limitations that need to be addressed to improve the accuracy and reliability of ROS measurements. The limits of detection of ROS by fluorescence probes need to be tracked. A change of perspective on the DCFH₂-DA test result is necessary because this probe does not react with H₂O₂, or even ROS directly as presented in Section 4. By adopting more comprehensive approaches scientists can gain deeper insights into the dynamics of cyanobacterial ROS and their impact on aquatic ecosystems. Of various selective methods to tract ROS (presented in reviews [3,94,96]) only a few were tested for cyanobacteria. Summary of recent advances of detection methods and probes for tracking ROS in biological systems can be found in refs. [72,107,108]. Numerous new selective probes and detection methods are available, which can be tested for ROS detection in phytoplankton. Therefore, studies on the application of new probes are urgent. In addition, studies need to be extended to filamentous cyanobacteria. The simultaneous measurement of natural and synthetic fluorescence of cells could be beneficial for strengthened statements. The next step in this research should be to investigate the impact of anthropogenic factors and climate change on ROS production by cyanobacteria.

CRediT authorship contribution statement

Hanna Lis: Writing – review & editing, Writing – original draft,

Investigation. **Klaudia Kwidzińska:** Writing – review & editing, Writing – original draft, Visualization, Investigation. **Katarzyna Bethke:** Writing – review & editing, Writing – original draft, Investigation. **Magda Caban:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Investigation, Funding acquisition, Conceptualization.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT in order to improve grammar and logical sequence in the article. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Magda Caban reports financial support was provided by National Science Centre Poland. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

Financial support was provided by the National Science Centre (Poland) under grants UMO-2019/35/B/NZ9/00253.

Data availability

No data was used for the research described in the article.

References

- [1] J. Jeffrey Morris, E.R. Zinser, Continuous hydrogen peroxide production by organic buffers in phytoplankton culture media, *J. Phycol.* 49 (2013) 1223–1228, <https://doi.org/10.1111/jpy.12123>.
- [2] J.J. Morris, A.L. Rose, Z. Lu, Reactive oxygen species in the world ocean and their impacts on marine ecosystems, *Redox Biol.* 52 (2022), <https://doi.org/10.1016/j.redox.2022.102285>.
- [3] G. Bartosz, Use of spectroscopic probes for detection of reactive oxygen species, *Clin. Chim. Acta* 368 (2006) 53–76, <https://doi.org/10.1016/j.cca.2005.12.039>.
- [4] A. Latifi, M. Ruiz, C.C. Zhang, Oxidative stress in cyanobacteria, *FEMS Microbiol. Rev.* 33 (2009) 258–278, <https://doi.org/10.1111/j.1574-6976.2008.00134.x>.
- [5] M. Mandal, M. Sarkar, A. Khan, M. Biswas, A. Masi, R. Rakwal, G.K. Agrawal, A. Srivastava, A. Sarkar, Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) in plants— maintenance of structural individuality and functional blend, *Advances in Redox Research* 5 (2022), <https://doi.org/10.1016/j.arres.2022.100039>.
- [6] S.S. Korshunov, J.A. Imlay, A potential role for periplasmic superoxide dismutase in blocking the penetration of external superoxide into the cytosol of Gram-negative bacteria, *Mol. Microbiol.* 43 (2002) 95–106, <https://doi.org/10.1046/j.1365-2958.2002.02719.x>.
- [7] P. Pospíšil, Production of reactive oxygen species by photosystem II as a response to light and temperature stress, *Front. Plant Sci.* 7 (2016), <https://doi.org/10.3389/fpls.2016.01950>.
- [8] Kumar Singh Prashant, Kumar Ajay, Kumar Singh Vipin, Kumar Shrivastava Alok (Eds.), *Advances in Cyanobacterial Biology*, first ed., Academic Press, 2020. <https://shop.elsevier.com/books/advances-in-cyanobacterial-biology/singh/978-0-12-819311-2>. (Accessed 24 March 2025).
- [9] A. Godrant, A.L. Rose, G. Sarthou, T. David Waite, New method for the determination of extracellular production of superoxide by marine phytoplankton using the chemiluminescence probes MCLA and red-CLA, *Limnol. Oceanogr.* Methods 7 (2009) 682–692, <https://doi.org/10.4319/lom.2009.7.682>.
- [10] F. Leunert, W. Eckert, A. Paul, V. Gerhardt, H.P. Grossart, Phytoplankton response to UV-generated hydrogen peroxide from natural organic matter, *J. Plankton Res.* 36 (2014) 185–197, <https://doi.org/10.1093/plankt/fbt096>.
- [11] E.R. Zinser, The microbial contribution to reactive oxygen species dynamics in marine ecosystems, *Environ. Microbiol. Rep.* 10 (2018) 412–427, <https://doi.org/10.1111/1758-2229.12626>.
- [12] P. Wardman, Factors important in the use of fluorescent or luminescent probes and other chemical reagents to measure oxidative and radical stress, *Biomolecules* 13 (2023), <https://doi.org/10.3390/biom13071041>.
- [13] A. Krieger-Liszak, Singlet oxygen production in photosynthesis, *J. Exp. Bot.* 56 (2004) 337–346, <https://doi.org/10.1093/jxb/erh237>.
- [14] A. Wilson, G. Ajlani, J.-M. Verbavatz, I. Vass, C.A. Kerfeld, D. Kirilovsky, A soluble carotenoid protein involved in phycobilisome-related energy dissipation in cyanobacteria, *Plant Cell* 18 (2006) 992–1007, <https://doi.org/10.1105/tpc.105.040121>.
- [15] R. Rachedi, M. Foglino, A. Latifi, Stress signaling in cyanobacteria: a mechanistic overview, *Life* 10 (2020) 312, <https://doi.org/10.3390/life10120312>.
- [16] S.S. Korshunov, J.A. Imlay, A potential role for periplasmic superoxide dismutase in blocking the penetration of external superoxide into the cytosol of Gram-negative bacteria, *Mol. Microbiol.* 43 (2002) 95–106, <https://doi.org/10.1046/j.1365-2958.2002.02719.x>.
- [17] K. Keyer, J.A. Imlay, Superoxide accelerates DNA damage by elevating free-iron levels, *Proc. Natl. Acad. Sci. U. S. A.* 93 (1996) 13635–13640, <https://doi.org/10.1073/pnas.93.24.13635>.
- [18] M. Tanaka, P.B. Chock, E.R. Stadtman, Oxidized messenger RNA induces translation errors, *Proc. Natl. Acad. Sci. U. S. A.* 104 (2007) 66–71, <https://doi.org/10.1073/pnas.0609737104>.
- [19] J.M. Diaz, S. Plummer, C. Tomas, C. Alves-De-Souza, Production of extracellular reactive oxygen species by phytoplankton: past and future directions, *J. Plankton Res.* 40 (2018) 655–666, <https://doi.org/10.1093/plankt/fby039>.
- [20] H. Yoon, H.C. Kim, S. Kim, Long-term seasonal and temporal changes of hydrogen peroxide from cyanobacterial blooms in fresh waters, *J. Environ. Manag.* 298 (2021) 113515, <https://doi.org/10.1016/j.jenvman.2021.113515>.
- [21] R.M. Cory, T.W. Davis, G.J. Dick, T. Johengen, V.J. Denef, M.A. Berry, S.E. Page, S.B. Watson, K. Yuhas, G.W. Kling, Seasonal dynamics in dissolved organic matter, hydrogen peroxide, and cyanobacterial blooms in Lake Erie, *Front. Mar. Sci.* 3 (2016), <https://doi.org/10.3389/fmars.2016.00054>.
- [22] C.M. Hansel, J.M. Diaz, Production of extracellular reactive oxygen species by marine biota, *Annual Review of Marine Science Annu. Rev. Mar. Sci.* 40 (2021) 177–200, <https://doi.org/10.1146/annurev-marine-041320.2021>.
- [23] Y.-Y. He, D.-P. Häder, UV-B-induced formation of reactive oxygen species and oxidative damage of the cyanobacterium *Anabaena* sp.: protective effects of ascorbic acid and N-acetyl-L-cysteine, *J. Photochem. Photobiol.* B 66 (2002) 115–124, [https://doi.org/10.1016/S1011-1344\(02\)00231-2](https://doi.org/10.1016/S1011-1344(02)00231-2).
- [24] Daekyung Kim, Tatsuya Oda, Atsushi Ishimatsu, Tsuyoshi Muramatsu, Ga; acturonic-acid-induced increase of superoxide production in Red Tide phytoplankton *Chattonella marina* and *Heterosigma akashiwo*, *Biosci. Biotechnol. Biochem.* 64 (2000) 911–914.
- [25] K. Cho, M. Ueno, Y. Liang, D. Kim, T. Oda, Generation of reactive oxygen species (ROS) by harmful algal bloom (HAB)-Forming phytoplankton and their potential impact on surrounding living organisms, *Antioxidants* 11 (2022), <https://doi.org/10.3390/antiox11020206>.
- [26] K. Bethke, K. Kropidowska, P. Stepnowski, M. Caban, Review of warming and acidification effects to the ecotoxicity of pharmaceuticals on aquatic organisms in the era of climate change, *Sci. Total Environ.* 877 (2023), <https://doi.org/10.1016/j.scitotenv.2023.162829>.
- [27] J.D. Willey, R.J. Kieber, G.J. Brooks Avery, *Effects of Rainwater Iron and Hydrogen Peroxide on Iron Speciation and Phytoplankton Growth in Seawater Near Bermuda*, 2004.
- [28] K. Wang, S. Garg, T.D. Waite, Light-mediated reactive oxygen species generation and iron redox transformations in the presence of exudate from the cyanobacterium *Microcystis aeruginosa*, *Environ. Sci. Technol.* 51 (2017) 8384–8395, <https://doi.org/10.1021/acs.est.7b01441>.
- [29] J.J. Morris, Z.I. Johnson, M.J. Szul, M. Keller, E.R. Zinser, Dependence of the cyanobacterium *Prochlorococcus* on hydrogen peroxide scavenging microbes for growth at the ocean's surface, *PLoS One* 6 (2011), <https://doi.org/10.1371/journal.pone.0016805>.
- [30] J.J. Morris, R. Kirkegaard, M.J. Szul, Z.I. Johnson, E.R. Zinser, Facilitation of robust growth of *Prochlorococcus* colonies and dilute liquid cultures by “helper” heterotrophic bacteria, *Appl. Environ. Microbiol.* 74 (2008) 4530–4534, <https://doi.org/10.1128/AEM.02479-07>.
- [31] M. Tichy, W. Vermaas, In vivo role of catalase-peroxidase in *Synechocystis* sp. Strain PCC 6803 (1999). <https://journals.asm.org/journal/jb>.
- [32] M. Drábková, W. Admiraal, B. Maršálek, Combined exposure to hydrogen peroxide and light-selective effects on cyanobacteria, green algae, and diatoms, *Environ. Sci. Technol.* 41 (2007) 309–314, <https://doi.org/10.1021/es060746i>.
- [33] Y. Yoshpe-Purer, Y. Henis, Factors affecting catalase level and sensitivity to hydrogen peroxide in *Escherichia coli*. <https://journals.asm.org/journal/aem>, 1976.
- [34] T. Tanaka, K. Kawasaki, S. Daimon, W. Kitagawa, K. Yamamoto, H. Tamaki, M. Tanaka, C.H. Nakatsu, Y. Kamagata, A hidden pitfall in the preparation of agar media undermines microorganism cultivability, *Appl. Environ. Microbiol.* 80 (2014) 7659–7666, <https://doi.org/10.1128/AEM.02741-14>.
- [35] J.M. Burns, W.J. Cooper, J.L. Ferry, D.W. King, B.P. DiMento, K. McNeill, C. J. Miller, W.L. Miller, B.M. Peake, S.A. Rusak, A.L. Rose, T.D. Waite, Methods for reactive oxygen species (ROS) detection in aqueous environments, *Aquat. Sci.* 74 (2012) 683–734, <https://doi.org/10.1007/s00027-012-0251-x>.
- [36] N. Soh, Recent advances in fluorescent probes for the detection of reactive oxygen species, *Anal. Bioanal. Chem.* 386 (2006) 532–543, <https://doi.org/10.1007/s00216-006-0366-9>.
- [37] R.P. Rastogi, S.P. Singh, D.P. Häder, R.P. Sinha, Detection of reactive oxygen species (ROS) by the oxidant-sensing probe 2',7'-dichlorodihydrofluorescein diacetate in the cyanobacterium *Anabaena variabilis* PCC 7937, *Biochem. Biophys. Res. Commun.* 397 (2010) 603–607, <https://doi.org/10.1016/j.bbrc.2010.06.006>.
- [38] S.P. Singh, R.P. Rastogi, D.P. Häder, R.P. Sinha, Temporal dynamics of ROS biogenesis under simulated solar radiation in the cyanobacterium *Anabaena variabilis* PCC 7937, *Protoplasma* 251 (2014) 1223–1230, <https://doi.org/10.1007/s00709-014-0630-3>.
- [39] R.P. Rastogi, S.P. Singh, A. Incharoensakdi, D.P. Häder, R.P. Sinha, Ultraviolet radiation-induced generation of reactive oxygen species, DNA damage and induction of UV-absorbing compounds in the cyanobacterium *Rivularia* sp. HKAR-4, *South Afr. J. Bot.* 90 (2014) 163–169, <https://doi.org/10.1016/j.sajb.2013.11.006>.
- [40] C.M. Hansel, C. Buchwald, J.M. Diaz, J.E. Ossolinski, S.T. Dyhrman, B.A.S. Van Mooy, D. Polyviou, Dynamics of extracellular superoxide production by *Trichodesmium* colonies from the Sargasso Sea, *Limnol. Oceanogr.* 61 (2016) 1188–1200, <https://doi.org/10.1002/lno.10266>.
- [41] Rajneesh, J. Pathak, A. Chatterjee, S. Singh, R. Sinha, Detection of reactive oxygen species (ROS) in cyanobacteria using the oxidant-sensing probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), *Bio Protoc* 7 (2017) 1–8, <https://doi.org/10.21769/BioProtoc.2545>.
- [42] J. Pathak, D. Kumar, D.K. Singh, H. Ahmed, V.K. Kannaujia, R.P. Sinha, Ultraviolet radiation and salinity-induced physiological changes and scytonemin induction in cyanobacteria isolated from diverse habitats, *Biointerface Res. Appl. Chem.* 12 (2022) 3590–3606, <https://doi.org/10.33263/BRIAC123.35903606>.
- [43] H. Ahmed, J. Pathak, Rajneesh, P.K. Sonkar, V. Ganesan, D.P. Häder, R.P. Sinha, Responses of a hot spring cyanobacterium under ultraviolet and photosynthetically active radiation: photosynthetic performance, antioxidative enzymes, mycosporine-like amino acid profiling and its antioxidative potentials, *3 Biotech* 11 (2021) 1–23, <https://doi.org/10.1007/s13205-020-02562-1>.
- [44] R. Kholssi, H. Lougraimzi, I. Moreno-Garrido, Influence of salinity and temperature on the growth, productivity, photosynthetic activity and intracellular ROS of two marine microalgae and cyanobacteria, *Mar. Environ. Res.* 186 (2023), <https://doi.org/10.1016/j.marenvres.2023.105932>.
- [45] P.R. Singh, J. Pathak, Rajneesh, H. Ahmed, D.P. Häder, R.P. Sinha, Physiological responses of the cyanobacterium *Synechocystis* sp. PCC 6803 under rhythmic light variations, *Photochem. Photobiol. Sci.* 22 (2023) 2055–2069, <https://doi.org/10.1016/j.psc.2023.04.029>.
- [46] G. Wang, S. Deng, J. Liu, C. Ye, X. Zhou, L. Chen, Cell damage caused by ultraviolet B radiation in the desert cyanobacterium *Phormidium tenue* and its recovery process, *Ecotoxicol. Environ. Saf.* 144 (2017) 315–320, <https://doi.org/10.1016/j.ecoenv.2017.06.024>.
- [47] Z. Lu, J. Sha, Y. Tian, X. Zhang, B. Liu, Z. Wu, Polyphenolic allelochemical pyrogallol acid induces caspase-3(like)-dependent programmed cell death in the cyanobacterium *Microcystis aeruginosa*, *Algal Res.* 21 (2017) 148–155, <https://doi.org/10.1016/j.algal.2016.11.007>.
- [48] B. Tiwari, S. Chakraborty, A.K. Srivastava, A.K. Mishra, Biodegradation and rapid removal of methyl parathion by the paddy field cyanobacterium *Fischerella* sp., *Algal Res.* 25 (2017) 285–296, <https://doi.org/10.1016/j.algal.2017.05.024>.

- [49] S. Chakraborty, B. Tiwari, S.S. Singh, A.K. Srivastava, A.K. Mishra, Differential physiological, oxidative and antioxidative responses of cyanobacterium *Anabaena sphaerica* to attenuate malathion pesticide toxicity, *Biocatal. Agric. Biotechnol.* 11 (2017) 56–63, <https://doi.org/10.1016/j.bcab.2017.05.011>.
- [50] J. Wang, J. Zhu, S. Liu, B. Liu, Y. Gao, Z. Wu, Generation of reactive oxygen species in cyanobacteria and green algae induced by allelochemicals of submerged macrophytes, *Chemosphere* 85 (2011) 977–982, <https://doi.org/10.1016/j.chemosphere.2011.06.076>.
- [51] P. Swapnil, A.K. Yadav, S. Srivastav, N.K. Sharma, S. Srikrishna, A.K. Rai, Biphasic ROS accumulation and programmed cell death in a cyanobacterium exposed to salinity (NaCl and Na₂SO₄), *Algal Res.* 23 (2017) 88–95, <https://doi.org/10.1016/j.algal.2017.01.014>.
- [52] E.S. Rueda, L. Borgnino, G. Bia, P.I. Gil, M. Bisbal, N. Pietrasiak, E.C. Mlewski, Responses to arsenic stress by the Andean benthic-extremophile cyanobacteria *Rivularia halophila*, *Algal Res.* 75 (2023), <https://doi.org/10.1016/j.algal.2023.103286>.
- [53] R.K. Sinha, J. Komenda, J. Knoppová, M. Sedlářová, P. Pospíšil, Small CAB-like proteins prevent formation of singlet oxygen in the damaged photosystem II complex of the cyanobacterium *Synechocystis* sp. PCC 6803, *Plant Cell Environ.* 35 (2012) 806–818, <https://doi.org/10.1111/j.1365-3040.2011.02454.x>.
- [54] T.V. Votyakova, I.J. Reynolds, Detection of hydrogen peroxide with Amplex Red: interference by NADH and reduced glutathione auto-oxidation, *Arch. Biochem. Biophys.* 431 (2004) 138–144, <https://doi.org/10.1016/j.abb.2004.07.025>.
- [55] D.S. Bilan, V.V. Belousov, Vivo Imaging of Hydrogen Peroxide with HyPer Probes, vol. 29, 2018, pp. 569–584, <https://doi.org/10.1089/ARS.2018.7540>. <https://Home.Liebertpub.Com/Ars>.
- [56] N. Bohmer, A. Rippl, S. May, A. Walter, M.B. Heo, M. Kwak, M. Roesslein, N. W. Song, P. Wick, C. Hirsch, Interference of engineered nanomaterials in flow cytometry: a case study, *Colloids Surf. B Biointerfaces* 172 (2018) 635–645, <https://doi.org/10.1016/j.colsurfb.2018.09.021>.
- [57] M. Sedlářová, L. Luhová, Re-evaluation of imaging methods of reactive oxygen and nitrogen species in plants and fungi: influence of cell wall composition, *Front. Physiol.* 8 (2017) 826, <https://doi.org/10.3389/fphys.2017.00826>.
- [58] R.K. Sinha, J. Komenda, J. Knoppová, M. Sedlářová, P. Pospíšil, Small CAB-like proteins prevent formation of singlet oxygen in the damaged photosystem II complex of the cyanobacterium *Synechocystis* sp. PCC 6803, *Plant Cell Environ.* 35 (2012) 806–818, <https://doi.org/10.1111/j.1365-3040.2011.02454.x>.
- [59] S. Duangthaisorn, E.J. Farrell, A.C. Alba-Rubio, P. Zelenay, D.S. Kim, Detection technologies for reactive oxygen species: fluorescence and electrochemical methods and their applications, *Biosensors (Basel)* 11 (2021), <https://doi.org/10.3390/Bios11020030>.
- [60] D.W. King, W.J. Cooper, S.A. Rusak, B.M. Peake, J.J. Kiddle, D.W. O'Sullivan, M. L. Melamed, C.R. Morgan, S.M. Theberge, Flow injection analysis of H₂O₂ in natural waters using acridinium ester chemiluminescence: method development and optimization using a kinetic model, *Anal. Chem.* 79 (2007) 4169–4176, <https://doi.org/10.1021/ac062228w>.
- [61] W.J. Cooper, J.K. Moegling, R.J. Kieber, J.J. Kiddle, A chemiluminescence method for the analysis of H₂O₂ in natural waters, *Mar. Chem.* 70 (2000) 191–200, [https://doi.org/10.1016/S0304-4203\(00\)00025-6](https://doi.org/10.1016/S0304-4203(00)00025-6).
- [62] X. Yuan, P.S. Nico, X. Huang, T. Liu, C. Ulrich, K.H. Williams, J.A. Davis, Production of hydrogen peroxide in groundwater at rifle, Colorado, *Environ. Sci. Technol.* 51 (2017) 7881–7891, <https://doi.org/10.1021/acs.est.6b04803>.
- [63] K.M. Sutherland, A. Coe, R.J. Gast, S. Plummer, C.P. Suffridge, J.M. Diaz, J. S. Bowman, S.D. Wankel, C.M. Hansel, Extracellular superoxide production by key microbes in the global ocean, *Limnol. Oceanogr.* 64 (2019) 2679–2693, <https://doi.org/10.1002/lno.11247>.
- [64] A.L. Rose, J.W. Moffett, D. Waite, Determination of superoxide in seawater using 2-Methyl-6-(4-methoxyphenyl)-3,7-dihydroimidazo[1,2-a]pyrazin-3(7H)-one chemiluminescence, *Anal. Chem.* 80 (2008) 1215–1227, <https://doi.org/10.1021/ac7018975>.
- [65] R.J. Schneider, K.L. Roe, C.M. Hansel, B.M. Voelker, Species-level variability in extracellular production rates of reactive oxygen species by diatoms, *Front. Chem.* 4 (2016), <https://doi.org/10.3389/fchem.2016.00005>.
- [66] A. Asserghine, A. Baby, S.T. Putnam, P. Qian, E. Gao, H. Zhao, J. Rodríguez-López, In situ detection of reactive oxygen species spontaneously generated on lead acid battery anodes: a pathway for degradation and self-discharge at open circuit, *Chem. Sci.* 14 (2023) 12292–12298, <https://doi.org/10.1039/D3SC04736A>.
- [67] J.M. Burns, W.J. Cooper, J.L. Ferry, D.W. King, B.P. DiMento, K. McNeill, C. J. Miller, W.L. Miller, B.M. Peake, S.A. Rusak, A.L. Rose, T.D. Waite, Methods for reactive oxygen species (ROS) detection in aqueous environments, *Aquat. Sci.* 74 (2012) 683–734, <https://doi.org/10.1007/s00027-012-0251-x>.
- [68] S.L. Hempel, G.R. Buettner, Y.Q. O'malley, D.A. Wessels, D.M. Flaherty, Original contribution dihydrofluorescein diacetate is superior for detecting intracellular oxidants: comparison with 2,7-dichlorodihydrofluorescein diacetate, 5-(and 6)-carboxy-2,7-dichlorodihydrofluorescein diacetate, and dihydrorhodamine 123, 1999.
- [69] C. Loetchutin, S. Kothan, S. Dechsupa, J. Meesungnoen, J.P. Jay-Gerin, S. Mankhetkorn, Spectrofluorometric determination of intracellular levels of reactive oxygen species in drug-sensitive and drug-resistant cancer cells using the 2',7'-dichlorofluorescein diacetate assay, *Radiat. Phys. Chem.* 72 (2005) 323–331, <https://doi.org/10.1016/j.radphyschem.2004.06.011>.
- [70] Z. Lu, J. Sha, Y. Tian, X. Zhang, B. Liu, Z. Wu, Polyphenolic allelochemical pyrogallol acid induces caspase-3(like)-dependent programmed cell death in the cyanobacterium *Microcystis aeruginosa*, *Algal Res.* 21 (2017) 148–155, <https://doi.org/10.1016/j.algal.2016.11.007>.
- [71] S. Mondal, S.P. Singh, Flow cytometry-based measurement of reactive oxygen species in cyanobacteria, *Bio Protoc* 12 (2022) 1–11, <https://doi.org/10.21769/BioProtoc.4417>.
- [72] M.R. Krishnendu, S. Singh, Reactive oxygen species: advanced detection methods and coordination with nanozymes, *Chem. Eng. J.* 511 (2025) 161296, <https://doi.org/10.1016/j.cej.2025.161296>.
- [73] D.K. Singh, J. Pathak, A. Pandey, Rajneesh, V. Singh, R.P. Sinha, Purification, characterization and assessment of stability, reactive oxygen species scavenging and antioxidative potentials of mycosporine-like amino acids (MAAs) isolated from cyanobacteria, *J. Appl. Phycol.* (2022) 3157–3175, <https://doi.org/10.1007/s10811-022-02832-w>.
- [74] S.P. Singh, R.P. Rastogi, D.P. Häder, R.P. Sinha, Temporal dynamics of ROS biogenesis under simulated solar radiation in the cyanobacterium *Anabaena variabilis* PCC 7937, *Protoplasma* 251 (2014) 1223–1230, <https://doi.org/10.1007/s00709-014-0630-3>.
- [75] M. You, X. You, J. Hu, X. Yang, W. Sun, Carbon nanotubes influence the toxic effects of chloramphenicol and tetracycline on cyanobacterium: *Synechocystis* sp. in different ways, *Environ. Sci. Nano* 8 (2021) 634–646, <https://doi.org/10.1039/d0en01204a>.
- [76] A. Aksmann, W. Pokora, A. Baścik-Remisiewicz, A. Dettlaff-Pokora, B. Wielgomas, M. Dziadziuszko, Z. Tukaj, Time-dependent changes in antioxidative enzyme expression and photosynthetic activity of *Chlamydomonas reinhardtii* cells under acute exposure to cadmium and anthracene, *Ecotoxicol. Environ. Saf.* 110 (2014) 31–40, <https://doi.org/10.1016/j.ecoenv.2014.08.005>.
- [77] M. Majewska, D. Harshkova, M. Guściora, A. Aksmann, Phytotoxic activity of diclofenac: evaluation using a model green alga *Chlamydomonas reinhardtii* with atrazine as a reference substance, *Chemosphere* 209 (2018) 989–997, <https://doi.org/10.1016/j.chemosphere.2018.06.156>.
- [78] A. Prasad, M. Sedlářová, P. Pospíšil, Singlet oxygen imaging using fluorescent probe Singlet Oxygen Sensor Green in photosynthetic organisms, *Sci. Rep.* 8 (2018), <https://doi.org/10.1038/s41598-018-31638-5>.
- [79] C. Flors, M.J. Fryer, J. Waring, B. Reeder, U. Bechtold, P.M. Mullineaux, S. Nonell, M.T. Wilson, N.R. Baker, Imaging the production of singlet oxygen in vivo using a new fluorescent sensor, Singlet Oxygen Sensor Green®, *J. Exp. Bot.* (2006) 1725–1734, <https://doi.org/10.1093/jxb/erj181>.
- [80] Y. Kambayashi, K. Ogino, Reestimation of Cypridina luciferin analogs (MCLA) as a chemiluminescence probe to detect active oxygen species—cautionary note for use of MCLA, *J. Toxicol. Sci.* 28 (2003) 139–148, <https://doi.org/10.2131/jts.28.139>.
- [81] J. Zielonka, B. Kalyanaram, Small-molecule luminescent probes for the detection of cellular oxidizing and nitrating species, *Free Radic. Biol. Med.* 128 (2018) 3–22, <https://doi.org/10.1016/j.freeradbiomed.2018.03.032>.
- [82] F. Arisawa, H. Tatsuzawa, Y. Kambayashi, H. Kuwano, K. Fujimori, M. Nakano, MCLA-dependent chemiluminescence suggests that singlet oxygen plays a pivotal role in myeloperoxidase-catalysed bactericidal action in neutrophil phagosomes, *Luminescence* 18 (2003) 229–238, <https://doi.org/10.1002/BIO.728>.
- [83] E.J. Swindle, J.A. Hunt, J.W. Coleman, A comparison of reactive oxygen species generation by rat peritoneal macrophages and mast cells using the highly sensitive real-time chemiluminescence probe phoxin: inhibition of antigen-induced mast cell degranulation by macrophage-derived hydrogen peroxide, *J. Immunol.* 169 (2002) 5866–5873, <https://doi.org/10.4049/JIMMUNOL.169.10.5866>.
- [84] P. Wardman, Factors important in the use of fluorescent or luminescent probes and other chemical reagents to measure oxidative and radical stress, *Biomolecules* (2023) 1041, <https://doi.org/10.3390/Biom13071041>. Vol. 13, Page 1041 13 (2023).
- [85] P. Wardman, Fluorescent and luminescent probes for measurement of oxidative and nitrosative species in cells and tissues: progress, pitfalls, and prospects, *Free Radic. Biol. Med.* 43 (2007) 995–1022, <https://doi.org/10.1016/J.FREERADBIOMED.2007.06.026>.
- [86] B. Kalyanaram, V. Darley-Usmar, K.J.A. Davies, P.A. Dennery, H.J. Forman, M. B. Grisham, G.E. Mann, K. Moore, L.J. Roberts, H. Ischiropoulos, Measuring reactive oxygen and nitrogen species with fluorescent probes: challenges and limitations, *Free Radic. Biol. Med.* 52 (2011) 1, <https://doi.org/10.1016/J.FREERADBIOMED.2011.09.030>.
- [87] H. Wang, J.A. Joseph, Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader, *Free Radic. Biol. Med.* 27 (1999) 612–616, [https://doi.org/10.1016/S0891-5849\(99\)00107-0](https://doi.org/10.1016/S0891-5849(99)00107-0).
- [88] M.G. Bonini, C. Rota, A. Tomasi, R.P. Mason, The oxidation of 2',7'-dichlorofluorescein to reactive oxygen species: a self-fulfilling prophecy? *Free Radic. Biol. Med.* 40 (2006) 968–975, <https://doi.org/10.1016/J.FREERADBIOMED.2005.10.042>.
- [89] M. Karlsson, T. Kurz, U.T. Brunk, S.E. Nilsson, C.I. Frennsson, What does the commonly used DCF test for oxidative stress really show? *Biochem. J.* 428 (2010) 183–190, <https://doi.org/10.1042/BJ20100208>.
- [90] X. Chen, Z. Zhong, Z. Xu, L. Chen, Y. Wang, 2',7'-Dichlorodihydrofluorescein as a fluorescent probe for reactive oxygen species measurement: forty years of application and controversy, *Free Radic. Res.* 44 (2010) 587–604, <https://doi.org/10.3109/10715761003709802>.
- [91] B. Kalyanaram, V. Darley-Usmar, K.J.A. Davies, P.A. Dennery, H.J. Forman, M. B. Grisham, G.E. Mann, K. Moore, L.J. Roberts, H. Ischiropoulos, Measuring reactive oxygen and nitrogen species with fluorescent probes: challenges and limitations, *Free Radic. Biol. Med.* 52 (2012) 1–6, <https://doi.org/10.1016/J.FREERADBIOMED.2011.09.030>.
- [92] M.P. Murphy, A. Holmgren, N.G. Larsson, B. Halliwell, C.J. Chang, B. Kalyanaram, S.G. Rhee, P.J. Thornalley, L. Partridge, D. Gems, T. Nyström,

- V. Belousov, P.T. Schumacker, C.C. Winterbourn, Unraveling the biological roles of reactive oxygen species, *Cell Metab.* 13 (2011) 361–366, <https://doi.org/10.1016/J.CMET.2011.03.010>.
- [93] C.C. Winterbourn, Reconciling the chemistry and biology of reactive oxygen species, *Nat. Chem. Biol.* 4 (5) (2008) 278–286, <https://doi.org/10.1038/nchembio.85>, 2008.
- [94] A. Gomes, E. Fernandes, J.L.F.C. Lima, Fluorescence probes used for detection of reactive oxygen species, *J. Biochem. Biophys. Methods* 65 (2005) 45–80, <https://doi.org/10.1016/j.jbbm.2005.10.003>.
- [95] C. Rota, C.F. Chignell, R.P. Mason, Evidence for free radical formation during the oxidation of 2'-7'-dichlorofluorescein to the fluorescent dye 2'-7'-dichlorofluorescein by horseradish peroxidase: possible implications for oxidative stress measurements, *Free Radic. Biol. Med.* 27 (1999) 873–881, [https://doi.org/10.1016/S0891-5849\(99\)00137-9](https://doi.org/10.1016/S0891-5849(99)00137-9).
- [96] C. Ortega-Villasante, S. Burén, A. Blázquez-Castro, Á. Barón-Sola, L.E. Hernández, Fluorescent in vivo imaging of reactive oxygen species and redox potential in plants, *Free Radic. Biol. Med.* 122 (2018) 202–220, <https://doi.org/10.1016/J.FREERADBIOMED.2018.04.005>.
- [97] P.R. Erickson, K.J. Moor, J.J. Werner, D.E. Latch, W.A. Arnold, K. McNeill, Singlet oxygen phosphorescence as a probe for triplet-state dissolved organic matter reactivity, *Environ. Sci. Technol.* 52 (2018) 9170–9178, https://doi.org/10.1021/ACS.EST.8B02379/ASSET/IMAGES/MEDIUM/ES-2018-02379T_M008.GIF.
- [98] V. Chaturvedi, R. Kumari, P. Sharma, A.K. Pati, Diverse fluorescent probe concepts for detection and monitoring of reactive oxygen species, *Chem. Asian J.* (2025) e202401524, <https://doi.org/10.1002/ASIA.202401524>.
- [99] K.J. Ong, T.J. MacCormack, R.J. Clark, J.D. Ede, V.A. Ortega, L.C. Felix, M.K. M. Dang, G. Ma, H. Fenniri, J.G.C. Veinot, G.G. Goss, Widespread nanoparticle-assay interference: implications for nanotoxicity testing, *PLoS One* 9 (2014) e90650, <https://doi.org/10.1371/JOURNAL.PONE.0090650>.
- [100] S. Onoue, N. Igarashi, S. Yamada, Y. Tsuda, High-throughput reactive oxygen species (ROS) assay: an enabling technology for screening the phototoxic potential of pharmaceutical substances, *J. Pharm. Biomed. Anal.* 46 (2008) 187–193, <https://doi.org/10.1016/J.JPBA.2007.09.003>.
- [101] T.V. Votyakova, I.J. Reynolds, Detection of hydrogen peroxide with Amplex Red: interference by NADH and reduced glutathione auto-oxidation, *Arch. Biochem. Biophys.* 431 (2004) 138–144, <https://doi.org/10.1016/J.ABB.2004.07.025>.
- [102] J. Serrano, M. Jové, J. Boada, M.J. Bellmunt, R. Pamplona, M. Portero-Otín, Dietary antioxidants interfere with Amplex Red-coupled-fluorescence assays, *Biochem. Biophys. Res. Commun.* 388 (2009) 443–449, <https://doi.org/10.1016/J.BBRC.2009.08.041>.
- [103] C. Galaup, C. Picard, F. Couderc, V. Gilard, F. Collin, Luminescent lanthanide complexes for reactive oxygen species biosensing and possible application in Alzheimer's diseases, *FEBS J.* 289 (2022) 2516–2539, <https://doi.org/10.1111/febs.15859>.
- [104] M. Ekstrand, M.G. Trajkovska, J. Perman-Sundelin, P. Fogelstrand, M. Adiels, M. Johansson, L. Mattsson-Hultén, J. Borén, M. Levin, Imaging of intracellular and extracellular ROS levels in atherosclerotic mouse aortas ex vivo: effects of lipid lowering by diet or atorvastatin, *PLoS One* 10 (2015), <https://doi.org/10.1371/JOURNAL.PONE.0130898>.
- [105] D. Campbell, V. Hurry, A.K. Clarke, P. Gustafsson, G. Öquist, Chlorophyll fluorescence analysis of cyanobacterial photosynthesis and acclimation, *Microbiol. Mol. Biol. Rev.* 62 (1998) 667–683, <https://doi.org/10.1128/MMBR.62.3.667-683.1998>.
- [106] T. Asaeda, M. Rahman, H.D.L. Abeynayaka, Hydrogen peroxide can be a plausible biomarker in cyanobacterial bloom treatment, *Sci. Rep.* 12 (2022) 12, <https://doi.org/10.1038/s41598-021-02978-6>.
- [107] H. Ghaedamini, D.-S. Kim, Recent advances in electrochemical detection of reactive oxygen species: a review, *Analyst* 150 (2025) 1490–1517, <https://doi.org/10.1039/D4AN01533A>.
- [108] E.M. Espinoza, J.J. Roise, I.-C. Li, R. Das, N. Murthy, Advances in imaging reactive oxygen species, *J. Nucl. Med.* 62 (2021) 457–461, <https://doi.org/10.2967/jnumed.120.245415>.

Publikacja – P5

Kwidzińska Klaudia*, Zalewska Martyna, Aksmann Anna, Kobos Justyna, Mazur-Marzec Hanna, Caban Magda, Multi-biomarker response of cyanobacteria *Synechocystis salina* and *Microcystis aeruginosa* to diclofenac, Journal of Hazardous Materials, 2024, 471, 134373, DOI: 10.1016/j.jhazmat.2024.134373.



Multi-biomarker response of cyanobacteria *Synechocystis salina* and *Microcystis aeruginosa* to diclofenac

Klaudia Kwidzińska^{a,*}, Martyna Zalewska^b, Anna Aksmann^b, Justyna Kobos^c,
Hanna Mazur-Marzec^c, Magda Caban^a

^a University of Gdansk, Faculty of Chemistry, Department of Environmental Analysis, ul. Wita Stwosza 63, 80-308 Gdańsk, Poland

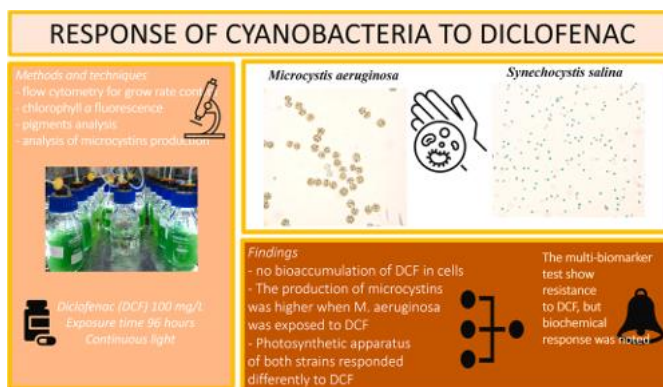
^b University of Gdansk, Faculty of Biology, Department of Plant Experimental Biology and Biotechnology, ul. Wita Stwosza 59, 80-308 Gdańsk, Poland

^c University of Gdansk, Faculty of Oceanography and Geography, Department of Marine Biology and Biotechnology, al. Marszałka Piłsudskiego 46, 81-378 Gdynia, Poland

HIGHLIGHTS

- *S. salina* and *M. aeruginosa* were exposed for 96-h to diclofenac (DCF).
- The 100 mg of DCF per L has no impact on grow rate and pigments concentration.
- The production of microcystins was higher when *M. aeruginosa* was exposed to DCF.
- Photosynthetic apparatus of both strains responded differently to DCF.
- The multi-biomarker test show resistance to DCF, but biochemical response was noted.

GRAPHICAL ABSTRACT



ARTICLE INFO

Keywords:

Pharmaceutical ecotoxicology
 Diclofenac
 Cyanobacteria
 Biomarkers of cyanobacteria stress
 Cyanotoxins

ABSTRACT

The cyanobacterial response to pharmaceuticals is less frequently investigated compared to green algae. Pharmaceuticals can influence not only the growth rate of cyanobacteria culture, but can also cause changes at the cellular level. The effect of diclofenac (DCF) as one of the for cyanobacteria has been rarely tested, and DCF has never been applied with cellular biomarkers. The aim of this work was to test the response of two unicellular cyanobacteria (*Synechocystis salina* and *Microcystis aeruginosa*) toward DCF (100 mg L⁻¹) under photoautotrophic growth conditions. Such endpoints were analyzed as cells number, DCF uptake, the change in concentrations of photosynthetic pigments, the production of toxins, and chlorophyll *a* in vivo fluorescence. It was noted that during a 96 h exposure, cell proliferation was not impacted. Nevertheless, a biochemical response was observed. The increased production of microcystin was noted for *M. aeruginosa*. Due to the negligible absorption of DCF into cells, it is possible that the biochemical changes are induced by an external signal. The application of non-standard biomarkers demonstrates the effect of DCF on microorganism metabolism without a corresponding

* Corresponding author.

E-mail address: klaudia.kwidzinska@phdstud.ug.edu.pl (K. Kwidzińska).

<https://doi.org/10.1016/j.jhazmat.2024.134373>

Received 4 December 2023; Received in revised form 14 February 2024; Accepted 19 April 2024

Available online 21 April 2024

0304-3894/© 2024 Elsevier B.V. All rights reserved.

effect on biomass. The high resistance of cyanobacteria to DCF and the stimulating effect of DCF on the secretion of toxins raise concerns for environment biodiversity.

1. Introduction

Cyanobacteria are primary producers that occur within every element of the environment, including extreme conditions such as high pollution or salinity, and extremes of temperature. The essential ecological role of cyanobacteria is undeniable, even though they can create blooms that are spatially and temporally problematic for people and the ecosystem. Several of these photosynthetic Gram-negative bacteria are cosmopolitan, as they can adapt to living under changeable conditions in the natural environment. Pharmaceuticals are one of the anthropogenic factors contributing to pollution exposure. Numerous pharmaceuticals are directly or indirectly introduced to the water environment [10]. In parallel, in some water bodies (for example, the Baltic Sea), the cyanobacterial bloom frequency and intensity have been increasing in recent years and have become an emerging regional problem [55]. This means that the microalgae coexist with pharmaceutical pollution, for which the net effect on the environment is unknown. Unicellular cyanobacteria have been found to be more tolerant to pharmaceuticals than eukaryotic algae [21], including nonsteroidal anti-inflammatory drugs (NSAIDs) [5]. Therefore, it is suggested that the accumulation of NSAIDs in waters can contribute to the expansion of cyanobacteria in natural ecosystems by pushing back eukaryotic algae [5]. The expansion of non-toxic and toxic cyanobacteria to a wide geographic range may have an impact on ecosystems, trophic cascades, and geochemical cycles [66]. Thereby, there is a need to investigate the non-target effect of pharmaceuticals, such as NSAIDs that are widely detected in water bodies on cyanobacteria.

Diclofenac (DCF) is detected in seas all over the world, including the Baltic Sea, the Mediterranean Sea, the Adriatic Sea, the Red Sea, and the North Pacific Ocean [2,69,7,8]. DCF is already known as an emerging pollutant in international scientific forums and by environmental stakeholders [25,40]. This is an NSAID that is often used without prescription, with a low rate of removal by conventional wastewater treatment plants (WWTPs) [33,34], and with proven effects on water organisms at environmentally relevant concentrations [36,51,62], but such knowledge is still limited to a few model species [68].

Essentially, there is an insufficient number of studies presenting the impact of pharmaceuticals on phytoplankton (around 4% of all ecotoxicology studies are centered on pharmaceuticals and personal care products), and most of them use green algae and diatoms [73]. Furthermore, the majority of studies were performed with the application of standard protocols using green algae as the targeted taxa. The test endpoint in the most popular protocol (OECD test number 201, titled “Freshwater Alga and Cyanobacteria, Growth Inhibition Test”) is the acute inhibition of algal growth (one of the mass-related markers) [54]. Modern ecotoxicology focuses on more discrete effects, such as subcellular-related endpoints. Unfortunately, the biomarkers of cyanobacteria cell stress are less well-known compared to green algae. In our previous work, cyanobacterium *Synechocystis salina* grow was found to be unaffected by NSAIDs at a high concentration, but no additional biomarkers were tested [35]. Several microorganisms were found to metabolize NSAIDs, often via unknown enzymes, which hypostatized the metabolic stimulation [51]. The modification of natural phytoplankton communities, namely, the reduction of phenotypic diversity after exposure to a mixture of pharmaceuticals at environmentally relevant concentrations [60], is another motivation for studying the markers of new emerging pollutants toward phytoplanktonic species.

The aim of the presented work was to test the selected physiological and biochemical responses of cyanobacteria toward pharmaceuticals of emerging concern. For this purpose, diclofenac (DCF) was chosen. Because the effect can be species dependent, two species of unicellular

cyanobacteria were tested, *S. salina* and *Microcystis aeruginosa*. They are often used as model cyanobacteria [3]. The main tasks were to test the changes in biochemical and physiochemical markers during the exposure in grow phase of culture. As well as the growth rate measurement, we decided to evaluate a new biochemical response in a basic process performed by microalgae – photosynthesis (the concentration of pigments and the chlorophyll *a* (Chl-*a*) fluorescence in vivo), the production of toxins and the consumption of nutrients.

2. Experimental

2.1. Chemicals

The standards of diclofenac sodium – DCF (CAS 15307–79-6), *N*-(2,6-dichlorophenyl)– 2-indolinone – IND-DCF (CAS)15362–40-4), 4'-hydroxydiclofenac – 4-OH-DCF (CAS 64118–84-9), 5-hydroxydiclofenac – 5-OH-DCF (CAS 69002–84-2), and diclofenac acyl-β-D-glucuronide – GLU-DCF (CAS 64118–81-6) were purchased from Sigma-Aldrich (Germany). HPLC grade acetonitrile (ACN), methanol (MeOH), and acetone were purchased from POCH S.A. (Poland), while ammonium acetate and glacial acetic acid were sourced from VWR International (USA).

Standards of chlorophyll *a* (CAS 479–61-8), echinenone (CAS 432–68-8), and β-carotene (CAS 7235–40-7) were purchased from DHI Lab Products (Denmark) in the form of ethanol or acetone solutions, and stored at – 20 °C. The standards of C-phycocyanin from *Spirulina* sp. (CAS 11016–15-2) and lysozyme from chicken egg (CAS 12650–88-3) were purchased from Sigma Aldrich (Germany).

2.2. Stock culture growth conditions

Both cyanobacteria species were obtained from the Culture Collection of Northern Poland (CCNP, Division of Marine Biotechnology, University of Gdansk, Poland). *S. salina* CCNP1104 (order of Synechococcales) was isolated from the Gulf of Gdansk in 2006. This picocyanobacterium does not form massive blooms but is a vital component of biocenosis. *M. aeruginosa* CCNP1101 (order of Chroococcales) was isolated from Vistula Lagoon in 2005. It is a freshwater, cosmopolitan species. In the Baltic, *Microcystis* can be observed in coastal waters and lagoons. A microscopy image of both tested cyanobacteria is presented in Fig. 1. The morphology of the cells is visibly different. The average size of *S. salina* is 2.2 μm, while *M. aeruginosa* is 5.6 μm. In the environment, *Microcystis* sp. lives in colonies, often surrounded by mucus. In a monoculture, they lose the ability to form colonies.

Stocks of the cyanobacterium *S. salina* were grown in BG11 growth medium prepared from a concentrate purchased from Merck (Germany). *M. aeruginosa* was grown in Z8 medium. The culture media were pre-aerated, and the pH was adjusted to 7.5 ± 0.2 . Both species of cyanobacteria were grown in 500 mL borosilicate bottles in a thermostat chamber (IL 65-W from Wamed, Poland) under constant illumination with a light intensity of $70 \pm 10 \mu\text{W m}^{-2} \text{s}^{-1}$ at a temperature of 23 °C. The cultures were constantly aerated and stirred using an air stream introduced by a silicone tube. The air was passed through a carbon filter (FW600, Wanza, Poland) and 0.2 μm PET sterile syringe filters (Chromafil, Germany). The experimental glasses were autoclaved for 20 min at 121 °C. Any manipulation with cultures was performed under sterile conditions using a laminar chamber UVC/T-M-AR (SIA Biosan, Poland). The cultures obtained from CCNP were non-axenic. Still, the cytometric analysis and microscopic observation did not indicate other bacteria.

2.3. Exposure test performance

The basis of the ecotoxicological test was the OECD 201 protocol. The modifications that we introduced included the use of a cyanobacteria specific medium and an extended duration from 72 to 96 h. The prolongation of exposure was motivated by fact that grow rate of cyanobacteria was lower than green algae, and growing phase of tested species was up to 5 days. Both cyanobacteria were exposed to DCF at a nominal concentration of 100 mg L^{-1} . The tests were carried out under the same temperature and light conditions as for the cultivation of stock cyanobacteria (Section 2.2). The test was carried out using 200 mL borosilicate glass bottles with 50 mL of culture inside. During the tests, the cyanobacteria were aerated and agitated with a stream of sterile air (the same as the stock cultures) to prevent the accumulation of cells in the solution top (*M. aeruginosa*) or bottle bottom (*S. salina*). The optical density (OD) was measured daily with a spectrophotometer (Thermo Scientific™ GENESYS™ 150, UK) at a characteristic wavelength.

There were five biotic control cultures (no DCF addition) and five DCF-exposed cultures per strain. The abiotic controls were solutions of DCF in sterile BG11 medium. DCF was added as a sodium salt (powder form) directly to the medium, and then inoculated with cyanobacteria. The addition of DCF salt did not change the pH of the medium. The optical density measured at 665 nm (OD_{665}), chlorophyll *a* specific wavelength at the start of the test was approximately 0.2. At the start and every 24 h, the appropriate volume was sampled from each test bottle for an analysis of the cyanobacteria growth and DCF stability. The pH of all samples was measured on the first and last days of the test using a pH meter (827 pH lab, Metrohm, Switzerland). Changes in the contents of nitrates, nitrites, phosphorus, and ammonium were investigated on the first and last days of the test using standard spectrophotometric methods (Supplementary Information Section S1). After the exposure, the rest of the volume of cyanobacteria was transferred to Falcon vials and centrifuged at $10,000g$ for 10 min at 20°C . The supernatant was discarded. The solid was lyophilized overnight (FreeZone 2.5 L, Lab-conco, USA). The biomasses from five repetitions of the control were pooled together, as well as the biomasses from the exposed cultures. The dry material was utilized for the subsequent analysis of selected markers.

2.4. Flow cytometry analysis of cyanobacterial cultures

Cell count was assessed via flow cytometry using a CytoFLEX 2L6C (Beckman Coulter) cytometer equipped with a blue 488 nm (50 mW) laser and a red 638 nm (50 mW) laser as an excitation source. Fluorescence from cyanobacterial chlorophyll (Chl) was detected with a 690 nm/50 nm bandpass emission filter (PC5.5 detector) after blue light excitation. Phycocyanin cyanobacterial fluorescence was detected with

a 660 nm bandpass filter (APC detector) after red light excitation. Forward light scatter (FSC) and side light scatter (SSC) were both detected from 488 nm blue light. The sample volume was $15 \mu\text{L}$. The sample flow rate was $30 \mu\text{L min}^{-1}$. A minimum of 10,000 events were recorded. Samples with higher cell concentrations than $30,000 \text{ cells } \mu\text{L}^{-1}$ were diluted with sterile water. Fluorescence-labeled beads (CytoFLEX Daily QC Fluorospheres, Beckman Coulter, USA) were used to standardize the machine with respect to green fluorescence. The SSC-to-FSC diagram was used to set the threshold.

2.5. Photosynthetic analysis

Chlorophyll *a* fluorescence O-J-I-P transients were recorded using a Handy PEA fluorimeter (Hansatech Ltd., UK) operated via PEA Plus V1.10 software. The analysis was performed on the first day, one hour after DCF application to the culture and on the last day of the test, immediately after culture suspension dilution to $\text{OD}_{665} = 0.2 (\pm 0.02)$ and transfer to glass vials (2 mL).

OJIP test parameters analyzed in this work include [41,42] F_v/F_m – the quantum yield of primary PSII photochemistry; ϕE_0 – the quantum yield of electron transport; ϕR_0 – the quantum yield of electron transport from Q_A^- to the PSI end electron acceptors; ϕD_0 – the quantum yield of energy dissipation; F_v/F_0 – the activity of the water-splitting complex on the donor side of PSII; RC_M – the fraction of active PSII reaction centers in the sample referring to time, when F_m (maximal fluorescence value under saturating light intensity and fully reduced state of Q_A pool) is reached; ψ_0 – the efficiency of moving the electron by the trapped exciton further than Q_A ; δR_0 – the efficiency with which an electron can move from the reduced intersystem electron acceptors to the PS I end electron acceptors; the specific energy fluxes per one active reaction center (RC) for absorption (ABS/RC), trapping (TR_0/RC), electron transport (ET_0/RC), and dissipation (DI_0/RC).

2.6. Diclofenac and metabolites analysis via HPLC-DAD

DCF was quantified via high-performance liquid chromatography with diode array detection (HPLC-DAD), together with the selected metabolites (4-OH-DCF, 5-OH-DCF, GLU-DCF, and IND-DCF). The samples were filtered using syringe filters ($0.2 \mu\text{m}$ PET) before analysis. The Nexera XR (Shimadzu, Kyoto, Japan) system was equipped with an SIL-20AC autosampler, an LC-20AD pump, a CTO-20AC column oven, and an SPD-M20A detector. The Gemini-NX column (C18, $5 \mu\text{m}$, 110 Å , $150 \times 4.6 \text{ mm}$, Phenomenex) with precolum was placed in an oven at a constant temperature of 27°C . The injection volume was set to $25 \mu\text{L}$. The autosampler oven temperature was set to 10°C . The mobile phase component A was 10 mM ammonium acetate with acetic acid (pH 4.0), while component B was ACN. The isocratic elution with 65% B was

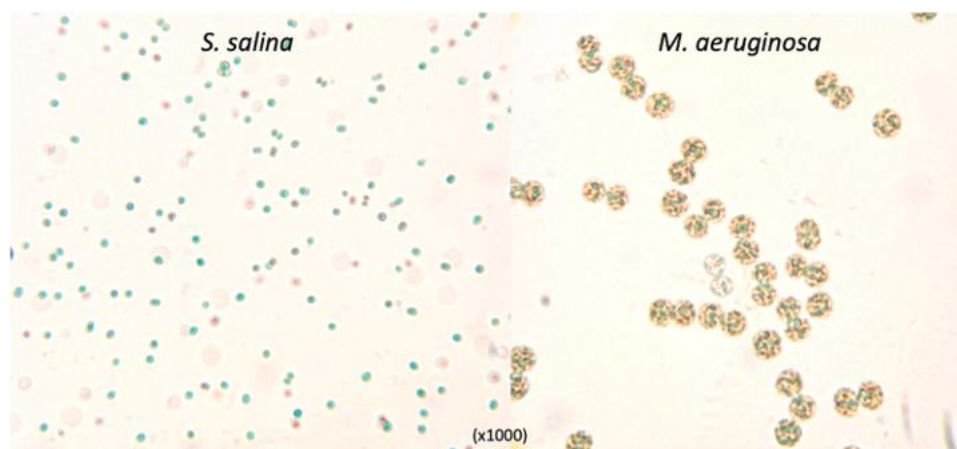


Fig. 1. Microscopy image of cyanobacteria: *Synechocystis salina* CCNP1104 (left) and *Microcystis aeruginosa* CCNP1101 (right) ($1000\times$ magnification).

applied for the separation of five analytes. The flow rate of the mobile phase was 1 mL min^{-1} . The DAD was set to measure the wavelength from 190 nm to 800 nm. The analytical wavelengths were 280 nm for DCF, GLU-DCF, 5-OH-DCF, and IND-DCF, and 270 nm for 4-OH-DCF. The calibration curve was prepared in a concentration range from 0.008 (LOQ) to 100 mg L^{-1} . The precision within this concentration range was lower than 4.7% (relative standard deviation), while the accuracy was between 92% and 101% for each analyte.

2.7. Pigments extraction and quantification

Extraction of hydrophobic pigments (chlorophylls and carotenoids) from cyanobacteria was performed on samples taken on the last day of each test (after 96 h exposure). The volume of 1 mL of culture was collected, and the cells were centrifuged at a speed of 20,000g for 4 min at ambient temperature (Centrifuge 5414, Eppendorf). Then, the supernatant was removed, and 1 mL of acetone solution with 50 mM ammonium acetate (90:10, v/v) was added. These samples were placed in homogenization plastic tubes, and two ceramic beads of size 2.8 mm and 10 mg of 0.1 mm glass microbeads (Ceramic Bead Media, OMNI, USA) were added. The samples were homogenized with a Bead Ruptor Elite (OMNI International, USA) using three cycles of 1 min each at 4 ms^{-1} ; between cycles, then the samples were cooled in ice water. The obtained samples were placed in a refrigerator (4°C) for 48 h.

The chromatographic analyses of chlorophylls and carotenoids were performed using HPLC-DAD (Nexera XR, Shimadzu). The separation of the pigments was performed using a ReproSil Gold analytical column (C18, $5 \mu\text{m}$, 120 Å , $150 \times 4.6 \text{ mm}$, Dr. Maisch, Germany) with a pre-column. The mobile phase component A was MeOH with 50 mM aq. ammonium acetate (80:20, v/v), while component B was MeOH with acetone (80:20, v/v). A gradient elution was applied for separation. Initially, the B-phase concentration was maintained at 30% (0–2.5 min), and then it was increased to 70% (2.5–30 min). After that, it increased to 100% (30–35 min) and was maintained for 20 min (35–55 min). After that, the concentration of phase B decreased to 30% (55–60 min), after which it remained at that level for 3 min (60–63 min). The flow rate of the mobile phase was 1 mL min^{-1} . The injection volume was $25 \mu\text{L}$. The calibration curves were prepared in a concentration range of 0.0053–0.0171 mg L^{-1} for chlorophyll *a*, 0.0030–0.0947 mg L^{-1} for echinenone, and 0.0052–0.0832 mg L^{-1} for β -carotene. The precision within this concentration range was lower than 9.65% (as a relative standard deviation). The accuracy ranged between 78.8% and 124.3%. The exact values of accuracy and precision for each analyte are listed in Table S1 in the Supplementary Information section. The concentrations in extracts found using HPLC-DAD were recalculated from the concentration of the target pigment to a single cell of cyanobacteria (with the cell concentration being determined via flow cytometric analysis).

The analysis of water-soluble pigments (phycobilins) was performed using the solid-liquid extraction technique. In preliminary studies (data not presented), the method of extraction was optimized to obtain the highest possible pigment concentration using both cyanobacterial strains. A volume of 0.9 mL of 0.1 M phosphate buffer (pH 7) was added to 3 mg of dry weight of cyanobacteria in plastic vials. Cell homogenization was performed in the same way as for the extraction of hydrophobic pigments. A volume of 0.1 mL of lysozyme (10 mg mL^{-1}) was added to the homogenized cells solution and heated at 37°C for 2 h. The samples were placed at 4°C for 48 h, then filtered and analyzed. Spectrophotometric semi-quantification was performed using phycocyanin from *Spirulina*. The purity of the material was 30–50% protein (Lowry method, specified by the producer); therefore, an exact determination was impossible. The standard curve was made based on phycocyanin over a concentration range of 0.0016–2.0000 mg L^{-1} (Fig. S1, Supplementary Information). Concentrations (three replicates) in the control and the exposed cultures were compared. The concentration of pigments was normalized to a single cell (the number of cells was known from cytometric analysis).

2.8. Analysis of microcystins produced by *M. aeruginosa*

S. salina has no known potential to produce any of the toxins; therefore, analysis of intracellular peptide toxins was performed only for *M. aeruginosa*. The samples (7 mg of lyophilized mass for the pooled biotic control and DCF-exposed cultures) were extracted in 1 mL of 75% MeOH in water via bath sonication for 1 min and vortexing for 5 min. After centrifugation, the supernatant was transferred to a chromatographic vial and analyzed with LC-MS/MS. The separation of extracts was performed in a Zorbax Eclipse XDB-C18 column ($4.61 \text{ mm} \times 150 \text{ mm}$; $5 \mu\text{m}$) (Agilent Technologies, Santa Clara, CA, USA) using an Agilent 1200 (Agilent Technologies, Waldbronn, Germany) chromatographic system coupled with a hybrid triple quadrupole/linear ion trap mass spectrometer (QTRAP5500, Applied Biosystems, Sciex, Concord, ON, Canada) as a detector. As a mobile phase, a mixture of 5% ACN in water (A) and 100% ACN (B), both with 0.1% formic acid, was used. During a 30 min chromatographic run (flow rate 0.6 mL min^{-1}), the gradient changed from 15% B to 99% B. The Turbo Ion Source operated at a capillary voltage of 5.5 kV and a source temperature of 550°C . The cone gas pressure and desolvation gas pressure were 60 psi, the declustering potential was 80 V, and the collision energy was $60 \pm 20 \text{ eV}$. To detect the compounds present in the samples, the untargeted, information-dependent acquisition (IDA) was used. The IDA threshold was set at 500,000 cps, and the m/z range was 500–1200. The scan rate was 2000 amu s^{-1} , and the linear ion trap fill time was 50 ms.

The structures of the detected microcystins were identified based on the fragmentation spectra of the pseudomolecular ions $[\text{M}+\text{H}]^+$. Where possible, the collected spectra were compared with the microcystins spectra available in published documents and databases [47,57,70,9]. Semi-quantitative analyses were based on the peak areas of the extracted ions (pseudomolecular ion m/z).

3. Results and discussion

3.1. Stability of diclofenac in an exposure environment

The graph presenting the DCF concentration quantified in the cultures during a 4-day-long exposition of the two cyanobacteria species can be found in Fig. 2. The mean concentration of DCF in the test bottles was $106.8 \pm 6.1 \text{ mg L}^{-1}$. DCF was stable, and a negligible change in concentration was obtained, both in the biotic and abiotic sets. There were statistically important differences between the concentrations of DCF in the biotic and abiotic sets obtained in *M. aeruginosa* after 48, 72, and 96 h, but having in mind the slightly different starting concentrations in the mentioned sets, these minor differences are reasonable. The changes of DCF concentration were a mean of -2.1% every 24 h, but there were no statistically important differences (t-test, $p > 0.05$) between the biotic and abiotic sets. It was also noted that despite the increase in cyanobacterial cell number during exposure, the decline of DCF was not noted (Fig. 2). This suggests that both cyanobacterial strains had no potential for the bioaccumulation of DCF as well as for metabolism. From the other side, the detoxication of DCF by the exclusion of native molecules from cell can be hypothesized (observed for example for antibiotics [4]), while the effect on photosynthesis (described later) was found after one hour of exposure, but not at the end.

On the HPLC-DAD chromatogram of the medium sampled on the last day, a few new low-intensity peaks were found (Fig. S2). One additional peak was noted for *M. aeruginosa*, and four for *S. salina*. In a case, of *S. salina* same peaks were noted for abiotic control, what mean that these peaks does not came from cyanobacteria. The targeted LC-MS (performance of the analysis in Supplementary Information Section S2 and Table S2) analysis shows that it was not the 4-OH-DCF metabolite.

The hypothesized lack of DCF penetration into the cyanobacterial cells and its adsorption onto cell external coatings is most probably

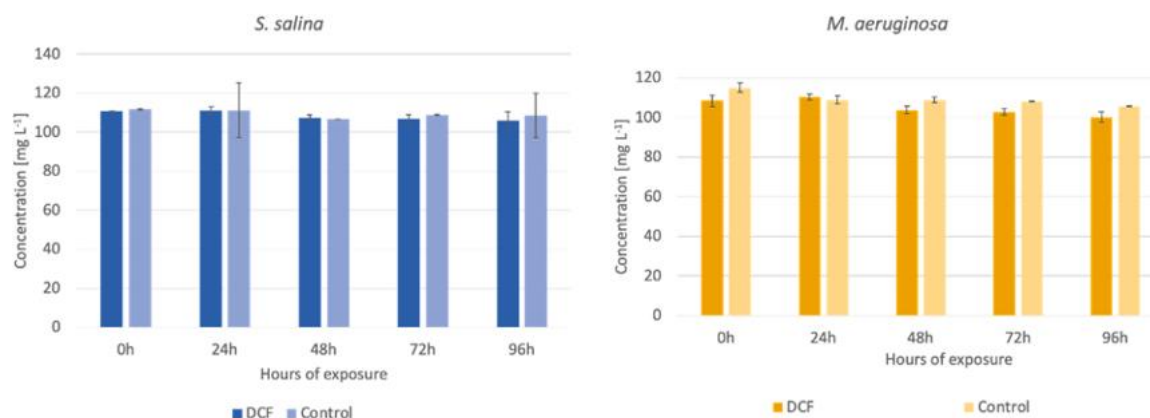


Fig. 2. Concentration of diclofenac (DCF) [mg L^{-1}] in abiotic control ($n = 2$) and exposed cultures ($n = 5$) of *S. salina* and *M. aeruginosa* during 96 h exposure.

caused by the negative charge carried by its molecules in the pH of the medium. Cyanobacterial cells are negatively charged in the natural pH of water [45,59]. Moreover, the lipophilicity of DCF changes with the pH because this molecule is a weak acid. The $\log K_{ow}$ of DCF is 4.51, and the $\log D$ in pH 8.5 is 0.75 [49], while the calculated $\log D$ in pH 9 is -0.3 . This means that with an increasing pH (the pH of the cultures after exposure was close to 9), the lipophilic character of DCF dramatically decreases, which lowers the potential to penetrate the membranes. Cyanobacteria are Gram-negative bacteria, and as such, they have an extracellular lipopolysaccharide barrier, which is often the reason for their impermeability to molecules [13,37]. The lipopolysaccharide barrier is thicker compared to other Gram-negative bacteria, and some species have an additional mucilaginous sheath [3]. The lack of bioaccumulation of DCF by both cyanobacterial species can cause a lack of biological response. On the other hand, external signals can induce secondary messengers that regulate homeostasis and help the cyanobacteria to survive in a changeable environment [1].

The zeta potential of green algae is negative [46] and comparable to cyanobacteria [45]. The *M. aeruginosa* cell surface was found to have more functional groups (mainly acidic, such as galacturonic acid; $1.60 \times 10^{-3} \text{ mol g}^{-1}$) compared to *C. vulgaris* ($0.22 \times 10^{-3} \text{ mol g}^{-1}$) [22]. Peptidoglycan in the cyanobacteria cell wall provides mechanical stability, permeability, prevention against drying, and resistance toward chemical substances, plus other probable roles that have not yet been found [26]. The general differences in the outer layer between cyanobacteria and green algae are then primarily influences for the DCF's

negligible potential for penetrating cyanobacterial cells compared to green algae cells (for example *Chlamydomonas reinhardtii*, which have potential to adsorb and bio-transform DCF [38]).

3.2. The growth of cyanobacterial cultures

The UV-Vis spectra of cyanobacterial cultures are presented in Fig. 3, and they correspond to the control culture on the last day of the experiment. The bands and peaks of phycobilins, carotenoids, and chlorophylls are marked. These spectra are in agreement with previously published spectra for tested cyanobacteria strains [71]. The OD values for *S. salina* and *M. aeruginosa* were measured using wavelengths of 635 nm (OD_{635}) and 625 nm (OD_{625}), respectively, which correspond to phycobilin's peak in the spectrum (Fig. 3) (note that a quantification using chlorophyll a specific wavelength yields the same results). The control cultures and DCF-impacted cultures yielded the same spectrum quality (data not shown). No other species of bacteria were observed in the cultures (checked via microscopic and cytometric analyses) after a 96 h incubation under test conditions.

The values of OD were measured daily in each bottle. Fig. 4 (parts A and B) presents a graph of mean OD for both cyanobacteria species tested in each test set. The exact values for each of the five repetitions are presented in Fig. S3 in the Supplementary Information section. It was determined that the cultures grew constantly, with the lowest growth rate occurring on the second day. On the fourth day, the OD was approximately five times higher compared to the start of the experiment.

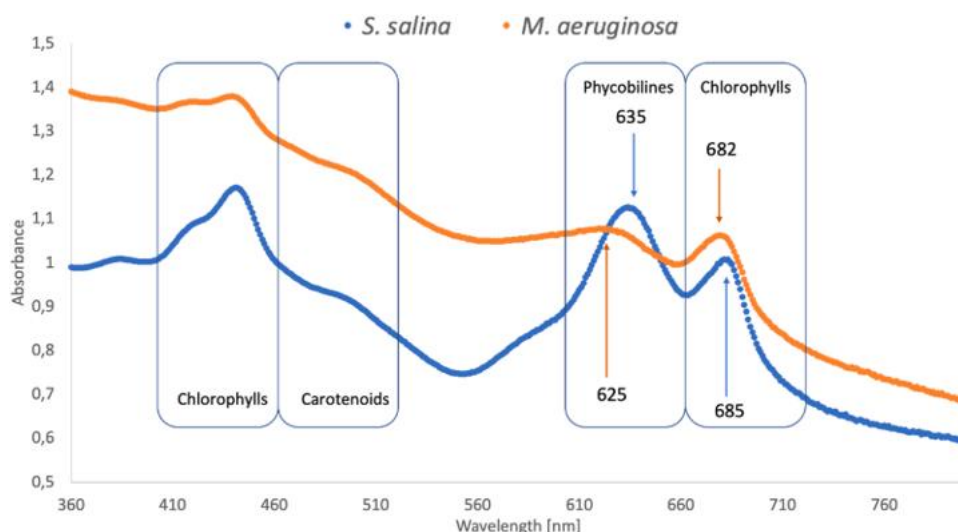


Fig. 3. The UV-Vis spectra of cyanobacteria *S. salina* and *M. aeruginosa* after 96 h incubation in growth medium under experimental conditions.

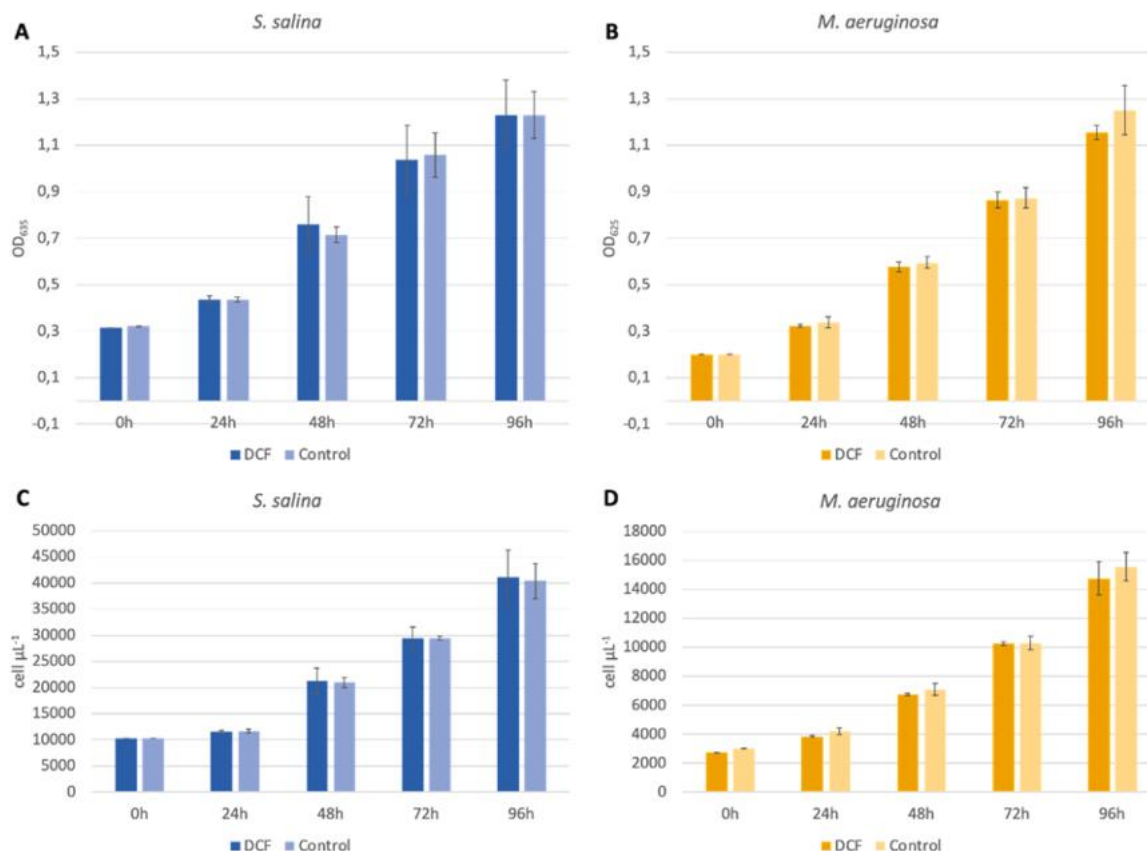


Fig. 4. The mean optical densities (ODs) measured for A. *S. salina* (OD₆₃₅) and B. *M. aeruginosa* (OD₆₂₅), in addition to mean cell concentrations of C. *S. salina* and D. *M. aeruginosa* at start (0 h) and every 24 h during 96 h exposure experiment of cultures impacted by diclofenac (DCF, n = 5) and control cultures (n = 5).

It is notable that DCF-impacted cultures and control cultures grew in the same manner. There were no statistical differences (t-test, $p = 0.05$) between those two sets, either for *S. salina* or *M. aeruginosa*. The consumption of nutrients was similar for the exposed and control cultures (Table S3, Supplementary Information). The pH values in each flask increased as an effect on cyanobacteria metabolism (Table S3).

The growth of culture was also checked via flow cytometry. The exposure experiment started with similar OD values, but the starting cell concentration was different for the different cyanobacteria strains. Cells of *S. salina* are smaller in size compared to *M. aeruginosa* (Fig. 1). The cell concentrations for *S. salina* were close to 10,000 and 40,000 per μL , respectively, at the start and at the end of the experiment (Fig. 4C). The corresponding values for *M. aeruginosa* were approximately 3,000 and 15,000 cells per μL (Fig. 4D). The exact concentrations of cells for each repetition, exposure time, and strain are presented in Fig. S4 in the Supplementary Information section. There was no statistically significant difference between the DCF-impacted and control cultures. Furthermore, the flow cytometry graphs showed no differences in median fluorescence between the DCF-impacted and control cultures on the fourth day of the experiment (Fig. 5 for *S. salina*, and Fig. S5 (Supplementary Information) for *M. aeruginosa*). It needs to be noted that the cytometer does not directly measure the cell itself, but the flowing objects. The cytometer counts dividing cyanobacteria cells as one object, but as can be seen in Fig. 1, the two newly divided cells are often together per specific time after division. Thereby, the numbers presented in Fig. 4 are a total number of cells. In our previous work [35], we presented the high correlation between OD and the cell concentration of *S. salina*, measured via flow cytometry, and assumed that the OD is sufficient for measuring the growth rate.

According to the OD and flow cytometry measurements, *S. salina* multiplied approximately four times, while *M. aeruginosa* multiplied

around 5.5 times during a four-day-long experiment. The daily growth rate was around 1.4, both for the DCF-impacted and the control cultures. The exact values of the growth rate, as determined using spectrophotometric technique and flow cytometry, are presented in Fig. S6 (Supplementary Information). The growth rate is often the base determinant of toxic effects and the factor for toxicity parameters calculations. DCF had no impact on growth in the case of the tested cyanobacteria species, what means that DCF does not disturb the main expression of cell metabolism – proliferation.

To compare the response to DCF of the target cyanobacteria with other species of phytoplankton, the published ecotoxicology data were collected, as in Table 1. The values of acute toxicity (EC_{50}) varied greatly between species and taxa, and they were reported over a range of 5 to 186 mg L^{-1} . The two tested diatoms showed the highest sensitivities toward DCF. What is interesting is that, in contrast to our study, *M. aeruginosa* responds to DCF at 100 mg L^{-1} during a 10-day-long exposure period through a 38% reduction in growth rate [5], which can be caused by differences in the given strain tested and the exposure time. From our experience, under the applied test conditions, the maximum time of a culture's growing phase was 5 days. The lack of presentations of acute toxicity data for cyanobacteria can be an effect of a trend away from publishing negative results, such as a lack of determination of the EC_{50} value.

High values of EC_{50} concentrations, and even LOEC and NOEC (Table 1), are unrealistic in the real environment, where pharmaceuticals are in the magnitude of ng to μg per L [16]. Nevertheless, such an assessment can help to understand the vulnerability of a species. The highest concentration allowed in ecotoxicological tests in accordance with the OECD 201 standard was used in our study. The toxicity of drugs at such concentrations is tested for both green algae [42], and cyanobacteria [15,5,74]. Ecotoxicological tests in which the acute response is

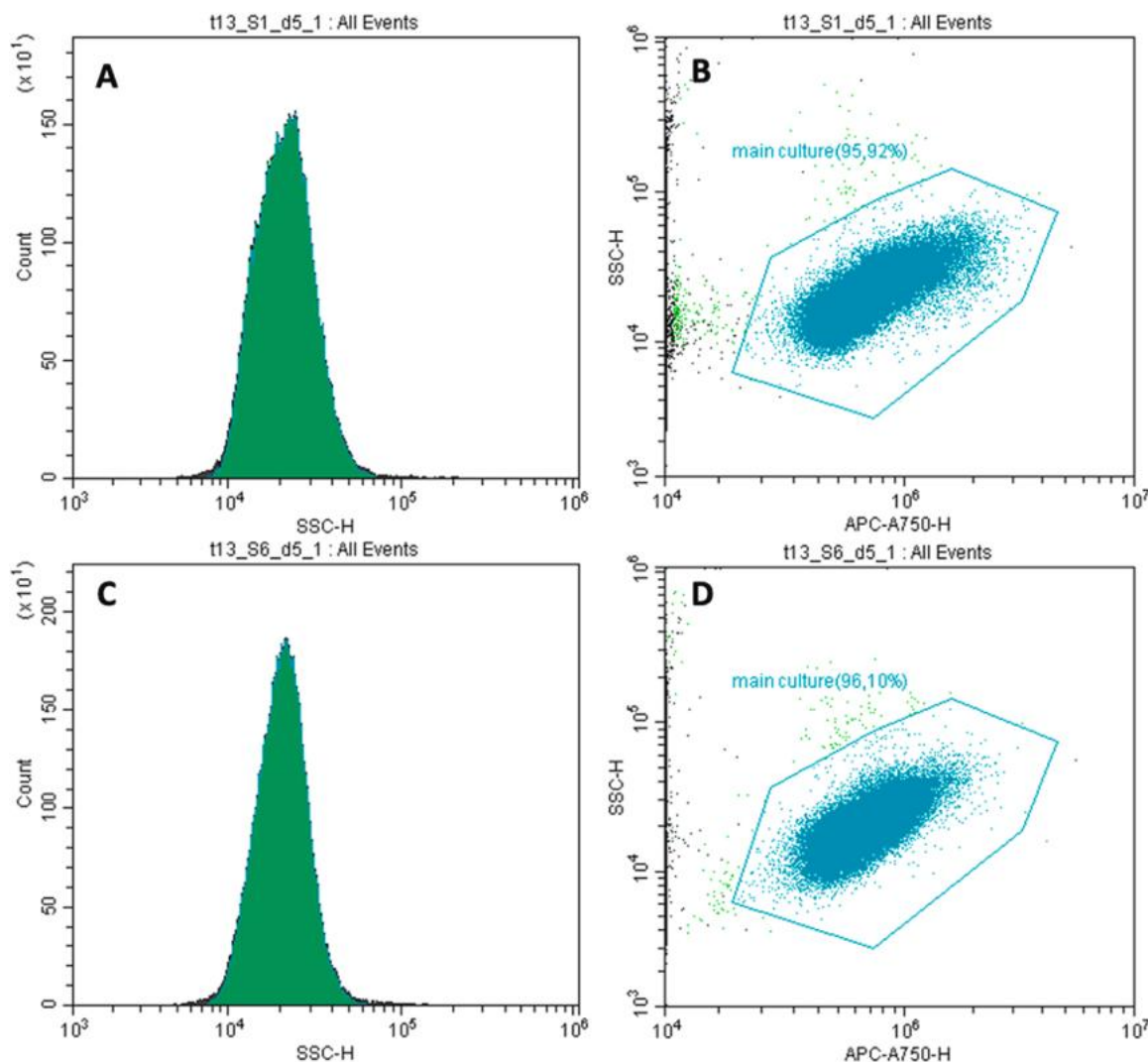


Fig. 5. Flow cytometry histograms and dot plots of diclofenac-exposed cultures (A and B) and control cultures (C and D) of *S. salina* on the fourth day of experiment.

determined are necessary to understand the mechanisms of influence of various substances on organisms. From the point of view of ecotoxicology, it is important to have an understanding of the hormetic response that is obtained from the stimulative effect resulting from a low concentration of xenobiotics. For example, a stimulative effect was noted for the growth of the cryptophyte *Cryptomonas ovata* when impacted by DCF over a concentration of lower than 10 mg L^{-1} [77]. Synthetic anesthetics over a low concentration ($0.1\text{--}1 \text{ }\mu\text{M}$) were found to stimulate *Synechococcus leopoliensis* [67]. In a preliminary study, we tested 10.0 mg L^{-1} and 0.1 mg L^{-1} , and no stimulative or toxic effects were noted for *S. salina* [35].

The strains that we tested have no potential for DCF bioremediation, but such effects were observed for phytoplankton at a ppm concentration of DCF. For example, DCF at a concentration of 25 mg L^{-1} stimulates the growth rates of *Chlorella sorokiniana*, *Chlorella vulgaris*, and *Scenedesmus obliquus* [18]. Furthermore, DCF was removed ($>79\%$) during cultivation. Similarly, *C. vulgaris*, *Nannochloropsis oculata*, *Scenedesmus acutus*, and *S. obliquus* were able to remove 59–92% of DCF (according to the sorption of the cell wall), starting at a concentration of $8.7 \text{ }\mu\text{g mL}^{-1}$ [61]. DCF had slight inhibitory effects on the growth of *Picocystis* sp. and *Graesiella* sp., which did not exceed 21% and 36%, respectively [6]. Recently, the potential for DCF degradation by *Chlamydomonas reinhardtii* was reported [38], demonstrating that in 96 h experiments, the algae were able to remove up to 37.7% of DCF from the

environment out of an initial concentration of 32.7 mg L^{-1} . Moreover, 38 transformation products, 18 of which were not previously reported, were detected, which suggests that a decrease in the level of DCF in the media could result not only from biosorption, but also from biotransformation. Several bacteria and fungi can remove DCF to some extent [52]. Unfortunately, there has been no work devoted to the degradation of DCF by cyanobacteria. In our work, this process was not observed.

3.3. Comparison of microcystins production

In *M. aeruginosa* CCNP1101 extract (both in biotic controls and DCF-impacted), nine microcystins (MCs) variants were detected (Table 2). As standards for only some of the toxins were available, to obtain comparable results for all the compounds, semi-quantitative analysis was performed. The concentrations of individual MCs were expressed as the chromatographic peak area to dry weight (mg). In the treated cyanobacteria, the production of all MCs variants was increased compared to the control. Within the time of the experiment, the increase was over a range of 9% (MC-LF) – 46% (MC-LW).

The increase in MCs production could result from a direct or indirect effect of DCF exposure. Microcystins belong to non-ribosomal peptides, and they are produced constitutively by some cyanobacterial strains, including those belonging to the genus *Microcystis*. So far, over 300 structural variants of the compounds have been identified [31].

Table 1

Selected reports presenting the toxic effects of diclofenac on phytoplankton species.

Taxon	Species	Acute toxicity endpoint value	Ref.
Chlorophytes	<i>Desmodesmus subspicatus</i> (freshwater)	EC ₅₀ 72 mg L ⁻¹ (96 h)	Cleuvers [12]
	<i>Pseudokirchneriella subcapitata</i> (freshwater)	LOEC 20 mg L ⁻¹ (96 h)	Ferrari et al. [20]
	<i>Dunaliella tertiolecta</i> (marine model organism)	EC ₅₀ 186 mg L ⁻¹ (96 h)	DeLorenzo, Fleming [14]
	<i>Pseudokirchneriella subcapitata</i> (freshwater)	EC ₅₀ 16.3 mg L ⁻¹ (96 h) NOEC 10.0 mg L ⁻¹ (96 h)	Ferrari et al. [19]
	<i>Desmodesmus communis</i> (freshwater)	60% growth inhibition in 10 days compared to control, DCF 100 mg L ⁻¹	Bácsi et al. [5]
	<i>Chlamydomonas reinhardtii</i> (freshwater)	EC ₅₀ 135 mg L ⁻¹ (96 h)	Majewska et al. [41]
	<i>Chlorella pyrenoidosa</i> (freshwater)	5-day exposure, positive effect in < 10 mg L ⁻¹ Significant metabolic disruption > 100 mg L ⁻¹	Zhang et al. [77]
Cryptophytes	<i>Cryptomonas ovata</i> (freshwater)	38% growth inhibition in 10 days compared to control, DCF 100 mg L ⁻¹	Bácsi et al. [5]
Diatoms	<i>Cyclotella meneghiniana</i> (freshwater)	EC ₅₀ 19.2 mg L ⁻¹ (96 h) NOEC 10.0 mg L ⁻¹ (96 h)	Ferrari et al. [19]
	<i>Skeletonema costatum</i> (marine water)	EC ₅₀ 5 mg L ⁻¹ (96 h)	Schmidt et al. [63]
Cyanobacteria	<i>Microcystis aeruginosa</i> (freshwater, estuaries)	38% growth inhibition in 10 days compared to control, DCF 100 mg L ⁻¹	Bácsi et al. [5]
	<i>Cylindrospermopsis raciborskii</i> (freshwater)	85% growth inhibition in 10 days compared to control, DCF 100 mg L ⁻¹	Bácsi et al. [5]

Table 2

Changes in the relative amounts of microcystin variants in freeze-dried biomass of *M. aeruginosa* CCNP1101 exposed to DCF.

Microcystins variant			Biotic control [area × 10 ⁷ / mg d.w.]	Exposure culture [area × 10 ⁷ / mg d.w.]	Percentage increase compared to control
Variant	Retention time [min]	m/z			
MC-LW	11.92	1025	0.68	1.00	46%
MC-MR	9.62	1013	3.16	4.20	33%
MC-LR	9.87	995	0.63	0.75	20%
MC-LF	12.16	986	0.75	0.81	9%
[DMAAdda ⁵] MC-LR	9.32	981a	1.69	2.2	31%
[Asp ³]MC-LR	9.64	981b	2.64	3.69	40%
MC-LL	12.18	952	1.65	2.32	41%
MC-LAib	11.58	924	3.92	5.24	34%
MC-LA	11.34	910	5.64	7.93	41%

Microcystins are potent eukaryotic protein phosphatases inhibitors, with acute effects on humans and animals [48]. Their role in the life of the producing organisms has also been explored, but no suggested hypotheses have been definitively proven [56]. Some authors, such as Zilliges et al., postulate that MCs might protect cyanobacteria against oxidative

stress [78]. The toxin-producing cyanobacteria were found to be more tolerant to low nutrient concentrations and hydrogen peroxide [30,56]. A lack of iron results in the activation of gene-encoded MCs. The comparison of toxic and non-toxic strains of *M. aeruginosa* shows that microcystin is part of this adaptation against oxidative stress [30]. It was also hypothesized that the microcystins may act as an extracellular signaling molecule in *Microcystis* sp. for controlling culture growth [53, 56]. Furthermore, these molecules are involved in carbon–nitrogen metabolism, and in redox control and perception. There are more results suggesting the role of cyanotoxins in primary metabolism [27]. In our experiment, the symptoms of stress in cyanobacteria cultures exposed to DCF were observed at the start of exposure (presented further in this report). The increase in MCs production could indicate the involvement of these compounds in the adaptation of cyanobacteria to DCF-induced stress. The question remains unanswered as to whether this is a direct reaction to the DCF or the effect of physiological changes (stress) caused by the presence of DCF.

The increase in MCs production by NSAIDs was never previously presented, but this was noted for two other antibiotics. Liu et al. determined that the secretion of microcystin by *M. aeruginosa* was affected by exposure to spiramycin and amoxicillin [39]. At environmentally relevant concentrations of 100 ng L⁻¹ to 1 mg L⁻¹, spiramycin reduced the total MCs content per cell and inhibited growth, while exposure to amoxicillin led to increases in the total MCs contents per cell and the percentage of extracellular MCs without affecting cyanobacterial growth.

3.4. Comparison of pigment contents

Fig. 6 presents the UV-Vis spectra (360–700 nm) of phycobilins extracts and carotenoids + chlorophylls extracts from two tested cyanobacteria species, *S. salina* and *M. aeruginosa* (control culture), and the color of the extracts after extraction (blue for phycobilins and green for lipophilic pigments). The spectra for both target species extracts are similar, meaning that their general compositions are comparable. In the spectrum of the phycobilins extract, the dominant peak corresponds to phycocyanin (max A at 621–623 nm), while in the carotenoid and chlorophylls extracts, the maximum intensity of absorbance was noted for chlorophyll *a* (432 nm, 663–666 nm).

Fig. 7 presents a bar graph with the concentrations of pigments being quantified for both species, expressed as fg of pigment per cell. The concentrations were different for each species, despite having similar values of OD (Fig. 4) and UV-Vis spectra (Fig. 6). The main reason for this is the fact that the number of cells was approximately five times higher for *S. salina* (Fig. 5). The highest concentration was quantified for chlorophyll *a*, while the concentration of β-carotene (14–46 fg cell⁻¹) was approximately nine times lower compared to chlorophyll *a* for both species. A similar β-carotene concentration per cell was previously noted for *M. aeruginosa*, and this was found to be dependent on the stage of the culture-growing phase [30]. It needs to be mentioned that the content of pigments found in our study is exclusive for the applied growing conditions, while the production of carotenoids is directed by both biotic and abiotic conditions [58].

The phycocyanin concentration needs to be interpreted with caution, given that the applied standard was not pure. Indeed, the literature data show that the concentration of this pigment is rather similar or higher compared to chlorophyll *a* in *Microcystis* sp. [30]. Still, the comparison of concentrations found in the control and exposed culture is justified.

No statistical differences were noted in the pigment contents between extracts of controls and DCF-impacted cultures. The concentration of pigments is reasonably taken as a biomarker of stress. The pathways of xanthophyll's biosynthesis from carotenenes are generally known [58], and the main enzymes have been determined. Carotenoids play roles as protection agents against saturating light and as quenchers of reactive oxygen species (ROS). In this sense, they can also protect cells from ROS resulting from stress. The photoactive and photoprotective

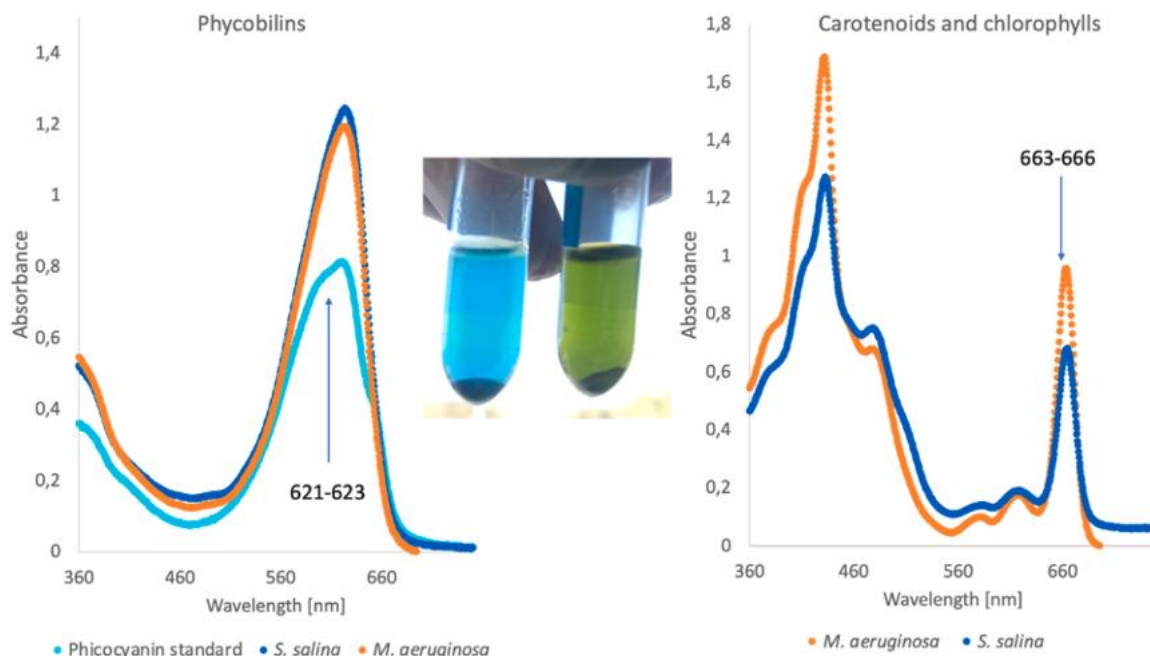


Fig. 6. The UV-Vis spectra of phycobilins (left) and carotenoids + chlorophylls (right) extracts from two tested cyanobacteria species, *S. salina* and *M. aeruginosa*. The graphic presents extracts of the pigments mentioned – blue for phycobilins and green for carotenoids + chlorophylls.

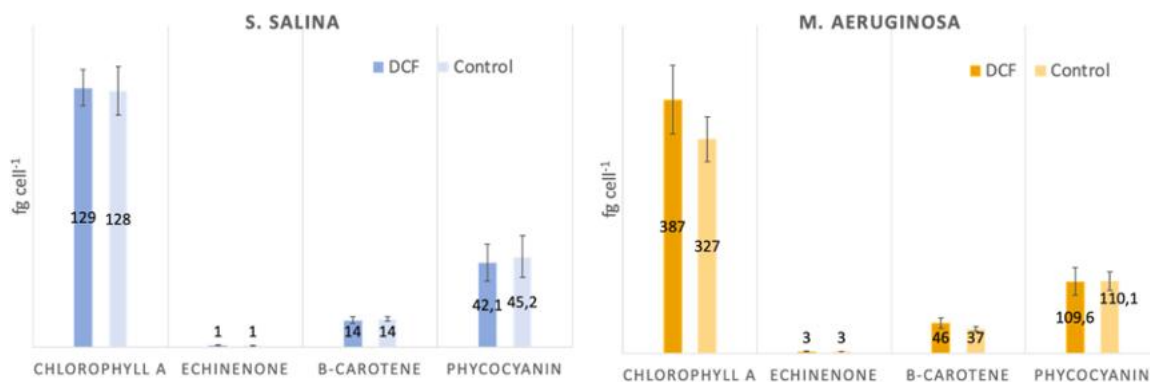


Fig. 7. The concentrations [fg cell⁻¹] of selected pigments in extracts of cyanobacteria *S. salina* and *M. aeruginosa* in control culture and culture impacted by diclofenac (DCF) at 96 h (phycocyanin concentration is semi-quantitative).

pigments were previously tested as markers of stress caused by pharmaceuticals. For example, the effects of anesthetics (lidocaine, dibucaine, procainamide, tetracaine, and procaine) to the ratio of phycobilins to chlorophyll in *Synechococcus leopoliensis* were determined [67] as a marker of photosynthetic oxygen evolution. It was noted that lidocaine supplementation enhanced photosynthetic oxygen evolution, but this did not appear to affect cyanobacterial pigment content. The NSAIDs (DCF, ibuprofen, diflunisal, mefenamic acid, and piroxicam) at a concentration of 1 mg L⁻¹ significantly lowered the growth rate and the chlorophyll *a* content in *Cylindrospermopsis raciborskii* after a 10-day exposure period [5]. In our study, the pigments content was not impacted by DCF, despite the observed impact of DCF on photosynthesis during the first few hours of the experiment (see Section 3.5.).

3.5. Impact on photosynthesis

The parameters of chlorophyll *a* fluorescence in vivo under the control conditions (Fig. 8, Table S4) of both strains were much lower than the parameters that were characteristic for higher plants and green algae, which is in line with the literature data [64]. Moreover, the parameters differed between the two strains, and in general, they reached

higher values in *M. aeruginosa* than in *S. salina*, which indicates that the photosynthetic apparatuses of both strains operate with different efficiencies. The high quantum yield of the primary photochemistry of PS II (Fv/Fm) and the high activity of the water-splitting complex (Fv/Fo) in *M. aeruginosa* suggested that this strain can utilize light energy more efficiently than *S. salina* can. This seems to mainly result from a large fraction of active PS II reaction centers (RCm) in *M. aeruginosa*. However, one active RC in this strain absorbed less energy than one active RC in *S. salina*, with the same trapping efficiency of absorbed energy (TRo/RC) in both organisms. Since the electron transport per one active RC (ETo/RC) in *S. salina* was of low efficiency, a significant portion of absorbed energy was dissipated in a non-photochemical manner (Dio/RC). Such differences in photosynthetic performance are not surprising, since a high photosynthetic variability is reported not only for different strains of cyanobacteria [44], but even for specific lineages of one strain [72,75]. Furthermore, Zavrel et al. (2017) reported that variations in a phenotype of *Synechocystis* sp. PCC 6803 substrains, namely, in the growth rate, photosynthesis efficiency, pigments composition, etc., are associated with different stress sensitivities. Taking additionally into account that the photosynthetic apparatus, especially PS II, seems to play an important role in cell acclimation (or the

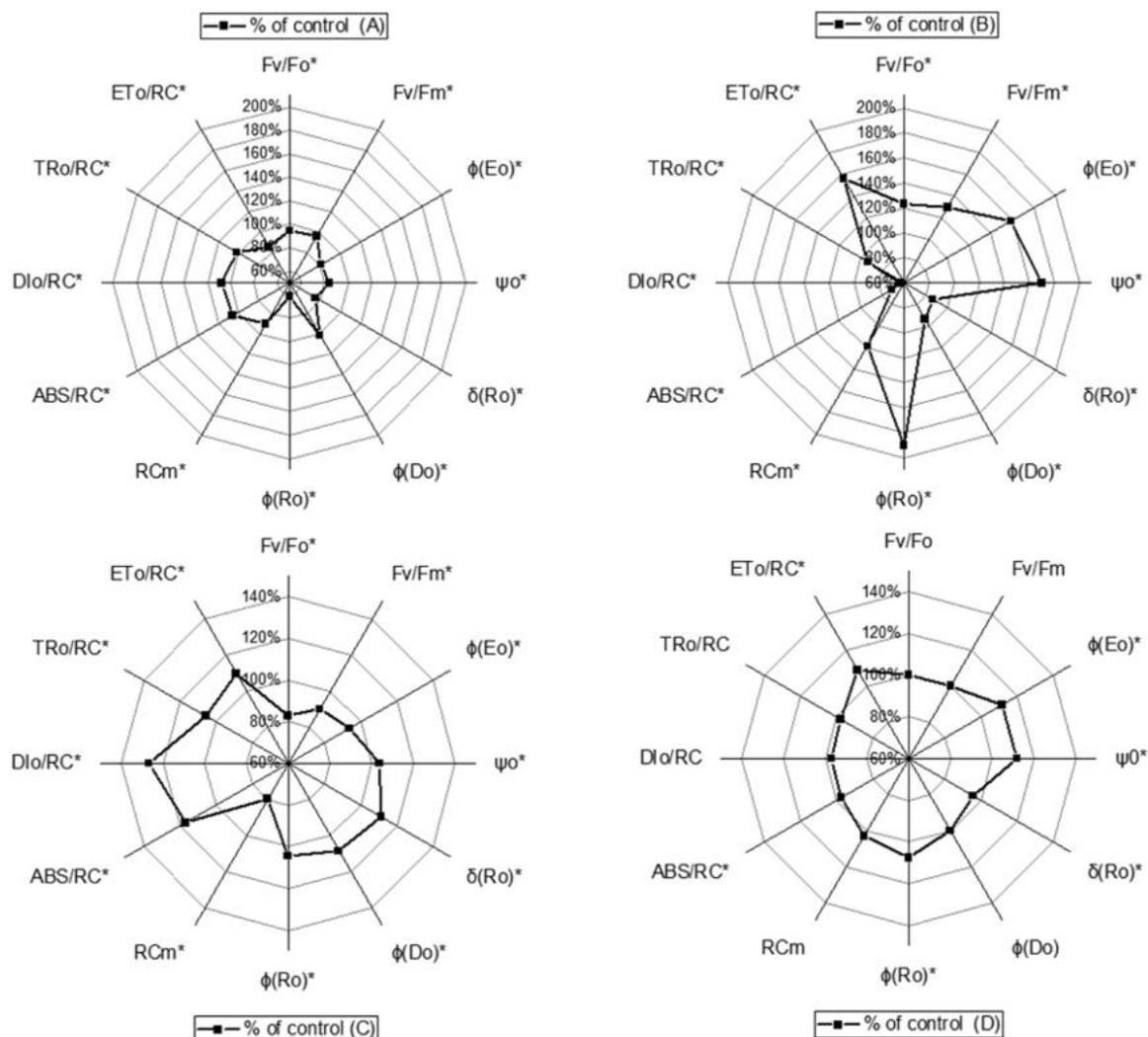


Fig. 8. The effect of diclofenac on the chlorophyll *a* fluorescence in vivo parameters in *M. aeruginosa* on the first (A) and last (B) days of the test, and in *S. salina* on the first (C) and last (D) days of the test, expressed as a percentage of control. The asterisk denotes a statistically significant difference between DCF and control treatments at the $p = 0.05$ level. The actual fluorescence values and their significance levels are shown in the [Supplementary Data, Table S4](#).

hormetic response) to stress factors [50], we can assume that the abovementioned differences in the photosynthetic characteristics of *M. aeruginosa* and *S. salina* may be one of the reasons for why the two strains reacted unequally to DCF (Fig. 8, Table S4).

In both strains, some effects of DCF action could already be seen after ~1 h of exposure. In *M. aeruginosa*, a significant decrease was observed for the quantum yield of primary photochemistry (Fv/Fm), the activity of the water-splitting complex (Fv/Fo), the quantum yield of electron transport (ϕ_{E0}), and the fraction of active PS II reaction centers (RCm), while in *S. salina*, it was mainly the electron transport beyond PS II to the end acceptors in photosystem I (ψ_0 , δ_{Ro} , and ϕ_{Ro}) that was inhibited. The specific energy flux by one active RC (ABS/RC, TRo/RC, ETo/RC, and Dlo/RC) in both strains increased, but the effect was more pronounced in *M. aeruginosa* as compared to *S. salina*.

The results described above indicate that after ~1 h of exposure to DCF, PS II was significantly affected in *M. aeruginosa*. A significant diminishment of the fraction of active PS II reaction centers was noticed, but this fraction of reaction centers of PS II, which remained active, seemed to function with high efficiency. Thus, it can be suggested that some PS II RCs were converted into “heat sinks”, i.e., RCs that do not reduce Q_a but instead convert excitation energy into heat (Tsimilli-Michael and Strasser, 2008). This protective mechanism, which allows for the safe dissipation of energy excess under stress conditions, has been

previously reported for DCF-treated *Chlamydomonas reinhardtii* [24,41]. Similarly to *M. aeruginosa*, and also in DCF-treated *C. reinhardtii*, the active RCs were able to function with high efficiency [41,42]. A significant effect of DCF on PS II functioning was reported, not only for green algae, but also for higher plants such as *Lemna minor* [23], *Zea mays*, and *Solanum lycopersicum* [65], which indicates that the DCF effect on PS II activity is commonly observed among different taxa of photosynthetic organisms. Nevertheless, in contrast to *M. aeruginosa*, in *S. salina*, PS II functioning was almost unaffected at the beginning of DCF treatment, while the electron transport beyond PS II to the end acceptors in photosystem I was inhibited. The observed “resistance” of PS II to low doses of DCF was previously reported in the literature for *Lemna minor* [36] and two green algae species, *Picocystis* sp. CINS 23 and *Graesiella* sp. CINS [6].

After 4 days of exposure to DCF, in *M. aeruginosa* cells, the fraction of PS II active reaction centers (RCm) returned to the control level, similarly to the quantum yield of primary photochemistry (Fv/Fm), the activity of the water-splitting complex (Fv/Fo), and the parameters of non-photochemical energy quenching (ϕ_{Do} , Dlo/RC). The parameters of electron transport (ϕ_{E0} , ψ_0 , and ϕ_{Ro}) reached 104–111% of the control value. In the case of *S. salina*, the fraction of PS II active reaction centers (RCm), the quantum yield of primary photochemistry (Fv/Fm), the activity of the water-splitting complex (Fv/Fo), the quantum yield of

electron transport ($\phi E0$), and the efficiency of moving the electron beyond Qa ($\Psi0$) significantly outranked the control values. The quantum yield of electron transport from Qa to the PS I end electron acceptors (ϕRo), which was significantly inhibited by DCF at the first stage of exposure (0 day), reached 175% of the control after 4 days, while the values for the parameters of non-photochemical energy quenching (ϕDo and DIo/RC) dropped to below the control level.

A comprehensive analysis of photosynthetic parameters after 4 days of exposure to DCF thus indicated that in both strains, some mechanisms of acclimation to the DCF-induced stress were activated, increasing the efficiency of the photosynthetic apparatus. In *M. aeruginosa*, the return of the fraction of PS II active reaction centers to the control level caused a recovery of the quantum yield of primary photochemistry and the activity of the water-splitting complex. The quantum yield of electron transport exceeded the control values, while non-photochemical energy quenching dropped as compared to the control. In the case of *S. salina*, acclimation processes caused the quantum yields of energy utilization in both PS II and PS I, in DCF-treated cells to significantly increase, as compared with day 0, and they even exceeded the control values. The most pronounced effects concerned the quantum yield of electron transport from Qa to the PS I end electron acceptors (ϕRo reached 175% of the control), and the parameters of non-photochemical energy quenching, which dropped significantly below the control level.

The acclimation of photosynthetic organisms to low doses of stressors, often considered to be connected with the hormetic response, is widely described in the literature [11,17]. However, studies describing the acclimation of algae and cyanobacteria to anthropogenic micropollutants are rather rare. Zhang et al. [77] reported on the stimulating effects of low doses of clofibric acid, ciprofloxacin, diclofenac, and carbamazepine on growth, chlorophyll accumulation, and lipid content in *Chlorella pyrenoidosa* cultures [77]. *M. aeruginosa* was found to be able to acclimate to low doses of chlorinated organophosphorus flame retardants (Cl-OPFRs) due to the upregulation of the photosynthetic cyclic electron transfer pathway and an increase in carotenoids synthesis [76]. The potentials of different phytoplankton species (*Dictyosphaerium chlorelloides*, *Scenedesmus intermedius*, *Tetraselmis suecica*, *M. aeruginosa*, *Phaeodactylum tricornutum*, *Emiliania huxleyi*, *Isochrysis galbana*, and *Monochrysis lutheri*) have been analyzed for acclimation and further adaptation to the herbicide simazine [29]. They have observed high diversities in the acclimation/adaptation potentials among those species, with cyanobacteria being the most sensitive to the herbicide. Such a discrepancy in the sensitivities/resistances of different microorganisms to stress factors is in line with our observations that despite the DCF influence on the photosynthetic processes in both strains, the photosynthetic apparatus of *M. aeruginosa* seems to be more susceptible to the drug and it has a lower potential to acclimate to the DCF-induced stress.

4. Conclusions

It was notable that the growth rates of two unicellular cyanobacteria originating from the Baltic Sea were the same over the biotic control culture and the exposed cultures, meaning that the proliferation of these cells was not affected by DCF. Flow cytometry analysis showed no fluorescence differences between the cell number at the start and at each day of exposure. Moreover, no statistical differences were noted between the pigment content in extracts of controls and DCF-exposed cultures. The DCF concentration was relatively stable, indicating that the cyanobacteria had no significant potential for bioaccumulation of this pollutant. Nevertheless, discrete effects were noted. Firstly, DCF increases intracellular microcystin production. In both strains, some effects of DCF action could already be seen in photosynthetic parameters after ~1 h of exposure, and after 4 days in both strains, some mechanisms of acclimation to the DCF-induced stress began, despite the lack of composition changes in the photosynthetic pigment. Considering that only trace amounts of DCF seem to enter the cell interiors, and that the

cells' responses to the pharmaceutical were very fast, it cannot be excluded that the signaling cascade leading to changes in the photosynthetic performance of the cyanobacteria commenced at the level of the cell membrane. Although this suggestion is supported by reports that cyanobacteria react to environmental stimuli by regulating their biochemical reactions and gene expression through two-component regulatory systems connected with the cell membrane [28,32,43], the problem still needs to be explored.

Compared to other phytoplankton taxa (Table 1), the unicellular cyanobacteria tested are less sensitive toward DCF, which gives them a competitive advantage over other organisms. The high resistance of cyanobacteria to DCF and the high DCF sensitivities of higher organisms (such as fish and crustaceans) may lead to a decrease in biodiversity in the aquatic environment. Furthermore, the increased amount of toxins secreted by cyanobacteria exposed to DCF will additionally affect higher aquatic organisms negatively.

The study of the biochemical responses of cyanobacteria to DCF shows the need for stress markers testing that are less obvious than enzymatic oxidative stress biomarkers and go beyond the standards of reference protocols. In a case of toxins producers it is crucial to investigate the probable stimulating influence on toxins production as non-target effect of biologically active substances.

Environmental implications

The presented research has shown that reference ecotoxicological tests do not provide us with sufficient information regarding the impacts of pollutants on cyanobacteria. Despite having no effects on the growth of cyanobacteria, DCF had stimulated the cyanobacterial for production of microcystins. Additionally, it has been noticed that cyanobacteria are more resistant to DCF than other phytoplankton species, and thus, they have an advantage in a polluted environment. Although the DCF concentration tested was not at environmental levels, testing the acute toxicity of DCF on cyanobacteria allows for an understanding of the mechanisms of its action in cyanobacteria.

CRediT authorship contribution statement

Justyna Kobos: Writing – original draft. **Anna Aksmann:** Writing – original draft. **Martyna Zalewska:** Writing – original draft, Investigation. **Klaudia Kwidzińska:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation. **Magda Caban:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Hanna Mazur-Marzec:** Writing – original draft, Investigation.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Magda Caban reports financial support was provided by National Science Centre Poland. Magda Caban reports a relationship with National Science Centre Poland that includes: funding grants. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgments

Financial support was provided by the National Science Center (Poland) under grants UMO 2019/35/B/NZ9/00253. The authors would

like to thank the Culture Collection of Northern Poland (CCNP, Institute of Oceanography, University of Gdansk, Poland) for providing cyanobacteria monocultures. We would like to thank the SHIM-POL A.M. Borzymowski company for making the LCMS-8050 triple quadrupole mass spectrometer (Shimadzu) available for research.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jhazmat.2024.134373](https://doi.org/10.1016/j.jhazmat.2024.134373).

References

- Agostoni, M., Montgomery, B.L., 2014. Survival strategies in the aquatic and terrestrial world: the impact of second messengers on cyanobacterial processes. *Life* 4, 745–769. <https://doi.org/10.3390/life4040745>.
- Ali, A.M., Sydnese, L.K., Alarif, W.M., Al-Lihaibi, S.S., Aly, M.M., Aanrud, S.G., Kallenborn, R., 2019. Diclofenac and two of its photooxidation products in the marine environment: their toxicology and occurrence in Red Sea coastal waters. *Environ Chem Ecotoxicol* 1, 19–25. <https://doi.org/10.1016/j.encc.2019.06.002>.
- Allaf, M.M., Peerhossaini, H., 2022. Cyanobacteria: model microorganisms and beyond. *Microorganisms* 10, 696. <https://doi.org/10.3390/microorganisms10040696>.
- Alotaibi, B.S., Khan, M., Shamim, S., 2021. Unraveling the underlying heavy metal detoxification mechanisms of bacillus species. *Microorganisms* 9. <https://doi.org/10.3390/microorganisms9081628>.
- Bácsi, I., B-Béres, V., Kókai, Z., Gonda, S., Novák, Z., Nagy, S.A., Vasas, G., 2016. Effects of non-steroidal anti-inflammatory drugs on cyanobacteria and algae in laboratory strains and in natural algal assemblages. *Environ Pollut* 212, 508–518. <https://doi.org/10.1016/j.envpol.2016.02.031>.
- Ben Ouada, S., Ben Ali, R., Cimetiere, N., Leboulanger, C., Ben Ouada, H., Sayadi, S., 2019. Biodegradation of diclofenac by two green microalgae: *Picocystis* sp. and *Gracilariopsis* sp. *Ecotoxicol Environ Saf* 186. <https://doi.org/10.1016/j.ecoenv.2019.109769>.
- Björklén, B., Ripszám, M., Haglund, P., Lindberg, R.H., Tysklind, M., Fick, J., 2018. Pharmaceutical residues are widespread in Baltic Sea coastal and offshore waters – screening for pharmaceuticals and modelling of environmental concentrations of carbamazepine. *Sci Total Environ* 633, 1496–1509. <https://doi.org/10.1016/j.scitotenv.2018.03.276>.
- Bonnefille, B., Gomez, E., Courant, F., Escande, A., Fenet, H., 2018. Diclofenac in the marine environment: a review of its occurrence and effects. *Mar Pollut Bull* 131, 496–506. <https://doi.org/10.1016/j.marpolbul.2018.04.053>.
- Bouaicha, N., Miles, C.O., Beach, D.G., Labidi, Z., Djabri, A., Benayache, N.Y., Nguyen-Quang, T., 2019. Structural diversity, characterization and toxicology of *Microcystis*. *Toxins* 11, 714. <https://doi.org/10.3390/toxins11120714>.
- Caban, M., Stepnowski, P., 2021. How to decrease pharmaceuticals in the environment? a review. *Environ Chem Lett* 19, 3115–3138. <https://doi.org/10.1007/s10311-021-01194-y>.
- Calabrese, E.J., Blain, R.B., 2009. Hormesis and plant biology. *Environ Pollut* 157, 42–48. <https://doi.org/10.1016/j.envpol.2008.07.028>.
- Clevers, M., 2004. Mixture toxicity of the anti-inflammatory drugs diclofenac, ibuprofen, naproxen, and acetylsalicylic acid. *Ecotoxicol Environ Saf* 59, 309–315. [https://doi.org/10.1016/S0147-6513\(03\)00141-6](https://doi.org/10.1016/S0147-6513(03)00141-6).
- De Almeida, Gonçalves, C., Figueredo, C.C., 2020. What we really know about the composition and function of microalgal cell coverings? - An overview. *Acta Bot Bras* 34, 599–614. <https://doi.org/10.1590/0102-33062020abb0309>.
- DeLorenzo, M.E., Fleming, J., 2008. Individual and mixture effects of selected pharmaceuticals and personal care products on the marine phytoplankton species *Dunaliella tertiolecta*. *Arch Environ Contam Toxicol* 54, 203–210. <https://doi.org/10.1007/s00244-007-9032-2>.
- Deng, C., Pan, X., Zhang, D., 2015. Influence of ofloxacin on photosystems I and II activities of *Microcystis aeruginosa* and the potential role of cyclic electron flow. *J Biosci Bioeng* 119, 159–164. <https://doi.org/10.1016/j.jbiosc.2014.07.014>.
- dos Santos, C.R., Arcanjo, G.S., de Souza Santos, L.V., Koch, K., Amaral, M.C.S., 2021. Aquatic concentration and risk assessment of pharmaceutically active compounds in the environment. *Environ Pollut* 290, 118049. <https://doi.org/10.1016/j.envpol.2021.118049>.
- Erofeeva, E.A., 2022. Environmental hormesis of non-specific and specific adaptive mechanisms in plants. *Sci Total Environ* 804, 150059. <https://doi.org/10.1016/j.scitotenv.2021.150059>.
- Escapa, C., Coimbra, R.N., Paniagua, S., García, A.I., Otero, M., 2016. Comparative assessment of diclofenac removal from water by different microalgae strains. *Algal Res* 18, 127–134. <https://doi.org/10.1016/j.algal.2016.06.008>.
- Ferrari, B., Mons, R., Vollat, B., Frayssé, B., Paxéus, N., Lo Giudice, R., Pollio, A., Garric, J., 2004. Environmental risk assessment of six human pharmaceuticals: Are the current environmental risk assessment procedures sufficient for the protection of the aquatic environment? *Environ Toxicol Chem* 23, 1344–1354. <https://doi.org/10.1897/03-246>.
- Ferrari, B., Paxéus, N., Giudice, R.L., Pollio, A., Garric, J., 2003. Ecotoxicological impact of pharmaceuticals found in treated wastewaters: Study of carbamazepine, clofibrac acid, and diclofenac. *Ecotoxicol Environ Saf* 55, 359–370. [https://doi.org/10.1016/S0147-6513\(02\)00082-9](https://doi.org/10.1016/S0147-6513(02)00082-9).
- Guo, R.X., Chen, J.Q., 2012. Phytoplankton toxicity of the antibiotic chlortetracycline and its UV light degradation products. *Chemosphere* 87, 1254–1259. <https://doi.org/10.1016/j.chemosphere.2012.01.031>.
- Hadjoudja, S., Deluchat, V., Baudu, M., 2010. Cell surface characterisation of *Microcystis aeruginosa* and *Chlorella vulgaris*. *J Colloid Interface Sci* 342, 293–299. <https://doi.org/10.1016/j.jcis.2009.10.078>.
- Hájková, M., Kummerová, M., Zezulka, Š., Babula, P., Váci, P., 2019. Diclofenac as an environmental threat: Impact on the photosynthetic processes of *Lemma minor* chloroplasts. *Chemosphere* 224, 892–899. <https://doi.org/10.1016/j.chemosphere.2019.02.197>.
- Harshkova, D., Liakh, I., Bialevich, V., Ondrejmišková, K., Aksmann, A., Bišová, K., 2021. Diclofenac alters the cell cycle progression of the green alga *Chlamydomonas reinhardtii*. *Cells* 10, 1936. <https://doi.org/10.3390/cells10081936>.
- Helcom, 2018. Diclofenac. HELCOM pre-core indicator report. Online. 1–29.
- Hoiczky, E., Hansel, A., 2000. Cyanobacterial cell walls: News from an unusual prokaryotic envelope. *J Bacteriol* 182, 1191–1199. <https://doi.org/10.1128/JB.182.5.1191-1199.2000>.
- Holland, A., Kinnear, S., 2013. Interpreting the possible ecological role(s) of cyanotoxins: compounds for competitive advantage and/or physiological aide? *Mar Drugs* 11, 2239–2258. <https://doi.org/10.3390/md11072239>.
- Hsiao, H.Y., He, Q., Van Waasbergen, L.G., Grossman, A.R., 2004. Control of photosynthetic and high-light-responsive genes by the histidine kinase DspA: Negative and positive regulation and interactions between signal transduction pathways. *J Bacteriol* 186, 3882–3888. <https://doi.org/10.1128/JB.186.12.3882-3888.2004>.
- Huertas, I.E., Rouco, M., López-Rodas, V., Costas, E., 2010. Estimating the capability of different phytoplankton groups to adapt to contamination: herbicides will affect phytoplankton species differently. *N Phytol* 188, 478–487. <https://doi.org/10.1111/j.1469-8137.2010.03370.x>.
- Jacinašević, F.R., Pacheco, A.B.F., Chow, F., Verissimo da Costa, G.C., Kalume, D. E., Rigonato, J., Schmidt, E.C., Sant'Anna, C.L., 2019. Different ecophysiological and structural strategies of toxic and non-toxic *Microcystis aeruginosa* (cyanobacteria) strains assessed under culture conditions. *Algal Res* 41, 101548. <https://doi.org/10.1016/j.algal.2019.101548>.
- Jones, M.R., Pinto, E., Torres, M.A., Dörr, F., Mazur-Marzec, H., Szubert, K., Tartaglione, L., Dell'Aversano, C., Miles, C.O., Beach, D.G., McCarron, P., Sivonen, K., Fewer, D.P., Jokela, J., Janssen, E.M.L., 2021. CyanoMetDB, a comprehensive public database of secondary metabolites from cyanobacteria. *Water Res* 196, 117017. <https://doi.org/10.1016/j.watres.2021.117017>.
- Junaid, M., Inaba, Y., Otero, A., Suzuki, I., 2021. Development of a reversible regulatory system for gene expression in the cyanobacterium *Synechocystis* sp. PCC 6803 by quorum-sensing machinery from marine bacteria. *J Appl Phycol* 33, 1651–1662. <https://doi.org/10.1007/s10811-021-02397-0>.
- Kapelewska, J., Kotowska, U., Karpínska, J., Kowalczyk, D., Arciszewska, A., Świrzydo, A., 2018. Occurrence, removal, mass loading and environmental risk assessment of emerging organic contaminants in leachates, groundwaters and wastewaters. *Microchem J* 137, 292–301. <https://doi.org/10.1016/j.microc.2017.11.008>.
- Kolecka, K., Gajewska, M., Stepnowski, P., Caban, M., 2019. Spatial distribution of pharmaceuticals in conventional wastewater treatment plant with Sludge Treatment Reed Beds technology. *Sci Total Environ* 647, 149–157. <https://doi.org/10.1016/j.scitotenv.2018.07.439>.
- Kropidłowska, K., Caban, M., 2023. Effect of salinity on the toxicity of diclofenac, ibuprofen and naproxen toward cyanobacterium *Synechocystis salina*. *Chemosphere* 338, 139521. <https://doi.org/10.1016/j.chemosphere.2023.139521>.
- Kummerová, M., Zezulka, Š., Babula, P., Triska, J., 2016. Possible ecological risk of two pharmaceuticals diclofenac and paracetamol demonstrated on a model plant *Lemma minor*. *J Hazard Mater* 302, 351–361. <https://doi.org/10.1016/j.jhazmat.2015.09.057>.
- Lambert, P.A., 2002. Cellular impermeability and uptake of biocides and antibiotics in Gram-positive bacteria and mycobacteria. *J Appl Microbiol* 92, 35–45. <https://doi.org/10.1046/j.1365-2672.92.5s1.7.x>.
- Liakh, I., Harshkova, D., Hrouzek, P., Bišová, K., Aksmann, A., Wielgomas, B., 2023. Green alga *Chlamydomonas reinhardtii* can effectively remove diclofenac from the water environment – a new perspective on biotransformation. *J Hazard Mater* 455, 131570. <https://doi.org/10.1016/j.jhazmat.2023.131570>.
- Liu, Y., Gao, B., Yue, Q., Guan, Y., Wang, Y., Huang, L., 2012. Influences of two antibiotic contaminants on the production, release and toxicity of microcystins. *Ecotoxicol Environ Saf* 77, 79–87. <https://doi.org/10.1016/j.ecoenv.2011.10.027>.
- Lonappan, L., Kaur, S., Kumar, R., Verma, M., Surampalli, R.Y., 2016. Diclofenac and its transformation products: environmental occurrence and toxicity - A review. *Environ Int* 96, 127–138. <https://doi.org/10.1016/j.envint.2016.09.014>.
- Majewska, M., Harshkova, D., Guściora, M., Aksmann, A., 2018. Phytotoxic activity of diclofenac: evaluation using a model green alga *Chlamydomonas reinhardtii* with atrazine as a reference substance. *Chemosphere* 209, 989–997. <https://doi.org/10.1016/j.chemosphere.2018.06.156>.
- Majewska, M., Harshkova, D., Pokora, W., Baścik-Remisiewicz, A., Tulodziecki, S., Aksmann, A., 2021. Does diclofenac act like a photosynthetic herbicide on green algae? *Chlamydomonas reinhardtii* synchronous culture-based study with atrazine as reference. *Ecotoxicol Environ Saf* 208, 111630. <https://doi.org/10.1016/j.ecoenv.2020.111630>.
- Marin, K., Suzuki, I., Yamaguchi, K., Ribbeck, K., Yamamoto, H., Kanesaki, Y., Hagemann, M., Murata, N., 2003. Identification of histidine kinases that act as

- sensors in the perception of salt stress in *Synechocystis* sp. PCC 6803. *Proc Natl Acad Sci USA* 100, 9061–9066. <https://doi.org/10.1073/pnas.1532302100>.
- [44] Martín-Clemente, E., Melero-Jiménez, I.J., Bañares-España, E., Flores-Moya, A., García-Sánchez, M.J., 2022. Photosynthetic performance in cyanobacteria with increased sulphide tolerance: an analysis comparing wild-type and experimentally derived strains. *Photosynth Res* 151, 251–263. <https://doi.org/10.1007/s11120-021-00882-8>.
- [45] Martinez, R.E., Pokrovsky, O.S., Schott, J., Oelkers, E.H., 2008. Surface charge and zeta-potential of metabolically active and dead cyanobacteria. *J Colloid Interface Sci* 323, 317–325. <https://doi.org/10.1016/j.jcis.2008.04.041>.
- [46] Matho, C., Schwarzenberger, K., Eckert, K., Keshavarzi, B., Walther, T., Steingroewer, J., Krujatz, F., 2019. Bio-compatible flotation of *Chlorella vulgaris*: Study of zeta potential and flotation efficiency. *Algal Res* 44, 101705. <https://doi.org/10.1016/j.algal.2019.101705>.
- [47] Mazur-Marzec, H., Browarczyk-Matusiak, G., Forycka, K., Kobos, J., Pliński, M., 2010. Morphological, genetic, chemical and ecophysiological characterisation of two *Microcystis aeruginosa* isolates from the Vistula Lagoon, southern Baltic. *Oceanologia* 52, 127–146. <https://doi.org/10.5697/oc.52-1.127>.
- [48] McLellan, N.L., Manderville, R.A., 2017. Toxic mechanisms of microcystins in mammals. *Toxicol Res (Camb)* 6, 391–405. <https://doi.org/10.1039/c7tx00043j>.
- [49] Mkaddem, H., Rosales, E., Pazos, M., Ben Amor, H., Sanromán, M.A., Mejjide, J., 2022. Anti-inflammatory drug diclofenac removal by a synthesized MgAl layered double hydroxide. *J Mol Liq* 359, 119207. <https://doi.org/10.1016/j.molliq.2022.119207>.
- [50] Moustakas, M., Moustaka, J., Sperdouli, I., 2022. Hormesis in photosystem II: a mechanistic understanding. *Curr Opin Toxicol* 29, 57–64. <https://doi.org/10.1016/j.cotox.2022.02.003>.
- [51] Mulikiewicz, E., Wolecki, D., Świacka, K., Kumirska, J., Stepnowski, P., Caban, M., 2021. Metabolism of non-steroidal anti-inflammatory drugs by non-target wild-living organisms. *Sci Total Environ* 791, 148251. <https://doi.org/10.1016/j.scitotenv.2021.148251>.
- [52] Navrozidou, E., Remmas, N., Melidis, P., Karpouzas, D.G., Tsimis, G., Ntougias, S., 2019. Biodegradation potential and diversity of diclofenac-degrading microbiota in an immobilized cell biofilter. *Processes* 7, 1–13. <https://doi.org/10.3390/pr7090554>.
- [53] Neilan, B.A., Pearson, L.A., Muenchhoff, J., Moffitt, M.C., Dittmann, E., 2013. Environmental conditions that influence toxin biosynthesis in cyanobacteria. *Environ Microbiol* 15, 1239–1253. <https://doi.org/10.1111/j.1462-2920.2012.02729.x>.
- [54] OECD, 2011. Test No. 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test, OECD Guidelines for the Testing of Chemicals, Section 2. <https://doi.org/https://doi.org/10.1787/9789264069923-en>.
- [55] Olofsson, M., Suikkanen, S., Kobos, J., Wasmund, N., Karlson, B., 2020. Basin-specific changes in filamentous cyanobacteria community composition across four decades in the Baltic Sea. *Harmful Algae* 91, 101685. <https://doi.org/10.1016/j.hal.2019.101685>.
- [56] Omid, A., Esterhuizen-Londt, M., Pflugmacher, S., 2018. Still challenging: the ecological function of the cyanobacterial toxin microcystin—What we know so far. *Toxin Rev* 37, 87–105. <https://doi.org/10.1080/15569543.2017.1326059>.
- [57] Overling, D., Toruńska-Sitarz, A., Katarzytę, M., Pilkaitytė, R., Gyratė, G., Mazur-Marzec, H., 2021. Characterization and diversity of Microcystins produced by cyanobacteria from the Curonian Lagoon (SE Baltic Sea). *Toxins (Basel)* 13, 838. <https://doi.org/10.3390/toxins13120838>.
- [58] Pagels, F., Vasconcelos, V., Guedes, A.C., 2021. Carotenoids from cyanobacteria: Biotechnological potential and optimization strategies. *Biomolecules* 11, 735. <https://doi.org/10.3390/biom11050735>.
- [59] Park, Y.H., Kim, S., Kim, H.S., Park, C., Choi, Y.E., 2020. Adsorption strategy for removal of harmful cyanobacterial species *Microcystis aeruginosa* using chitosan fiber. *Sustain* 12, 4587. <https://doi.org/10.3390/su12114587>.
- [60] Pomati, F., Jokela, J., Castiglioni, S., Thomas, M.K., Nizzetto, L., 2017. Water-borne pharmaceuticals reduce phenotypic diversity and response capacity of natural phytoplankton communities. *PLoS One* 12, 1–18. <https://doi.org/10.1371/journal.pone.0174207>.
- [61] Sánchez-Sandoval, D.S., González-Ortega, O., Vazquez-Martínez, J., García de la Cruz, R.F., Soria-Guerra, R.E., 2022. Diclofenac removal by the microalgae species *chlorella vulgaris*, *nannochloropsis oculata*, *scenedesmus acutus*, and *scenedesmus obliquus*. *3 Biotech* 12, 1–16. <https://doi.org/10.1007/s13205-022-03268-2>.
- [62] Saravanan, M., Karthika, S., Malarvizhi, A., Ramesh, M., 2011. Ecotoxicological impacts of clofibrac acid and diclofenac in common carp (*Cyprinus carpio*) fingerlings: Hematological, biochemical, ionoregulatory and enzymological responses. *J Hazard Mater* 195, 188–194. <https://doi.org/10.1016/j.jhazmat.2011.08.029>.
- [63] Schmidt, W., O'Rourke, K., Hernan, R., Quinn, B., 2011. Effects of the pharmaceuticals gemfibrozil and diclofenac on the marine mussel (*Mytilus* spp.) and their comparison with standardized toxicity tests. *Mar Pollut Bull* 62, 1389–1395. <https://doi.org/10.1016/j.marpolbul.2011.04.043>.
- [64] Schuurmans, R.M., van Alphen, P., Schuurmans, J.M., Matthijs, H.C.P., Hellingwerf, K.J., 2015. Comparison of the photosynthetic yield of cyanobacteria and green algae: different methods give different answers. *PLoS One* 10, e0139061. <https://doi.org/10.1371/journal.pone.0139061>.
- [65] Siemieniuk, A., Ludynia, M., Rudnicka, M., 2021. Response of two crop plants, *Zea mays* L. and *Solanum lycopersicum* L., to diclofenac and naproxen. *Int J Mol Sci* 22, 8856. <https://doi.org/10.3390/ijms22168856>.
- [66] Sukenik, A., Quesada, A., Salmaso, N., 2015. Global expansion of toxic and non-toxic cyanobacteria: effect on ecosystem functioning. *Biodivers Conserv* 24, 889–908. <https://doi.org/10.1007/s10531-015-0905-9>.
- [67] Suzuki, T., Nakasato, K., Shapiro, S., Pomati, F., Neilan, B.A., 2004. Effects of synthetic local anaesthetics on the growth of the cyanobacterium *Synechococcus leopoliensis*. *J Appl Phycol* 16, 145–152. <https://doi.org/10.1023/B:JAPH.0000044776.04977.00>.
- [68] Świacka, K., Michnowska, A., Maculewicz, J., Caban, M., Smolarz, K., 2021. Toxic effects of NSAIDs in non-target species: a review from the perspective of the aquatic environment. *Environ Pollut* 273. <https://doi.org/10.1016/j.envpol.2020.115891>.
- [69] UNESCO, HELCOM, 2017. Pharmaceuticals in the aquatic environment of the Baltic Sea region in the aquatic environment of the Baltic Sea region - A status report. In: UNESCO Emerg. Pollut. Water Ser. 1. UNESCO Publ., Paris.
- [70] Varriale, F., Tartaglione, L., Zervou, S.-K., Miles, C.O., Mazur-Marzec, H., Triantis, T.M., Kaloudis, T., Hiskia, A., Dell'Aversano, C., 2023. Untargeted and targeted LC-MS and data processing workflow for the comprehensive analysis of oligopeptides from cyanobacteria. *Chemosphere* 311, 137012. <https://doi.org/10.1016/j.chemosphere.2022.137012>.
- [71] Wojtasiewicz, B., Stoń-Egier, J., 2016. Bio-optical characterization of selected cyanobacteria strains present in marine and freshwater ecosystems. *J Appl Phycol* 28, 2299–2314. <https://doi.org/10.1007/s10811-015-0774-3>.
- [72] Wu, Z.-X., Song, L., 2008. Physiological comparison between colonial and unicellular forms of *Microcystis aeruginosa* Kütz. (Cyanobacteria). *Phycologia* 47, 98–104. <https://doi.org/10.2216/07-49.1>.
- [73] Xin, X., Huang, G., Zhang, B., 2021. Review of aquatic toxicity of pharmaceuticals and personal care products to algae. *J Hazard Mater* 410, 124619. <https://doi.org/10.1016/j.jhazmat.2020.124619>.
- [74] Yisa, A.G., Chia, M.A., Sha'aba, R.I., Gauji, B., Gadzama, I.M.K., Oniye, S.J., 2021. The antibiotic ciprofloxacin alters the growth, biochemical composition, and antioxidant response of toxin-producing and non-toxin-producing strains of *Microcystis*. *J Appl Phycol* 33, 2145–2155. <https://doi.org/10.1007/s10811-021-02485-1>.
- [75] Zavřel, T., Očenášová, P., Červený, J., 2017. Phenotypic characterization of *Synechocystis* sp. PCC 6803 substrains reveals differences in sensitivity to abiotic stress. *PLoS One* 12, e0189130. <https://doi.org/10.1371/journal.pone.0189130>.
- [76] Zhang, X., Ai, S., Wei, J., Yang, X., Huang, Y., Hu, J., Wang, Q., Wang, H., 2022. Biphasic effects of typical chlorinated organophosphorus flame retardants on *Microcystis aeruginosa*. *Ecotoxicol Environ Saf* 241, 113813. <https://doi.org/10.1016/j.ecoenv.2022.113813>.
- [77] Zhang, Yonggang, Guo, J., Yao, T., Zhang, Yalei, Zhou, X., Chu, H., 2019. The influence of four pharmaceuticals on *Chlorella pyrenoidosa* culture. *Sci Rep* 9, 1–10. <https://doi.org/10.1038/s41598-018-36609-4>.
- [78] Zilliges, Y., Kehr, J.C., Meissner, S., Ishida, K., Mikkat, S., Hagemann, M., Kaplan, A., Börner, T., Dittmann, E., 2011. The cyanobacterial hepatotoxin microcystin binds to proteins and increases the fitness of *Microcystis* under oxidative stress conditions. *PLoS One* 6, e17615. <https://doi.org/10.1371/journal.pone.0017615>.

Publikacja – P6

Kwidzińska Klaudia, Bethke Katarzyna, Caban Magda*, Growth Inhibition and Beyond: Hormetic and Environmentally Relevant Effects of Pharmaceuticals on Cosmopolitan Cyanobacteria, Aquatic Toxicology, 2026, 108035
<https://doi.org/10.1016/j.aquatox.2026.108035>.



Growth inhibition and beyond: hormetic and environmentally relevant effects of pharmaceuticals on cosmopolitan cyanobacteria

Klaudia Kwidzińska, Katarzyna Bethke, Magda Caban*

University of Gdansk, Department of Environmental Analysis, Faculty of Chemistry, 63 Wita Stwosza St., 80-308, Gdansk, Poland

ARTICLE INFO

Keywords:

Pharmaceuticals pollutants
Cyanobacteria
Antibiotic resistance
Growth stimulation
Hormetic effect

ABSTRACT

Pharmaceutical residues are increasingly detected in aquatic environments as a result of continuous anthropogenic inputs. These compounds represent emerging contaminants capable of affecting primary producers, including cyanobacteria, which play key ecological roles in freshwater systems and are often responsible for harmful algal blooms. This review critically examines experimental studies evaluating the effects of pharmaceuticals on *Synechococcus* sp. and *Microcystis aeruginosa* - two cosmopolitan and widely investigated cyanobacteria. The available evidence indicates that pharmaceutical exposure can significantly influence cyanobacterial physiology in a species- and concentration-dependent manner. High concentrations of antibiotics typically inhibit cyanobacterial growth, whereas subinhibitory concentrations can induce hormesis, characterized by stimulated proliferation at low doses and inhibition at higher doses. Repeated exposure may additionally promote the development of antibiotic tolerance and resistance. Importantly, the bloom-forming species *M. aeruginosa* may respond to pharmaceutical-induced stress by increasing the production and release of microcystins even when growth inhibition is limited. Environmentally relevant concentrations of certain antibiotics, particularly fluoroquinolones, may therefore simultaneously stimulate cyanobacterial growth and toxin production, potentially altering microbial community structure and favouring bloom development. Because cyanobacteria in natural systems are exposed to complex contaminant mixtures, environmental risk assessment remains challenging. Further investigation of mixture toxicity is required to better evaluate the ecological implications of pharmaceutical pollution in aquatic ecosystems. Hormetic responses to pharmaceuticals require further investigation because they may trigger ecological effects whose long-term consequences remain uncertain.

1. Introduction

Environmental contamination by pharmaceuticals constitutes an increasingly significant global challenge. Advances in medical science, population growth, and rising life expectancy have led to a steady increase in the production and consumption of medicinal products. Intensive animal husbandry and aquaculture, driven by growing food demand, also contribute substantially to pharmaceutical emissions (Audebrand et al., 2016). Pharmaceutical pollution primarily affects the aquatic environment, where these compounds enter through multiple pathways. For most pharmaceuticals, effluent discharge from wastewater treatment plants represents the principal source (Caban and Stepnowski, 2021). Although many pharmaceuticals undergo degradation within days or weeks, their continuous input into aquatic systems results in pseudo-persistence, raising considerable ecological concern.

Pharmaceuticals could potentially exert significant effects on aquatic organisms, given that they are designed to elicit biological activity at low concentrations. However, most of these impacts on aquatic species at environmentally relevant concentrations have been investigated under laboratory conditions (Khan and Barros, 2023; Pinto et al., 2022). Antibiotics are of particular concern, not only because they are among the most frequently detected pharmaceutical classes in the environment, but also due to their role in promoting the spread of antimicrobial resistance (Arpin-Pont et al., 2016). Analgesic and anti-inflammatory drugs, such as diclofenac, ibuprofen, and paracetamol, are also commonly detected in surface waters (Huynh et al., 2023; Ortúzar et al., 2022). Their widespread occurrence is largely attributable to high consumption rates, over-the-counter availability, and extensive use in both human and veterinary medicine (Lakshmi et al., 2024). Even at low concentrations, these compounds have been shown to induce toxic effects in various aquatic organisms, including fish, am-

* Corresponding author.

E-mail address: magda.caban@ug.edu.pl (M. Caban).

<https://doi.org/10.1016/j.aquatox.2026.108035>

0166-445/© 20XX

phibians, and molluscs (Swiacka et al., 2021). Reported effects include organ damage, impaired hatching, oxidative stress, neurotoxicity, and endocrine disruption (OECD, 2019a). In addition, anticancer agents, psychotropic drugs, hormonal pharmaceuticals, estrogens, and β -blockers have also been detected in aquatic environments (Gworek et al., 2020). These substances are associated with diverse potential adverse effects in aquatic organisms, including behavioural alterations, feminization, phytotoxicity, organ damage, and metabolic disturbances. The term "potential" reflects the fact that these adverse effects depend heavily on exposure conditions, such as the specific concentration of the pharmaceutical and the duration of exposure. Additionally, biological sensitivity varies significantly across different species, meaning an effect observed in one organism may not occur in another. Thus, using "potential" acknowledges the scientifically established risk without claiming that every pharmaceutical automatically causes harm to all aquatic life under all natural conditions.

The occurrence of pharmaceutical residues in aquatic ecosystems poses potential direct and indirect risks to human health. Certain compounds can bioaccumulate in aquatic organisms, potentially leading to chronic exposure of human through the consumption of fish and seafood (OECD, 2019a; Puckowski et al., 2016). Environmental and human health are intrinsically interconnected (Shetty et al., 2023). Therefore, comprehensive investigation of the ecological impacts of pharmaceuticals is imperative (Eapen et al., 2024). This includes research on both higher trophic-level organisms, and primary producers such as phytoplankton. The second one form the foundation of aquatic food webs and play a critical role in primary production, nutrient and energy cycling, and oxygen generation, thereby underpinning the functioning and stability of entire aquatic ecosystems. Among primary producers, eukaryotic microalgae and cyanobacteria constitute distinct groups of photosynthetic microorganisms. Although cyanobacteria were historically referred to as "blue-green algae", they are photosynthetic prokaryotes and are not taxonomically classified as microalgae. However, both groups are sometimes considered together in ecological studies due to their functional similarities. (Daneshvar et al., 2021; Miazek and Brozek-Pluska, 2019). In addition, in selected studies both green algae and cyanobacteria are considered as microalgae due to ecological similarities (Vonshak and Torzillo, 2003).

Cyanobacteria, commonly referred to as blue-green algae, are oxygenic photoautotrophic prokaryotes that constitute a substantial component of freshwater and marine phytoplankton communities (Hoiczky and Hansel, 2000). Phylogenetically, cyanobacteria are recognized as the earliest oxygenic photosynthesizers, and their metabolic activity has been pivotal in shaping Earth's atmosphere, contributing significantly to global oxygen production (Sánchez-Baracaldo et al., 2022). Cyanobacteria are characterized by considerable ecological plasticity. As Gram-negative bacteria with diverse physiological capabilities, they can colonize oligotrophic and otherwise extreme environments. Their evolutionary persistence over approximately 3 billion years is attributed to a suite of adaptive traits, including resistance to desiccation, tolerance to high temperatures, and the capacity to withstand elevated salinity and acidity. Moreover, their ability to exploit a broad spectrum of photosynthetically active radiation, coupled with nitrogen fixation in diazotrophic species, enhances their competitive advantage under nutrient-limited conditions (Mazur-Marzec et al., 2009).

Despite their ecological importance, cyanobacteria are also associated with adverse environmental impacts. Under favourable conditions, particularly in nutrient-enriched waters, they can form harmful algal blooms (HABs) that disrupt ecosystem functioning. Such blooms contribute to hypoxia through increased organic matter production and subsequent microbial decomposition, thereby exacerbating eutrophication processes (Huisman et al., 2018). In addition, numerous bloom-forming species synthesize and release cyanotoxins, including hepatotoxins, neurotoxins, and cytotoxins, which pose significant risks to

aquatic biota, terrestrial animals, and human health (Catherine et al., 2013).

Given their ubiquitous distribution in aquatic environments, cyanobacteria are continuously exposed to a wide range of anthropogenic contaminants, including pharmaceuticals and other emerging pollutants (dos Santos Costa et al., 2021). Considering their central role in biogeochemical cycling, primary production, and water quality, as well as their economic relevance in the context of ecosystem services and water resource management, comprehensive assessment of cyanobacterial responses to environmental contaminants is essential for accurate ecological risk evaluation and sustainable ecosystem management.

Pharmaceuticals comprise a structurally and functionally heterogeneous group of compounds, characterized by wide variability in physicochemical properties, modes of action, and environmental fate. Despite their frequent detection in aquatic systems, the prioritization of pharmaceutical toxicity with respect to cyanobacteria remains inadequately addressed. Current evidence indicates that cyanobacteria exhibit pronounced sensitivity to antibiotics, which is mechanistically consistent with their prokaryotic cellular organization and the conservation of antibiotic molecular targets (e.g., ribosomal subunits and DNA gyrase) (Ando et al., 2007; Wan et al., 2024; Wang et al., 2018).

An unresolved and ecologically relevant issue concerns the potential stimulatory effects of sublethal or environmentally relevant concentrations of pharmaceuticals. Hormetic or growth-promoting responses have been documented for several classes of antibiotics, including β -lactams (amoxicillin, ampicillin), fluoroquinolones (enrofloxacin), sulfonamides (sulfamethoxazole, sulfisoxazole), and macrolides (erythromycin, spiramycin) (Chen et al., 2025; Fang and Liu, 2023; Liu et al., 2016; Wang et al., 2019; Zhang et al., 2020), at the environmentally relevant concentrations. Comparable stimulatory effects have also been reported for non-steroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen (Sha'aba et al., 2023), as well as for local anaesthetics, including lidocaine, procainamide, tetracaine, dibucaine, and procaine (Suzuki et al., 2004). Such biphasic dose-response patterns may potentially enhance cyanobacterial growth, alter community structure, and potentially facilitate bloom development under environmentally realistic exposure scenarios. Consequently, elucidating concentration-dependent effects of pharmaceuticals on cyanobacteria is essential for accurate ecological risk assessment and for understanding their role in bloom dynamics in contaminated aquatic systems.

Accordingly, the objective of this review is to synthesize and critically evaluate the current knowledge regarding the effects of pharmaceuticals on cyanobacteria. The literature analysis focuses on two cosmopolitan and ecologically relevant species, *Microcystis aeruginosa* and *Synechococcus* sp., which are among the most extensively investigated cyanobacteria in ecotoxicological research. Their broad distribution, ecological significance, and the availability of experimental data enable comparative assessment and facilitate toxicity prioritization across pharmaceutical classes. Previous reviews (Table 1) have summarized the impacts of pharmaceuticals on cyanobacteria and phytoplankton communities (Couto et al., 2022; dos Santos Costa et al., 2021; Guo et al., 2015; Hejna et al., 2022; Kock et al., 2023; Lu et al., 2021; Miazek and Brozek-Pluska, 2019; Obaid et al., 2023; Xin et al., 2021), among other organisms. However, the novelty of the present review lies in its integrative and comparative analysis of pharmaceutical toxicity specifically toward *Microcystis aeruginosa* and *Synechococcus* sp. The study compiles and systematically evaluates reported EC₅₀ values, emphasizing inter-study variability and discrepancies in toxicity estimates for individual compounds. In addition, particular attention is devoted to the effects observed at environmentally relevant concentrations, including evidence of stimulatory or hormetic responses at sublethal exposure levels. By contextualizing experimental toxicity data within measured or predicted environmental concentrations, this review advances a more ecologically realistic risk characterization framework. This per-

Table 1

List of available (March 2026) reviews in ecotoxicological effects of pharmaceuticals toward photosynthetic microorganisms, among other organisms.

Photosynthetic group	Title	Year	Ref.
Microalgae and cyanobacteria	Analysis, Fate and Removal of Pharmaceuticals in the Water Cycle - Chapter 3.3 Ecotoxicity of pharmaceuticals	2007	(Jones et al., 2007)
Microalgae and cyanobacteria	Do Pharmaceuticals Pose a Threat to Primary Producers?	2015	(Guo et al., 2015)
Cyanobacteria	Cyanobacteria As Bioindicators And Bioreporters Of Environmental Analysis In Aquatic Ecosystems	2015	(Mateo et al., 2015)
Microalgae	Effect of PHRs and PCPs on Microalgal Growth, Metabolism and Microalgae-Based Bioremediation Processes: A Review	2019	(Miazeck and Brozek-Pluska, 2019)
Microalgae	Review Of Aquatic Toxicity Of Pharmaceuticals And Personal Care Products To Algae	2020	(Xin et al., 2021)
Cyanobacteria	The Link Between Pharmaceuticals And Cyanobacteria: A Review Regarding Ecotoxicological, Ecological, And Sanitary Aspects	2021	(dos Santos Costa et al., 2021)
Microalgae and cyanobacteria	The Toxic Effects Of Antibiotics On Freshwater And Marine Photosynthetic Microorganisms: State Of The Art	2021	(Sharma et al., 2021)
Microalgae	Review Of Aquatic Toxicity Of Pharmaceuticals And Personal Care Products To Algae	2021	(Xin et al., 2021)
Microalgae and cyanobacteria	Pollutant Toxicology With Respect To Microalgae And Cyanobacteria	2021	(Lu et al., 2021)
Microalgae and cyanobacteria	Environmental Fate And Effects Assessment Of Human Pharmaceuticals: Lessons Learnt From Regulatory Data	2021	(Schwarz et al., 2021)
Microalgae and cyanobacteria	Occurrence and Effects of Antimicrobials Drugs in Aquatic Ecosystems	2021	(Fernandez et al., 2021)
Microalgae	Toxic Effect of Antibiotics on Freshwater Algal Systems and the Mechanisms	2021	(Roy and Suresh, 2021)
Algae	of Toxicity: A Review Pharmaceuticals in the Aquatic Environment: A Review on Ecotoxicology and the Remediation Potential of Algae	2022	(Hejna et al., 2022)
Microalgae and aquatic macrophytes	The Potential Of Algae And Aquatic Macrophytes In The Pharmaceutical And Personal Care Products (PCPs) Environmental Removal: A Review	2022	(Couto et al., 2022)
Microalgae and cyanobacteria	An Overview of the Impact of Pharmaceuticals on Aquatic Microbial Communities	2022	(Pinto et al., 2022)
Diatom	Emerging Challenges Of The Impacts Of Pharmaceuticals On Aquatic Ecosystems: A Diatom Perspective	2023	(Kock et al., 2023)
Microalgae	Review on Toxicity and Removal of Pharmaceutical Pollutants Using Immobilised Microalgae	2023	(Obaid et al., 2023)
Cyanobacteria	Do Pharmaceuticals Affect Microbial Communities In Aquatic Environments? A Review	2023	(Świacka et al., 2023)
Microalgae and cyanobacteria	How Do Freshwater Microalgae And Cyanobacteria Respond To Antibiotics?	2023	(Le et al., 2023)
Microalgae, aquatic and terrestrial plants	Drugs In The Environment - Impact On Plants: A Review	2024	(Pawłowska and Biczak, 2024)
Microalgae and cyanobacteria	Assessment Of Ecological Risks Posed By Veterinary Antibiotics In European Aquatic Environments: A Comprehensive Review And Analysis	2024	(Albarano et al., 2024)

Table 1 (continued)

Photosynthetic group	Title	Year	Ref.
Microalgae	Aquatic Ecotoxicology of Antiretrovirals: A Review	2025	(Souza-Silva et al., 2025)
Cyanobacteria	Effects of Antibiotics at Environmental Concentrations on Cyanobacteria and its Mechanisms: A Review Focusing on Hormesis	2025	(Wang and Tao, 2025)
Cyanobacteria	Interactions Between Cyanobacteria And Emerging Contaminants In Aqueous Environments	2026	(Yang et al., 2026)

spective addresses a critical gap in conventional ecotoxicological assessments, which predominantly emphasize growth inhibition endpoints while often overlooking subinhibitory effects. Based on the synthesis of available studies, conventional ecotoxicological assessments focused on EC₅₀ based growth inhibition may overlook environmentally relevant concentrations that do not inhibit cyanobacterial growth but can instead stimulate proliferation and toxin production, representing a potential ecological risk.

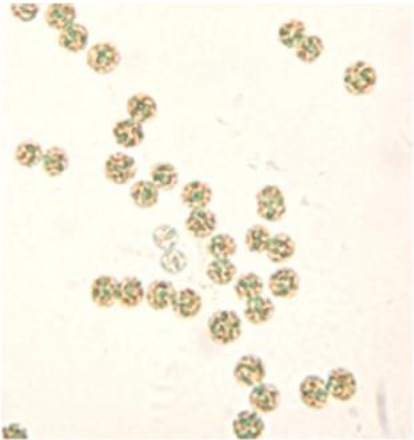
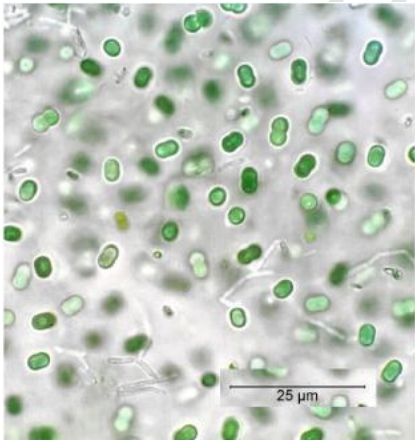
2. *Microcystis* and *Synechococcus* as cosmopolitan cyanobacteria species

Cosmopolitan cyanobacterial taxa exhibit a global distribution, occurring across a broad range of latitudinal gradients and climatic regimes. Their global distribution is caused by broad ecological tolerance (eurytopic behaviour, including wide temperature, light, salinity, and pH ranges), high physiological plasticity, enabling acclimation to fluctuating environmental conditions, and efficient dispersal mechanisms, such as transport via water currents, wind, migratory birds, or anthropogenic vectors. In addition, traits such as rapid reproduction and buoyancy regulation may contribute to bloom formation (Kromkamp et al., 1988). Furthermore, toxin production in certain cyanobacterial taxa may offer a competitive advantage under specific conditions, aiding in population maintenance rather than guaranteeing survival.

A representative example is *Microcystis aeruginosa*, which has been reported to bloom on every continent except Antarctica (Zurawell et al., 2005). Its cell diameters typically range from 2 to 7 µm (Table 2). In natural environments, cells are commonly embedded within mucilaginous colonies composed of extracellular polymeric substances (EPS), which confer structural integrity and provide protection against grazing pressure and other environmental stressors (Le et al., 2022). This species inhabits diverse aquatic ecosystems, with a marked predominance in freshwater environments, including ponds, lakes, rivers, and lagoons (Harke et al., 2016). Under eutrophic conditions, *M. aeruginosa* frequently outcompetes co-occurring phytoplankton through a combination of physiological plasticity, buoyancy regulation via gas vacuoles, and efficient nutrient uptake strategies. These traits facilitate the formation of dense HABs. During bloom events, selected strains of *M. aeruginosa* that are capable of producing microcystins can substantially increase the biosynthesis of these toxins (Cai et al., 2025; Pineda-Mendoza et al., 2016). Microcystins are a class of cyclic hepatotoxic heptapeptides that pose significant ecological and public health risks (Ye et al., 2026). Owing to its ecological dominance, bloom-forming capacity, and the ability of certain strains to produce microcystins, *M. aeruginosa* is among the most extensively studied cyanobacterial species in aquatic ecology and limnology (Reynolds et al., 1980). In this review, we found reports only on *M. aeruginosa* among *Microcystis* sp.

Another broadly distributed and intensively investigated cyanobacterial taxon is *Synechococcus* sp. *Synechococcus* is one of the most abundant photoautotrophic picoplankton in the marine ecosystem with nearly 20% contribution to oceanic primary production (Flombaum et al., 2013). In only one study, the 46 strains of *Synechococcus* were iso-

Table 2
The basic characteristics and differences between *Microcystis* sp. and *Synechococcus* sp. (EPS - extracellular polymeric substances).

Feature	<i>Microcystis</i> sp.	<i>Synechococcus</i> sp.
Exemplary species	<i>M. aeruginosa</i>	<i>Synechococcus leopoliensis</i> (former name <i>Synechococcus elongatus</i>)
Microscopic pictures		
Cell size	Own photo 2-7 µm Colony size 3-40 mm in diameter Thick EPS	and Guiry, 2020) < 2 µm (picophytoplankton) (Callieri, 2008) Thin EPS
Morphology	Unicellular or colony forming (Singh et al., 2020). Colonial morphology varies from small aggregates to large, irregular mucilaginous masses. Gas vacuoles that enable vertical migration within the water column, optimizing access to light and nutrients (Kromkamp et al., 1988).	Unicellular. Cells are usually solitary, spherical to ovoid, and lack large mucilaginous colonial structures.
Habitat	Freshwater, lagoons, especially in eutrophic systems (Harke et al., 2016).	Freshwater, coastal, and saline waters. Occupies both oligotrophic and mesotrophic systems.
Toxin productions	Microcystins (Harke et al., 2016; Krishnan and Mou, 2021).	Seldom form toxic blooms (Stockner et al., 2000, chap. 7). Can produce cylindrospermopsin and anatoxin-a (Gin et al., 2021).
Ecology	Can be a nuisance and harm other organisms; forms dense harmful algal blooms (HABs), often surface scums; capable of dominating phytoplankton biomass.	Can reach high cell densities but typically does not form macroscopic surface scums; contributes substantially to picophytoplankton biomass and primary production.
Other characteristics	Low cell surface-area-to-volume ratio - high storage capacity	High cell surface-area-to-volume ratio - high nutrient uptake; Easily adapted, ubiquitous organism

lated in the Baltic Sea and showed considerable variation in their cell size and pigmentation phenotypes (red, pink and blue-green strains) (Haverkamp et al., 2009). In the literature reviewed, *Synechococcus leopoliensis* and *Synechococcus elongatus* were the target of research. However, *S. leopoliensis* is largely an outdated taxonomic name, and many strains previously assigned to it are now classified as *S. elongatus* based on molecular phylogeny (Golden, 2019). The freshwater cyanobacterium *S. elongatus* includes several laboratory strains previously classified under different names, including *S. leopoliensis* and *Anacystis nidulans*. Taxonomic revisions based on phylogenetic and genetic analyses demonstrated that these strains belong to the same lineage now recognized as *S. elongatus* (Robertson et al., 2001). Members of this genus are unicellular picocyanobacteria and are classified within the picoplankton owing to their small cell size, typically ranging from approximately 0.6-2 µm in diameter. Cells are generally spherical to ovoid and exhibit a high surface-area-to-volume ratio, which enhances nutrient uptake efficiency in oligotrophic environments.

Species of *Synechococcus* display pronounced ecological versatility. Their tolerance to a wide spectrum of physicochemical stressors - including elevated pH, temperature variability, and fluctuations in light intensity - combined with high specific growth rates, enables them to compete effectively with other phytoplankton taxa and, under favourable conditions, to dominate microbial communities. Picocyanobacteria, such as *Synechococcus*, have been reported to dominate following H₂O₂ treatment in lake systems, suggesting a high level of resistance to oxidative stress (Hancock et al., 2024).

Representatives of this genus occupy an extensive range of aquatic habitats, encompassing freshwater, estuarine, coastal, and open-ocean systems (Dvořák et al., 2015). Their broad thermal niche permits persis-

tence across extreme temperature gradients, from geothermal springs to polar marine environments (Papke et al., 2003). They play a significant ecological role, especially in oceans, where they are among the key primary producers. *Synechococcus* sp. is an important experimental model organism that grows well in various culture types, allowing for easy cultivation under laboratory conditions (Nägeli, 1849). *S. elongatus* (picture in Table 2) is a reference test organism included in the Organisation for Economic Co-operation and Development (OECD) Test Guidelines 201 (OECD, 2011).

Microcystis sp. and *Synechococcus* sp. can co-occur during cyanobacterial bloom events (Te et al., 2023). Their co-existence is shaped, in part, by interspecific competition for limiting nutrients, particularly nitrogen (Tan et al., 2019a) and phosphorus (Tan et al., 2019b).

Notably, microcystin production by toxin-producing strains of *M. aeruginosa* can be mitigated through co-cultivation with rapidly growing, non-toxic cyanobacteria. For example, *S. elongatus* UTEX 2973 has been shown to significantly reduce both the biomass of toxin-producing strains *M. aeruginosa* and their microcystin production (Lee et al., 2024). This suppression is likely mediated by competitive interactions for limiting nutrients and light, as well as potential allelopathic or metabolic interference mechanisms. Such findings highlight the potential application of fast-growing, benign cyanobacterial strains as biological control agents for managing harmful algal blooms and associated cyanotoxin risks.

In summary (Table 2), *M. aeruginosa* is a colony-forming, bloom-dominant, toxin-producing cyanobacterium primarily associated with eutrophic freshwater systems and characterized by buoyancy regulation and EPS-mediated protection (Le et al., 2022). In contrast, *Synechococcus* sp. comprises small, mostly solitary picocyanobacteria with

high nutrient uptake efficiency, broad habitat distribution, and a major role in global primary production, particularly in oligotrophic aquatic ecosystems. Their contrasting morphologies and ecological strategies underpin distinct yet sometimes overlapping niches in aquatic microbial communities.

3. Ecotoxicological test protocols and hormetic response

Ecotoxicological studies are designed to evaluate the effects of chemical substances on individual organisms or defined groups of organisms. These investigations are generally categorized into laboratory and field studies. Most ecotoxicological research on cyanobacteria is conducted under laboratory conditions, where environmental variables are strictly controlled and experiments typically focus on a single species or strain. Such controlled settings enable precise dose-response assessments and mechanistic insights. In contrast, field studies assess impacts at the community or consortium level under environmentally realistic conditions, where multiple biotic interactions and fluctuating abiotic factors introduce greater complexity and variability (Te et al., 2023; Xiong et al., 2017). Both approaches are valid in ecotoxicology.

Based on exposure duration, ecotoxicological assays are commonly classified as acute or chronic. Acute tests involve short-term exposures, typically at relatively high concentrations, and are designed to capture immediate or sublethal effects. Chronic tests, in contrast, extend over longer periods - often at lower concentrations of pollutant - and aim to detect effects on long-term endpoints, such as biomarkers of stress or reproduction. In cyanobacterial ecotoxicology, however, the distinction between acute and chronic exposure is not always clear-cut. There is no universally standardized exposure threshold across all ecotoxicological frameworks, as classifications often depend on exposure duration relative to an organism's generation time, the experimental endpoints evaluated, and guidelines such as those from the OECD. In rapidly proliferating microalgae or cyanobacteria during the exponential growth phase, standard test durations (e.g., a 72 h exposure in OECD test guidelines) encompass multiple cell generations. Consequently, such multi-generational durations allow physiological adaptation and compensatory responses to occur within timeframes that might otherwise be considered short-term for slower-growing organisms. For instance, Lee et al. (2022) categorized a 72 h exposure as chronic due to the multi-generational timeframe for cyanobacteria, while an 8 h exposure was evaluated as acute.

To ensure comparability and regulatory acceptance of ecotoxicological data, standardized test guidelines have been developed that define experimental design, exposure conditions, endpoints, and data evaluation procedures in a harmonized manner. A one of the principal body responsible for establishing such standards is the OECD. The guideline most widely applied in ecotoxicological testing of cyanobacteria is OECD Test Guideline 201, entitled: 'Freshwater Alga and Cyanobacteria, Growth Inhibition Test' (OECD, 2011). This protocol describes a standardized method for assessing the effects of chemical substances on the growth of freshwater microalgae and cyanobacteria. The test is designed to evaluate growth inhibition over a short-term exposure period (72-96 h), and is often considered acute, although it captures effects across multiple cell generations. Protocol OECD 201 specifies recommended test species, defines the physicochemical and biological conditions under which the assay must be conducted, and details key methodological parameters. These include test duration, composition of the growth medium, light and temperature regimes, procedures for biomass quantification (e.g., cell density or optical density (OD) measurements), criteria for test validity, use of reference substances to confirm test performance, and standardized approaches for statistical analysis and endpoint calculation (e.g., EC₅₀, NOEC where applicable).

In addition to OECD 201, other standardized protocols are occasionally employed, although far less frequently. These include guidelines issued by the International Organization for Standardization (ISO), such

as ISO 8692, 2012 (ISO 8692, 2012), entitled: 'Water quality - Fresh water algal growth inhibition test with unicellular green algae'. This standard outlines procedures comparable to OECD 201 but are primarily focused on unicellular green algae. Another relevant framework is provided by the United States Environmental Protection Agency (US EPA), specifically OPPTS 850.5400, entitled: 'Algal Toxicity, Tiers I and II' (United States Environmental Protection Agency, 1996), which describes tiered testing approaches for evaluating algal toxicity under regulatory contexts in the United States.

Despite the availability of these harmonized protocols, adherence in the scientific literature is not always strict. Modifications to exposure conditions, test duration, endpoints, or analytical methods are common, particularly in academic research. Such deviations, while sometimes justified by specific research objectives, contribute to substantial variability in reported toxicity values, even for the same species and pharmaceutical compounds, thereby complicating cross-study comparisons and meta-analytical assessments.

The most commonly measured test endpoint is the increase in cyanobacterial biomass/cell density, assessed spectrophotometrically by OD, cytometrically by counting cell numbers, or indirectly by determining chlorophyll *a* extracted from cells. The biomass change is then recalculated to growth rate (per day). The final toxic/stimulative effect is calculated by comparison of growth rate for control and exposed cultures. Additionally, physiological and biochemical changes can be examined, including oxidative stress indicators such as reactive oxygen species (ROS) production, malondialdehyde (MDA) content, and antioxidant enzyme activities (peroxidase (POD), catalase (CAT), superoxide dismutase (SOD), glutathione (GSH), and glutathione S-transferase (GST)). Photosynthetic efficiency is evaluated using the Fv/Fm parameter. The content of photosynthetic pigments, including chlorophylls, carotenoids, and phycobilins, is also measured. Biochemical composition changes in cells - such as lipid, protein, and carbohydrate content - are assessed, as well as the production and release of cyanotoxins. However, none of the mentioned reference protocols demand biomarker analysis.

Multiple ecotoxicological studies across algae species have documented low-dose stimulation and high-dose inhibition, a hallmark of hormesis. Reviews in the environmental literature show that hormetic responses occur frequently in freshwater and marine algal taxa exposed to a wide range of contaminants (herbicides, antibiotics, nanoparticles, and other pollutants) (Cedergreen et al., 2007). For example, recent research indicates that low concentrations of antibiotics (1×10^{-4} – 2×10^{-4} mg L⁻¹) such as sulfamethoxazole can stimulate *M. aeruginosa* cell density, with higher concentrations inhibiting growth (Liu et al., 2017). Antibiotics also induce hormetic response in green algae (Ma et al., 2021). Low-dose stimulation of the growth of the harmful alga, *Prymnesium parvum*, by glyphosate and glyphosate-based herbicides was also reported (Dabney and Patiño, 2018). Certain halogenated organic pollutants, including quinolones (which are structurally related to fluoroquinolone antibiotics), have induced marked hormetic stimulation of *M. aeruginosa* proliferation at lower doses (Yueheng Zhang et al., 2022).

There are several mechanistic bases of hormesis in phototrophic microorganisms. Hormetic responses are often associated with redox signalling, when low-level ROS generation acting as signalling molecules (Calabrese et al., 2011; Foyer and Noctor, 2005). In addition, hormesis can be affected by activation of antioxidant defences, for example by upregulation of CAT, SOD, or GSH-related systems (Mittler, 2002; Sharma et al., 2012). The increased chlorophyll or carotenoid synthesis under mild stress was also observed as photosynthetic acclimation (Ahmad et al., 2013; Demmig-Adams and Adams, 1996). Stress-induced gene expression (for example, induction of heat shock proteins and repair enzymes) also contributes to hormetic outcomes (Gao et al., 2013). In general, it is probable that hormesis occurs for chemicals with multiple molecular targets in a single cell (Calabrese and Baldwin, 2002).

The hormetic effect can be also caused by so-called compensatory cell division (overcompensation following slight metabolic disruption) (Calabrese et al., 2007). Finally, hormetic responses reflect both direct stimulatory or overcompensation responses to damage induced by relatively low doses of pollutant (Calabrese, 2013). The mechanisms of hormetic response of cyanobacteria to antibiotics is reviewed by Wang and Tao (2025).

The hormetic effects complicate risk assessment because they produce non-monotonic concentration-response curves, challenging classical toxicological models (Mattson and Calabrese, 2010). However, the investigation of hormetic effect is crucial for cyanobacteria, because it can contribute to algal blooms (Yueheng Zhang et al., 2022). Standard endpoints (e.g., EC₅₀) may fail to capture ecologically relevant sub-stimulatory effects. As most research on plants focus on adverse effects, while hormesis is normally only commented on as outliers relative to the sigmoid dose-response curve (Cedergreen et al., 2007). In standard protocols, the hormetic response is challenging to assess. According to OECD 201, any significant growth stimulation needs to be reported. However, most standard software (like Probit or Logit models) assumes a monotonic decrease (a straight downward curve). If hormesis is present, these models will give you an "ill-fit" or an incorrect EC₅₀. To fix this, researchers often use the Brain-Cousens model, which is a modified logistic equation specifically designed to account for that "hump" at the start of the curve (Brain and Cousens, 1989; Klátyik et al., 2024). If the low-dose group grows significantly higher than the control, determining the NOEC becomes tricky. Usually, regulators treat the stimulatory dose as "no adverse effect," but it can complicate the statistical power of the test (Environment Canada, 2007). If the stimulation is extreme (e.g., >20% higher than control), may indicate an issue with the test medium or that the test substance is being used as a primary carbon/nutrient source, which could invalidate the "toxicity" aspect of the study (EFSA PPR Panel, 2013; OECD, 2019b). As presented in Table 3, the observed stimulative effect can be classified according to its extend. The true hormetic response is observed after about 10 to 20% enhancement in grow rate, while higher stimulation is rather an effect of nutrient enrichment by tested substance.

The experimental design for determination and tracking of hormesis has already been proposed by Pozuelo-Campos et al. (2023). It is indicated that a traditional setup of 5-6 concentrations with a dilution factor of 2 or 3 lacks sufficient statistical power to detect subtle deviations (stimulation) in the sub-inhibitory range. They recommend increasing

the number of concentrations to at least 8-12 and increasing dose density below the predicted EC₁₀ threshold. It is also recommended that measurements be taken every 24 hours, as hormesis is a dynamic process (Calabrese, 2001). During the adaptive phase (early stress response), an immediate metabolic surplus is frequently observed. The optimal measurement window spans from 48 to 72 h, representing the peak phase of compensation and stimulation of cell division (Calabrese, 2007). In the late phase (96 h to 7 days), stimulation may subside due to nutrient depletion in the test vessel or transition into delayed toxicity (Zhu et al., 2013).

The model for hormesis determination are under development and improvement (Zhu et al., 2013). Reporting hormetic effects in ecotoxicity studies on cyanobacteria is critical because environmental concentrations of pharmaceuticals often fall within low-dose ranges that paradoxically stimulate rather than inhibit cyanobacterial cell growth and division. This sub-lethal stimulation can accelerate harmful algal blooms and trigger the overproduction of dangerous cyanotoxins, posing severe risks to aquatic ecosystems and human health. Consequently, documenting these non-monotonic dose-response patterns prevents the underestimation of ecological risks that standard linear models typically fail to capture.

4. Effect of pharmaceuticals on cyanobacteria

4.1. Antibiotics and cyanobacterial antibiotics resistance

Antibiotics are pharmaceutical agents used against bacterial infections and, as such, can be particularly toxic to cyanobacteria, as they are classified as bacteria based on their cellular structure (Castenholz, 2015). However, cyanobacteria possess several important features that distinguish them from other bacteria. One of these is the structure of the cell wall. Based on cell wall structure, bacteria are divided into Gram-positive and Gram-negative groups. Gram-positive bacteria are characterized by a multilayered peptidoglycan sheath with a thickness of 20-40 nm (approximately 50% of the weight of walls), whereas Gram-negative bacteria have a significantly thinner cell wall measuring 2-6 nm (10%-20% of wall mass) (Allison and Lambert, 2024; Rogers et al., 1980). Cyanobacteria are classified as Gram-negative bacteria; however, their cell wall structure exhibits feature of both types. Despite the overall Gram-negative structure, cyanobacteria have a substantially thicker peptidoglycan layer. The cyanobacterial cell wall may also differ in the degree of cross-linking between peptidoglycan chains, as reported for *Synechocystis* sp. strain PCC 6714, in which the degree of cross-linking is significantly higher (56-63%) than that typically observed in Gram-negative bacteria (20-30%) (Hoiczky and Hansel, 2000). Thus, in this respect, cyanobacterial cells resemble Gram-positive bacterial cells more closely. These structural differences may influence the toxicity of specific antibiotics toward cyanobacteria compared to other Gram-negative bacteria. Antibiotics differ in their mechanism of action. They can cause cell death or be bacteriostatic agents, which inhibit microbial reproduction (Balaban et al., 2019). The primary mechanisms of antibiotic action include inhibition of cell wall synthesis, disruption of cell membrane structure and function, inhibition of protein synthesis, or blockage of key metabolic pathways, among others. Antibiotics belonging to the same structural class exhibit similar properties and, consequently, similar mechanisms of action. Therefore, their toxicity toward particular types of microorganisms may also be comparable (Etebu and Ibemologi, 2016). The most well-known molecular classes of antibiotics include beta-lactams, macrolides, tetracyclines, quinolones, aminoglycosides, sulfonamides, and glycopeptides (Van Hoek et al., 2011). The mechanisms of action of these pharmaceuticals, the spectrum of activity, and EC₅₀ value ranges are summarized in Table S1. The literature reviewed in this study (Tables 4 and 5) indicates that antibiotics are the most extensively studied group of pharmaceuticals and exhibit the highest toxicity toward

Table 3
The hormesis in ecotoxicological stimulation effect framework.

Observed stimulative effect	Scientific interpretation	Regulatory or methodological reference
0-10% stimulation	Statistical noise / low hormesis. Minor variation within the biological range of the control.	OECD 201 (2011): Section 9 notes that only "statistically significant" stimulation must be reported.
10-20% stimulation	True hormetic response. A compensatory biological stimulus due to low-level chemical stress.	Cedergreen et al. (2007) define principles of hormesis. Brain and Cousens (1989) defines the biphasic model. Klátyik et al. (2024) documents SOD/CAT enzyme activation at these levels.
> 20% stimulation	Nutrient enrichment / masking. The test substance likely provides N, P, or C, overriding the medium's balance.	Environment Canada (2007) warns that excess nutrients mask toxicity. OECD GD 23 (2019b) addresses "difficult substances" that act as nutrients.
Non-monotonic curve	Incompatibility with linear models. Standard Probit/Logit models will yield an "ill-fit" or inflated EC ₅₀ .	EFSA PPR Panel (2013) recommends specific tiered modelling (like Brain-Cousens) for non-monotonic curves.

Table 4

Impact of pharmaceuticals on *Synechococcus* sp. (n.t.- not tested; ROS – reactive oxygen species; POD peroxidase, CAT - catalase; SOD - superoxide dismutase; GSH - glutathione, and GST - glutathione S-transferase; MDA - malondialdehyde).

Cyanobacteria species (names given in report)	Pharmaceutical class and representative	Concentrations tested	Test condition			Impact on growth (measurement)	Biochemical markers tested	Ref.
			Exposure time	A. light intensity [$\mu\text{mol m}^{-2} \text{s}^{-1}$]; B. photoperiod (day/night); C. temp.; D. medium	Reference protocol			
<i>Synechococcus leopoliensis</i>	Antibiotic - Fluoroquinolone Enrofloxacin	2×10^{-4} - 2×10^{-2} mg L ⁻¹	96 h	A, B, C - not given D. BG11 medium	Not applied	Growth inhibition at 1.0×10^{-2} - 2.0×10^{-2} mg L ⁻¹ Growth stimulation at 2×10^{-4} - 5×10^{-3} mg L ⁻¹ (OD ₆₈₀)	photosynthesis biomass lipid productivity ultra-structure metabolomic profile	(Fang and Liu, 2023)
<i>Synechococcus leopoliensis</i>	Antibiotic - Fluoroquinolone Ofloxacin	Not specified	96 h	A. 116.2 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 16/8 h; C. 28 °C; D. Bold's medium;	Not applied	Acute toxicity: EC ₅₀ = 1.6×10^{-2} mg L ⁻¹ Chronic toxicity: NOEC = 5×10^{-3} mg L ⁻¹ (OD ₅₅₀ and cell count)	n.t.	(Ferrari et al., 2004)
<i>Synechococcus leopoliensis</i>	Antibiotic - Fluoroquinolone Norfloxacin	Not specified	144 h	A. 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 24/24 h C, D – not given	OECD 201	NOEC = 1.6×10^{-1} mg L ⁻¹ EC ₅₀ = 6.3×10^{-1} mg L ⁻¹ MIC > 5.0 mg L ⁻¹ (OD ₆₅₅)	n.t.	(Ando et al., 2007)
<i>Synechococcus</i> sp.		Not specified	144 h	A. 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 24/24 h; C, D – not given	OECD 201	NOEC = 1.6×10^{-1} mg L ⁻¹ EC ₅₀ = 6.3×10^{-1} mg L ⁻¹ MIC > 5.0 mg L ⁻¹ (OD ₆₅₅)	n.t.	(Ando et al., 2007)
<i>Synechococcus leopoliensis</i>	Antibiotic - Fluoroquinolone Ciprofloxacin	2×10^{-4} - 2×10^{-2} mg L ⁻¹	96 h	A, B, C – not given; D. BG11 medium	Not applied	Growth stimulation at 2×10^{-4} - 2×10^{-2} mg L ⁻¹ (OD ₆₈₀)	photosynthesis biomass lipid productivity ultra-structure metabolomic profile	(Fang and Liu, 2023)
<i>Synechococcus leopoliensis</i>	Antibiotic - Beta-lactam Amoxicillin	5×10^{-5} -50 mg L ⁻¹	96 h	A. 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 24/24 h; C. 28 °C D – not given	Not used	NOEC = 7.8×10^{-4} mg L ⁻¹ LOEC = 1.56×10^{-3} mg L ⁻¹ EC ₅₀ = 2.2×10^{-3} mg L ⁻¹ (OD ₅₅₀)	n.t.	(Andreozzi et al., 2004)
<i>Synechococcus</i> sp.	Antibiotic - Beta-lactam Ampicillin	Not specified	144 h	A. 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 24/24 h; C, D – not given	OECD 201	NOEC = 3.1×10^{-4} mg L ⁻¹ EC ₅₀ = 6.9×10^{-3} mg L ⁻¹ MIC = 2.0×10^{-2} mg L ⁻¹ (OD ₆₅₅)	n.t.	(Ando et al., 2007)
<i>Synechococcus elongatus</i>		Not specified	24 h	A. ~ 55 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B – not given; C. 28 °C D. BG11 medium;	OECD 201 and EPS 1/RM/25	EC ₅₀ = 5.40×10^{-2} mg L ⁻¹ (phycocyanin fluorescence)	n.t.	(Le Page et al., 2019)
<i>Synechococcus leopoliensis</i>		Not specified	144 h	A. 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 24/24 h; C, D – not given	OECD 201	NOEC = 2.5×10^{-2} mg L ⁻¹ EC ₅₀ = 8.3×10^{-2} mg L ⁻¹ MIC = 2.0×10^{-1} mg L ⁻¹ (OD ₆₅₅)	n.t.	(Ando et al., 2007)
<i>Synechococcus leopoliensis</i>		Not specified	24 h	A. ~ 55 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B – not given C. 28 °C D. BG11 medium	OECD 201 and EPS 1/RM/25	EC ₅₀ = 2.75×10^{-2} mg L ⁻¹ (phycocyanin fluorescence)	n.t.	(Le Page et al., 2019)
<i>Synechococcus</i> sp.		Not specified	24 h	A. ~ 55 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B – not given; C. 28 °C D. BG11 medium	OECD 201 and EPS 1/RM/25	EC ₅₀ = 6.66×10^{-2} mg L ⁻¹ (phycocyanin fluorescence)	n.t.	(Le Page et al., 2019)
<i>Synechococcus</i> sp.	Antibiotic - Beta-lactam Cefazolin	Not specified	24 h	A. ~ 55 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B – not given; C. 28 °C D. BG11 medium	OECD 201 and EPS 1/RM/25	EC ₅₀ = 2.832×10^{-1} mg L ⁻¹ (phycocyanin fluorescence)	n.t.	(Le Page et al., 2019)

(continued on next page)

Table 4 (continued)

Cyanobacteria species (names given in report)	Pharmaceutical class and representative	Concentrations tested	Test condition			Impact on growth (measurement)	Biochemical markers tested	Ref.
			Exposure time	A. light intensity [$\mu\text{mol m}^{-2} \text{s}^{-1}$]; B. photoperiod (day/night); C. temp.; D. medium	Reference protocol			
<i>Synechococcus elongatus</i>		Not specified	24 h	A. $\sim 55 \mu\text{mol m}^{-2} \text{s}^{-1}$; B – not given; C. 28 °C; D. BG11 medium	OECD 201 and EPS 1/RM/25	$\text{EC}_{50} = 2.380 \times 10^{-1} \text{ mg L}^{-1}$ (phycocyanin fluorescence)	n.t.	(Le Page et al., 2019)
<i>Synechococcus leopoliensis</i>		Not specified	24 h	A. $\sim 55 \mu\text{mol m}^{-2} \text{s}^{-1}$; B – not given; C. 28 °C; D. BG11 medium	OECD 201 and EPS 1/RM/25	$\text{EC}_{50} = 1.341 \times 10^{-1} \text{ mg L}^{-1}$ (phycocyanin fluorescence)	n.t.	(Le Page et al., 2019)
<i>Synechococcus elongatus</i>		Not specified	24 h	A. $\sim 55 \mu\text{mol m}^{-2} \text{s}^{-1}$; B – not given; C. 28 °C; D. BG11 medium	OECD 201 and EPS 1/RM/25	$\text{EC}_{50} = 7.54 \times 10^{-2} \text{ mg L}^{-1}$ (phycocyanin fluorescence)	n.t.	(Le Page et al., 2019)
<i>Synechococcus leopoliensis</i>		Not specified	24 h	A. $\sim 55 \mu\text{mol m}^{-2} \text{s}^{-1}$; B – not given; C. 28 °C; D. BG11 medium	OECD 201 and EPS 1/RM/25	$\text{EC}_{50} = 3.10 \times 10^{-2} \text{ mg L}^{-1}$ (phycocyanin fluorescence)	n.t.	(Le Page et al., 2019)
<i>Synechococcus</i> sp.		Not specified	24 h	A. $\sim 55 \mu\text{mol m}^{-2} \text{s}^{-1}$; B – not given; C. 28 °C; D. BG11 medium	OECD 201 and EPS 1/RM/25	$\text{EC}_{50} = 9.79 \times 10^{-2} \text{ mg L}^{-1}$ (phycocyanin fluorescence)	n.t.	(Le Page et al., 2019)
<i>Synechococcus leopoliensis</i>	Antibiotic - Sulfonamide Sulfamethoxazole	Not specified	96 h	A. $116.2 \mu\text{mol m}^{-2} \text{s}^{-1}$; B. 16/8 h; C. 28 °C; D. Bolda's medium	Not used	Acute toxicity: $\text{EC}_{50} = 26.8 \text{ mg L}^{-1}$ Chronic toxicity: $\text{NOEC} = 5.9 \times 10^{-3} \text{ mg L}^{-1}$ (OD ₅₅₀ and cell count)	n.t.	(Ferrari et al., 2004)
<i>Synechococcus</i> sp.		5×10^{-4} and $1 \times 10^{-2} \text{ mg L}^{-1}$	168 h	A. $\sim 90 \mu\text{mol m}^{-2} \text{s}^{-1}$ (constant); B – not given; C. 22 °C; D. BG11 medium	Not used	Growth inhibition 63% at $1.0 \times 10^{-3} \text{ mg L}^{-1}$ (OD ₆₈₀ and cell count)	chlorophyll <i>a</i> , <i>b</i> carotenoids psbA (photosynthesis gene) chlorophyll metabolism Tocopherol Photosynthetic pathways	(Yibo Zhang et al., 2022)
<i>Synechococcus</i> sp.		5×10^{-3} – $5 \times 10^{-2} \text{ mg L}^{-1}$	264 h	A. $\sim 50 \mu\text{mol m}^{-2} \text{s}^{-1}$ (constant); B - not given; C. 25 °C; D. BG11 medium	OECD 201	$\text{EC}_{50} = 1.56 \times 10^{-2} \text{ mg L}^{-1}$ (cell count)	chlorophyll <i>a</i> , <i>b</i> carotenoids photosynthesis (Fv/Fm) ROS (MDA, SOD, CAT, GPx, GST, GSH)	(Zhang et al., 2023)
<i>Synechococcus leopoliensis</i>	Antibiotic - Sulfonamide Sulfamethoxazole	7.8×10^{-2} – 5 mg L^{-1}	96 h	A. $90 \mu\text{mol m}^{-2} \text{s}^{-1}$ (constant); B – not given; C. 24 °C; D. BBM medium	US-EPA 797.1050	$\text{EC}_{50} = 2.834 \text{ mg L}^{-1}$ (cell count)	n.t.	(De Liguoro et al., 2010)
<i>Synechococcus leopoliensis</i>	Antibiotic - Sulfonamide Sulfaguanidine	2.5–40 mg L^{-1}	96 h	A. $90 \mu\text{mol m}^{-2} \text{s}^{-1}$ (constant); B – not given; C. 24 °C; D. BBM medium	US-EPA 797.1050	$\text{EC}_{50} = 16.594 \text{ mg L}^{-1}$ (cell count)	n.t.	(De Liguoro et al., 2010)
<i>Synechococcus</i> sp.	Antibiotic - Tetracycline Oxytetracycline	Not specified	144 h	A. $50 \mu\text{mol m}^{-2} \text{s}^{-1}$; B. 24/24 h; C - not given; D – not given	OECD 201	$\text{NOEC} = 2.5 \times 10^{-1} \text{ mg L}^{-1}$ $\text{EC}_{50} = 2.0 \text{ mg L}^{-1}$ $\text{MIC} = 16 \text{ mg L}^{-1}$ (OD ₆₅₅)	n.t.	(Ando et al., 2007)
<i>Synechococcus leopoliensis</i>		Not specified	144 h	A. $50 \mu\text{mol m}^{-2} \text{s}^{-1}$; B. 24/24 h; C - not given; D – not given	OECD 201	$\text{NOEC} = 2.5 \times 10^{-1} \text{ mg L}^{-1}$ $\text{EC}_{50} = 1.1 \text{ mg L}^{-1}$ $\text{MIC} = 16 \text{ mg L}^{-1}$ (OD ₆₅₅)	n.t.	(Ando et al., 2007)

(continued on next page)

Table 4 (continued)

Cyanobacteria species (names given in report)	Pharmaceutical class and representative	Concentrations tested	Test condition			Impact on growth (measurement)	Biochemical markers tested	Ref.
			Exposure time	A. light intensity [$\mu\text{mol m}^{-2} \text{s}^{-1}$]; B. photoperiod (day/night); C. temp.; D. medium	Reference protocol			
<i>Synechococcus elongatus</i>	Antibiotic - Macrolide Erythromycin	Not specified	24 h	A. $\sim 55 \mu\text{mol m}^{-2} \text{s}^{-1}$; B – not given; C. 28 °C; D. BG11 medium	OECD 201 and EPS 1/RM/25	$\text{EC}_{50} = 6.33 \times 10^{-2} \text{ mg L}^{-1}$ (phycocyanin fluorescence)	n.t.	(Le Page et al., 2019)
<i>Synechococcus leopoliensis</i>		Not specified	24 h	A. $\sim 55 \mu\text{mol m}^{-2} \text{s}^{-1}$; B – not given; C. 28 °C; D. BG11 medium	OECD 201 and EPS 1/RM/25	$\text{EC}_{50} = 6.39 \times 10^{-2} \text{ mg L}^{-1}$ (phycocyanin fluorescence)	n.t.	(Le Page et al., 2019)
<i>Synechococcus sp.</i>		Not specified	24 h	A. $\sim 55 \mu\text{mol m}^{-2} \text{s}^{-1}$; B - not given; C. 28 °C; D. BG11 medium	OECD 201 and EPS 1/RM/25	$\text{EC}_{50} = 5.98 \times 10^{-2} \text{ mg L}^{-1}$ (phycocyanin fluorescence)	n.t.	(Le Page et al., 2019)
<i>Synechococcus sp.</i>		Not specified	144 h	A. $50 \mu\text{mol m}^{-2} \text{s}^{-1}$; B. 24/24 h; C – not given; D – not given	OECD 201	$\text{NOEC} = 7.8 \times 10^{-3} \text{ mg L}^{-1}$ $\text{EC}_{50} = 2.3 \times 10^{-1} \text{ mg L}^{-1}$ $\text{MIC} = 2.0 \text{ mg L}^{-1}$ (OD ₆₅₅)	n.t.	(Ando et al., 2007)
<i>Synechococcus leopoliensis</i>		Not specified	144 h	A. $50 \mu\text{mol m}^{-2} \text{s}^{-1}$; B. 24/24 h; C – not given; D – not given	OECD 201	$\text{NOEC} = 2.0 \times 10^{-3} \text{ mg L}^{-1}$ $\text{EC}_{50} = 1.6 \times 10^{-1} \text{ mg L}^{-1}$ $\text{MIC} = 2.0 \text{ mg L}^{-1}$ (OD ₆₅₅)	n.t.	(Ando et al., 2007)
<i>Synechococcus elongatus</i>	Antibiotic - Macrolide Azithromycin	Not specified	24 h	A. $\sim 55 \mu\text{mol m}^{-2} \text{s}^{-1}$; B – not given; C. 28 °C; D. BG11 medium	OECD 201 and EPS 1/RM/25	$\text{EC}_{50} = 1.74 \times 10^{-2} \text{ mg L}^{-1}$ (phycocyanin fluorescence)	n.t.	(Le Page et al., 2019)
<i>Synechococcus sp.</i>		Not specified	24 h	A. $\sim 55 \mu\text{mol m}^{-2} \text{s}^{-1}$; B – not given; C. 28 °C; D. BG11 medium	OECD 201 and EPS 1/RM/25	$\text{EC}_{50} = 3.38 \times 10^{-2} \text{ mg L}^{-1}$ (phycocyanin fluorescence)	n.t.	(Le Page et al., 2019)
<i>Synechococcus leopoliensis</i>		Not specified	24 h	A. $\sim 55 \mu\text{mol m}^{-2} \text{s}^{-1}$; B – not given; C. 28 °C; D. BG11 medium	OECD 201 and EPS 1/RM/25	$\text{EC}_{50} = 2.35 \times 10^{-2} \text{ mg L}^{-1}$ (phycocyanin fluorescence)	n.t.	(Le Page et al., 2019)
<i>Synechococcus leopoliensis</i>	Antibiotic - Diaminopyrimidine Trimethoprim	Not specified	144 h	A. $50 \mu\text{mol m}^{-2} \text{s}^{-1}$; B – not given; C. 24/24 h; D – not given	OECD 201	$\text{NOEC} = 13 \text{ mg L}^{-1}$ $\text{EC}_{50} > 200 \text{ mg L}^{-1}$ $\text{MIC} > 200 \text{ mg L}^{-1}$ (OD ₆₅₅)	n.t.	(Ando et al., 2007)
<i>Synechococcus sp.</i>		Not specified	144 h	A. $50 \mu\text{mol m}^{-2} \text{s}^{-1}$; B – not given; C. 24/24 h; D – not given	OECD 201	$\text{NOEC} = 50 \text{ mg L}^{-1}$ $\text{EC}_{50} > 200 \text{ mg L}^{-1}$ $\text{MIC} > 200 \text{ mg L}^{-1}$ (OD ₆₅₅)	n.t.	(Ando et al., 2007)
<i>Synechococcus leopoliensis</i>	Antibiotic - Amphenicol Thiamphenicol	Not specified	144 h	A. $50 \mu\text{mol m}^{-2} \text{s}^{-1}$; B – not given; C. 24/24 h; D – not given	OECD 201	$\text{NOEC} = 1.6 \times 10^{-1} \text{ mg L}^{-1}$ $\text{EC}_{50} = 3.6 \times 10^{-1} \text{ mg L}^{-1}$ $\text{MIC} = 2.5 \text{ mg L}^{-1}$ (OD ₆₅₅)	n.t.	(Ando et al., 2007)
<i>Synechococcus sp.</i>		Not specified	144 h	A. $50 \mu\text{mol m}^{-2} \text{s}^{-1}$; B – not given; C. 24/24 h; D – not given	OECD 201	$\text{NOEC} = 1.6 \times 10^{-1} \text{ mg L}^{-1}$ $\text{EC}_{50} = 6.7 \times 10^{-1} \text{ mg L}^{-1}$ $\text{MIC} = 5.0 \text{ mg L}^{-1}$ (OD ₆₅₅)	n.t.	(Ando et al., 2007)
<i>Synechococcus leopoliensis</i>	NSAIDs Diclofenac	Not specified	96 h	A. $116.2 \mu\text{mol m}^{-2} \text{s}^{-1}$; B. 16/8 h; C. 28 °C; D. Bold's Medium;	Not applied	Acute toxicity: $\text{EC}_{50} = 14.50 \text{ mg L}^{-1}$ Chronic toxicity: $\text{NOEC} = 10.00 \text{ mg L}^{-1}$ (OD ₅₅₀ and cell count)	n.t.	(Ferrari et al., 2004)
<i>Synechococcus elongatus</i>		100-1000 mg L^{-1}	240 h	A – not given; B. 24/24 h; C. 28 °C; D. Allen's medium	Not applied	Slight impact on growth (OD ₆₆₃ and cell count)	n.t.	(Bácsi et al., 2016)

(continued on next page)

Table 4 (continued)

Cyanobacteria species (names given in report)	Pharmaceutical class and representative	Concentrations tested	Test condition			Impact on growth (measurement)	Biochemical markers tested	Ref.
			Exposure time	A. light intensity [$\mu\text{mol m}^{-2} \text{s}^{-1}$]; B. photoperiod (day/night); C. temp.; D. medium	Reference protocol			
<i>Synechococcus elongatus</i>	NSAIDs Ibuprofen	100-1000 mg L ⁻¹	240 h	A – not given; B. 24/24 h; C. 28 °C; D. Allen's medium	Not applied	Slight impact on growth (OD ₆₆₃ and cell count)	n.t.	(Bácsi et al., 2016)
<i>Synechococcus elongatus</i>	NSAIDs Diflunisal	100-1000 mg L ⁻¹	240 h	A – not given; B. 24/24 h; C. 28 °C; D. Allen's medium	Not applied	Slight impact on growth (OD ₆₆₃ and cell count)	n.t.	(Bácsi et al., 2016)
<i>Synechococcus elongatus</i>	NSAIDs Mefenamic acid	100-1000 mg L ⁻¹	240 h	A – not given; B. 24/24 h; C. 28 °C; D. Allen's medium	Not applied	Slight impact on growth (OD ₆₆₃ and cell count)	n.t.	(Bácsi et al., 2016)
<i>Synechococcus elongatus</i>	NSAIDs Piroxicam	100-1000 mg L ⁻¹	240 h	A – not given; B. 24/24 h; C. 28 °C; D. Allen's medium	Not applied	Slight impact on growth (OD ₆₆₃ and cell count)	n.t.	(Bácsi et al., 2016)
<i>Synechococcus leopoliensis</i>	Anesthetics Lidocaine	2.34×10^{-2} -2.34 mg L ⁻¹	48 h	A. 20 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 24/24 h; C. 30 °C; D. Detmer medium (with modification)	Not applied	Growth stimulation (80-90%) at 2.34×10^{-2} - 2.34×10^{-1} mg L ⁻¹ 10 μM - no effect > 10 μM - growth inhibition (OD ₆₀₀)	photosynthetic oxygen evolution ratio of phycobilin to chlorophyll <i>a</i> phytohormones	(Suzuki et al., 2004)
<i>Synechococcus leopoliensis</i>	Anaesthetics Procaine	2.36×10^{-2} -2.36 mg L ⁻¹	40 h	A. 20 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 24/24 h; C. 30 °C; D. Detmer medium (with modification)	Not applied	Growth stimulation (25-30%) at 2.36×10^{-2} - 2.36×10^{-1} mg L ⁻¹ 10 μM - no effect > 10 μM - growth inhibition (OD ₆₀₀)		(Suzuki et al., 2004)
<i>Synechococcus leopoliensis</i>	Anesthetic Dibucaine	2.34×10^{-2} – 2.34 mg L ⁻¹	48 h	A. 20 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 24/24 h; C. 30 °C; D. Detmer medium (with modification)	Not applied	Growth stimulation at 2.34×10^{-2} - 2.34×10^{-1} mg L ⁻¹ growth inhibition at 2.34×10^{-1} mg L ⁻¹ (OD ₆₀₀)		(Suzuki et al., 2004)
<i>Synechococcus leopoliensis</i>	Anesthetic Procainamide	2.35×10^{-2} -2.35 mg L ⁻¹	32 h	A. 20 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 24/24 h; C. 30 °C; D. Detmer medium (with modification)	Not applied	Growth stimulation at 2.35×10^{-2} - 2.35×10^{-1} mg L ⁻¹ 10 μM – no effect > 10 μM - growth inhibition (OD ₆₀₀)		(Suzuki et al., 2004)
<i>Synechococcus leopoliensis</i>	Anesthetic Tetracaine	2.64×10^{-2} – 2.64 mg L ⁻¹	32 h	A. 20 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 24/24 h; C. 30 °C; D. Detmer medium (with modification)	Not applied	Growth stimulation at 2.64×10^{-2} - 2.64×10^{-1} mg L ⁻¹ 10 μM – no effect > 10 μM - growth inhibition (OD ₆₀₀)		(Suzuki et al., 2004)
<i>Synechococcus leopoliensis</i>	Antineoplastic Imatinib mesylate	1.04-16.0 mg L ⁻¹	72 h	A. 80-120 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 24/24 h; C. 24 °C; D – not given	OECD 201	EC ₅₀ = 5.36 mg L ⁻¹ EC ₂₀ = 3.81 mg L ⁻¹ EC ₁₀ = 3.12 mg L ⁻¹ LOEC = 4.00 mg L ⁻¹ NOEC = 3.84 mg L ⁻¹ (flow cytometry)	n.t.	(Brezovšek et al., 2014)
<i>Synechococcus leopoliensis</i>	Antineoplastic Cisplatin	3×10^{-2} -32.0 mg L ⁻¹	72 h	A. 80-120 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 24/24 h; C. 24 °C; D – not given	OECD 201	EC ₅₀ = 6.7×10^{-1} mg L ⁻¹ EC ₂₀ = 1.3×10^{-1} mg L ⁻¹ EC ₁₀ = 5×10^{-2} mg L ⁻¹ LOEC = 3.1×10^{-1} mg L ⁻¹ NOEC = 1.0×10^{-1} mg L ⁻¹ (flow cytometry)	n.t.	(Brezovšek et al., 2014)
<i>Synechococcus leopoliensis</i>	Antineoplastic Etoposide	3.37-351.1 mg L ⁻¹	72 h	A. 80-120 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 24/24 h; C. 24 °C; D – not given	OECD 201	NOEC = 351.05 mg L ⁻¹ (flow cytometry)	n.t.	(Brezovšek et al., 2014)

(continued on next page)

Table 4 (continued)

Cyanobacteria species (names given in report)	Pharmaceutical class and representative	Concentrations tested	Test condition	Impact on growth (measurement)		Biochemical markers tested	Ref.
				Exposure time	A. light intensity [$\mu\text{mol m}^{-2} \text{s}^{-1}$]; B. photoperiod (day/night); C. temp.; D. medium	Reference protocol	
<i>Synechococcus leopoliensis</i>	Antineoplastic 5-fluorouracil	3.8×10^{-2} –12.8 mg L ⁻¹	72 h	A. 80–120 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 24/24 h; C. 24 °C; D – not given	OECD 201	EC ₅₀ = 1.20 mg L ⁻¹ EC ₂₀ = 2.9×10^{-1} mg L ⁻¹ EC ₁₀ = 1.3×10^{-1} mg L ⁻¹ LOEC = 3.9×10^{-1} mg L ⁻¹ NOEC = 1.2×10^{-1} mg L ⁻¹ (flow cytometry)	n.t. (Brezovšek et al., 2014)
<i>Synechococcus</i> sp.	Beta-blocker Propranolol	Not specified	96 h	A. 116.2 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 16/8 h.; C. 28 °C; D. Bold's medium	Not applied	Acute toxicity: EC ₅₀ = 6.68×10^{-1} mg L ⁻¹ Chronic toxicity: NOEC = 3.5×10^{-1} mg L ⁻¹ (OD ₅₅₀ and cell count)	n.t. (Ferrari et al., 2004)
<i>Synechococcus</i> sp.	Antiepileptics Carbamazepine	Not specified	96 h	A. 116.2 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 16/8 h.; C. 28 °C; D. Bold's medium	Not applied	Acute toxicity: EC ₅₀ = 33.60 mg L ⁻¹ Chronic toxicity: NOEC = 17.5 mg L ⁻¹ (OD ₅₅₀ and cell count)	n.t. (Ferrari et al., 2004)
<i>Synechococcus</i> sp.	Fibrate Clofibrac acid	Not specified	96 h	A. 116.2 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 16/8 h.; C. 28 °C; D. Bold's medium	Not applied	Acute toxicity: EC ₅₀ = 40.20 mg L ⁻¹ Chronic toxicity: NOEC = 23.5 mg L ⁻¹ (OD ₅₅₀ and cell count)	n.t. (Ferrari et al., 2004)

cyanobacteria. Concentrations of pharmaceuticals, mostly antibiotics, causing 50% growth inhibition (EC₅₀) for *M. aeruginosa* and *Synechococcus* sp. are presented in Fig. 1.

In the following sections, environmental concentrations are compared with EC₅₀ values to evaluate possible environmental risk. Beyond direct toxicological effects, the second major negative impact of antibiotic presence - the proliferation of antimicrobial resistance must also be considered. The antibiotics are an emerging environmental problem due to global antibiotic resistance occurrence (Larsson and Flach, 2022). However, reporting the presence of this phenomenon is likely to be problematic. In some cases, reduced sensitivity after only two consecutive exposures is described as the development of "resistance". However, based on the available evidence, terms such as adaptive response, acclimation, or increased tolerance would be more appropriate unless stable resistance is demonstrated (e.g., increased MIC, persistence of the phenotype after antibiotic withdrawal, maintenance over multiple generations, or identification of resistance mechanisms).

Cyanobacteria represent a crucial yet frequently overlooked component of the aquatic resistome, functioning simultaneously as sensitive non-target organisms and potential reservoirs of antimicrobial resistance (Manganelli et al., 2024). Evaluating their response to pharmaceutical contamination requires a strict differentiation between physiological (phenotypic) tolerance and true genetic resistance. Physiological resistance in cyanobacteria predominantly relies on phenotypic plasticity, including the secretion of extracellular polymeric substances (EPS), upregulation of non-specific efflux pumps or metabolic shifts (Dias et al., 2019, 2015). These mechanisms allow populations to acclimate to ambient chemical stress without alterations to their primary DNA sequence (Dias et al., 2015). In contrast, genetic resistance involves permanent, heritable alterations such as target-site point mutations or the horizontal gene transfer (HGT) of antibiotic resistance genes (ARGs). Notably, genomic and phenotypic profiling of freshwater cyanobacteria, such as *Planktothrix* spp., reveals a frequent disconnect between genotype and phenotype. Strains often display high phenotypic tolerance despite lacking classical ARGs, underscoring the dominance of non-specific physiological defense strategies in natural populations (Dias et al., 2019).

This physiological defense is closely linked to hormesis - a biphasic dose-response phenomenon where exposure to sub-inhibitory, low-dose

concentration of antibiotics stimulates cellular processes rather than inhibiting them. Hormetic responses induce low-level oxidative stress defenses, bolster membrane repair mechanisms, and enhance overall cellular vitality (as described in Section 3). While hormesis itself is a transient, non-heritable physiological state, it plays a pivotal role in the emergence of permanent resistance. By mitigating acute toxicity and maintaining population viability under continuous sub-lethal exposure, hormesis widens the temporal window during which cyanobacteria can undergo genetic adaptation.

The ultimate evolutionary pathway to resistance is fundamentally dictated by exposure kinetics: short-term high-concentration shocks versus chronic low-concentration regimes. Direct exposure to high, lethal antibiotic concentrations creates a severe population bottleneck, selecting almost exclusively for rare, single-step mutations that often carry substantial metabolic fitness costs. Conversely, long-term exposure to sub-inhibitory (sub-MIC) antibiotic levels (which mirror actual concentrations found in contaminated aquatic environments) drives the step-wise evolution of high-level genetic resistance (Wistrand-Yuen et al., 2018). Under chronic low-level exposure, cyanobacteria iteratively accumulate low-cost, compensatory, and synergistic mutations. This step-wise selection allows cells to reach high MIC thresholds while maintaining ecological competitiveness. Furthermore, this evolutionary dynamic does not occur in isolation; global environmental changes, such as climate warming, act synergistically with sub-lethal antibiotic exposure to promote collateral antibiotic resistance in cyanobacteria (Wang et al., 2024). Deciphering these complex interactions between physiological acclimation, hormetic stress responses, and long-term evolutionary selection is vital for incorporating cyanobacteria into the broader One Health perspective on global antimicrobial resistance (Manganelli et al., 2024).

4.1.1. Fluoroquinolone

Fluoroquinolones are among the most extensively studied classes of antibiotics in both clinical and environmental research due to their broad-spectrum antibacterial activity and high environmental persistence. Structurally, members of this class share a bicyclic core derived from 4-quinolone (Andriole, 2005). Fluoroquinolones are widely used in both human and veterinary medicine due to their broad-spectrum antibacterial activity. Their antibacterial activity is mediated through

Table 5

Impact of pharmaceuticals on *M. aeruginosa* (n.t. - not tested; MC – microcystins; ROS – reactive oxygen species; POD - peroxidase, CAT - catalase; SOD - superoxide dismutase; GSH - glutathione, and GST - glutathione S-transferase; MDA - malondialdehyde).

Pharmaceutical class and representative	Concentrations tested	Test condition			Impact on growth (measurement)	Biochemical markers tested	Ref.
		Exposure time	A. Light intensity [$\mu\text{mol m}^{-2} \text{s}^{-1}$]; B. photoperiod (day/night); C. temp.; D. medium	Reference protocol			
Antibiotic - Fluoroquinolone Enrofloxacin	Growth rate: 12-120 mg L^{-1} microcystins release: 5.61×10^{-2} -1.4025 mg L^{-1}	96 h/336 h	A – not given; B. 12/12 h; C. 25- 34 °C; D. BG 11 medium	Not applied	$\text{EC}_{50} = 56.10 \text{ mg L}^{-1}$ Growth stimulation at 12 and 36 mg L^{-1} (after 144 h) (OD ₆₈₀)	MC	(Xia et al., 2023)
	Not specified	120 h	A. 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 12/12 h; C. 20 °C; D. Allen's medium	Not applied	$\text{EC}_{50} = 4.9 \times 10^{-2} \text{ mg L}^{-1}$ (fluorescence)	n.t.	(Robinson et al., 2005)
Antibiotic - Fluoroquinolone Norfloxacin	5×10^{-2} -5 mg L^{-1}	72 h/ 288 h (co-culture with <i>S. quadricauda</i>)	A. 42 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 12/12 h; C. 25 °C; D. modified HGZ-145 medium	Not applied	$\text{EC}_{50} = 1.687 \times 10^{-2} \text{ mg L}^{-1}$ (OD ₆₆₃)	ROS ($\text{O}_2^{\cdot-}$, SOD) MC	(J. J. Li et al., 2021)
	3.19×10^{-3} -3.19 mg L^{-1}	96 h	A. 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 12/12; C. 25 °C; D. BG11 medium	Not applied	$\text{EC}_{50} = 2.18 \text{ mg L}^{-1}$ (OD ₆₈₀)	combination with two other antibiotics	(Zhao et al., 2023)
	Not specified	144 h	A. 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 24 h; C – not given; D – not given	OECD 201	NOEC = $1.6 \times 10^{-3} \text{ mg L}^{-1}$ $\text{EC}_{50} = 6.2 \times 10^{-2} \text{ mg L}^{-1}$ MIC = 0.20 mg L^{-1} (OD ₆₅₅)	n.t.	(Ando et al., 2007)
	1.5×10^{-3} -1.6 mg L^{-1}	312 h	A. 16 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 14/10 h; C. 20 °C; D. Z8 medium	OECD 201	Growth inhibition: 100 % at $1.5 \times 10^{-3} \text{ mg L}^{-1}$ 95 % at $3 \times 10^{-3} \text{ mg L}^{-1}$ 80 % at $6 \times 10^{-3} \text{ mg L}^{-1}$ 30 % at $1.2 \times 10^{-2} \text{ mg L}^{-1}$ 25 % at $2.5 \times 10^{-2} \text{ mg L}^{-1}$ 20 % at 5×10^{-2} -1.6 mg L^{-1} (OD ₄₅₀)	n.t.	(Dias et al., 2015)
	Not specified	120 h	A. 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 12/12 h; C. 20 °C; D. Allen's medium	Not applied	$\text{EC}_{50} = 7.9 \times 10^{-3} \text{ mg L}^{-1}$ (fluorescence)	n.t.	(Robinson et al., 2005)
Antibiotic - Fluoroquinolone Levofloxacin	1×10^{-4} -1 mg L^{-1}	96 h	A. 37.5 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 12/12 h; C. 25 °C; D. BG11 medium	Not applied	$\text{EC}_{50} = 4.376 \times 10^{-1} \text{ mg L}^{-1}$ (cell count)	photosynthesis phycobiliprotein ROS (MDA)	(Wu et al., 2022)
Antibiotic - Fluoroquinolone Lomefloxacin	Not specified	120 h	A. 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (12/12 h; B – not given; C. 20 °C; D. Allen's medium	Not applied	$\text{EC}_{50} = 1.86 \times 10^{-1} \text{ mg L}^{-1}$ (fluorescence)	n.t.	(Robinson et al., 2005)

(continued on next page)

Table 5 (continued)

Pharmaceutical class and representative	Concentrations tested	Test condition			Impact on growth (measurement)	Biochemical markers tested	Ref.
		Exposure time	A. Light intensity [$\mu\text{mol m}^{-2} \text{s}^{-1}$]; B. photoperiod (day/night); C. temp.; D. medium	Reference protocol			
Antibiotic - Fluoroquinolone Ciprofloxacin	1×10^{-4} -100 mg L^{-1}	96 h	A. 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$ B. 16/8 h; C. 23 °C; D. BG11 medium	OECD 201	$\text{EC}_{50} = 2.33 \times 10^{-2} \text{ mg L}^{-1}$ (strain EAWAG 198) $\text{EC}_{50} = 3.956 \text{ mg L}^{-1}$ (strain LE3) (cell count)	ROS (H_2O_2 , MDA, POD, GST)	(Yisa et al., 2021)
	Not specified	72 h	According to OECD 201	OECD 201	$\text{EC}_{50} = 5 \times 10^{-3} \text{ mg L}^{-1}$ (biomass)	biodegradation	(Halling-Sorensen et al., 2000)
	3.31×10^{-3} -3.31 mg L^{-1}	96 h	A. 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$ B. 12/12 h; C. 25 °C; D. BG11 medium	Not applied	$\text{EC}_{50} = 5.73 \times 10^{-1} \text{ mg L}^{-1}$ (OD_{680})	effects of combination with two other antibiotics	(Zhao et al., 2023)
	Growth rate: 30-150 mg L^{-1} MC release: 4.98×10^{-2} -1.2450 mg L^{-1}	96h/336 h	A – not given; B. Light 12/12 h; C. 25- 34 °C; D. BG 11 medium	Not applied	$\text{EC}_{50} = 49.80 \text{ mg L}^{-1}$ ($\text{OD}_{680}/96\text{h}$)	MC	(Xia et al., 2023)
	1×10^{-3} -8 $\times 10^{-3} \text{ mg L}^{-1}$	432 h	A. 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 12/12 h; C. 25 °C; D. BG11 medium		Growth inhibition (strain depending) 71.75 % and 41.13% at $8 \times 10^{-3} \text{ mg L}^{-1}$ (OD_{680})	MC C:N ratio amino acid content (leucine, tyrosine, arginine) mcyB gene expression	(Wan et al., 2024)
	2.1×10^{-3} - $2.94 \times 10^{-2} \text{ mg L}^{-1}$	96 h	A. 98 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 12/12 h; C. 25 °C; D – not given	OECD 201 (with modification)	$\text{EC}_{50} = 1.72 \times 10^{-2} \text{ mg L}^{-1}$ $\text{EC}_{10} = 3.2 \times 10^{-3} \text{ mg L}^{-1}$ (chlorophyll <i>a</i> and cell count)	n.t.	(Azevedo et al., 2019)
	1×10^{-5} - $2 \times 10^{-4} \text{ mg L}^{-1}$	360 h	A. 60 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 16/8 h; C. 25 °C; D. BG 11 medium	Not applied	Growth stimulation at 5×10^{-5} - $2 \times 10^{-4} \text{ mg L}^{-1}$ (cell count)	chlorophyll <i>a</i> photosynthesis (Fv/Fm) MC ROS - SOD proteomics effects of combination with sulfamethoxazole	(Liu et al., 2017)
	Not specified	120 h	A. 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ B. 12/12 h; C. 20 °C; D. Allen's medium	Not applied	$\text{EC}_{50} = 1.7 \times 10^{-2} \text{ mg L}^{-1}$ (fluorescence)	n.t.	(Robinson et al., 2005)
	1.8×10^{-4} - $1.3 \times 10^{-2} \text{ mg L}^{-1}$	96 h	A. 27 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B – not given; C – not given; D. different medium at pH 7.2-9.0	OECD 201	$\text{EC}_{50} = 6.53 \times 10^{-4} \text{ mg L}^{-1}$ (cell count)	impact of medium pH	(Q. Zhang et al., 2022)
Antibiotic Fluoroquinolone Ofloxacin	Not specified	120 h	A. 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 12/12 h; C. 20 °C; D. Allen's medium	Not applied	$\text{EC}_{50} = 2.1 \times 10^{-2} \text{ mg L}^{-1}$ (fluorescence)	n.t.	(Robinson et al., 2005)

(continued on next page)

Table 5 (continued)

Pharmaceutical class and representative	Concentrations tested	Test condition			Impact on growth (measurement)	Biochemical markers tested	Ref.
		Exposure time	A. Light intensity [$\mu\text{mol m}^{-2} \text{s}^{-1}$]; B. photoperiod (day/night); C. temp.; D. medium	Reference protocol			
	3.61×10^{-2} -36.1 mg L^{-1}	96h	A. 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 12/12 h; C. 25 °C; D. BG11 medium	Not applied	$\text{EC}_{50} = 6.54 \text{ mg L}^{-1}$ (OD ₆₈₀)	effect of combination with two other antibiotics	(Zhao et al., 2023)
	1×10^{-1} -100 mg L^{-1}	12 h	A. 30 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 12/12 h; C. 25 °C; D. BG 11 medium	Not applied	n.d.	photosynthesis pigments (chlorophyll <i>a</i> and carotenoids)	(Deng et al., 2015)
AntibioticFluoroquinolone Cinafloxacin	Not specified	120 h	A. 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 12/12 h; C. 20 °C; D. Allen's medium	Not applied	$\text{EC}_{50} = 1.03 \times 10^{-1} \text{ mg L}^{-1}$ (fluorescence)	n.t.	(Robinson et al., 2005)
AntibioticFluoroquinolone Flumequine	9.4×10^{-2} -3.69 $\times 10^{-1} \text{ mg L}^{-1}$	168 h	A. 42 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 24 h; C. 21 °C; D. ISO medium with modification	ISO (1989)	$\text{EC}_{50} = 1.59 \times 10^{-1} \text{ mg L}^{-1}$ (OD ₄₃₀ and OD ₆₇₁)	n.t.	(Holten Lützhøft et al., 1999)
	1×10^{-4} -10 mg L^{-1}	24 h	A. 10 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 16/8 h; C. 20 °C; D. Dutch Standard Water	Not applied	Growth inhibition $\text{EC}_{50} > 8.8 \text{ mg L}^{-1}$ (Fv/Fm)	n.t.	(van der Grinten et al., 2010)
	Not specified	120 h	A. 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 12/12 h; C. 20 °C; D. Allen's medium	Not applied	$\text{EC}_{50} = 1.960 \text{ mg L}^{-1}$ (OD ₄₃₀ and OD ₆₇₁)	n.t.	(Robinson et al., 2005)
AntibioticFluoroquinolone Sarafloxacin	4×10^{-3} -9 $\times 10^{-3} \text{ mg L}^{-1}$	168 h	A. 42 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 24 h; C. 21 °C; D. ISO medium with modification	ISO (1989)	$\text{EC}_{50} = 1.5 \times 10^{-2} \text{ mg L}^{-1}$ (OD ₄₃₀ and OD ₆₇₁)	n.t.	(Holten Lützhøft et al., 1999)
AntibioticQuinolone Oxolinic acid	6.0×10^{-2} -5.40 $\times 10^{-1} \text{ mg L}^{-1}$	168 h	A. 42 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (24 h); B – not given; C. 21 °C; D. ISO medium with modification	ISO (1989)	$\text{EC}_{50} = 1.80 \times 10^{-1} \text{ mg L}^{-1}$ (OD ₄₃₀ and OD ₆₇₁)	n.t.	(Holten Lützhøft et al., 1999)
AntibioticQuinolone Nalidixic acid	1.5×10^{-3} -1.6 mg L^{-1}	312 h	A. 16 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 14/10 h; C. 20 °C; D. Z8 medium	OECD 201	No effect (OD ₄₅₀)	n.t.	(Dias et al., 2015)
Antibiotic Beta-lactam Ampicillin	Not specified	144 h	A. 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 24/24 h; C – not given; D – not given	OECD 201	$\text{EC}_{50} = 2 \times 10^{-4} \text{ mg L}^{-1}$ (OD ₆₅₅)	n.t.	(Ando et al., 2007)

(continued on next page)

Table 5 (continued)

Pharmaceutical class and representative	Concentrations tested	Test condition			Impact on growth (measurement)	Biochemical markers tested	Ref.
		Exposure time	A. Light intensity [$\mu\text{mol m}^{-2} \text{s}^{-1}$]; B. photoperiod (day/night); C. temp.; D. medium	Reference protocol			
Antibiotic Beta-lactam Amoxicillin	4.5×10^{-3} – $7 \times 10^{-3} \text{ mg L}^{-1}$	168 h	A. 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 12/12 h; C. 25 °C; D. BG11 medium	Not applied	$\text{EC}_{50} = 5.79 \times 10^{-3} \text{ mg L}^{-1}$ (cell count)	MC K^{+} and Mg^{2+} leakage combined effect of antibiotic mixture	(Wang et al., 2018)
	$3 \times 10^{-4} \text{ mg L}^{-1}$	672 h	A. 50.49 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 12/12 h; C. 25 °C; D. BG11 medium	Not applied	Growth stimulation at $3 \times 10^{-4} \text{ mg L}^{-1}$ (cell count)	MC ROS photosynthesis	(Wang et al., 2019)
	6×10^{-3} – $1.4 \times 10^{-2} \text{ mg L}^{-1}$	28 days	A. 70–80 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B – not given; C. 20 °C; D. BG11 medium	Not applied	Growth inhibition (~80%) at each concentration after 7 days (cell count)	MC biodegradation	(Baselga-Cervera et al., 2019)
	9×10^{-4} – $3.8 \times 10^{-3} \text{ mg L}^{-1}$	168 h	A. 42 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 24 h; C. 21 °C; D. ISO medium with modification	ISO (1989)	$\text{EC}_{50} = 3.7 \times 10^{-3} \text{ mg L}^{-1}$ (OD ₄₃₀ and OD ₆₇₁)	n.t.	(Holten Lützhøft et al., 1999)
	1×10^{-4} – $3 \times 10^{-4} \text{ mg L}^{-1}$	720 h	A. 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 16/8 h; C. 25 °C; D. BG11 medium	Not applied	Growth stimulation at 1×10^{-4} – $3 \times 10^{-4} \text{ mg L}^{-1}$ (48 to 240–336 h) (cell count)	MC photosynthesis proteomic response	(Liu et al., 2016)
	2×10^{-4} – $5 \times 10^{-4} \text{ mg L}^{-1}$	168 h	A. 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 16/8 h; C. 25 °C; D. BG11 medium with modification	Not applied	Low nitrogen concentration: No impact on growth Medium nitrogen concentration: Growth stimulation at $5 \times 10^{-4} \text{ mg L}^{-1}$ High nitrogen concentration: Growth stimulation at 2×10^{-4} – $5 \times 10^{-4} \text{ mg L}^{-1}$ (cell count)	nitrogen-dependent toxicity ROS (GST, GSH)	(Liu et al., 2015b)
	1×10^{-4} – $6 \times 10^{-4} \text{ mg L}^{-1}$	168 h	A. 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 16/8 h; C. 25 °C; D. BG11 medium with modification	Not applied	Low nitrogen concentration: Growth inhibition Medium nitrogen concentration: Growth stimulation at $6 \times 10^{-4} \text{ mg L}^{-1}$ High nitrogen concentration: Growth stimulation at 1×10^{-4} – $6 \times 10^{-4} \text{ mg L}^{-1}$ (cell count)	photosynthesis MC protein synthesis and polysaccharides	(Liu et al., 2015a)
	6.75×10^{-3} – $9 \times 10^{-3} \text{ mg L}^{-1}$	168 h	A. 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 16/8; C. 25 °C; D. BG11 medium	Not applied	$\text{EC}_{50} = 8.03 \times 10^{-3} \text{ mg L}^{-1}$ (cell count)	MC impact of nitrogen availability on toxicity	(Liu et al., 2012a)
	5×10^{-5} – $1 \times 10^{-3} \text{ mg L}^{-1}$	168 h	A. 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 16/8 h; C. 25 °C; D. BG11 medium	Not applied	No effect on the growth (cell count)	ROS (SOD, POD, CAT), biodegradation ability	(Liu et al., 2012b)

(continued on next page)

Table 5 (continued)

Pharmaceutical class and representative	Concentrations tested	Test condition			Impact on growth (measurement)	Biochemical markers tested	Ref.
		Exposure time	A. Light intensity [$\mu\text{mol m}^{-2} \text{s}^{-1}$]; B. photoperiod (day/night); C. temp.; D. medium	Reference protocol			
Antibiotic Beta-lactam Cefazidime	1.5×10^{-3} - 1.6 mg L^{-1}	312 h	A. $16 \mu\text{mol m}^{-2} \text{s}^{-1}$; B. 14/10 h; C. 20°C ; D. Z8 medium	OECD 201	Growth inhibition ~80% at $1.2 \times 10^{-2} \text{ mg L}^{-1}$ MIC = 1×10^{-1} - 1.6 mg L^{-1} (strain-dependent) (OD ₄₅₀)	n.t.	(Dias et al., 2015)
	1.5×10^{-3} - 1.6 mg L^{-1}	312 h	A. $16 \mu\text{mol m}^{-2} \text{s}^{-1}$; B. 14/10 h; C. 20°C ; D. Z8 medium	OECD 201	Growth inhibition: 50 % at $5 \times 10^{-2} \text{ mg L}^{-1}$ (OD ₄₅₀)	n.t.	(Dias et al., 2015)
	1.5×10^{-3} - 1.6 mg L^{-1}	312 h	A. $16 \mu\text{mol m}^{-2} \text{s}^{-1}$; B. 14/10 h; C. 20°C ; D. Z8 medium	OECD 201	Growth inhibition: 50 % at $1 \times 10^{-1} \text{ mg L}^{-1}$ (OD ₄₅₀)	n.t.	(Dias et al., 2015)
Antibiotic Beta-lactam Ceftriaxone	1.5×10^{-3} - 1.6 mg L^{-1}	312 h	A. $16 \mu\text{mol m}^{-2} \text{s}^{-1}$; B. 14/10 h; C. 20°C ; D. Z8 medium	OECD 201	Growth inhibition: 50 % at $1 \times 10^{-1} \text{ mg L}^{-1}$ (OD ₄₅₀)	n.t.	(Dias et al., 2015)
Antibiotic Sulfonamide Sulfadiazine	2.0×10^{-2} - $1.80 \times 10^{-1} \text{ mg L}^{-1}$	168 h	A. $42 \mu\text{mol m}^{-2} \text{s}^{-1}$; B. 24 h; C. 21°C ; D. ISO medium with modification	ISO (1989)	EC ₅₀ = $1.35 \times 10^{-1} \text{ mg L}^{-1}$ (OD ₄₃₀ and OD ₆₇₁)	n.t.	(Holten Lützhøft et al., 1999)
Antibiotic Sulfonamide Sulfamethoxazole	1×10^{-4} - 10 mg L^{-1}	24 h	A. $10 \mu\text{mol m}^{-2} \text{s}^{-1}$; B. 16/8 h; C. 20°C ; D. Dutch Standard Water	Not applied	EC ₅₀ = $5.5 \times 10^{-1} \text{ mg L}^{-1}$ (Fv/Fm)	n.t.	(van der Grinten et al., 2010)
	1×10^{-5} - $2 \times 10^{-4} \text{ mg L}^{-1}$	360 h	A. $60 \mu\text{mol m}^{-2} \text{s}^{-1}$; B. 16/8 h; C. 25°C ; D. BG11 medium	Not applied	Growth stimulation at 1×10^{-4} - $2 \times 10^{-4} \text{ mg L}^{-1}$ (cell count)	chlorophyll <i>a</i> photosynthesis (Fv/Fm) MC ROS (SOD) proteomics combined effect with ciprofloxacin	(Liu et al., 2017)
	5×10^{-4} - $1.25 \times 10^{-1} \text{ mg L}^{-1}$	168 h	A. $37 \mu\text{mol m}^{-2} \text{s}^{-1}$; B. 12/12 h; C. 25°C ; D. BG11 medium	Not applied	Growth inhibition at concentrations $\geq 1.25 \times 10^{-2} \text{ mg L}^{-1}$ (cell count)	chlorophyll <i>a</i> fluorescence esterase activity integrity and potential of the cytoplasmic membrane cytometric analysis ROS MC	(Zhang et al., 2020)
	20-200 mg L^{-1}	840 h	A. $50 \mu\text{mol m}^{-2} \text{s}^{-1}$; B. 12/12 h; C. 25°C ; D. BG11	Not applied	Significant growth inhibition at 20-200 mg L^{-1} (OD ₆₈₀)	chlorophyll <i>a</i> ROS (SOD, CAT, MDA)	(Dong et al., 2020)
	1×10^{-2} - 50 mg L^{-1}	168 h	A. $92.4 \mu\text{mol m}^{-2} \text{s}^{-1}$; B. 12/12 h; C. 25°C ; D. BG11 medium	Not applied	Growth stimulation at $1 \times 10^{-2} \text{ mg L}^{-1}$ Growth inhibition at $\geq 1 \text{ mg L}^{-1}$ (cell count)	chlorophyll <i>a</i> phycobiliproteins ROS (SOD, CAT, MDA) ascorbate peroxidase glutathione reductase total protein content	(Chen et al., 2025)

(continued on next page)

Table 5 (continued)

Pharmaceutical class and representative	Concentrations tested	Test condition			Impact on growth (measurement)	Biochemical markers tested	Ref.
		Exposure time	A. Light intensity [$\mu\text{mol m}^{-2} \text{s}^{-1}$]; B. photoperiod (day/night); C. temp.; D. medium	Reference protocol			
Antibiotic Sulfonamide Sulfisoxazole	1×10^{-2} -50 mg L^{-1}	168 h	A. 92.4 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. (12/12 h); C. 25 °C; D. BG11 medium	Not applied	Grow stimulation at $1 \times 10^{-2} \text{ mg L}^{-1}$ Growth inhibition at $\geq 1 \text{ mg L}^{-1}$ (cell count)	chlorophyll <i>a</i> phycobiliproteins ROS (SOD, CAT, MDA) ascorbate peroxidase glutathione reductase total protein content	(Chen et al., 2025)
Antibiotic Sulfonamide Sulfadimethoxine	Not specified	144 h	A. 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 24/24 h; C – not given; D – not given	OECD 201	NOEC = 250 mg L^{-1} EC ₅₀ = 500 mg L^{-1} MIC = 2000 mg L^{-1} (OD ₆₅₅)	n.d.	(Ando et al., 2007)
Antibiotic Tetracycline Oxytetracycline	Not specified	144 h	A. 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 24/24 h; C – not given; D – not given	OECD 201	NOEC = $3.1 \times 10^{-2} \text{ mg L}^{-1}$ EC ₅₀ = $2.3 \times 10^{-1} \text{ mg L}^{-1}$ MIC = 4.0 mg L^{-1} (OD ₆₅₅)	n.d.	(Ando et al., 2007)
	4.0×10^{-2} -3.60 $\times 10^{-1} \text{ mg L}^{-1}$	168 h	A. 42 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 24 h; C. 21 °C; D. ISO medium with modification	ISO (1989)	EC ₅₀ = $2.07 \times 10^{-1} \text{ mg L}^{-1}$ (OD ₄₃₀ and OD ₆₇₁)	n.d.	(Holten Lützhøft et al., 1999)
	1×10^{-4} -10 mg L^{-1}	24 h	A. 10 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 16/8 h; C. 20 °C; D. Dutch Standard Water	Not applied	EC ₅₀ = 5.4 mg L^{-1} (Fv/Fm)	n.d.	(van der Grinten et al., 2010)
	1×10^{-2} ; 2×10^{-2} ; 5×10^{-2} ; and $1 \times 10^{-1} \text{ mg L}^{-1}$	672 h	A. 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B – not given; C. 28 °C; D. BG11 medium	OECD 201	Low growth inhibition 9.0-12.6% at 2×10^{-2} - $5 \times 10^{-2} \text{ mg L}^{-1}$ (after 456 h) (OD ₆₈₅)	MC combined with other antibiotic	(Ye et al., 2020)
	1×10^{-5} -8 $\times 10^{-3} \text{ mg L}^{-1}$	240/264 h	A. 80 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 24 h; C. 22 °C; D. BG11 medium	Not used	Growth inhibition: 8 mg L^{-1} 93%; 4 mg L^{-1} 92% No effect at concentration: 1×10^{-2} - $1 \times 10^{-1} \text{ mg L}^{-1}$ (OD ₆₈₀)	photosynthesis (Fv/Fm) pheophytin chlorophyll <i>a</i>	(Siedlewicz et al., 2020)
Antibiotic Macrolides Erythromycin	Not specified	144 h	A. 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 24/24 h; C – not given; D – not given	OECD 201	EC ₅₀ = $2.3 \times 10^{-2} \text{ mg L}^{-1}$ NOEC = $1.0 \times 10^{-2} \text{ mg L}^{-1}$ MIC = $3.2 \times 10^{-1} \text{ mg L}^{-1}$ (OD ₆₅₅)	n.d.	(Ando et al., 2007)
	1×10^{-3} -1.50 $\times 10^{-1} \text{ mg L}^{-1}$	96 h	A. 35 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 12/12 h; C. 25 °C; D. BG11 medium	Not applied	EC ₅₀ = $2.297 \times 10^{-2} \text{ mg L}^{-1}$ First exposure: EC ₅₀ = $2.95 \times 10^{-2} \text{ mg L}^{-1}$ Second exposure: EC ₅₀ = $5.9 \times 10^{-2} \text{ mg L}^{-1}$ Third exposure: EC ₅₀ = $8.1 \times 10^{-2} \text{ mg L}^{-1}$ (cell count)	photosynthesis ROS (MDA, SOD, CAT) MC antibiotic resistance analysis of transcriptional responses cells	(Wu et al., 2020)

(continued on next page)

Table 5 (continued)

Pharmaceutical class and representative	Concentrations tested	Test condition			Impact on growth (measurement)	Biochemical markers tested	Ref.
		Exposure time	A. Light intensity [$\mu\text{mol m}^{-2} \text{s}^{-1}$]; B. photoperiod (day/night); C. temp.; D. medium	Reference protocol			
Antibiotic Macrolides Clarithromycin	5×10^{-4} - $5 \times 10^{-1} \text{ mg L}^{-1}$	168 h	A. $37 \mu\text{mol m}^{-2} \text{s}^{-1}$; B. 12/12 h; C. 25°C ; D. BG11 medium	Not applied	Growth inhibition at 2×10^{-1} and $5 \times 10^{-1} \text{ mg L}^{-1}$ Growth stimulation at 5×10^{-4} and $5 \times 10^{-3} \text{ mg L}^{-1}$ (after 24 and 96 h) (cell count)	chlorophyll <i>a</i> fluorescence esterase activity integrity and potential of the cytoplasmic membrane cytometric analysis ROS; MC photosynthesis	(Zhang et al., 2020)
	1×10^{-1} - 25 mg L^{-1}	12 h	A. $30 \mu\text{mol m}^{-2} \text{s}^{-1}$; B. 12/12 h; C. 25°C ; D. BG11 medium	Not applied	n.d.	photosynthesis	(Deng et al., 2014)
	1×10^{-4} - $1 \times 10^{-1} \text{ mg L}^{-1}$	96 h	A. $37.5 \mu\text{mol m}^{-2} \text{s}^{-1}$; B. 12/12 h; C. 25°C ; D. BG11 medium	Not applied	$\text{EC}_{50} = 4.331 \times 10^{-2} \text{ mg L}^{-1}$ (cell count)	photosynthesis phycobiliprotein ROS (MDA)	(Wu et al., 2022)
	1×10^{-4} - 10 mg L^{-1}	24 h	A. $10 \mu\text{mol m}^{-2} \text{s}^{-1}$; B. 16/8 h; C. 20°C ; D. Dutch Standard Water	Not applied	$\text{EC}_{50} = 2.9 \times 10^{-1} \text{ mg L}^{-1}$ (Fv/Fm)	n.t.	(van der Grinten et al., 2010)
	2.5×10^{-4} - $2.5 \times 10^{-3} \text{ mg L}^{-1}$	168 h	A. $40 \mu\text{mol m}^{-2} \text{s}^{-1}$; B. 16/8 h; C. 25°C ; D. BG11 medium	Not applied	$\text{EC}_{50} = 8.7 \times 10^{-4}$ - $1.38 \times 10^{-3} \text{ mg L}^{-1}$ (nitrogen-dependent) (cell count)	MC impact of nitrogen availability on toxicity	(Liu et al., 2012a)
	5×10^{-5} - $1 \times 10^{-3} \text{ mg L}^{-1}$	168 h	A. $40 \mu\text{mol m}^{-2} \text{s}^{-1}$; B. 16/8 h; C. 25°C ; D. BG11 medium	Not applied	Growth inhibition at 5×10^{-4} - $1 \times 10^{-3} \text{ mg L}^{-1}$ (cell count)	ROS (SOD, POD, CAT), biodegradation	(Liu et al., 2012b)
	$3 \times 10^{-4} \text{ mg L}^{-1}$	30 days	A. $50 \mu\text{mol m}^{-2} \text{s}^{-1}$; B. 12/12 h; C. 25°C ; D. BG11 medium	Not applied	Growth stimulation at concentration $3 \times 10^{-4} \text{ mg L}^{-1}$ (cell count)	MC ROS photosynthesis	(Wang et al., 2019)
Antibiotic Macrolide Azithromycin	2.5×10^{-4} - $2.5 \times 10^{-3} \text{ mg L}^{-1}$	168 h	A. $40 \mu\text{mol m}^{-2} \text{s}^{-1}$; B. 12/12 h; C. 25°C ; D. BG11 medium	Not applied	$\text{EC}_{50} = 1.14 \times 10^{-3} \text{ mg L}^{-1}$ (cell count)	MC K^+ and Mg^{2+} leakage combined effect of antibiotic mixture	(Wang et al., 2018)
	3×10^{-4} and $6 \times 10^{-4} \text{ mg L}^{-1}$	240 h	A. $50 \mu\text{mol m}^{-2} \text{s}^{-1}$; B. 12/12 h; C. 24°C ; D. ASM-1 medium	Not applied	Growth stimulation at concentration 3×10^{-4} and $6 \times 10^{-4} \text{ mg L}^{-1}$	MC photosynthesis	(Celano et al., 2026)

(continued on next page)

Table 5 (continued)

Pharmaceutical class and representative	Concentrations tested	Test condition			Impact on growth (measurement)	Biochemical markers tested	Ref.
		Exposure time	A. Light intensity [$\mu\text{mol m}^{-2} \text{s}^{-1}$]; B. photoperiod (day/night); C. temp.; D. medium	Reference protocol			
Antibiotic Macrolide Roxithromycin	3×10^{-5} and $8 \times 10^{-3} \text{ mg L}^{-1}$	168 h	A. 27 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 12/12 h; C. 25 °C; D. BG-11 medium	Not applied	Growth stimulation at concentration 3×10^{-4} and $1 \times 10^{-3} \text{ mg L}^{-1}$ Growth inhibition 5×10^{-3} and $8 \times 10^{-3} \text{ mg L}^{-1}$	MC RNA-seq transcriptomic analysis	(Xin et al., 2022)
Antibiotic Diaminopyrimidines Trimethoprim	Not specified	144 h	A. 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 24 h; C – not given; D – not given	OECD 201	NOEC = 100 mg L^{-1} EC₅₀ = 150 mg L^{-1} MIC = 200 mg L^{-1} (OD ₆₅₅)	n.t.	(Ando et al., 2007)
	1×10^{-4} -10 mg L^{-1}	24 h	A. 10 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 16/8 h; C. 20 °C; D. Dutch Standard Water	Not applied	EC₅₀ = 6.9 mg L^{-1} (Fv/Fm)	n.t.	(van der Grinten et al., 2010)
	100 – 132 mg L^{-1}	168 h	A. 42 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 24 h; C. 21 °C; D. ISO medium with modification	ISO (1989)	EC₅₀ = 112 mg L^{-1} (OD ₄₃₀ and OD ₆₇₁)	n.t.	(Holten Lützhøft et al., 1999)
Antibiotic Aminoglycoside Kanamycin	1.5×10^{-3} -1.6 mg L^{-1}	312 h	A. 16 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 14/10 h; C. 20 °C; D. Z8 medium	OECD 201	Growth inhibition: 100 % at $1.5 \times 10^{-3} \text{ mg L}^{-1}$ 95 % at $3 \times 10^{-3} \text{ mg L}^{-1}$ 90 % at $6 \times 10^{-3} \text{ mg L}^{-1}$ 80 % at 12×10^{-2} – $2.5 \times 10^{-2} \text{ mg L}^{-1}$ 75 % at $5 \times 10^{-2} \text{ mg L}^{-1}$ 60 % at $1 \times 10^{-1} \text{ mg L}^{-1}$ 30 % at $2 \times 10^{-1} \text{ mg L}^{-1}$ 20 % at 0.4-1.6 mg L^{-1} (OD ₄₅₀)	n.t.	(Dias et al., 2015)
Antibiotic Aminoglycoside Gentamycin	1.5×10^{-3} -1.6 mg L^{-1}	312 h	A. 16 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 14/10 h; C. 20 °C; D. Z8 medium	OECD 201	Growth inhibition 50 % at $2 \times 10^{-1} \text{ mg L}^{-1}$ (OD ₄₅₀)	n.t.	(Dias et al., 2015)
Antibiotic Aminoglycoside Streptomycin	1×10^{-4} -10 mg L^{-1}	24 h	A. 10 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 16/8 h; C. 20 °C; D. Dutch Standard Water	Not used	EC₅₀ = 3.4 $\times 10^{-2} \text{ mg L}^{-1}$ (Fv/Fm)	n.t.	(van der Grinten et al., 2010)
	5×10^{-2} -1 mg L^{-1}	96 h	A. ~70 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 12/12 h; C. 25 °C; D. BG11 medium		EC₅₀ = 2.9 $\times 10^{-1} \text{ mg L}^{-1}$ (cell density)	photosynthetic pigments (chlorophyll a, phycobilins) ROS (SOD, POD, CAT, MDA) photosynthesis genes	(Qian et al., 2012)
Antibiotic Amphenicol Thiamphenicol	Not specified	144 h	A. 50 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 24/24 h; C – not given; D – not given	OECD 201	EC₅₀ = 3.2 $\times 10^{-1} \text{ mg L}^{-1}$ (OD ₆₅₅)	n.t.	(Ando et al., 2007)

(continued on next page)

Table 5 (continued)

Pharmaceutical class and representative	Concentrations tested	Test condition			Impact on growth (measurement)	Biochemical markers tested	Ref.
		Exposure time	A. Light intensity [$\mu\text{mol m}^{-2} \text{s}^{-1}$]; B. photoperiod (day/night); C. temp.; D. medium	Reference protocol			
Antibiotic/Tetracycline Tetracycline	1.5×10^{-3} - 1.6 mg L^{-1}	312 h	A. $16 \mu\text{mol m}^{-2} \text{s}^{-1}$; B. 14/10 h; C. 20°C ; D. Z8 medium	OECD 201	Growth inhibition 50 % at $5 \times 10^{-2} \text{ mg L}^{-1}$ (OD ₄₅₀)	n.t.	(Dias et al., 2015)
	0.05 - 1 mg L^{-1}	144 h	A. $28 \mu\text{mol m}^{-2} \text{s}^{-1}$; B. 12/12 h; C. 25°C ; D. BG11 medium	OECD 201	Growth inhibition at 0.1 - 1 mg L^{-1} Growth stimulation at 0.05 mg L^{-1} (OD/cell count)	biomass photosynthesis (Fv/Fm) ROS (SOD and MDA) antibiotic resistance	(Yang et al., 2013)
	1×10^{-2} ; 2×10^{-2} ; 5×10^{-2} ; and $1 \times 10^{-1} \text{ mg L}^{-1}$	672 h	A. $40 \mu\text{mol m}^{-2} \text{s}^{-1}$; B – not given; C. 28°C ; D. BG11 medium	OECD 201	Low growth stimulation 3.7 - 8.6% at 1×10^{-2} - $5 \times 10^{-2} \text{ mg L}^{-1}$ (after 456 h) (OD ₆₈₅)	MC combined with other antibiotic	(Ye et al., 2020)
Antibiotic - Tetracycline Chlortetracycline	1×10^{-2} ; 2×10^{-2} ; 5×10^{-2} ; and $1 \times 10^{-1} \text{ mg L}^{-1}$	672 h	A. $40 \mu\text{mol m}^{-2} \text{s}^{-1}$; B – not given; C. 28°C ; D. BG11 medium	OECD 201	Not significant effect on the growth (OD ₆₈₅)	MC combined with other antibiotic	(Ye et al., 2020)
NSAIDs Diclofenac	100 mg L^{-1}	240 h	A – not given; B – 24/24h; C – 28°C ; D – not given	Not applied	Growth inhibition 38% compared to control (OD ₆₆₃ and cell count)	n.t.	(Bácsi et al., 2016)
	100 mg L^{-1}	96 h	A – not given; B. 24/24h; C. 23°C ; D. Z8 medium	OECD 201	No effect on the growth (OD ₆₂₅ and cell count)	MC photosynthesis	(Kwidzińska et al., 2024)
NSAIDs Ibuprofen	100 mg L^{-1}	240 h	A – not given; B. 24/24h; C. 28°C ; D – not given	Not applied	Growth inhibition 7.6% (OD ₆₆₃ and cell count)	n.t.	(Bácsi et al., 2016)
	1.0×10^{-2} - 10 mg L^{-1}	96 h	A. $40 \mu\text{mol m}^{-2} \text{s}^{-1}$; B. 16/8 h; C – not given; D. BG11 medium	OECD 201	EC₅₀ = $1.836 \times 10^{-1} \text{ mg L}^{-1}$ and $3.6804 \times 10^{-1} \text{ mg L}^{-1}$ (strain-dependent) growth stimulation at 0.01 mg L^{-1} (after 24h/LE3 strain) (cell count)	chlorophyll <i>a</i> ; ROS (POD, GST, MDA); biochemical composition (total carbohydrates, proteins, and lipids)	(Sha'aba et al., 2023)
NSAIDs Diflunisal	100 mg L^{-1}	240 h	A. $116.2 \mu\text{mol m}^{-2} \text{s}^{-1}$; B. 16/8 h; C – not given; D – not given	Not used	Significant effect on the growth (OD ₆₆₃ and cell count)	n.t.	(Bácsi et al., 2016)
NSAIDs Mefenamic acid	100 mg L^{-1}	240 h	A. $116.2 \mu\text{mol m}^{-2} \text{s}^{-1}$; B. 16/8 h; C – not given; D – not given	Not used	Growth inhibition 27% compared to control (OD ₆₆₃ and cell count)	n.t.	(Bácsi et al., 2016)
NSAIDs Piroxicam	100 mg L^{-1}	240 h	A. $116.2 \mu\text{mol m}^{-2} \text{s}^{-1}$; B. 16/8 h; C – not given; D – not given	Not used	Significant effect on the growth (OD ₆₆₃ and cell count)	n.t.	(Bácsi et al., 2016)

(continued on next page)

Table 5 (continued)

Pharmaceutical class and representative	Concentrations tested	Test condition			Impact on growth (measurement)	Biochemical markers tested	Ref.
		Exposure time	A. Light intensity [$\mu\text{mol m}^{-2} \text{s}^{-1}$]; B. photoperiod (day/night); C. temp.; D. medium	Reference protocol			
Antiseptic Chlorhexidine	9.48×10^{-2} - $2.358 \times 10^{-1} \text{ mg L}^{-1}$	96 h	A. 98 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 12/12 h; C. 25 °C; D – not given	OECD 201 (with modification)	$\text{EC}_{50} = 2.064 \times 10^{-1} \text{ mg L}^{-1}$ $\text{EC}_{10} = 1.085 \times 10^{-1} \text{ mg L}^{-1}$ (chlorophyll <i>a</i> and cell count)	n.t.	(Azevedo et al., 2019)
Antidepressants Sertraline	2.5×10^{-2} - $2.0 \times 10^{-1} \text{ mg L}^{-1}$	72 h	A. 46 $\mu\text{mol m}^{-2} \text{s}^{-1}$; B. 12/12 h; C. 25 °C; D. BG11	Not used	Growth inhibition: 10 % at $5.0 \times 10^{-2} \text{ mg L}^{-1}$ 27 % at $1 \times 10^{-1} \text{ mg L}^{-1}$ 69.1% at $2 \times 10^{-1} \text{ mg L}^{-1}$ (OD ₆₈₀)	n.t.	(Yang et al., 2019)

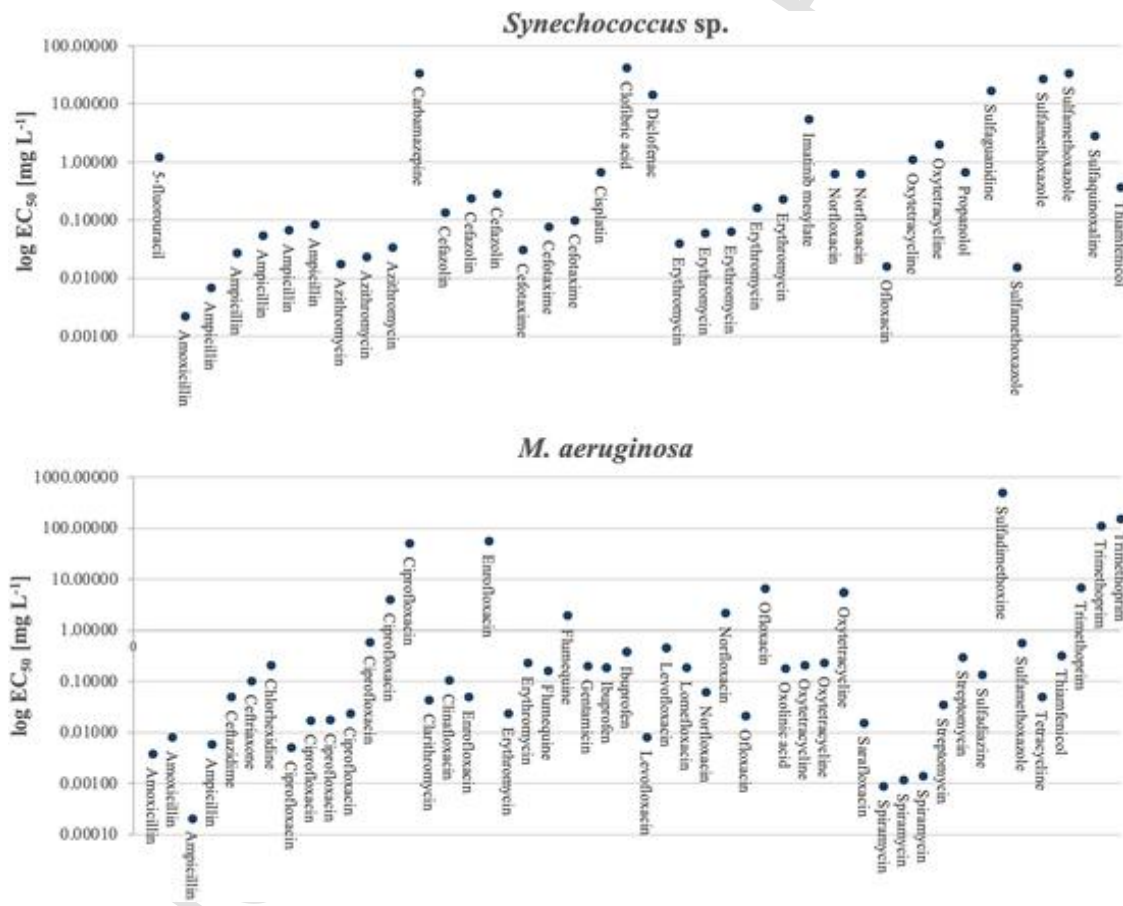


Fig. 1. EC₅₀ values for *M. aeruginosa* and *Synechococcus* sp. exposed to pharmaceuticals

inhibition of bacterial DNA synthesis via targeting two essential type II topoisomerases: DNA gyrase (the primary target in Gram-negative bacteria) and topoisomerase IV (the primary target in Gram-positive bacteria). Inhibition of these enzymes stabilizes the DNA-enzyme cleavage complex, prevents re-ligation of double-stranded DNA breaks, and ultimately leads to irreversible chromosomal damage and bacterial cell death. Fluoroquinolones exhibit concentration-dependent bactericidal activity.

Owing to their chemical stability and low biodegradability, fluoroquinolones exhibit high environmental persistence and are not efficiently removed by conventional wastewater treatment processes (Thai et al., 2023). Consequently, these compounds are frequently detected in wastewater treatment plant effluents as well as in sewage sludge, which is often applied to agricultural soils as fertilizer. Through these pathways, fluoroquinolones are introduced into aquatic systems and terrestrial environments (Al Masud et al., 2024). As consequence, fluoroquinolones are frequently detected in aquatic environments. Their con-

centrations in WWTP influents and effluents have been reported up to 6.453×10^{-3} and 9.347×10^{-3} mg L⁻¹, respectively, while concentrations in surface waters range from below detection limits to 2.888×10^{-3} mg L⁻¹ (Pauletto and De Liguoro, 2024). Concentrations in the mg L⁻¹ range have occasionally been documented. Such elevated concentrations are generally associated with proximity to pharmaceutical manufacturing facilities or intensive aquaculture operations (Pauletto and De Liguoro, 2024).

Current evidence (Fig. 2) indicates that cyanobacteria are highly sensitive to fluoroquinolones and that environmentally realistic exposure levels can exert both inhibitory and stimulatory effects, depending on concentration.

The data compiled in Tables 4 and 5 highlight the considerable variability in the EC₅₀ values reported for fluoroquinolones. Substantial variability was observed not only among different fluoroquinolones, but also between studies investigating the same compound and cyanobacterial species. In the case of *M. aeruginosa*, the reported EC₅₀ values showed high variability, ranging from 6.53×10^{-4} to 56.10 mg L⁻¹ (Table 5). Substantial differences were observed not only among different fluoroquinolones, but also between studies investigating the same compound. For example, for enrofloxacin, the EC₅₀ determined by Xia et al. (2023) was 56.10 mg L⁻¹, whereas Robinson et al. (2005) (Robinson et al., 2005) reported a value of 4.9×10^{-2} mg L⁻¹. Similarly, for flumequine, EC₅₀ values ranged from 1.59×10^{-1} mg L⁻¹ (Holten Lützhøft et al., 1999) to 1.96 mg L⁻¹ (Robinson et al., 2005). Comparable discrepancies were observed for levofloxacin (7.9×10^{-3} mg L⁻¹ (Robinson et al., 2005) vs. 4.376×10^{-1} mg L⁻¹ (Wu et al., 2022)) and ofloxacin (2.1×10^{-2} mg L⁻¹ (Robinson et al., 2005) vs. 6.54 mg L⁻¹ (Zhao et al., 2023)). Ciprofloxacin was the most frequently studied fluoroquinolone, with its effects on *M. aeruginosa* investigated in nine independent studies (Azevedo et al., 2019; Halling-

Sorensen et al., 2000; Liu et al., 2017; Robinson et al., 2005; Wan et al., 2024; Xia et al., 2023; Yisa et al., 2021; Q. Zhang et al., 2022; Zhao et al., 2023). The variability in EC₅₀ values for ciprofloxacin was particularly pronounced, ranging from 5.0×10^{-3} mg L⁻¹ (Halling-Sørensen, 2000) to 49.80 mg L⁻¹ (Xia et al., 2023). The second most frequently studied fluoroquinolone was norfloxacin, for which EC₅₀ values ranged from 1.687×10^{-2} mg L⁻¹ (J. J. Li et al., 2021) to 2.18 mg L⁻¹ (Zhao et al., 2023).

Such pronounced discrepancies in ecotoxicological test results, despite all studies reporting the same toxicity endpoint (EC₅₀), may result from differences in exposure duration, experimental guidelines and protocols, growth assessment methods, toxicity endpoints, cyanobacterial strains, and culture conditions. Therefore, direct comparisons of the reported EC₅₀ values should be interpreted with caution, as they were obtained under different experimental conditions (see Section 6 for a detailed discussion). Substantial differences were observed not only among different fluoroquinolones, but also between studies investigating the same compound.

Additionally, intraspecific variability in susceptibility among *M. aeruginosa* strains may have contributed to the observed discrepancies. For example, in the study conducted by Yisa et al. (2021) ecotoxicological assays were performed on two *M. aeruginosa* strains under standardized experimental conditions using identical growth assessment methods. Despite these controlled parameters, substantial inter-strain differences in susceptibility to ciprofloxacin were observed. The EC₅₀ value for strain EAWAG 198 (2.33×10^{-2} mg L⁻¹) was more than approximately 200-fold lower than that determined for strain LE3 (3.956 mg L⁻¹), indicating substantial inter-strain variability in susceptibility to ciprofloxacin.

Fluoroquinolones may stimulate cyanobacterial growth at environmentally relevant concentrations, as demonstrated for ciprofloxacin,

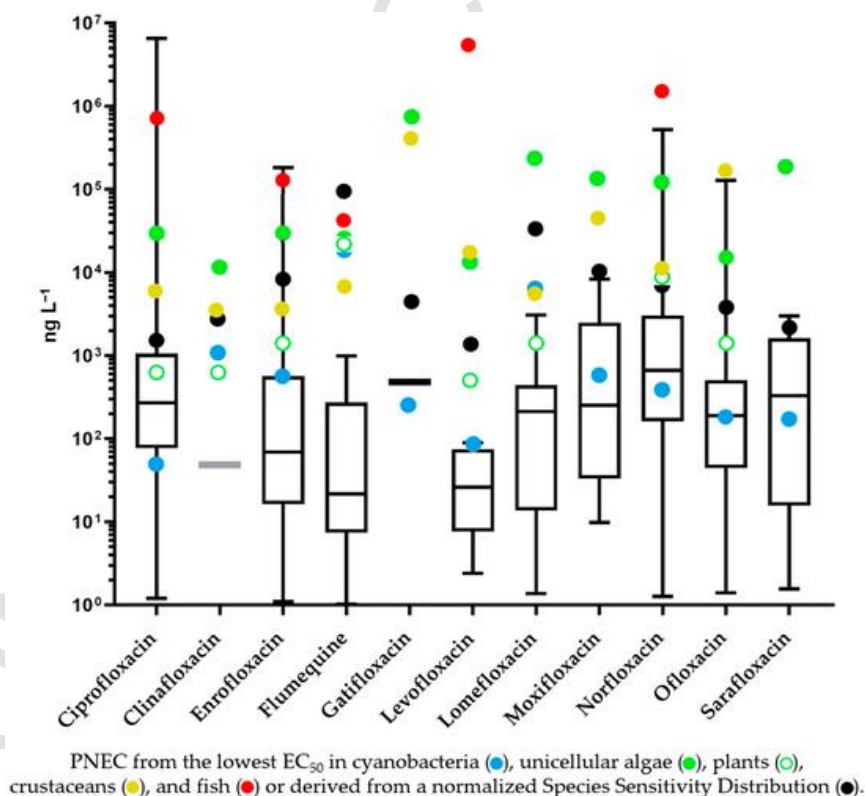


Fig. 2. Box plot of the highest concentrations of fluoroquinolones detected in the freshwater environment compared with the PNEC. Reproduced from Pauletto and De Liguoro (2024), Journal of Xenobiotics, 14, 717–752. <https://doi.org/10.3390/jox14020042>. Licensed under CC BY 4.0. (<https://www.mdpi.com/2039-4713/14/2/42>)

which enhanced the growth of *M. aeruginosa* at concentrations of 5×10^{-5} - 2×10^{-4} mg L⁻¹ (Liu et al., 2017). In contrast, stimulation induced by enrofloxacin was observed only under prolonged exposure (≥ 7 days) and at relatively high concentrations (12-36 mg L⁻¹), indicating a time-dependent and less typical response rather than classical low-dose hormesis (Xia et al., 2023). This suggests that the observed stimulation in the case of enrofloxacin may result from adaptive or compensatory responses rather than direct hormetic stimulation.

Beyond impact on the growth, fluoroquinolones induce significant physiological and biochemical alterations. Xia et al. (2023) demonstrated that prolonged exposure to sublethal concentrations of enrofloxacin (5.61×10^{-2} -1.4025 mg L⁻¹) and ciprofloxacin (4.98×10^{-2} -1.245 mg L⁻¹) resulted in a substantial increase in microcystin production. After four days of exposure, microcystin levels were approximately 50-fold higher than in control cultures. With extended exposure (8-14 days), toxin production declined relative to the peak response but remained approximately threefold higher than in unexposed controls.

Further evidence suggests that fluoroquinolones may induce significant biochemical alterations in *M. aeruginosa* cells. Yisa et al. (2021) demonstrated that exposure to ciprofloxacin induces oxidative stress in *M. aeruginosa*. After 96 h of exposure, treated cells exhibited significantly elevated intracellular hydrogen peroxide (H₂O₂) concentrations relative to controls. This increase in ROS was accompanied by alterations in antioxidant defence systems, including changes in peroxidase and GST activities, indicating disruption of cellular redox homeostasis. These findings demonstrate that ciprofloxacin can disrupt the cellular redox balance in *M. aeruginosa* and trigger antioxidant defence responses. Biochemical changes were also observed by Wu et al. (2022) after exposure to levofloxacin. This antibiotic significantly reduced photosynthetic efficiency at concentrations of 5×10^{-1} and 1 mg L⁻¹ and inhibited chlorophyll a synthesis at concentrations of 1×10^{-2} - 1 mg L⁻¹, as well as carotenoid synthesis at concentrations of 5×10^{-2} - 1 mg L⁻¹. Levofloxacin induced oxidative stress; the researchers observed a significant increase in ROS (138.9%) and MAD (132.7%) after exposure to 1×10^{-1} mg L⁻¹. In the study conducted by Deng et al. (2015), biochemical alterations in *M. aeruginosa* cells were observed following exposure to ofloxacin. Cyanobacterial cells exposed to 1×10^{-1} mg L⁻¹ of ofloxacin exhibited an increase in chlorophyll a and carotenoid contents by 23% and 21%, respectively, along with enhanced photosynthetic efficiency.

Compared with *M. aeruginosa*, substantially fewer ecotoxicological studies have examined fluoroquinolone effects on *Synechococcus* sp. The sensitivity of *Synechococcus* sp. has been evaluated for four fluoroquinolones: ofloxacin, norfloxacin, ciprofloxacin, and enrofloxacin. Among these compounds, ofloxacin exhibited the highest toxicity, with the lowest reported EC₅₀ value (1.6×10^{-2} mg L⁻¹) (Ferrari et al., 2004), whereas norfloxacin was the least toxic (EC₅₀ 6.3×10^{-1} mg L⁻¹) (Ando et al., 2007).

Studies by Fang and Liu (2023) further elucidated the effects of ciprofloxacin and enrofloxacin on *Synechococcus* sp. Cyanobacterial cultures were exposed to concentrations ranging from 2×10^{-4} to 2.0×10^{-2} mg L⁻¹ for four days. In the case of enrofloxacin, a biphasic (hormetic) response was observed. Higher concentrations (1.0×10^{-2} - 2.0×10^{-2} mg L⁻¹) significantly inhibited cell growth, whereas lower concentrations (2×10^{-4} - 5×10^{-3} mg L⁻¹) stimulated growth relative to controls. In addition to growth endpoints, multiple physiological and biochemical parameters were assessed, including lipid production, photosynthetic performance, and metabolic activity associated with bioaccumulation processes. Changes in photosynthetic activity paralleled growth responses, with inhibition at higher concentrations and stimulation at lower concentrations. All these results suggest induction of multi-pathway stress in cyanobacteria cell. Moreover, the study demonstrated that *Synechococcus* sp. possesses the capacity to bioaccumulate

and partially biodegrade fluoroquinolones, indicating a potential role in the environmental fate and transformation of these compounds.

Environmental exposure to fluoroquinolones - particularly from treated wastewater and aquaculture effluents, where concentrations peak - is hypothesized to potentially disrupt cyanobacterial communities. For example, enrofloxacin, a veterinary fluoroquinolone, has been shown to stimulate the growth of *Synechococcus* at environmentally relevant concentrations (2×10^{-4} mg L⁻¹) (Fang and Liu, 2023). Such growth stimulation at subinhibitory concentrations may disrupt microbial community structure and potentially contribute to the proliferation of cyanobacterial blooms. Moreover, exposure to enrofloxacin at 5.6×10^{-2} mg L⁻¹ concentrations has been reported to enhance the production and release of hepatotoxic microcystins by *M. aeruginosa*, posing additional risks to aquatic organisms and water quality (Xia et al., 2023). Experimental evidence further suggests that fluoroquinolone concentrations of 1×10^{-3} mg L⁻¹, used as a conservative estimate of environmental exposure in surface waters, may pose a risk to *M. aeruginosa* (Robinson et al., 2005). Notably, predicted no-effect concentrations (PNECs) for fluoroquinolones are lowest for cyanobacteria compared with other aquatic taxa (Al Masud et al., 2024), indicating their high sensitivity. However, verifying such hypothesis that fluoroquinolones can disrupt the phytoplankton communities requires experiments conducted under conditions that closely mimic real-world environments.

Collectively, current evidence indicates that cyanobacteria are highly sensitive to fluoroquinolones and that environmentally realistic exposure levels can exert both inhibitory and stimulatory effects, depending on concentration, exposure duration, and strain-specific susceptibility.

4.1.2. Beta-lactams

Beta-lactam antibiotics account for approximately half of the total global antibiotic production (Elander, 2003). A defining structural feature of this class is the presence of a β -lactam ring, which is essential for their bactericidal activity. The antibacterial mechanism of β -lactams involves the covalent binding of antibiotic molecules to enzymes embedded in the bacterial cytoplasmic membrane (Table S1). These enzymes, known as penicillin-binding proteins (PBPs), are so named because of their structural and biochemical affinity for β -lactam compounds. PBPs play a critical role in the biosynthesis and remodelling of bacterial peptidoglycan. Consequently, inhibition of their enzymatic activity disrupts cell wall assembly, compromises cell wall integrity, and ultimately leads to osmotic lysis and bacterial cell death (Lima et al., 2020). Environmental concentrations of β -lactam antibiotics are generally lower than those of many other antibiotic classes because the β -lactam ring is chemically unstable and undergoes rapid hydrolysis and photodegradation in aquatic environments (Arsand et al., 2018). Nevertheless, β -lactam antibiotics have been detected in various aquatic matrices, with reported concentrations reaching 3.34×10^{-1} mg L⁻¹ in wastewater, 1.375×10^{-2} mg L⁻¹ in surface water, 1.22×10^{-3} mg L⁻¹ in groundwater, and 3.462×10^{-3} mg L⁻¹ in drinking water (information from <https://www.umweltbundesamt.de/en/database-pharmaceuticals-in-the-environment-1>).

Among β -lactam antibiotics, ampicillin was one of the most frequently investigated compounds in the reviewed studies (Tables 4 and 5). In experiments conducted by Ando et al. (2007), ampicillin exhibited high toxicity toward *M. aeruginosa*, with an EC₅₀ value of 2×10^{-4} mg L⁻¹ (144 h exposure; growth assessed based on OD₆₅₅). In contrast, Wang et al. (2019) reported growth stimulation of *M. aeruginosa* at 3×10^{-4} mg L⁻¹, a concentration very close to the EC₅₀ value reported by Ando et al. (2007), highlighting substantial variability in the observed responses. In turn, Wang et al. (2018) reported lower toxicity, with an EC₅₀ value of 5.79×10^{-3} mg L⁻¹ (168 h exposure; growth assessed based on chlorophyll a content). Furthermore, Baselga-Cervera et al. (2019) demonstrated that the growth of *M. aeruginosa* exposed to

ampicillin at concentrations ranging from 6×10^{-3} to 1.4×10^{-2} mg L⁻¹ was inhibited by approximately 80% across the tested concentration range. In the case of *Synechococcus*, the observed toxicity of ampicillin was also variable. Ando et al. (2007) reported EC₅₀ values of 8.3×10^{-2} and 6.9×10^{-3} mg L⁻¹ for two simultaneously examined strains. Comparable EC₅₀ values were reported for four *Synechococcus* strains, for which growth was assessed via phycocyanin fluorescence measurements, ranging from 2.75×10^{-2} to 6.66×10^{-2} mg L⁻¹ (Le Page et al., 2019).

Amoxicillin is another frequently investigated β -lactam antibiotic. The EC₅₀ value determined by Andreozzi et al. (2004) for *Synechococcus* was 2.2×10^{-3} mg L⁻¹. In the case of *M. aeruginosa*, EC₅₀ values ranged from 3.7×10^{-3} mg L⁻¹ (Holten Lützhøft et al., 1999) to 8.03×10^{-3} mg L⁻¹ (Liu et al., 2012a). Furthermore, Dias et al. (2015) demonstrated that amoxicillin inhibited the growth of *M. aeruginosa* by approximately 80% after exposure to 1.2×10^{-2} mg L⁻¹, while the minimum inhibitory concentration (MIC) ranged from 1×10^{-1} to 1.6 mg L⁻¹ depending on the tested strain.

Another β -lactam antibiotic, cefotaxime, exhibited comparable toxicity, with EC₅₀ values for *Synechococcus* ranging from 3.10×10^{-2} to 9.79×10^{-2} mg L⁻¹ depending on the strain. In contrast, the same study reported that cefazolin showed markedly lower toxicity toward *Synechococcus*, with EC₅₀ values ranging from 0.134 to 2.83×10^{-1} mg L⁻¹, also depending on the tested strain (Le Page et al., 2019).

β -lactam antibiotics also may induce hormetic responses in cyanobacteria. For example, stimulation of *M. aeruginosa* growth was observed following exposure to amoxicillin at concentrations of 1×10^{-4} - 3×10^{-4} mg L⁻¹ (Liu et al., 2016). A comparable stimulatory effect was reported after a 7-day exposure to amoxicillin at concentrations of 2×10^{-4} - 5×10^{-4} mg L⁻¹ under varying nitrogen conditions, with the magnitude of growth stimulation increasing with rising antibiotic concentration in each culture (Liu et al., 2015a, 2015b). Moreover, amoxicillin exposure induced biochemical responses in *M. aeruginosa*, including enhanced production and release of cyanotoxins during a 30-day exposure period at concentrations of 1×10^{-4} - 3×10^{-4} mg L⁻¹ (Liu et al., 2016). This suggests that in locations with high wastewater discharge, there is a real risk of cyanobacterial stimulation and a consequent increase in microcystin production.

Overall, the available data indicate substantial variability in cyanobacterial responses to β -lactam antibiotics, both between species and experimental conditions, with effects ranging from strong growth inhibition to stimulation at low concentrations. This variability may be even more pronounced under natural environmental conditions. The toxicity of β -lactam antibiotics may differ between cyanobacteria cultivated under controlled laboratory conditions and those occurring in natural environments. Laboratory cultures of *Microcystis* sp. often become more dispersed or form smaller colonies over time, although some strains may retain their colonial morphology during prolonged cultivation under laboratory conditions (Otsuka et al., 2000; Zhang et al., 2007). Colonial cyanobacteria often possess a thicker extracellular polysaccharide matrix than disaggregated cells. This structural difference may act as a protective barrier, potentially reducing β -lactam penetration and resulting in lower apparent toxicity in colonial cyanobacteria.

4.1.3. Sulphonamides

Sulphonamides constitute one of the oldest classes of synthetic antibiotics. Their primary mechanism of action involves inhibition of folic acid biosynthesis through competitive inhibition of the enzyme dihydropteroate synthase (Table S1). This enzyme is essential for the synthesis of dihydropteroate, a precursor of tetrahydrofolate, which is required for the biosynthesis of purines, pyrimidines, and ultimately DNA. Consequently, sulphonamides suppress bacterial growth by disrupting nucleotide synthesis pathways (Tacie et al., 2017). Due to their broad antibacterial activity and relatively low cost, these compounds

are widely used in both human and veterinary medicine, including applications as feed additives in animal production systems (Ovung and Bhattacharyya, 2021). Bacteria have developed multiple strategies to cope with sulphonamide exposure. These include the development of antibiotic resistance mechanisms, as well as metabolic processes such as co-metabolic transformation and partial or complete mineralization of the compounds (Nunes et al., 2020). Cyanobacteria are also capable of developing resistance to antibiotics, including sulphonamides, through adaptive physiological and genetic responses (Wang et al., 2020; Xin et al., 2024). Among sulphonamides, sulfamethoxazole is currently one of the most widely used compounds and is frequently administered in combination with trimethoprim to enhance antibacterial efficacy (Van Boeckel et al., 2014).

Primary producers such as algae and aquatic plants are generally considered the most sensitive aquatic organisms to sulphonamides because these antibiotics interfere with folate biosynthesis pathways (Cheong et al., 2020). Ecotoxicological risk assessments have indicated that several sulphonamides, including sulfadiazine, sulfamethoxazole, and sulfamethazine, may pose considerable risks to aquatic ecosystems (Duan et al., 2022). Based on species sensitivity distribution analyses, ecological risk thresholds for sulphonamides in aquatic environments have been estimated at approximately 9.4×10^{-2} mg L⁻¹ (Fu et al., 2024).

Sulfonamides exhibited variable toxicity toward cyanobacteria across species, compounds, and even within the same species exposed to the same pharmaceutical. The EC₅₀ values determined for sulfamethoxazole and sulfaguanidine toward *Synechococcus* were 2.834 mg L⁻¹ and 16.594 mg L⁻¹, respectively (De Liguoro et al., 2010). Sulfamethoxazole has been the most extensively studied sulfonamide, and the EC₅₀ values reported for *Synechococcus* vary considerably for this antibiotic. According to Ferrari et al. (2004), the EC₅₀ for *S. leopoliensis* is 26.8 mg L⁻¹. In contrast, Wu et al. (2022) reported that exposure to 1×10^{-3} mg L⁻¹ reduced the growth of *Synechococcus* sp. by 63%, while Zhang et al. (2023) determined an EC₅₀ value of 1.56×10^{-2} mg L⁻¹.

Sulfamethoxazole induced a hormetic effect toward *M. aeruginosa*. Exposure to higher concentrations inhibited growth, as observed at concentrations of 11.25×10^{-3} - 1.25×10^{-1} mg L⁻¹ (Zhang et al., 2020), 20 - 200 mg L⁻¹ (Dong et al., 2020), and 1 - 50 mg L⁻¹ (Chen et al., 2025), while van der Grinten et al. (2010) reported an EC₅₀ value of 5.5×10^{-1} mg L⁻¹. In contrast, exposure to very low concentrations stimulated cyanobacterial growth. For example, Liu et al. (2017) observed growth stimulation following exposure to concentrations of 1×10^{-4} - 2×10^{-4} mg L⁻¹. A hormetic effect was also reported by Chen et al. (2025) who observed growth stimulation at 1×10^{-2} mg L⁻¹ for both sulfamethoxazole and sulfisoxazole, whereas exposure to higher concentrations clearly inhibited growth (50 mg L⁻¹ reduced growth by 29.3% and 38.1%, respectively).

The toxicity of sulfadiazine toward *M. aeruginosa* was similar to that of sulfamethoxazole, with an EC₅₀ value of 1.35×10^{-1} mg L⁻¹ (Holten Lützhøft et al., 1999). Among the tested sulfonamides, sulfadimethoxine was the least toxic to *M. aeruginosa*, with an EC₅₀ value of 500 mg L⁻¹ (Ando et al., 2007).

The molecular response to sulfonamides was also assessed, including ROS (MDA, SOD, CAT, GPx, GST, GSH), carotenoid content, photosynthetic efficiency (Fv/Fm) and psbA expression, as well as changes in chlorophyll and tocopherol metabolism, photosynthetic pathways and folic acid biosynthesis (Tables 4 and 5).

Overall, the available data indicate pronounced variability in cyanobacterial responses to sulphonamides, which appears to be influenced by both compound-specific properties and experimental conditions.

The substantial variability in EC₅₀ values reported for sulphonamide toxicity toward cyanobacteria may partly result from changes in the chemical speciation of these compounds under different environmental

pH conditions. The dominant molecular form of a compound at a given pH is determined by its pK_a values, which reflect the tendency of the molecule to donate or accept protons. Sulphonamides typically possess two protonation/deprotonation sites and therefore exhibit multiple pK_a values. For example, sulfamethoxazole has a pK_{a2} of 5.81, meaning that at this pH the compound exists in approximately equal proportions of neutral and anionic forms (Simon et al., 2011). As pH increases, deprotonation increases, and the molecule becomes predominantly anionic, reaching nearly 100% anionic form at around pH 8. Conversely, decreasing pH favours the neutral form, which predominates at approximately pH 4. At pH values below 1.39 (pK_{a1}), sulfamethoxazole begins to adopt a cationic form, which becomes increasingly dominant as pH decreases further. These changes in molecular speciation can influence membrane permeability and therefore affect the bioavailability and toxicity of sulphonamides (Białk-Bielińska et al., 2011; Lis et al., 2019). However, this phenomenon is not limited to this class of compounds. Fluoroquinolones and tetracyclines contain both acidic and basic functional groups in their structure, and the pK_a values of these groups, depending on the pH of the environment, allow these antibiotics to exist in acidic, basic, and zwitterionic forms (Hsu et al., 2018; Vasudevan et al., 2009). This is not characteristic of sulfonamides, which, within the pH range of natural waters, typically occur as neutral molecules or anions, while the zwitterionic form is rather uncommon. Macrolides, on the other hand, as weak bases, will mostly tend to occur as protonated forms at pH levels similar to those of natural waters (Mandic, 2014). Beta-lactam antibiotics, due to their varied structure and the presence of various additional ionizing groups, have different pH-dependent speciation profiles, which even differ among individual representatives of this class of antibiotics (Klein et al., 2021). As was reviewed (Bethke et al., 2023), the pH of water is an important modulator of ecotoxicology of pharmaceuticals.

The substantial variability in EC_{50} values reported for sulphonamide toxicity toward cyanobacteria may partly result from changes in the chemical speciation of these compounds under different environmental pH conditions. The dominant molecular form of a compound at a given pH is determined by its pK_a values, which reflect the tendency of the molecule to donate or accept protons. Sulphonamides typically possess two protonation/deprotonation sites and therefore exhibit multiple pK_a values. For example, sulfamethoxazole has a pK_{a2} of 5.81, meaning that at this pH the compound exists in approximately equal proportions of neutral and anionic forms (Simon et al., 2011). As pH increases, deprotonation increases, and the molecule becomes predominantly anionic, reaching nearly 100% anionic form at around pH 8. Conversely, decreasing pH favours the neutral form, which predominates at approximately pH 4. At pH values below 1.39 (pK_{a1}), sulfamethoxazole begins to adopt a cationic form, which becomes increasingly dominant as pH decreases further. These changes in molecular speciation can influence membrane permeability and therefore affect the bioavailability and toxicity of sulphonamides (Białk-Bielińska et al., 2011; Lis et al., 2019). However, this phenomenon is not limited to this class of compounds. Fluoroquinolones and tetracyclines contain both acidic and basic functional groups in their structure, and the pK_a values of these groups, depending on the pH of the environment, allow these antibiotics to exist in acidic, basic, and zwitterionic forms (Hsu et al., 2018; Vasudevan et al., 2009). Macrolides, on the other hand, as weak bases, will mostly tend to occur as protonated forms at pH levels similar to those of natural waters. Beta-lactam antibiotics, due to their varied structure and the presence of various additional ionizing groups, have different pH-dependent speciation profiles, which even differ among individual representatives of this class of antibiotics (Klein et al., 2021). As was reviewed (Bethke et al., 2023), the pH of water is an important modulator of ecotoxicology of pharmaceuticals.

However, in many ecotoxicological studies the pH of the culture medium is not reported, despite its potential influence on toxicity outcomes. This limitation is particularly relevant for cyanobacterial sys-

tems, as cyanobacterial growth can substantially increase the pH of the surrounding medium, often reaching values above 10 during active growth phases (Kropidłowska and Caban, 2023). In addition to pH, experimental factors such as culture medium composition, initial cell density, and exposure duration may also significantly influence observed toxicity responses.

Although cyanobacteria share several characteristics with green algae and are taxonomically classified as bacteria, their responses to antibiotic exposure may differ substantially from those of both bacterial and algal organisms. For example, Zhang et al. (2023) investigated the effects of sulfamethoxazole on *Synechococcus* and the green alga *Raphidocelis subcapitata*. Their results demonstrated that *Synechococcus* is considerably more sensitive to sulfamethoxazole than green algae, with EC_{50} values of 1.56×10^{-2} mg L⁻¹ for cyanobacteria and 8.62×10^{-1} mg L⁻¹ for green algae after four days of exposure. High concentrations of sulfamethoxazole negatively affected photosynthesis in both organisms and were associated with reductions in chlorophyll *a* content.

Further molecular-level analyses revealed that the mechanism of sulfamethoxazole toxicity in *Synechococcus* may differ from its classical antibacterial mode of action. Transcriptomic analyses performed by Yibo Zhang et al. (2022) following exposure to 5×10^{-3} and 1×10^{-2} mg L⁻¹ sulfamethoxazole showed that folic acid synthesis was not significantly inhibited in *Synechococcus*. Instead, the antibiotic primarily affected pathways related to porphyrin and chlorophyll metabolism, photosynthesis, selenium compound metabolism, and tocopherol biosynthesis.

4.1.4. Tetracycline

Tetracyclines represent a widely used class of antibiotics in both human and veterinary medicine. Similar to sulphonamides, these compounds have historically been incorporated into animal feed to promote growth and improve feed efficiency in livestock production. Tetracyclines exhibit broad-spectrum antibacterial activity against both Gram-positive and Gram-negative bacteria (Roberts, 1996). They belong to a group of bacteriostatic antibiotics whose primary molecular target is the bacterial ribosome. Their mode of action involves inhibition of protein synthesis through binding to the 30S ribosomal subunit, thereby preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor site and ultimately blocking translation (Table S1). However, the widespread use of tetracyclines has led to the increasing emergence of bacterial resistance, which is primarily acquired through the development and horizontal transfer of resistance genes (Chopra and Roberts, 2001).

Tetracyclines include both naturally occurring compounds and synthetic derivatives. Naturally occurring tetracyclines were originally isolated from *Streptomyces aureofaciens* and *Streptomyces rimosus*, yielding compounds such as chlortetracycline and oxytetracycline. Numerous semi-synthetic derivatives have subsequently been developed, including metacycline, doxycycline, and minocycline, as well as fully synthetic compounds such as rolitetracycline and lymecycline.

Typical concentrations of tetracyclines are 0.20×10^{-5} mg L⁻¹ in European surface waters (average contamination range) (Antos et al., 2024), 1.56×10^{-6} - 8.789×10^{-5} mg L⁻¹ for oxytetracycline in river water in China (Li et al., 2018), and 5×10^{-6} - 9×10^{-6} mg L⁻¹ tetracycline measured in surface waters near animal farming areas (Javid et al., 2016). However, higher levels (μg L⁻¹) can occur locally near pollution sources such as livestock farms or wastewater discharge (for example 2.79×10^{-3} - 3.597×10^{-2} mg L⁻¹ in surface runoff from manure-amended agricultural soils after rainfall (Huang et al., 2022)). Tetracyclines are generally well adsorbed to soil and sediments due to chelation with metal ions and clay minerals (Boxall et al., 2004). They therefore have low mobility in soil but may be transported via particle-bound runoff. In addition, they are well biosorbed and biodegradable (Amangelsin et al., 2023).

Among tetracyclines, oxytetracycline is the compound most frequently investigated in ecotoxicological studies involving cyanobacteria. Ando et al. (2007) determined EC_{50} values for oxytetracycline against *M. aeruginosa* and two strains of *Synechococcus*, reporting values of 2.3×10^{-1} mg L⁻¹, 1.1 mg L⁻¹, and 2.0 mg L⁻¹, respectively. These values are consistent with earlier findings by Holten Lützhöft et al. (1999), who reported an EC_{50} value of 2.07×10^{-1} mg L⁻¹ for oxytetracycline toward *M. aeruginosa*. In contrast, van der Grinten et al. (2010) reported substantially lower toxicity, with an EC_{50} value of 5.4 mg L⁻¹. However, this discrepancy may be explained by methodological differences, as their experiment lasted only 24 hours and employed a different toxicity endpoint compared with other studies.

More detailed investigations of oxytetracycline toxicity toward *M. aeruginosa* were conducted by Siedlewicz et al. (2020). In that study, cyanobacterial cultures were exposed to concentrations ranging from 1×10^{-2} to 8.00 mg L⁻¹, and effects on growth, photosynthetic efficiency, and chlorophyll *a* fluorescence were evaluated. The results showed that oxytetracycline had no significant effect on cyanobacterial growth at concentrations between 1×10^{-2} and 1 mg L⁻¹. However, exposure to higher concentrations (4 and 8 mg L⁻¹) resulted in substantial growth inhibition, reaching 92% and 93%, respectively, after five days of exposure compared with control cultures. Similarly, chlorophyll *a* content decreased markedly, by 49% and 85% at 4 and 8 mg L⁻¹, respectively. The efficiency of photosystem II (PSII) was also significantly reduced at these highest tested concentrations. A study conducted by Ye et al. (2020) also investigated biochemical changes. The results showed that exposure of *M. aeruginosa* to oxytetracycline caused only a slight reduction in growth at a concentration of 2×10^{-2} mg L⁻¹ (by 12.5% after 19 days of exposure). In contrast, oxytetracycline significantly stimulated microcystins production, with an increase of 48.5% after 6 days and 83.9% after 9 days at the same concentration.

The toxicity of tetracycline toward *M. aeruginosa* appears to be comparable to that of oxytetracycline. Wang et al. (2022) reported an EC_{50} value of 5.4×10^{-1} mg L⁻¹ for tetracycline-induced growth inhibition. Similar results were reported by Dias et al. (2015), who additionally evaluated the sensitivity of nine *M. aeruginosa* strains and observed no significant differences in their responses to tetracycline exposure.

Evidence also indicates that cyanobacteria may rapidly develop adaptive responses to tetracycline exposure. Yang et al. (2013) demonstrated that *M. aeruginosa* could acquire resistance following repeated exposure to tetracycline. During the second exposure, significantly lower levels of growth inhibition were observed compared with the initial exposure. Similar adaptive responses were observed in the photosynthetic apparatus. During the first exposure, high tetracycline concentrations (5×10^{-1} and 1 mg L⁻¹) caused pronounced inhibition of photosystem II efficiency, whereas no significant effect on photosynthetic performance was detected during the second exposure.

The development of resistance was also reflected in oxidative stress responses. Yang et al. (2013) assessed oxidative stress using MDA concentration and SOD activity. MDA is a lipid peroxidation product commonly used as a biomarker of ROS-induced cellular damage, whereas SOD represents a key antioxidant enzyme responsible for detoxifying superoxide radicals. During the first tetracycline exposure, *M. aeruginosa* exhibited elevated MDA levels relative to control cultures, indicating increased oxidative stress. In contrast, during the second exposure MDA concentrations decreased while SOD activity increased substantially across all tested concentrations, suggesting enhanced antioxidant capacity and improved cellular defence against ROS. Notably, similar adaptive responses were not observed in the green algae examined in the same study.

Comparable adaptive responses have also been reported for other tetracyclines. Lu et al. (2015) investigated the effects of chlortetracycline on *M. aeruginosa* and observed evidence of resistance development following repeated exposure. Specifically, the EC_{50} value increased from 14.13 mg L⁻¹ during the first exposure to 29.56 mg L⁻¹ during the

second exposure. Changes in the photosynthetic system followed a similar pattern: photosystem II efficiency was strongly inhibited at 48 mg L⁻¹ and significantly inhibited at 24 mg L⁻¹ during the first exposure, whereas such inhibition was not observed during the second exposure. As in the tetracycline study, reduced oxidative stress during the second exposure was indicated by lower MDA concentrations.

In natural aquatic ecosystems, cyanobacteria compete with algae and other phytoplankton groups. The presence of antibiotics such as tetracyclines may disrupt ecological interactions by selectively affecting certain taxa and thereby altering community structure. Zhou et al. (2022) examined co-cultures of *M. aeruginosa* and the green alga *Chlamydomonas microspheara* exposed to tetracycline and oxytetracycline. Their results demonstrated that *M. aeruginosa* gained a competitive advantage in the presence of tetracyclines. Oxytetracycline promoted cyanobacterial growth at concentrations of 1×10^{-1} and 2 mg L⁻¹, whereas the growth of *C. microspheara* was strongly inhibited at all tested concentrations.

Differences in sensitivity between cyanobacteria and green algae have also been reported in other studies. In Microtox-based assays evaluating photosystem II performance, the green alga *Raphidocelis subcapitata* was found to be more sensitive to oxytetracycline than cyanobacteria (van der Grinten et al., 2010). However, when compared with the cyanobacterium *M. aeruginosa*, the difference in sensitivity was relatively small for the green alga *Pseudokirchneriella subcapitata*, with EC_{50} values of 2.3×10^{-1} mg L⁻¹ and 3.42×10^{-1} mg L⁻¹, respectively (Ando et al., 2007).

4.1.5. Macrolides

Macrolides are a widely used class of bacteriostatic antibiotics. Their primary mechanism of action involves inhibition of protein synthesis through reversible binding to the 50S subunit of the bacterial ribosome (Table S1). This interaction blocks peptide chain elongation during translation and consequently suppresses bacterial cell growth (Wu et al., 2020).

Macrolides are among the most frequently detected antibiotics in rivers and wastewater effluents worldwide (aus der Beek et al., 2016; Wilkinson et al., 2022). Typical concentrations are $\sim 1 \times 10^{-5}$ - 3×10^{-4} mg L⁻¹ for erythromycin, about 5×10^{-6} - 5×10^{-4} mg L⁻¹ for clarithromycin, and 1.326×10^{-3} to 3.340 mg L⁻¹ for azithromycin near discharge zones (González-Pleiter et al., 2013; Luo et al., 2014; Ribeiro Trisotto et al., 2025). The increased concentrations of macrolides were noted in the discharge zone in rivers during the COVID pandemic and post-pandemic event (Chen et al., 2021; Ribeiro Trisotto et al., 2025). The NOECs for algae often are about 1×10^{-2} - 5×10^{-2} mg L⁻¹ and 2×10^{-2} - 1×10^{-1} mg L⁻¹ for erythromycin and clarithromycin (Guo et al., 2016; Li et al., 2022). The comparison of the reported environmental concentration and laboratory NOEC values, the base of the risk assessment, lead the conclusion that the presence of antibiotics in natural water is a treat for selected algae species. From the other side, the antibiotic resistance genes are mostly found in the cyanobacteria occupied the wastewater (Dias et al., 2019) near discharge zones (González-Pleiter et al., 2013; Luo et al., 2014; Ribeiro Trisotto et al., 2025), what mean that at these areas they can be more tolerant than laboratory study shows.

Erythromycin is one of the most extensively studied macrolide antibiotics and was originally isolated from the bacterium *Saccharopolyspora erythraea*. Among the macrolides reported in the literature analysed in this review, erythromycin has been investigated most frequently in relation to cyanobacteria (Tables 4 and 5). Ecotoxicological studies indicate that erythromycin exhibits relatively high toxicity toward *M. aeruginosa*. Reported EC_{50} values are highly consistent between studies, with values of 2.3×10^{-2} mg L⁻¹ (Ando et al., 2007) and 2.297×10^{-2} mg L⁻¹ (Wu et al., 2020). However, at low concentrations, erythromycin may also stimulate cyanobacterial growth. In a study by Zhang et al. (2020b) a hormetic effect was observed in *M.*

aeruginosa. Erythromycin at concentrations of 5×10^{-4} – 5×10^{-3} mg L⁻¹ stimulated both cyanobacterial growth and photosynthetic activity, whereas exposure to higher concentrations (2×10^{-1} – 5×10^{-1} mg L⁻¹) resulted in growth inhibition. Moreover, exposure to erythromycin over a broader concentration range (5×10^{-4} – 5×10^{-1} mg L⁻¹) increased the production and release of microcystins as well as the generation of ROS, leading to damage of cyanobacterial cell membranes.

Other studies have demonstrated that erythromycin can impair the photosynthetic apparatus of cyanobacteria. Deng et al. (2014) reported that the efficiency of photosystem I decreased significantly following exposure to 1×10^{-1} mg L⁻¹ erythromycin, whereas photosystem II was affected only at higher concentrations ranging from 5 to 25 mg L⁻¹. Similarly, Wu et al. (2020) observed reductions in photosynthetic efficiency in *M. aeruginosa* following erythromycin exposure. Their study also demonstrated that erythromycin induced oxidative stress, as indicated by significant increases in MDA, ROS, and SOD activity at certain exposure concentrations. However, at higher concentrations (1.50×10^{-1} mg L⁻¹), SOD activity was inhibited. Erythromycin exposure also affected microcystin production: concentrations between 1.0×10^{-2} and 6.0×10^{-2} mg L⁻¹ stimulated toxin synthesis, whereas higher concentrations (1.0×10^{-1} – 1.5×10^{-1} mg L⁻¹) resulted in inhibition.

The potential for *M. aeruginosa* to develop resistance to erythromycin has also been investigated. Wu et al. (2020) demonstrated that adaptive responses to antibiotic exposure may occur, suggesting that cyanobacteria may gradually reduce their sensitivity to erythromycin under repeated exposure conditions.

In the case of cyanobacteria from the genus *Synechococcus*, EC₅₀ values determined for erythromycin were more variable. Ando et al. (2007) reported EC₅₀ values of 2.3×10^{-1} and 1.6×10^{-1} mg L⁻¹ for two different *Synechococcus* strains. In contrast, Le Page et al. (2019) found EC₅₀ values that were approximately one order of magnitude lower for three strains: 6.33×10^{-2} mg L⁻¹ for *S. elongatus*, 6.39×10^{-2} mg L⁻¹ for *S. leopoliensis*, and 5.98×10^{-2} mg L⁻¹ for *Synechococcus* sp. Erythromycin also induces hormetic responses in *Synechococcus*. Fang and Liu, (2023) demonstrated that exposure to low concentrations (2×10^{-4} – 5×10^{-3} mg L⁻¹) stimulated growth and photosynthetic efficiency, whereas exposure to 2×10^{-2} mg L⁻¹ resulted in reduced growth rates and decreased photosynthetic performance. The same study also showed that erythromycin affected lipid production in cyanobacterial cells. Additionally, *Synechococcus* exhibited the ability to biodegrade erythromycin, achieving degradation efficiencies of 48.72–64.71% within 96 hours.

Hormetic responses were also observed following exposure of *M. aeruginosa* to the macrolide roxithromycin. Environmentally relevant concentrations of 3×10^{-4} and 1×10^{-3} mg L⁻¹ significantly stimulated cyanobacterial growth and microcystin production, whereas higher concentrations (5×10^{-3} and 8×10^{-3} mg L⁻¹) inhibited both responses. In addition, extracellular microcystin concentrations increased with increasing roxithromycin concentration, indicating enhanced toxin release despite growth inhibition at higher concentrations (Xin et al., 2022).

A similar response was reported for another macrolide antibiotic, azithromycin. Celano et al. (2026) demonstrated that environmentally relevant concentrations of azithromycin (3×10^{-4} and 6×10^{-4} mg L⁻¹) significantly stimulated the growth of *M. aeruginosa* and increased total microcystin content. Although the cellular microcystin quota gradually decreased over time, the total microcystin content remained higher than in the control cultures due to the increased cyanobacterial biomass. Similar observations were reported by Li et al. (2023), who demonstrated a hormetic concentration–response relationship for azithromycin, characterized by growth stimulation at low concentrations and growth inhibition at higher concentrations. The effects of azithromycin have also been investigated in cyanobacteria of the genus *Synechococcus*. Le Page et al. (2019) reported values of 1.74×10^{-2} mg

L⁻¹ for *S. elongatus*, 3.38×10^{-2} mg L⁻¹ for *Synechococcus* sp., and 2.35×10^{-2} mg L⁻¹ for *S. leopoliensis*.

Another widely used macrolide antibiotic is spiramycin, which is commonly employed in the treatment of upper and lower respiratory tract infections and toxoplasmosis. Early ecotoxicological assessments indicated high toxicity of spiramycin toward cyanobacteria, with an EC₅₀ value of 5×10^{-3} mg L⁻¹ (Halling-Sørensen, 2000). More detailed studies were later conducted by Liu et al. (2012a), who examined not only growth inhibition but also the effect of spiramycin on microcystin production in *M. aeruginosa*. The EC₅₀ value determined in this study was 1.15×10^{-3} mg L⁻¹, indicating a toxicity level comparable to that reported previously (Liu et al., 2012a). Exposure to spiramycin resulted in a decrease in total microcystin content relative to control cultures.

Further experiments investigated the effects of spiramycin at environmentally relevant concentrations (5×10^{-5} – 1×10^{-3} mg L⁻¹) on oxidative stress responses and the ability of *M. aeruginosa* to degrade the antibiotic (Liu et al., 2012b). Spiramycin exposure increased SOD activity while inhibiting POD and CAT enzyme activities. In addition, cyanobacteria could degrade between 12.5% and 32.9% of spiramycin within seven days. Subsequent research demonstrated that the toxicity of spiramycin toward *M. aeruginosa* may depend strongly on environmental conditions (Liu et al., 2015c). For example, exposure experiments conducted at concentrations of 1×10^{-4} – 4×10^{-4} mg L⁻¹ showed that the toxicity of spiramycin was influenced by nitrogen availability in the growth medium.

The sensitivity of cyanobacteria to spiramycin may also depend on their morphological state. Wang et al. (2019) demonstrated that the growth stimulation rate and microcystin content following exposure to spiramycin (3×10^{-4} mg L⁻¹) were significantly higher in unicellular *M. aeruginosa* than in colonial forms. Furthermore, interactions with other antibiotics may modify toxicity. For example, mixtures of spiramycin and ampicillin produced effects ranging from synergistic to antagonistic depending on their relative concentrations, significantly affecting *M. aeruginosa* growth and altering the quantity of toxins produced and released (Wang et al., 2018).

The toxicity of another macrolide antibiotic, tylosin, has also been investigated in cyanobacteria. Reported EC₅₀ values for *M. aeruginosa* vary considerably, ranging from 2.9×10^{-1} mg L⁻¹ (van der Grinten et al., 2010) to 3.4×10^{-2} mg L⁻¹ (Halling-Sørensen et al., 2000). These discrepancies are likely attributable to differences in experimental design, including the endpoints used (photosynthetic activity versus growth inhibition) and the duration of exposure (24 h versus 72 h). In addition, the sensitivity of *M. aeruginosa* to clarithromycin has been assessed, with an EC₅₀ value of 4.331×10^{-2} mg L⁻¹ reported. Clarithromycin exposure also altered photosynthetic pigment content and induced oxidative stress responses in cyanobacterial cells (Wu et al., 2022). In addition, for another macrolide antibiotic, azithromycin, EC₅₀ values have been determined for cyanobacteria of the genus *Synechococcus*. Le Page et al. (2019) reported values of 1.74×10^{-2} mg L⁻¹ for *S. elongatus*, 3.38×10^{-2} mg L⁻¹ for *Synechococcus* sp., and 2.35×10^{-2} mg L⁻¹ for *S. leopoliensis*.

Overall, the available data indicate that macrolides exhibit relatively high toxicity toward cyanobacteria, with consistent EC₅₀ values across studies, while also inducing hormetic and adaptive responses depending on concentration and exposure conditions.

4.1.6. Other antibiotics

Much of the existing literature has focused on trimethoprim, a synthetic antibiotic belonging to the diaminopyrimidine class. Trimethoprim exerts its antibacterial activity through inhibition of dihydrofolate reductase, thereby interfering with folate metabolism and nucleic acid synthesis.

Reported EC₅₀ values for trimethoprim show considerable variation across studies and test conditions. For *M. aeruginosa*, EC₅₀ values of 150 mg L⁻¹ (OD) (Ando et al., 2007), 112 mg L⁻¹ (OD) (Halling-Sørensen et

al., 2000; Holten Lützhøft et al., 1999) and 6.2 mg L^{-1} (Fv/Fm) (van der Grinten et al., 2010) have been reported. Dias et al. (2015) further demonstrated that cyanobacterial growth was not significantly affected at trimethoprim concentrations up to 1.6 mg L^{-1} , the highest concentration tested in their study. Such pronounced differences in EC_{50} values are likely attributable to differences in the selected endpoints.

The variability among reported toxicity thresholds can be attributed to methodological differences among studies. Ando et al. (2007) evaluated cyanobacterial growth inhibition using chlorophyll *a* content extracted from centrifuged cells after 72 h of exposure. In contrast, Holten Lützhøft et al. (1999) quantified chlorophyll content fluorometrically using a luminescence spectrophotometer at the end of a 7-day exposure period. Van der Grinten et al. (2010) employed photosystem II efficiency, expressed as the Fv/Fm fluorescence ratio, as the toxicity endpoint during a 24-h exposure period. Differences in exposure duration, physiological endpoints, and analytical methodologies likely contributed to the discrepancies in reported EC_{50} values. No EC_{50} value of trimethoprim was determined for *Synechococcus* spp. in Ando et al. (2007), however, the EC_{50} was estimated to exceed 200 mg L^{-1} , indicating relatively low acute toxicity toward this genus.

Another group of antibiotics that has been studied in relation to cyanobacteria are aminoglycosides. Their mode of action involves binding to the 30S ribosomal subunit and inhibiting protein synthesis, which leads to the suppression of cell growth. These antibiotics were toxic to *M. aeruginosa*. Among the aminoglycoside antibiotics tested, EC_{50} values were determined only for streptomycin. These values differed and were $3.4 \times 10^{-2} \text{ mg L}^{-1}$ (van der Grinten et al., 2010) and $2.9 \times 10^{-1} \text{ mg L}^{-1}$ (Qian et al., 2012). Kanamycin caused a reduction in growth at slightly higher concentrations (approx. 1×10^{-1} – $4 \times 10^{-1} \text{ mg L}^{-1}$), and a similar level of toxicity was observed for gentamicin, which inhibited growth by about 50% at a concentration of $2 \times 10^{-1} \text{ mg L}^{-1}$ (Dias et al., 2015).

The sensitivity of cyanobacteria to other antibiotics has also been investigated. For example, the EC_{50} of thiamphenicol was reported as $3.2 \times 10^{-1} \text{ mg L}^{-1}$ for *M. aeruginosa* and $3.6 \times 10^{-1} \text{ mg L}^{-1}$ and $6.7 \times 10^{-2} \text{ mg L}^{-1}$ for two *Synechococcus* strains (Ando et al., 2007), suggesting greater susceptibility of cyanobacteria to this compound compared with trimethoprim.

4.2. Analgesic and anti-inflammatory drugs

Anaesthetics and non-steroidal anti-inflammatory drugs (NSAIDs) are widely used pharmaceuticals that are commonly available over the counter in many countries. Consequently, they are frequently detected in aquatic environments (Świacka et al., 2022). Its concentration, especially at hot spots, are much higher compared to antibiotics, and reached up to several to dozen mg L^{-1} (Singh and Pflugmacher, 2026; Stec and Astel, 2024). Despite their widespread occurrence, these compounds remain relatively understudied with respect to cyanobacteria, as these microorganisms are not their primary biological targets. Nevertheless, increasing evidence indicates that NSAIDs can influence cyanobacterial growth and physiology.

Ferrari et al. (2004) demonstrated that diclofenac, one of the most frequently detected NSAIDs in natural waters, significantly reduced the growth of *S. leopoliensis*, with an EC_{50} value of 14.5 mg L^{-1} . In contrast, Bácsi et al. (2016) reported that when the same compound was tested on a different *Synechococcus* strain, no significant growth inhibition was observed even at very high concentrations (100 – 1000 mg L^{-1}). In the case of *M. aeruginosa*, exposure to 100 mg L^{-1} diclofenac for 10 days resulted in a 38 % reduction in growth relative to control cultures (Bácsi et al., 2016). Diclofenac has also been investigated in greater detail with respect to *M. aeruginosa*. In a study by Kwizzińska et al. (2024), the effects of diclofenac on cell density (OD), cell abundance (determined by flow cytometry), photosynthetic efficiency, and pigment content were examined. After 96 h of exposure to 100 mg L^{-1}

diclofenac, no significant reduction in growth rate was observed. However, the compound induced pronounced physiological and biochemical changes. A significant decrease in photosystem II efficiency was detected, indicating impaired photosynthetic activity. Moreover, diclofenac stimulated the production of microcystins.

Another frequently detected NSAID in aquatic environments is ibuprofen. Bácsi et al. (2016) evaluated the effects of ibuprofen on the growth of *M. aeruginosa* and *Synechococcus*, using chlorophyll *a* content as an indicator of biomass. Their results showed that ibuprofen at a concentration of 100 mg L^{-1} did not exert toxic effects on *M. aeruginosa* during a 10-day exposure period, making it the only one among the five tested NSAIDs that did not exhibit toxicity under the experimental conditions. Similarly, ibuprofen was the least toxic compound tested for *Synechococcus*, causing only 6 % growth inhibition during the same exposure period.

A more comprehensive assessment of ibuprofen toxicity was conducted by Sha'aba et al. (2023), who investigated its effects (1×10^{-2} – 10 mg L^{-1}) on growth, biochemical composition, and antioxidant responses in two strains of *M. aeruginosa*. The study revealed a hormetic response in one strain. Specifically, ibuprofen stimulated the growth of *M. aeruginosa* LE3 at a concentration of $1 \times 10^{-2} \text{ mg L}^{-1}$ after 24 h of exposure. At higher concentrations, however, growth inhibition was observed in both strains, with EC_{50} values of $1.836 \times 10^{-1} \text{ mg L}^{-1}$ for strain LE3 and $3.680 \times 10^{-1} \text{ mg L}^{-1}$ for strain EAWAG 198. The effects of ibuprofen on chlorophyll *a* content were strain dependent. No significant changes were observed in strain LE3, whereas in strain EAWAG 198 a concentration-dependent decrease was detected, except at $1 \times 10^{-1} \text{ mg L}^{-1}$, where a stimulatory effect was observed. Ibuprofen exposure also induced concentration-dependent oxidative stress, as evidenced by elevated MDA levels and increased activity of antioxidant enzymes. Furthermore, ibuprofen altered the biochemical composition of cyanobacterial cells, affecting the content of proteins, lipids, and carbohydrates, and both the production of cyanotoxins. This response was strain-dependent: in strain LE3, ibuprofen stimulated cyanotoxin production across the tested concentration range, whereas in strain EAWAG 198 toxin production increased at lower concentrations but decreased at concentrations exceeding $2 \times 10^{-1} \text{ mg L}^{-1}$. The study also included green algae, which were found to be more sensitive to ibuprofen than cyanobacteria.

The effects of several additional NSAIDs, including diflunisal, mefenamic acid, and piroxicam, have also been evaluated (Bácsi et al., 2016). None of these compounds significantly affected the growth of *Synechococcus*. In contrast, they inhibited the growth of *M. aeruginosa* by approximately 30 % relative to control cultures.

Procaine is a local anaesthetic that acts by blocking voltage-gated sodium channels and is commonly used in dental procedures. The effects of procaine and its derivatives on growth, photosynthetic O_2 evolution, and the ratio of phycobilins to chlorophyll *a* were investigated in *Synechococcus* cells by Suzuki et al. (2004). The study examined several compounds, including procaine, procainamide, tetracaine, lidocaine, and dibucaine, at concentrations ranging from 1×10^{-1} to 10 μM . Low concentrations (1×10^{-1} – 1 μM) of these synthetic local anaesthetics enhanced photoautotrophic growth. Among them, lidocaine exhibited the strongest stimulatory effect, followed by procainamide, tetracaine, dibucaine, and procaine. The observed increase in growth rate was comparable to that induced by similar concentrations of auxins, which are widely used as plant growth stimulators. It was also observed that the rate of oxygen release by cyanobacteria exposed to lidocaine at concentrations of 1×10^{-1} and 1 mM was three times higher than in the control and when exposed to 10 mM of the drug. However, no effect of lidocaine on changes in the ration of phicobilins to chlorophyl aof phy-cobilins produced was observed.

As reviewed, studies indicate that the presence of NSAIDs in aquatic environments may disrupt ecosystem functioning by altering the population dynamics and physiological performance of phytoplankton, po-

tentially affecting higher trophic levels within aquatic food webs (Bácsi et al., 2016; Chia et al., 2021; Grzesiuk et al., 2018). This selective pressure may promote cyanobacterial dominance or blooms, which can destabilize aquatic ecosystems.

Particularly concerning consequence of NSAID exposure is its influence on the production and release of microcystins, which are highly toxic to co-occurring aquatic organisms. These potent cyanotoxins may be stimulated by exposure to widely used pharmaceuticals such as ibuprofen and diclofenac at concentrations that do not significantly inhibit cyanobacterial growth. This response is considered part of the cellular defence mechanism against oxidative stress induced by these compounds (Holland and Kinnear, 2013; Lis et al., 2026). Under stress conditions, microcystins may be released into the surrounding environment, where they can function as antioxidants (Kurmayer, 2011) and facilitate colony formation (Gan et al., 2012), thereby increasing the resilience of cyanobacterial populations to environmental stressors.

4.3. Antineoplastic drugs

Antineoplastic drugs (cytostatic or chemotherapeutic agents) are designed to inhibit the uncontrolled proliferation of cancer cells. Their mechanisms of action involve interference with DNA replication, transcription, and repair processes, as well as disruption of cellular signalling pathways that regulate cell growth and division (Mahnik et al., 2004). Due to their increasing consumption and incomplete removal during wastewater treatment, these pharmaceuticals have been identified as emerging contaminants in aquatic environments (Castellano-Hinojosa et al., 2023; Wormington et al., 2020).

Environmental concentrations of antineoplastic drugs are generally lower than those reported for many other classes of pharmaceuticals (Nassour et al., 2020). The maximum concentration of antineoplastic drugs detected in receiving waters was $7.62 \times 10^{-4} \text{ mg L}^{-1}$ (D. Li et al., 2021). Despite their relatively low concentrations, these compounds remain environmentally relevant due to their high biological activity and inherent toxicity. Moreover, many antineoplastic drugs exhibit high chemical stability and persistence in aquatic environments (Toński et al., 2021), which may enhance their potential ecological impact. Studies investigating the effects of antineoplastic drugs on phytoplankton at environmentally relevant concentrations remain very limited. One of the few available studies was conducted by Van de Perre et al. (2022), who evaluated the effects of cyclophosphamide on a freshwater mesocosm at concentrations close to the highest levels reported in the environment (5×10^{-4} – $5 \times 10^{-2} \text{ mg L}^{-1}$). Changes in the phytoplankton community structure were observed at $5 \times 10^{-3} \text{ mg L}^{-1}$, whereas adverse effects at the population level were reported for, among others, the cyanobacterium *Oscillatoria* sp., for which the NOEC was $< 5 \times 10^{-4} \text{ mg L}^{-1}$.

The effects of four antineoplastic drugs, 5-fluorouracil, cisplatin, etoposide, and imatinib mesylate on the cyanobacterium *Synechococcus* were investigated by Brezovšek et al. (2014). Among the tested compounds, cisplatin exhibited the highest toxicity, with an EC_{50} value of $6.7 \times 10^{-1} \text{ mg L}^{-1}$. Although cyanobacteria are not the intended biological targets of this drug, its mechanism of action involves the formation of intra- and interstrand cross-links in DNA, thereby inhibiting DNA replication and transcription. The second most toxic compound was 5-fluorouracil ($\text{EC}_{50} = 1.2 \text{ mg L}^{-1}$), an antimetabolite belonging to the class of pyrimidine analogues. This compound interferes with nucleic acid metabolism by incorporating into DNA and RNA molecules and inhibiting DNA synthesis (Longley et al., 2003). 5-Fluorouracil and its derivatives are among the most widely used anticancer drugs in chemotherapy.

Imatinib mesylate exhibited moderate toxicity, with an EC_{50} value of 5.36 mg L^{-1} (Brezovšek et al., 2014). This compound acts as a selective inhibitor of several tyrosine kinases and is widely used in the treatment of certain forms of leukaemia (Hochhaus et al., 2017). In contrast,

etoposide showed the lowest toxicity, with no observed inhibition of cyanobacterial growth within the tested concentration range ($\text{NOEC} = 351.05 \text{ mg L}^{-1}$). Etoposide acts by inhibiting topoisomerase II, which leads to the accumulation of DNA strand breaks and ultimately induces cell death (Pommier, 2013).

Importantly, the toxicity thresholds determined in laboratory experiments were higher than the concentrations currently detected in aquatic environments, suggesting that individual anticancer drugs may not pose an immediate ecological risk under present environmental conditions.

4.4. Beta-blockers

Beta-blockers are a class of pharmaceuticals widely prescribed for the treatment of cardiovascular disorders, including hypertension, arrhythmias, and heart failure. Due to their extensive consumption and incomplete removal during wastewater treatment processes, these compounds are frequently detected in aquatic environments at concentrations ranging from a level of ng L^{-1} to $\mu\text{g L}^{-1}$ (Maszkowska et al., 2014a, 2014b). Consequently, they have attracted increasing attention as emerging contaminants of potential ecological concern.

The ecotoxicological effects of propranolol, a representative non-selective β -adrenergic receptor antagonist, were investigated by Ferrari et al. (2004) in a 96-h growth inhibition test. The study demonstrated that propranolol inhibited the growth of cyanobacteria, with an EC_{50} value of $6.68 \times 10^{-1} \text{ mg L}^{-1}$ and a NOEC of $3.50 \times 10^{-1} \text{ mg L}^{-1}$. These findings suggest that elevated concentrations of propranolol, particularly in areas influenced by wastewater effluents, may pose a potential risk to phytoplankton communities. \$

The precise mechanism underlying propranolol toxicity in microalgae has not yet been fully elucidated. However, it has been suggested that the compound may interfere with cell membrane integrity and photosynthetic processes (Neuwoehner and Escher, 2011), potentially leading to impaired cellular metabolism and reduced growth (Dzionek, 2025). In a study on river biofilms (containing algae and cyanobacteria), which evaluated three β -blockers (propranolol, metoprolol, and atenolol) showed that propranolol significantly inhibited photosynthetic activity in biofilm algae (Bonnineau et al., 2010). Generally, propranolol is reported to be the strongest inhibitor of microalgal growth with mostly lower than 1 mg L^{-1} (Miaze and Brozek-Pluska, 2019). The propranolol risk assessment, based on the results of over 600 research papers, showed that median EC_{50} values across aquatic species are about 6.64 mg L^{-1} , indicating generally low acute toxicity at environmental concentrations (mean $7.1 \times 10^{-6} \text{ mg L}^{-1}$) (Sumpter et al., 2021). However, the authors emphasize that sublethal and community-level effects remain insufficiently studied.

To date, no studies have specifically investigated the effects of β -blockers on the cyanobacterium *M. aeruginosa*. Overall, the current knowledge regarding the impact of β -blockers on cyanobacteria remains very limited, representing a significant gap in the ecotoxicological literature, particularly considering the widespread use of these pharmaceuticals and their global occurrence in aquatic environments (Love et al., 2024).

4.5. Mixtures of pharmaceuticals

In aquatic environments, organisms are frequently exposed to mixtures of contaminants rather than single compounds. However, evaluating mixture toxicity is remarkably difficult due to the vast, almost infinite number of pharmaceutical combinations found in aquatic and terrestrial ecosystems. Researchers face significant hurdles in modelling non-linear dose-response relationships, accounting for varying environmental transformations, and untangling complex mechanisms where pharmaceuticals may amplify or suppress each other's biological impacts.

Wang et al. (2022) investigated the effects of binary mixtures of tetracycline, sulfamethoxazole, and sulfadiazine on *M. aeruginosa*. Their results demonstrated that mixture interactions varied depending on the antibiotic combination and concentration. The mixture of sulfadiazine and tetracycline exhibited synergistic effects at low concentrations but antagonistic effects at higher concentrations. In contrast, mixtures containing sulfamethoxazole (sulfamethoxazole-sulfadiazine and sulfamethoxazole-tetracycline) showed synergistic effects across all tested concentrations.

Studies investigating mixtures of three anticancer drugs (5-fluorouracil, imatinib mesylate, and etoposide) demonstrated additive or synergistic toxicity toward *Synechococcus* (EC_{50} -mixture = 38.77 mg L⁻¹) and the green alga *Pseudokirchneriella subcapitata* (EC_{50} -mixture = 12.57 mg L⁻¹) (Brezovšek et al., 2014; Elersek et al., 2016). These studies also indicated that green algae exhibit higher sensitivity to anticancer drugs than cyanobacteria.

Stimulatory effects at environmentally relevant concentrations have also been observed for mixtures of pharmaceuticals. In a study by Jiang et al. (2021) (Jiang et al., 2021), stimulation of *M. aeruginosa* growth was reported following exposure to a mixture of antibiotics (amoxicillin, ciprofloxacin, spiramycin, sulfamethoxazole, and tetracycline) at environmentally relevant concentrations (ng L⁻¹ levels). The authors also reported biochemical changes, including increased microcystin production and alterations in gene activity. Notably, the effects observed for mixtures were more pronounced than those for individual compounds, suggesting that the environmental impact of pharmaceuticals may be amplified under realistic exposure conditions.

In a similar study on *M. aeruginosa*, exposure to a mixture of sulfamethoxazole, tetracycline, roxithromycin, and ofloxacin (2.0×10^{-4} , 8.0×10^{-4} , and 1.2×10^{-3} mg L⁻¹) significantly altered the physiology of the cyanobacteria (Feng et al., 2025). The presence of these antibiotics enhanced the production of extracellular polymeric substances (EPS), while notably increasing maximum biomass by 169% to 219% and colony size by 22% to 37%. These findings demonstrate that cyanobacterial blooms - characterized by increases in biomass, colony size, and EPS levels - can be stimulated by environmentally relevant concentrations of antibiotic mixtures.

5. Stimulative effect of pharmaceuticals

Some pharmaceuticals have been shown to stimulate cyanobacterial growth. The concentration ranges inducing stimulation in *M. aeruginosa* and *Synechococcus* sp. (Tables 4 and 5), together with concentrations detected in surface waters (aus der Beek et al., 2016), are presented in Fig. 3. Growth stimulation was observed at concentrations ranging from 5×10^{-4} to 36 mg L⁻¹ (after prolonged exposure) for *M. aeruginosa* and from 2×10^{-4} to 2.64×10^{-1} mg L⁻¹ for *Synechococcus*. In the case of *M. aeruginosa*, the compounds inducing stimulation were primarily antibiotics, except for ibuprofen, whereas for *Synechococcus* these included local anaesthetics as well as several antibiotics. Importantly, concentrations inducing stimulation were within environmentally relevant ranges (presented in Fig. 3) and, in most cases, were lower than the maximum concentrations of these pharmaceuticals detected in aquatic environments.

Such a stimulative effect may have important ecological consequences at the ecosystem level, as they may contribute to the increasing occurrence of cyanobacterial blooms. These blooms pose a significant ecological threat, leading to changes in water quality, biodiversity, and overall ecosystem functioning. Their development is often associated with oxygen depletion resulting from biomass decomposition, which may lead to hypoxic or anoxic conditions, fish mortality, and loss of benthic organisms (Carmichael and Boyer, 2016). In addition, blooms can disrupt trophic interactions by reducing plankton diversity and altering community structure, thereby decreasing ecosystem stability. The decomposition of cyanobacterial biomass further contributes to nu-

trient release and organic matter accumulation, intensifying eutrophication processes and promoting further bloom development. Moreover, exposure to such concentrations of pharmaceuticals may enhance microcystin production in *M. aeruginosa* (Liu et al., 2017; Wang et al., 2018; Xia et al., 2023), which are toxic to aquatic organisms and may bioaccumulate in food webs.

Investigating the effects of pharmaceuticals on cyanobacteria at environmentally relevant concentrations remains a significant research gap. Existing studies have focused primarily on antibiotics, while compounds from other therapeutic groups remain poorly understood, particularly at low concentrations. Experimental designs in ecotoxicology are often based on effects observed at higher concentrations, which elicit clear biological responses, leading to the exclusion of lower, environmentally relevant ranges. As a result, compounds that do not exhibit measurable effects at higher concentrations are rarely investigated at lower levels, despite the possibility that they may induce different responses, including stimulatory effects or biochemical changes. Furthermore, ecotoxicological studies predominantly focus on adverse outcomes such as growth inhibition, mortality, or EC_{50} determination, which may not fully capture the actual ecological risks associated with pharmaceutical contamination in aquatic environments. Therefore, expanding research to include environmentally relevant concentrations and considering both inhibitory and stimulatory effects is essential. This may lead to an underestimation of the actual impact of pharmaceuticals on the functioning of aquatic ecosystems.

6. Discrepancies in ecotoxicological study results

Among the results compiled in the present study (Table 4, Table 5 and Fig. 1), significant discrepancies are observed in the determined EC_{50} values and other toxicity parameters for the same species exposed to identical pharmaceuticals. For instance, in the case of *M. aeruginosa* exposed to oxytetracycline, studies conducted by Ando et al. (2007) and Holten Lützhof et al. (1999) reported EC_{50} values of approximately 2×10^{-1} mg L⁻¹, whereas van der Grinten et al. (2010) reported an EC_{50} of 5.4 mg L⁻¹ when photosynthetic activity was used as the endpoint in a 24-hour test. Discrepancies in ecotoxicological assessment were very large in the case of ciprofloxacin, which was the most frequently studied pharmaceutical. The EC_{50} values determined for *M. aeruginosa* ranged from 5×10^{-3} mg L⁻¹ to 49.80 mg L⁻¹. Significant differences were observed even when comparing studies conducted in accordance with OECD 201 guidelines. In these studies, the EC_{50} values were 5×10^{-3} mg L⁻¹ (Halling-Sorensen et al., 2000), 1.72×10^{-2} (Azevedo et al., 2019), 2.33×10^{-2} mg L⁻¹ and 3.956 mg L⁻¹ in the same study for two different strains of *M. aeruginosa* (Yisa et al., 2021). These differences may result, among other factors, from variations in ecotoxicological testing conditions, such as the type, intensity, and duration of illumination, aeration, mixing method, temperature, and the composition of the culture medium. As is presented in Table 4 and Table 5, test conditions varied in order of light, temperature, and duration. These factors have direct impact on the growth of cultures. In addition, not all authors follow the reference guidelines (presented in Section 2). Of course, this is reasonable if the main aim of the test is beyond the EC_{50} determination.

Another reason for discrepancies in results could be the methodology used to measure the growth rate of the culture (Breuer et al., 2016). In most studies, growth is determined by measuring OD, but at different wavelengths. Measurement at different wavelengths may yield varying responses because a given stressor can stimulate or reduce the production of specific pigment groups present in cyanobacteria. This error can be avoided by determining growth based on the number of cells per 1 µL of culture solution using flow cytometry, however, this method also has limitations. Firstly, it can only be applied to unicellular cyanobacteria, and secondly, cells that are diplococci (in the process of division) are counted as single cells. According to Breuer et al. (2016), the best

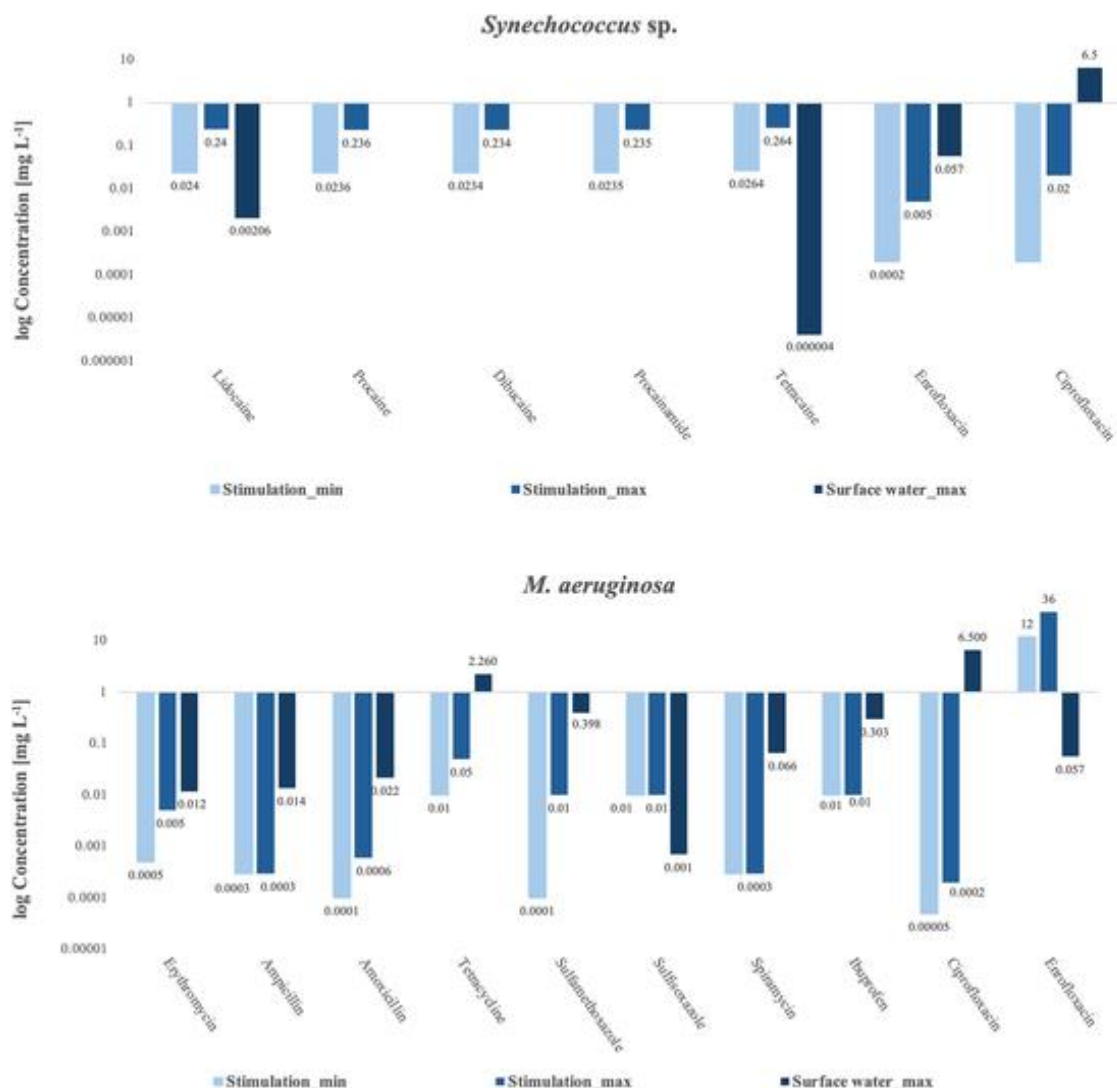


Fig. 3. Comparison of concentrations inducing growth stimulation in cyanobacteria with maximum concentrations reported in surface waters. Data on surface water concentrations were obtained from the UBA database “Pharmaceuticals in the Environment”.

among four tested growth rate methods in terms of sensitivity and usability is delayed fluorescence.

Optical microscopy-based approaches also remain an important method for assessing cyanobacterial growth. These methods provide reliable information on cell abundance, particularly when measurements based on optical density or pigment content are affected by physiological or pigmentation changes in the cells. A major advantage of optical microscopy is that it enables the observation of morphological alterations, including changes in cell shape, size, and other structural features, for example, the presence of heterocysts in cyanobacteria. However, these approaches also have several limitations, as they are time-consuming and highly operator-dependent. Another method used to assess biomass increase in cyanobacterial ecotoxicological studies is biovolume determination. This approach is particularly useful for filamentous or colonial cyanobacteria, for which simple cell counting may not adequately reflect changes in biomass. It provides a reliable and ecologically meaningful estimate of biomass by accounting for cell size and also enables the observation of morphological alterations, including changes in cell shape, size, and volume (Hawkins et al., 2005; Hillebrand et al., 1999). Nevertheless, biovolume determination also has several limitations, including difficulties associated with filament

fragmentation, the selection of appropriate geometric models for biovolume estimation, as well as being time-consuming and operator-dependent.

According to OECD guideline 201, it is required to demonstrate a correlation between the measured parameter and dry biomass (OECD, 2011). This principle aims to standardize results and enable comparison of parameters measured across different studies. However, this process is time-consuming and requires the availability of a relatively large amount of biological material.

Moreover, it has been demonstrated that significant differences in toxicity can occur within a single species among different strains. In a study conducted by Dias et al. (2015), nine distinct strains of *M. aeruginosa* were exposed to various antibiotics. The study determined the minimum inhibitory concentration (MIC). For some antibiotics, MIC values varied between strains. The greatest variability was observed for amoxicillin, with MIC values ranging from 1×10^{-1} to 1.6 mg L^{-1} .

In addition, among the wide range of pharmaceuticals present in the environment, only antibiotics and a limited number of other groups have been investigated. This indicates a significant knowledge gap regarding the ecotoxicity of these substances in the two studied cos

mopolitan species. In addition, mixture toxicity is only indicated in selected papers (Elsek et al., 2016; Jiang et al., 2021).

7. Conclusions

Previous reviews summarized in Table 1 consistently indicate that pharmaceuticals pose a substantial risk to microalgae, with cyanobacteria appearing particularly sensitive to antibiotics. Certain compounds, especially antibiotics and anticancer drugs, can directly inhibit cyanobacterial growth at environmentally relevant concentrations. Direct comparisons of the sensitivity of *M. aeruginosa* and *Synechococcus* sp. were not possible based on the available data, and even within a single species considerable variability in reported EC₅₀ values has been observed. Based on the reviewed experimental studies, it can be concluded that pharmaceuticals present in aquatic environments may exert significant hormetic effects on cyanobacteria. However, these effects are highly case-specific and observed only in laboratory conditions. These findings underscore the need for refined environmental risk assessment frameworks incorporating sublethal endpoints, hormetic responses, and intraspecific variability.

It has also been reported that cyanobacteria may develop resistance following repeated exposure to pharmaceuticals that are otherwise toxic. Consequently, the potential development of genetical antibiotic resistance in cyanobacteria inhabiting relatively pristine environments requires further investigation to improve the reliability of ecological risk assessments for in situ species. Sub-inhibitory concentrations of fluoroquinolones within environmentally relevant ranges may have particularly important ecological implications for cyanobacteria. Such concentrations may not only stimulate cyanobacterial growth but also enhance toxin production. These responses may potentially alter microbial community composition and promote cyanobacterial bloom formation. Future studies should therefore focus on the effects of pharmaceuticals at environmentally relevant concentrations, including both growth responses and biochemical changes, particularly toxin production. The special attention should therefore be given to environment subjected to continuous low doses of antibiotics, such as hot spots of wastewater discharge.

In natural aquatic systems, cyanobacteria are exposed not to individual compounds but to complex mixtures of pharmaceuticals and other contaminants, which makes environmental risk assessment particularly challenging. Mixture toxicity thus remains an important area requiring further investigation. Additionally, substantial discrepancies in experimental results have been reported for the same pharmaceutical tested against the same cyanobacterial species, highlighting the need for more standardized experimental methodologies on the one hand, and searching for the source of this variability in the protocols used. In conclusion, pharmaceutical contamination represents an emerging environmental factor that may simultaneously suppress cyanobacterial growth through toxic stress while, under certain conditions, promoting bloom formation and toxin production. This dual role underscores the complexity of pharmaceutical - cyanobacteria interactions and their potential consequences for aquatic ecosystem stability.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used ChatGPT to improve grammar and logical sequence in the article. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

CRedit authorship contribution statement

Klaudia Kwidzińska: Writing – review & editing, Writing – original draft, Visualization, Investigation, Conceptualization. **Katarzyna**

Bethke: Writing – original draft. **Magda Caban:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

Financial support was provided by the National Science Centre (Poland) under grants UMO-2019/35/B/NZ9/00253.

Data availability

No data was used for the research described in the article.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2026.108035.

References

- Ahmad, A., Azooz, M.N., Prasad, S.I., 2013. Oxidative Stress and Antioxidative Responses in Cyanobacteria, Ecophysiology and Responses of Plants Under Salt Stress. Springer, New York. <https://doi.org/10.1007/978-1-4614-4747-4>.
- Al Masud, M.A., Shin, W.S., Septian, A., Samaraweera, H., Khan, I.J., Mohamed, M.M., Billah, M.M., López-Maldonado, E.A., Rahman, M.M., Islam, A.R.M.T., 2024. Exploring the environmental pathways and challenges of fluoroquinolone antibiotics: a state-of-the-art review. Sci. Total Environ. 926, 171944. <https://doi.org/10.1016/j.scitotenv.2024.171944>.
- Albarano, L., Guadalupe, E., Suarez, P., Maggio, C., La, A., Iovine, R., Lofrano, G., Guida, M., Vaiano, V., Carotenuto, M., Libralato, G., 2024. Assessment of ecological risks posed by veterinary antibiotics in European aquatic environments: a comprehensive review and analysis. Sci. Total Environ. 954, 176280. <https://doi.org/10.1016/j.scitotenv.2024.176280>.
- Allison, D.G., Lambert, P.A., 2024. Chapter 31 - Modes of action of antibacterial agents, Molecular Medical Microbiology. Elsevier, pp. 597–614. <https://doi.org/10.1016/B978-0-12-818619-0.00133-7>.
- Amangelsin, Y., Semenova, Y., Dadar, M., Aljofan, M., Björklund, G., 2023. The impact of tetracycline pollution on the aquatic environment and removal strategies. Antibiotics 12, 440. <https://doi.org/10.3390/antibiotics12030440>.
- Ando, T., Nagase, H., Eguchi, K., Hirooka, T., Nakamura, T., Miyamoto, K., Hirata, K., 2007. A novel method using cyanobacteria for ecotoxicity test of veterinary antimicrobial agents. Environ. Toxicol. Chem. 26, 601–606. <https://doi.org/10.1897/06-195R.1>.
- Andreozzi, R., Caprio, V., Ciniglia, C., De Champdoré, M., Lo Giudice, R., Marotta, R., Zuccato, E., 2004. Antibiotics in the environment: occurrence in Italian STPs, fate, and preliminary assessment on algal toxicity of amoxicillin. Environ. Sci. Technol. 38, 6832–6838. <https://doi.org/10.1021/es049509a>.
- Andriole, V.T., 2005. The quinolones: past, present, and future. Clin. Infect. Dis. 41, S113–S119. <https://doi.org/10.1086/428051>.
- Antos, J., Piosik, M., Ginter-Kramarczyk, D., Zembrzuska, J., Kruszelnicka, I., 2024. Tetracyclines contamination in European aquatic environments: a comprehensive review of occurrence, fate, and removal techniques. Chemosphere 353, 141519. <https://doi.org/10.1016/j.chemosphere.2024.141519>.
- Arpin-Pont, L., Martínez-Bueno, M.J., Gomez, E., Fenet, H., 2016. Occurrence of PPCPs in the marine environment: a review. Environ. Sci. Pollut. Res. 23, 4978–4991. <https://doi.org/10.1007/s11356-014-3617-x>.
- Arsand, J.B., Hoff, R.B., Jank, L., Meirelles, L.N., Silvia Díaz-Cruz, M., Pizzolato, T.M., Barceló, D., 2018. Transformation products of amoxicillin and ampicillin after photolysis in aqueous matrices: identification and kinetics. Sci. Total Environ. 642, 954–967. <https://doi.org/10.1016/j.scitotenv.2018.06.122>.
- aus der Beek, T., Weber, F.A.A., Bergmann, A., Hickmann, S., Ebert, I., Hein, A., Küster, A., 2015. Pharmaceuticals in the environment-Global occurrences and perspectives. Environ. Toxicol. Chem. 35, 823–835. <https://doi.org/10.1002/etc.3339>.
- Azevedo, F.C.R., Vaz, I.C.D., Barbosa, F.A.R., Magalhães, S.M.S., 2019. Toxicological effects of ciprofloxacin and chlorhexidine on growth and chlorophyll a synthesis of freshwater cyanobacteria. Brazilian J. Pharm. Sci. 55, 1–11. <https://doi.org/10.1590/s2175-97902019000217661>.
- Bácsi, I., B-Béres, V., Kókai, Z., Gonda, S., Novák, Z., Nagy, S.A., Vasas, G., 2016. Effects of non-steroidal anti-inflammatory drugs on cyanobacteria and algae in laboratory strains and in natural algal assemblages. Environ. Pollut. 212, 508–518. <https://doi.org/10.1016/j.envpol.2016.02.031>.
- Balaban, N.Q., Helaine, S., Lewis, K., Ackermann, M., Aldridge, B., Andersson, D.I.,

- Brynildsen, M.P., Bumann, D., Camilli, A., Collins, J.J., Dehio, C., Fortune, S., Ghigo, J.M., Hardt, W.D., Harms, A., Heinemann, M., Hung, D.T., Jenal, U., Levin, B.R., Michiels, J., Storz, G., Tan, M.W., Tenson, T., Van Melderen, L., Zinkernagel, A., 2019. Definitions and guidelines for research on antibiotic persistence. *Nat. Rev. Microbiol.* 17, 441–448. <https://doi.org/10.1038/s41579-019-0196-3>.
- Baselga-Cervera, B., Cordoba-Diaz, M., García-Balboa, C., Costas, E., López-Rodas, V., Cordoba-Diaz, D., 2019. Assessing the effect of high doses of ampicillin on five marine and freshwater phytoplankton species: a biodegradation perspective. *J. Appl. Phycol.* 31, 2999–3010. <https://doi.org/10.1007/s10811-019-01823-8>.
- Bethke, K., Kropidłowska, K., Stepnowski, P., Caban, M., 2023. Review of warming and acidification effects to the ecotoxicity of pharmaceuticals on aquatic organisms in the era of climate change. *Sci. Total Environ.* 877, 162829. <https://doi.org/10.1016/j.scitotenv.2023.162829>.
- Białk-Bielińska, A., Stolte, S., Arning, J., Uebbers, U., Bösch, A., Stepnowski, P., Matzke, M., 2011. Ecotoxicity evaluation of selected sulfonamides. *Chemosphere* 85, 928–933. <https://doi.org/10.1016/j.chemosphere.2011.06.058>.
- Bonnineau, C., Guasch, H., Proia, L., Ricart, M., Geislinger, A., Romaní, A.M., Sabater, S., 2010. Fluvial biofilms: a pertinent tool to assess β -blockers toxicity. *Aquat. Toxicol.* 96, 225–233. <https://doi.org/10.1016/j.aquatox.2009.10.024>.
- Boxall, A.B.A., Fogg, L.A., Blackwell, P.A., Kay, P., Pemberton, E.J., Croxford, A., 2004. Veterinary medicines in the environment. *Rev. Environ. Contam. Toxicol.* 180, 1–91. https://doi.org/10.1007/0-387-21729-0_1.
- Brain, P., Cousens, R., 1989. An equation to describe dose responses where there is stimulation of growth at low doses. *Weed Res.* 29, 93–96. <https://doi.org/10.1111/j.1365-3180.1989.tb00845.x>.
- Breuer, F., Dören, L., Ebke, K.-P., 2016. Comparison of four measuring techniques to assess growth inhibition in standardized tests with seven freshwater algae and cyanobacteria. *Toxicol. Environ. Chem.* 98, 848–859. <https://doi.org/10.1080/02772248.2016.1139116>.
- Brezovšek, P., Eleršek, T., Filipič, M., 2014. Toxicities of four anti-neoplastic drugs and their binary mixtures tested on the green alga *Pseudokirchneriella subcapitata* and the cyanobacterium *Synechococcus leopoliensis*. *Water Res.* 52, 168–177. <https://doi.org/10.1016/j.watres.2014.01.007>.
- Caban, M., Stepnowski, P., 2021. How to decrease pharmaceuticals in the environment? a review. *Environ. Chem. Lett.* 19, 3115–3138. <https://doi.org/10.1007/s10311-021-01194-y>.
- Cai, L., Chen, C., Wang, B., Xie, G., Wang, B., Li, X., Wang, W., 2025. Metabolomic analysis of both microcystin-producing and microcystin-free *M. aeruginosa* strains in response to exogenous microcystin exposure. *Water* 17, 993. <https://doi.org/10.3390/w17070993>.
- Calabrese, E., 2007. Hormesis: why it is important to toxicology and toxicologists. *Environ. Toxicol. Chem. preprint*. 1. <https://doi.org/10.1897/07-541>.
- Calabrese, E.J., 2013. Biphasic dose responses in biology, toxicology and medicine: accounting for their generalizability and quantitative features. *Environ. Pollut.* 182, 452–460. <https://doi.org/10.1016/j.envpol.2013.07.046>.
- Calabrese, E.J., 2001. Overcompensation stimulation: a mechanism for hormetic effects. *Crit. Rev. Toxicol.* 31, 425–470. <https://doi.org/10.1080/20014091111749>.
- Calabrese, E.J., Bachmann, K.A., Bailer, A.J., Bolger, P.M., Borak, J., Cai, L., Cedergreen, N., Cherian, M.G., Chiueh, C.C., Clarkson, T.W., Cook, R.R., Diamond, D.M., Doolittle, D.J., Dorato, M.A., Duke, S.O., Feinendegen, L., Gardner, D.E., Hart, R.W., Hastings, K.L., Hayes, A.W., Hoffmann, G.R., Ives, J.A., Jaworski, Z., Johnson, T.E., Jonas, W.B., Kaminski, N.E., Keller, J.G., Klaunig, J.E., Knudsen, T.B., Kozumbo, W.J., Lettieri, T., Liu, S.Z., Maisseu, A., Maynard, K.I., Masoro, E.J., McClellan, R.O., Mehendale, H.M., Mothersill, C., Newlin, D.B., Nigg, H.N., Oehme, F.W., Phalen, R.F., Philbert, M.A., Rattan, S.I.S., Riviere, J.E., Rodricks, J., Sapolsky, R.M., Scott, B.R., Seymour, C., Sinclair, D.A., Smith-Sonneborn, J., Snow, E.T., Spear, L., Stevenson, D.E., Thomas, Y., Tubiana, M., Williams, G.M., Mattson, M.P., 2007. Biological stress response terminology: integrating the concepts of adaptive response and preconditioning stress within a hormetic dose-response framework. *Toxicol. Appl. Pharmacol.* 222, 122–128. <https://doi.org/10.1016/j.taap.2007.02.015>.
- Calabrese, E.J., Baldwin, L.A., 2002. Applications of hormesis in toxicology, risk assessment and chemotherapeutics. *Trends Pharmacol. Sci.* 23, 331–337. [https://doi.org/10.1016/S0165-6147\(02\)02034-5](https://doi.org/10.1016/S0165-6147(02)02034-5).
- Calabrese, E.J., Stanek, E.J., Nascarella, M.A., 2011. Evidence for hormesis in mutagenicity dose-response relationships. *Mutat. Res. - Genet. Toxicol. Environ. Mutagen.* 726, 91–97. <https://doi.org/10.1016/j.mrgentox.2011.04.006>.
- Callieri, C., 2008. Picophytoplankton in freshwater ecosystems: the importance of small-sized phototrophs. *Freshw. Rev.* 1, 1–28. <https://doi.org/10.1608/frj.1.1.1>.
- Carmichael, W.W., Boyer, G.L., 2016. Health impacts from cyanobacteria harmful algae blooms: implications for the North American Great Lakes. *Harmful Algae* 54, 194–212. <https://doi.org/10.1016/j.hal.2016.02.002>.
- Castellano-Hinojosa, A., Gallardo-Altamirano, M.J., González-López, J., González-Martínez, A., 2023. Anticancer drugs in wastewater and natural environments: a review on their occurrence, environmental persistence, treatment, and ecological risks. *J. Hazard. Mater.* 447, 130818. <https://doi.org/10.1016/j.jhazmat.2023.130818>.
- Castenholz, R.W., 2015. General Characteristics of the Cyanobacteria. <https://doi.org/10.1002/9781118960608.cbm00019>.
- Catherine, Q., Susanna, W., Isidora, E.S., Mark, H., Aurélie, V., Jean-François, H., 2013. A review of current knowledge on toxic benthic freshwater cyanobacteria - Ecology, toxin production and risk management. *Water Res.* 47, 5464–5479. <https://doi.org/10.1016/j.watres.2013.06.042>.
- Cedergreen, N., Streibig, J.C., Kudsk, P., Mathiasen, S.K., Duke, S.O., 2007. The occurrence of hormesis in plants and algae. *Dose-Response* 5, 150–162. <https://doi.org/10.2203/dose-response.06-008.cedergreen>.
- Celano, M.R., Vilar, M.C.P., Pacheco, A.B.F., e Azevedo, de O, S.M.F., 2026. Azithromycin at environmentally relevant concentrations increases the growth and microcystin content in the cyanobacterium *M. aeruginosa*. *Water. Air. Soil Pollut.* 237. <https://doi.org/10.1007/s11270-025-08662-z>.
- Chen, J., Qin, B., Han, Y., Yang, S., 2025. Sulfamethoxazole and sulfisoxazole stress on growth, photosynthesis, cell structure and oxidation system in *M. aeruginosa*. *Algal Res.* 87, 103880. <https://doi.org/10.1016/j.algal.2024.103880>.
- Chen, X., Lei, L., Liu, S., Han, J., Li, R., Men, J., Li, L., Wei, L., Sheng, Y., Yang, L., Zhou, B., Zhu, L., 2021. Occurrence and risk assessment of pharmaceuticals and personal care products (PPCPs) against COVID-19 in lakes and WWTP-river-estuary system in Wuhan, China. *Sci. Total Environ.* 792, 148352. <https://doi.org/10.1016/j.scitotenv.2021.148352>.
- Cheong, M.S., Seo, K.H., Chohra, H., Yoon, Y.E., Choe, H., Kantharaj, V., Lee, Y.B., 2020. Influence of sulfonamide contamination derived from veterinary antibiotics on plant growth and development. *Antibiotics* 9, 456. <https://doi.org/10.3390/antibiotics9080456>.
- Chia, M.A., Lorenzi, A.S., Ameh, I., Dauda, S., Cordeiro-Araújo, M.K., Agee, J.T., Okpanachi, I.Y., Adesalu, A.T., 2021. Susceptibility of phytoplankton to the increasing presence of active pharmaceutical ingredients (APIs) in the aquatic environment: a review. *Aquat. Toxicol.* 234, 105809. <https://doi.org/10.1016/j.aquatox.2021.105809>.
- Chopra, I., Roberts, M., 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol. Mol. Biol. Rev.* 65, 232–260. <https://doi.org/10.1128/mmr.65.2.232-260.2001>.
- Couto, E., Assemany, P.P., Carneiro, G.C.A., Soares, D.C.F., 2022. The potential of algae and aquatic macrophytes in the pharmaceutical and personal care products (PPCPs) environmental removal: a review. *Chemosphere* 302, 134808. <https://doi.org/10.1016/j.chemosphere.2022.134808>.
- Dabney, B.L., Patiño, R., 2018. Low-dose stimulation of growth of the harmful alga, *Prymnesium parvum*, by glyphosate and glyphosate-based herbicides. *Harmful Algae* 80, 130–139. <https://doi.org/10.1016/j.hal.2018.11.004>.
- Daneshvar, E., Sik Ok, Y., Tavakoli, S., Sarkar, B., Shaheen, S.M., Hong, H., Luo, Y., Rinklebe, J., Song, H., Bhatnagar, A., 2021. Insights into upstream processing of microalgae: a review. *Bioresour. Technol.* 329, 124870. <https://doi.org/10.1016/j.biortech.2021.124870>.
- De Liguoro, M., Di Leva, V., Gallina, G., Faccio, E., Pinto, G., Pollio, A., 2010. Evaluation of the aquatic toxicity of two veterinary sulfonamides using five test organisms. *Chemosphere* 81, 788–793. <https://doi.org/10.1016/j.chemosphere.2010.07.003>.
- Demmig-Adams, B., Adams, W.W., 1996. The role of xanthophyll cycle carotenoids in the protection of photosynthesis. *Trends Plant Sci.* 1, 21–26. [https://doi.org/10.1016/S1360-1385\(96\)80019-7](https://doi.org/10.1016/S1360-1385(96)80019-7).
- Deng, C., Pan, X., Zhang, D., 2015. Influence of ofloxacin on photosystems I and II activities of *M. aeruginosa* and the potential role of cyclic electron flow. *J. Biosci. Bioeng.* 119, 159–164. <https://doi.org/10.1016/j.jbiosc.2014.07.014>.
- Deng, C.N., Zhang, D.Y., Pan, X.L., 2014. Toxic effects of erythromycin on photosystem I and II in *M. aeruginosa*. *Photosynthetica* 52, 574–580. <https://doi.org/10.1007/s11099-014-0063-4>.
- Dias, E., Oliveira, M., Jones-Dias, D., Vasconcelos, V., Ferreira, E., Manageiro, V., Caniça, M., 2015. Assessing the antibiotic susceptibility of freshwater cyanobacteria spp. *Front. Microbiol.* 6, 1–11. <https://doi.org/10.3389/fmicb.2015.00799>.
- Dias, E., Oliveira, M., Manageiro, V., Vasconcelos, V., Caniça, M., 2019. Deciphering the role of cyanobacteria in water resistome: hypothesis justifying the antibiotic resistance (phenotype and genotype) in *Planktothrix* genus. *Sci. Total Environ.* 652, 447–454. <https://doi.org/10.1016/j.scitotenv.2018.10.167>.
- Dong, X., Sun, S., Jia, R., Xu, L., Hou, W., Lu, N., Wang, M., Zhao, Q., Hou, L., 2020. Effects of sulfamethoxazole exposure on the growth, antioxidant system of *Chlorella vulgaris* and *M. aeruginosa*. *Bull. Environ. Contam. Toxicol.* 105, 358–365. <https://doi.org/10.1007/s00128-020-02952-4>.
- dos Santos Costa, R., Quadra, G.R., de Oliveira Souza, H., do Amaral, V.S., Navoni, J.A., 2021. The link between pharmaceuticals and cyanobacteria: a review regarding ecotoxicological, ecological, and sanitary aspects. *Environ. Sci. Pollut. Res.* 28, 41638–41650. <https://doi.org/10.1007/s11356-021-14698-5>.
- Duan, W., Cui, H., Jia, X., Huang, X., 2022. Occurrence and ecotoxicity of sulfonamides in the aquatic environment: a review. *Sci. Total Environ.* 820, 153178. <https://doi.org/10.1016/j.scitotenv.2022.153178>.
- Dvořák, P., Pouličková, A., Hašler, P., Belli, M., Casamatta, D.A., Papini, A., 2015. Species concepts and speciation factors in cyanobacteria, with connection to the problems of diversity and classification. *Biodivers. Conserv.* 24, 739–757. <https://doi.org/10.1007/s10531-015-0888-6>.
- Dzionek, A., 2025. β -Blockers in the environment: challenges in understanding their persistence and ecological impact. *Molecules* 30, 4630. <https://doi.org/10.3390/molecules30234630>.
- Eapen, J.V., Thomas, S., Antony, S., George, P., Antony, J., 2024. A review of the effects of pharmaceutical pollutants on humans and aquatic ecosystem. *Explor. Drug Sci.* 484–507. <https://doi.org/10.37349/eds.2024.00058>.
- EFSA PPR Panel, 2013. Guidance on tiered risk assessment for plant protection products for aquatic organisms in edge-of-field surface waters. *EFSA J.* 11. <https://doi.org/10.2903/j.efsa.2013.3290>.
- Elander, R.P., 2003. Industrial production of β -lactam antibiotics. *Appl. Microbiol. Biotechnol.* 61, 385–392. <https://doi.org/10.1007/s00253-003-1274-y>.
- Eleršek, T., Milavec, S., Korošec, M., Brezovšek, P., Negreira, N., Zonja, B., de Alda, M.L., Barceló, D., Heath, E., Ščančar, J., Filipič, M., 2016. Toxicity of the mixture of selected antineoplastic drugs against aquatic primary producers. *Environ. Sci. Pollut. Res.* 23, 14780–14790. <https://doi.org/10.1007/s11356-015-6005-2>.
- Environment Canada, 2007. Biological test method: growth inhibition test using a freshwater alga. *Environmental Canada. Report EPS 1/RM/25. Environmental Science and Technology Centre Science and Technology Branch Environment Canada.*

- Etebu, E., Ibemologi, A., 1997. Antibiotics: classification and mechanisms of action with emphasis on molecular perspectives. *Int. J. Appl. Microbiol. Biotechnol. Res.* 96, 535–541. <https://doi.org/10.1161/01.CIR.96.2.535>.
- Fang, Y., Liu, Y., 2023. Efficient degradation of ciprofloxacin and erythromycin coupled to elevated lipid synthesis in *Synechococcus* sp. and *Chroococcus* sp. *Algal Res.* 74, 103231. <https://doi.org/10.1016/j.algal.2023.103231>.
- Feng, G., Duan, Z., Wu, L., Gao, Y., Zhang, Y., Li, F., Meng, X.Z., 2025. Environmental-level antibiotics disrupt *Microcystis* stoichiometry: an overlooked risk in the context of cyanobacterial harmful algal blooms. *Harmful Algae* 144, 102839. <https://doi.org/10.1016/j.hal.2025.102839>.
- Fernandez, R., Colás-Ruiz, N.R., Bolívar-Anillo, H.J., Anfuso, G., Hampel, M., 2021. Occurrence and effects of antimicrobials drugs in aquatic ecosystems. *Sustain* 13, 13428. <https://doi.org/10.3390/su132313428>.
- Ferrari, B., Mons, R., Vollat, B., Frayse, B., Paxéus, N., Lo Giudice, R., Pollio, A., Garric, J., 2004. Environmental risk assessment of six human pharmaceuticals: are the current environmental risk assessment procedures sufficient for the protection of the aquatic environment? *Environ. Toxicol. Chem.* 23, 1344–1354. <https://doi.org/10.1897/03-246>.
- Flombaum, P., Gallegos, J.L., Gordillo, R.A., Rincón, J., Zabala, L.L., Jiao, N., Karl, M.D., Li, W.K.W., Lomas, M.W., Veneziano, D., Vera, C.S., Vrugt, J.A., Martiny, A.C., 2013. Present and future global distributions of the marine cyanobacteria *Prochlorococcus* and *Synechococcus*. *Proc. Natl. Acad. Sci.* 110, 9824–9829. <https://doi.org/10.1073/pnas.1307701110>.
- Foyer, C.H., Noctor, G., 2005. Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. *Plant Cell* 17, 1866–1875. <https://doi.org/10.1105/tpc.105.033589>.
- Fu, B., Li, E., Yan, Y., Jiang, S., Wu, Y., Ma, Y., 2024. Ecological criteria for antibiotics in aquatic environments based on species sensitivity distribution. *Ecotoxicol. Environ. Saf.* 287, 117261. <https://doi.org/10.1016/j.ecoenv.2024.117261>.
- Gan, N., Xiao, Y., Zhu, L., Wu, Z., Liu, J., Hu, C., Song, L., 2012. The role of microcystins in maintaining colonies of bloom-forming *Microcystis* spp. *Environ. Microbiol.* 14, 730–742. <https://doi.org/10.1111/j.1462-2920.2011.02624.x>.
- Gao, Q., Garcia-Pichel, F., Castenholz, R.W., 2013. Molecular ecology of thermophilic cyanobacteria in Yellowstone hot springs. *Microb. Ecol.* 66, 592–607. <https://doi.org/10.1007/s00248-013-0220-y>.
- Gin, K.Y.-H., Sim, Z.Y., Goh, K.C., Kok, J.W.K., Te, S.H., Tran, N.H., Li, W., He, Y., 2021. Novel cyanotoxin-producing *Synechococcus* in tropical lakes. *Water Res.* 192, 116828. <https://doi.org/10.1016/j.watres.2021.116828>.
- Golden, S.S., 2019. The international journeys and aliases of *Synechococcus elongatus*. *New Zeal. J. Bot.* 57, 70–75. <https://doi.org/10.1080/0028825X.2018.1551805>.
- González-Pleiter, M., Gonzalo, S., Rodea-Palmares, I., Leganés, F., Rosal, R., Boltes, K., Marco, E., Fernández-Piñas, F., 2013. Toxicity of five antibiotics and their mixtures towards photosynthetic aquatic organisms: implications for environmental risk assessment. *Water Res.* 47, 2050–2064. <https://doi.org/10.1016/j.watres.2013.01.020>.
- Grzesiuk, M., Spijkerman, E., Lachmann, S.C., Wacker, A., 2018. Environmental concentrations of pharmaceuticals directly affect phytoplankton and effects propagate through trophic interactions. *Ecotoxicol. Environ. Saf.* 156, 271–278. <https://doi.org/10.1016/j.ecoenv.2018.03.019>.
- Guiry, M.D., Guiry, G.M., 2020. *AlgaeBase*. J. World-wide Electron. Publ. Natl. Univ. Ireland, Galw. WWW.Document.
- Guo, J., Boxall, A., Selby, K., 2015. Do pharmaceuticals pose a threat to primary producers? *Crit. Rev. Environ. Sci. Technol.* 45, 2565–2610. <https://doi.org/10.1080/10643389.2015.1061873>.
- Guo, J., Selby, K., Boxall, A.B.A., 2016. Effects of antibiotics on the growth and physiology of chlorophytes, cyanobacteria, and a diatom. *Arch. Environ. Contam. Toxicol.* 71, 589–602. <https://doi.org/10.1007/s00244-016-0305-5>.
- Gworek, B., Kijewska, M., Zaborowska, M., Wrzosek, J., Tokarz, L., Chmielewski, J., 2020. Occurrence of pharmaceuticals in aquatic environment—a review. *Desalin. Water Treat.* 184, 375–387. <https://doi.org/10.5004/dwt.2020.25325>.
- Halling-Sørensen, B., 2000. Algal toxicity of antibacterial agents used in intensive farming. *Chemosphere* 40, 731–739. [https://doi.org/10.1016/S0045-6535\(99\)00445-2](https://doi.org/10.1016/S0045-6535(99)00445-2).
- Halling-Sørensen, B., Holten Lützhøft, H.C., Andersen, H.R., Ingerslev, F., 2000. Environmental risk assessment of antibiotics: comparison of mecillinam, trimethoprim and ciprofloxacin. *J. Antimicrob. Chemother.* 46, 53–58. https://doi.org/10.1093/jac/46.suppl_1.53.
- Hancock, T.L., Dahedl, E.K., Kratz, M.A., Urakawa, H., 2024. *Synechococcus* dominance induced after hydrogen peroxide treatment of *Microcystis* bloom in the Caloosahatchee River, Florida. *Environ. Pollut.* 345, 123508. <https://doi.org/10.1016/j.envpol.2024.123508>.
- Harke, M.J., Steffen, M.M., Gobler, C.J., Otten, T.G., Wilhelm, S.W., Wood, S.A., Paerl, H.W., 2016. A review of the global ecology, genomics, and biogeography of the toxic cyanobacterium, *Microcystis* spp. *Harmful Algae* 54, 4–20. <https://doi.org/10.1016/j.hal.2015.12.007>.
- Haverkamp, T.H.A., Schouten, D., Doeleman, M., Wollenzien, U., Haverkamp, T.H.A., Schouten, D., Doeleman, M., Wollenzien, U., Huisman, J., Stal, L.J., 2009. Colorful microdiversity of *Synechococcus* strains (picocyanobacteria) isolated from the Baltic Sea. *ISME J.* 3, 397–408. <https://doi.org/10.1038/ismej.2008.118>.
- Hawkins, P.R., Holliday, J., Kathuria, A., Bowling, L., 2005. Change in cyanobacterial biovolume due to preservation by Lugol's Iodine. *Harmful Algae* 4, 1033–1043. <https://doi.org/10.1016/j.hal.2005.03.001>.
- Hejna, M., Kapuścińska, D., Akemann, A., 2022. Pharmaceuticals in the aquatic environment: a review on eco-toxicology and the remediation potential of algae. *Int. J. Environ. Res. Public Health* 19, 7717. <https://doi.org/10.3390/ijerph19137717>.
- Hillebrand, H., Dürselen, C., Kirschtel, D., Pollinger, U., Zohary, T., 1999. Biovolume calculation for pelagic and benthic microalgae. *J. Phycol.* 35, 403–424. <https://doi.org/10.1046/j.1529-8817.1999.3520403.x>.
- Hochhaus, A., Larson, R.A., Guillot, F., Radich, J.P., Branford, S., Hughes, T.P., Baccarani, M., Deininger, M.W., Cervantes, F., Fujihara, S., Ortmann, C.-E., Menssen, H.D., Kantarjian, H., O'Brien, S.G., Druker, B.J., 2017. Long-term outcomes of imatinib treatment for chronic myeloid leukemia. *N. Engl. J. Med.* 376, 917–927. <https://doi.org/10.1056/nejmoa1609324>.
- Hoiczyk, E., Hansel, A., 2000. Cyanobacterial cell walls: news from an unusual prokaryotic envelope. *J. Bacteriol.* 182, 1191–1199. <https://doi.org/10.1128/jb.182.5.1191-1199.2000>.
- Holland, A., Kinnear, S., 2013. Interpreting the possible ecological role(s) of cyanotoxins: compounds for competitive advantage and/or physiological aide? *Mar. Drugs* 11, 2239–2258. <https://doi.org/10.3390/md11072239>.
- Holten Lützhøft, H.C., Halling-Sørensen, B., Jørgensen, S.E., 1999. Algal toxicity of antibacterial agents applied in Danish fish farming. *Arch. Environ. Contam. Toxicol.* 36, 1–6. <https://doi.org/10.1007/s002449900435>.
- Hsu, L.-C., Liu, Y.-T., Syu, C.-H., Huang, M.-H., Tzou, Y.-M., Teah, H.Y., 2018. Adsorption of tetracycline on Fe (hydr)oxides: effects of pH and metal cation (Cu 2+, Zn 2+ and Al 3+) addition in various molar ratios. *R. Soc. Open Sci.* 5, 171941. <https://doi.org/10.1098/rsos.171941>.
- Huang, X., Chen, C., Zeng, Q., Ding, D., Gu, J., Mo, J., 2022. Field study on loss of tetracycline antibiotics from manure-applied soil and their risk assessment in regional water environment of Guangzhou, China. *Sci. Total Environ.* 827, 154273. <https://doi.org/10.1016/j.scitotenv.2022.154273>.
- Huisman, J., Codd, G.A., Paerl, H.W., Ibelings, B.W., Verspagen, J.M.H., Visser, P.M., 2018. Cyanobacterial blooms. *Nat. Rev. Microbiol.* 16, 471–483. <https://doi.org/10.1038/s41579-018-0040-1>.
- Huynh, N.C., Nguyen, T.T.T., Nguyen, D.T.C., Van Tran, T., 2023. Occurrence, toxicity, impact and removal of selected non-steroidal anti-inflammatory drugs (NSAIDs): a review. *Sci. Total Environ.* 898, 165317. <https://doi.org/10.1016/j.scitotenv.2023.165317>.
- ISO 8692, 2012. Water quality—Fresh water algal growth inhibition test with unicellular green algae 2012, 3.
- Javid, A., Mesdaghinia, A., Nasser, S., Mahvi, A.H., Alimohammadi, M., Gharibi, H., 2016. Assessment of tetracycline contamination in surface and groundwater resources proximal to animal farming houses in Tehran, Iran. *J. Environ. Heal. Sci. Eng.* 14, 1–5. <https://doi.org/10.1186/s40201-016-0245-z>.
- Jiang, Y., Liu, Y., Zhang, J., 2021. Mechanisms for the stimulatory effects of a five-component mixture of antibiotics in *M. aeruginosa* at transcriptomic and proteomic levels. *J. Hazard. Mater.* 406, 124722. <https://doi.org/10.1016/j.jhazmat.2020.124722>.
- Jones, O.A.H., Voulvoulis, N., Lester, J.N., 2007. Ecotoxicity of pharmaceuticals. *Compr. Anal. Chem.* 50, 387–424. [https://doi.org/10.1016/S0166-526X\(07\)50012-7](https://doi.org/10.1016/S0166-526X(07)50012-7).
- Khan, A.H.A., Barros, R., 2023. Pharmaceuticals in water: risks to aquatic life and remediation strategies. *Hydrobiologia* 2, 395–409. <https://doi.org/10.3390/hydrobiology2020026>.
- Klátyk, S., Takács, E., Barócsi, A., Lenk, S., Kocsányi, L., Darvas, B., Székács, A., 2024. Hormesis, the individual and combined phytotoxicity of the components of glyphosate-based formulations on algal growth and photosynthetic activity. *Toxics* 12, 257. <https://doi.org/10.3390/toxics12040257>.
- Klein, A.R., Sarri, E., Kelch, S.E., Basinski, J.J., Vaidya, S., Aristilde, L., 2021. Probing the fate of different structures of beta-lactam antibiotics: hydrolysis, mineral capture, and influence of organic matter. *ACS Earth Sp. Chem.* 5, 1511–1524. <https://doi.org/10.1021/acsearthspacechem.1c00064>.
- Kock, A., Glanville, H.C., Law, A.C., Stanton, T., Carter, L.J., Taylor, J.C., 2023. Emerging challenges of the impacts of pharmaceuticals on aquatic ecosystems: a diatom perspective. *Sci. Total Environ.* 878, 162939. <https://doi.org/10.1016/j.scitotenv.2023.162939>.
- Krishnan, A., Mou, X., 2021. A brief review of the structure, cytotoxicity, synthesis, and biodegradation of microcystins. *Water (Switzerland)* 13, 2147. <https://doi.org/10.3390/w13162147>.
- Kromkamp, J., Botterweg, J., Mur, L.R., 1988. Buoyancy regulation in *M. aeruginosa* grown at different temperatures. *EMS Microbiol. Ecol.* 53, 231–237. <https://doi.org/10.1111/j.1574-6968.1988.tb02669.x>.
- Kropidłowska, K., Caban, M., 2023. Effect of salinity on the toxicity of diclofenac, ibuprofen and naproxen toward cyanobacterium *Synechocystis salina*. *Chemosphere* 338, 139521. <https://doi.org/10.1016/j.chemosphere.2023.139521>.
- Kurmayer, R., 2011. The toxic cyanobacterium *nostoc* sp. strain 152 produces highest amounts of microcystin and nostophycin under stress conditions. *J. Phycol.* 47, 200–207. <https://doi.org/10.1111/j.1529-8817.2010.00931.x>.
- Kwidzińska, K., Zalewska, M., Akemann, A., Kobos, J., Mazur-Marzec, H., Caban, M., 2024. Multi-biomarker response of cyanobacteria *Synechocystis salina* and *M. aeruginosa* to diclofenac. *J. Hazard. Mater.* 471, 134373. <https://doi.org/10.1016/j.jhazmat.2024.134373>.
- Lakshmi, D., Vijaya, G., Murali, V., 2024. From prescription to pollution: the ecological consequences of NSAIDs in aquatic ecosystems. *Toxicol. Rep.* 13, 101775. <https://doi.org/10.1016/j.toxrep.2024.101775>.
- Larsson, D.G.J., Flach, C.-F., 2022. Antibiotic resistance in the environment. *Nat. Rev. Microbiol.* 20, 257–269. <https://doi.org/10.1038/s41579-021-00649-x>.
- Le Page, G., Gunnarsson, L., Trznadel, M., Wedgwood, K.C.A., Baudrot, V., Snape, J., Tyler, C.R., 2019. Variability in cyanobacteria sensitivity to antibiotics and implications for environmental risk assessment. *Sci. Total Environ.* 695, 133804. <https://doi.org/10.1016/j.scitotenv.2019.133804>.
- Van Le, V., Srivastava, A., Ko, S.R., Ahn, C.Y., Oh, H.M., 2022. Microcystin colony formation: extracellular polymeric substance, associated microorganisms, and its application. *Bioresour. Technol.* 360, 127610. <https://doi.org/10.1016/j.biortech.2022.127610>.

- j.biortech.2022.127610.
- Van Le, V., Tran, Q.-G., Ko, S.-R., Lee, S.-A., Oh, H.-M., Kim, H.-S., Ahn, C.-Y., 2023. How do freshwater microalgae and cyanobacteria respond to antibiotics? Crit. Rev. Biotechnol. 43, 191–211. <https://doi.org/10.1080/07388551.2022.2026870>.
- Lee, H., Xu, V., Diao, J., Zhao, R., Chen, M., Moon, T.S., Liu, H., Parker, K.M., Jun, Y.S., Tang, Y.J., 2024. The use of a benign fast-growing cyanobacterial species to control microcystin synthesis from *M. aeruginosa*. Front. Microbiol. 15. <https://doi.org/10.3389/fmicb.2024.1461119>.
- Lee, S.H., Jung, K., Chung, J., Lee, Y.W., 2022. Comparative study of algae-based measurements of the toxicity of 14 manufactured nanomaterials. Int. J. Environ. Res. Public Health 19, 5853. <https://doi.org/10.3390/ijerph19105853>.
- Li, D., Chen, H., Liu, H., Schlenk, D., Mu, J., Lacorte, S., Ying, G.G., Xie, L., 2021. Anticancer drugs in the aquatic ecosystem: environmental occurrence, ecotoxicological effect and risk assessment. Environ. Int. 153, 106543. <https://doi.org/10.1016/j.envint.2021.106543>.
- Li, J., Li, W., Liu, K., Guo, Y., Ding, C., Han, J., Li, P., 2022. Global review of macrolide antibiotics in the aquatic environment: sources, occurrence, fate, ecotoxicity, and risk assessment. J. Hazard. Mater. 439, 129628. <https://doi.org/10.1016/j.jhazmat.2022.129628>.
- Li, J.J., Chao, J.J., McKay, R.M.L., Xu, R.B., Wang, T., Xu, J., Zhang, J.L., Chang, X.X., 2021. Antibiotic pollution promotes dominance by harmful cyanobacteria: a case study examining norfloxacin exposure in competition experiments. J. Phycol. 57, 677–688. <https://doi.org/10.1111/jpy.13133>.
- Li, M., Zhou, H., Ye, M., Xu, X., Pang, L., Zhao, Z., Xuan, Y., 2023. Interactions between typical antibiotics and *M. aeruginosa* in aquatic environment. CLEAN – Soil, Air, Water 51. <https://doi.org/10.1002/clen.202200298>.
- Li, Y., Fang, J., Yuan, X., Chen, Y., Yang, H., Fei, X., 2018. Distribution characteristics and ecological risk assessment of tetracyclines pollution in the Weihe River, China. Int. J. Environ. Res. Public Health 15, 1803. <https://doi.org/10.3390/ijerph15091803>.
- Lima, L.M., Silva, B.N.M.da, Barbosa, G., Barreiro, E.J., 2020. β -lactam antibiotics: an overview from a medicinal chemistry perspective. Eur. J. Med. Chem. 208, 112829. <https://doi.org/10.1016/j.ejmech.2020.112829>.
- Lis, H., Kwidzińska, K., Bethke, K., Caban, M., 2026. Cyanobacteria as generators of reactive oxygen species – a review of analysis methods and case studies. Talanta 297, 128648. <https://doi.org/10.1016/j.talanta.2025.128648>.
- Lis, H., Stepnowski, P., Caban, M., 2019. Salinity and pH as factors affecting the passive sampling and extraction of pharmaceuticals from water. J. Sep. Sci. 42, 2949–2956. <https://doi.org/10.1002/jssc.201900346>.
- Liu, Y., Chen, S., Zhang, J., Gao, B., 2016. Growth, microcystin-production and proteomic responses of *M. aeruginosa* under long-term exposure to amoxicillin. Water Res. 93, 141–152. <https://doi.org/10.1016/j.watres.2016.01.060>.
- Liu, Y., Chen, S., Zhang, J., Li, X., Gao, B., 2017. Stimulation effects of ciprofloxacin and sulphonamethoxazole in *M. aeruginosa* and isobaric tag for relative and absolute quantitation-based screening of antibiotic targets. Mol. Ecol. 26, 689–701. <https://doi.org/10.1111/mec.13934>.
- Liu, Y., Chen, X., Zhang, J., Gao, B., 2015a. Hormesis effects of amoxicillin on growth and cellular biosynthesis of *M. aeruginosa* at different nitrogen levels. Microb. Ecol. 69, 608–617. <https://doi.org/10.1007/s00248-014-0528-9>.
- Liu, Y., Gao, B., Yue, Q., Guan, Y., Wang, Y., Huang, L., 2012a. Influences of two antibiotic contaminants on the production, release and toxicity of microcystins. Ecotoxicol. Environ. Saf. 77, 79–87. <https://doi.org/10.1016/j.ecoenv.2011.10.027>.
- Liu, Y., Guan, Y., Gao, B., Yue, Q., 2012b. Antioxidant responses and degradation of two antibiotic contaminants in *M. aeruginosa*. Ecotoxicol. Environ. Saf. 86, 23–30. <https://doi.org/10.1016/j.ecoenv.2012.09.004>.
- Liu, Y., Wang, F., Chen, X., Zhang, J., Gao, B., 2015b. Cellular responses and biodegradation of amoxicillin in *M. aeruginosa* at different nitrogen levels. Ecotoxicol. Environ. Saf. 111, 138–145. <https://doi.org/10.1016/j.ecoenv.2014.10.011>.
- Liu, Y., Wang, F., Chen, X., Zhang, J., Gao, B., 2015c. Influence of coexisting spiramycin contaminant on the harm of *M. aeruginosa* at different nitrogen levels. J. Hazard. Mater. 285, 517–524. <https://doi.org/10.1016/j.jhazmat.2014.11.027>.
- Longley, D.B., Harkin, D.P., Johnston, P.G., 2003. 5-Fluorouracil: mechanisms of action and clinical strategies. Nat. Rev. Cancer 3, 330–338. <https://doi.org/10.1038/nrc1074>.
- Love, D., Slovitsky, M., Costa, K.A., Megarani, D., Mehdi, Q., Colombo, V., Ivantsova, E., Subramaniam, K., Bowden, J.A., Bisesi, J.H., Martyniuk, C.J., 2024. Toxicity risks associated with the beta-blocker metoprolol in marine and freshwater organisms: a review. Environ. Toxicol. Chem. 43, 2530–2544. <https://doi.org/10.1002/etc.5981>.
- Lu, L., Wu, Y., Ding, H., Zhang, W., 2015. The combined and second exposure effect of copper (II) and chlortetracycline on fresh water algae, *Chlorella pyrenoidosa* and *M. aeruginosa*. Environ. Toxicol. Pharmacol. 40, 140–148. <https://doi.org/10.1016/j.etap.2015.06.006>.
- Lu, T., Zhang, Q., Zhang, Z., Hu, B., Chen, J., Chen, J., Qian, H., 2021. Pollutant toxicology with respect to microalgae and cyanobacteria. J. Environ. Sci. (China) 99, 175–186. <https://doi.org/10.1016/j.jes.2020.06.033>.
- Luo, Y., Guo, W., Ngo, H.H., Nghiem, L.D., Hai, F.I., Zhang, J., Liang, S., Wang, X.C., 2014. A review on the occurrence of micropollutants in the aquatic environment and their fate and removal during wastewater treatment. Sci. Total Environ. 473–474, 619–641. <https://doi.org/10.1016/j.scitotenv.2013.12.065>.
- Ma, Z., Yang, F., Ren, J., Fan, R., Duan, Q., Guo, J., Guo, J., 2021. Growth inhibition and oxidative stress in two green algal species exposed to erythromycin. J. Am. Water Resour. Assoc. 57, 628–637. <https://doi.org/10.1111/1752-1688.12915>.
- Mahniki, S.N., Rizovski, B., Fuerhacker, M., Mader, R.M., 2004. Determination of 5-fluorouracil in hospital effluents. Anal. Bioanal. Chem. 380, 31–35. <https://doi.org/10.1007/s00216-004-2727-6>.
- Mandic, Z., 2014. Voltammetric study of the partitioning of macrolide antibiotics at the water/nitrobenzene interface. Relationship to the pharmacokinetic profiling of macrolides. ADMET DMPK 2. <https://doi.org/10.5599/admet.2.3.46>.
- Manganelli, M., Testai, E., Codd, G.A., 2024. The complex relationship between cyanobacteria and antibiotics/antimicrobial resistance in the environment: an emerging factor in the one health vision on antimicrobial resistance. Adv. Oceanogr. Limnol. 15. <https://doi.org/10.4081/aiol.2024.12754>.
- Maszkowska, J., Stolte, S., Kumirska, J., Łukaszewicz, P., Mioduszevska, K., Puckowski, A., Caban, M., Wagil, M., Stepnowski, P., Białk-Bielin'ska, A., Białk-Bielin'ska, A., 2014a. Beta-blockers in the environment: part I. Mobility and hydrolysis study. Sci. Total Environ. 493, 1112–1121. <https://doi.org/10.1016/j.scitotenv.2014.06.023>.
- Maszkowska, J., Stolte, S., Kumirska, J., Łukaszewicz, P., Mioduszevska, K., Puckowski, A., Caban, M., Wagil, M., Stepnowski, P., Białk-Bielin'ska, A., Białk-Bielin'ska, A., 2014b. Beta-blockers in the environment: part II. Ecotoxicity study. Sci. Total Environ. 493, 1122–1126. <https://doi.org/10.1016/j.scitotenv.2014.06.039>.
- Mateo, P., Leganés, F., Perona, E., Loza, V., Fernández-Piñas, F., 2015. Cyanobacteria as bioindicators and bioreporters of environmental analysis in aquatic ecosystems. Biodivers. Conserv. 24, 909–948. <https://doi.org/10.1007/s10531-015-0903-y>.
- Mattson, M.P., Calabrese, E.J., 2010. Hormesis: a revolution in biology. Toxicology and Medicine. Humana Totowa, New York. <https://doi.org/10.1007/978-1-60761-495-1>.
- Mazur-Marzec, H., Pliński, M., Mazur-Marzec, H., Pliński, M., 2009. Do toxic cyanobacteria blooms pose a threat to the Baltic ecosystem? Oceanologia 51, 293–319.
- Miazek, K., Brozek-Pluska, B., 2019. Effect of phrs and pcps on microalgal growth, metabolism and microalgae-based bioremediation processes: a review. Int. J. Mol. Sci. 20, 2492. <https://doi.org/10.3390/ijms20102492>.
- Mittler, R., 2002. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 12, 281. <https://doi.org/10.3390/antiox12020281>.
- Nägeli, C., 1849. Gattungen einzelliger Algen, physiologisch und systematisch bearbeitet.
- Nassour, C., Barton, S.J., Nabhani-Gebara, S., Saab, Y., Barker, J., 2020. Occurrence of anticancer drugs in the aquatic environment: a systematic review. Environ. Sci. Pollut. Res. 27, 1339–1347. <https://doi.org/10.1007/s11356-019-07045-2>.
- Neuwöhner, J., Escher, B.I., 2011. The pH-dependent toxicity of basic pharmaceuticals in the green alga *Scenedesmus vacuolatus* can be explained with a toxicokinetic ion-trapping model. Aquat. Toxicol. 101, 266–275. <https://doi.org/10.1016/j.aquatox.2010.10.008>.
- Nunes, O.C., Manaia, C.M., Kolvenbach, B.A., Corvini, P.F.-X., 2020. Living with sulfonamides: a diverse range of mechanisms observed in bacteria. Appl. Microbiol. Biotechnol. 104, 10389–10408.
- Obaid, Z.H., Salman, J.M., Kadhim, N.F., 2023. Review on toxicity and removal of pharmaceutical pollutants using immobilised microalgae. Ecol. Eng. Environ. Technol. 24, 44–60. <https://doi.org/10.12912/27197050/166013>.
- OECD, 2019a. Pharmaceutical Residues in Freshwater: Hazards and Policy Responses, OECD Studies on Water. OECD Publishing, Paris. <https://doi.org/10.1787/c936f42d-en>.
- OECD, 2019b. Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures. Paris. <https://doi.org/10.1787/0ed2f88e-en>.
- OECD, 2011. Test No. 201: freshwater alga and cyanobacteria, growth inhibition test, OECD guidelines for the testing of chemicals. Section 2. <https://doi.org/10.1787/9789264069923-en>.
- Ortizur, M., Esterhuizen, M., Olicón-Hernández, D.R., González-López, J., Aranda, E., 2022. Pharmaceutical pollution in aquatic environments: a concise review of environmental impacts and bioremediation systems. Front. Microbiol. 13, 1–25. <https://doi.org/10.3389/fmicb.2022.869332>.
- Otsuka, S., Suda, S., Li, R., Matsumoto, S., Watanabe, M.M., 2000. Morphological variability of colonies of microcystis morphospecies in culture. J. Gen. Appl. Microbiol. 46, 39–50. <https://doi.org/10.2323/jgam.46.39>.
- Ovung, A., Bhattacharyya, J., 2021. Sulfonamide drugs: structure, antibacterial property, toxicity, and biophysical interactions. Biophys. Rev. 13, 259–272. <https://doi.org/10.1007/s12551-021-00795-9>.
- Papke, R.T., Ramsing, N.B., Mary, M., Ward, D.M., 2003. Geographical isolation in hot spring cyanobacteria. Environ. Microbiol. 5, 650–659. <https://doi.org/10.1046/j.1462-2920.2003.00460.x>.
- Pauletto, M., De Liguoro, M., 2024. A review on fluoroquinolones' toxicity to freshwater organisms and a risk assessment. J. Xenobiotics 14, 717–752. <https://doi.org/10.3390/jox14020042>.
- Pawłowska, B., Biczak, R., 2024. Drugs in the environment-impact on plants: a review. Environ. Toxicol. Pharmacol. 111, 104557. <https://doi.org/10.1016/j.etap.2024.104557>.
- Pineda-Mendoza, R.M., Zúñiga, G., Martínez-Jerónimo, F., 2016. Microcystin production in *M. aeruginosa*: effect of type of strain, environmental factors, nutrient concentrations, and N:P ratio on *mcyA* gene expression. Aquat. Ecol. 50, 103–119. <https://doi.org/10.1007/s10452-015-9559-7>.
- Pinto, I., Simões, M., Gomes, I.B., 2022. An Overview of the impact of pharmaceuticals on aquatic microbial communities. Antibiotics 11, 1700. <https://doi.org/10.3390/antibiotics11121700>.
- Pommier, Y., 2013. Drugging topoisomerases: lessons and challenges. ACS Chem. Biol. 8, 82–95. <https://doi.org/10.1021/cb300648v>.
- Pozuelo-Campos, S., Casero-Alonso, V., Amo-Salas, M., 2023. Optimal designs for detecting and characterizing hormesis in toxicological tests. Chemom. Intell. Lab. Syst. 235, 104753. <https://doi.org/10.1016/j.chemolab.2023.104753>.
- Puckowski, A., Mioduszevska, K., Łukaszewicz, P., Borecka, M., Caban, M., Maszkowska, J., Stepnowski, P., 2016. Bioaccumulation and analytics of pharmaceutical residues in the environment: a review. J. Pharm. Biomed. Anal. 127, 232–255. <https://doi.org/10.1016/j.jpba.2016.02.049>.
- Qian, H., Li, J., Pan, X., Sun, Z., Ye, C., Jin, G., Fu, Z., 2012. Effects of streptomycin on growth of algae *Chlorella vulgaris* and *M. aeruginosa*. Environ. Toxicol. 27, 229–237. <https://doi.org/10.1002/tox.20636>.

- Reynolds, C.S., Jaworski, G.H.M., Cmiech, H.A., Leedale, G.F., 1981. On the annual cycle of the blue-green alga *M. aeruginosa* Kütz. emend. Elenkin. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 293, 419–477. <https://doi.org/10.1098/rstb.1981.0081>.
- Ribeiro Trisotto, L.F., Figueredo, C.C., Gomes, M.P., 2025. Rivers at risks: the interplay of “COVID kit” medication misuse and urban waterway contaminants. Chemosphere 370, 143933. <https://doi.org/10.1016/j.chemosphere.2024.143933>.
- Roberts, M.C., 1996. Tetracycline resistance determinants: mechanisms of action, regulation of expression, genetic mobility, and distribution. FEMS Microbiol. Rev. 19, 1–24. [https://doi.org/10.1016/0168-6445\(96\)00021-6](https://doi.org/10.1016/0168-6445(96)00021-6).
- Robertson, B.R., Tezuka, N., Watanabe, M.M., 2001. Phylogenetic analyses of *Synechococcus* strains (cyanobacteria) using sequences of 16S rDNA and part of the phycocyanin operon reveal multiple evolutionary lines and reflect phycobilin content. Int. J. Syst. Evol. Microbiol. 51, 861–871. <https://doi.org/10.1099/00207713-51-3-861>.
- Robinson, A.A., Belden, J.B., Lydy, M.J., 2005. Toxicity of fluoroquinolone antibiotics to aquatic organisms. Environ. Toxicol. Chem. 24, 423–430. <https://doi.org/10.1897/04-210R.1>.
- Rogers, J., Perkkins, H.R., Wars, J.B., 1980. Microbial cell walls and membranes.
- Roy, B., Suresh, P.K., 2021. Toxic effect of antibiotics on freshwater algal systems and the mechanisms of toxicity: a review. Nat. Environ. Pollut. Technol. 20. <https://doi.org/10.46488/NEPT.2021.v20i04.023>.
- Sánchez-Baracaldo, P., Bianchini, G., Wilson, J.D., Knoll, A.H., 2022. Cyanobacteria and biogeochemical cycles through Earth history. Trends Microbiol. 30, 143–157. <https://doi.org/10.1016/j.tim.2021.05.008>.
- Schwarz, S., Gildemeister, D., Hein, A., Schröder, P., Bachmann, J., 2021. Environmental fate and effects assessment of human pharmaceuticals: lessons learnt from regulatory data. Environ. Sci. Eur. 33, 68. <https://doi.org/10.1186/s12302-021-00503-0>.
- Sha'aba, R.I., Chia, M.A., Gana, Y.A., Alhassan, A.B., Gadzama, I.M.K., 2023. The growth, biochemical composition, and antioxidant response of microcystis and chlorella are influenced by Ibuprofen. Environ. Sci. Pollut. Res. 30, 13118–13131. <https://doi.org/10.1007/s11356-022-22837-9>.
- Sharma, L., Siedlewicz, G., Pazdro, K., 2021. The toxic effects of antibiotics on freshwater and marine photosynthetic microorganisms: state of the art. Plants 10, 591. <https://doi.org/10.3390/plants10030591>.
- Sharma, P., Jha, A.B., Dubey, R.S., Pessarakli, M., 2012. Reactive oxygen species, oxidative damage, and antioxidant defense mechanism in plants under stress conditions. J. Bot. 2012, 1–26. <https://doi.org/10.1155/2012/217037>.
- Shetty, S.S., D, D., S, H., Sonkusare, S., Naik, P.B., Kumari, N., S, Madhyastha, H., 2023. Environmental pollutants and their effects on human health. Heliyon 9, e19496. <https://doi.org/10.1016/j.heliyon.2023.e19496>.
- Siedlewicz, G., Żak, A., Sharma, L., Kosakowska, A., Pazdro, K., 2020. Effects of oxytetracycline on growth and chlorophyll a fluorescence in green algae (*Chlorella vulgaris*), diatom (*Phaeodactylum tricornutum*) and cyanobacteria (*M. aeruginosa* and *Nodularia spumigena*). Oceanologia 62, 214–225. <https://doi.org/10.1016/j.oceano.2019.12.002>.
- Simon, A., Price, W., Nghiem, L.D., 2011. Implications of membrane fouling toward the removal of the pharmaceutical sulfamethoxazole by nanofiltration processes. J. Zhejiang Univ. Sci. A 12, 575–582. <https://doi.org/10.1631/jzus.A1000469>.
- Singh, D.S., Pflugmacher, S., 2026. Ecotoxicological assessments of over-the-counter NSAID (diclofenac, ibuprofen, aspirin) and antipyretic (acetaminophen) pharmaceuticals: a review of their toxicity effects on aquatic crustaceans. Integr. Environ. Assess. Manag. 22, 47–83. <https://doi.org/10.1093/iteam/vjaf082>.
- Singh, U., Laxmi, Singh, A.K., Asthana, R.K., 2020. Bioactive molecules from microalgae and constraints in commercialization. In: Sinha, R.P., Hader, D.-P. (Eds.), Natural Bioactive Compounds: Technological Advancements. Academic Press, pp. 143–164. <https://doi.org/10.1016/B978-0-12-820655-3.00007-0>.
- Souza-Silva, G., Bispo, V.A., Alcantara, M.D., Vieira Martins Starling, M.C., de Souza, C.R., Jardim, L.T.R., Miranda, M., Nunes, K.P., de Jesus Pereira, C.A., Mol, M.P.G., et al., 2025. Aquatic ecotoxicology of antiretrovirals: a review. Environments 12, 384. <https://doi.org/10.3390/environments12100384>.
- Stec, M., Astel, A., 2024. Occurrence and toxicological assessment of six non-steroidal anti-inflammatory drugs (NSAIDs) in a wastewater treatment plant in Słupsk (Poland). Ecohydrol. Hydrobiol. 24, 523–534. <https://doi.org/10.1016/j.ecohyd.2024.04.007>.
- Stockner, J.G., Callieri, C., National, I., Cronberg, G., 2000. Picoplankton and Other Non-Bloom-Forming Cyanobacteria in Lakes, The Ecology of Cyanobacteria. Springer, Dordrecht, pp. 195–231. <https://doi.org/10.1007/0-306-46855-7>.
- Sumpter, J.P., Runnalls, T.J., Donnachie, R.L., Owen, S.F., 2021. A comprehensive aquatic risk assessment of the beta-blocker propranolol, based on the results of over 600 research papers. Sci. Total Environ. 793, 148617. <https://doi.org/10.1016/j.scitotenv.2021.148617>.
- Suzuki, T., Nakasato, K., Shapiro, S., Pomati, F., Neilan, B.A., 2004. Effects of synthetic local anaesthetics on the growth of the cyanobacterium *Synechococcus leopoliensis*. J. Appl. Phycol. 16, 145–152. <https://doi.org/10.1023/B:JAPH.0000044776.04977.00>.
- Świacka, K., Maculewicz, J., Kowalska, D., Caban, M., Smolarz, K., Świeżak, J., 2022. Presence of pharmaceuticals and their metabolites in wild-living aquatic organisms – current state of knowledge. J. Hazard. Mater. 424, 127350. <https://doi.org/10.1016/J.JHAZMAT.2021.127350>.
- Świacka, K., Maculewicz, J., Kowalska, D., Grace, M.R., 2023. Do pharmaceuticals affect microbial communities in aquatic environments? a review. Front. Environ. Sci. 10, 1–15. <https://doi.org/10.3389/fenvs.2022.1093920>.
- Świacka, K., Michnowska, A., Maculewicz, J., Caban, M., Smolarz, K., 2021. Toxic effects of NSAIDs in non-target species: a review from the perspective of the aquatic environment. Environ. Pollut. 273, 115891. <https://doi.org/10.1016/j.envpol.2020.115891>.
- Tacic, A., Nikolic, V., Nikolic, L., Savic, I., 2017. Antimicrobial sulfonamide drugs. Adv. Technol. 6, 58–71. <https://doi.org/10.5937/savteh1701058t>.
- Tan, X., Gu, H., Ruan, Y., Zhong, J., Parajuli, K., Hu, J., 2019a. Effects of nitrogen on interspecific competition between two cell-size cyanobacteria: *M. aeruginosa* and *Synechococcus* sp. Harmful Algae 89, 101661. <https://doi.org/10.1016/j.hal.2019.101661>.
- Tan, X., Gu, H., Zhang, X., Parajuli, K., Duan, Z., 2019b. Effects of phosphorus on interspecific competition between two cell-size cyanobacteria: *Synechococcus* sp. and *M. aeruginosa*. Bull. Environ. Contam. Toxicol. 102, 231–238. <https://doi.org/10.1007/s00128-018-2527-x>.
- Te, S.H., Kok, J.W.K., Luo, R., You, L., Sukarji, N.H., Goh, K.C., Sim, Z.Y., Zhang, D., He, Y., Gin, K.Y.H., 2023. Coexistence of *Synechococcus* and *Microcystis* blooms in a tropical urban reservoir and their links with microbiomes. Environ. Sci. Technol. 57, 1613–1624. <https://doi.org/10.1021/acs.est.2c04943>.
- Thai, V.A., Dang, V.D., Thuy, N.T., Pandit, B., Vo, T.K.Q., Khedulkar, A.P., 2023. Fluoroquinolones: fate, effects on the environment and selected removal methods. J. Clean. Prod. 418, 137762. <https://doi.org/10.1016/j.jclepro.2023.137762>.
- Toński, M., Dołzonek, J., Stepnowski, P., Białk-Bielińska, A., 2021. Hydrolytic stability of anticancer drugs and one metabolite in the aquatic environment. Environ. Sci. Pollut. Res. 28, 57939–57951. <https://doi.org/10.1007/s11356-021-14360-0>.
- United States Environmental Protection Agency, 1996. Ecological Effects Test Guidelines OPPTS 850.5400: Algal Toxicity, Tiers I and II (EPA 712-C-96-164). Washington, DC.
- Van Boeckel, T.P., Gandra, S., Ashok, A., Caudron, Q., Grenfell, B.T., Levin, S.A., Laxminarayan, R., 2014. Global antibiotic consumption 2000 to 2010: an analysis of national pharmaceutical sales data. Lancet Infect. Dis. 14, 742–750. [https://doi.org/10.1016/S1473-3099\(14\)70780-7](https://doi.org/10.1016/S1473-3099(14)70780-7).
- Van de Perre, D., Li, D., Yao, K.-S., Lei, H.-J., Van den Brink, P.J., Ying, G.-G., 2022. The effects of the chemotherapy drug cyclophosphamide on the structure and functioning of freshwater communities under sub-tropical conditions: a mesocosm study. Sci. Total Environ. 806, 150678. <https://doi.org/10.1016/j.scitotenv.2021.150678>.
- van der Grinten, E., Pikkemaat, M.G., van den Brandhof, E.J., Stroomberg, G.J., Kraak, M.H.S., 2010. Comparing the sensitivity of algal, cyanobacterial and bacterial bioassays to different groups of antibiotics. Chemosphere 80, 1–6. <https://doi.org/10.1016/j.chemosphere.2010.04.011>.
- Van Hoek, A.H.A.M., Mevius, D., Guerra, B., Mullany, P., Roberts, A.P., Aarts, H.J.M., 2011. Acquired antibiotic resistance genes: an overview. Front. Microbiol. 2, 203. <https://doi.org/10.3389/fmicb.2011.00203>.
- Vasudevan, D., Bruland, G.L., Torrance, B.S., Upchurch, V.G., MacKay, A.A., 2009. pH-dependent ciprofloxacin sorption to soils: interaction mechanisms and soil factors influencing sorption. Geoderma 151, 68–76. <https://doi.org/10.1016/j.geoderma.2009.03.007>.
- Vonshak, A., Torzillo, G., 2003. Environmental stress physiology. Handb. Microalgal Cult. 57–82. <https://doi.org/10.1002/9780470995280.ch4>.
- Wan, L., Huang, R., Zhou, Y., Guo, J., Jiao, Y., Gao, J., 2024. Effects of ciprofloxacin on the production and composition of cellular microcystins in *M. aeruginosa*. Toxics 12, 759. <https://doi.org/10.3390/toxics12100759>.
- Wang, J., Tao, Y., 2025. Effects of antibiotics at environmental concentrations on cyanobacteria and its mechanisms: a review focusing on hormesis. Curr. Pollut. Rep. 11, 61. <https://doi.org/10.1007/s40726-025-00390-6>.
- Wang, Y., Gao, C., Qu, Z., Li, M., 2022. The combined toxicity of binary mixtures of antibiotics against the cyanobacterium *Microcystis* is dose-dependent: insight from a theoretical nonlinear combined toxicity assessment method. Environ. Sci. Pollut. Res. 29, 11612–11624. <https://doi.org/10.1007/s11356-021-16594-4>.
- Wang, Z., Chen, Q., Hu, L., Wang, M., 2018. Combined effects of binary antibiotic mixture on growth, microcystin production, and extracellular release of *M. aeruginosa*: application of response surface methodology. Environ. Sci. Pollut. Res. 25, 736–748. <https://doi.org/10.1007/s11356-017-0475-3>.
- Wang, Z., Chen, Q., Zhang, J., Dong, J., Ao, Y., Wang, M., Wang, X., 2019. Long-term exposure to antibiotic mixtures favors microcystin synthesis and release in *M. aeruginosa* with different morphologies. Chemosphere 235, 344–353. <https://doi.org/10.1016/j.chemosphere.2019.06.192>.
- Wang, Z., Chen, Q., Zhang, J., Guan, T., Chen, Y., Shi, W., 2020. Critical roles of cyanobacteria as reservoir and source for antibiotic resistance genes. Environ. Int. 144, 106034. <https://doi.org/10.1016/j.envint.2020.106034>.
- Wang, Z., Chen, Q., Zhang, J., Xu, H., Miao, L., Zhang, T., Liu, D., Zhu, Q., Yan, H., Yan, D., 2024. Climate warming promotes collateral antibiotic resistance development in cyanobacteria. Water Res. 256, 121642. <https://doi.org/10.1016/j.watres.2024.121642>.
- Wilkinson, J.L., Boxall, A.B.A., Kolpin, D.W., Leung, K.M.Y., Lai, R.W.S., Galban-Malag, C., Adell, A.D., Mondon, J., Metian, M., Marchant, R.A., Bouzas-Monroy, A., Cuni-Sanchez, A., Coors, A., Carriquiriborde, P., Rojo, M., Gordon, C., Cara, M., Moerman, C., Al, E., 2022. Pharmaceutical pollution of the world's rivers. Pnas 119, 1–10. <https://doi.org/10.1073/pnas.2113947119/-/DCSupplemental.Published>.
- Wistrand-Yuen, E., Knopp, M., Hjort, K., Koskineniemi, S., Berg, O.G., Andersson, D.I., 2018. Evolution of high-level resistance during low-level antibiotic exposure. Nat. Commun. 9. <https://doi.org/10.1038/s41467-018-04059-1>.
- Wormington, A.M., De María, M., Kurita, H.G., Biesi, J.H., Denslow, N.D., Martyniuk, C.J., 2020. Antineoplastic agents: environmental prevalence and adverse outcomes in aquatic organisms. Environ. Toxicol. Chem. 39, 967–985. <https://doi.org/10.1002/etc.4687>.
- Wu, Y., Ding, H., Wan, L., Zhang, W., Zhang, Y., Yang, L., Zhao, C., 2022. Single and combined toxic effects of clarithromycin and levofloxacin on *M. aeruginosa*. Environ. Pollut. Bioavailab. 34, 482–495. <https://doi.org/10.1080/26395940.2022.2130825>.
- Wu, Y., Wan, L., Zhang, W., Ding, H., Yang, W., 2020. Resistance of cyanobacteria *M. aeruginosa* to erythromycin with multiple exposure. Chemosphere 249, 126147.

- <https://doi.org/10.1016/j.chemosphere.2020.126147>.
- Xia, Y., Xie, Q.M., Chu, T.J., 2023. Effects of enrofloxacin and ciprofloxacin on growth and toxin production of *M. aeruginosa*. *Water (Switzerland)* 15, 3580. <https://doi.org/10.3390/w15203580>.
- Xin, R., Yu, X., Fan, J., 2022. Physiological, biochemical and transcriptional responses of cyanobacteria to environmentally relevant concentrations of a typical antibiotic—roxithromycin. *Sci. Total Environ.* 814, 152703. <https://doi.org/10.1016/j.scitotenv.2021.152703>.
- Xin, R., Zhang, Y., Zhang, K., Yang, Y., Ma, Y., Niu, Z., 2024. Investigation of the antimicrobial susceptibility patterns of marine cyanobacteria in Bohai Bay: cyanobacteria may be important hosts of antibiotic resistance genes in marine environment. *Sci. Total Environ.* 909, 168516.
- Xin, X., Huang, G., Zhang, B., 2021. Review of aquatic toxicity of pharmaceuticals and personal care products to algae. *J. Hazard. Mater.* 410, 124619. <https://doi.org/10.1016/j.jhazmat.2020.124619>.
- Xiong, J.Q., Kurade, M.B., Jeon, B.H., 2017. Ecotoxicological effects of enrofloxacin and its removal by monoculture of microalgal species and their consortium. *Environ. Pollut.* 226, 486–493. <https://doi.org/10.1016/j.envpol.2017.04.044>.
- Yang, W., Tang, Z., Zhou, F., Zhang, W., Song, L., 2013. Toxicity studies of tetracycline on *M. aeruginosa* and *Selenastrum capricornutum*. *Environ. Toxicol. Pharmacol.* 35, 320–324. <https://doi.org/10.1016/j.etap.2013.01.006>.
- Yang, Y., Zhang, X., Tan, L., Xin, R., Ma, Y., Niu, Z., 2026. Interactions between cyanobacteria and emerging contaminants in aqueous environments. *Aquat. Toxicol.* 290, 107621. <https://doi.org/10.1016/j.aquatox.2025.107621>.
- Yang, Z., Lu, T., Zhu, Y., Zhang, Q., Zhou, Z., Pan, X., Qian, H., 2019. Aquatic ecotoxicity of an antidepressant, sertraline hydrochloride, on microbial communities. *Sci. Total Environ.* 654, 129–134. <https://doi.org/10.1016/j.scitotenv.2018.11.164>.
- Ye, J., Huang, C., Shang, A., Xu, C., Wu, L., 2020. Characteristics of toxin production and release in *M. aeruginosa* exposed to three tetracycline antibiotics. *Environ. Sci. Pollut. Res.* 27, 16798–16805. <https://doi.org/10.1007/s11356-020-08253-x>.
- Ye, S., Ip, J.C., Yang, Y., Han, B., Zhou, G., 2026. Ecological risks of microcystins and the implications on their adverse effects to freshwater ecosystems under multiple temperature scenarios. *Environ. Chem. Ecotoxicol.* 8, 1563–1570. <https://doi.org/10.1016/j.enceco.2026.02.017>.
- Yisa, A.G., Chia, M.A., Sha'aba, R.I., Gauji, B., Gadzama, I.M.K., Oniye, S.J., 2021. The antibiotic ciprofloxacin alters the growth, biochemical composition, and antioxidant response of toxin-producing and non-toxin-producing strains of *Microcystis*. *J. Appl. Phycol.* 33, 2145–2155. <https://doi.org/10.1007/s10811-021-02485-1>.
- Zhang, M., Kong, F., Tan, X., Yang, Z., Cao, H., Xing, P., 2007. Biochemical, morphological, and genetic variations in *M. aeruginosa* due to colony disaggregation. *World J. Microbiol. Biotechnol.* 23, 663–670. <https://doi.org/10.1007/s11274-006-9280-8>.
- Zhang, M., Steinman, A.D., Xue, Q., Zhao, Y., Xu, Y., Xie, L., 2020. Effects of erythromycin and sulfamethoxazole on *M. aeruginosa*: cytotoxic endpoints, production and release of microcystin-LR. *J. Hazard. Mater.* 399, 123021. <https://doi.org/10.1016/j.jhazmat.2020.123021>.
- Zhang, Q., Demeestere, K., De Schampelaere, K.A.C., 2022. A bioavailability model to predict the impact of pH and dissolved organic carbon on ciprofloxacin ecotoxicity to the cyanobacterium *M. aeruginosa*. *Environ. Toxicol. Chem.* 41, 2835–2847. <https://doi.org/10.1002/etc.5454>.
- Zhang, Y., Gao, Q., Liu, S.S., Tang, L., Li, X.G., Sun, H., 2022. Hormetic dose-response of halogenated organic pollutants on *M. aeruginosa*: joint toxic action and mechanism. *Sci. Total Environ.* 829, 154581. <https://doi.org/10.1016/j.scitotenv.2022.154581>.
- Zhang, Y., He, D., Bu, Z., Li, Y., Guo, J., Li, Q., 2022. The transcriptomic analysis revealed sulfamethoxazole stress at environmentally relevant concentration on the mechanisms of toxicity of cyanobacteria *Synechococcus* sp. *J. Environ. Chem. Eng.* 10, 107637. <https://doi.org/10.1016/j.jece.2022.107637>.
- Zhang, Y., Li, M., Chang, F., Yi, M., Ge, H., Fu, J., Dang, C., 2023. The distinct resistance mechanisms of cyanobacteria and green algae to sulfamethoxazole and its implications for environmental risk assessment. *Sci. Total Environ.* 854, 158723. <https://doi.org/10.1016/j.scitotenv.2022.158723>.
- Zhao, Y., Huang, Y., Hu, S., Xu, T., Fang, Y., Liu, H., Xi, Y., Qu, R., 2023. Combined effects of fluoroquinolone antibiotics and organophosphate flame retardants on *M. aeruginosa*. *Environ. Sci. Pollut. Res.* 30, 53050–53062. <https://doi.org/10.1007/s11356-023-25974-x>.
- Zhou, X., Jiang, X., Chen, J., Gao, P., 2022. Interspecific competition between *M. aeruginosa* and *Chlamydomonas microspora* stressed by tetracyclines. *Environ. Sci. Pollut. Res.* 29, 83262–83272. <https://doi.org/10.1007/s11356-022-21537-8>.
- Zhu, X.W., Liu, S.S., Qin, L.T., Chen, F., Liu, H.L., 2013. Modeling non-monotonic dose-response relationships: model evaluation and hormetic quantities exploration. *Ecotoxicol. Environ. Saf.* 89, 130–136. <https://doi.org/10.1016/j.ecoenv.2012.11.022>.
- Zurawell, R.W., Chen, H., Burke, J.M., Prepas, E.E., 2005. Hepatotoxic cyanobacteria: a review of the biological importance of microcystins in freshwater environments. *J. Toxicol. Environ. Heal. Part B* 8, 1–37. <https://doi.org/10.1080/10937400500889412>.

Manuskrypt – M1

Kwidzińska Klaudia, Caban Magda*, Re-evaluation of dichlorodihydrofluorescein and dichlorofluorescein diacetates applicability for cyanobacteria cells staining, Algal Research (wysłano do recenzji). IF 5,0 (Q1)

**Re-evaluation of dichlorodihydrofluorescein and dichlorofluorescein
diacetates applicability for cyanobacteria cells staining**

Klaudia Kwidzińska, Magda Caban *

*University of Gdańsk, Faculty of Chemistry, Department of Environmental Analysis, ul. Wita
Stwosza 63, 80-308 Gdańsk, Poland*

***Corresponding author:** Phone (+48 58) 523 5208; E-mail address: magda.caban@ug.edu.pl

Abstract

Fluorescein derivatives, particularly dichlorodihydrofluorescein diacetate (DCFH₂-DA) and dichlorofluorescein diacetates (DCF-DA), are among the most widely used probes for detecting oxidative stress and cellular viability in aquatic ecotoxicology. However, their methodological reliability in microorganisms remains controversial. This study provides the first comprehensive evaluation of the feasibility, chemical stability, and cellular interactions of DCF, DCF-DA, and DCFH₂-DA in the unicellular cyanobacterium *Synechococcus salina*. Using a multi-technique approach combining spectrophotometry, flow cytometry (FCM), and high-performance liquid chromatography with diode-array detection (HPLC-DAD), we systematically tracked probe stability in BG11 medium, sorption/desorption kinetics, and cellular responses under methyl viologen-induced oxidative stress. Contrary to established literature asserting the broad utility of DCFH₂-DA for intracellular reactive oxygen species (ROS) monitoring, our comparative analysis demonstrates that DCFH₂-DA is unsuitable for *S. salina*, failing to yield detectable fluorescence in the FITC channel during FCM analysis. Conversely, DCF-DA demonstrated effective cellular entry and internal deacetylation, serving as a reliable marker for membrane permeability and deacetylation processes. Ultimately, this work establishes a critical baseline for aquatic ecotoxicology, demonstrating that unvalidated DCFH₂-DA usage generates severe chemical artifacts and offering a robust analytical framework to ensure data integrity in cyanobacterial risk assessment.

Keywords: cyanobacteria; dichlorodihydrofluorescein diacetate; cell staining; reactive oxygen species; flow cytometry

1. Introduction

Reactive oxygen species (ROS) are constantly produced in all cell types as a product of normal metabolic processes. Oxidative stress occurs when the balance between the generation and scavenging of ROS and other oxidants is disturbed. In photosynthetic organisms, such as cyanobacteria, oxidation and reduction processes are closely linked to photosynthesis and respiration [1]. These processes are modulated by fluctuating abiotic factors, including light quality and quantity, pH, salinity, temperature, nutrient limitation, and chemical stressors [2]. Consequently, understanding the impact of stressors on oxidative stress is essential for assessing biochemical biomarkers.

The probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH₂-DA) is an established cell stain for tracking intracellular ROS in various cell types due to its simplicity of use and compatibility with fluorescence microscopy (FM) and flow cytometry (FCM). The accepted mechanism of this probe is based on its initial uptake by the cell, followed by deacetylation to 2',7'-dichlorodihydrofluorescein (DCFH₂), and final oxidation to the highly fluorescent product 2',7'-dichlorofluorescein (DCF). Despite uncertainties regarding the exact mechanism [3], several pathways have been proposed [4], suggesting that multiple competitive reactions are involved in the generation of the fluorescent product. Several unknowns remain associated with this process [5]. For instance, it is unclear whether DCFH₂-DA can penetrate cell walls bidirectionally. It is also uncertain whether the deacetylation occurs inside or outside the cells, and whether the probe resides intracellularly or remains on the cell surface. Furthermore, the fluorescence response under stress may change not only due to elevated ROS levels but also as a result of low esterase activity. Researchers have reported that DCFH₂ does not directly react with hydrogen peroxide (H₂O₂) and that intracellular iron, heme, or peroxidases are required as catalysts, or that peroxynitrite-derived radicals are involved in this process [6]. Hence, without a catalyst, DCFH₂ is not a useful probe for the two most common ROS in cells, namely H₂O₂ and hydroxyl radicals [4,7]. Consequently, the results may reflect the levels of catalysts rather than the oxidants themselves. It has also been demonstrated that oxidants other than ROS react more rapidly with DCFH₂. Furthermore, it was observed that the one-electron oxidized intermediate, the DCFH radical, reacts with oxygen to generate superoxide O₂^{•-}, leading to the formation of H₂O₂ and subsequent amplification of the fluorescence signal [6,8,9]. This auto-oxidation artifact reduces the cognitive value of the results. Thereby, some authors compare the application of DCFH₂-DA to a "self-fulfilling prophecy" [9]. DCFH₂-DA probes possess additional well-known shortcomings that have been repeatedly highlighted by numerous

authors (for further details, see [8]). Ultimately, the application of DCFH₂-DA as a fluorescent probe remains controversial, as summarized in several reviews [3,5,10]. In standard application protocols, the response to a stressor is represented by an increase in fluorescence in stressed cells compared to control cells; however, contrary to frequent claims by researchers, this does not provide quantitative values of intracellular ROS.

Despite the aforementioned uncertainties, several experimental studies have utilized DCFH₂-DA to track oxidative stress in cyanobacteria (exemplary studies presented in **Table 1**) induced by physicochemical or chemical stressors across various strains. The protocols employed were generally straightforward, mostly relying on the direct addition of DCFH₂-DA to cyanobacterial cultures, followed by a 60-minute incubation and subsequent analysis via FM or FCM. These procedures were largely adapted from protocols originally developed for eukaryotic cells. However, it is crucial to verify the applicability of DCFH₂-DA specifically to cyanobacterial cell types, given the high diversity in their cell wall compositions [11]. To date, the applicability of DCFH₂-DA for ROS tracking in cyanobacterial cells has not been systematically investigated. Consequently, there is a critical knowledge gap, as the fundamental applicability, chemical stability, and potential artifacts of DCFH₂-DA in cyanobacterial systems have never been systematically or rigorously investigated.

Compounds structurally similar to DCFH₂-DA are also applied to cyanobacterial cells, albeit for different purposes. Dichlorofluorescein diacetate (DCF-DA) and fluorescein diacetate (F-DA) are nonpolar, nonfluorescent, lipophilic molecules capable of freely diffusing across plasma membranes. Inside the cells, enzymatic hydrolysis occurs (analogous to DCFH₂-DA), yielding the fluorescent product DCF. The quantification of cellular DCF fluorescence has been reported as a sensitive technique to evaluate non-specific cellular esterase activity and, consequently, "cell viability." A similar esterase-mediated conversion occurs when F-DA is hydrolyzed to its fluorescent derivative, fluorescein, which can be detected by FCM [12]. The F-DA stain has been widely used to assess cyanobacterial metabolic activity [13–15]. In this study, DCF-DA as more lipophilic analogue was undertaken. Similar to DCFH₂-DA, the application of DCF-DA to cyanobacterial cells has not been deeply investigated, and it appears that the same mechanical uncertainties (**Figure 1**) apply to DCF-DA.

To address these unresolved methodological challenges, this study provides the first comprehensive evaluation of the feasibility of staining cyanobacterial cells with DCFH₂-DA for oxidative stress detection and with DCF-DA for cell viability analysis. The novelty of approach lies in the simultaneous application of complementary analytical techniques - namely spectrophotometry, FCM, and high-performance liquid chromatography with diode-array

detection (HPLC-DAD), to systematically track the fate, stability, and transformation of these probes. A study involving DCF, DCF-DA, and DCFH₂-DA was performed using the unicellular cyanobacterium *Synechococcus salina*. The experiments aimed to evaluate the stability of the probes in BG11 growth medium, as well as their sorption and desorption characteristics. Additionally, the methyl viologen was applied as reference substance with oxidative stress properties. Ultimately, this work establishes a critical baseline for interpreting fluorometric data in cyanobacterial research, separating true biological responses from chemical artifacts.

Table 1. Exemplary reports with 2',7'-dichlorodihydrofluorescein diacetate (DCFH₂-DA) application to track ROS production by cyanobacteria

Cyanobacteria stressor	Staining procedure	Ref.
<i>Microcystis aeruginosa</i> Antibiotics erythromycin and sulfamethoxazole	12 µmol L ⁻¹ final conc. DCFH ₂ -DA; incubation 37 °C for 90 min, then cells harvested and resuspended in PBS	[16]
<i>Microcystis flos-aquae</i> Antibiotic erythromycin	Cells harvested and resuspended in pure medium, then 10 µmol L ⁻¹ DCFH ₂ -DA added, incubation 20 min 37 °C, then cells washed with pure medium	[17]
<i>Microcystis aeruginosa</i> Antibiotics clarithromycin and levofloxacin	Cells harvested and resuspended in pure water, then 10 mmol/L DCFH ₂ -DA added, incubation in dark 30 min	[18]
<i>Microcystis aeruginosa</i> NaCl stress	No data	[19]
<i>Anabaena variabilis</i> PCC 7937 UV light	Stock solution of 2 mM (w/v) DCFH ₂ -DA in ethanol, 5 µmol L ⁻¹ final conc. 60 min staining, lack of info on culture density	[20]
<i>Anabaena fertilissima</i> salinity	Cells pelleted, exposed to 5 µM DCFH ₂ -DA per 45 min, then washed with PBS (pH 7.0)	[21]
<i>Synechococcus elongatus</i> PCC, filamentous <i>Fremyella diplosiphon</i> <i>Nostoc</i> sp. UV light	2 mM DCFH ₂ -DA in ethanolic stock solution; removal of grow medium, suspension of cells in PBS medium, addition of 0-40 µM DCFH ₂ -DA; 60 min incubation in dark; FC analysis	[22,23]
<i>Synechococcus</i> sp. Salinity and temperature	10 ⁴ cells mL ⁻¹ , DCFH ₂ -DA stock solution (10 mM) in PBS DCFH ₂ -DA was used at 80 µM final concentration (0.8% DMSO); incubation 30 min	[24]

<i>Synechocystis</i> sp. UV-A, UV-B, PAR	DCFH ₂ -DA stock solution in ethanol Final conc. 50 µM; cells harvested, washed with water, resuspended in phosphate buffer (pH 7.0), incubation 60 min in dark	[25]
<i>Microcystis aeruginosa</i> pyrogalllic acid	10 µM DCFH ₂ -DA working conc. Cells harvested, washed with PBS buffer, resuspended in PBS (pH 7.0); incubation 60 min	[26]

2. Experimental

2.1. Chemicals

The BG11 growth medium concentrate was purchased from Merck. Two distinct standards of DCFH₂-DA, along with individual standards of DCF-DA and DCF, were obtained; their detailed quality and purity specifications are presented in **Table S1** (Supplementary Information). Was noted that the names and abbreviations applied by supplier sometimes are misleading; thereby, the CAS number need to be checked before purchased. Acetone, acetonitrile (ACN), and methanol (MeOH), all of HPLC grade, were purchased from Avantor Performance Materials Poland S.A. (Poland). Fluorophore stock solutions were prepared in either acetone or ACN at a concentration of 1 mg mL⁻¹ and stored at -20 °C. Working solutions were prepared daily in the appropriate solvents, although stability tests indicated that solutions in ACN remained sufficiently stable for at least one week. To prevent photodegradation, all manipulations involving the fluorescent dyes were strictly performed under reduced light conditions at a controlled room temperature 22 °C. The methyl viologen (MV) was purchased from Sigma-Aldrich.

2.2. Cyanobacteria strains cultivation

The cyanobacterial strains used in this study, *S. salina* (strain CCNP 1104), *Microcystis aeruginosa* (CCNP 1101), *Nodularia spumigena* (CCNP 1401), and *Anabaena cylindrica* (CCNP 1405), were obtained from the Culture Collection of Northern Poland (CCNP, Division of Marine Biotechnology, University of Gdańsk, Poland). These strains were originally isolated from the Baltic Sea or the Vistula Lagoon, as detailed by Mazur-Marzec et al. (2015). *S. salina* and *M. aeruginosa* were cultivated in BG11 growth medium, while *A. cylindrica* and *N. spumigena* in Z8 medium. Most of experiments presented in this work was with *S. salina*. Other species were applied at the last experiment to check variability of results with strains.

The stock cultures of cyanobacteria were grown in borosilicate bottles in a thermostat chamber

(PHYTO700, Biogenet, Poland) under constant light intensity of $35 \pm 5 \mu\text{M m}^{-2} \text{s}^{-1}$ at a temperature of 23°C , and constant aeration. The inoculation to fresh medium was performed weekly to maintain exponential growth of cultures. Any manipulation with cultures was conducted under a laminar chamber UVC/T-M-AR (SIA Biosan, Poland).

2.3. Flow cytometry and staining

Cell count was assessed by flow cytometry using a CytoFLEX 2L6C (Beckman Coulter) cytometer equipped with a blue 488 nm (50 mW) laser, and a red 638 nm (50 mW) laser as an excitation source. Fluorescence from cyanobacterial chlorophyll (Chl) was detected with a 690 nm/50 nm bandpass emission filter (PC5.5 detector) after blue light excitation. Phycocyanin cyanobacterial fluorescence was detected with a 660 nm bandpass filter (APC detector) after red light excitation. DCF fluorescence was detected at 525/40 nm bandpass emission filter (FITC detector) after blue light extinction. Forward light scatter (FSC) and side light scatter (SSC) were both detected from 488 nm blue light. The SSC-to-FSC diagram was used to set the threshold. The sample volume was 15 μL . The sample flow rate was $30 \mu\text{L min}^{-1}$. A minimum of 10,000 events were recorded. Fluorescence-labeled beads (CytoFLEX Daily QC Fluorospheres, Beckman Coulter, USA) were used to standardize the machine with respect to green fluorescence. For the FCM measurements, cells concentration was adjusted to 3,500-5,000 cell μL^{-1} . The geomean of fluorescence of histograms groups was taken as results (MFI - mean fluorescence intensity).

2.4. Stability, sorption and desorption of stains test

The hydrolysis (de-esterification) of DCFH₂-DA and DCF-DA was tested in fresh BG11 medium and BG11 medium from stock culture (centrifuged and filtered by syringe filters PET, 0.2 μm pore size), as predicted that they could have different psychochemical parameters. For this purpose, 2 mL of growth medium was added to quartz cuvette, blank was measured and then the 100 μL of stain solution (DCF, DCF-DA, or DCFH₂-DA, 1 mg mL^{-1} , in ACN) was added. The UV-Vis spectra (spectrophotometer Genesys150, Thermo Scientific) was measured every 5-10 min up to 60 min, then at 120 min and 180 min. The HPLC-DAD analysis for determination of stain concentration was performed parallelly.

The sorption of DCF, DCF-DA, and DCFH₂-DA by *S. salina* cells was tested as follow. The culture in growing phase and high density (about 4,000 cells per μL) was used. One part of culture was centrifuged, and obtained solution filtered by syringe filter (PET, 0.2 μm pore size).

The obtained solution had pH 9.7 (such high pH of cyanobacteria culture is normal situation, as noted in previous study [28]). The 10 mL volume of filtered medium (abiotic control) or cyanobacteria culture was added to the glass vial. Then the 250 μ L of DCF, DCF-DA, or DCFH₂-DA (1 mg mL⁻¹, in ACN) or ACN (solvent control) was added. The samples was shaken (100 rpm) per 60 min without the light in ambient temperature (22 °C). The first sample was taken from abiotic controls just after addition of fluoresceines to obtain reference concentration at start of experiment. After 60 min, the next samples was taken from abiotic controls (to get information about hydrolysis rate). Parallely, the 1 mL of cyanobacteria culture samples was sampled to Eppendorf tubes, centrifuged, filtered and liquid phase was analyzed by HPLC. The samples with cells were analyzed by FCM, after 10 times dilution in BG11 adjusted to pH 8.6. The three technical samples were performed.

The desorption of DCF and DCF-DA was performed using *S. salina* culture. To the 10 mL culture, the 250 μ L of DCF or DCF-DA solutions (1 mg mL⁻¹, in ACN) were added. After 60 min the samples were taken and analyzed by FCM after 10 times dissolution in BG11. Then, 1 mL of culture was taken, centrifuged and liquid discharged. The 1 mL of BG11 medium was added to pellet, and whole sample was vortex mixed to obtain suspended cells. After 1 min and 10 min of vortex mixing (what correspond the 1 min and 10 min desorption), the FCM analysis were performed.

2.5. HPLC analysis

Analysis of DCF, DCF-DA and DCFH₂-DA was performed by the high-performance liquid chromatography (HPLC) with diode array detection (DAD). The Nexera XR system (Shimadzu) with Gemini NX-C18 column (150 mm length, 4.6 mm internal diameter, 5 μ m particle size, Phenomenex) was used for this purpose. The mobile phase was mixture of A. 10 mM ammonium acetate buffer (pH 3.5) and B. acetonitrile (ACN). The gradient of elution was: 0 min 80:20=A:B, 6 min 10:90=A:B, 8 min 10:90=A:B, 10 min 80:20=A:B, 12 min 80:20=A:B. Flow rate was 1 mL min⁻¹. DAD measure spectrum between 190 and 800 nm. Column oven was set to 30 °C, while autosampler oven to 10 °C. The retention time (and analytical wavelength) was as follow: DCF 6.4 min (283 nm and 514 nm), DCF-DA 7.48 min (259 nm), DCFH₂-DA 7.91 min (299 nm). The chromatogram of mixture analysis (each compound 10 mg L⁻¹) is presented in **Figure S1** (Supplementary Information, SI). The quantification was performed by external standard method. The linear response (regression coefficient 0.9990-0.9997) was tested between 0.012 to 100.000 mg L⁻¹ (nine calibration points). The precision

was between 0.51 to 5.09 %, while accuracy between 82.0 to 112.1 %. The quantification limit (LOQ) was between 0.05-0.39 mg L⁻¹, while detection limit (LOD) was LOQ/3, with signal to noise (S/N) >3.

2.6. Test of stains using plate reader and four cyanobacteria species

The FCM is a great technique to measure the fluorescence of unicellular cells, but inadequate for filamentous strains. Thereby we decided to test the plate reader for detection of fluorescence as addition to FCM reading. Furthermore, such detection is often applied in reports presented in **Table 1**. In addition to this, we test how the cells washing after staining using fresh growth buffer affect the obtained fluorescence. The Victor X (Perkin Elmer) reader was applied. In preliminary study, two kind of 96-well black plate was tested – with transparent bottom and black bottom. With the first one the absorbance can be measured, but the fluorescence was often out of detector range. The samples for this test were prepared in 1.5 mL Eppendorf tubes by mixing of 990 µL of four tested cyanobacteria strains with the 10 µL of DCF-DA or DCFH₂-DA solutions. The incubation time was 60 min. Then some tubes were vortexed (20,000 rpm, 5 min) to separate the cells from buffer. The cells were resuspended with fresh growth medium BG11 adjusted to pH 8.0. Then, 100 µL of culture was transferred to 96-well black bottom plates. The following measurements were taken:

- empty well
- BG11 growth medium
- Biotic control (no stains)
- DCF-DA stained cells with no washing step
- DCF-DA stained cells with washing step
- DCFH₂-DA stained cells with no washing step
- DCFH₂-DA stained cells with washing step
- DCF-DA in BG11 growth medium (no cells)
- DCFH₂-DA in BG11 growth medium (no cells)

The fluorescence was measured using protocol given by manufacturers for fluorescein: 0.1 s measurement, top measurement on black bottom plate, excitation 485 nm, and emission 535 nm. To the measurement, plate cover was taken down. The time between application of cultures to plate to fluorescence measurement need to be as short as possible because the fast cell settling or aggregation, depending of species. In addition, the reaction of deacetylation can continued during the sample handling. In our case, the time after the end of staining to the sample reading was about 5 min.

2.7. The oxidative stress by methyl viologen

The methyl viologen (MV) was applied as model pesticide with proven mechanism of ROS production [29]. The stock solution of MV (1 mg 100 mL⁻¹) was prepared directly in BG11 growth medium, as well as exposure solution (1 mg L⁻¹). The test was performed in plastics wells, where 2 mL of MV solution and 2 mL of *S. salina* culture (about 4,000 cells per μ L, FCM analyzed) were mixed. The 1-h long exposure was performed in the light. After exposure, the staining by DCFH₂-DA and DCF-DA was performed according to procedure presented in **Section 2.3**. The biotic control without MV was also measured.

3. Results

3.1. Stability of fluoresceines in BG11 medium with and without cyanobacteria cells

Figure 1 presents the UV-Vis spectra of DCF, DCF-DA, and DCFH₂-DA in fresh BG-11 medium and cell-free culture filtrate ('culture BG-11') over a 60-min period following probe addition. A 60-min incubation period reflects standard practice for DCFH₂-DA staining (**Table 1**). For the filtered culture medium, measurements were extended to 180 min. DCF proved to be stable in both solutions regardless of the medium type. HPLC-DAD analysis confirmed a constant DCF concentration of 30.6 mg L⁻¹ at both the start and end of the stability assay. Notably, its UV-Vis spectrum differed between fresh BG-11 and culture BG-11. This spectral shift is attributable to pH differences: fresh BG-11 medium has a pH of 5.7, whereas the filtered culture medium reached a pH of 9.7. Fluorescein derivatives are well known to exhibit pH-dependent spectral properties [30].

The UV-Vis spectrum of DCF-DA in fresh BG-11 medium changed over 60 min, characterized by a decrease in peak intensity near 260 nm. A similar trend was observed in the culture filtrate, alongside the emergence of a new absorption peak around 514 nm. HPLC analysis of the culture filtrate revealed a reduction in DCF-DA concentration from 37.02 mg L⁻¹ to 34.09 mg L⁻¹ after 60 min, and ultimately to 30.20 mg L⁻¹ at 180 min. In fresh BG-11, the concentration decreased slightly from 37.04 mg L⁻¹ to 35.04 mg L⁻¹ over 60 min. The new spectral peak corresponds to DCF generated via the deacetylation of DCF-DA. The formation of DCF was confirmed by HPLC analysis, reaching a concentration of 6.06 mg L⁻¹ at the end of the 180-min incubation in the culture filtrate. Conversely, no DCF peaks were detected on chromatograms or spectra obtained in fresh BG-11. Because DCF results from the complete hydrolysis of both ester

groups of DCF-DA, these findings demonstrate that DCF-DA undergoes alkaline hydrolysis, with 8% of the initial DCF-DA being converted to DCF within 60 min.

A similar trend was observed for DCFH₂-DA, characterized by a time-dependent decrease in concentration in both media and the emergence of a new absorption peak in the spectrum of the culture filtrate. In the culture BG-11 medium, the DCFH₂-DA concentration decreased substantially from 26.46 mg L⁻¹ to 9.05 mg L⁻¹ within 60 min, reaching 2.33 mg L⁻¹ at 180 min. This indicates that rapid probe hydrolysis in the BG-11 medium is highly likely during standard staining procedures. The products of this hydrolysis yielded two distinct HPLC peaks (discussed in detail below). Furthermore, a DCF peak emerged in the spectrum after 180 min (**Figure 1**), corresponding to a concentration of 0.96 mg L⁻¹.

Formation of a white precipitate was observed in fresh BG-11 spiked with DCFH₂-DA, indicating low solubility in this acidic medium. Reduced aqueous solubility and partial precipitation of the probe have been reported previously [31]. Although the precise pK_a value of the carboxyl group in DCFH₂-DA is not documented, it is likely close to 4 (typical for aromatic carboxylic acids), implying lower solubility under acidic conditions than in alkaline media. The initial concentration was 26.46 mg L⁻¹, which dropped to 14.97 mg L⁻¹ after 60 min (a 43% reduction). No additional peaks were detected on the chromatograms, confirming the absence of hydrolysis at pH 5.7. Thus, limited solubility was the sole cause of the decreased DCFH₂-DA concentration in fresh BG-11 at pH 5.7.

This pH-dependent hydrolysis (de-esterification) was previously reported in a study tracking the oxidative capacity of thermally excited pyroelectric powders using DCFH₂-DA [31]. The authors identified three distinct pH regimes: regime I with minimal or insufficient hydrolysis (pH 5.3-7.2), regime II with partial hydrolysis (pH 7.2-8.7), and regime III with substantial hydrolysis of DCFH₂-DA (pH > 8.7). These findings align closely with the high rate of alkaline hydrolysis observed in our study. Consequently, a substantial loss of DCFH₂-DA is highly likely during standard 60-min staining procedures in alkaline media. Phosphate-buffered saline (PBS), which typically maintains a physiological pH of 7.2–7.4, is frequently used for cell staining (**Table 1**). Thus, probe depletion in PBS can also be significant, driven not only by spontaneous hydrolysis but also by limited aqueous solubility.

The exact chemical structure of the two transformation products resulting from DCFH₂-DA hydrolysis in alkaline medium remains to be fully elucidated. **Figure 2** presents overlaid chromatograms (extracted at 259 nm) of a DCFH₂-DA solution at the start of the assay (specifically, HPLC-DAD analysis initiated approximately 5 min post-preparation), as well as after 60 min and 180 min. Two additional peaks corresponding to transformation products (TPs)

were observed. **Figure 2** also displays the UV-Vis spectra of these chromatographic peaks. Transformation product 1 (TP1) and product 2 (TP2) exhibited higher polarity than the parent DCFH₂-DA, as evidenced by their shorter retention times on the reversed-phase column (6.77 min for TP1 and 6.08 min for TP2). We propose that TP1 represents the mono-deacetylated derivative of DCFH₂-DA, whereas TP2 corresponds to fully deacetylated DCFH₂. However, the lack of commercial reference standards precludes definitive confirmation of this hypothesis. Similarly, two additional peaks were observed for DCF-DA: one was unequivocally identified as DCF using an analytical standard, while the other likely corresponds to a mono-deacetylated intermediate (TP1, RT = 7.22 min), though its intensity was too low to acquire a reliable UV-Vis spectrum.

Next, the stability of the stains in media containing *S. salina* cells was evaluated under the same conditions as the cell-free hydrolysis assay. **Table 2** summarizes the concentrations of DCF, DCF-DA, and DCFH₂-DA at the start of the experiment and after 60 min of staining. First, the DCF concentration was negligibly reduced during exposure to cyanobacterial cells, suggesting limited cellular uptake or outer-membrane sorption. DCF concentrations remained below 1.6 mg L⁻¹ after 60 min of staining with both DCF-DA and DCFH₂-DA, regardless of the presence of cyanobacterial cells. Extracellular DCF generated from the spontaneous hydrolysis of DCF-DA and DCFH₂-DA most likely exhibits low cell-membrane permeability. Therefore, any cellular fluorescence observed in the FITC channel during FCM analysis is presumably caused by DCF produced intracellularly via the enzymatic hydrolysis of diacetylated fluoresceins.

The detection of DCF in DCFH₂-DA samples suggests an oxidative environment within the growth medium. This can be attributed to two main factors: direct auto-oxidation of DCFH₂-DA by dissolved oxygen [31] and metal-catalyzed oxidation involving trace iron or other transition metal ions present in BG-11. Alternatively, a synergistic effect of both mechanisms may occur. Cyanobacterial cells exhibited strong uptake or removal of DCF-DA. While only 9.9% of DCF-DA underwent spontaneous hydrolysis in cell-free BG-11 medium over 60 min (**Table 2**), 96% of the initial amount was depleted in the presence of cells. As the octanol-water partition coefficient (logP) of DCF-DA is not documented in the literature, it remains uncertain whether the compound primarily adsorbs onto the cell wall matrix or accumulates intracellularly. However, both scenarios contribute to a positive fluorescence signal in FCM analysis. Finally, approximately 47% of DCFH₂-DA was depleted via hydrolysis or precipitation in the culture medium, while cell interaction accounted for a further 20% reduction. After 60 min, both TP1 and TP2 were detected in the medium independently of the presence of cyanobacterial cells.

Table 2. Concentration of fluoresceines-based stains [mg L^{-1}] in sorption test with and without (abiotic control) cells of *S. salina*

Analyte	Start of test	60 min with cyanobacteria cells	60 min abiotic control (filtered BG11)
DCF	29.90 mg L^{-1}	29.28 mg L^{-1}	29.85 mg L^{-1}
DCF-DA	23.60 mg L^{-1}	0.96 mg L^{-1} (96.0% reduction) DCF 1.60 mg L^{-1}	21.25 mg L^{-1} (9.9 % reduction) DCF 1.50 mg L^{-1}
DCFH ₂ -DA	17.10 mg L^{-1}	5.83 mg L^{-1} (65.9% reduction) DCF 0.78 mg L^{-1}	9.12 (46.7% reduction) DCF 0.81 mg L^{-1}

3.2. Verification of stains sorption

FCM has rarely been applied to detect reactive oxygen species (ROS) in cyanobacteria [2,32,33]. Furthermore, the intrinsic fluorescence of cyanobacterial pigments is often overlooked, despite offering a valuable additional indicator of physiological stress. FCM is a robust technique enabling the simultaneous analysis of photopigments (chlorophylls and phycocyanin) and the tracking of cyanobacteria within complex phytoplankton communities [34]. Therefore, in this study, both cellular autofluorescence (APC channel) and stain-derived fluorescence (FITC channel) were monitored simultaneously. A microplate reader was deliberately excluded from this part of the study, as it measures total sample fluorescence - combining both dissolved and cell-bound fractions, whereas evaluating cellular uptake requires selective monitoring of the cell-bound fraction alone.

Figure 3 presents the flow cytometry histograms and dot plots for selected detection channels. The SSC/FSC plots (**Figure 3A**) and the MFI in the APC channel (**Figure 3B**) were comparable between stained and unstained cells (exact values are provided in **Figure S2**, SI). In contrast, a pronounced difference was observed in the geometric mean of the FITC channel (**Figure 3C**), as well as in the SSC/FITC signal distribution between stained and unstained cells. The highest MFI values were recorded for DCF (approximately 15,700), whereas for DCFH₂-DA, the MFI

remained virtually identical to both the biotic and solvent controls. This demonstrates that staining with the DCFH₂-DA probe was unsuccessful. Potential reasons for this lack of signal include rapid probe hydrolysis in the alkaline BG-11 medium, precipitation, poor cellular uptake, or an absence of intracellular oxidation. The experiment was independently repeated by both authors at least twice over a one-year period using different reagent batches (**Table S1**, SI). All trials confirmed that DCFH₂-DA-stained *S. salina* exhibited MFI values in the FITC channel close to those of the biotic control, whereas DCF-DA application consistently yielded a clear increase in cellular fluorescence.

These findings suggest that DCFH₂-DA may not accurately reflect natural intracellular ROS activity due to the absence of MFI signal amplification between stained and unstained cells. Furthermore, it remains questionable whether DCF-DA staining truly reflects enzymatic intracellular deacetylation or simply mirrors the hydrolytic nature of the intracellular environment. It can be hypothesized that the DCF fluorescence observed immediately after 60 min arises from rapid enzymatic hydrolysis of the dye rather than spontaneous aqueous hydrolysis. Additionally, the fluorescence detected following DCF-DA staining likely resulted from residual DCF present in the culture medium, as confirmed by HPLC analysis. DCF exhibits high MFI (**Figure 3**), and its presence in the DCF-DA working solution in BG-11 medium was detected by both HPLC (**Table 2**) and UV-Vis spectroscopy (**Figure 2**). Although cellular sorption of DCF appears to be negligible (**Table 2**), the logarithmic scale of the cytograms in **Figure 3** indicates that even minor amounts of DCF adsorbed onto the cell surface could induce a statistically significant increase in MFI. The following experiments were specifically designed to address these methodological uncertainties.

A critical remaining question concerned the stability of the FITC signal in cells stained with DCF and DCF-DA. To evaluate potential dye desorption and signal stability, we conducted an experiment involving a 60-min sorption phase followed by 1-min and 10-min desorption steps. The 1-min desorption step was designed to simulate standard post-staining sample washing (**Table 1**). DCFH₂-DA was omitted from this test as it exhibited no positive staining (**Figure 3**). **Figure 4** shows the FCM results for the FITC channel, with precise MFI values compiled in **Figure S3** (SI). Staining with DCF clearly resolved two distinct cell sub-populations: FITC-A and FITC-B (**Figures 3** and **Figure 4**). For DCF-DA, the MFI of the FITC-A population remained stable following 60 min of sorption and the subsequent 1-min and 10-min washing steps (25,000–28,000 MFI). Conversely, for DCF, the MFI of the FITC-A sub-population was approximately 29,000 post-sorption, but rapidly dropped to 17,000 after just 1 min of washing with fresh BG-11 medium (pH 8.3), decreasing further to approximately 4,600 after 10 min.

This rapid washout strongly suggests that DCF was predominantly bound to the cell surface. The stable MFI observed in DCF-DA-stained cells indicates true intracellular accumulation followed by enzymatic deacetylation. The FITC-B population exhibited exceptionally high MFI after 60 min of DCF sorption (~430,000), but virtually vanished following washing with BG-11. It can be speculated that this group corresponds to weak, multi-layered adsorption of DCF onto the cell surface. Notably, centrifugation of DCF-stained cells proved technically challenging, suggesting that adsorbed DCF may form a negatively charged outer layer on the cell surface, inducing electrostatic repulsion between cells.

3.3. The possibilities to detect stained fluorescence by application of plate reader

First, it should be noted that a microplate reader measures total sample fluorescence rather than exclusively cellular fluorescence. Consequently, any deacetylated DCF present in the extracellular solution will lead to an overestimation of the signal. Nevertheless, utilizing a microplate reader enabled the comparative analysis of both unicellular species (*S. salina*, *M. aeruginosa*) and filamentous cyanobacterial strains (*A. cylindrica*, *N. spumigena*). **Figure 5** presents the fluorescence measurements obtained via top-reading microplate spectroscopy for DCFH₂-DA and DCF-DA. Empty wells and the growth medium (BG-11) alone yielded baseline fluorescence values below 80 FU (fluorescence units). Unstained cultures exhibited baseline FU values ranging from 158 to 393, primarily attributed to light scattering and photosynthetic pigment autofluorescence overlap in the FITC detection channel. When DCFH₂-DA was added to acellular BG-11 medium, fluorescence increased by approximately 80% compared to the biotic control (reaching up to 350 FU), except in the case of *M. aeruginosa*, where values remained comparable. In contrast, DCF-DA in acellular BG-11 medium produced a substantial fluorescence signal of approximately 13,700 FU.

Furthermore, post-staining cell washing led to a marked decrease in culture fluorescence: 46-fold for *S. salina*, 30-fold for *A. cylindrica*, 15-fold for *N. spumigena*, and 7-fold for *M. aeruginosa*. A comparable reduction in FU following cell washing was observed for DCFH₂-DA. These findings indicate that the majority of the signal in non-washed samples derived from extracellular dye deacetylation within the growth medium, as described in **Section 3.1**. Another factor contributing to this signal drop may be the technical challenge of harvesting unicellular species. Parallel optical density measurements revealed a noticeable loss of cell density during washing for *S. salina* (14–22%) and *M. aeruginosa* (40–47%), whereas density loss was negligible for the filamentous strains (**Figure S4**, SI). For *M. aeruginosa*, a fraction of cells visibly accumulated at the liquid surface following centrifugation (**Figure S5**, SI). Microscopic

evaluation confirmed structural alterations in these buoyant cells (**Figure S5**, SI); specifically, pelleted cells lost their characteristic intracellular gas vacuoles, indicating that centrifugation exerts mechanical stress. Consequently, quantitative cell recovery of unicellular strains from low-density cultures was unachievable.

Nonetheless, the cell density loss during centrifugation alone cannot account for the full reduction in fluorescence. As shown in **Figure 4**, FITC fluorescence in *S. salina* cells remained unchanged after washing away excess dye, whereas **Figure 5** demonstrates the opposite trend. This discrepancy arises because FCM measures exclusively cellular fluorescence, whereas a microplate reader detects the combined signal from both cellular and extracellular compartments. Overall, these results demonstrate that when employing a microplate reader, medium replacement post-staining is critical to eliminate artifacts caused by spontaneous dye hydrolysis in the assay medium (**Table 2** and **Figure 1**). Consequently, while microplate reading is accessible for high-throughput screening, single-cell techniques such as flow cytometry are strongly recommended for delicate, vacuolated unicellular cyanobacteria (such as *M. aeruginosa*) to circumvent centrifugation-induced artifacts and biomass loss.

3.4. The application of diacetylated strains to measure cells stress

Figure 6 presents the median fluorescence intensity (MFI, FITC channel) of *S. salina* cells stained with both diacetylated dyes alongside the biotic control. As expected based on earlier observations (**Figure 3**), DCFH₂-DA staining did not yield an increase in cellular fluorescence compared to the biotic control (both ~780 MFI). Notably, even exposure to MV, a well-known ROS-generating toxicant, failed to elevate the MFI. Conversely, staining with DCF-DA substantially increased the MFI by 608% relative to the biotic control. However, subsequent exposure to MV (1 h, light conditions) led to a 52% reduction in MFI. This decline is hypothetically attributed to the impairment of intracellular esterase activity or potential loss of membrane integrity following MV treatment.

MV (paraquat) is a non-selective herbicide whose light-dependent generation of ROS represents the primary driver of toxicity in photoautotrophs [35,36] although its toxicity extends to heterotrophic organisms as well [37]. While oxidative stress and enzymatic capacity are deeply intertwined, our focus in *S. salina* was predominantly directed toward evaluating enzymatic activity. The physiological impact of MV on cyanobacteria was previously assessed in *Synechococcus elongatus* using DCFH₂-DA [38], where cells were stained for 30 min, washed, and exposed to 50 μ M MV under illumination. After subtracting background autofluorescence, the authors reported a ~10% increase in normalized fluorescence in MV-

466 treated cells. In contrast, we observed no such response in *S. salina*, a discrepancy likely
467 stemming from significant differences in experimental protocols.

468 Literature reports on DCFH₂-DA efficacy in cyanobacteria remain highly variable. For instance,
469 MV exposure exceeding 0.2 mg L⁻¹ elevated ROS levels over 20-fold in *M. aeruginosa* [39],
470 whereas a 5 µM MV treatment in *Synechocystis* sp. produced only a ~10% increase after 6 h
471 (using 10 µM DCFH₂-DA with a BG-11 wash step). Unfortunately, brief methodological
472 descriptions in existing literature hinder rigorous protocol comparisons. Furthermore, while
473 DCF-DA application in cyanobacteria has not been previously reported, it shares structural
474 similarities with the more polar FDA. FDA has been successfully used in select green
475 microalgae [40] and *M. aeruginosa* under heat stress [41], yet it proved unsuitable for
476 *Synechococcus elongatus* PCC 7942 under similar thermal conditions [42]. Taken together,
477 these findings confirm that neither DCFH₂-DA nor DCF-DA can be considered universally
478 applicable fluorophores for cyanobacteria, emphasizing the critical need for species- and
479 protocol-specific validation prior to toxicity testing.

4. Conclusion

482 This study highlights critical methodological constraints regarding the use of diacetylated
483 fluorescent probes (DCFH₂-DA and DCF-DA) in cyanobacterial research. Our results
484 demonstrate that DCFH₂-DA is unsuitable for evaluating intracellular ROS generation in *S.*
485 *salina*, as it undergoes rapid spontaneous hydrolysis in alkaline growth media (BG-11, pH >
486 8.5) and suffers from limited aqueous solubility, leading to a complete absence of cellular signal
487 amplification. Conversely, while DCF-DA yields high fluorescence, the signal is heavily
488 confounded by extracellular dye deacetylation and non-specific, electrostatic surface binding
489 of DCF to the cyanobacterial cell wall. Crucially, comparison between analytical techniques
490 revealed that microplate readers detect combined extracellular and cellular fluorescence,
491 making post-staining medium replacement imperative. However, for unicellular species with
492 gas vacuoles (e.g., *M. aeruginosa*), centrifugation-based washing causes substantial cell loss
493 and structural damage. Consequently, single-cell techniques such as flow cytometry are
494 strongly recommended over microplate assays to accurately differentiate true intracellular dye
495 processing from extracellular and surface-adsorbed artifacts.

496 DCF-DA proved applicable for staining *S. salina* due to its effective intracellular uptake and
497 subsequent internal deacetylation. While DCF-DA cannot track ROS directly, it serves as a
498 valuable probe for assessing intracellular deacetylation and membrane permeability, although
499 whether this process is driven by enzymatic esterase activity or the internal pH environment

remains to be clarified. In conclusion, despite previous reports asserting the utility of DCFH₂-DA, its application in cyanobacterial research must be approached with caution and rigorously validated.

Acknowledgement

Financial support was provided by the National Science Center (Poland) under grants UMO-2019/35/B/NZ9/00253. The authors would like to thank the Culture Collection of Northern Poland (CCNP, Institute of Oceanography, University of Gdansk, Poland) for providing cyanobacteria monocultures.

Declaration of generative AI and AI-assisted technologies in the manuscript preparation process

During the preparation of this work, the authors used Google's Gemini in order to edit language, correct grammar, and improve the overall readability of the text. After using this tool, the authors thoroughly reviewed and edited the content as necessary and take full responsibility for the content of the publication.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Klaudia Kwidzińska: Writing – review & editing, Writing – original draft, Methodology, Investigation, Data Curation. **Magda Caban:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

References

- [1] A. Latifi, M. Ruiz, C.C. Zhang, Oxidative stress in cyanobacteria, FEMS Microbiol. Rev. 33 (2009) 258–278. <https://doi.org/10.1111/j.1574-6976.2008.00134.x>.
- [2] S. Mondal, S.P. Singh, Flow Cytometry-based Measurement of Reactive Oxygen Species in Cyanobacteria, Bio. Protoc. 12 (2022) 1–11. <https://doi.org/10.21769/BioProtoc.4417>.

- [3] H. Lis, K. Kwidzińska, K. Bethke, M. Caban, Cyanobacteria as generators of reactive oxygen species – A review of analysis methods and case studies, *Talanta* 297 (2026). <https://doi.org/10.1016/j.talanta.2025.128648>.
- [4] P. Wardman, Fluorescent and luminescent probes for measurement of oxidative and nitrosative species in cells and tissues: Progress, pitfalls, and prospects, *Free Radic. Biol. Med.* 43 (2007) 995–1022. <https://doi.org/10.1016/j.freeradbiomed.2007.06.026>.
- [5] X. Chen, Z. Zhong, Z. Xu, L. Chen, Y. Wang, 2',7'-Dichlorodihydrofluorescein as a fluorescent probe for reactive oxygen species measurement: Forty years of application and controversy, *Free Radic. Res.* 44 (2010) 587–604. <https://doi.org/10.3109/10715761003709802>.
- [6] B. Kalyanaraman, M. Hardy, R. Podsiadly, G. Cheng, J. Zielonka, Recent developments in detection of superoxide radical anion and hydrogen peroxide: Opportunities, challenges, and implications in redox signaling, *Arch. Biochem. Biophys.* 617 (2017) 38–47. <https://doi.org/10.1016/j.abb.2016.08.021>.
- [7] C. von Sonntag, H.-P. Schuchmann, [4] Suppression of hydroxyl radical reactions in biological systems: Considerations based on competition kinetics, in: 1994: pp. 47–56. [https://doi.org/10.1016/S0076-6879\(94\)33007-7](https://doi.org/10.1016/S0076-6879(94)33007-7).
- [8] P. Wardman, Factors Important in the Use of Fluorescent or Luminescent Probes and Other Chemical Reagents to Measure Oxidative and Radical Stress, *Biomolecules* 13 (2023). <https://doi.org/10.3390/biom13071041>.
- [9] M.G. Bonini, C. Rota, A. Tomasi, R.P. Mason, The oxidation of 2',7'-dichlorofluorescein to reactive oxygen species: A self-fulfilling prophesy?, *Free Radic. Biol. Med.* 40 (2006) 968–975. <https://doi.org/10.1016/j.freeradbiomed.2005.10.042>.
- [10] N. Soh, Recent advances in fluorescent probes for the detection of reactive oxygen species, *Anal. Bioanal. Chem.* 386 (2006) 532–543. <https://doi.org/10.1007/s00216-006-0366-9>.
- [11] M. Sedlářová, L. Luhová, Re-evaluation of imaging methods of reactive oxygen and nitrogen species in plants and fungi: Influence of cell wall composition, *Front. Physiol.* 8 (2017) 826. <https://doi.org/10.3389/fphys.2017.00826>.
- [12] N.M. Franklin, M.S. Adams, J.L. Stauber, R.P. Lim, Development of an improved rapid enzyme inhibition bioassay with marine and freshwater microalgae using flow cytometry, *Arch. Environ. Contam. Toxicol.* 40 (2001) 469–480. <https://doi.org/10.1007/s002440010199>.
- [13] P. Doppler, R. Kriechbaum, O. Spadiut, High-throughput characterization of the filamentous cyanobacterium *Anabaena* sp. using flow cytometry, *J. Microbiol. Methods* 199 (2022) 106510. <https://doi.org/10.1016/j.mimet.2022.106510>.
- [14] S. Geary, G. Ganf, J. Brookes, The use of FDA and flow cytometry to measure the metabolic activity of the cyanobacteria, *Microcystis aeruginosa*, *SIL Proceedings*, 1922-2010 26 (1998) 2367–2369. <https://doi.org/10.1080/03680770.1995.11901173>.
- [15] J. Li, D. Ou, L. Zheng, N. Gan, L. Song, Applicability of the fluorescein diacetate assay for metabolic activity measurement of *Microcystis aeruginosa* (Chroococcales, Cyanobacteria), *Phycological Res.* 59 (2011) 200–207. <https://doi.org/10.1111/j.1440-1835.2011.00618.x>.
- [16] M. Zhang, A.D. Steinman, Q. Xue, Y. Zhao, Y. Xu, L. Xie, Effects of erythromycin and sulfamethoxazole on *Microcystis aeruginosa*: Cytotoxic endpoints, production and

- release of microcystin-LR, J. Hazard. Mater. 399 (2020).
<https://doi.org/10.1016/j.jhazmat.2020.123021>.
- [17] J. Wan, P. Guo, X. Peng, K. Wen, Effect of erythromycin exposure on the growth, antioxidant system and photosynthesis of *Microcystis flos-aquae*, J. Hazard. Mater. 283 (2015) 778–786. <https://doi.org/10.1016/j.jhazmat.2014.10.026>.
- [18] Y. Wu, H. Ding, L. Wan, W. Zhang, Y. Zhang, L. Yang, C. Zhao, Single and combined toxic effects of clarithromycin and levofloxacin on *Microcystis aeruginosa*, Environmental Pollutants and Bioavailability 34 (2022) 482–495.
<https://doi.org/10.1080/26395940.2022.2130825>.
- [19] L. Chen, F. Mao, G.C. Kirumba, C. Jiang, M. Manefield, Y. He, Changes in metabolites, antioxidant system, and gene expression in *Microcystis aeruginosa* under sodium chloride stress, Ecotoxicol. Environ. Saf. 122 (2015) 126–135.
<https://doi.org/10.1016/j.ecoenv.2015.07.011>.
- [20] R.P. Rastogi, S.P. Singh, D.P. Häder, R.P. Sinha, Detection of reactive oxygen species (ROS) by the oxidant-sensing probe 2',7'-dichlorodihydrofluorescein diacetate in the cyanobacterium *Anabaena variabilis* PCC 7937, Biochem. Biophys. Res. Commun. 397 (2010) 603–607. <https://doi.org/10.1016/j.bbrc.2010.06.006>.
- [21] P. Swapnil, A.K. Yadav, S. Srivastav, N.K. Sharma, S. Srikrishna, A.K. Rai, Biphasic ROS accumulation and programmed cell death in a cyanobacterium exposed to salinity (NaCl and Na₂SO₄), Algal Res. 23 (2017) 88–95.
<https://doi.org/10.1016/j.algal.2017.01.014>.
- [22] S. Mondal, S.P. Singh, Flow Cytometry-based Measurement of Reactive Oxygen Species in Cyanobacteria, Bio. Protoc. 12 (2022) 1–11.
<https://doi.org/10.21769/BioProtoc.4417>.
- [23] . Rajneesh, J. Pathak, A. Chatterjee, S. Singh, R. Sinha, Detection of Reactive Oxygen Species (ROS) in Cyanobacteria Using the Oxidant-sensing Probe 2',7'-Dichlorodihydrofluorescein Diacetate (DCFH-DA), Bio. Protoc. 7 (2017) 1–8.
<https://doi.org/10.21769/bioprotoc.2545>.
- [24] R. Kholssi, H. Lougraimzi, I. Moreno-Garrido, Influence of salinity and temperature on the growth, productivity, photosynthetic activity and intracellular ROS of two marine microalgae and cyanobacteria, Mar. Environ. Res. 186 (2023) 105932.
<https://doi.org/10.1016/j.marenvres.2023.105932>.
- [25] P.R. Singh, J. Pathak, Rajneesh, H. Ahmed, D.P. Häder, R.P. Sinha, Physiological responses of the cyanobacterium *Synechocystis* sp. PCC 6803 under rhythmic light variations, Photochemical and Photobiological Sciences 22 (2023) 2055–2069.
<https://doi.org/10.1007/s43630-023-00429-x>.
- [26] Z. Lu, J. Sha, Y. Tian, X. Zhang, B. Liu, Z. Wu, Polyphenolic allelochemical pyrogallol acid induces caspase-3(like)-dependent programmed cell death in the cyanobacterium *Microcystis aeruginosa*, Algal Res. 21 (2017) 148–155.
<https://doi.org/10.1016/j.algal.2016.11.007>.
- [27] H. Mazur-Marzec, A. Błaszczuk, A. Felczykowska, N. Hohlfield, J. Kobos, A. Toruńska-Sitarz, P. Devi, S. Montalvão, L. D'souza, P. Tammela, A. Mikosik, S. Bloch, B. Nejman-Faleńczyk, G. Węgrzyn, Baltic cyanobacteria – a source of biologically active compounds, Eur. J. Phycol. 50 (2015) 343–360.
<https://doi.org/10.1080/09670262.2015.1062563>.
- [28] K. Kwidzińska, M. Zalewska, A. Aksmann, J. Kobos, H. Mazur-Marzec, M. Caban, Multi-biomarker response of cyanobacteria *Synechocystis salina* and *Microcystis aeruginosa*

- to diclofenac, *J. Hazard. Mater.* 471 (2024).
<https://doi.org/10.1016/j.jhazmat.2024.134373>.
- [29] J. Ezequiel, M.R. Nitschke, M. Laviale, J. Serôdio, J.C. Frommlet, Concurrent bioimaging of microalgal photophysiology and oxidative stress, *Photosynth. Res.* 155 (2023) 177–190. <https://doi.org/10.1007/s11120-022-00989-6>.
- [30] N. Klonis, W.H. Sawyer, Spectral properties of the prototropic forms of fluorescein in aqueous solution, *J. Fluoresc.* 6 (1996) 147–157. <https://doi.org/10.1007/BF00732054>.
- [31] S. Raufeisen, M. Stelter, P. Braeutigam, Pyrocatalysis-The DCF assay as a pH-robust tool to determine the oxidation capability of thermally excited pyroelectric powders, *PLoS One* 15 (2020) 1–16. <https://doi.org/10.1371/journal.pone.0228644>.
- [32] R. Kholssi, H. Lougraimzi, I. Moreno-Garrido, Influence of salinity and temperature on the growth, productivity, photosynthetic activity and intracellular ROS of two marine microalgae and cyanobacteria, *Mar. Environ. Res.* 186 (2023) 105932. <https://doi.org/10.1016/j.marenvres.2023.105932>.
- [33] Z. Lu, J. Sha, Y. Tian, X. Zhang, B. Liu, Z. Wu, Polyphenolic allelochemical pyrogallol acid induces caspase-3(like)-dependent programmed cell death in the cyanobacterium *Microcystis aeruginosa*, *Algal Res.* 21 (2017) 148–155. <https://doi.org/10.1016/j.algal.2016.11.007>.
- [34] B. Poniedzialek, H.I. Falfushynska, P. Rzymiski, Flow cytometry as a valuable tool to study cyanobacteria: A mini-review, *Limnological Review* 17 (2017) 89–95. <https://doi.org/10.1515/limre-2017-0009>.
- [35] F. Cui, M. Brosché, A. Shapiguzov, X.Q. He, J.P. Vainonen, J. Leppälä, A. Trotta, S. Kangasjärvi, J. Salojärvi, J. Kangasjärvi, K. Overmyer, Interaction of methyl viologen-induced chloroplast and mitochondrial signalling in *Arabidopsis*, *Free Radic. Biol. Med.* 134 (2019) 555–566. <https://doi.org/10.1016/j.freeradbiomed.2019.02.006>.
- [36] G.C. Whitlam, G.A. Cold, Photoinhibition of photosynthesis in the cyanobacterium *Microcystis aeruginosa*, *Planta* 157 (1983) 561–566. <https://doi.org/10.1007/BF00396889>.
- [37] M. Morimyo, Isolation and characterization of methyl viologen-sensitive mutants of *Escherichia coli* K-12, *J. Bacteriol.* 170 (1988) 2136–2142. <https://doi.org/10.1128/jb.170.5.2136-2142.1988>.
- [38] K. Tanaka, G. Shimakawa, S. Nakanishi, Time-of-day-dependent responses of cyanobacterial cellular viability against oxidative stress, *Sci. Rep.* 10 (2020) 1–10. <https://doi.org/10.1038/s41598-020-77141-8>.
- [39] F. Bai, Y. Jia, C. Yang, T. Li, Z. Wu, J. Liu, L. Song, Multiple physiological response analyses aid the understanding of sensitivity variation between *Microcystis aeruginosa* and *Chlorella* sp. under paraquat exposures, *Environ. Sci. Eur.* 31 (2019). <https://doi.org/10.1186/s12302-019-0255-4>.
- [40] D. Wang, F.L. de los Reyes, J.J. Ducoste, Microplate-Based Cell Viability Assay as a Cost-Effective Alternative to Flow Cytometry for Microalgae Analysis, *Environ. Sci. Technol.* 57 (2023) 21200–21211. <https://doi.org/10.1021/acs.est.3c05675>.
- [41] J. Li, L. Song, Applicability of the MTT assay for measuring viability of cyanobacteria and algae, specifically for *Microcystis aeruginosa* (Chroococcales, Cyanobacteria), *Phycologia* 46 (2007) 593–599. <https://doi.org/10.2216/07-11.1>.
- [42] S. Yewalkar, T. Wu, D. Kuan, H. Wang, D. Li, A. Johnson, D. Posarac, S. Duff, X.T. Bi, Applicability of differential fluorescein diacetate and propidium iodide fluorescence

671 staining for monitoring algal growth and viability, Waste Dispos. Sustain. Energy 1
1 672 (2019) 199–206. <https://doi.org/10.1007/s42768-019-00014-y>.
2 673
3
4 674
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Figure captions

Figure 1. Spectral evolution (UV-Vis) of DCF, DCF-DA, and DCFH₂-DA over time (60 min, or 180 min for DCFH₂-DA) after dissolution in two different matrices: fresh BG11 growth medium (pH 5.7) and filtered culture supernatant of *S. salina* (pH 9.7)

Figure 2. HPLC-DAD chromatograms (extracted at 259 nm) of DCFH₂-DA solution after dissolution in culture-derived BG11 medium at 5 min, 60 min and 180 min, with UV-Vis spectra of the three chromatographic peaks and proposed structures for the transformation products (TP1 and TP2).

Figure 3. Flow cytometric cytograms of cyanobacterial cultures: biotic control, solvent control, and cells stained for 60 min with dichlorofluorescein (DCF), dichlorofluorescein diacetate (DCF-DA), and dichlorodihydrofluorescein diacetate (DCFH₂-DA). The APC channel corresponds to phycocyanin autofluorescence, while the FITC channel corresponds to DCF fluorescence

Figure 4. Flow cytometry analysis (geometric mean MFI, FITC channel) of DCF and DCF-DA sorption and desorption kinetics on *Synechocystis salina* cells

Figure 5. Microplate reader fluorescence measurements (FU) of cyanobacterial cultures (*A. cylindrica*, *N. spumigena*, *S. salina*, and *M. aeruginosa*) following 60 min of staining with DCF-DA or DCFH₂-DA, with and without a post-staining washing step. Controls include empty wells, growth medium (BG11), unstained

Figure 6. Flow cytometry analysis (geometric mean MFI, FITC channel) of *S. salina* cells after 60 min staining with DCFH₂-DA or DCF-DA, with and without prior exposure of *S. salina* to methyl viologen (MV)

Figure 1

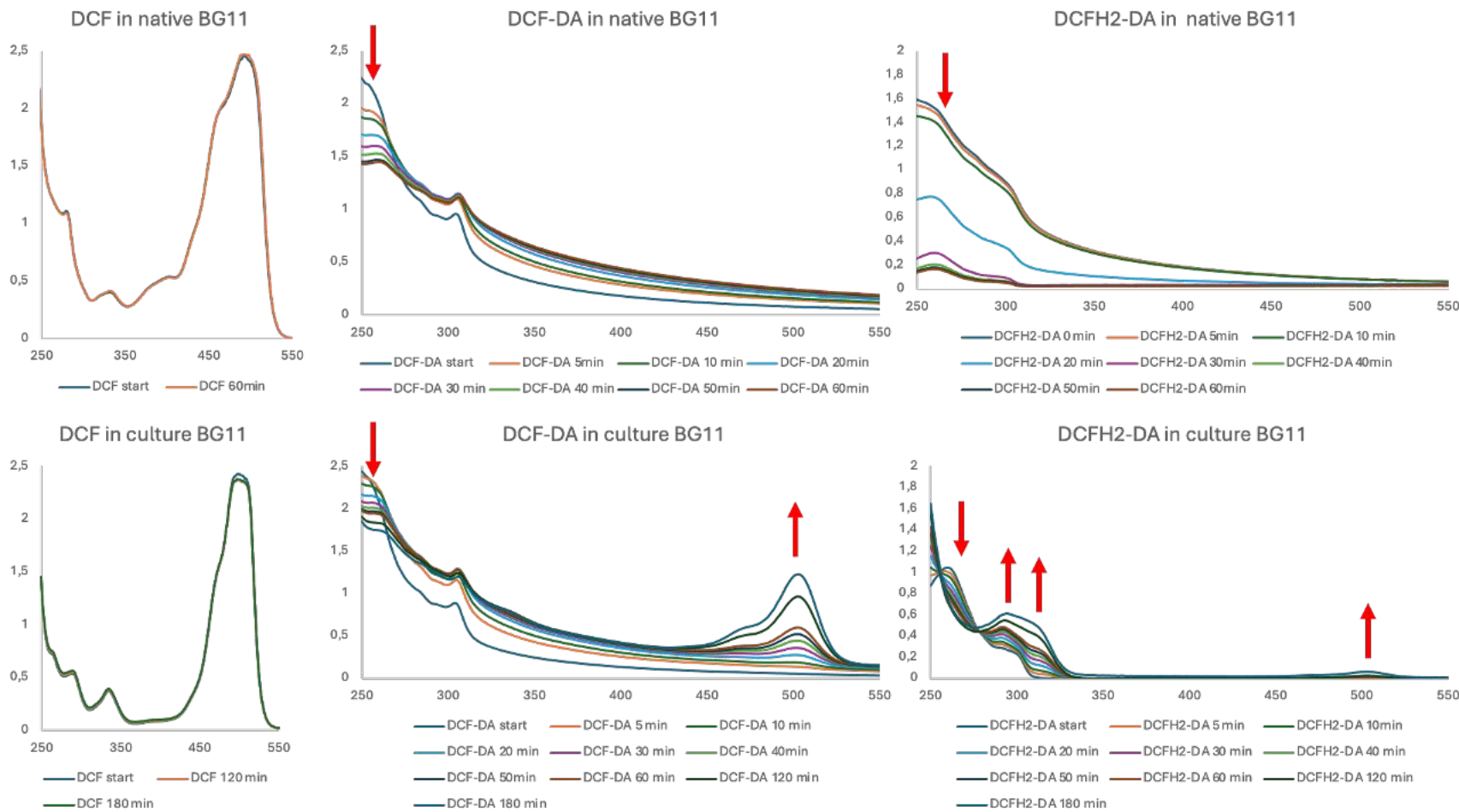


Figure 2

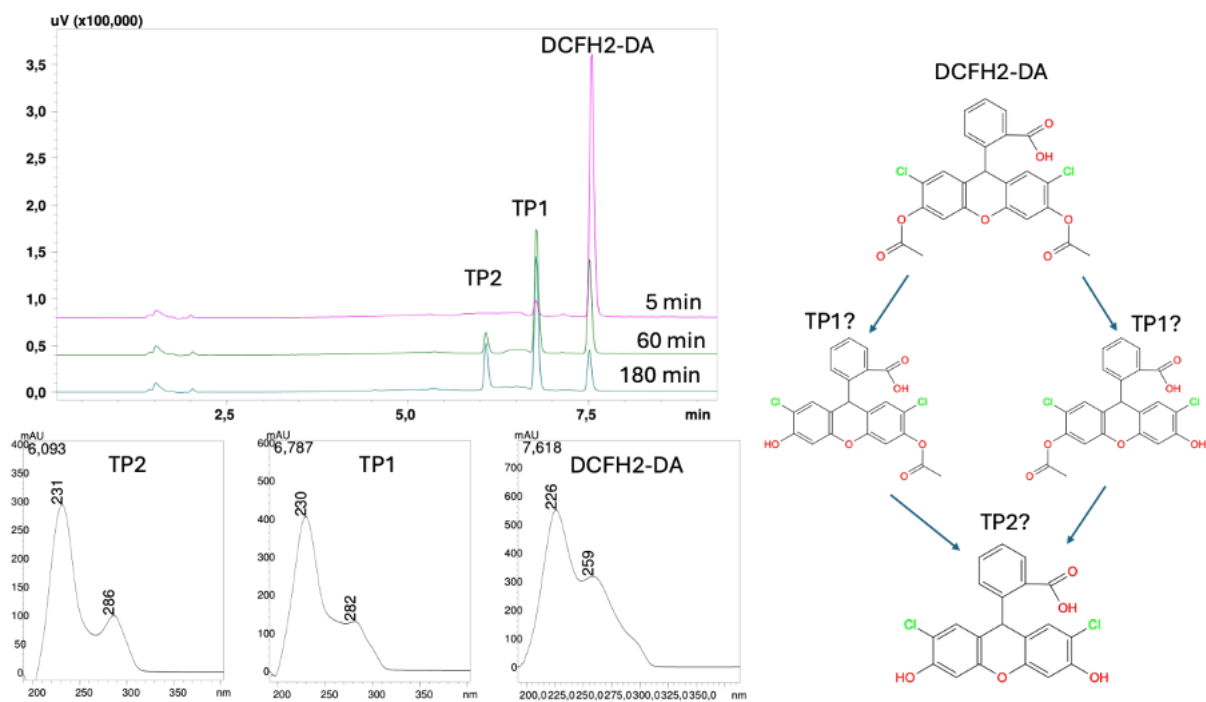


Figure 3

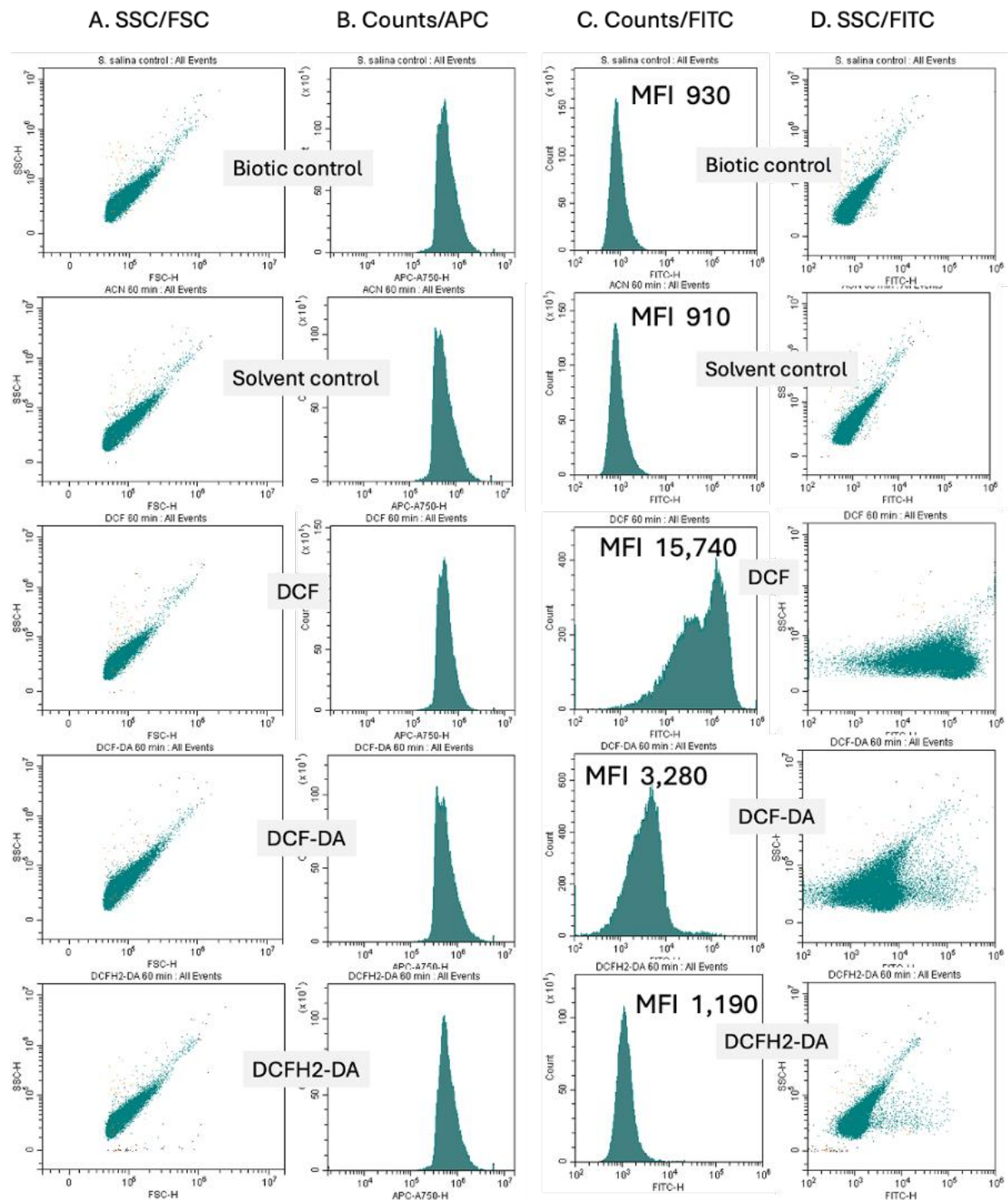


Figure 4

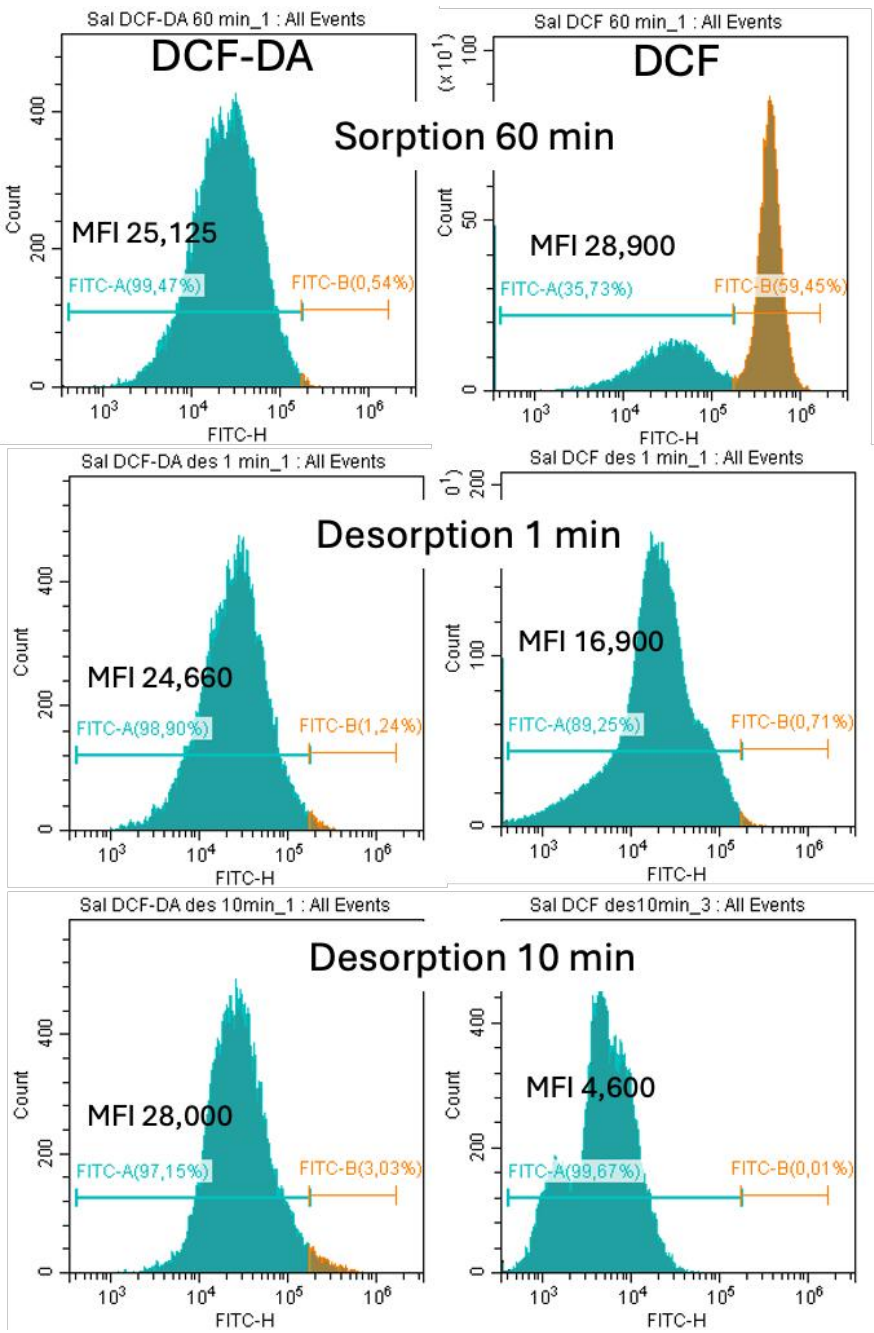


Figure 5

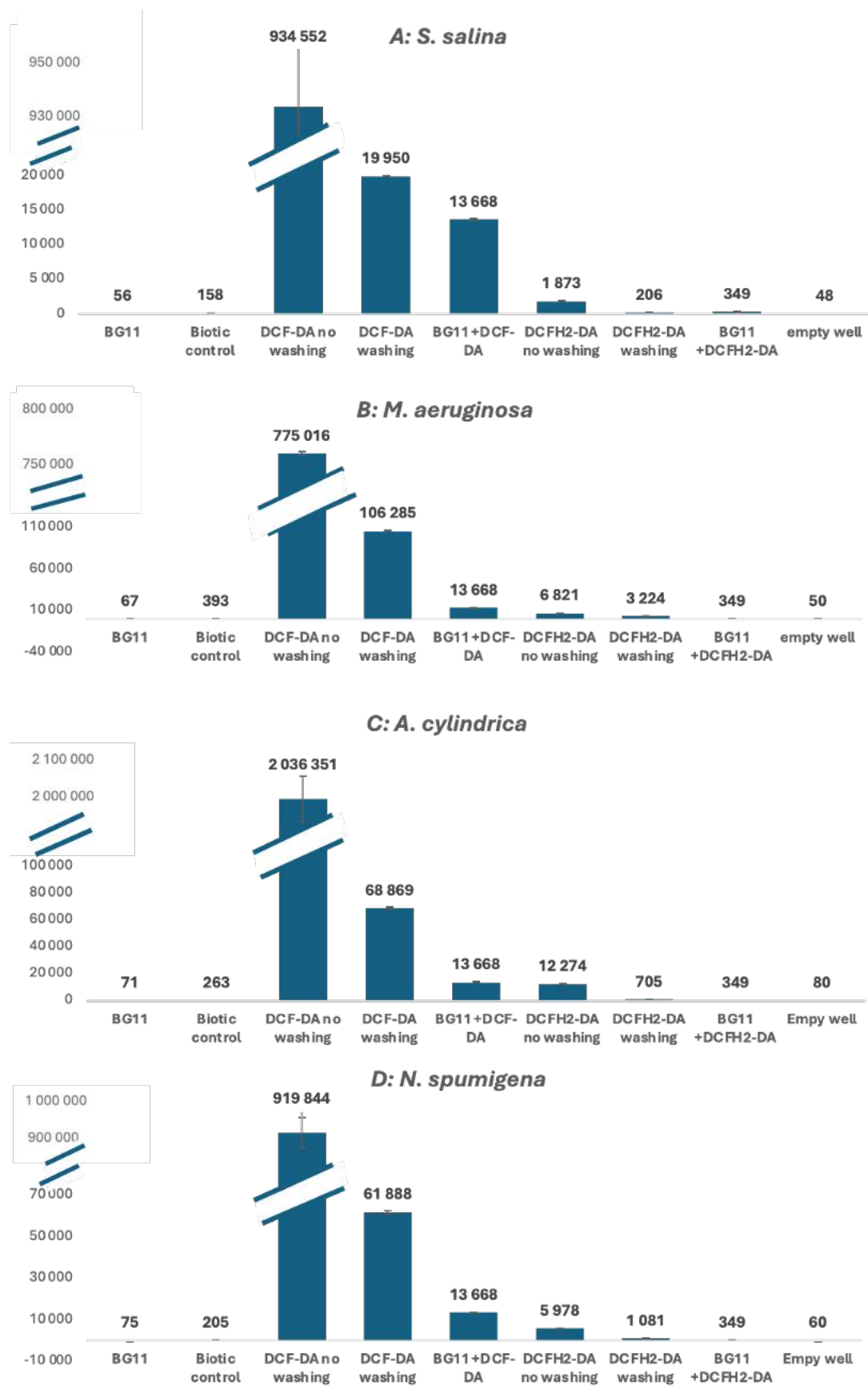
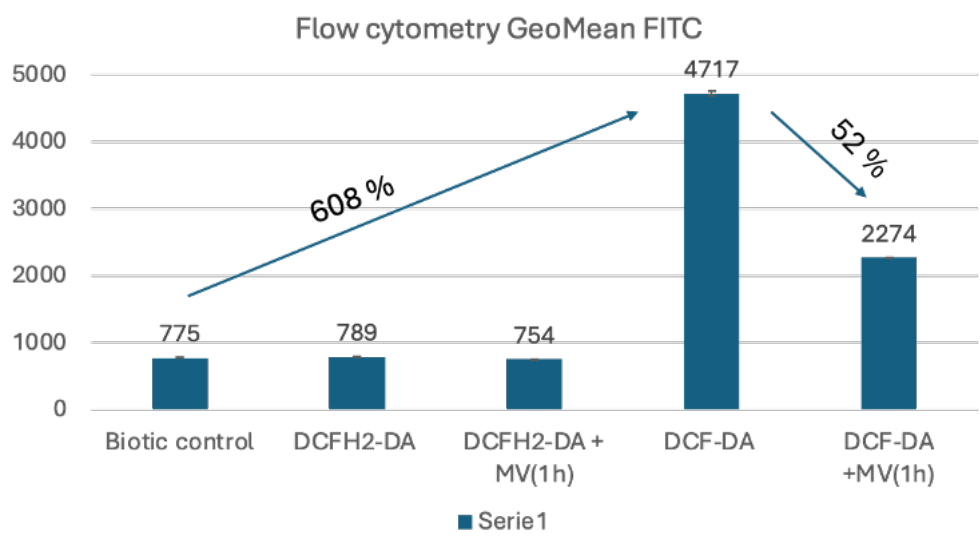


Figure 6



SUPPLEMENTARY INFORMATION

to

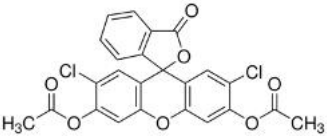
Re-evaluation of dichlorodihydrofluorescein and dichlorofluorescein diacetates applicability for cyanobacteria cells staining

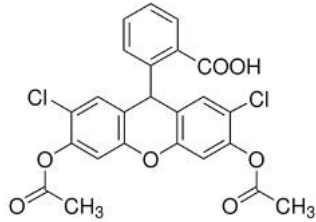
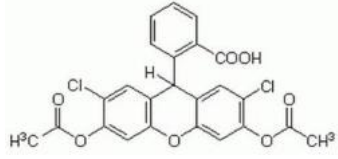
Klaudia Kwidzińska, Magda Caban *

University of Gdansk, Faculty of Chemistry, Department of Environmental Analysis, ul. Wita Stwosza 63, 80-308 Gdańsk, Poland

***Corresponding author:** Phone (+48 58) 523 5208; E-mail address: magda.caban@ug.edu.pl

Table S1. Data on purchased fluoresceins for tests

Name on package	Information from package or webpage	Purity	Supplier given names	Supplier description of use
2',7'-dichlorofluorescein diacetate DCF-DA	Merck, Germany 35845-5G MW 485.27 CAS 2044-85-1 $C_{24}H_{14}Cl_2O_7$ 	$\geq 95\%$	1 DCFHDA 2',7'-Dichlorofluorescein diacetate, Diacetyldichlorofluorescein	2',7'-Dichlorofluorescein diacetate is predominantly used to detect reactive oxygen (ROS) intermediates and quantify the overall oxidative stress in cells. 2',7'-Dichlorofluorescein diacetate, also known as DCFHDA or Dcfh-DA, is a sensitive cell-permeable redox probe. The lipophilic non-fluorescent Dcfh-DA readily crosses the cell membrane through passive diffusion followed by deacetylation. The deacetylated product is an oxidant sensitive 2',7'-dichlorofluorescein (DCHF). DCHF is oxidized later to form highly fluorescent DCF, which is measured. Reference entitled "2',7'-Dichlorodihydrofluorescein Diacetate" (doi 10.3791/60682) show application of 2',7'-Dichlorodihydrofluorescein Diacetate
2',7'-dichlorodihydrofluorescein diacetate DCFH2-DA	Sigma-Aldrich, Israel D6883-50MG MW 487.29 $C_{24}H_{16}Cl_2O_7$ CAS 4091-99-0	$\geq 97\%$	2 2',7'-Dichlorofluorescein Diacetate, H_2DCFDA , 2',7'-Dichlorodihydrofluorescein Diacetate	2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) is an oxidation-sensitive fluorescent dye used to detect the intracellular reactive oxygen species (ROS) level. DCFH-DA is a cell-permeable non-fluorescent probe, that can be hydrolyzed by intracellular esterase to DCFH, which forms fluorescent DCF when oxidized by the intracellular

				ROS. 2',7'-Dichlorofluorescein diacetate is suitable for use in: sensitive and rapid quantitation of oxygen-reactive species in response to oxidative metabolism (...).
2',7'-dichlorofluorescein diacetate DCFH2-DA	Merck, China 287810-100MG CAS not provided on vial, 4091-99-0 on webpage MW 487.3 C ₂₄ H ₁₆ Cl ₂ O ₇ 	≥94.0 %	2',7'-Dichlorofluorescein Diacetate, H ₂ DCFDA, 2',7'- Dichlorodihydrofluorescein Diacetate	Cell-permeable fluorogenic probe that is useful for the detection of reactive oxygen species (ROS) and nitric oxide (•NO) and for the determination of the degree of overall oxidative stress.
2',7'-dichlorofluorescein DCF	Sigma Aldrich, Germany 35848-5G	≥90.0 %	DCF, Dichlorofluorescein	2',7'-Dichlorofluorescein is used as fluorescent probe to measure ROS (Reactive Oxygen Species) for biological studies [ref https://pubmed.ncbi.nlm.nih.gov/20370560/]

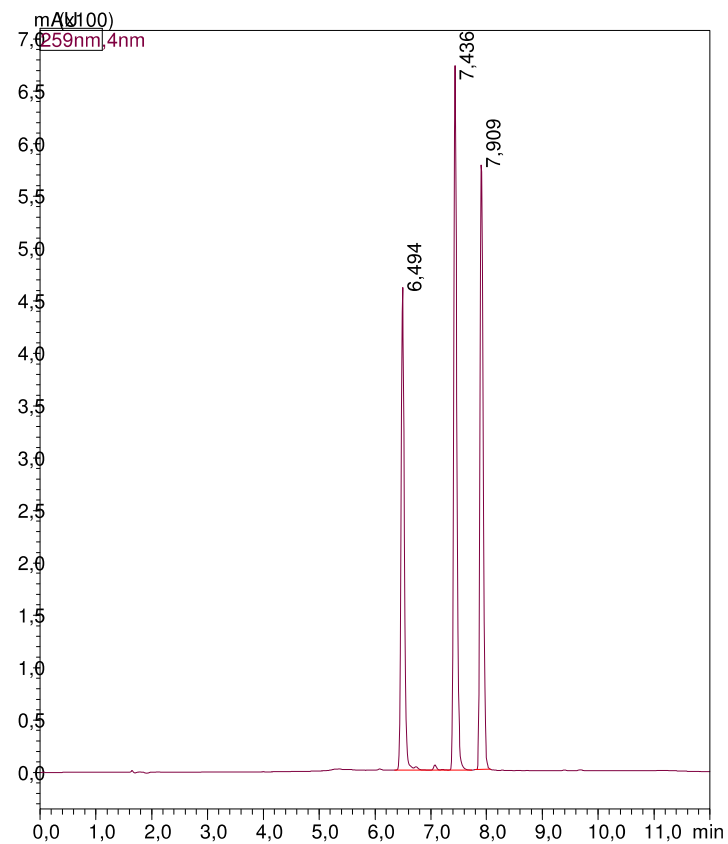


Figure S1. The chromatogram HPLC-DAD extracted for 259 nm of analysis of dichlorofluorescein (RT 6.49 min), dichlorofluorescein diacetate (RT 7.44 min) and dichlorodihydrofluorescein diacetate (RT 7.91 min)

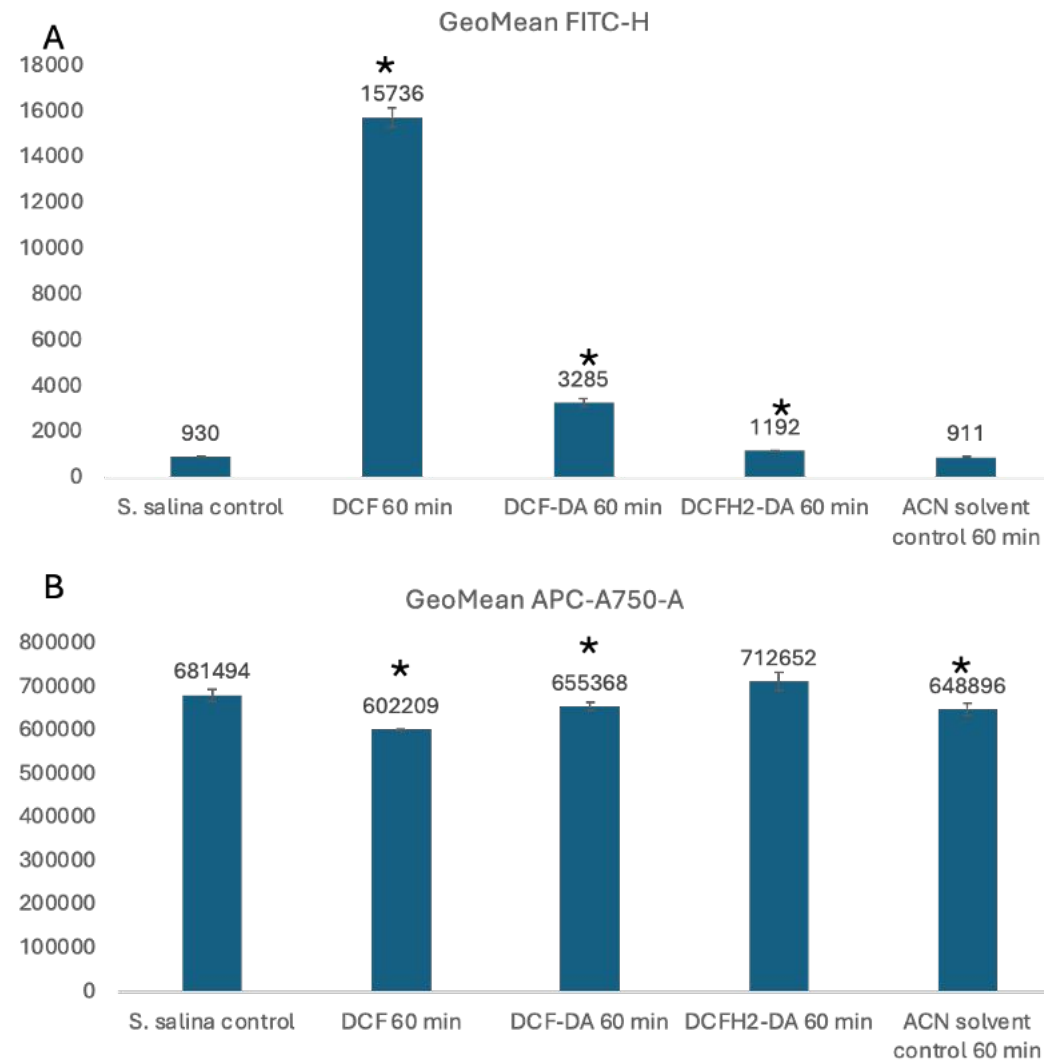


Figure S2. The geomean MFI in the FITC and APC canals of cyanobacterium *S. salina* stained by DCF, DCF-DA and DCFH2-DA per 60 min and for control culture

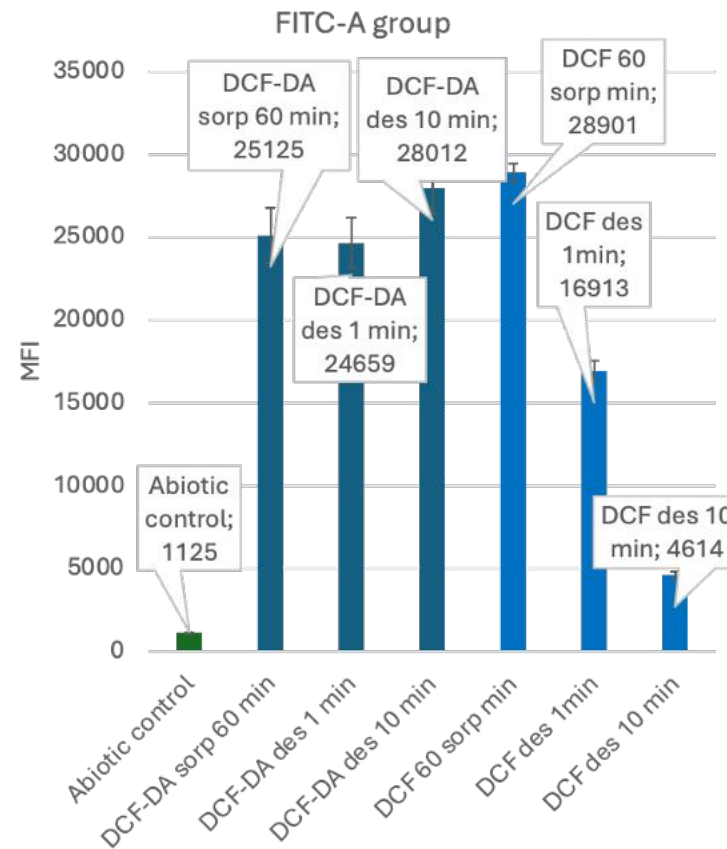


Figure S3. The geomean of MFI for FITC canal of cells not stained (biotic control) and DCF-DA and DCF stained *Synechocystis salina* cells after 60 min staining and after 1 min and 10 min long desorption

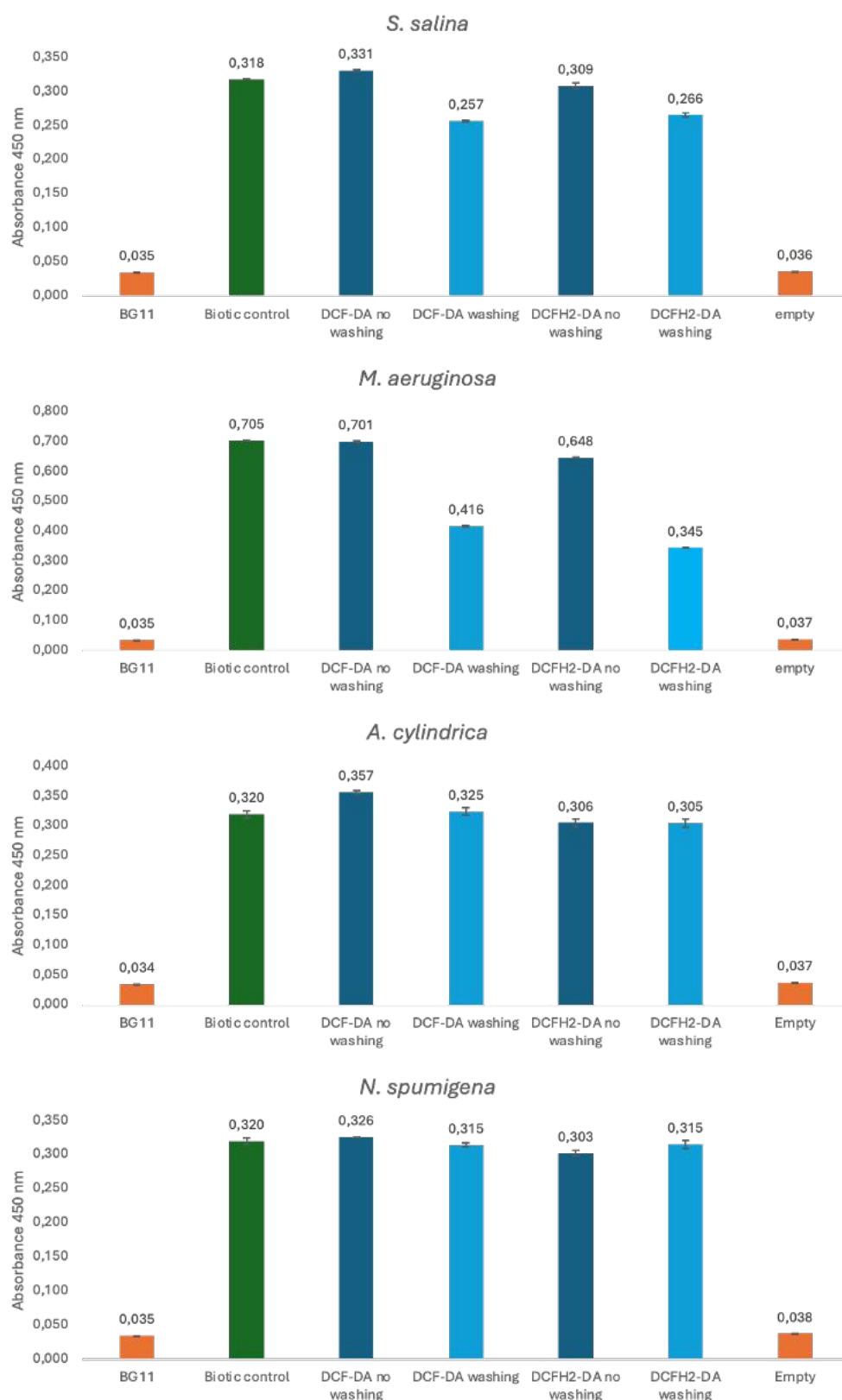


Figure S4. The optical density measured by plate reader in the samples in the 96-well plate: BG11 growth medium, cyanobacteria (4 species) culture without stains (green), DCF-DA or DCFH2-DA stained culture with (light blue) or without (dark blue) washing after staining, and empty well

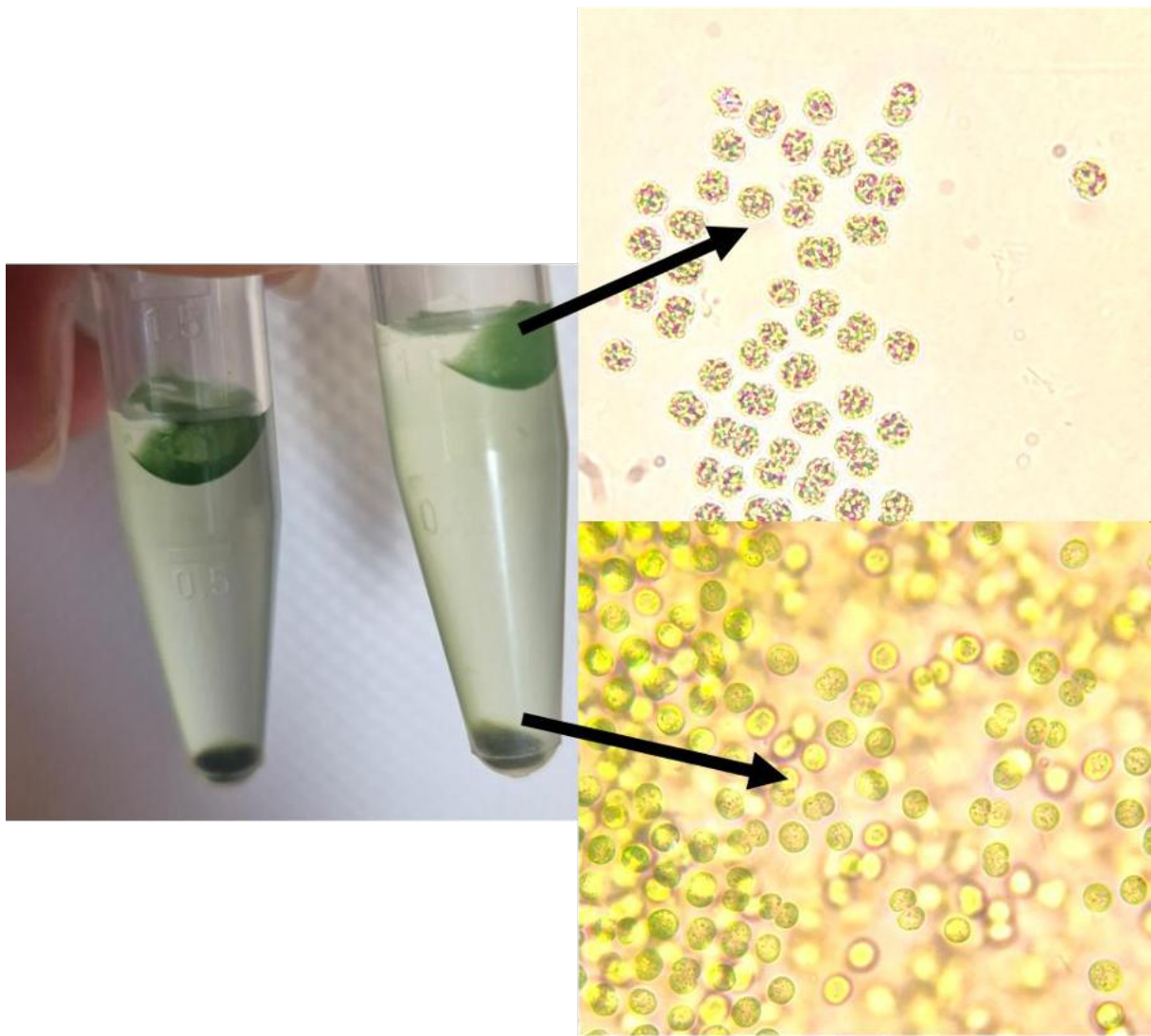


Figure S5. The picture of Eppendorf tube after centrifugation (20,000 rpm, 5 min) of *M. aeruginosa* culture, and the microscopic (oil, x1000) picture of top and bottom collected fractions of cells

Manuskrypt – M2

Kwidzińska Klaudia*, Zalewska Martyna, Aksmann Anna, Caban Magda, Nitrogen availability determines diclofenac toxicity in the diazotrophic cyanobacterium *Anabaena cylindrica* (wysłano odpowiedź na recenzję). IF 4,6 (Q1)

Nitrogen availability determines diclofenac toxicity in the diazotrophic cyanobacterium

Anabaena cylindrica

Klaudia Kwidzińska^{1*}, Martyna Zalewska², Anna Aksmann², Magda Caban¹

¹*University of Gdansk, Faculty of Chemistry, Department of Environmental Analysis, ul. Wita Stwosza 63, 80-308 Gdańsk, Poland*

²*University of Gdansk, Faculty of Biology, Department of Plant Experimental Biology and Biotechnology, ul. Wita Stwosza 59, 80-308 Gdańsk, Poland*

***Corresponding author:** Phone (+48 58) 523 5203; E-mail address:

klaudia.kwidzinska@phdstud.ug.edu.pl

Abstract

Diazotrophic cyanobacteria are capable of fixing atmospheric nitrogen, enabling their growth under nitrogen-depleted conditions. Because nitrogen fixation is a highly energy-demanding process that involves extensive metabolic and physiological reprogramming, it may increase susceptibility to pollutant-induced stress. However, this hypothesis has never been experimentally tested. In the present study, the effect of diclofenac (DCF) on the growth and biochemical changes of diazotrophic cyanobacterial cells was investigated under different levels of nitrogen availability, namely nitrate-repleted and nitrate-depleted conditions. The results demonstrated a strong relationship between the toxicity of DCF toward *Anabaena cylindrica* and nitrogen availability in the water. DCF (nominal 100 mg L⁻¹, 216 h) caused no significant growth inhibition under nitrate-repleted conditions (3.2%) but inhibited growth by 64.3% under nitrogen depletion. The 2,6-dichloroaniline was detected as a photodegradation product of DCF for the first time in an ecotoxicological study involving phototrophs. Although DCF affected the photochemical efficiency of photosystem II (PSII) in *A. cylindrica* to a similar degree regardless of nitrogen availability, the cyanobacterium employed contrasting coping strategies depending on nutrient levels. While nitrogen deprivation alone induced an increase in photoprotective carotenoids, the combination of nitrogen deprivation and DCF exposure was associated with pronounced alterations in pigment composition. Overall, the results demonstrate that nitrogen availability is an important modifier of pollutant toxicity in diazotrophic microorganisms.

Keywords: pharmaceutical ecotoxicology; diclofenac; cyanobacteria; biochemical biomarkers; photosynthesis; nitrogen availability

1. Introduction

Cyanobacteria are among the oldest photosynthetic organisms on Earth and play a key role in the functioning of aquatic ecosystems. Their ecological success results from numerous adaptive mechanisms that enable them to survive under diverse, often extreme environmental conditions, including high and low temperatures, nutrients deprivation, limited light availability, and high salinity (Allaf and Peerhossaini, 2022; Yadav et al., 2022). One of the key adaptive traits that has evolved in some cyanobacterial species is the ability to fix atmospheric nitrogen under conditions of limited availability of bioavailable forms of nitrogen. This capability is found, among others, in filamentous *Anabaena cylindrica* (Meeks et al., 1977; Wolk et al., 1976). These make this species widespread and capable of forming blooms, which may produce cyanotoxins that pose a threat to human and animal health (Bouma-Gregson et al., 2017).

Nitrogen availability is one of the most important factors determining the growth and metabolic activity of cyanobacteria. Excessive nitrate concentrations in aquatic environments may lead to eutrophication, promoting the intensive growth of cyanobacteria, and the formation of blooms. In contrast, nitrogen deficiency limits the growth of these organisms and, in diazotrophic species, induces atmospheric nitrogen fixation (Srivastava et al., 2013). N₂ fixation is a highly energy-demanding process and is associated with substantial metabolic and physiological changes in cyanobacterial cells. In response to nitrogen deficiency, specialized cells called heterocysts are formed along cyanobacterial filaments. These cells provide a microoxic environment for nitrogenase, enabling the fixation of N₂ into ammonia. This process also requires the reorganization of photosynthetic apparatus, including the inactivation of photosystem II (PSII) in heterocysts, which maintains the oxygen-depleted conditions necessary for nitrogenase activity. Such a considerable energetic investment may influence the ability of cyanobacterial cells to respond to additional stressors, including chemical stress (Flores and Herrero, 2005; Herrero et al., 2001). It is possible that the increased energy demand

limits the resources available for the activation of defense mechanisms, such as antioxidant production and the repair of damaged cellular structures. Consequently, the toxicity of chemical contaminants may depend not only on their concentration but also on nitrogen availability, which determines whether diazotrophic cyanobacteria rely on N₂ fixation.

In addition to natural environmental stressors, aquatic ecosystems are increasingly exposed to a wide variety of anthropogenic pollutants. Under natural conditions, aquatic organisms are typically exposed to multiple chemical compounds simultaneously; therefore, their response may depend on the co-occurrence of various environmental factors. One group of pollutants of particular concern is pharmaceuticals, which due to their widespread use and incomplete removal at wastewater treatment plants are now under regular monitoring in surface waters. Diclofenac (DCF) is one of the most commonly detected nonsteroidal anti-inflammatory drugs (NSAIDs) and exhibits varied toxicity toward aquatic organisms (Lee et al., 2011; Świacka et al., 2021). In our previous studies, two species of unicellular cyanobacteria (*Synechocystis salina* and *Microcystis aeruginosa*) exhibited high tolerance to DCF under laboratory conditions in a full-nitrogen growth medium (Kropidłowska and Caban, 2023; Kwidzińska et al., 2024). However, under realistic environmental conditions, nitrogen availability may be limited. Unfortunately, the influence of nitrogen availability on pharmaceutical toxicity in diazotrophic cyanobacteria has not been investigated to date. As nitrogen availability naturally fluctuates in aquatic ecosystems, cyanobacteria may encounter pharmaceutical contaminants under physiological conditions that are not represented in standard laboratory toxicity tests.

Therefore, the aim of this study was to investigate the effect of DCF on the growth and physiological response of the diazotrophic cyanobacterium *A. cylindrica* under nitrogen deprivation and its full nitrogen availability conditions. Despite growth rate as biomass related biomarker, photosynthetic efficiency, cell morphology, and the content of photosynthetic pigments, including chlorophylls, and carotenoids were analyzed. This work aims to fill a gap

in the assessment of pharmaceutical toxicity in cyanobacteria, as it is the first study to investigate the role of nitrogen deprivation in modulating pharmaceutical toxicity. This study contributes to a better understanding of how physiological state of cyanobacteria cells under nitrogen deprivation influences the toxicity of emerging contaminants, thereby improving the environmental relevance of ecotoxicological studies and supporting more realistic environmental risk assessment.

2. Experimental

2.1. Chemicals

The standards of diclofenac sodium (DCF) (CAS 15307-79-6), *N*-(2,6-dichlorophenyl)-2-indolinone (CAS 15362-40-4), 4'-hydroxydiclofenac (CAS 64118-84-9), 5-hydroxydiclofenac (CAS 69002-84-2), and diclofenac acyl- β -D-glucuronide (CAS 64118-81-6) were purchased from Sigma-Aldrich (Germany). Standard of 2,6-dichloroniline (2,6-DCA) was purchased from LGC Standards (Canada). HPLC grade acetonitrile, methanol, and acetone were purchased from POCH S.A. (Poland), while ammonium acetate and glacial acetic acid were sourced from VWR International (USA).

Pigment identification and quantification were performed using analytical standards of 4-ketomyxoxanthophyll, aphanizophyll, myxoxanthophyll, canthaxanthin, β -cryptoxanthin, Chl *a*, echinenone, pheophytin, and β -carotene (DHI Lab Products, Denmark). The standards were supplied as ethanol or acetone solutions.

2.2. Microorganisms and culture growth conditions

The cyanobacterial strain *A. cylindrica* (CCNP 1405) was obtained from the Culture Collection of Northern Poland (CCNP, Division of Marine Biotechnology, University of Gdansk, Poland). *A. cylindrica* (order *Nostocales*) was isolated from the Bay of Puck in 2005. Their vegetative

cells are cylindrical (1.8-3.5 μm long), forming long filaments (in monoculture). In natural environments, this species forms trichomes that develop into mats. A microscopic image of *A. cylindrica* is presented in **Figure 1**. Heterocysts responsible for N_2 fixation are formed only in media lacking bioavailable nitrogen. In intact filaments, heterocysts are generally distributed at intervals along the trichome, whereas heterocysts appearing terminal in the micrographs may represent intercalary heterocysts exposed at the ends of fragmented filaments. Heterocysts are typically separated by approximately 20–30 vegetative cells and usually contain cyanophycin granules at their poles adjacent to a vegetative cell (Kumar et al., 2010a). Cyanophycin, a chemically large aminoacid polymer, is the main storage material of carbon and nitrogen. A detailed description of *A. cylindrica* is provided by Mazur-Marzec et al., 2015.

Non-axenic cultures of *A. cylindrica* were grown in standard Z8 growth medium (Z8) and Z8 growth medium without nitrates (Z8₀). Cyanobacteria stock culture was maintained in 500 mL borosilicate bottles at 23°C under a constant light intensity of 70 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in a FITO700 phytotron (Biogenet, Poland). Cyanobacteria cultures were continuously aerated with purified air. Air was supplied through a silicone tube, passed through a carbon filter and syringe filters (PET material, 0.2 μm , Chromafil, Germany). Glassware and tools needed for cyanobacterial culture were autoclaved at 121°C for 20 min, and all manipulations with the cyanobacterial culture were performed under sterile conditions in a UVC/T-M-AR chamber (Biosan, Poland).

2.3. Exposure tests performance

Exposure tests were performed using *A. cylindrica* cultured in Z8 and Z8₀ media. Both experimental variants were conducted simultaneously using cyanobacteria obtained from the same stock culture. Before the experiment, the cyanobacteria were acclimated to the test conditions for at least 14 days in nitrate-free growth medium with minimum three-time inoculation to Z8₀ medium. After the acclimation period, microscopic observations were performed to confirm the presence of heterocysts.

A. cylindrica was exposed to DCF at a nominal starting concentration of 100 mg L⁻¹ for 216 h. Such concentration was found not to inhibit growth of two other species of cyanobacteria in previous study (Kwidzińska et al., 2024). The DCF was added as sodium salt directly into growth medium with no need for additional solvent. Each experimental variant included five replicates for both biotic control (without DCF) and DCF-exposed cultures. The cyanobacteria were cultured in 250 mL borosilicate bottles containing 150 mL of culture. Culture conditions, including temperature, light intensity, and aeration, were the same as those described in Section 2.2. Cyanobacterial growth was measured daily by determining the optical density at 680 nm (OD₆₈₀) after replacing the evaporated water by autoclaved deionized water and vigorous shaking of bottle for even distribution of filamentous cells. The average specific growth rate was calculated for each replicate over the entire 216 h exposure period in accordance with OECD Guideline 201. For each treatment and control group, the mean average specific growth rate and standard deviation were calculated. Growth inhibition was calculated according to OECD Guideline 201 based on the average specific growth rate. At the end of exposure, the pH was monitored.

To evaluate the differences between the two nitrogen regimes at each individual time point, an independent samples Student's t-test was performed. Prior to analysis, data were checked for normality using the Shapiro-Wilk test and for homogeneity of variance using Levene's test. Statistical significance was set at $\alpha = 0.05$. The statistical analyses were conducted using Microsoft Excel. Differences in the average specific growth rate between DCF-exposed and control cultures were evaluated using the Mann–Whitney U test, separately for each nitrogen regime (Z8 and Z8₀).

2.4. Photosynthetic analysis

Chlorophyll *a* fluorescence transients were recorded at the end of the exposure period using a Handy PEA fluorimeter (Plant Efficiency Analyzer, Hansatech Instruments, UK) according to the manufacturer's instructions. OJIP test parameters were subsequently calculated using PEA Plus (V1.10) software. Prior to analysis, samples from each bottle were diluted to obtain the same density ($OD_{665} = 0.2 \pm 0.02$) and transferred to glass vials (2 mL). Measurements were carried out at room temperature using a saturating actinic light pulse of 3000 μmol (photons) $\text{m}^{-2} \cdot \text{s}^{-1}$. The parameters analyzed in this work are presented in **Table 1**.

Table 1. Chlorophyll *a* fluorescence parameters determined in this work

Photochemical efficiency and PSII reaction center parameters	
F_V/F_M	quantum yield of primary PSII photochemistry
F_V/F₀	the activity of the water-splitting complex on the donor side of PSII
RC_M	fraction of active PSII reaction centers in the sample referring to time, when F _M (maximal fluorescence value under saturating light intensity and fully reduced state of Q _A pool) is reached
Specific energy fluxes expressed per active PSII reaction centre (RC)	
ABS/RC	absorption flux
DI₀/RC	dissipated energy flux
TR₀/RC	trapped energy flux
ET₀/RC	electron transport flux
RE₀/RC	PSI acceptor-side electron flux
Quantum yields and efficiencies	
ψ₀	the efficiency of moving the electron by the trapped exciton further than Q _A
φE₀	the quantum yield of the electron transport
φR₀	the quantum yield of the electron transport from Q _A ⁻ to the PSI end electron acceptors
φD₀	the quantum yield of energy dissipation

δR_0	the efficiency with which an electron can move from the reduced intersystem electron acceptors to the PS I end electron acceptors;
--------------	--

182

183 Statistical analyses were performed using STATISTICA software (version 13, TIBCO Software
184 Inc.). For all tests, the level of significance was set at $\alpha = 0.05$.

185 **2.5. Chemical quantification and statistics**

186 *Stability of DCF*

187 DCF and its major metabolites (4-hydroxydiclofenac, 5-hydroxydiclofenac, indoline, and
188 diclofenac glucuronide) were analyzed in growth medium by HPLC-DAD according to the
189 method described in our previous study (Kwidzińska et al., 2024). Samples (1 mL) were
190 collected on each day of the exposure test from cyanobacterial cultures and abiotic controls
191 containing nominal 100 mg L⁻¹ DCF in Z8 and Z8₀ media, respectively. The samples were then
192 centrifuged (in plastic vials, 10,000 rpm per 2 min) and filtered (PES syringe filters, 0.2 µm,
193 Chromafil, Germany) into brown-glass chromatographic vials.

194 *Photosynthetic pigments*

195 To evaluate changes in the profile of hydrophobic pigments, 1 mL of cyanobacterial culture
196 was collected from each biological replicate (n = 5) at the end of each of the three independent
197 experiments. The samples were centrifuged, and the supernatant was discarded. Chlorophylls
198 and carotenoids were extracted using a mixture of acetone and 50 mM ammonium acetate
199 (90:10, v/v) and analyzed by HPLC-DAD according to the procedure described in our previous
200 study (Kwidzińska et al., 2024; Kwidzińska and Caban, 2026). Pigments were identified based
201 on their retention times and UV-Vis absorption spectra by comparison with the corresponding
202 analytical standards.

203 Pigment contents were expressed as ratios relative to Chl *a* to account for slight inter-replicate
204 variations in harvested biomass and to specifically evaluate cellular pigment stoichiometry.

Absolute pigment concentrations in extracts (mg L⁻¹) are provided in **Table S1 - SI**. The normalization was performed according to the following equation:

$$R = \frac{C_p}{C_{Chl\ a}}$$

where C_p is the concentration of the analyzed pigment in the extract and C_{Chl a} is the Chl *a* concentration in the same extract.

For each experimental group, the mean normalized pigment content was calculated from five biological replicates. Relative changes (P) in pigment composition were then expressed as:

$$P = \frac{\overline{R_A}}{\overline{R_B}} \times 100\%$$

Where $\overline{R_A}$ and $\overline{R_B}$ are the mean normalized pigment contents (pigment/Chl *a*) of the compared groups. Depending on the analysis, the ratios were calculated either between biotic controls grown in Z8₀ and Z8 media (Biotic Control Z8₀/Z8) or between DCF-exposed cultures and the corresponding biotic controls grown in the same cultivation medium (DCF/Control). The final values are presented as the mean ± standard deviation of three independent experiments.

All R ratios values are expressed as the mean ± standard deviation (SD) of three independent biological replicates (n = 3, presented in **Table S2 - SI**). To evaluate the main effects of the growth medium (Z8 vs. Z8₀), diclofenac treatment (biotic control vs. DCF), and their interaction on the levels of individual pigments, a Two-Way Analysis of Variance (ANOVA) was performed. To control the Type I error rate associated with multiple testing across seven distinct pigments, the raw *p*-values were adjusted using the Benjamini-Hochberg False Discovery Rate (FDR) procedure. Results were considered statistically significant at an FDR-adjusted *p*-value (*q*-value) < 0.05.

To assess global shifts in the overall pigment profiles, multivariate statistical approaches were employed. Data were standardized, and Principal Component Analysis (PCA) was conducted

to visualize the clustering of samples and identify the primary pigments driving the separation between experimental groups. Furthermore, to statistically validate the multivariate response, Permutational Multivariate Analysis of Variance (PERMANOVA) based on Euclidean distance matrices with 999 permutations was utilized. All statistical analyses and visualizations were executed using Python 3 (incorporating *statsmodels*, *scikit-learn*, and *scikit-bio* libraries).

Detection of volatile products

The gas chromatography with mass detection (GC-MS, low resolution, single quadrupole, electron impact (EI) ion source) was applied for tracking volatile chemicals at the end of exposure. The analysis was prompted by the noticeable odor detected in bottles containing DCF. The abiotic control, biotic control, and exposed cultures were analyzed. Volatile organic compounds were extracted using head-space solid phase microextraction (HS-SPME) technique. The fiber adsorbent phase was a mixed mode (DVB/CAR/PDMS, 50/30 μm , 2 cm on stableflex, 24 Ga, supplied by Supelco, Germany). The fibers were cleaned via incubation in a hot injector before sampling. Ten milliliters of sample were introduced into 20 mL headspace vials, thereby head space was 10 mL. A magnetic stirrer bar and 2.5 g of NaCl were added, and as fast as possible the vial was capped by metal caps with hole and silicone. The fiber was introduced to head space through pre-holed septum. The equilibration and sampling took 30 minutes. After this time, the fiber was retracted into the holder and transferred to the GC-MS laboratory in an N_2 -purged vial.

The holder was inserted into the GC injector, and the fiber was pushed out. The analysis was started immediately, and the desorption time was 3 minutes. The GC parameters were as follows: column – Zebron ZB-SemiVolatiles (30 m L x 0.25 mm ID x 0.25 μm df, Phenomenex), injector temperature 200 $^{\circ}\text{C}$, splitless injection per 1 minute, pressure of He 100 kPa, gradient of temperature (start 30 $^{\circ}\text{C}$ per 1 min, then 16 $^{\circ}\text{C}$ per min to 230 $^{\circ}\text{C}$, finally 1 min

in 230 °C). The MS operated in the scan mode (45-450 m/z, 0.3 seconds per scan, 5 min solvent cut time) or in selected ion monitoring (SIM). The interface temperature was 235 °C. The EI operated in 70 eV at 200 °C. The m/z values for 2,6-DCA were selected based on the analysis of the standard (% of normalized intensity): 161 (100%), 163 (25%), 90 (15%), 63 (12%), and 52 (10%). The analysis of 2,6-DCA was performed qualitatively to detect its presence at the end of the exposure period; however, full quantification was beyond the scope of this study and was not performed. The blank sample was Z8₀ growth medium; the positive control was 2,6-DCA (1 mg L⁻¹) in Z8₀. Both the biotic control and DCF-exposed colonies were analyzed in order of 2,6-DCA presence.

3. Results

3.1. Determined effect on growth

Cyanobacteria cultured in Z8 and Z8₀ exhibited different growth kinetics, as reflected by the changes in OD₆₈₀ over the cultivation period (**Figure S2**). In Z8, intensive biomass increase was observed during the first 96-144 h of cultivation, followed by a stationary phase in which OD₆₈₀ values remained relatively stable. In contrast, cultures grown in Z8₀ exhibited a slower growth and a longer growth phase, lasting approximately 168 h. The lower growth rate observed in Z8₀ was the reason for extending the exposure period to 216 h, compared with the 72-h specified in OECD Guideline 201 for testing growth inhibition in microalgae.

DCF exposure markedly inhibited the growth of *A. cylindrica* under nitrate-deplete conditions, whereas no substantial effect on growth was observed under nitrate-replete conditions. The average specific growth rate calculated over the entire 216 h exposure period was higher in Z8 than in Z8₀, reaching 0.23 and 0.18 d⁻¹, respectively (**Figure 2**). DCF exposure resulted in only a slight decrease in the growth rate in Z8, from 0.23 to 0.22 d⁻¹. In contrast, in Z8₀, the growth

rate decreased from 0.18 d⁻¹ in the control to 0.06 d⁻¹ following DCF exposure. The difference between DCF-exposed and control cultures was not statistically significant in Z8 (Mann–Whitney U test, $p = 0.154$), whereas it was statistically significant in Z8₀ ($p < 0.001$).

These results indicate that DCF had no apparent effect on the growth of *A. cylindrica* growing in Z8. The DCF/Control ratio remained close to 100% throughout the experiment, indicating comparable growth of the DCF-exposed and control cultures (**Figure 3**). Growth inhibition, calculated according to OECD Guideline 201 based on the average specific growth rate, was 3.24%. The DCF/Control ratio was used to reduce the variability in absolute OD₆₈₀ values observed among the three independent experiments (shown in **Figure S2**).

A different response was observed in cultures grown in Z8₀. At the beginning of the exposure test, the DCF/Control ratio remained close to 100% (89.8-102.2%); however, it gradually decreased thereafter (**Figure 3**). After 144 h, the DCF/Control ratio decreased to approximately 50% and reached approximately 40% at the end of the exposure period (216 h), indicating a marked reduction in the growth of DCF-exposed cultures. Growth inhibition, calculated according to OECD Guideline 201 based on the average specific growth rate, was 64.3%.

The microscopic pictures were taken at the end of the exposure. The 5 random pictures are presented in **Figure S1 - SI**. It was unable to determine differences in the filament's length or heterocysts presence between exposure culture and biotic controls, because in both sample types, the single, double and multicellular filaments were observed.

3.2. Observed changes in pH

The pH measured at the end of the exposure experiment differed markedly between the cultivation conditions (**Figure 4**). In cultures grown in the standard Z8, the pH (mean value) increased to 9.68 in the DCF-exposed cultures and 9.40 in the corresponding biotic controls. In

contrast, the abiotic control remained close to neutral (pH 7.66). A similar trend was observed in the nitrate-depleted medium (Z8₀), although the pH values were substantially lower. The final pH reached 7.18 in the DCF-exposed cultures and 6.12 in the biotic controls, whereas the abiotic control remained at pH 7.55.

Overall, cultures that grown in the standard Z8 medium exhibited higher pH values than cultures grown in Z8₀, consistent with their greater biomass production and more intensive photosynthetic activity. Was previously noted, that the grow medium pH is increased in bottles with cyanobacteria growing in optimal condition (Kwidzińska et al., 2024; Nyholm and Källqvist, 1989).

3.3. Stability of diclofenac

DCF concentration was measured daily in *A. cylindrica* cultures exposed to DCF and in the corresponding abiotic controls (Z8 and Z8₀, both containing nominal 100 mg L⁻¹ DCF). A gradual decrease in DCF concentration was observed in all experimental variants throughout the 216-h exposure period (**Figure 5**). In the cyanobacterial cultures, DCF concentration (mean values, n=3) decreased by 23% in the standard Z8 and by 24% in Z8₀. In the corresponding abiotic controls, the decrease was lower, reaching 12% in the Z8 and 13% in the Z8₀.

An additional chromatographic peak with a retention time (RT) of approximately 4.0 min and a maximum UV absorption at 268 nm was detected exclusively in DCF-exposed *A. cylindrica* cultures (**Figure 6**). The peak first appeared after 24 h of exposure, and its intensity increased progressively throughout the experiment. The increase was more pronounced in cultures grown in the standard Z8 than in the Z8₀. This peak was not detected in the corresponding biotic controls or abiotic controls, indicating that it was formed only in the presence of both cyanobacterial biomass and DCF. Currently, it remains unclear what kind of compound was

generated during the experiment and whether its origin is cyanobacterial or a result of DCF transformation.

In the bottles with DCF, the characteristic scent was noted when bottles were opened at the end of experiment. Thereby, was decided to analyze the volatile compounds using HS-SPME-GC-MS techniques for identification of compounds. As we noted in previous ecotoxicological experiments with DCF in our laboratory (data not presented), the irradiation of DCF solution results in the creation of 2,6-DCA as a degradation product. The scent of this substance is characteristic, and the same was noted in experiments with *A. cylindrica*. The HS-SPME-GC-MS results prove the present of this substance in DCF-containing bottles (verified by the analytical standard, **Figure 7**). The detection of 2,6-DCA in spiked medium Z8₀ (compound with retention time 8.7 min on SIM chromatogram presented in **Figure 7A**) proves that the applied method of its analysis work. The detection of this compound was also observed in bottles with cyanobacterium and DCF (nominal 100 mg L⁻¹) (part B of **Figure 7**), but not in biotic control (part C of **Figure 7**). The 2,6-DCA was detected independent to cyanobacteria presence, while its peak appear in chromatograms from the abiotic control with Z8₀ with DCF, but without the *A. cylindrica* (part D of **Figure 7**). The same observation was made for sets with Z8.

Need to be added that 2,6-DCA was also analyzed using HPLC-DAD. However, in the applied chromatographic system both 2,6-DCA and DCF give the same retention time. Thereby, only application of GC-MS can prove the presence of 2,6-DCA in tested samples.

3.4. Effect on photosynthetic pigments

The following pigments were detected: Chl *a*, canthaxanthin, β -cryptoxanthin, aphanizophyll/myxoxanthophyll (determined together), 4-ketomyxoxanthophyll, echinenone, β -carotene, and pheophytin, a degradation product of chlorophyll. This observation is with

agreement with our previous study of cyanobacterial pigments quality analysis (Kwidzińska and Caban, 2026). Nitrogen deficiency resulted in an increased relative content (expressed as Biotic Control Z8₀/Z8 ratio) of most of the analyzed carotenoids (**Figure 8**). The relative contents of 4-ketomyxoxanthophyll, canthaxanthin, aphanizophyll/myxoxanthophyll, β-cryptoxanthin, and β-carotene increased by 69%, 64%, 30%, 27%, and 17%, respectively, compared with cultures grown in the standard Z8. Echinenone was the only carotenoid whose content decreased, showing an approximately 10% reduction under nitrogen-deficient conditions. In addition, the pheophytin content increased by approximately 39%.

The overall pigment composition of the cyanobacterial cultures was significantly altered by the interaction between the growth medium and DCF exposure, as confirmed by PERMANOVA ($F = 8.705$, $p = 0.0040$) (**Table S3 - SI**). While the presence of DCF exerted a significant independent effect on the pigment profiles ($F = 5.620$, $p = 0.0170$), the growth medium alone had a marginal impact ($F = 3.599$, $p = 0.0580$). PCA effectively modelled this variance, with the first two principal components explaining 90.9% of the total dataset variability (PC1: 79.3%, PC2: 11.6%) (**Figure 9A**). The PCA biplot revealed a distinct spatial separation of the DCF-treated samples cultivated in the Z8₀ medium, which clustered distantly from both the biotic control groups and the DCF-treated Z8 cultures along the primary axis. Vector loadings indicated that this prominent shift in the Z8₀ + DCF group was primarily driven by elevated relative concentrations of 4-ketomyxoxanthophyll, cantaxanthin, and pheophytin (**Figure 9B**). The separation of this group is with accordance with relative pigment increase in this experimental set (**Figure 8**).

Two-way ANOVA with Benjamini-Hochberg FDR correction demonstrated that DCF exposure significantly upregulated the synthesis of all tested pigments (**Table 2**). A significant interaction between the medium and the treatment was identified for three specific stress-responsive compounds: 4-ketomyxoxanthophyll ($q = 0.0047$), cantaxanthin ($q = 0.0155$), and pheophytin

($q = 0.0295$). For these pigments, the DCF-induced accumulation was vastly amplified in the Z8₀ medium compared to the Z8 medium, confirming the trends observed in the multivariate analysis.

Table 2. Two-way ANOVA results with Benjamini-Hochberg FDR correction for individual cyanobacterial pigments. *Legend:* Raw p -values and FDR-adjusted q -values for the main effects of growth medium (Z8 vs. Z8₀), diclofenac exposure (Control vs. DCF), and their interaction across individual pigments. Bold values indicate statistical significance after false discovery rate control ($q < 0.05$).

Pigment	Factor: Medium (p / q)	Factor: DCF (p / q)	Interaction: Medium × DCF (p / q)
4-ketomyxoxanthophyll	0.0003 / 0.0024	0.0002 / 0.0014	0.0007 / 0.0047
Canthaxanthin	0.0019 / 0.0051	0.0019 / 0.0068	0.0044 / 0.0155
Pheophytin	0.0022 / 0.0051	0.0051 / 0.0119	0.0127 / 0.0295
Aphanizophyll / Myxoxanthin	0.0271 / 0.0324	0.0312 / 0.0312	0.0434 / 0.0759
β-carotene	0.0257 / 0.0324	0.0149 / 0.0174	0.6716 / 0.6716
β-cryptoxanthin	0.0278 / 0.0324	0.0072 / 0.0126	0.3972 / 0.4634
Echinenone	0.4101 / 0.4101	0.0149 / 0.0174	0.3192 / 0.4468

For the remaining pigments, the interaction effect was not statistically significant, indicating independent responses to the experimental factors. DCF treatment consistently increased the relative abundance of β-carotene ($q = 0.0174$), β-cryptoxanthin ($q = 0.0126$), aphanizophyll/myxoxanthin ($q = 0.0312$), and echinenon ($q = 0.0174$). The type of culture medium also independently influenced the baseline levels of most pigments ($q < 0.05$), with the

notable exception of echinenone, which remained unaffected by the medium type ($q = 0.4101$) and responded exclusively to the presence of DCF.

3.5. Photosynthetic efficiency

To assess the effects of DCF and nitrogen availability on photosynthetic performance of *A. cylindrica*, fast chlorophyll *a* fluorescence kinetics (OJIP test) were analyzed (**Table S4**). At the end of the experiment, the maximal photosynthetic efficiency (F_v/F_m) of the control cultures reached 0.49 in Z8 and 0.48 in nitrate-depleted Z8₀. Although slightly lower F_v/F_m values were observed under nitrogen-depleted conditions, the difference between the control cultures was not statistically significant. In DCF-exposed cultures, F_v/F_m showed contrasting responses depending on nitrogen availability. In Z8-grown cultures of *A. cylindrica*, F_v/F_m decreased by approximately 2% compared with the control, whereas in Z8₀-grown cultures it increased by approximately 2%.

In general, in DCF-exposed cultures, the values of the analyzed photosynthetic parameters remained within a narrow range compared with controls. Under nitrate-repleted conditions (**Figure 10A**), the measured parameters ranged from 96 to 105% of control values, depending on the analyzed parameter. Under nitrogen-depleted conditions (**Figure 10B**), DCF exposed cultures of *A. cylindrica* exhibited values ranging from 91 to 109% of the respective controls. Specifically, in N-depleted conditions the fraction of active reaction centres (RC_m) increased relative to control and statistically significant decreases were observed in specific fluxes calculated per reaction centre (RC) including absorbed photon flux for PS II antenna chlorophylls (ABS/RC), trapped excitation flux leading to Q_A reduction (TR_0/RC), maximum electron transport flux further than Q_A^- (ET_0/RC), and electron transport flux from PS II to the final electron acceptors at the PS I end (RE_0/RC). In line with this, probability with which a

PS II trapped electron is transferred from Q_A to Q_B (Ψ_0) was significantly lower than in control, whereas probability with which an electron from Q_B is transferred until PSI acceptors (δR_0) significantly increased. In both nitrogen availability conditions, DCF exposure resulted in statistically significant changes in RE_0/RC parameter compared with the controls.

4. Discussion

4.1. Effect on growth

The obtained results indicate that the effect of DCF on the growth of *A. cylindrica* strongly depends on nitrogen availability. Under standard culture conditions, DCF (nominal 100 mg L⁻¹) had no significant effect on cyanobacterial growth, with growth inhibition of only 3.2%. A similar response was previously observed for the Baltic cyanobacterial species *S. salina* and *M. aeruginosa*, whose growth was also unaffected by exposure to DCF at the same concentration (Kwidzińska et al., 2024). In contrast, under nitrate-depleted conditions, DCF significantly inhibited the growth of *A. cylindrica*, with growth inhibition reaching 64.3%. It should be stressed that the concentration used in this study (100 mg L⁻¹) is significantly higher than ambient environmental levels of DCF, which typically range from ng to µg L⁻¹ in aquatic ecosystems. While this high exposure limits direct extrapolation to field conditions, it served as an acute testing model to demonstrate that nitrogen availability acts as a critical modifier of pharmaceutical toxicity. Limit test concentration of 100 mg L⁻¹ was chosen in accordance with OECD Guideline 201 and our previous study (Kropidłowska et al., 2026). While this approach effectively demonstrated the modulating role of nitrogen availability, full concentration - response profiling (EC₅₀, NOEC) under nitrogen depletion warrants further investigation. This response may be related to the altered physiological state of cells growing without bioavailable source of nitrogen. *A. cylindrica* can fix atmospheric nitrogen; however, this process is highly energy-demanding and requires substantial changes in cellular metabolism

and the functioning of the photosynthetic apparatus. In response to nitrogen deprivation, some vegetative cells distributed along the filament differentiate into heterocysts, specialized cells responsible for atmospheric nitrogen fixation (heterocysts marked in **Figure 1**). Heterocysts contain nitrogenase, an enzyme that catalyzes the reduction of N_2 to ammonia, which can subsequently be incorporated into cellular metabolism (Bauersachs et al., 2014; Magnuson and Cardona, 2016). To achieve this, the heterocyst cell wall undergoes extensive remodeling and becomes enriched with additional polysaccharide and glycolipid layers that restrict oxygen diffusion into the cell (Bauersachs et al., 2014). These physiological changes might potentially influence the susceptibility of cyanobacterial cells to additional environmental stressors, a hypothesis that could account for not only growth inhibition but also changes in the physiological biomarkers described later in this study. It must be emphasized that nitrogen removal induces a complex physiological state encompassing both general nitrogen deprivation responses (e.g., degradation of photosynthetic proteins, shifts in C/N stoichiometry) and the energy-demanding activation of N_2 fixation in heterocysts (Flores and Herrero, n.d.; Kumar et al., 2010b). Therefore, the pronounced toxicity of DCF observed in Z80 reflects the combined burden of nitrogen starvation stress and the metabolic reorganization required for diazotrophy, rather than N_2 fixation in isolation.

The influence of nitrogen availability on the response of cyanobacteria to pharmaceuticals has been reported by Liu et al., 2015a; however, for non-diazotrophic cyanobacterium. The authors demonstrated that the response of *M. aeruginosa* to spiramycin depended on the nitrogen content of the culture medium. At a low nitrate concentration (0.5 mg L^{-1}), environmentally relevant concentrations of spiramycin ($0.1\text{-}0.4 \text{ } \mu\text{g L}^{-1}$) did not significantly affect cyanobacterial growth but stimulated the production and release of microcystins. In contrast, at higher nitrate concentrations ($\geq 5 \text{ mg L}^{-1}$), spiramycin at the same concentrations inhibited the growth of *M. aeruginosa* and affected the expression of genes associated with nitrogen

463 metabolism and the functioning of the photosynthetic apparatus (*ntcA*, *mcyB*, *psbA*, and *rbcL*).
464 A similar relationship with nitrogen availability was also reported for *M. aeruginosa* exposed
465 to antibiotic amoxicillin (Liu et al., 2015b). The authors also demonstrated that nitrogen
466 availability influenced the levels of reactive oxygen species (ROS) and the biodegradation of
467 amoxicillin. Although the response reported by Liu et al., 2015b differed from that observed in
468 the present study, both studies indicate that nitrogen availability is an important factor
469 modifying the response of cyanobacteria to pharmaceutical contaminants, both for diazotrophic
470 and non-diazotrophic cyanobacterium. In *M. aeruginosa*, the effect of nitrogen depended on the
471 type and concentration of the antibiotic, whereas in *A. cylindrica*, nitrogen deprivation clearly
472 increased the susceptibility of the cells to DCF.

473 Previous studies on the effects of N₂ fixation into anthropogenic organic pollutants toxicity
474 were restricted to pesticides and soil cyanobacteria species. For example, the *Nostoc* sp.
475 cyanobacteria isolated from paddy soil were exposed to endosulfan in concentration 5, 10, and
476 20 mg L⁻¹ of BG11 growth medium without nitrates (Prasad et al., 2011). After six-day
477 treatment, the grow inhibition was noticed in addition to photosynthetic oxygen evolution
478 reduction (up to 50%) and nitrogen fixation inhibition (up to 60%, depending on dose). In
479 another work, the *Anabaena* PCC 7119 was subjected to treatment for another chlorinated
480 pesticide trichlorfon (10 to 300 mg L⁻¹) per 4 days (Orus et al., 1990). Such effect was noted as
481 inhibition of N₂ fixation, irregular division of cells, increase in cell volume, and lower number
482 of cells per filament. Was summarized that primary action of target pesticide is N₂ fixation. In
483 the newest article, the thiamethoxam, broad-spectrum neonicotinoid insecticide, was tested in
484 order of *Nostoc* sp. PCC7120 biochemical response (Zhang et al., 2026). The cell density was
485 measurable lower at the highest tested concentration of 20 mg L⁻¹ after 12-day exposure. At
486 concentration lower than 10 mg L⁻¹ the hormetic effect was noted. Transmission Electron
487 Microscopy (TEM) analysis revealed that in treated groups, heterocyst envelopes were blurred,

membranes broken, and intracellular structures chaotic. However, none of these studies compared the effects under standard growth conditions and nitrogen deprivation.

4.2. Changes in pH

The reasons for pH shift with cyanobacterial grow is explained in the work of Nyholm and Källqvist, 1989. Under initial growth conditions, cyanobacteria primarily absorb gaseous CO₂. As the colony grows, this resource becomes insufficient, and they begin to assimilate bicarbonates. The process of bicarbonate uptake generates OH⁻ ions, which increases the solution's pH. In addition, the nitrate assimilation leads to strong alkalization of the external environment due to the need to maintain the acid-base balance of the cell (Brewer and Goldman, 1976). When cyanobacteria take up NO₃⁻ from the medium, they use a symport (co-transport) mechanism with protons (H⁺) or an antiport (exchange) mechanism with OH⁻ to maintain the cell's electroneutrality. This change causes the loss of protons from the surrounding environment, which directly increases the medium's pH. In Z8₀, intensive energy metabolism (requiring large amounts of ATP and cellular respiration in heterocysts) and the secretion of organic acids or extracellular mucus cause a decrease in the pH of the medium (acidification) compared to the variant with nitrates (Rippka et al., 1979; Subhashini and Kaushik, 1981). In addition, the assimilation of the produced ammonium ions in heterocytes results in the formation of an equivalent amount of H⁺ ions (Nyholm and Källqvist, 1989).

Researchers studying *Anabaena* demonstrate that NO₃⁻ transport is closely linked to the movement of hydrogen or hydroxide ions (Herrero and Flores, 2019). They show that as an *Anabaena* colony intensively consumes nitrate from the medium, it undergoes a rapid alkylation of the environment. Versions grown without mineral nitrogen do not exhibit this alkaline spike because the nitrate symport mechanism remains inactive. Such a situation was observed in our study. The pH was increased in Z8 with cyanobacteria, independent of DCF presence - what additionally proves that this pollutant has no effect on *A. cylindrica* metabolism and growth. In

the Z8₀, the sample with biotic control decreases the pH - from 7.55 to 6.12 (**Figure 4**), and such acidification is expected. However, the grow medium with DCF has changed pH only slightly - from 7.55 to 7.18. The low reduction in pH indicate the suppressed N₂ fixation, and, as effect, lower grow. This observation is with agreement with observed effects on culture density.

4.3. Stability of DCF

DCF concentration decreased in both abiotic controls and *A. cylindrica* cultures (**Figure 4**). However, the reduction was greater in the presence of cyanobacterial biomass (23-24%) than in the corresponding abiotic controls (~10%). The decrease observed in the abiotic controls may be attributed to photolytic degradation, which has previously been reported for DCF (Buser et al., 1998; Nosek and Zhao, 2024). The greater reduction in DCF concentration observed in *A. cylindrica* cultures suggests that processes associated with the presence of cyanobacterial biomass contributed to DCF removal from the medium. However, it should be noted that the pH of the culture medium was considerably higher in the cyanobacterial cultures than in the abiotic controls, which may also have contributed to the observed decrease in DCF concentration. Therefore, based on the present study, it is not possible to determine the mechanism responsible for the greater reduction in DCF concentration in the presence of biomass. Despite approximately sevenfold higher *A. cylindrica* biomass in the Z8 than in the Z8₀, the decrease in DCF concentration was nearly identical in both experimental variants. This suggests that DCF removal was not proportional to cyanobacterial biomass. Therefore, bioaccumulation was unlikely to be the dominant mechanism responsible for the observed decrease in DCF concentration. However, the greater toxicity of DCF in Z8₀ can be connected with the higher cellular uptake of DCF.

In our previous study involving *M. aeruginosa* and *S. salina*, the decrease in DCF concentration in cyanobacterial cultures was comparable to that observed in the corresponding abiotic

controls, indicating no evidence of substantial bioaccumulation or biotransformation of DCF by these cyanobacteria (Kwidzińska et al., 2024). In the present study, the difference between *A. cylindrica* cultures and the abiotic controls was more pronounced, although still relatively small, and therefore does not allow any definitive conclusions regarding the mechanism of DCF removal. What is interesting, during 12-days exposure of *Anabaena* PCC 7119 to pesticide trichlorfon no concentration of trichlorfon in cell-free and cell-inoculated culture media was noted (Orus et al., 1990). This indicates that pesticide was not absorbed, and the reported deleterious effect on N₂ fixation enzymes was not direct.

Several green microalgae, including *Chlorella sorokiniana*, *Chlorella vulgaris*, and *Chlamydomonas reinhardtii*, have been reported to remove DCF from the culture medium through processes such as biosorption, bioaccumulation, and biotransformation (Majewska et al., 2021; Sánchez-Sandoval et al., 2022). These differences may result from the distinct cell wall structure of green microalgae and cyanobacteria, which influences both the sorption properties and the permeability of DCF into the cells. This may partly explain the higher DCF removal efficiency reported for some green microalgae as well as their greater susceptibility to DCF compared with cyanobacteria.

The part of decline in DCF concentration can be derived from production of 2,6-DCA - volatile chemicals detected by GC-MS (**Figure 7**). The photodegradation of DCF in water is well documented, as well as obtained products (Gackowska et al., 2025; Iovino et al., 2017). However, most reports present the water-soluble compounds (detected by LC-MS), while volatile chemicals were not analyzed. In our work 2,6-DCA was detected as a product of DCF photo-transformation. This compound was previously found in the test of sonochemical degradation of DCF (Ziylan et al., 2014), and studies on oxidation and photooxidation process (Dadban Shahamat et al., 2019; Myint Sein et al., 2008; Najafinejad et al., 2026). In addition, DCF could be degraded by native microbial soil consortia with 2,6-DCA production (Facey et

al., 2018). However, with our best knowledge, 2,6-DCA was never previously detected in laboratory ecotoxicological tests. Chloroanilines are toxic for water organisms, and can be created via degradation of various pesticides, pharmaceuticals, and dyes (Rebelo et al., 2023). However, only 4-chloroaniline and 3,4-dichloroaniline were tested in order of ecotoxicity. Thereby we are unable to state what is contribution of 2,6-DCA into total toxicity observed for *A. cylindrica*. In addition, in our test system, the culture was aerated, thereby the volatile compounds are constantly blown out of the bottle.

4.4. Photosynthetic pigments alteration

Changes in the ratio of pigments can be a valuable biomarker of stress in photosynthetic cells. Carotenoids play a crucial role in the functioning of the photosynthetic apparatus by participating in both light harvesting and the protection of photosystems against photoinhibition and oxidative stress through quenching of excited chlorophyll states and scavenging of ROS. In addition, they modulate membrane microviscosity, thereby contributing to the maintenance of membrane integrity and proper cellular function (Zakar et al., 2016). In cyanobacteria, carotenoid content is influenced by various environmental stressors, including high light intensity, light quality, temperature, pH, salinity, and nutrient availability (Pagels et al., 2021). Moreover, both the nitrogen source and its concentration in the growth medium have been shown to affect carotenoid composition (Takaichi and Mochimaru, 2007). In the present study, nitrogen deprivation resulted in an increase in the content of all analyzed carotenoids, except for a slight decrease in echinenone (**Figure 8**). The observed changes in the carotenoid profile may reflect different levels of oxidative stress. Under nitrogen deprivation alone, the relative content of most carotenoids increased, which may indicate the activation of photoprotective mechanisms aimed to protect from oxidative stress.

Exposure to DCF under nitrate-replete conditions markedly altered the relative carotenoid content with respect to Chl *a* (**Figure 9**). Since the biosynthetic pathways of chlorophyll and carotenoids are closely interconnected and share common metabolic precursors, disturbances in one of these pathways may trigger cascading changes in the biosynthesis of the remaining pigments (Takaichi et al., 2005). Analysis of the changes (**Figure 9**) together with the carotenoid biosynthetic pathway (**Figure 11**) indicates that the greatest decreases were observed for carotenoids synthesized at the terminal steps of the individual biosynthetic branches, namely canthaxanthin and 4-ketomyxoxanthophyll (named as (3*S*,2'*S*)-4-ketomyxol 2'-fucoside in **Figure 11**). At the same time, minor growth of *A. cylindrica* was observed under these culture conditions, indicating substantial physiological disturbance. It is conceivable that the combined effect of nitrogen deprivation and DCF contributed to a reduction in N₂ fixation efficiency, which might subsequently have constrained overall protein synthesis. Consequently, this could indirectly affect the abundance of enzymes involved in carotenoid biosynthesis, which could explain the greatest changes observed for carotenoids representing the end products of the individual biosynthetic branches. However, this potential mechanism warrants further verification through direct enzymatic and gene expression assays.

In the present study, cellular pigment dynamics were evaluated primarily through ratios normalized to Chl *a*. This normalization strategy was used to decouple specific metabolic shifts (for example photoprotective carotenoid upregulation) from overall changes in culture growth and harvested biomass, which naturally exhibit minor inter-replicate variability (as visualized in **Figure 2**). However, a potential limitation of this approach is that Chl *a* biosynthesis itself is susceptible to stress induced by both nitrogen deprivation and pharmaceutical toxicity. Consequently, an observed increase in a pigment/Chl *a* ratio could theoretically stem either from active secondary pigment synthesis or from a preferential degradation of Chl *a*. To provide

full transparency and account for this boundary condition, absolute pigment yields were also evaluated (**Table S1 - SI**).

4.5. Photosynthetic efficiency modulation

As the OJIP test method is based on the induction of fast Chl *a* fluorescence, the measured parameters mainly reflect PS II activity, including photosynthetic efficiency, electron transport capacity, and the functionality of PS II acceptor side (Stirbet and Govindjee, 2011). Thus, in the present study, the recorded fluorescence signal primarily originates from vegetative cells of *A. cylindrica*, as heterocyst observed under N-depleted conditions (**Figure 1**) contain an inactive PS II and contribute negligibly to Chl *a* fluorescence emission (Malatinszky et al., 2017; Nozue et al., 2017).

Previous studies have demonstrated that nitrogen availability can strongly influence cyanobacterial photosynthetic performance (Li et al., 2018), with N deprivation resulting in reduced F_v/F_m values. However, in the present study, the F_v/F_m parameter derived from Chl *a* fluorescence *in vivo* measurements exhibited only slight alterations (with changes not exceeding 2%) after 192 h of DCF exposure under both nitrate-repleted and nitrate-depleted conditions, suggesting that the maximum photochemical efficiency of PS II was affected by DCF to a comparable extend regardless N availability. This is further suggested by the absence of significant differences between F_v/F_m values in DCF-treated cultures grown in Z8 and Z8₀.

When the treatments were considered individually, the obtained results indicated that DCF exerted only a limited effect on the photosynthetic performance of *A. cylindrica* grown in nitrate-containing Z8. Under these conditions, a slight reduction in the density of active reaction centres (RC) was observed, accompanied by increased energy dissipation per reactive centre (DI_0/RC) and decreased activity of the water-splitting complex (F_v/F_0). This response of the photosynthetic apparatus (PS II) to DCF is consistent with previous findings for the model

green alga *Chlamydomonas reinhardtii*, in which exposure to DCF at a concentration corresponding to the EC_{50} (35 mg L^{-1}) induced a similar alteration in photosynthetic performance (Majewska et al., 2018; 2021). In the cited study, the toxic effects of DCF were attributed to transformation of active RCs into silent ones, so called “heat sinks”, which enhance thermal energy dissipation and prevent oxidative damage (Bai et al., 2019). Considering that a similar protective response of cyanobacterial photosynthetic apparatus was previously observed in our study involving *S. salina* and *M. aeruginosa* (Kwidzińska et al., 2024), this mechanism may also contribute to the acclimation response of *Anabaena* to DCF-induced stress when N availability is not a limiting factor.

Conversely, under N-depleted conditions, the photosynthetic response to DCF differed. *A. cylindrica* cultures grown in $Z8_0$ responded to DCF exposure by increasing the density of active RCs. This was accompanied by a decrease in specific fluxes per active RCs including ABS/RC , TR_0/RC , DI_0/RC and ET_0/RC . These changes suggest a possible redistribution of excitation energy among a larger number of active RCs, resulting in lower energy pressure on individual RC.

The contrasting responses of *A. cylindrica* to DCF under different N regimes may be related to distinct physiological states of cells and the availability of resources for maintaining and repairing photosynthetic apparatus. A related study revealed that sufficient N availability was a crucial factor for antioxidant production by in the *M. aeruginosa* exposed to 17β -estradiol (Bai et al., 2019). Moreover, under N-deprivation, heterocystous species such as *A. cylindrica* redistribute cellular resources between vegetative cells and heterocysts. These processes may affect nitrogen allocation towards antioxidant defense and maintenance of photosynthetic apparatus. Thus, under N-deprivation, these adjustments may favour a larger pool of active RCs while reducing the pressure on individual PS II RCs.

5. Conclusions

This work proves that the toxicity of DCF toward target cyanobacteria is dependent on the nitrogen source and its form in the growth medium. Under nitrate-repleted conditions, DCF (100 mg L⁻¹) did not affect the growth of *A. cylindrica*, whereas under nitrogen deprivation (required N₂-fixation) it caused 64.3% growth inhibition. This was also connected with the lower carotenoids fraction compared to Chl *a*. In addition, the changes in photosynthesis were noted. The effect of DCF on the maximum photochemical efficiency of PS II (F_v/F_m) in *A. cylindrica* is similar regardless of nitrogen regime. Under nitrate-repleted conditions, the cyanobacterium protects itself from oxidative damage by reducing the density of active reaction centers and increasing thermal energy dissipation.

The finding can have reflection in the environmental risk assessment. While all ecotoxicological studies are performed with the standard medium with full availability of nutrients, the obtained data can have a significant lack of reliability. The induction of pollutants toxicity by nitrate-repleted conditions needs to be investigated not only for emerging pollutants, but also the chemicals frequently found in the environment for which actual risk assessment shows low risk. While 100 mg L⁻¹ serves as a proof-of-concept threshold based on OECD recommendations, full multi-concentration testing across wider gradients remains an essential direction for future research.

Acknowledgments

Financial support was provided by the National Science Center (Poland) under grants UMO 2019/35/B/NZ9/00253. The authors would like to thank the Culture Collection of Northern Poland (CCNP, Institute of Oceanography, University of Gdansk, Poland) for providing cyanobacteria monocultures.

685 **Declaration of Competing Interests**

686 The authors declare that they have no known competing financial interests or personal
687 relationships that could have appeared to influence the work reported in this paper.

688

689 **CRediT authorship contribution statement**

690 Klaudia Kwidzińska: Writing – original draft, Visualization, Methodology, Investigation,
691 Validation, Writing – review and editing. Martyna Zalewska: Writing – original draft,
692 Investigation, Visualization, Validation, Writing – review and editing. Anna Aksmann: Writing
693 – original draft, Writing – review and editing. Magda Caban: Conceptualization, Writing –
694 original draft, Writing – review and editing, Methodology, Investigation, Visualization,
695 Validation, Funding acquisition, Project administration, Supervision.

696

697 **Declaration of generative AI use**

698 During the preparation of this work, the authors used ChatGPT (OpenAI) to assist with
699 language editing. After using this tool, the authors reviewed and edited the content as needed
700 and take full responsibility for the content of the published article.

References

- Allaf, M.M., Peerhossaini, H., 2022. Cyanobacteria: Model microorganisms and beyond. *Microorganisms* 10, 696. <https://doi.org/10.3390/microorganisms10040696>
- Bai, L., Cao, C., Wang, C., Zhang, H., Deng, J., Jiang, H., 2019. Response of bloom-forming cyanobacterium *Microcystis aeruginosa* to 17 β -estradiol at different nitrogen levels. *Chemosphere* 219, 174–182. <https://doi.org/10.1016/j.chemosphere.2018.11.214>
- Bauersachs, T., Stal, L.J., Grego, M., Schwark, L., 2014. Temperature induced changes in the heterocyst glycolipid composition of N₂ fixing heterocystous cyanobacteria. *Org. Geochem.* 69, 98–105. <https://doi.org/10.1016/j.orggeochem.2014.02.006>
- Bouma-Gregson, K., Power, M.E., Bormans, M., 2017. Rise and fall of toxic benthic freshwater cyanobacteria (*Anabaena* spp.) in the Eel River: Buoyancy and dispersal. *Harmful Algae* 66, 79–87. <https://doi.org/10.1016/j.hal.2017.05.007>
- Brewer, P.G., Goldman, J.C., 1976. Alkalinity changes generated by phytoplankton growth. *Limnol. Oceanogr.* 21, 108–117. <https://doi.org/10.4319/lo.1976.21.1.0108>
- Buser, H.R., Poiger, T., Müller, M.D., 1998. Occurrence and fate of the pharmaceutical drug diclofenac in surface waters: Rapid photodegradation in a lake. *Environ. Sci. Technol.* 32, 3449–3456. <https://doi.org/10.1021/es980301x>
- Dadban Shahamat, Y., Zazouli, M.A., Zare, M.R., Mengelizadeh, N., 2019. Catalytic degradation of diclofenac from aqueous solutions using peroxymonosulfate activated by magnetic MWCNTs-CoFe₃O₄ nanoparticles. *RSC Adv.* 9, 16496–16508. <https://doi.org/10.1039/c9ra02757b>
- Facey, S.J., Nebel, B.A., Kontny, L., Allgaier, M., Hauer, B., 2018. Rapid and complete degradation of diclofenac by native soil microorganisms. *Environ. Technol. Innov.* 10, 55–61. <https://doi.org/10.1016/j.eti.2017.12.009>
- Flores, E., Herrero, A., 2005. Nitrogen assimilation and nitrogen control in cyanobacteria. *Biochem. Soc. Trans.* 33, 164–167. <https://doi.org/10.1042/BST0330164>
- Gackowska, A., Studziński, W., Kudlek, E., Przybyłek, M., 2025. Environmental fate and ecotoxicity of diclofenac degradation products generated by photo-assisted advanced oxidation processes. *J. Hazard. Mater.* 489, 137708. <https://doi.org/10.1016/j.jhazmat.2025.137708>
- Herrero, A., Muro-Pastor, A.M., Flores, E., 2001. Nitrogen control in cyanobacteria. *J. Bacteriol.* 183, 411–425. <https://doi.org/10.1128/JB.183.2.411-425.2001>
- Herrero, A., Flores, E., 2019. Genetic responses to carbon and nitrogen availability in *Anabaena*. *Environ. Microbiol.* <https://doi.org/10.1111/1462-2920.14370>
- Iovino, P., Chianese, S., Canzano, S., Prisciandaro, M., Musmarra, D., 2017. Photodegradation of diclofenac in wastewaters. *Desalination Water Treat.* 61, 293–297. <https://doi.org/10.5004/dwt.2016.11063>

738 Kropidłowska, K., Caban, M., 2023. Effect of salinity on the toxicity of diclofenac, ibuprofen
739 and naproxen toward cyanobacterium *Synechocystis salina*. Chemosphere 338, 139521.
740 <https://doi.org/doi.org/10.1016/j.chemosphere.2023.139521>

741 Kumar, K., Mella-Herrera, R.A., Golden, J.W., 2010a. Cyanobacterial heterocysts. Cold
742 Spring Harb. Perspect. Biol. 2. <https://doi.org/10.1101/cshperspect.a000315>

743 Kwidzińska, K., Caban, M., 2026. Extraction and separation of cyanobacterial photosynthetic
744 and photoprotective pigments: comparison of practical and accessible methodologies.
745 Eur. J. Phycol. 61, 322–338. <https://doi.org/10.1080/09670262.2026.2682186>

746 Kwidzińska, K., Zalewska, M., Aksmann, A., Kobos, J., Mazur-Marzec, H., Caban, M., 2024.
747 Multi-biomarker response of cyanobacteria *Synechocystis salina* and *Microcystis*
748 *aeruginosa* to diclofenac. J. Hazard. Mater. 471.
749 <https://doi.org/10.1016/j.jhazmat.2024.134373>

750 Lee, J., Ji, K., Lim Kho, Y., Kim, P., Choi, K., 2011. Chronic exposure to diclofenac on two
751 freshwater cladocerans and Japanese medaka. Ecotoxicol. Environ. Saf. 74, 1216–1225.
752 <https://doi.org/10.1016/j.ecoenv.2011.03.014>

753 Li, X., Li, W., Zhai, J., Wei, H., 2018. Effect of nitrogen limitation on biochemical
754 composition and photosynthetic performance for fed-batch mixotrophic cultivation of
755 microalga *Spirulina platensis*. Bioresour. Technol. 263, 555–561.
756 <https://doi.org/10.1016/j.biortech.2018.05.046>

757 Liu, Y., Wang, F., Chen, X., Zhang, J., Gao, B., 2015a. Influence of coexisting spiramycin
758 contaminant on the harm of *Microcystis aeruginosa* at different nitrogen levels. J.
759 Hazard. Mater. 285, 517–524. <https://doi.org/10.1016/j.jhazmat.2014.11.027>

760 Liu, Y., Wang, F., Chen, X., Zhang, J., Gao, B., 2015b. Cellular responses and biodegradation
761 of amoxicillin in *Microcystis aeruginosa* at different nitrogen levels. Ecotoxicol.
762 Environ. Saf. 111, 138–145. <https://doi.org/10.1016/j.ecoenv.2014.10.011>

763 Magnuson, A., Cardona, T., 2016. Thylakoid membrane function in heterocysts. Biochim.
764 Biophys. Acta Bioenerg. <https://doi.org/10.1016/j.bbabbio.2015.10.016>

765 Majewska, M., Harshkova, D., Pokora, W., Baścik-Remisiewicz, A., Tułodziecki, S.,
766 Aksmann, A., 2021. Does diclofenac act like a photosynthetic herbicide on green algae?
767 *Chlamydomonas reinhardtii* synchronous culture-based study with atrazine as reference.
768 Ecotoxicol. Environ. Saf. 208, 111630. <https://doi.org/10.1016/j.ecoenv.2020.111630>

769 Malatinszky, D., Steuer, R., Jones, P.R., 2017. A comprehensively curated genome-scale two-
770 cell model for the heterocystous cyanobacterium *Anabaena* sp. PCC 7120. Plant Physiol.
771 173, 509–523. <https://doi.org/10.1104/pp.16.01487>

772 Mazur-Marzec, H., Błaszczuk, A., Felczykowska, A., Hohlfeld, N., Kobos, J., Toruńska-
773 Sitarz, A., Devi, P., Montalvão, S., D'souza, L., Tammela, P., Mikosik, A., Bloch, S.,
774 Nejman-Faleńczyk, B., Węgrzyn, G., 2015. Baltic cyanobacteria – a source of
775 biologically active compounds. Eur. J. Phycol. 50, 343–360.
776 <https://doi.org/10.1080/09670262.2015.1062563>

777 Meeks, J.C., Wolk, C.P., Thomas, J., Lockau, W., Shaffer, P.W., Austin, S.M., Chien, W.S.,
778 Galonsky, A., 1977. The pathways of assimilation of $^{13}\text{NH}_4^+$ by the cyanobacterium,
779 *Anabaena cylindrica*. Journal of Biological Chemistry 252, 7894–7900.
780 [https://doi.org/10.1016/S0021-9258\(17\)41049-0](https://doi.org/10.1016/S0021-9258(17)41049-0)

781 Myint Sein, M., Zedda, M., Tuerk, J., Schmidt, T.C., Golloch, A., Von Sonntag, C., 2008.
782 Oxidation of Diclofenac with Ozone in Aqueous Solution 42, 6656–6662.
783 <https://doi.org/https://doi.org/10.1021/es8008612>

784 Najafinejad, M.S., Fenti, A., Sokhansanj, A., Falco, G., Iovino, P., Chianese, S., Musmarra,
785 D., 2026. Diclofenac degradation through electro-oxidation using a continuous reactor
786 equipped with meshed electrodes: removal efficiency, degradation pathways and reactor
787 modelling. J. Environ. Chem. Eng. 14, 121637.
788 <https://doi.org/10.1016/j.jece.2026.121637>

789 Nosek, K., Zhao, D., 2024. Transformation products of diclofenac: Formation, occurrence,
790 and toxicity implication in the aquatic environment. Water Res. 266, 122388.
791 <https://doi.org/10.1016/j.watres.2024.122388>

792 Nozue, S., Katayama, M., Terazima, M., Kumazaki, S., 2017. Comparative study of thylakoid
793 membranes in terminal heterocysts and vegetative cells from two cyanobacteria,
794 *Rivularia* M-261 and *Anabaena variabilis*, by fluorescence and absorption spectral
795 microscopy. Biochim. Biophys. Acta Bioenerg. 1858, 742–749.
796 <https://doi.org/10.1016/j.bbabbio.2017.05.007>

797 Nyholm, N., Källqvist, T., 1989. Methods for growth inhibition toxicity tests with freshwater
798 algae. Environ. Toxicol. Chem. 8, 689–703. <https://doi.org/10.1002/etc.5620080807>

799 Orus, M.I., Marco, E., Martinez, E., 1990. Effect of Trichlorfon on N_2 -Fixing
800 Cyanobacterium *Anabaena* PCC 7119. Arch. Environ. Contam. Toxicol 19, 297–301.
801 <https://doi.org/doi.org/10.1007/BF01054968>

802 Pagels, F., Vasconcelos, V., Guedes, A.C., 2021. Carotenoids from cyanobacteria:
803 Biotechnological potential and optimization strategies. Biomolecules 11, 735.
804 <https://doi.org/10.3390/biom11050735>

805 Prasad, S.M., Zeeshan, M., Kumar, D., 2011. Toxicity of endosulfan on growth,
806 photosynthesis, and nitrogenase activity in two species of Nostoc (*Nostoc muscorum* and
807 *Nostoc calcicola*). Toxicol. Environ. Chem. 93, 513–525.
808 <https://doi.org/10.1080/02772248.2010.542157>

809 Rebelo, D., Antunes, S.C., Rodrigues, S., 2023. The Silent Threat: Exploring the Ecological
810 and Ecotoxicological Impacts of Chlorinated Aniline Derivatives and the Metabolites on
811 the Aquatic Ecosystem. J. Xenobiot. 13, 604–614. <https://doi.org/10.3390/jox13040038>

812 Rippka, R., Deruelles, J., Waterbury, J.B., Herdman, M., Stanier, R.Y., 1979. Generic
813 Assignments, Strain Histories and Properties of Pure Cultures of Cyanobacteria. Journal
814 of General Microbiol 111, 1–61. <https://doi.org/doi.org/10.1099/00221287-111-1-1>

815 Sánchez-Sandoval, D.S., González-Ortega, O., Vazquez-Martínez, J., García de la Cruz, R.F.,
816 Soria-Guerra, R.E., 2022. Diclofenac removal by the microalgae species *Chlorella*

817 *vulgaris*, *Nannochloropsis oculata*, *Scenedesmus acutus*, and *Scenedesmus obliquus*. 3
818 Biotech 12, 210. <https://doi.org/10.1007/s13205-022-03268-2>

819 Srivastava, A.K., Rai, A.N., Neilan, B.A. (Eds.), 2013. Stress biology of cyanobacteria:
820 molecular mechanisms to cellular responses. CRC Press, Boca Raton.

821 Stirbet, A., Govindjee, 2011. On the relation between the Kautsky effect (chlorophyll a
822 fluorescence induction) and Photosystem II: Basics and applications of the OJIP
823 fluorescence transient. J. Photochem. Photobiol. B 104, 236–257.
824 <https://doi.org/10.1016/j.jphotobiol.2010.12.010>

825 Subhashini, D., Kaushik, B.D., 1981. Amelioration of sodic soils with blue-green algae. Aust.
826 J. Soil Res. 19, 361–367. <https://doi.org/doi.org/10.1071/SR9810361>

827 Świacka, K., Michnowska, A., Maculewicz, J., Caban, M., Smolarz, K., 2021. Toxic effects
828 of NSAIDs in non-target species: A review from the perspective of the aquatic
829 environment. Environmental Pollution 273, 115891.
830 <https://doi.org/10.1016/j.envpol.2020.115891>

831 Takaichi, S., Mochimaru, M., 2007. Carotenoids and carotenogenesis in cyanobacteria:
832 Unique ketocarotenoids and carotenoid glycosides. Cellular and Molecular Life Sciences
833 64, 2607–2619. <https://doi.org/10.1007/s00018-007-7190-z>

834 Takaichi, S., Mochimaru, M., Maoka, T., Katoh, H., 2005. Myxol and 4-ketomyxol 2'-
835 fucosides, not rhamnosides, from *Anabaena* sp. PCC 7120 and *Nostoc punctiforme* PCC
836 73102, and proposal for the biosynthetic pathway of carotenoids. Plant Cell Physiol. 46,
837 497–504. <https://doi.org/10.1093/pcp/pci049>

838 Wolk, C.P., Thomas, J., Shaffer, P.W., Austin, S.M., Galonsky, A., 1976. Pathway of nitrogen
839 metabolism after fixation of ¹³N-labeled nitrogen gas by the cyanobacterium, *Anabaena*
840 *cylindrica*. Journal of Biological Chemistry 251, 5027–5034.
841 [https://doi.org/https://doi.org/10.1016/S0021-9258\(17\)33216-7](https://doi.org/https://doi.org/10.1016/S0021-9258(17)33216-7)

842 Yadav, P., Singh, R.P., Rana, S., Joshi, D., Kumar, D., Bhardwaj, N., Gupta, R.K., Kumar,
843 A., 2022. Mechanisms of Stress Tolerance in Cyanobacteria under Extreme Conditions.
844 Stresses 2, 531–549. <https://doi.org/10.3390/stresses2040036>

845 Zakar, T., Laczko-Dobos, H., Toth, T.N., Gombos, Z., 2016. Carotenoids assist in
846 cyanobacterial photosystem II assembly and function. Front. Plant Sci. 7, 295.
847 <https://doi.org/10.3389/fpls.2016.00295>

848 Zhang, J., Zhu, S., Xu, X., Chai, Y., Shao, J., Lu, G., Zeng, J., Bai, M., Chen, A., 2026. The
849 ecotoxicological effects of thiamethoxam on photosynthesis and nitrogen fixation in
850 cyanobacteria: From inhibition to recovery. J. Environ. Sci. (China) 162, 676–686.
851 <https://doi.org/10.1016/j.jes.2025.05.077>

852 Ziylan, A., Dogan, S., Agopcan, S., Kidak, R., Aviyente, V., Ince, N.H., 2014. Sonochemical
853 degradation of diclofenac: Byproduct assessment, reaction mechanisms and
854 environmental considerations. Environmental Science and Pollution Research 21, 5929–
855 5939. <https://doi.org/10.1007/s11356-014-2514-7>

856

857

858 **Figure captions**

859 **Figure 1.** Microscopic (x1000) picture of *Anabaena cylindrica* cultured in standard Z8 medium (A) and
860 cultured in Z8 medium without nitrates (heterocysts marked by black arrows, cyanophycin granules
861 marked by blue arrows).

862 **Figure 2.** Average specific growth rate of *A. cylindrica* under nitrate-replete and nitrate-depleted
863 conditions with and without DCF exposure. Data represent mean $\pm$ SD (n = 3, independent experiments).

864 **Figure 3.** Relative growth (DCF/Control ratio (%)) for *A. cylindrica* cultures exposed to nominal 100
865 mg L⁻¹ diclofenac in standard Z8 medium and Z8₀. Data represent mean $\pm$ SD (n=3, independent
866 experiments). Asterisks above individual time points indicate statistically significant differences
867 between Z8 and Z8₀ treatments (*p < 0.05, Student's t-test)

868 **Figure 4.** Final pH values of *A. cylindrica* cultures and abiotic controls after 216 h of exposure
869 in standard Z8 and medium without nitrates - Z8₀ media. Values are presented as mean $\pm$ SD
870 (n = 3, independent experiments).

871 **Figure 5.** Diclofenac (DCF) concentration in *Anabaena cylindrica* cultures and corresponding
872 abiotic controls grown in Z8 and Z8₀ media, measured every 24 h throughout the exposure
873 period. Values represent the mean $\pm$ SD (n = 3, independent experiments).

874 **Figure 6.** Representative HPLC-DAD chromatograms of culture medium collected from *A. cylindrica*
875 cultures exposed to diclofenac (DCF, RT 12 min) in Z8 and Z8₀ media after 24 and 216 h.
876 Chromatograms of the corresponding abiotic controls (24 and 216 h) and biotic controls (216 h) are
877 shown for comparison.

878 **Figure 7.** The SIM (selected ions monitoring) chromatograms of Z8₀ growth medium: A. spiked with
879 analytical standard of 2,6-dichloroaniline (2,6-DCA), B. with *A. cylindrica* culture exposed to
880 diclofenac at nominal 100 mg L⁻¹, C. with *A. cylindrica* culture (biotic control, not exposed to
881 diclofenac), and D. abiotic control spiked with diclofenac at 100 mg L⁻¹ (without *A. cylindrica*)

Figure 8. Effect of nitrate deficiency on the relative carotenoid and pheophytin composition of *A. cylindrica*. Values are expressed as the ratio of pigment to chlorophyll *a* content in biotic controls grown in standard medium Z8 and medium with nitrate omission - Z8₀ (data are presented as mean $\pm$ SD (n = 3, independent experiments)).

Figure 9. Principal Component Analysis (PCA) and pigment response of cyanobacteria to diclofenac (DCF) treatment in Z8 and Z8₀ growth media. (A) PCA biplot showing the separation of cyanobacterial pigment profiles based on growth medium (Z8 vs. Z8₀) and exposure to diclofenac (Control vs. DCF). Red vectors represent individual pigment loadings. (B) Relative changes in key stress-responsive pigments (4-ketomyxoxanthophyll, pheophytin, and cantaxanthin) expressed as a percentage ratio to chlorophyll *a* [%]. Values represent mean $\pm$ SD (n = 3, independent experiments).

Figure 10. OJIP test parameters for DCF exposed cultures of *A. cylindrica* presented as the mean of 2 independent experiments and expressed as a percentage of the control. A - culture exposed to DCF grown in Z8 medium; B - culture exposed to DCF and grown in Z8₀ medium. Asterisks indicate significant differences at $\alpha = 0.05$.

Figure 11. Carotenoid biosynthetic pathway in cyanobacteria. Carotenoids analyzed in the present study are highlighted with red boxes. The carotenoids (3R,2'S)-myxol 2'-fucoside (4) and (3S,2'S)-4-ketomyxol 2'-fucoside (2) are referred to throughout this study as myxoxanthophyll and 4-ketomyxoxanthophyll, respectively. Modified from (Takaichi and Mochimaru, 2007).

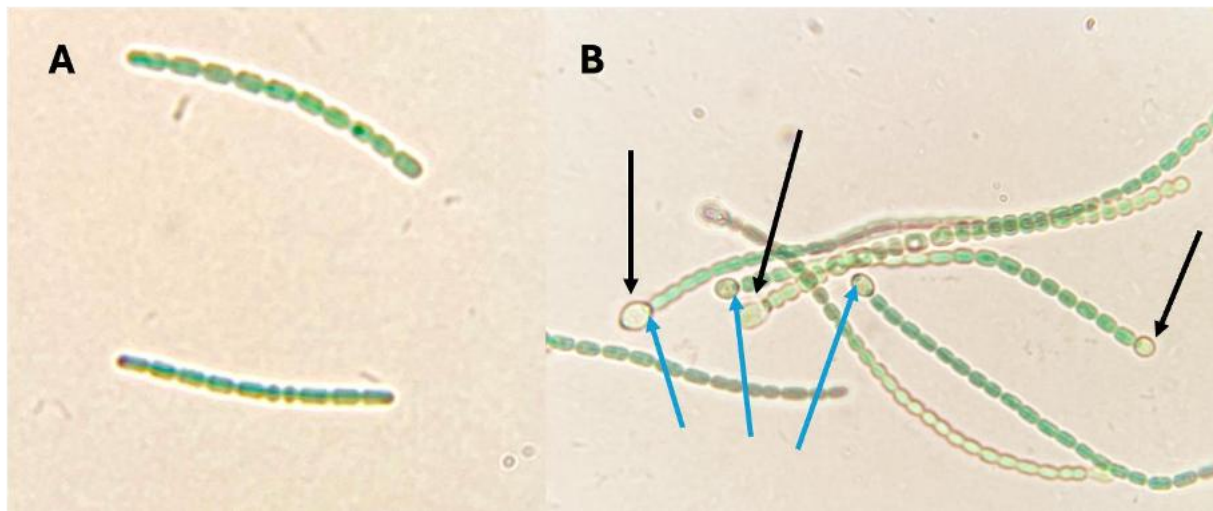


Figure 2

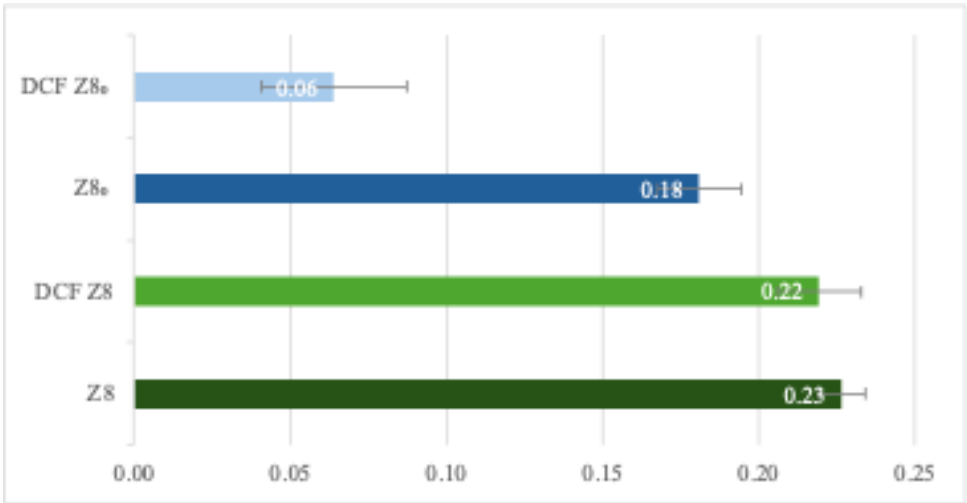
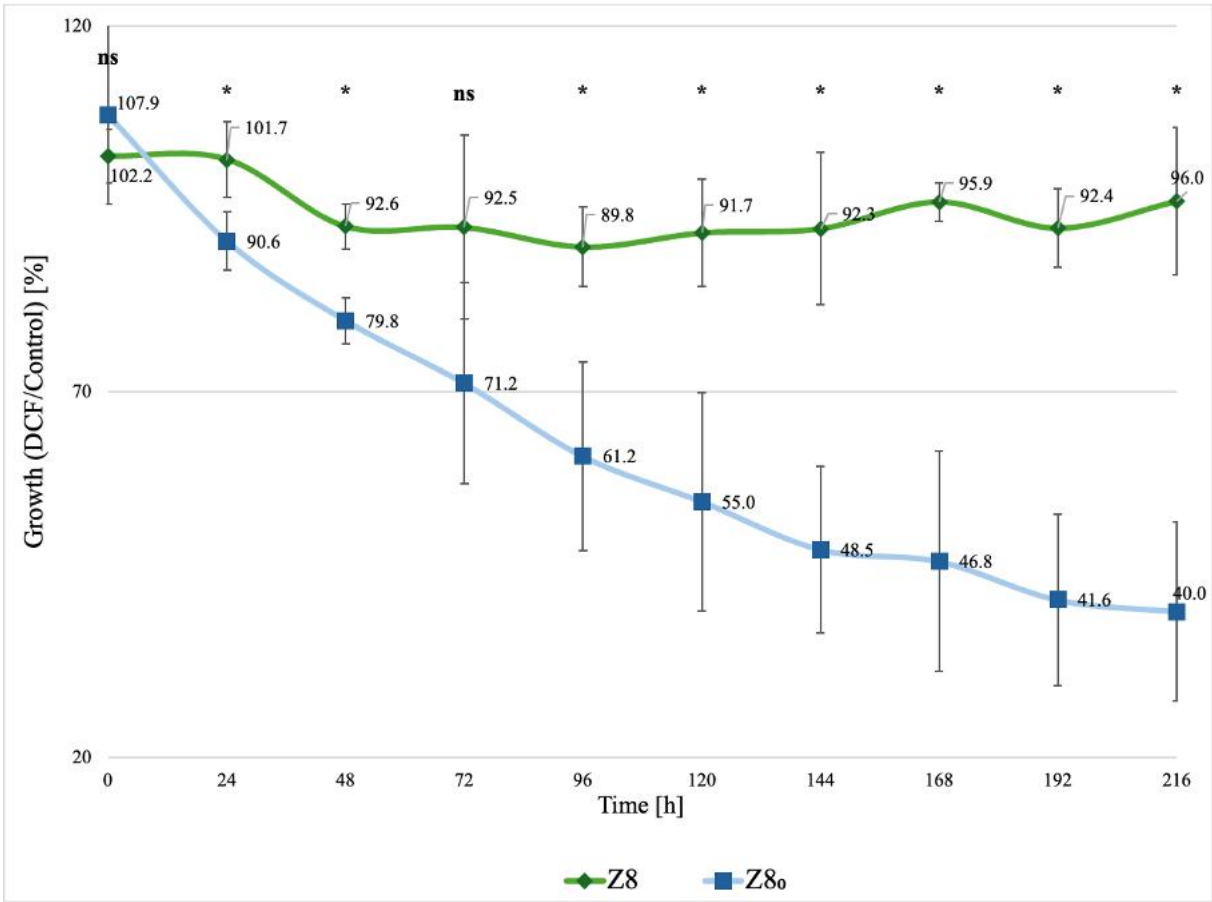


Figure 3



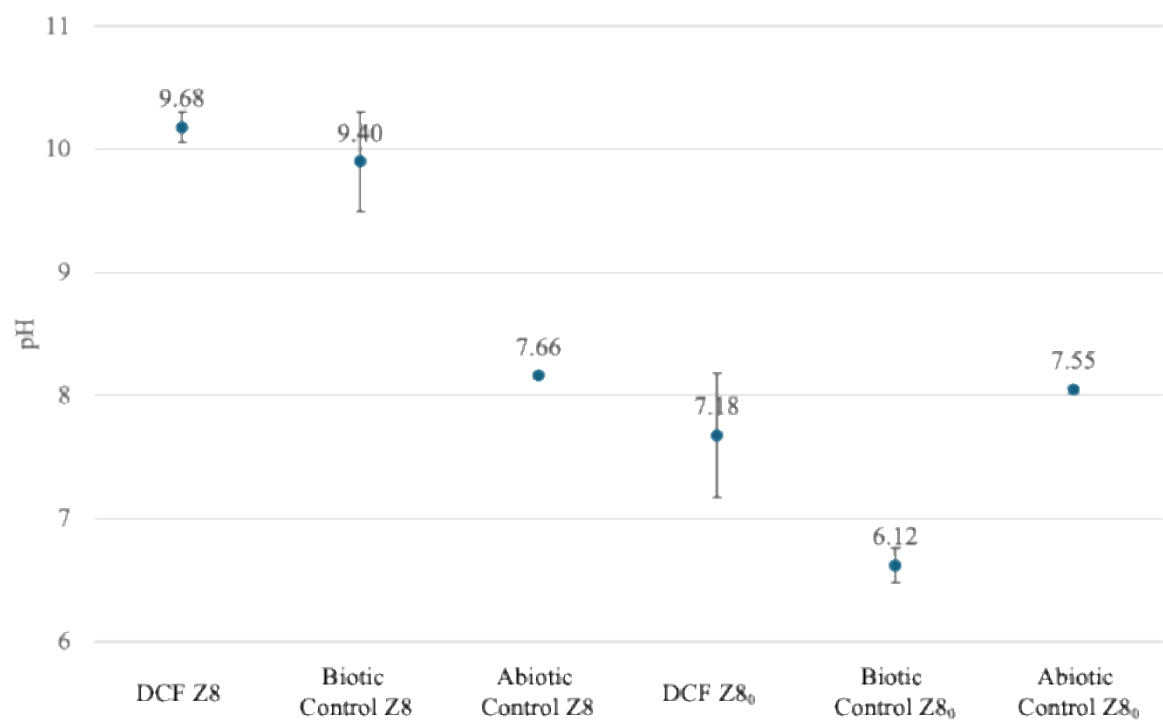
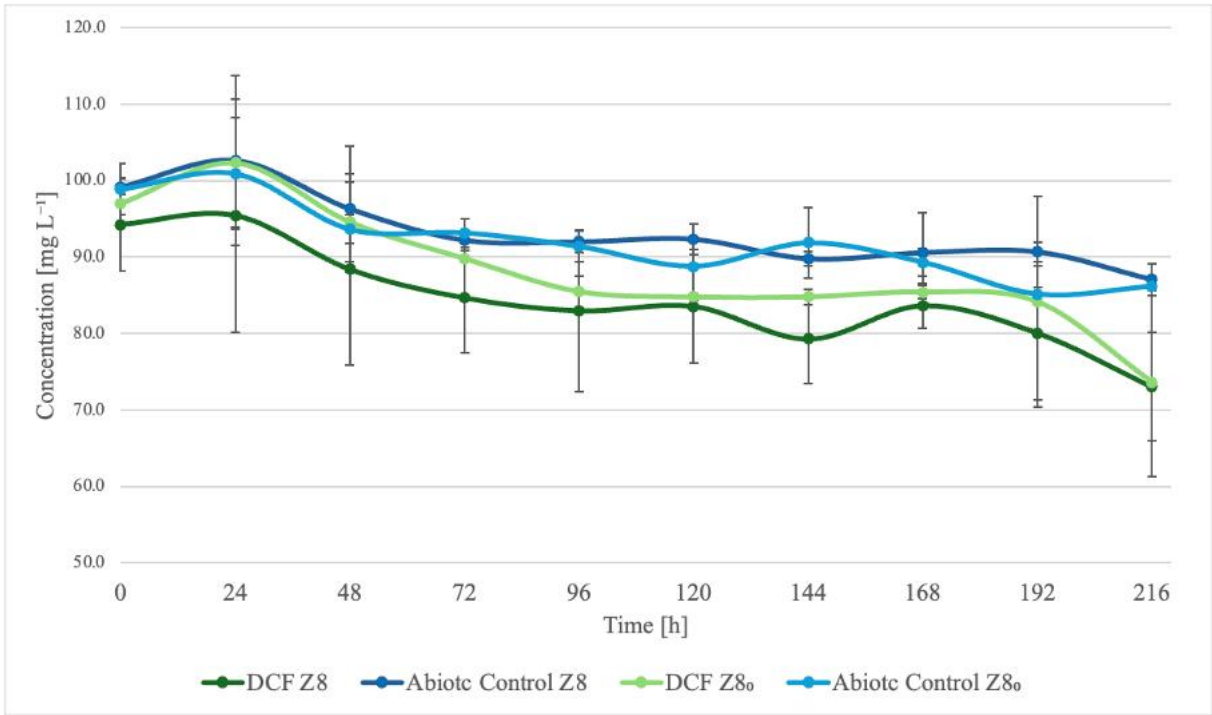
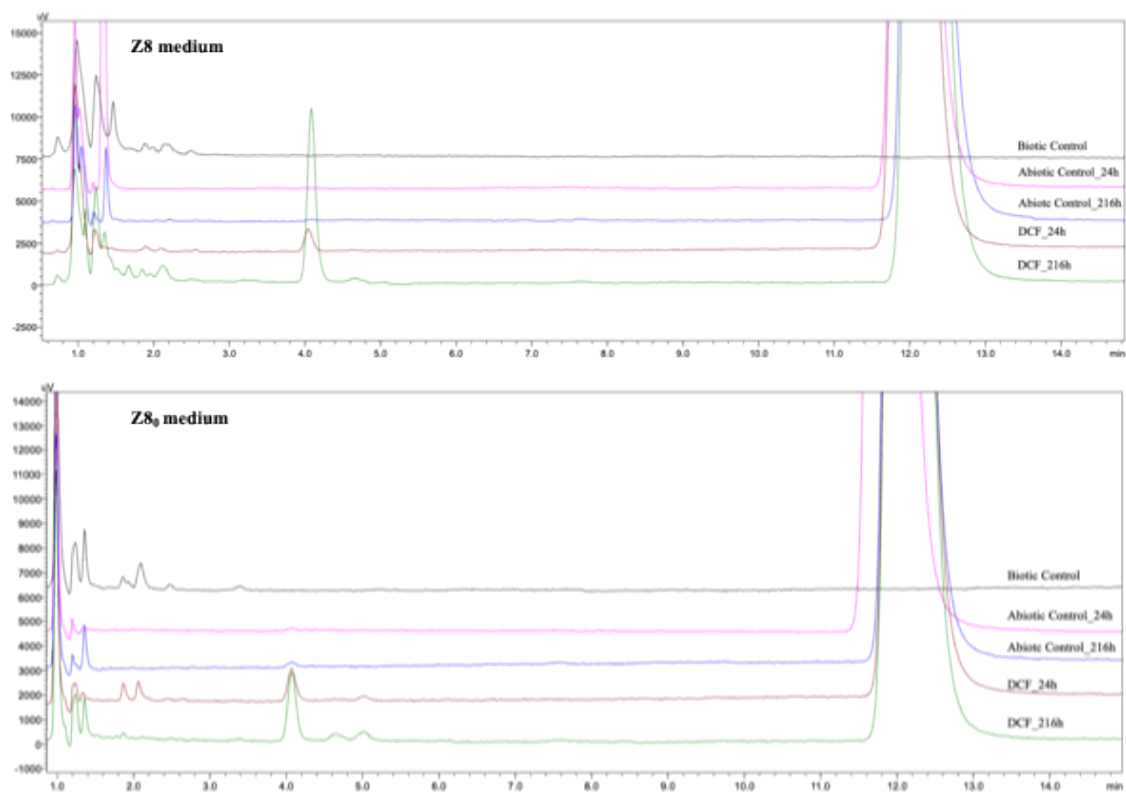


Figure 5





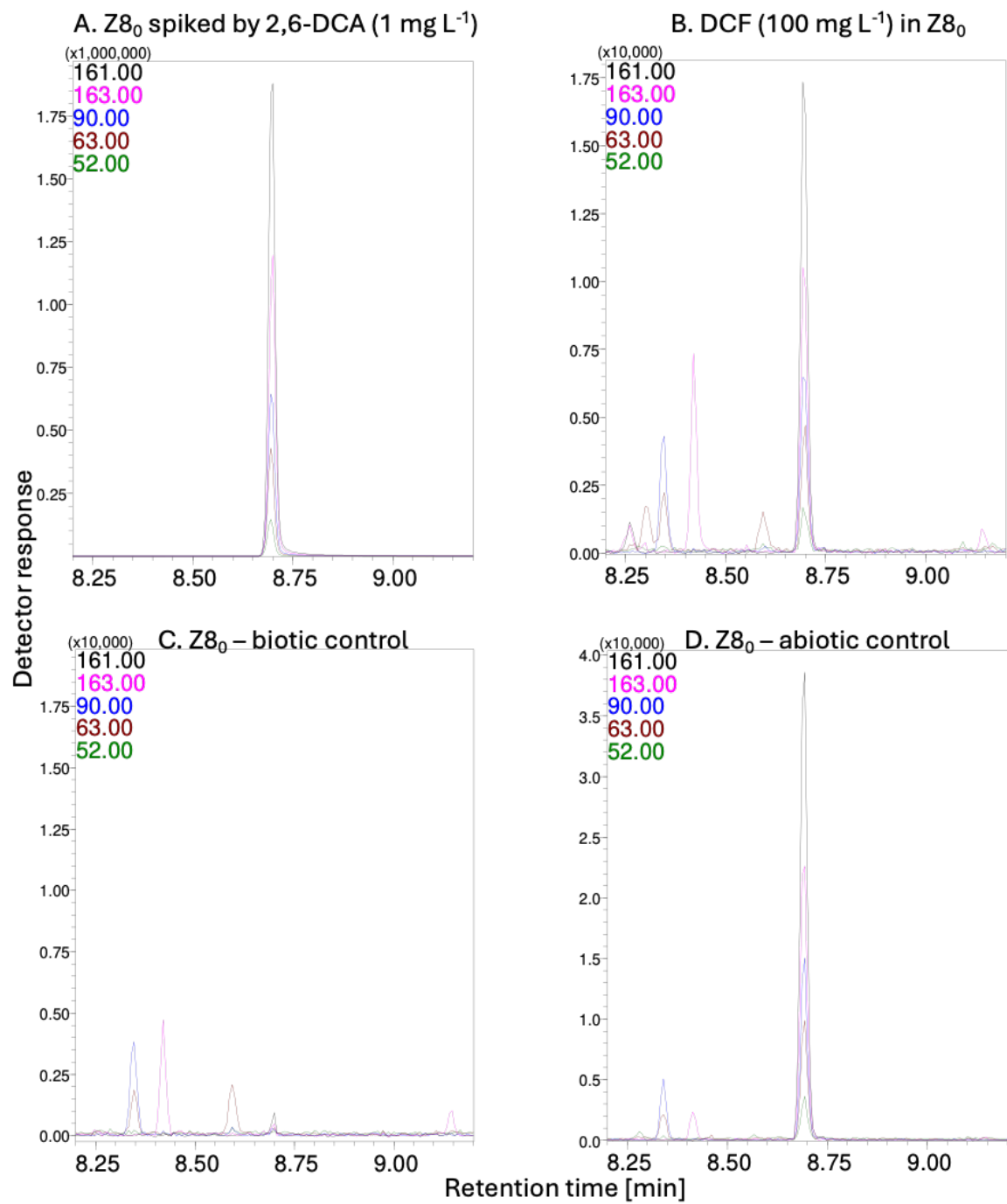
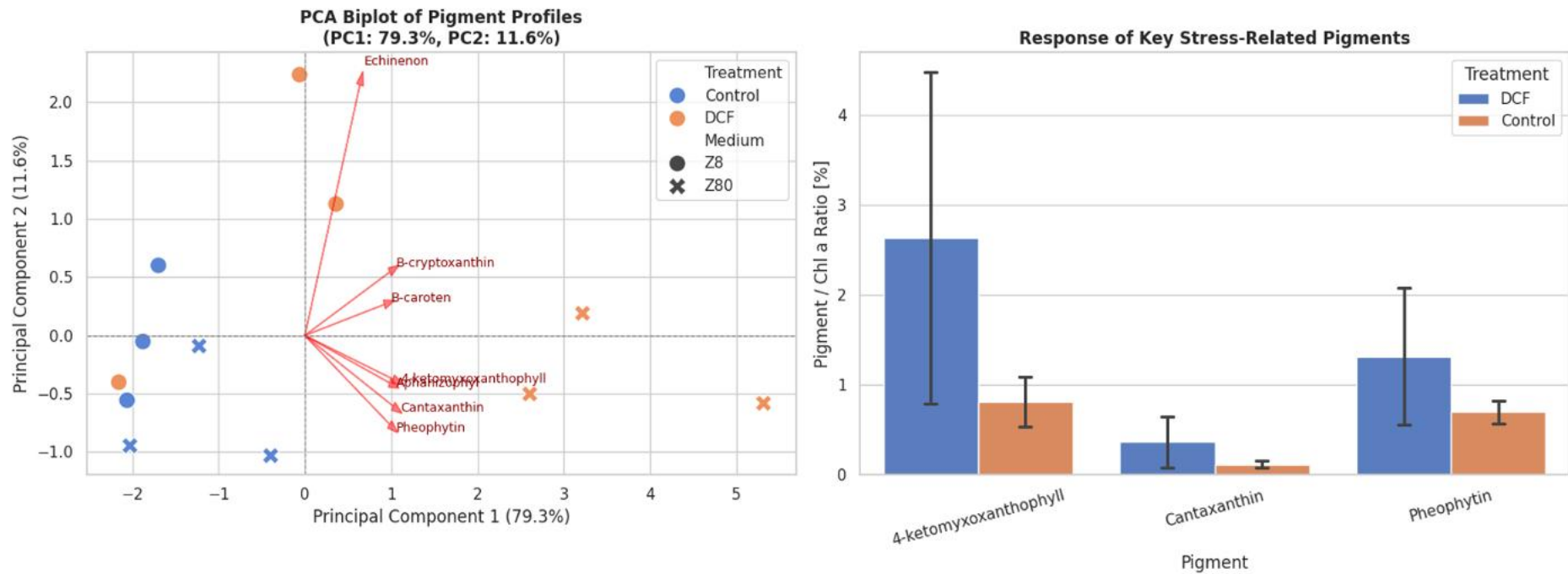
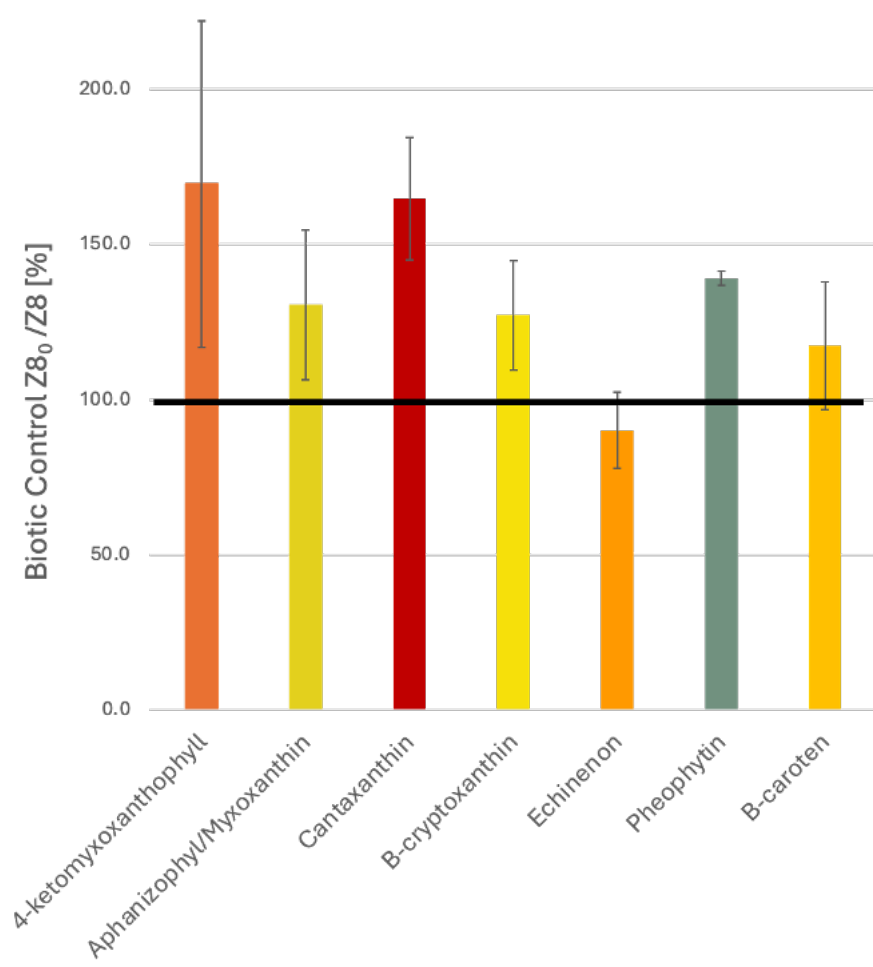
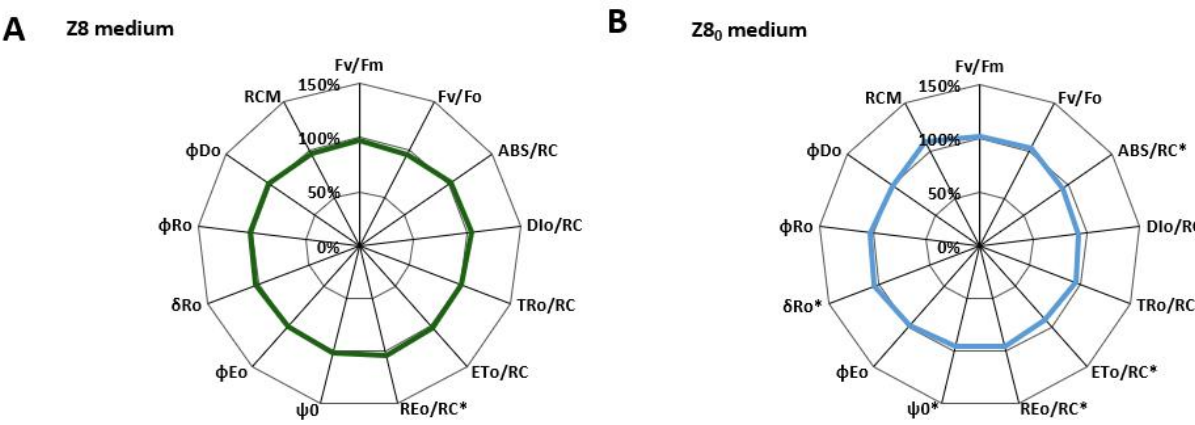


Figure 9



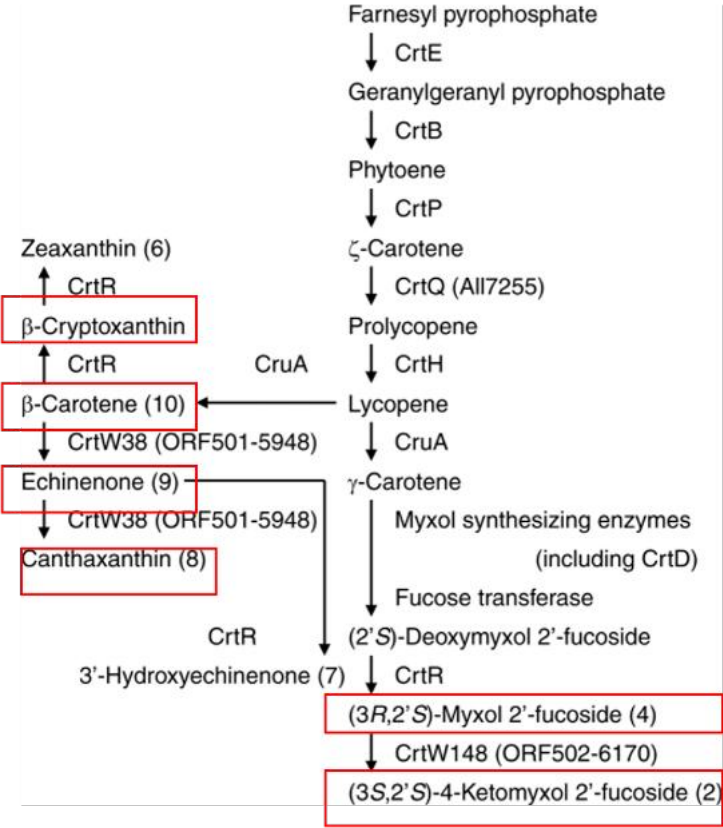




B

Z8₀ medium

Parameter	Value (%)
Fv/Fm	100
Fv/Fo	100
ABS/RC*	100
DIo/RC	100
TRo/RC*	100
ETIo/RC*	100
REo/RC*	100
ψ0*	100
φEo	100
δRo*	100
φRo	100



Supplementary Information to

Nitrogen availability determines diclofenac toxicity in the diazotrophic cyanobacterium

Anabaena cylindrica

Klaudia Kwidzińska^{1*}, Martyna Zalewska², Anna Aksmann², Magda Caban¹

¹*University of Gdansk, Faculty of Chemistry, Department of Environmental Analysis, ul. Wita Stwosza 63, 80-308 Gdańsk, Poland*

²*University of Gdansk, Faculty of Biology, Department of Plant Experimental Biology and Biotechnology, ul. Wita Stwosza 59, 80-308 Gdańsk, Poland*

***Corresponding author:** Phone (+48 58) 523 5208; E-mail address:
klaudia.kwidzinska@ug.edu.pl

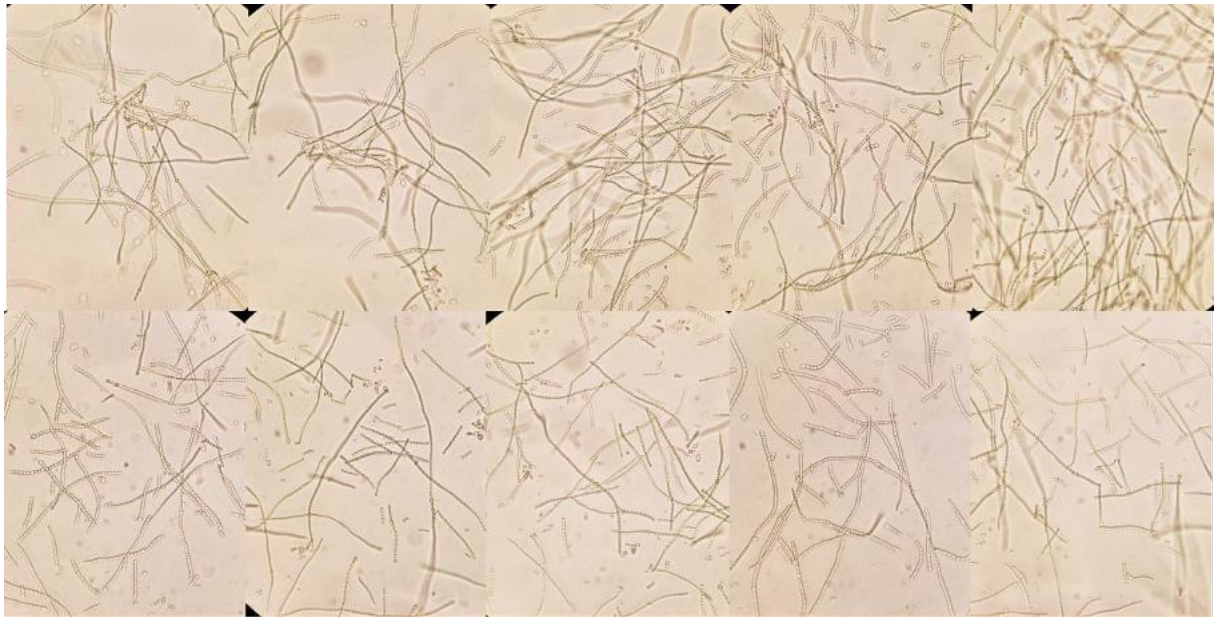


Figure S1. The microscopic photos (x400) of A1-5 culture of *A. cylindrica* in Z80 growth medium and B1-5 culture of *A. cylindrica* in Z80 growth medium exposed to diclofenac 100 mg L⁻¹.

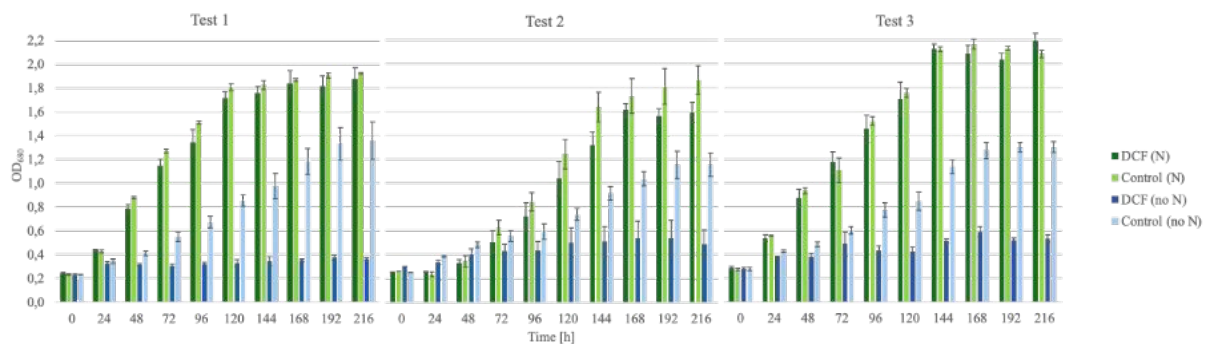


Figure S2. Growth of *A. cylindrica* exposed to 100 mg L⁻¹ diclofenac (DCF) in standard Z8 medium (N) and Z8₀ medium (without nitrates), expressed as optical density at 680 nm (OD₆₈₀). Representative results from independent experiments (test 1 to 3). Values are presented as mean ± SD (n = 5 biological replicates for each of independent experiments 1 to 3).

Table S1. Photosynthetic pigment content in a 1 mL culture sample collected at the end of the test (n = 3, independent experiments)

Sample	Control Z8		DCF Z8		Control Z8 ₀		DCF Z8 ₀	
Pigment	Concentration [mg L ⁻¹]	SD	Concentration [mg L ⁻¹]	SD	Concentration [mg L ⁻¹]	SD	Concentration [mg L ⁻¹]	SD
4-ketomyxoxanthophyll	0.095	0.03	0.110	0.04	0.066	0.01	0.085	0.05
Aphanizophyll/Myxoxanthin	0.810	0.03	0.785	0.09	0.539	0.04	0.253	0.13
Cantaxanthin	0.018	0.01	0.015	0.00	0.011	0.00	0.011	0.00
B-cryptoxanthin	0.024	0.01	0.033	0.01	0.021	0.00	0.009	0.00
Chlorophyll a	21.800	13.81	11.790	3.03	7.844	1.93	2.038	1.07
Echinenon	1.280	0.59	0.847	0.12	0.471	0.16	0.151	0.08
Pheophytin	0.075	0.02	0.081	0.02	0.063	0.02	0.035	0.01
B-caroten	2.845	1.18	3.327	0.32	2.125	0.28	0.780	0.42

Table S2. Selected pigment concentrations (mean $\pm$ SD) expressed as pigment to chlorophyll *a* ratio [%]

Pigment	Z8 Control	Z8 + DCF	Z80 Control	Z80 + DCF
4-ketomyxoxanthophyll	0.73 $\pm$ 0.23	1.03 $\pm$ 0.61	0.90 $\pm$ 0.35	4.23 $\pm$ 0.64
Aphanizophyll / Myxoxanthin	6.83 $\pm$ 1.62	7.07 $\pm$ 2.53	7.17 $\pm$ 1.42	12.67 $\pm$ 1.85
Canthaxanthin	0.10 $\pm$ 0.00	0.13 $\pm$ 0.06	0.13 $\pm$ 0.06	0.60 $\pm$ 0.17
b-cryptoxanthin	0.20 $\pm$ 0.00	0.30 $\pm$ 0.10	0.27 $\pm$ 0.06	0.43 $\pm$ 0.06
Echinenone	6.67 $\pm$ 0.38	7.37 $\pm$ 1.04	6.00 $\pm$ 0.35	7.43 $\pm$ 0.29
Pheophytin	0.60 $\pm$ 0.00	0.70 $\pm$ 0.00	0.80 $\pm$ 0.10	1.93 $\pm$ 0.55
b-carotene	21.13 $\pm$ 7.41	29.07 $\pm$ 4.47	28.00 $\pm$ 4.28	38.57 $\pm$ 3.79

Table S3. PERMANOVA results for global pigment profile shifts (Euclidean distance, 999 permutations). *Legend:* Pseudo-F statistics and raw *p*-values assessing the overall multivariate response of cyanobacterial pigment composition to diclofenac treatment (DCF), growth medium (Z8 vs. Z8₀), and their combined interaction ($\alpha = 0.05$).

Source of Variation	Pseudo- <i>F</i>	Raw <i>p</i> -value	Conclusion ($\alpha=0.05$)
Treatment (DCF)	5.620	0.0170	Significant DCF effect
Medium	3.599	0.0580	Not significant
Interaction / Combined Groups	8.705	0.0040	Significant interaction

74 **Table S4.** OJIP test parameters expressed as mean $\pm$ standard deviation (SD). Asterisks indicate statistically significant differences at $\alpha = 0.05$
75 (Welch's t-test and Mann-Whitney U test).

Z8 medium													
	F_V/F_M	F_V/F_0	ABS/R C	DI ₀ /RC	TR ₀ /RC	ET ₀ /RC	RE ₀ /RC	ψ_0	ϕE_0	δR_0	ϕR_0	ϕD_0	RC _M
Control	0.491 $\pm$ 0.049	0.98 $\pm$ 0.19	4.93 $\pm$ 0.61	2.54 $\pm$ 0.56	2.391 $\pm$ 0.054	0.821 $\pm$ 0.080	0.617 $\pm$ 0.017	0.344 $\pm$ 0.040	0.171 $\pm$ 0.036	0.757 $\pm$ 0.067	0.127 $\pm$ 0.017	0.509 $\pm$ 0.049	230 $\pm$ 48
DCF	0.480 $\pm$ 0.048	0.94 $\pm$ 0.18	5.068 $\pm$ 0.666	2.66 $\pm$ 0.61	2.404 $\pm$ 0.061	0.84 $\pm$ 0.11	0.649* $\pm$ 0.048	0.351 $\pm$ 0.053	0.171 $\pm$ 0.041	0.781 $\pm$ 0.088	0.130 $\pm$ 0.020	0.520 $\pm$ 0.048	221 $\pm$ 40
Z8 ₀ medium													
	F_V/F_M	F_V/F_0	ABS/R C	DI ₀ /RC	TR ₀ /RC	ET ₀ /RC	RE ₀ /RC	ψ_0	ϕE_0	δR_0	ϕR_0	ϕD_0	RC _M
Control	0.480 $\pm$ 0.025	0.93 $\pm$ 0.09	5.06 $\pm$ 0.35	2.64 $\pm$ 0.30	2.421 $\pm$ 0.050	0.99 $\pm$ 0.08	0.852 $\pm$ 0.042	0.410 $\pm$ 0.028	0.197 $\pm$ 0.016	0.859 $\pm$ 0.032	0.169 $\pm$ 0.012	0.520 $\pm$ 0.025	221 $\pm$ 28
DCF	0.488 $\pm$ 0.032	0.96 $\pm$ 0.13	4.74* $\pm$ 0.38	2.44 $\pm$ 0.34	2.301* $\pm$ 0.052	0.908* $\pm$ 0.026	0.821* $\pm$ 0.022	0.395* $\pm$ 0.014	0.193 $\pm$ 0.018	0.904* $\pm$ 0.014	0.174 $\pm$ 0.014	0.512 $\pm$ 0.032	241 $\pm$ 39