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Review of the doctoral dissertation of M.Sc. Zofia Konarzewska titled:

"Allelopathy in different phenotypes of the picoplanktonic cyanobacteria Synechococcus sp.: A key feature determining the structure of phytoplankton communities in the Baltic Sea"

Completed under the supervision of dr Aldo Barreiro Felpeto, Blue Biotechnology, Environment and Health Research Group, Interdisciplinary Centre of Marine and Environmental Research (CIIMAR), University of Porto, and the auxiliary supervisor, dr hab. Sylwia Śliwińska-Wilczewska, Department of Marine Ecosystems Functioning Faculty of Oceanography and Geography University of Gdańsk.

One of the fundamental questions in plankton ecology is how so many species can coexist in a relatively homogeneous environment with a limited variety of resources, and not follow the principle of competitive exclusion. This phenomenon, known as the paradox of the plankton, was first addressed by G. E. Hutchinson in 1961. Since the 1960s, aquatic ecologists have proposed dozens of solutions to this paradox, drawing on constantly changing environmental conditions and their gradients, as well as concepts such as game theory, chaos theory, and stochastic processes. One of the factors that was corroborated in these solutions more recently is allelopathy. However, allelopathy began to be studied in terrestrial environments and the context of plant relationships. Studies on allelopathy in the aquatic environment have a relatively short history, initially linked to research on macrophytes exerting allelopathic effects on phytoplankton. The production of allelopathic substances by algae and cyanobacteria, as well as their allelopathic effects, began to be studied in greater depth in the 21st century.

Cyanobacteria produce hundreds of secondary metabolites, but only a small fraction can be classified as allelochemicals. Among them are cyclic and non-cyclic peptides, polyketides, alkaloids, phenols, and chlorinated aromatic compounds. Still, the allelopathy among phytoplankton remains understudied on the community level. One of the organisms that exert allelopathic effects on other phytoplankton taxa is picocyanobacteria, with the most common genus in freshwaters and brackish environments being *Synechococcus*. However, studies of these organisms are challenging due to their extremely small size.

M.Sc. Zofia Konarzewska, the author of the PhD dissertation presented for review, became involved in *Synechococcus* research as early as 2019. She is the coauthor of four publications between 2019 and 2021, and the first author of five publications that comprise her doctoral dissertation. Three of those publications have already been published in reputable international journals with an IF between 2.6 and 4.9, and two others are awaiting reviews.

The presented PhD thesis aimed to investigate the role of *Synechococcus* allelopathy in the structuring of the phytoplankton community in the Baltic Sea. The PhD thesis consists of an introduction that includes an abstract, justifications for the studies, hypotheses and aims of the studies, and the methodology used in the experimental and fieldwork. The next part of the dissertation comprises published papers and manuscripts submitted for review, while the final part involves verification of the stated hypotheses and their implications for the diversity and functioning of phytoplankton in the Baltic Sea.

In her research, M.Sc. Zofia Konarzewska stated three hypotheses:

H1 Abiotic factors that promote the growth of *Synechococcus* phenotypes enhance their allelopathic activity.

H2 Allelopathy exhibited by different *Synechococcus* phenotypes affects co-occurring phytoplankton species. The allelopathic effect varies depending on the *Synechococcus* phenotype and the target phytoplankton species.

H3 The strength of allelopathy from *Synechococcus* phenotypes influences plankton community diversity: Low allelopathic strength is associated with lower diversity due to dominance by the strongest competitors. Intermediate allelopathic intensity increases community diversity through coexistence between allelopathic weak competitors and sensitive, stronger competitors. High allelopathic intensity reduces diversity due to dominance by *Synechococcus* phenotypes.

To verify these hypotheses, Ms. Zofia Konarzewska established four research goals and conducted various laboratory experiments on different scales, utilizing diverse methodologies, as well as ecological modeling and field studies. The thesis is well thought out, starting with an examination of intra-genus interactions, an analysis of the relationship between different *Synechococcus* phenotypes, and the influence of distinct phenotypes in short-term allelopathic assays using cell-free filtrate additions from three picoplankton cultures. In the second publication, the author examined the impact of various abiotic factors on the growth and allelopathic inhibition of the studied phenotypes of *Synechococcus*. The third publication tested inter-genera interactions and the allelopathic effects of *Synechococcus* on other cyanobacteria and eukaryotic algae, while the fourth and fifth publications focused on community-level interactions.

Publication 1 revealed that abiotic factors, light, temperature, and, to some extent, salinity, significantly influence the allelopathic interaction between the studied phenotypes of picocyanobacteria. Publication 2 – The paper revealed the impact of nutrient concentrations, specifically nitrate (NO_3^-) and phosphate (PO_4^{3-}), on the interactions among the studied phenotypes of Synechococcus. The paper also demonstrated the production of microcystin MC-LR by the three examined phenotypes of picocyanobacteria. In this paper, the graphical experimental setting worked exceptionally well, making the experiments easy to follow. The results presented in publication 3 revealed the allelopathic effect of three studied phenotypes of *Synechococcus* on 18 species of cyanobacteria, green algae, and diatoms, demonstrating that the green algae were the most susceptible to allelochemicals. The GH-MS analysis revealed in turn the presence of allelochemicals that had not been detected before in Synechococcus strains. In phenotype 1, the most abundant compound was eicosane, 10-methyl as, while in phenotype 2, the oxime-methoxy-phenyl dominated the compounds. In phenotype 3, the silanediol dimethyl, was the most abundant. These compounds have been previously shown to exhibit antibacterial, antifungal, and antialgal properties, with silanediol dimethyl, a member of a new zinc-binding group, also participating in the induction of apoptosis.

The results of publication 4 demonstrated that allelopathy exerted by Synechococcus induces oscillatory coexistence in marine phytoplankton. Long-term experimental and modeling results with two sets of four co-culturing phytoplankton species demonstrated the oscillatory coexistence of the studied species without competitive extinction, as expected based on classic competition experiments, such as those proposed by Tilman (1977). Publication 5 investigated the structuring effect of various allelopathic impacts generated by *Synechococcus*, ranging from low to high strengths, on the phytoplankton community composition, abundance, and diversity in continuous cultures under nitrate limitation.

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M.Sc. Zofia Konarzewska verified positively most of the hypotheses and included interpretations and implications of these hypotheses in a chapter summarizing the results of the doctoral thesis. Based on the results of the 1st and 2nd publications, the author positively verified H1 in the parts regarding the influence of temperature, light, and, to some extent, nutrient availability and limitations. However, the part that tested salinity as one of the abiotic factors influencing allelopathy was not fully supported by the results. The author stated that the salinity, in a broader range, should be further analyzed before the results are precise.

Hypothesis 2 was tested in publication 3. In this study, the author explored the impact of the cell-free filtrate of three different phenotypes of *Synechococcus* on co-occurring phytoplankton taxa. The author demonstrated that Type 1 *Synechococcus* had the strongest though variable influence, mainly causing inhibition of the studied parameters, but also in a few cases stimulation. Strain from Type 2 had mostly negative influences or no effect, with only one case of a positive impact. In contrast, the third strain, representing Type 3, exhibited the lowest impact, mostly negative, with positive effects only in the case of cyanobacteria species. From the examined phytoplankton taxa, green algae were the most susceptible to the allelopathic effects, while diatoms were less susceptible, but the effect differed with the *Synechococcus* type. Based on these results, the authors drew meaningful conclusions regarding the relationships between picocyanobacteria and diatoms, as well as the possible limitations of spring diatom blooms, particularly during warm springs.

The results of publications 4 and 5 allowed the author to verify the third hypothesis positively. The authors revealed that the allelopathic effects of *Synechococcus* on examined phytoplankton taxa resulted in oscillatory coexistence of studied taxa, with no exclusion of species that was the worst competitor for resources, contrary to the classic Tillman (1977) experiment. A model simulation further verified this observation. In the following paper, the authors investigated the various strengths of allelopathy exerted by *Synechococcus* on the diversity of coexisting taxa. Similarly, to the intermediate disturbance hypothesis, the author demonstrated that the medium strength of allelopathy exerted by *Synechococcus*, as assessed based on the abundance of *Synechococcus*, supported the highest diversity (Shannon diversity index and species richness of the investigated species. The authors concluded that the strength of *Synechococcus* allelopathy may influence the diversity and structure of phytoplankton taxa in the Baltic Sea. They also pointed out that, with ongoing global warming and the predicted increase in *Synechococcus* abundance, its allelopathic effect on other phytoplankton taxa is expected to increase over time.

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M.Sc. Zofia Konarzewska finished her doctoral dissertation with conclusions that summarized all the results.

Comments, questions, and suggestions to the presented papers:

1) Publication 1 revealed significant differences in the abundance of studied phenotypes in co-cultures, as well as lower differences in the cell-free filtrate experiments. In a few cases, the experiments with cell-free filtrates yielded results opposite to those of the co-culture trials. This suggests that, in the co-culture experiments, interactions other than allelopathy were at play; however, the author has not commented on this in the discussion.
2) Publication 2 – Studies on the production of microcystins MC-LR by Synechococcus began in the first decade of the 21st century; however, their presence has not been confirmed in various locations for a long time, despite the ubiquity of *Synechococcus*. Thus, the laboratory identification of MC-LR production by the phenotypes presented in this thesis is a fascinating result. However, the author employed only ELISA analyses for this identification and quantification. The ELISA assay is known to yield false-positive and sometimes false-negative results. As a semi-quantitative measurement, the tests are sensitive to salinity, for which they should be calibrated. Therefore, it would be beneficial to perform an HPLC analysis to verify the presence of microcystins.
3) Although the author described methods for enumerating the studied phytoplankton taxa in each paper in the introduction to the thesis, I have not found which techniques were used to enumerate the various phenotypes in publication 2. This was probably added in the final version of this work.
4) Question to publication 3. Why do various species of green algae react differently to the presence of Synechococcus allelochemical, with inhibition or stimulation of growth? Cyanobacteria and diatoms generally exhibited similar directional changes. Interestingly, in the case of two cyanobacteria and two diatom species, the authors did not observe a decrease in growth rate. However, in some instances, they observed a reduction in chlorophyll *a* fluorescence, as well as a decrease in the concentrations of chlorophyll *a* and carotenoids. Could the author give some interpretation of these results?
5) My next question is, why did the author use such low light intensities (10 µmol photons m^{-2} s^{-1}) in the experiments? Picocyanobacteria are known to utilize light efficiently due to their small size. They have a lower packing effect (a self-shading of photosynthetic pigments in cells) than larger cells, and lower costs of absorbing and transforming light into chemical

energy than in larger cells. With this, picocyanobacteria have an advantage over larger cyanobacteria and algae in low-light conditions, which could influence the results, especially in trials presented in papers 4 and 5.

6) In paper 4, the explanations of the ratios 5, 18, and 40 in the first panel and 0.8, 5, and 26 in the second panel in Figures 2, 3, and 6 were not provided. They should be included in the figure caption.
7) I miss the phylogenetic identification of the strains in the studies in the dissertation. Studies concerning the phylogenetic relationships between Synechococcus-like cyanobacteria have a long history. It was shown that some differently pigmented picocyanobacteria may be closely phylogenetically related, while some similarly pigmented phenotypes may belong to different lineages. Information about their relatedness could shed some light on the relationships and allelopathic capabilities between the phenotypes.

Ms. Zofia Konarzewska provided her research with plenty of novel and valuable information concerning the allelopathy of Synechococcus and its effects on various levels, from the intra-genus to the inter-genera and the level of complex community. The doctoral thesis presents five publications, along with well-written introductory and summary sections. Summarizing, the doctoral dissertation I am evaluating presents a scientifically and methodologically coherent work that raises important issues regarding the allelopathy of *Synechococcus* as one of the driving forces responsible for the coexistence of many phytoplankton species without competitive exclusion adding a new solution to explain the paradox of the phytoplankton My questions and comments do not undermine the high substantive value of the presented work and represent just a contribution to the academic discussion.

Having presented the above, I state that the evaluated doctoral thesis fully meets the requirements for doctoral dissertations specified in Art. 187 of the Act of July 20, 2018, Law on higher education and science, uniform text: Journal of Laws of 2021, item 478 on academic degrees and scientific titles and on degrees and titles in the field of art. On this basis, I appeal to the High Scientific Council of the Discipline of Earth and Environmental Science to accept the dissertation and admit M.Sc. Zofia Konarzewska, to the following stages of the doctoral process. Considering the comprehensiveness of the research conducted, the wide range of ecophysiological research techniques employed, and the significance of the results and conclusions, I propose that the dissertation be distinguished.

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