

Biological Activities of Compounds Produced by Microorganisms Encountered in Cyanobacterial Blooms and Cultures

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Cyanobacteria, also called the blue-green algae, are a group of organisms considered as potent producers of bioactive compounds. The pioneering studies of Richard E. Moore at the University of Hawaii, started in the 1970s and carried out through the early 2000s, revealed numerous bioactive secondary metabolites exhibiting anticancer activity in several cancer cell lines, toxicity towards macroscopic life forms and an anti-infection activity. Further studies over the last decade have continued this research, both in the field of chemical structures and the biological activities of cyanobacterial secondary metabolites. Nowadays, over a dozen of classes of bioactive compounds isolated from the cyanobacteria have been characterized, including alkaloids, polyketides, peptides, fatty acids, amides or lactones [1,2]. However, a vast majority of secondary metabolites has been obtained from a limited number of cyanobacterial species, especially those inhabiting waters in the tropical climate. In contrast, the Baltic Sea, a rich source of cyanobacteria of different features, is still underexplored in this field [1,2].

The Baltic Sea is characterized by unique ecological features, resulting from a slow water exchange with the rest of the World Ocean. At the same time, the Baltic Sea is continuously supplied with freshwater (ca. 660 km³ per year) coming from more than 250 rivers. This creates the brackish water conditions, with average salinity of approximately 6–8 ‰, which is very low, compared to the salinity of the ocean (ca. 35 ‰). It would be highly stressful for most marine organisms to live under such severe conditions. Only a limited number of species has succeeded to colonize this specific environment, since salinity is too low for most of the Atlantic and North Sea species, while still too high for the freshwater species. Nevertheless, a selected group of both, marine and freshwater cyanobacteria, has adapted to these brackish conditions. It is possible, that the survival of these organisms in the Baltic Sea is enhanced by production of low molecular weight compounds, a well-known cyanobacterial adaptation strategy, enabling them to survive in a wide range of both physical and chemical conditions. These metabolites may be also involved in interactions with co-occurring organisms, from viruses and bacteria to marine animals [2].

The vast majority of low molecular weight bioactive compounds produced by cyanobacteria has been isolated and identified using conventional techniques, based on analysis of cell extracts prepared from these organisms grown in the laboratory. This method was successfully applied in the isolation and identification of compounds with potential use as bio-fertilizers, herbicides and insecticides, as well as potential immunosuppressive, antimicrobial, and anticancer agents [2,3]. It is worth noting that the techniques based on analysis of cell extracts have significant limitations. A

major part of secondary metabolites derived from cyanobacteria can be produced only in specific physiological conditions, which are difficult or impossible to reproduce under the laboratory conditions. Moreover, the extraction yield of the individual compounds obtained from samples of cyanobacteria grown in the laboratory may be rather low, and thus this approach might be very expensive and inefficient [3].

Nowadays, it is known that under laboratory conditions it is possible to culture only about 1-5% of consortia of microorganisms obtained from environmental samples [4]. This represents a significant limitation in the use of new techniques for the isolation of bioactive compounds from previously unknown organisms based on their cultivation in the laboratory. In the last two decades, there has been a tendency to replace or supplement the conventional methods for testing the biodiversity of microbial populations with the techniques independent of cultivation [4]. One of the most popular methods to study complex biological activities of organisms present in the environmental samples is Metagenomics. It combines various techniques of molecular biology, allowing the study of biodiversity of organisms that cannot be grown under laboratory conditions and can assess their potential use in biotechnology and pharmacology [4,5,6]. Metagenomics is based on the analysis of a metagenomic library. Preparation of the metagenomic library comprises the following steps: (i) a complex isolation of eDNA (*environmental Deoxyribonucleic Acid*) directly from the environmental samples, (ii) cloning of the DNA fragments into an appropriate vector, selected based on the size and type of the analyzed fragments of genetic material, and depending on the bacterial host to be utilized (iii) transfer of the pool of obtained plasmids (referred to as a metagenomic library) into the host [4,5,6]. Metagenomic libraries can be analyzed using methods based on an expected function or sequence. The first one involves the screening of a metagenomic library to find the desired activity, such as antibacterial or enzymatic activities. The latter strategy uses an eDNA sequence analysis to locate the specific fragment of genetic material [4].

The primary aim of my PhD thesis was the acquisition of information on the biological activities of microorganisms encompassed in the cultures and blooms of cyanobacteria isolated from the Baltic Sea. In the conducted experiments, I combined the classical methods of analysis of cell extracts obtained from the cyanobacterial culture, and a modern molecular biology method - metagenomics, that enables testing of environmental samples without the cultivation of marine microorganisms in the laboratory. This experimental design allowed for a comprehensive analysis of the cultures and blooms of cyanobacteria in the context of their potential use in biotechnology and medicine.

First, the main goal was completed based on the analysis of enzymatic and antibacterial activities of 27 strains of cyanobacteria isolated from the Baltic Sea, belonging to the CCNP collection (*Culture Collection of Northern Poland*) located at the Department of Oceanography and

Geography at the University of Gdansk. To this end, I studied the enzymatic and antimicrobial activities with the use of the conventional methods based on analysis of aqueous and ethanol extracts prepared from cyanobacteria cultured under laboratory conditions [2].

I performed experiments exploring the enzyme activity with the use of a commercially available API ZYM test (bioMérieux), enabling the simultaneous examination of 19 enzyme activities, including enzymes catalyzing hydrolysis of proteins (trypsin, α -chymotrypsin, leucine arylamidase, valine arylamidase, cystine arylamidase), enzymes that catalyze the hydrolytic reaction of ester linkages (esterase C4, C8 esterase, lipase C14), enzymes catalyzing hydrolysis of carbohydrates (α -galactosidase, β -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase and α -fucosidase), and the enzymes catalyzing dephosphorylation (alkaline phosphatase, acid phosphatase, naphthol-AS-BI-phosphohydrolase) [2]. My study has demonstrated that aqueous extracts of cyanobacteria isolated from the Baltic Sea are a rich source of enzymes. Among the 19 enzymatic activities, the most common were alkaline phosphatase, acid phosphatase, esterase (C4 and C8) and naphthol-AS-BI-phosphohydrolase, as they were detected in 21-27 extracts of cyanobacteria [2]. In contrast, I identified cystine arylamidase, lipase C14, α -galactosidase, β -galactosidase and β -glucuronidase activities in a relatively few strains (1-3). I also observed significant differences in the number of enzymatic activities in individual strains of cyanobacteria. Aqueous extracts from *Microcystis aeruginosa* CCNP1102, *Synechocystis salina* CCNP1104, *Nodularia spumigena* CCNP1401 and CCNP1402, and *Anabaena* sp. CCNP1406 proved to be the richest in enzymatic activities (11 to 15 active enzymes), whereas in *Spirulina subsalsa* CCNP1310, *Lyngbya* cf. *aestuarii* CCNP1314, CCNP1316, *N. spumigena* CCNP1403 and *Anabaena* sp. CCNP1416, I detected only 2 to 4 active enzymes [2]. The data obtained constitute the first extensive report on the enzymatic activities of cyanobacteria from the Baltic Sea. Until then, only several specific enzymes and their inhibitors or activators have been mainly analyzed. Interestingly, a similar richness of enzyme activities and the differences in their occurrence within one type of blue-green algae were observed in the study of phenotypic and genotypic variability of *Microcystis* strains. This indicates that both, the number and the type of enzymatic activity are strictly specific to and dependent on a particular strain of cyanobacteria [2].

I investigated the antibacterial activity of both, aqueous and ethanol extracts, prepared from the Baltic cyanobacteria, with the application of the microdilution method. I analyzed the effect of the compounds present in these extracts against Gram-positive (*Micrococcus luteus* and *Staphylococcus aureus*) and Gram-negative (*Serratia marcescens* and *Pseudomonas aeruginosa*) bacteria. I cultivated the selected strains for 16 hours with the addition of the extracts at a concentration range of 0.01-1 mg mL⁻¹. Then, I measured the optical density of the culture, and I compared it to the control, which was the culture of bacteria without the addition of the extract. The

obtained results indicated that the compounds included in both, the aqueous and ethanol extracts of *Microcystis aeruginosa*, strongly inhibit the growth of all tested bacteria: *Staphylococcus aureus* DMB-PR-1-20 (MIC₅₀ = 42 µg ml⁻¹), *Micrococcus luteus* DMB-pR1-1 (MIC₅₀ = 42 µg ml⁻¹), *Serratia marcescens* DMBpR-1-2 (MIC₅₀ = 74 µg ml⁻¹), and *Pseudomonas aeruginosa* DMB-PR-1-23 (MIC₅₀ = 168 µg ml⁻¹). The same strains demonstrated a slightly lower sensitivity to the extracts obtained from *Anabaena* sp. CCNP1406 (MIC₅₀ = 250-300 µg ml⁻¹), and (except of *P. aeruginosa*) to the compounds produced by *S. salina* CCNP1104 (MIC₅₀ = 250-300 µg ml⁻¹) and *M. aeruginosa* CCNP1103 (MIC₅₀ = 165 µg ml⁻¹). Interestingly, extracts prepared from 13 out of the 27 strains of marine cyanobacteria, stimulated the growth of at least one of the examined bacteria by at least 50% compared to the control, wherein the aqueous and ethanol extracts obtained from the *Cyanobacterium* sp. CCNP1105 and *Leptolyngbya* sp. CCNP1302 stimulated the growth of all bacteria, with the exception of *P. aeruginosa*. This clearly demonstrated that the active compounds produced by cyanobacteria isolated from the Baltic Sea strongly modulate the growth of Gram-positive and Gram-negative bacteria [2].

The next stage of my PhD thesis research was concerned with analysis of potential anticancer activity of the Baltic cyanobacteria. I conducted this study using an *in vitro* breast cancer model cell line MCF-7. First, I tested the impact of 22 ethanol and aqueous extracts obtained from 11 strains of Baltic cyanobacteria on the MCF-7 cell line viability after 24-hour and 48-hour exposure, at a concentration range of 25-400 µg ml⁻¹. This experiment has shown that ethanol extracts from *Microcystis aeruginosa* CCNP1102, *Pseudanabaena* sp. CCNP1311 and *Pseudanabaena* cf. *galeata* CCNP1313 strongly inhibit the viability of breast carcinoma cells, with IC₅₀ values of 100, 50 and 100 µg ml⁻¹, respectively. These extracts were selected for further studies to determine the molecular mechanism of their cytotoxic effect on tumor cells [1].

To determine whether a reduction of viability of tumor cells is associated with apoptosis, the levels of the apoptotic marker - PARP-1 polymerase (*Poly ADP-ribose polymerase*) were analyzed with the application of the Western blotting technique. This experiment demonstrated that all three extracts induce the caspase-dependent cleavage of the PARP-1 protein, but its extent in relation to the concentration used was different for each extract. The CCNP1102 ethanol extract increased the level of the cleaved form of the marker, in a dose-dependent manner, to about 4-folds when compared to the control at a concentration of 50 µg ml⁻¹, and it remained at this level at higher concentrations of the extract. The CCNP1311 extract induced the PARP-1 cleavage in a dose-dependent manner, reaching a 3.6 fold increase in the cleaved PARP when compared with control at the highest concentration used. In case of CCNP1313, I observed the highest level of cleaved PARP, which was 6-8 times higher than in control at all concentrations of the extract tested.

In order to gain insight into changes in the morphology of the MCF-7 cells after treatment with the extracts of cyanobacteria, I analyzed the ultrastructure of the cells using the transmission

electron microscopy. In samples treated with $100 \mu\text{g ml}^{-1}$ of CCNP1102 or CCNP1311, I observed typical necrotic cells with cytoplasmic swelling, moderate chromatin condensation, swelling of the organelles and rupture of the cytoplasmic membrane. On the contrary, cells treated with the CCNP1313 extract did not display the morphology characteristic for necrosis. However, they shrank and their cytoplasm was dense, which may indicate early stages of apoptosis [1].

Finally, in order to verify whether the active compounds contained in extracts obtained from the Baltic cyanobacteria induce the death of tumor cells, I conducted an analysis of apoptotic and necrotic fraction in the MCF-7 cell line after exposure to selected ethanol extracts, at a concentration range of $25\text{-}100 \mu\text{g ml}^{-1}$, using flow cytometry. The effect of CCNP1102 and CCNP1311 extracts on induction of apoptosis was moderate. It reached 15% for cells treated with the extract at concentrations of $75\text{-}100 \mu\text{g ml}^{-1}$. Interestingly, both extracts increased the necrotic fraction of cancer cells up to 80% at the highest concentration used. Apoptosis was observed predominantly in the cells treated with the extract obtained from *Pseudanabaena cf. galeata* CCNP1313 and it reached the level of 60% of the cells at a concentration of $100 \mu\text{g ml}^{-1}$ [1].

To determine whether the cytotoxic activity of bioactive compounds present in extracts from the Baltic cyanobacteria is characteristic only for the MCF-7 breast cancer cell line, or universal for all cancer cells, I examined the effect of ethanol extracts from *Microcystis aeruginosa* CCNP1102, *Pseudanabaena sp.* CCNP1311 and *Pseudanabaena cf. galeata* CCNP1313 on HeLa cervical cancer cells' viability. I conducted an analogous experiment using the healthy human skin fibroblast model cells HDFa (*Human Dermal Fibroblast adult*). The results clearly show that the extracts of the Baltic cyanobacteria inhibit the viability of both, MCF-7 and HeLa cancer cells, in a time- and dose-dependent manner, with a statistically insignificant effect on the healthy cells (HDFa). It is worth noting, that the LC-MS/MS analysis of cyanobacterial extracts indicated the presence of previously unknown low molecular weight bioactive compounds which are probably responsible for the induction of death in cancer cells. In the context of the potential use of these compounds in anticancer therapy, the necrosis induction is extremely important. To date, the vast majority of anticancer drugs have been directed to induction of apoptosis. However, over time, the tumor cells acquire resistance to therapy. Thus, it is necessary to search for the bioactive compounds inducing non-apoptotic forms of cell death. Moreover, recent reports indicate that the process of necrosis in the tumor cells induces the immune response, which may be a factor supporting the anticancer therapy [1].

The final stage of my PhD thesis implementation was the analysis of antibacterial and anticancer activities of selected organisms, present in the blooms and cultures of cyanobacteria isolated from the Baltic Sea, with the application of metagenomics [3]. Metagenomic study complemented the previous experiments based on the analysis of cell extracts obtained from cyanobacteria cultured in the laboratory. In my research, I used the functional analysis of

metagenomics. The main advantage of this method is the certainty that the factor holding the desired activity is correctly biosynthesized, and thus it can be produced by a foreign organism. This aspect is particularly important in the context of reports indicating that the expression of foreign genes in the most commonly used bacterial host in metagenomic studies - *Escherichia coli* - is limited to approximately 40% [5]. Based on the results obtained in the investigation of biological activities of the Baltic cyanobacteria extracts, I constructed three metagenomic libraries that included the DNA sequences isolated from: (i) a bloom of *Nodularia* sp., collected in the Gdańsk Bay, Baltic Sea (Poland) in June 2009; (ii) a bloom of *Nodularia* sp. and *Aphanizomenon flos-aquae*, collected in the Gdańsk Bay, Baltic Sea (Poland) in May 2011; (iii) cultures of 5 marine cyanobacteria, isolated from the Baltic Sea (deposited in the Culture Collection of Northern Poland, CCNP, at the University of Gdańsk, Poland) and cultured in the laboratory: *Microcystis aeruginosa* CCNP1101, *Microcystis aeruginosa* CCNP1102, *Microcystis aeruginosa* CCNP1103, *Anabaena* sp. CCNP1406, *Synechocystis salina* CCNP1104 [5].

In order to analyze the metagenomic libraries carrying DNA isolated from cultures and blooms of cyanobacteria, I designed and conducted an experiment allowing examination of the gene library for anticancer and antibacterial activities. This is the first study with the application of the metagenomic analysis focusing on the anticancer activity, and also the first study using the microdilution method to find the antibacterial activity within a metagenomic library. In my experiments, I used two hundred randomly selected clones with an average size of the insert of 40,000 bp, for each library constructed [5].

The results obtained in the library screening for potential antibacterial activity revealed that 1% [% v / v] concentration of the extract obtained from clone 123 from the library no. 2 (clone 123-2), induces growth inhibition of *Serratia marcescens* and *Escherichia coli* BL21 by 45% and 40%, respectively [5].

Although for medical applications it is useful to identify the antibacterial activities, for biotechnological purposes it is often desirable to stimulate the growth of bacteria which are employed to express recombinant genes and synthesize particular products [3]. My previous results have shown that the selected strains of cyanobacteria isolated from the Baltic Sea stimulate the growth of Gram-negative bacteria [2]. Therefore, I designed experiments to determine whether the genes carried on a plasmid in the individual clones have a positive impact on the growth of the bacterial host - *Escherichia coli*. As a control, I used clone 105-2 containing the fragment of the *E. coli* genome. Interestingly, I have observed that in the presence of clones 123-3 and 129-3, the generation time at mid-exponential phase of growth was 29 minutes for the host cell, while for the control it was 36 minutes. This experiment confirms that expression of some genes isolated from cyanobacteria stimulates the growth of *E. coli*, which can be potentially used for the construction of new strains of this bacterium for a biotechnological application [3].

The next stage of the metagenomic analysis was the gene library screening for anticancer activity. For this purpose, I examined the effect of 370 clones from each library on the viability of the MCF-7 breast cancer cell line. The results of the experiment have demonstrated that clone 123-3 significantly inhibits the breast cancer cell survival, with minimal impact on healthy human fibroblasts HDFa [3]. Selected clones from the gene libraries, indicating presence of the desired biological activities, were sent for sequencing using NGS (*Next Generation Sequencing*) techniques. Sequencing results were analyzed using the Blast software and genes potentially responsible for the occurrence of the tested biological activities were listed and described [3].

In the framework of this PhD thesis, I focused on the analysis of biological activity of microorganisms present in the cultures and blooms of cyanobacteria isolated from the Baltic Sea, the presence of the compounds produced by these organisms with anticancer and enzymatic activities, as well as those modulating growth of both, Gram-positive and Gram-negative bacteria [1,2,3]. Low molecular weight compounds, as well as genes potentially responsible for the occurrence of the biological activities, have been indicated [1,2,3]. This PhD thesis has allowed to enhance our knowledge on the biodiversity of cyanobacteria inhabiting the Baltic Sea, and has also demonstrated the effectiveness of combining traditional techniques that study biological activities based on the analysis of cell extracts with a modern tool in molecular biology, which is metagenomics.

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