

THE PRODUCTION AND RELEASE OF ALLELOPATHIC COMPOUNDS BY BALTIC CYANOBACTERIA

Sylvia Śliwińska, Maria Skauradszun, Elżbieta Niemirycz, Adam Latała

University of Gdansk, Institute of Oceanography, Av. Piłsudskiego 46, 81-378 Gdynia, Poland

Abstract

*Allelopathic interactions of phytoplankton may cause the dominance of different species of algae and cyanobacteria which result in massive blooms in freshwater, brackish and marine ecosystems. Some species of microalgae are able to produce secondary metabolites that may be harmful to microorganisms, phyto- and zooplankton, crustaceans, fish and even humans. In this study, the influence of allelochemicals on the growth and cell morphology of green alga *Chlorella vulgaris* was investigated by the addition of cell-free filtrate of Baltic cyanobacteria *Nodularia spumigena* and *Synechococcus* sp. cultures grown under $10 \mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, 20°C and 8 psu. Harmfulness and toxicity of cyanobacterial cell-free filtrate was tested using Microtox 500, U.S Microbics Inc. These findings suggest that nitrogen-fixing *N. spumigena* and picocyanobacteria *Synechococcus* sp. can have negative influence on growth and cell morphology of tested green algae *C. vulgaris* and this effect was caused by released allelochemicals, which were nontoxic to bacteria *Vibrio fischeri*.*

Key words: allelopathy, allelochemicals, Baltic Sea, blooms, cyanobacteria

1. INTRODUCTION

The composition and biomass of phytoplankton are essential for the functioning of the food web of aquatic ecosystems. Allelopathic interactions between cyanobacteria and algae may influence the structure of phytoplankton and cause the formation of massive blooms in many freshwater, brackish and marine ecosystems (Legrand et al. 2003; Weissbach et al. 2010; Žak et al. 2012). Species forming massive blooms have become a serious problem, both ecologically and economically (Anderson & Garrison 1997). The cyclical changes in taxonomic composition and biomass of phytoplankton groups can be observed every year. Depending on the season and the availability of nutrients blooms of cyanobacteria, dinoflagellates, green algae and diatoms may appear (Stal et al. 2003).

The mass occurrence of cyanobacteria may have different implications for the surrounding ecosystem. The massive blooms can cause death or paralysis of aquatic organisms and reduce water quality (Stal et al. 2003). The massive blooms are developing in coastal and brackish waters. They are often mostly monospecific, suggesting the existence of a significant mechanism that causes their great advantage over other phytoplankton species. Blooms of cyanobacteria in the Baltic Sea consist of two groups - large, filamentous cyanobacteria and picocyanobacteria (Stal et al. 2003). One of the most important species that can form toxic blooms in the Baltic Sea is filamentous *N. spumigena*. However, the less recognized nontoxic picoplanktonic cyanobacteria may constitute up to 80 % of the total biomass of cyanobacteria and 50 % of the total primary production in cyanobacterial bloom (Stal et al. 2003).

Production of active organic compounds is an important adaptation, by which some species of phytoplankton can achieve competitive advantage over other primary producers (Legrand et al. 2003). The production of allelopathic compounds by phytoplankton was recognized in several groups of phytoplankton such as cyanobacteria, dinoflagellates, prymnesiophytes, raphidiophytes, green algae and diatoms (Subba Rao & Smith 1995; Gross 2003; Chiang et al. 2004; Suikkanen et al. 2004). Some of the species forming blooms produce secondary metabolites that may be harmful to microorganisms, crustaceans, fish and even humans (Anderson & Garrison 1997; Granéli & Hansen 2006).

The main aim of this work was to estimate the allelopathic interaction of dominant, in the summer period, Baltic cyanobacteria *N. spumigena* and *Synechococcus* sp. on green algae *C. vulgaris*. In this study, the influence of allelopathic compounds on the growth and cell morphology of analyzed green algae was investigated by addition of cyanobacterial cell-free filtrate. Additionally, the harmfulness and toxicity of cyanobacterial filtrate was tested by determining the bioluminescence of the indicator bacteria *V. fischeri* after exposure to allelopathic substances.

2. MATERIALS AND METHODS

2.1. Algal material and culture conditions

The experiments were conducted on filamentous cyanobacterium *Nodularia spumigena* (BA-15), picocyanobacterium *Synechococcus* sp. (BA-124) and green algae *Chlorella vulgaris* (BA-80). The strains were isolated from the coastal zone of the Gulf of Gdańsk (southern Baltic Sea) and are maintained as unialgal cultures in the Culture Collection of Baltic Algae (<http://ccba.ug.edu.pl>) at the Institute of Oceanography, University of Gdańsk, Poland (Latała 2003; Latała et al. 2006). Tests on the “batch cultures” were carried out in 100 ml glass Erlenmeyer flasks containing sterilized f/2 medium (Guillard 1975). The media were prepared from Baltic water with salinity of about 8 psu, which was filtered through glass fiber filters (Whatman GF/C). The analyzed strains were incubated under a 16:8 h light:dark cycle at 20 °C and PAR irradiances of 10 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. The cultures were acclimated for 7 days, afterwards actively growing cultures were used as a source of inoculum for the establishment of the allelopathic experiment.

2.2. Experiment setup

Allelopathic interaction was determined by using the modified method proposed by Suikkanen et al. (2004). Allelopathic interaction was studied by adding the cell-free filtrate obtained from cyanobacterial culture to tested green algae. The filtrate was filtered through Whatman GF/C filters. Next the cell-free filtrate ($V = 2 \text{ ml}$) was added to Erlenmeyer flasks containing tested algae ($V = 20 \text{ ml}$). In all experiments, the ratio of cyanobacteria to target species in the Erlenmeyer flasks was adjusted to 1:1 based on the chlorophyll *a* contents of the cultures thus the proportion of cyanobacteria in relation to target algae was equal to 80:80 $\mu\text{g chl } a \text{ l}^{-1}$. Control samples were prepared by adding mineral medium f/2 with a volume equal to the added cell-free filtrate obtained from cultures of cyanobacteria.

2.3. Measurements of culture density

After 1, 3 and 7 days of exposure, the target species' culture density was determined. Culture density was determined by the number of cells and optical density (OD). The number of cells was measured microscopically using Bürker chamber and OD was measured spectrophotometrically at 750 nm with a Multiskan GO Thermo Scientific UV-VIS spectrophotometer. The results of cell number and OD cultures were used to determine the linear correlation between the measured parameters. Determined relationships were afterwards used to estimate the number of cells in the cultures tested only on the measurements of the OD. Tests were conducted in triplicate and all analyzed green algae were obtained from early exponential growth phase.

2.4. Determination of cell morphology

On the seventh day of the experiment photographs of cell morphology of green algae *C. vulgaris* were taken by the light microscope Nikon Eclipse 80i. The photographs presented differences between the control and green algae after exposure to cell-free filtrate obtained from cultures of donor cyanobacteria.

2.5. Determination of harmfulness and toxicity of cyanobacterial cell-free filtrate

Harmfulness and toxicity of cyanobacterial cell-free filtrate was tested using Microtox 500, U.S Microbics Inc. This test method is based on the decrease of bioluminescence which is an effect of metabolic inhibition of the indicator bacteria (*Vibrio fischeri*) after exposure to toxic substances. Microtox 500 analyzer consists of a photometer and an incubator. The photometer is capable of measuring light in the visible range (400-700 nm) produced by the bacteria, while the incubator maintained the luminescent bacteria and test sample at a constant temperature (Johnson 2005). Harmfulness and toxicity of cyanobacterial cell-free filtrate was determined by the protocol 81.9 % Screening Test according to the instructions (Azur Environmental Ltd. 1998). This test allows observing a reduction in the luminescence of the bacteria compared with the control and gives results expressed as percentage of this reduction (Percentage Effect - PE). Based on this test it can be concluded that the sample was toxic ($\text{PE} \geq 20 \%$) or nontoxic ($\text{PE} < 20 \%$).

2.6. Statistical analyses

Analysis of variance (ANOVA) was used to test differences in all analyzed parameters between the target algae cultures treated with cyanobacterial cell-free filtrates and the control, over the experimental period. A post hoc test (Tukey's HSD) was used to show which treatments significantly differed from the control and from each other. All tests are in a significance level of $P = 0.05$, and data are reported as means $\pm$ SD. The statistical analyses were performed using the SSP Statistica® 10.

3. RESULTS

3.1. Measurements of culture density

The results showed that Baltic cyanobacteria *Synechococcus* sp. and *N. spumigena* significantly decreased ($p < 0.05$) the number of cells of green algae *C. vulgaris* compared to their control (Fig. 1). The greatest decrease of growth of *C. vulgaris* was observed after addition of cell-free filtrate obtained from *Synechococcus* sp. The longer was the exposure time, the slower growth of analyzed green algae in comparison with control. On the seventh day of the experiment the minimum cells response of *C. vulgaris* cultures treated with culture filtrates of *Synechococcus* sp. and *N. spumigena* constituted respectively 77 % and 91 % of their control (100 %).

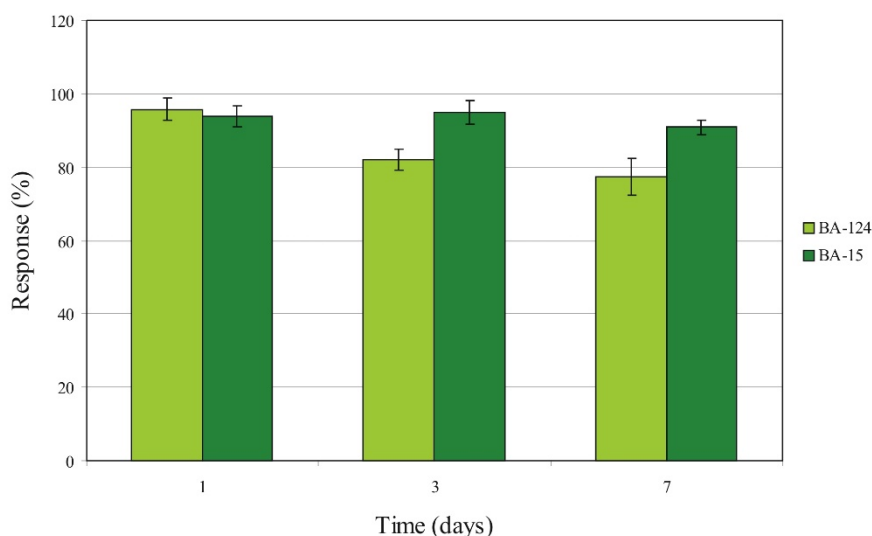


Fig. 1. The effects of filtrate of *Synechococcus* sp. (BA-124) and *N. spumigena* (BA-15) on growth of *C. vulgaris* after 1, 3 and 7 days of exposition to the filtrates, expressed as the response (%) of culture density in relation to the control ($n=3$, mean $\pm$ SD).

3.2. Determination of cell morphology

In this work we also showed that addition of cell-free filtrate from cyanobacterial cultures had harmful effect on cell morphology of *C. vulgaris*. Figure 2 describes morphological changes of green algae *C. vulgaris* after 7 days of exposure to cell-free filtrate obtained from analyzed Baltic cyanobacteria *Synechococcus* sp. and *N. spumigena*. In this study we reported that the cyanobacterial cell-free filtrate causes damage to cell membranes and other cell organelles. Additionally, it was observed that the filtrate obtained from cyanobacteria caused deformation of *C. vulgaris* cells or cell lysis and reduced their pigmentation compared to control cultures that grew only on mineral medium.

3.3. Determination of toxicity of cyanobacterial cell-free filtrate

In this study the harmfulness and toxicity of cyanobacterial cell-free filtrate was tested using Microtox 500, U.S Microbics Inc. (Tab. 1). The study showed that the cell-free filtrate obtained from the culture of cyanobacteria *Synechococcus* sp. and *N. spumigena* was nontoxic to the bacteria *V. fischeri*. Only for *Synechococcus* sp. after 5 minutes of incubation a small decrease of bioluminescence was observed. This experiment indicated the effect of metabolic inhibition of the bacteria (*V. fischeri*) after exposure to the filtrate. However, this effect was below the value of 20 % and classified the filtrate as non-toxic.

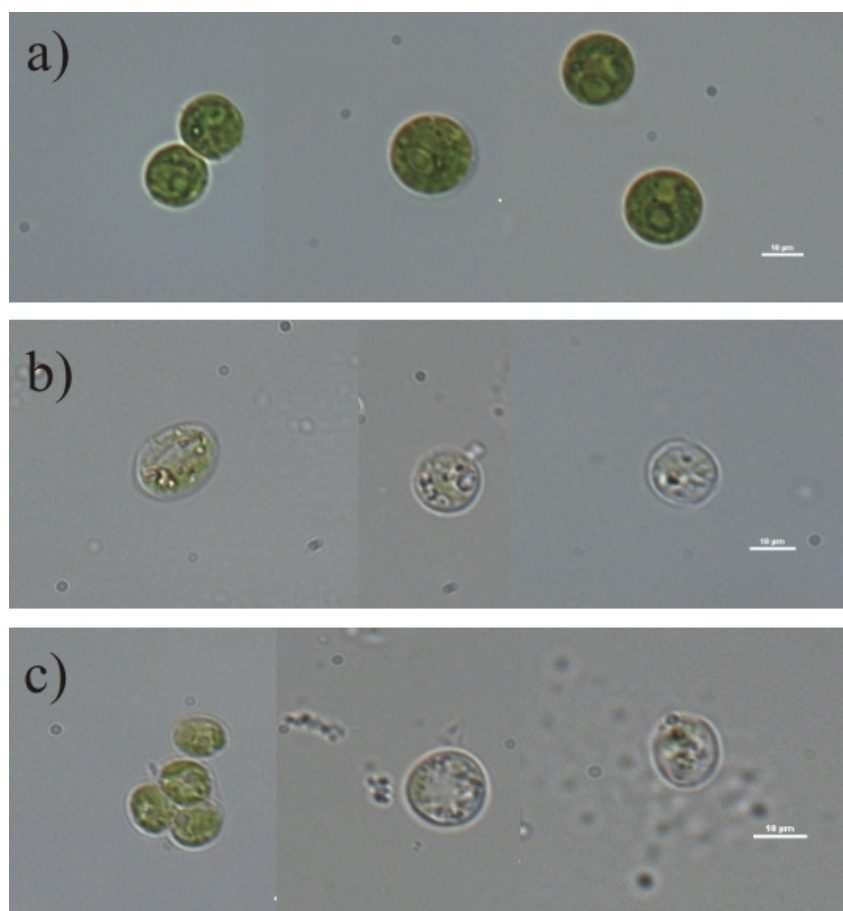


Fig. 2. Cell morphology of green algae *C. vulgaris* in control (a) and after 7 days of exposure to cell-free filtrate obtained from cultures of *N. spumigena* (b) and *Synechococcus* sp. (c).

Tab. 1. PE (%) of bioluminescence of bacteria *V. fischeri* after 5 and 15 min of exposition to cell-free filtrate obtained from the culture of cyanobacteria *Synechococcus* sp. (BA-124) and *N. spumigena* (BA-15), compared to the control (n=3, mean±SD).

	Strains	
	BA-15	BA-124
Time (min)	PE (%)	
5	-2.423 ± 1.052	5.526 ± 2.465
15	-17.330 ± 1.245	-3.853 ± 1.681

4. DISCUSSION

Various compounds are excreted into the environment during algal growth or decay of cells. However, for many of them the biological or physiological activity has not been described. These compounds, which can act positively or negatively and thus affect the structure of the ecosystem, are called allelochemicals. In a previous study Leflaive & Ten-Hage (2007) showed that the allelopathic compounds may constitute an important group of bioactive components containing toxic and nontoxic compounds. These compounds may have antialgal, antibiotic and antifungal properties (Gross 2003; Legrand et al. 2003; Leflaive & Ten-Hage 2007). Some of these may also affect interspecies competition or deter predators (Turner et al. 1997).

The occurrence of allelopathic interactions is often reported in cyanobacteria and algae, but the precise mode of action of secreted compounds is still poorly recognized. In natural phytoplankton communities it is difficult to demonstrate direct evidence of allelopathic interactions. It is therefore important to characterize allelopathy under controlled experimental conditions in order to examine the nature of the released substances and their mode of action on target organisms. In this work we have shown that the addition of cell-free filtrate from Baltic

N. spumigena and *Synechococcus* sp. cultures had negative impact on growth and cell morphology of *C. vulgaris*. The greatest decrease of growth of *C. vulgaris* was observed after the addition of cell-free filtrate obtained from *Synechococcus* sp. and constituted about 77 % of their control. Suikkanen et al. (2006) indicate that *Anabaena flos-aquae* and *N. spumigena* filtrates significantly decreased the cell number of *Rhodomonas* sp., compared to the control. This inhibition was already observed after the first day of the experiment. *A. flos-aquae* extract inhibited the number of cells significantly more than that from *N. spumigena*. By day 6, *A. flos-aquae* and *N. spumigena* filtrates had decreased the cell number of *Rhodomonas* sp. by 29 % and 14 %, respectively. Some authors show that cyanobacteria have negative effect on green algae (Suikkanen et al. 2006; Gantar et al. 2008). Gantar et al. (2008) have shown that the lipophilic extract obtained from *Fischerella* sp. inhibited green algae *Chlorella* sp. Moreover, inhibitory effects exhibited *Anabaena torulosa* against the coccal green algae *Scenedesmus acutus*, the cyanobacterium *Anabaena cylindrica*, and the volvocalean *Pandorina morum*. Furthermore, *Nostoc muscorum* inhibited *Scenedesmus acutus* and the *P. morum* (Schagerl et al. 2002). Žak et al. (2012) has described an interesting research where *N. spumigena* showed an inhibitory, stimulatory and no effect on *C. vulgaris*, depending on the initial density of the target species. Authors suggested that different reactions to cyanobacterial filtrate could be caused by changes in bioactive compounds concentration in cultures. Additionally, Kearns & Hunter (2000) also observed a stimulatory effect of *Chlamydomonas reinhardtii* on the blue-green algae *A. flos-aquae*, which was described and explained as a positive effect of extracellular products secreted by green algae.

The destruction of the cell membrane is a possible mode of action of allelopathic compounds (Gantar et al. 2008). This study demonstrated that addition of cell-free filtrate from Baltic cyanobacteria has harmful effect on cell morphology of *C. vulgaris*. Figure 2 presented morphological changes of green algae *C. vulgaris* after 7 days of exposure to cell-free filtrate obtained from *N. spumigena* and *Synechococcus* sp. It was observed that the filtrate obtained from cyanobacteria caused damage to cell membranes and other cell organelles, deformation of cells and reduction of pigmentation or, in final effect, cell lysis of *C. vulgaris* compared to control cultures that grew on mineral medium f/2. Similar results were presented by Valdor & Aboal (2007), where morphological changes under the influence of an extract of cyanobacteria *Geitlerinema* sp., *Oscillatoria* sp. and *Phormidium* sp. were observed under light and electron microscopy in cyanobacteria *Scytonema* sp., *Pseudocapsa* sp., *Nostoc* sp., and green algae *Klebsormidium* sp. In their research, cells and filaments of *Scytonema* sp. were extracted, forming granules or were empty. They also observed fragmentation of trichomes and the increasing number of heterocyst and cytoplasmic inclusions in cells. For *Pseudocapsa* sp. and *Nostoc* sp. authors noted a tendency for separation of the filaments into single cells, and found many dead and empty cells. Moreover, trichomes were colorless, divided into fragments or deformed. Also *Klebsormidium* sp. showed a strong tendency to fragmentation. In addition, ultrastructural research have shown that extract obtained from *Fischerella* sp., after 24 h of exposure resulted in structural and morphological changes in cells of *Chlamydomonas* sp. (Gantar et al. 2008). Authors, using electron microscope, demonstrated the degradation of thylakoid and cell membranes and loss of cell organelles, including nucleus. Furthermore, Fistarol et al. (2004) demonstrated that the filtrate from the dinoflagellate *Alexandrium tamarense* caused cell lysis, loss of pigmentation, increase in the number of empty cells and formation of temporary cysts in *Scrippsiella trochoidea*. However Žak et al. (2012) found that cell-free filtrate obtained from *Anabaena variabilis* and *N. spumigena* has no influence on *C. vulgaris* viability and morphological changes of analyzed green algae was not observed.

Production of allelopathic compounds is a common feature of most species forming massive blooms of phytoplankton. Unfortunately, reasons for the synthesis of these compounds remain unknown (Legrand et al. 2003; Gross 2003). It is possible that their primary function is to deter predators and inhibition of co-occurring species of phytoplankton (Turner et al. 1997). The use of biological tests is the first step in identifying which fraction or group of components is responsible for the allelopathic effects (Fistarol et al. 2004). In Table 1 we showed that the cell-free filtrate obtained from the culture of cyanobacteria *Synechococcus* sp. and *N. spumigena* was nontoxic to the bacteria *V. fischeri* because this effect was below the value of 20 % (Persoone 2003). This is the first attempt to use these methods to determine the toxicity of the cyanobacterial cell-free filtrate and more research is necessary to verify this phenomenon. The production and release of allelopathic compounds is an interesting adaptation, in which substances are secreted by coexisting species. Different strains of cyanobacteria are known from production of intra- and extra-cellular metabolites with diverse biological activities, such as antialgal (Kaya et al. 2002), antibacterial (Isnansetyo et al. 2003), antifungal (Kundim et al. 2003) and antiviral activity (Shih et al. 2003; Noaman et al. 2004). The effect of allelochemicals on bacterial abundance and production has been reported in a few studies (Uronen et al. 2007; Weissbach et al. 2010). Weissbach et al. (2010) indicate that allelochemicals produced by *Alexandrium tamarense* have no direct negative impact on bacteria in treatments containing single and continuous high and low concentrated additions of lytic and non-lytic *Alexandrium* supernatant during 96 h. Moreover, bacterial biomass and production did not differ from the control with K-medium in any of the treatments. Furthermore, Martins et al. (2008) shows that none of the tested

extracts of the *Synechocystis* sp. and *Synechococcus* sp. strains showed inhibitory effect on Gram-negative bacteria. Antibacterial activity of cyanobacteria was particularly evident in Gram positive bacteria (Pushparaj et al. 1999; Kreitlow et al. 1999). This finding can be related to the fact that most Gram-negative bacteria are resistant to harmful substances in the environment due to the barrier of lipopolysaccharides of their outer membrane (Dixon et al. 2004). The difference in toxicity between Gram positive and Gram negative bacteria can also be explained by the mechanism of toxicity that involves different functions in two types of cells namely different permeability to the cyanobacteria compounds. In spite of the nontoxic effect of cyanobacterial extracts on Gram-negative bacteria, authors suspect that components are changing the permeability of the cell's outer membrane (Martins et al. 2008). This effect can be enhanced by antibiotics, particularly hydrophobic ones, as demonstrated in the cyanobacterial toxins microcystin-RR (Dixon et al. 2004).

Cyanobacteria and algae are known to produce and release secondary metabolites that may negatively affect coexisting organisms. Conducting further research on allelopathic compounds and the mechanisms of their effects on the surrounding ecosystem may be important in understanding the phenomenon of the appearance of massive blooms of phytoplankton in many aquatic ecosystems, including the Baltic Sea.

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