## ABSTRACT

The term "allelopathy" was introduced to science in 1937 by Hans Molisch which used the concept of allelopathy to identify negative or positive impact of chemicals secreted by a plant on the growth of neighboring plants (Molisch, 1937). In 1996, the International Allelopathy Society (IAS, 1996) has expanded this definition by adding to it any inhibitory or stimulatory process in which chemical substances secreted by the various organisms interact with the ecosystem (Legrand et al., 2003). Allelopathy in the aquatic environment can cause or facilitate the dominance of certain species of cyanobacteria, micro- and macroalgae and higher plants. Field studies and laboratory experiments have shown that allelopathy occurs in all marine, brackish and freshwater habitats (Gross, 2003). In addition, primary producers are able to produce many of the active compounds. Recent studies indicated that some species of phytoplankton are able to produce and release secondary metabolites that affect the growth and development of other organisms (e.g. Granéli and Johansson, 2003a, 2003b; Fistarol et al., 2005; Kubanek et al., 2005; Suikkanen et al., 2005; Antunes et al., 2012; Żak et al., 2012). The production of allelopathic compounds by phytoplankton was identified in several taxonomic groups such as cyanobacteria, dinoflagellates, prymnesiophytes, green algae and diatoms (Subba Rao and Smith, 1995; Gross, 2003; Legrand et al., 2003).

Allelopathy may be one of the factors contributing to the formation and maintenance of cyanobacterial blooms (Suikkanen et al., 2004), which strongly affect coastal marine ecosystems and cause economic problems for commercial aquaculture (Stal et al., 2003). Furthermore, some cyanobacteria produce harmful compounds which can have negative effects on plants, animals and even humans (Suikkanen et al., 2005). Over the past few decades, the world's coastal waters have experienced an increase in the number of harmful algal bloom events. Allen et al. (2006) described that blooms are occurring in more areas than ever before and new massive blooms are reported regularly. Generally, the blooms of cyanobacteria that develop each summer in the freshwater and brackish ecosystems are composed of two different groups: the large, colony-forming, filamentous $N_2$-fixing cyanobacteria (e.g. *Nodularia spumigena*) and small-sized picocyanobacteria from the genus *Synechococcus* and *Synechocystis*. Surprisingly, the picocyanobacteria fraction may comprise as much as 80% of the total cyanobacterial biomass and contribute as much as 50% of the total primary production of a cyanobacterial bloom (Kahru et al., 1994; Stal et al., 2003; Jasser, 2006; Jodłowska and Śliwińska, 2014). The increasing cyanobacterial blooms are changing the structure of the aquatic communities in the Baltic Sea (Stal et al., 2003; Suikkanen et al., 2005). Among cyanobacteria, allelopathy seems to be especially common in freshwater species (Gross, 2003), but there is also recent evidence on allelopathic inhibition of cryptophyte and diatom growth by

brackish-water cyanobacteria (Suikkanen et al., 2005). It has been suggested that the ecological role of allelochemicals of cyanobacteria from the Baltic Sea is to maintain their dominance after a critical cell concentration has been formed due to environmental factors (Suikkanen et al., 2005). Therefore it is essential to characterize allelopathic activity under different experimental conditions and their mode of action on target organisms (Smith and Doan 1999; Gross, 2003).

The number of reports about the allelopathic effects of cyanobacteria has been steadily increasing. However, the impact of environmental factors and mode of action of allelopathic compounds produced by Baltic cyanobacteria on microalgae remains unknown. The main aim of this work was to estimate the allelopathic interaction of the Baltic cyanobacteria *Synechococcus* sp. and *N. spumigena* on the green algae *C. vulgaris* and *O. submarina* and the diatom *S. marinoi* and *B. paxillifer*. Moreover, in this work the scope of the phenomenon of allelopathy between the cyanobacteria *Synechococcus* sp. and *N. spumigena* was determined. In this study, the influence of allelopathic compounds on the analyzed species was investigated by addition of cell-free filtrate of *Synechococcus* sp. and *N. spumigena* cultures grown under different light, temperature and availability of nutrients. These studies demonstrated tolerance and sensitivity of the analyzed organisms to allelopathic compounds produced by cyanobacteria growing on different environmental factors similar to those occurring in the surface water on the Baltic Sea during the summer period. It is believed that the presence of allelopathic interaction is common among the phytoplankton, but the precise mode of action and characteristics of secreted compounds are still unknown. In this study, the influence of allelopathic compounds on the growth, chlorophyll *a* fluorescence and photosynthesis performance of analyzed species was investigated by single and repeated addition of cell-free filtrate of cyanobacterial cultures obtained from the exponential and stationary phase of growth. Providing new information on the extent of the effect of allelopathic cyanobacteria may be important for a better understanding of the worldwide intensifying phenomenon of the emergence of massive blooms of cyanobacteria in many aquatic ecosystems.

The experiments were conducted on the cyanobacteria *Synechococcus* sp. (BA-124) and *Nodularia spumigena* (BA-15), the green algae *Chlorella vulgaris* (BA-80) and *Oocystis submarina* (BA-01) and the diatom *Skeletonema marinoi* (BA-98) and *Bacillaria paxillifer* (BA-14). 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 (CCBA) (http://ccba.ug.edu.pl) at the Institute of Oceanography, University of Gdańsk, Poland (Latała, 2003; Latała et al., 2006). The tests on the "batch cultures" were carried out in 25 ml glass Erlenmeyer flasks containing sterilized f/2 medium (Guillard, 1975). The media were prepared from Baltic water with a salinity of about 8 psu, which was filtered through glass fiber filters (Whatman

GF/C). The cyanobacteria strains were incubated under a 16:8 h light:dark cycle at three PAR irradiances (10, 100 and 190 µmol photons·$m^{-2}$·$s^{-1}$), three temperatures (15, 20 and 25°C) and different availability of nutrients (nutrient-sufficient NP, nitrogen-deficient -N or phosphorus-deficient -P). The cultures were acclimated to each culture condition for 7 days, afterwards actively growing cultures were used as a source of inoculum for the establishment of the allelopathic experiment. Target organisms were grown in constant conditions of 10 µmol photons·$m^{-2}$·$s^{-1}$, 20°C, and nutrient-sufficient NP.

Allelopathic interactions were determined by using the modified method proposed by Suikkanen et al. (2004). The allelopathic interaction was studied by adding the single and repeated cell-free filtrate obtained from a cyanobacterial culture to the tested organisms. The donor cyanobacteria culture was filtered through Whatman GF/C filters. In all experiments, the ratio of donor to target species in Erlenmeyer flasks was adjusted to 1:1 based on the chlorophyll *a* content (final chlorophyll *a* concentration in the experimental cultures was 0,8 µg chl *a* $ml^{-1}$). The cell-free filtrate (V=2 ml) was added first day of experiment to 25 ml Erlenmeyer flasks containing the tested cyanobacteria (V=20 ml). Control samples were prepared by adding mineral medium f/2 with a volume equal to the added cell-free filtrate. To simulate the effects of continuously released cyanobacterial allelochemicals on target species, cyanobacterial filtrates were added daily to the target cultures for one week. The first addition was made as described above. Subsequent additions were made by removing 2 ml of test volume, used for cell counts each day, and replacing it with an equal volume of fresh filtrate or control medium. Tests were conducted in triplicate. Donor cyanobacteria were obtained from exponential and stationary growth phase and all analyzed target species were obtained from early exponential growth phase. Culture density was determined by the number of cells and optical density (OD). The number of cells was counted using Bürker chamber and OD was measured spectrophotometrically at 750 nm with a Multiskan GO Thermo Scientific UV-VIS spectrophotometer. The results of cell counts and respective OD measurements were then used to determine the linear correlation between them for each species. Determined relationships were subsequently used to estimate the number of cells in the experimental cultures after the $1^{st}$, $3^{rd}$ and $7^{th}$ day of the diatom exposure to the cyanobacterial filtrate. Chlorophyll *a* fluorescence was measured with a Pulse Amplitude Modulation (PAM) fluorometer FSM1 Hansatech, using 594 nm amber modulating beam with 4 step frequency control as a measuring light. Analyzed species were taken for chlorophyll fluorescence analysis after 0 (1 h), $1^{st}$, $3^{rd}$ and $7^{th}$ day of exposure to the filtrate. Before measurements, each sample taken from the culture was filtered through 13 mm glass fiber filters (Whatman GF/C). Before starting the experiment the filter sample was adapted in the dark for about 15 minutes. The maximum PSII quantum efficiency ($F_v/F_m$) was calculated

(Campbell et al., 1998). The measurements of oxygen evolution were carried out on the 7$^{th}$ day of the experiment by using Clark-type oxygen electrode (Chlorolab 2, Hansatech). Dark respiration was estimated from $O_2$ uptake by cells incubated in the dark. Experimental data were fitted to the photosynthesis-irradiance curve using equation (Jassby and Platt, 1976) and Statistica® 10 software. Analysis of variance (ANOVA) was used to test for 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. Data were reported as mean ± standard deviation (SD). The statistical analyses were performed using the Statistica® 10 software.

The influence of abiotic and biotic factors on the production and release of allelopathic compounds is relatively poorly understood, because the study has focused mainly on the allelopathic interactions between the organisms. Several studies have indicated that the selection of the donor and target organisms, physiological state of cells, amount of cell-free filtrate, cell density, heterotrophic bacteria and the potential role of toxins present in the cyanobacterial cell-free filtrate can influence the impact of allelopathy, but relatively few studies precisely define the effect of biotic factors on the production and release of allelopathic compounds into the environment (Gross, 2003; Antunes et al., 2012). Moreover, several studies have demonstrated that abiotic factors such as light intensity, temperature, pH and nutrient deficiencies may affect the production of allelopathic compounds (Gross, 2003; Noaman et al., 2004; Antunes et al., 2012).

These studies showed that the biotic factors such as selection of the donor and target organisms, physiological state of cells and amount of cell-free filtrate are affecting the cyanobacterial allelopathy. All tested cyanobacteria were found to be allelopathic to some of the target algae. Each target species responded differently to the addition of cyanobacterial cell-free filtrates: *C. vulgaris* and *S. marinoi* was negatively affected by all cyanobacteria, even with one filtrate addition only, *B. paxillifer* showed a certain tolerance, being affected by all cyanobacteria only after repeated filtrate additions and *O. submarina* was resistant to all cyanobacterial filtrates. Specific group characteristics, e.g. membrane permeability, may contribute to the susceptibility of phytoplankton species to allelochemicals. This might be one explanation for the sensitivity of *C. vulgaris* and *S. marinoi* compared with the other species tested. Moreover, some coevolutionary aspects may have contributed to the observed effects. *S. marinoi,* typical in the phytoplankton spring bloom in the Baltic Sea, may not regularly encounter mass occurrences of cyanobacteria in the natural conditions. Therefore, the group may not have evolved any resistance mechanisms to cyanobacterial allelochemicals. A relatively uninvestigated factor that may affect the sensitivity of cells to environmental chemicals is that of cell size. The different size of target species showed

different responses to cyanobacterial allelochemicals. “Large” cells were less susceptible to allelopathic compounds. *B. paxillifer* (50 µm) and *O. submarina* (20 µm) cells were also less affected by cyanobacterial allelochemicals than smaller *C. vulgaris* and *S. marinoi* (5 µm) and it appears that either physiological differences between size classes or more efficient transport of the chemical into smaller cells leads to the observed differences. Generally, the filtrate of the *Synechococcus* sp. and *N. spumigena* culture in exponential phase had a negative allelopathic effect on target species, whereas the stationary phase culture filtrate did not affect either of the species significantly. In the natural environment, phytoplankton are constantly exposed to chemicals released by other species, but in batch culture experiments involving just a single filtrate addition, the effect may be lost after some time of exposure. In the present study, the responses of the least sensitive species *O. submarina* and *B. paxillifer* to cyanobacterial filtrates were approximately the same irrespective of the number of filtrate additions. The effect of increasing exposure to fresh filtrate was most clearly shown by the response of *C. vulgaris* and *S. marinoi* to cyanobacterial filtrate. For *C. vulgaris*, the effect of repeated cyanobacterial filtrate additions was stronger than the effect of a single addition (71% and 69% compared to 32% and 58% decrease in cell numbers on seven days of exposure with filtrate additions obtain from *Synechococcus* sp. and *N. spumigena*, respectively). This study shown that some Baltic cyanobacteria are able to inhibit other phytoplankton species under certain biotic factors. Some species are more sensitive to these compounds than others, thus the increasing cyanobacterial blooms may have the capacity to change the structure of some phytoplankton communities in the Baltic Sea.

In this work a wide range of abiotic factors such as light intensity, temperature and availability of nutrients was selected to determine the cyanobacterial allelopathy under optimal and more stressful conditions for the analyzed organisms. It was shown that high light and temperature and nutrient-sufficient affected the cyanobacteria *Synechococcus* sp. and *N. spumigena* by increasing its production of allelochemicals. Furthermore this result showed that light affected the most the donor species by increasing its production of allelochemicals. Generally, the highest decrease in cell number of target species was observed after the addition of cell-free filtrate obtained from cyanobacteria grown at 190 µmol photons$\cdot m^{-2} \cdot s^{-1}$, 25°C and nutrient-sufficient NP. The longer the exposure time the lower response of the target species. On the seventh day of the experiment, the minimum cell response of the most sensitive species *S. marinoi*, after addition of cell-free filtrate obtained from *Synechococcus* sp. and *N. spumigena*, constituted respectively 32% and 18% of control. Additionally, in the current study, the parallel influence of allelopathic compounds between the cyanobacteria *Synechococcus* sp. and *N. spumigena* were investigated by addition of cell-free filtrate of cyanobacterial cultures grown under different light conditions. It was

shown that light affected the picocyanobacterium *Synechococcus* sp. by increasing its production of allelochemicals. On the first day of the experiment, the minimum cell response of *N. spumigena* cultures, after addition of cell-free filtrate, constituted 89%, while on the seventh day of experiment the minimum cells response constituted 67% of control. Moreover, the study indicated that *N. spumigena* had no similar allelopathic effect on picocyanobacterium *Synechococcus* sp. The results demonstrated for the first time that the common Baltic picocyanobacterium *Synechococcus* sp. affects coexisting filamentous cyanobacteria *N. spumigena* negatively. Finally, all these findings indicated that the Baltic cyanobacteria reveals allelopathic activity, and that the production of allelopathic substances depends on the abiotic factors such as light intensity, temperature and availability of nutrients. Furthermore these study indicate, that light intensity affected the most of all analyzed factors the donor Baltic cyanobacteria by increasing its production of allelochemicals.

The precise mode of action of allelopathic compounds remains relatively poorly known due to methodological difficulties. It is therefore important to characterize the allelopathic effects in controlled experimental conditions, in order to examine the nature of released substances and their mode of action on the targets organisms. In the present work, the parallel influence of allelopathic compounds on the growth, chlorophyll *a* fluorescence and photosynthesis performance between the donor cyanobacteria *Synechococcus* sp. and *N. spumigena* on target species was investigated. It was demonstrated that the photosynthetic parameter $P_m$ of the studied species was negatively affected due to the addition of the cyanobacterial cell-free filtrate after 7 days of incubation. The strongest effect of cyanobacterial allelochemicals on the maximum photosynthetic rate $P_m$ on *S. marinoi* was observed when the cell-free filtrate obtained from *Synechococcus* sp. and *N. spumigena* grown at 190 µmol photons·$m^{-2}$·$s^{-1}$ was added, and it constituted 41% and 43%, respectively. Moreover, cell-free filtrate obtained from *Synechococcus* sp. and *N. spumigena* generally had no effect on the α parameter of target species. The maximum quantum yield of PSII is frequently used as a proxy for photosynthetic efficiency and consequently as a general measure of cell stress. The effects of cyanobacterial cell-free filtrate on chlorophyll *a* fluorescence after 1 hour and 1$^{st}$, 3$^{rd}$ and 7$^{th}$ days of incubation are determined. These studies indicated that the longer exposure to the filtrate, the lower the response of fluorescence parameter $F_v/F_m$ for target species. The addition of cell-free filtrate from *Synechococcus* sp. and *N. spumigena* cultures grown under varied light, temperature and availability of nutrients significantly inhibits the values of $F_v/F_m$. The strongest allelopathic effect on the parameter $F_v/F_m$ on *S. marinoi* was observed when the cell-free filtrate obtained from *Synechococcus* sp. and *N. spumigena* was added, and it constituted 14% and 27%, respectively. Moreover, at the seventh day of experiment, after addition the cell free-filtrate obtained from picocyanobacterium *Synechococcus* sp., the minimum response of *N. spumigena* was 62% for

growth, 45% for $F_v/F_m$ and 35% for $P_m$. However, the study showed that *N. spumigena* had no allelopathic effect on *Synechococcus* sp. Inhibition of photosynthesis by cyanobacterial cell-free filtrate appeared to parallel the negative effects on growth of target species. Species whose growth was strongly suppressed by cyanobacterial filtrate (*C. vulgaris, S. marinoi* and *N. spumigena*) also experienced substantial reduction in photosynthetic efficiency. Because of the sensitivity and speed of this assay, decreases in PSII efficiency may provide information about when target species are most susceptible to allelopathy. Finally, compounds exuded by Baltic *Synechococcus* sp. and *N. spumigena* inhibited the efficiency of PSII and photosynthesis performance of tested species, suggesting a new potential mechanism for allelopathy.

Allelopathic interactions of phytoplankton may be one of the key factors that influence the dominance of different taxa and cause massive blooms in freshwater, brackish and marine ecosystems around the world. However, despite the seriousness of the problem, reports of the occurrence and consequences of allelopathic effects of cyanobacteria in aquatic ecosystems are still insufficient. Therefore, it is important to conduct a more intensive study of this phenomenon. These studies indicated that the common Baltic cyanobacteria affect coexisting organisms negatively. Basing on the study's results, it was examined that the picocyanobacterium *Synechococcus* sp. and nitrogen-fixing cyanobacterium *N. spumigena* reveals allelopathic activity on the photosynthesis and chlorophyll fluorescence which results in the inhibition of growth of target microalgae and cyanobacteria. These studies have indicated that the biotic factors such as selection of the donor and target organisms, physiological state of cells and amount of cell-free filtrate affecting the cyanobacterial allelopathy. Moreover these findings indicated that the production of allelopathic substances by analyzed cyanobacteria is regulated by different abiotic factors such as light, temperature and availability of nutrients. To evaluate the significance of the phenomenon of allelopathy, we need to study the varied biotic and abiotic factors affecting the production and secretion of active allelopathic compounds in more detail. A better understanding of the factors affecting the release of allelopathic compounds and the modes of their action on organisms and the surrounding ecosystem may be important in explaining the phenomenon of the emergence of massive blooms of algae in many aquatic ecosystems. Understanding the mechanisms and consequences of the impact of allelopathic cyanobacteria in aquatic ecosystems should therefore be considered essential and fundamental for future research.