Allelopathic interactions between *Synechococcus* sp. and *Nodularia spumigena* under different light conditions

SYLWIA ŚLIWIŃSKA-WILCZEWSKA*, FILIP PNIEWSKI and ADAM LATALA

University of Gdansk, Institute of Oceanography, Department of Marine Ecosystems Functioning, Av. Pilsudskiego 46, 81-378 Gdynia, Poland
E. Mail: ocessl@ug.edu.pl

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ABSTRACT

The mutual influence of allelopathic compounds on the growth, chlorophyll fluorescence and photosynthesis of cyanobacteria *Synechococcus* sp. and *Nodularia spumigena* by single and repeated addition of cell-free filtrate (CFF) of cyanobacterial cultures grown under different light conditions was investigated. The effects on growth, fluorescence parameter ($F_v/F_m$) and the photosynthetic parameter ($P_m$) of *N. spumigena* were amplified by repeated filtrate additions of *Synechococcus* CFF compared to a single addition. But *N. spumigena* CFF had no allelopathic effects on *Synechococcus* sp. The picocyanobacterium decreased the coexisting filamentous, bloom forming cyanobacterium and the production of allelopathic substances increased with light intensity.

Key words: Allelopathy, allelochemicals, chlorophyll fluorescence, cyanobacteria, filtrate, growth, light, *Nodularia spumigena*, picocyanobacteria, photosynthesis performance, *Synechococcus*

INTRODUCTION

Certain species of phytoplankton such as cyanobacteria, dinoflagellates, prymnesiophytes, green algae and diatoms produce and release secondary metabolites (Allelochemicals) that affect the growth and development of other organisms (16,27,31,41,43). Allelopathy may be a factor contributing to the formation and maintenance of cyanobacterial blooms (37), which strongly affect the coastal marine ecosystems and cause economic problems for commercial aquaculture. In the past few decades, the world’s coastal waters have experienced an increasing number of harmful algal blooms (18). Generally, the cyanobacterial blooms that develop during the summer in freshwater and brackish ecosystems are composed of two different groups of cyanobacteria: (i) A large, colony-forming, filamentous N₂-fixing cyanobacteria (*Nodularia* sp., *Aphanizomenon* sp., *Anabaena* sp.) and (ii) a small-sized picocyanobacteria (*Synechococcus* sp. and *Synechocystis* sp.). The picocyanobacteria may comprise as much as 80% of the total cyanobacterial biomass and contributes 50% to the total primary production of cyanobacterial blooms (21,36).

*Correspondence Author
The influence of abiotic and biotic factors on the production and release of allelopathic compounds is however poorly understood (13). Temperature, pH, phosphate concentration, incubation period, medium constituents and light intensity are known to influence the production of antimicrobial chemicals (1,8,13,14,30). The mode of action of allelopathic compounds is also poorly understood because it is difficult to demonstrate direct evidence of allelopathy in natural phytoplankton communities. Hence, it is essential to characterize allelopathic activity and their mode of action on target organisms under controlled experimental conditions (16,27).

The present study was aimed to determine the scope of allelopathy between the cyanobacteria, namely *Synechococcus* sp. and *N. spumigena*, the species dominant during the summer in the Baltic Sea. The influence of allelopathic compounds on the growth, chlorophyll fluorescence and photosynthetic performance of analyzed species by single and repeated addition of CFF of cyanobacterial cultures grown under different light conditions in the laboratory was examined.

**MATERIALS AND METHODS**

**Culture Conditions**

The experiments were conducted with the picocyanobacterium *Synechococcus* sp. (BA-124) and the filamentous cyanobacterium *N. spumigena* (BA-15). The strains were isolated from the coastal zone of the Gulf of Gdańsk (southern Baltic Sea), where both constitute the live planktonic flora. These cyanobacterial strains were 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 (26).

Batch cultures (Vol. 20 ml) were grown in sterilized f/2 medium (17) in 25 ml glass Erlenmeyer flasks. Media were prepared from Baltic Sea water (Salinity: 8 psu), previously filtered through Whatman GF/C filters. The flasks were gently shaken once daily for 15 min at 200 RPM during the experiment. The donor cyanobacterial cultures were incubated under a 16:8 h of light:dark cycle at 20°C and three PAR irradiances: 10, 100 and 190 µmol photons·m⁻²·s⁻¹. Target cyanobacteria were grown in constant conditions and low light intensity for growth and photosynthesis [20°C and 8 psu, under a 16:8 h light:dark cycle at 10 µmol photons·m⁻²·s⁻¹] (19,22,23). The intensity of PAR was measured using a LI-COR LI-189 quantum-meter with a cosine collector. The cyanobacterial cultures (Vol. 60 ml) were acclimatized in 100 ml glass Erlenmeyer flasks for each culture condition for 7 days before using them in allelopathic experiments. Then they served as inoculum for test cultures, where the initial number of cells were 10⁶·ml⁻¹ for *Synechococcus* sp. or 10⁵·ml⁻¹ for *N. spumigena*. Test cultures (Vol. 60 ml) were grown in 3 replications in 100 ml glass Erlenmeyer flasks and were incubated for one week at each culture condition. After wards we measured the chlorophyll a content of the donor and target cyanobacteria and added a suitable amount of f/2 medium to obtain 0.8 µg chl a·ml⁻¹. Then the target cyanobacteria (Vol.20 ml) were transferred in 3 replications to 25 ml glass Erlenmeyer flasks and using them for allelopathic experiments.
Experimental setup

Allelopathic interactions were determined by using the modified method of Suikkanen et al. (37) by exposing the target cyanobacterium to CFF of the donor cyanobacterial strain. Prior to the experiments, the concentrations of major inorganic nutrients (nitrate and phosphate) were measured in the cyanobacterial cultures by using spectrophotometric methods described by Grasshoff (15). The concentrations of nitrate and phosphate in the cyanobacterial filtrates were adjusted to the same level as in the f/2 growth medium. Therefore, both the treatments and the controls received the same amount of nitrate and phosphate (data not shown).

The allelopathic interaction was studied by adding single or repeated doses of CFF from a cyanobacterial cultures to *Synechococcus* sp. or *N. spumigena* under test. The donor cyanobacterial culture was filtered through Macherey-Nagel MN GF-5 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 $a$: 0.8 µg chl a·ml$^{-1}$). The CFF (V=2 ml) was added on the first day of experiment to the Erlenmeyer flasks containing the cyanobacterium under test (V=20 ml). Controls were prepared by adding equal volume of mineral medium f/2.

To simulate the effects of continuously released cyanobacterial allelochemicals on target species, cyanobacterial filtrates were added daily to the target cultures for one week. Additions were made by removing 2 ml of test volume for cell counts and replacing it with an equal volume of fresh CFF or control medium. All tests were conducted in triplicate.

Culture density

Culture density was determined by the number of cells using a Bürker chamber and optical density (OD) was measured spectrophotometrically at 750 nm using a Multiskan GO Thermo Scientific UV-VIS spectrophotometer. For *N. spumigena* this formed the basis of determining the correlation coefficient ($r$: 0.98) and the linear correlation (y: 1243137.94x-6092.73, where y: Number of filament units and x: OD.). The number of filament units in the test cultures was determined from the calibration curve (22) and later the filament units were converted into number of cells. For *Synechococcus* sp. it was easy to determine the correlation coefficients ($r$: 0.99) and the linear correlations between the number of cells and OD (y: 92720000x-400000, where, y: number of cells and x: OD.). The cell concentrations in the test cultures were estimated based on the calibration curves (24). Determined relationships were subsequently used to estimate the number of cells in the experimental cultures on 0 (1 h of exposure), 1$^{st}$, 3$^{rd}$ and 7$^{th}$ day after the exposure of the target cyanobacterium to the cyanobacterial filtrate.

Chlorophyll $a$ fluorescence

Chlorophyll $a$ fluorescence of cyanobacterium under test was measured using a Pulse Amplitude Modulation (PAM) fluorometer FSM1 Hansatech using a 594 nm amber modulating beam with 4 step frequency control as a measuring light on 0 (1 h of exposure), 1$^{st}$, 3$^{rd}$ and 7$^{th}$ day of exposure to the filtrate. Before measurements, each sample from the culture was filtered through 13 mm glass fiber filters (Whatman GF/C) and adapted in the dark for about 15 min. The maximum PSII quantum efficiency ($F_{v}/F_{m}$) was calculated (4).
Photosynthesis

Oxygen evolution was measured on the 7th day by using Clark-type oxygen electrode (Chlorolab 2, Hansatech). at 20°C, with a cooling system (LAUDA E100). Illumination was by a high intensity probe-type light array with 11 red LED’s centered on 650 nm. Irradiance was measured with a quantum sensor Quantitherm (Hansatech). Dark respiration was determined from O2 uptake by cells incubated in the dark (20). Experimental data were fitted to the photosynthesis-irradiance curve using equation (20) and Statistica® 10 software.

Statistical analyses

Analysis of variance (ANOVA) was used to test the differences in all analyzed parameters between the target algal cultures treated with cyanobacterial CFF and the control over the experimental period. A post hoc test (Tukey’s HSD) was used to show which treatments for growth and fluorescence parameter significantly differed from the control and from each other. Data are reported as mean ± standard deviation (SD). Levels of significance were: *p < 0.05; **p < 0.01; ***p < 0.001. The statistical analyses were performed using the Statistica® 10 software.

RESULTS AND DISCUSSION

Effects of cyanobacterial filtrates on growth

The allelopathic effect of Synechococcus sp. significantly decreased the number of cells of N. spumigena (p = 0.000 for single filtrate addition and p = 0.045 for repeated filtrate addition) (Fig. 1A). The single or repeated addition of CFF from Synechococcus sp. grown under 3 different light conditions, inhibited the growth of N. spumigena and light affected the donor species by increasing its allelopathic activity. The filtrate from Synechococcus sp. inhibited N. spumigena, while the control showed active growth during the experiment (data not shown). After the seventh day, for a single filtrate addition from Synechococcus sp. grown at 10 and 100 µmol photons-m-2-s-1 the percent of culture density was 78% (p = 0.000) and 70% (p = 0.000), respectively, whereas for repeated filtrate addition, the percent of culture density was 82% (p = 0.077) and 73% (p = 0.001), respectively. The highest drop in growth was seen after the addition of single and repeated CFF from Synechococcus sp. grown at 190 µmol photons-m-2-s-1. On the 7th day of the experiment, the minimum cell response of N. spumigena, after the single and repeated addition of CFF was 67% (p = 0.000) and 72% (p = 0.000), respectively.

The results of the effects of single and repeated addition of CFF of N. spumigena grown at different light intensities (10–190 µmol photons-m-2-s-1), on the growth of Synechococcus sp. after 1, 3 and 7 days of exposure to the filtrates, (expressed as percent of culture density in relation to the control) are presented in Fig. 1B. It is seen that addition of the filtrate did not significantly affect Synechococcus sp. (p = 0.877 for single filtrate addition and p = 0.988 for repeated filtrate addition) and no significant difference between single and repeated additions was seen. Repeated addition of CFF from N. spumigena grown at 190 µmol photons-m-2-s-1 showed a slight inhibitory effect on the growth of Synechococcus sp. which on the 7th day of exposure was about 91% but not statistically significant (p > 0.05).
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Biological tests are the first step in understanding and identifying allelopathy occurring among many species of cyanobacteria (11). The present study was aimed at finding the effect of single and multiple addition of cell-free filtrate from the picocyanobacterium *Synechococcus* sp., on filamentous cyanobacterium *N. spumigena*. The results show, for the first time, that picocyanobacterium *Synechococcus* sp. affects the filamentous cyanobacteria *N. spumigena* negatively and that the production of allelopathic substances by the former is influenced by light intensity.

Earlier it was reported that other cyanobacteria have a negative effect on microalgae (12,33,34). Schlegel et al. (34) found that out of approximately two hundred cyanobacterial isolates, only members of the genera *Fischerella*, *Nostoc* and *Calothrix* displayed bioactivity against the green algae. Schagerl et al. (33) described the inhibitory effects of *Anabaena torulosa* against the green algae *Scenedesmus acutus*, the cyanobacterium *Anabaena cylindrica*, and the volvocalean *Pandorina morum*. Gantar et al. (12) found that the extract from *Fischerella* sp. inhibited the green algae *Chlorella* sp.

The stimulatory effect of *Chlamydomonas reinhardtii* on the cyanobacteria *Anabaena flos-aquae* was reported (25) and explained as a positive effect of extracellular products secreted by the green algae. Żak et al. (42) demonstrated that Baltic *N. spumigena* had different allelopathic effects on *C. vulgaris*, and this impact depended on the phase of growth of the analyzed cyanobacteria and the initial density of the target green algae.

In this study, it was noted that the test species showed variable response to single and repeated addition of culture filtrates. The responses of growth and fluorescence of target *Synechococcus* sp. to *N. spumigena* filtrates were almost the same irrespective of the amount of filtrate added. However, the photosynthetic parameter $P_{m}$ was inhibited or

![Figure 1. Effect of singe and multiple addition of CFF from *Synechococcus* sp. and *N. spumigena* grown at 10, 100 and 190 µmol photons·m$^{-2}$·s$^{-1}$ on the growth of target cyanobacteria. Growth is expressed as the % culture density in relation to the control. The values refer to means (n = 3, mean ± SD). Asterisk indicates significant difference compared with control.](image)
stimulated depending on the addition of the filtrate. On the other hand, with *N. spumigena*, it was noticed that repeated addition of filtrate had a stronger influence on chlorophyll fluorescence and photosynthesis than a single addition. Further, the growth inhibition was similar irrespective of the amount of filtrate added. Suikkanen *et al.* (37) have also reported such similarly with *Aphanizomenon flos-aquae* and *Thalassiosira weissflogii*. After a single filtrate addition from *A. flos-aquae*, the cell numbers of *T. weissflogii* decreased initially but increased to original levels after two days. This suggested that the recovery of *T. weissflogii* growth after a single filtrate addition may be due to its ability to metabolise the allelochemicals (40).

Of the total phytoplankton biomass in the seas, the autotrophic picoplankton constitutes up to 90% and up to 43% in fresh waters (21, 36). This suggests that the picocyanobacterium *Synechococcus* sp. is able to produce allelopathic substances that affect negatively the coexisting bloom forming cyanobacterium *N. spumigena*. Additionally, the picocyanobacterium was resistant to allelochemicals released by the filamentous, brackish cyanobacterium. Therefore, these results make an important contribution to the knowledge about the phenomenon of allelopathy between cyanobacteria *Synechococcus* sp. and *N. spumigena*. Our study confirms the hypothesis proposed by Stal *et al.* (36) that the picocyanobacteria keep the diazotrophic cyanobacteria in check by their allelopathic activity.

**Effects of cyanobacterial filtrates on chlorophyll fluorescence**

The effects of picocyanobacterial CFF on chlorophyll *a* fluorescence after 0 (1h), 1, 3 and 7 days of incubation are shown in Fig. 2. The fluorescence parameter *F*/*F*<sub>m</sub> was different on single and repeated addition of the CFF from *Synechococcus* sp. It was seen that a single addition of CFF from *Synechococcus* sp. cultures grown under varying light had no significant effect on *F*/*F*<sub>m</sub> (*p > 0.05*). However, when the target organism was exposed to repeated additions of CFF, the value of *F*/*F*<sub>m</sub> was 59% (*p = 0.000*), 57% (*p = 0.000*), and 45% (*p = 0.000*), respectively on the third day of experiment for the 10, 100 and 190 µmol photons·m<sup>-2</sup>·s<sup>-1</sup>.

The effect of single or repeated addition of CFF from *N. spumigena* grown under different light intensities (10–190 µmol photons·m<sup>-2</sup>·s<sup>-1</sup>) on the fluorescence parameter *F*/*F*<sub>m</sub> of *Synechococcus* sp. after 0 (1h), 1, 3 and 7 days of exposure is shown in Fig. 2B. Single or repeated addition of filtrate from *N. spumigena* did not affect the fluorescence parameter *F*/*F*<sub>m</sub> of *Synechococcus* sp. (*p > 0.05*).

Factors that contribute to increased production and secretion of allelopathic compounds have not been studied in detail, although it is believed that light, temperature, nutrients and the genetic potential, may have an impact on the occurrence of allelopathy in aquatic environment (7, 27). Therefore, in this work a wide range of light intensities were selected to determine its effect on allelopathy to other organisms. It was found that high light intensities affected increased allelopathic activity of *Synechococcus* sp. However, *N. spumigena* had no similar allelopathic effect on *Synechococcus* sp. It is reported earlier that light conditions influence the production of allelopathic compounds in other cyanobacteria. Antunes *et al.* (1) showed that the production of allelopathic compounds by the *Cylindrospermopsis raciborskii* was modulated by light and temperature. The highest
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Figure 2. Effect of single and multiple addition of CFF from *Synechococcus* sp. and *N. spumigena* grown at 10, 100 and 190 µmol photons-m⁻²-s⁻¹ on the fluorescence parameter of target cyanobacteria. \(F_v/F_m\) is the quantum yield of PSII equivalent to the potential oxygen production. The values refer to means (n = 3, mean ± SD). * Indicates significant difference compared with control.

Light intensity (90 µmol photons-m⁻²-s⁻¹) treatment resulted in a significant growth inhibition of *A. falcatus*. In this species the production of saxitoxin follows a circadian rhythm (5) and the concentrations of both intracellular and extracellular toxin was related to light intensities (8).

Nakai *et al.* (29) reported that in cyanobacterial assays using *Microcystis aeruginosa*, the reduction in the maximum PSII quantum yield and associated growth inhibition, was by eight allelochemicals (five polyphenols – catechin, eugenin, ellagic acid, gallic acid and pyrogalllic acid and three fatty acids – nonanoic acid, cis-6-octadecenoic acid and cis-9-octadecenoic acid) released by *Myriophyllum spicatum*. It was also found that the reduction in PSII activity was the major mechanism responsible for the growth inhibition of *M. aeruginosa* by the eight allelochemicals at 25-75 µmol m⁻² s⁻¹. With the addition of eight allelochemicals, the reduction in specific growth rate at 25 µmol m⁻² s⁻¹ was 1.5 times that at 75 µmol m⁻² s⁻¹. Also the observed trend of stronger growth inhibition at lower light intensities agree with observations of atrazine-mediated growth inhibition in *M. aeruginosa* (6), *Oscillatoria limnetica* (3), and *Selenastrum capricornutum* (28). Nakai *et al.* (29) explained that the increased growth inhibition at lower light intensities was perhaps the result of changes in the degradation behaviors of the eight allelochemicals and/or changes in the sensitivity of *M. aeruginosa* to these allelochemicals.
However, when the influence of the environmental factors on allelopathy are examined, not only the change in allelochemicals production but also that the sensitivity of the target organism to the allelochemicals should be considered. The present work shows that the tested cyanobacteria showed different sensitivities to the addition of CFF. Bährs et al. (2) found the growth of the three tested cyanobacterial species (Synechocystis sp. Nostoc sp. and M. aeruginosa) was sensitive to p-benzoquinone (PBQ) than the growth of the green alga Pseudokirchneriella subcapitata, Monoraphidium braunii, and Desmodesmus armatus. This suggested that cyanobacterial species are more sensitive to algicidal organic compounds than eukaryotes. Suikkanen et al. (38) found that cyanobacteria may either stimulate or inhibit natural phytoplanktons, depending on the target species. The variation in responses may be caused by morphological and physiological differences in each of the species (37). As reported by Figueredo et al. (10), we believe that these differences in species responses can help explain phytoplankton community structure and succession in many aquatic ecosystems.

**Effects of cyanobacterial filtrates on the photosynthetic response**

The photosynthetic irradiance response (P-E) curves for N. spumigena treated with CFF from Synechococcus sp. grown at 10, 100 and 190 µmol photons·m⁻²·s⁻¹ are presented in Fig. 3. It is seen that the filamentous cyanobacterium is sensitive to the picocyanobacterium CFF (p < 0.05). The influence of the cyanobacterial CFF on the maximum photosynthetic rate (Pₘ) is shown in Fig. 4. The lowest values of Pₘ in N. spumigena were observed after repeated addition of CFF, similar to that observed in the case of growth and chlorophyll fluorescence, from Synechococcus sp. grown at 190 µmol photons·m⁻²·s⁻¹, and were respectively about 49% lower than in the control (p = 0.001).

The experiments also comprised measurements of the P-E curves for single and repeated additions of CFF from N. spumigena grown at different light intensities (10–190 µmol photons·m⁻²·s⁻¹) on Synechococcus sp. after 7 days of exposure. There was no significant influence of the filtrate from N. spumigena grown at the highest light intensity on the Pₘ of Synechococcus sp. (p > 0.05). However, a single addition of the filtrate from the donor cyanobacterium grown at 100 µmol photons·m⁻²·s⁻¹ significantly inhibited Pₘ of the Synechococcus sp. and the value was 75% (p = 0.003). Surprisingly, repeated addition of the filtrate from N. spumigena grown at 10 and 100 µmol photons·m⁻²·s⁻¹ significantly affected the Pₘ of Synechococcus sp. and stimulated it to reach 113%, p = 0.017 and 114%, p = 0.029, respectively.

The precise mode of action of allelopathic compounds remains unknown due to methodological difficulties. In the present work, the fluorescence parameter Fₘ/Fₘ in N. spumigena and the photosynthetic parameter Pₘ in N. spumigena were amplified by repeated filtrate additions compared to a single filtrate addition. However, N. spumigena had no significant effect on Synechococcus sp.

The maximum quantum yield of PSII is frequently used as a proxy for photosynthetic efficiency and consequently as a general measure of cell stress. A decrease in PSII efficiency can result from a variety of factors including allelopathy (32). Besides the filtrate-induced growth inhibition, a short-term reduction of the fluorescence parameter
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Figure 3. P-E curves for single and multiple addition of CFF from *Synechococcus* sp. and *N. spumigena* grown at 10, 100 and 190 µmol photons-m⁻²-s⁻¹ on target cyanobacteria. Measurements were made after 7 days of exposure.

Figure 4. Effect of single and multiple addition of CFF from *Synechococcus* sp. and *N. spumigena* grown at 10, 100 and 190 µmol photons-m⁻²-s⁻¹ on the $P_m$ (µmolO₂/cell·10⁹/h) of target cyanobacteria. Measurements were made at 7 days after exposure. The values are means (n = 3, mean ± SD). * indicates significant difference compared with control.
Fv/Fm (Fig. 2) was also seen. However, while growth inhibition continued, Fv/Fm recovered in tested N. spumigena after a 3rd day of filtrate exposure (Fig. 2). Hence, long-term growth inhibition cannot be caused by the short-term reduction of photosynthetic efficiency. Further, the recovery of Fv/Fm showed that the cells were viable, even when growth inhibition and reduction in oxygen evolution (Fig. 3) occurred. A similar effect was seen by Bährs et al. (2) who reported that while growth inhibition continued, the rETR recovered after a minimum of 24 h in all tested species except Synechocystis sp. However, in M. aeruginosa, which was the most sensitive species concerning growth inhibition by PBQ, there was no detrimental effect on rETR. These findings suggest multiple effects of cyanobacterial filtrates on target organisms.

On the other hand, Prince et al. (32) found that all species examined (Skeletonema costatum, Akashiwo cf. sanguinea, Amphora sp., Asterionellopsis glacialis, and Prorocentrum minimum) displayed a decrease in photosynthetic efficiency within 1 h of exposure to Karenia brevis bloom extract, with S. costatum being the intensely affected. It was found that the efficiency of PSII measured after 1-h exposure to allelopathic extracts was a more sensitive indicator of target organisms stress than the effects on growth measured over 2-4 d. Because of the sensitivity and speed of this assay, decreases in PSII efficiency provides information as to when the tested species are most susceptible to allelopathy.

There are a few reports that allelopathic compounds may affect photosynthesis in target organisms. Smith and Doan (35) suggested that bioactive compounds of cyanobacteria are directed at biochemical targets involved in photosynthesis. Sukenik et al. (39) found that the cyanobacterium Microcystis sp. inhibits photosynthesis in the freshwater dinoflagellate Peridinium gatunense by affecting the carbonic anhydrase activity. Bährs et al. (2) reported that in Synechocystis sp., gross oxygen production by M. aeruginosa was not affected by exposure to the EC90 concentration of PBQ. These results agree with the measurements of rETR. Suikkanen et al. (37) reported that in the natural environment, phytoplankton is constantly exposed to chemicals released by other species; however, in batch culture experiments involving a single filtrate addition, the effect may be lost after some time of exposure due to the degradation of the chemicals.

Despite the seriousness of the problem, information on the occurrence and consequences of allelopathic effects of cyanobacteria in marine, brackish and freshwater ecosystems is incomplete. Therefore, a better understanding of the factors affecting the release of allelopathic compounds and the mechanisms of their effects on target organisms and the surrounding ecosystem is important to explain the emergence of massive algal blooms.

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