Allelopathic activity of the picocyanobacterium *Synechococcus* sp. on coexisting microalgae

*Sylwia Śliwińska-Wilczewska*, *Aldo Barreiro Felpeto*<sup>B,D</sup>, *Jakub Maculewicz*<sup>A</sup>, *Amanda Sobczyk*<sup>A</sup>, *Vitor Vasconcelos*<sup>B,C</sup> and *Adam Latała*<sup>A</sup>

<sup>A</sup>University of Gdansk, Institute of Oceanography, Av. Pilsudskiego 46, 81-378 Gdynia, Poland

<sup>B</sup>Interdisciplinary Center of Marine and Environmental Research–CIMAR/CIIAMAR, University of Porto, Av. General Norton de Matos s/n, 4450-208 Matosinhos, Portugal

<sup>C</sup>Department of Biology, Faculty of Sciences, Porto University. Rua do Campo Alegre, 4069-007 Porto, Portugal

<sup>D</sup>Corresponding author. Email: aldo.barreiro@gmail.com

**Abstract.** The production and release of allelopathic compounds is an important adaptation by which some species of cyanobacteria can achieve a competitive advantage over other primary producers. In this study, we tested the allelopathic activity of the picocyanobacterium *Synechococcus* sp. against the following coexisting microalgae: *Porphyridium purpureum*, *Stichococcus bacillaris*, *Prymnesium parvum* and *Nitzschia dissipata*. We demonstrated that both addition of *Synechococcus* sp. cell-free filtrate and co-culture inhibited the growth of *P. purpureum* and *S. bacillaris*. Conversely, the growth of *P. parvum* was positively affected. By contrast, *N. dissipata* was unaffected by either the picocyanobacterial filtrate or co-culture. These results showed that *Synechococcus* sp. allelopathy should be considered when estimating the potential interactions between picocyanobacteria and coexisting microalgae in aquatic ecosystems.
**Introduction**

Allelopathy can be found in aquatic environments and primary producers can release active compounds which can affect the functioning of the marine, brackish and freshwater ecosystems (e.g., Fistarol et al. 2004; Ji et al. 2011; Poulson-Ellestad et al. 2014; Brutemark et al. 2015). Allelopathy is also considered among the potential drivers of massive blooms throughout many aquatic environments. The number of reports on the allelopathic effects of cyanobacteria and microalgae has been steadily increasing (e.g., Barreiro and Hairston 2013; Żak and Kosakowska 2015; Dias et al. 2017; Wang et al. 2017).

Picocyanobacteria play an important role in aquatic ecosystems (Beardall 2008; Callieri 2010; Costa et al. 2015; Worden and Wilken 2016) but little is known about their allelopathic activity against coexisting microalgae. In previous works, we demonstrated the allelopathic effect of *Synechococcus* sp. on diatom *Navicula perminuta* (Śliwińska-Wilczewska et al. 2016) and we also showed a significant inhibitory effect of cell-free filtrate obtained from *Synechococcus* sp. against the filamentous cyanobacteria *Nostoc* sp. and *Phormidium* sp. (Śliwińska-Wilczewska et al. 2017a) and against different groups in a natural plankton community (Śliwińska-Wilczewska et al. 2017b). There are also some reports of allelopathic effects such as growth inhibition or stimulation caused by other species of cyanobacteria (e.g., Suikkanen et al. 2004, 2005; Antunes et al. 2012; Barreiro and Vasconcelos 2014; Rzymski et al. 2014) but there is no information about allelopathic potential of *Synechococcus* sp. on coexisting microalgae like *Porphyridium purpureum*, *Stichococcus bacillaris*, *Prymnesium*...
parvum and Nitzschia dissipata. The issue of picocyanobacterial allelopathy needs more researcher’s attention, since this group is the major contributor to marine primary production.

The effect of allelochemicals depends on the nature of the interaction between donor and target organisms (direct cell to cell contact, no contact) and activity of the chemical compounds responsible of this interaction. In aquatic ecosystems, donor organisms may affect the target species in different ways, and, in most cases, allelopathic compounds can reduce the growth rate and eventually cause the death of targeted organisms (Rzymski et al. 2014). However, it is difficult to find direct evidence of allelopathic interactions between cyanobacteria and microalgae in natural communities (see Keating 1977, 1978). Before this can be achieved, it is very important to characterize allelopathic interactions under controlled laboratory conditions, in order to investigate in detail the nature of released substances and their impact on target organisms.

The main aim of this study was to determine the influence of allelopathic compounds produced by picocyanobacterium Synechococcus sp. on growth of Porphyridium purpureum, Stichococcus bacillaris, Prymnesium parvum and Nitzschia dissipata in both co-cultures and bioassays employing cell-free filtrates. These species are widely geographically distributed and relevant in terms of abundance in phytoplankton communities, so allelopathic interactions between them could play a significant role in aquatic ecosystems. Based on this, further research could be done to identify the chemical structures of the allelopathic compounds involved in the interaction, as well as their mechanisms and modes of action on target organisms.

Materials and methods

Material and culture conditions
The experiments were conducted with the picocyanobacterium *Synechococcus* sp. (BA-124) and the following microalgae: the red algae *Porphyridium purpureum* (MA-03), the green algae *Stichococcus bacillaris* (BA-09), the golden algae *Prymnesium parvum* (AA-69) and the diatom *Nitzschia dissipata* (BA-40). These strains were isolated from the coastal zone of the Norwegian Sea and the Baltic Sea and were maintained as unispecies cultures in the Culture Collection of Baltic Algae (CCBA) at the Institute of Oceanography, University of Gdańsk, Poland.

Cultures were grown in f/2 medium (Guillard 1975) in 25-mL glass Erlenmeyer flasks that were swirled daily during the experiment. Culture media was prepared with Baltic Sea water filtered through glass microfiber filters (Whatman GF/C) and autoclaved. The salinity was 8 PSU, as measured with a salinometer (inoLab Cond Level 1, Weilheim in Oberbayern, Germany). The picocyanobacteria and microalgae were incubated under a 16:8 h light:dark cycle at Photosynthetically Active Radiation (PAR) irradiance 10 μmol photons m$^{-2}$s$^{-1}$ and temperature 20°C. The intensity of PAR was measured using a quantum-meter (LI-COR, Nebraska, USA) with a cosine collector.

Prior to the experiments, the concentrations of macronutrients (i.e., nitrate, N-NO$_3$ and P-PO$_4$ orthophosphates) in the picocyanobacterial cultures were measured to set them to the standard levels of f/2 medium. Nutrients were determined using spectrophotometric methods as described by Grasshoff (1976). Then, the concentrations of N-NO$_3$ and P-PO$_4$ in the controls and all treatments were adjusted to the same level as in the f/2 growth medium. The microalgae in the controls showed active growth during the course of the experiment. Therefore, the effects of major nutrients, microelements and vitamin limitations in the control and allelochemical treatments can be excluded. On the first and the last days of the experiment, the pH of all experimental flasks was measured using a pH-meter (Elmetron CP-
401, Zabrze, Poland). pH values were similar across treatments and durations and ranged from 8.2 to 8.6.

Test of the allelopathic effect of cell-free filtrates

Allelopathic effects were tested following a modified version of the method proposed by Suikkanen et al. (2004). Cultures of the donor picocyanobacterium *Synechococcus* sp. and the target microalgae were maintained in active growth during 7 days in standard conditions (see above) and then employed in the experiments. The picocyanobacterium culture was gently filtered through a 0.45-µm filter (Macherey-Nagel MN GF-5) using a vacuum pump (400 mbar). The filtrate obtained was analyzed under an epifluorescence microscope (Nikon Eclipse 80i, Nikon, Tokyo, Japan) in order to confirm the absence of picocyanobacteria cells.

Experimental treatments were prepared by adding 4 mL of this cell-free filtrate to 25-mL Erlenmeyer flasks containing 20 mL of cell suspensions of the target microalgae. These cell suspensions were obtained from culture aliquots. Controls were prepared by adding 4 mL of filtered f/2 medium to 25-mL Erlenmeyer flasks containing 20 mL of cell suspensions of the same microalgae species. These tests were conducted in triplicate. In all experiments, the ratio of donor picocyanobacteria to target species was adjusted to 1:1 based on the chlorophyll *a* (Chl *a*) content (the initial Chl *a* concentration in the experimental cultures was 0.8 µg Chl *a* mL⁻¹). The experiments lasted 7 days.

Test of the allelopathic effect in co-cultures

Allelopathic interactions in co-cultures were determined following a modified version of the method proposed by Ji et al. (2011). Employed cultures maintained in the conditions described above, 4 mL aliquots from the donor picocyanobacteria were added to 25-mL Erlenmeyer flasks containing 20 mL of cell suspensions of the target microalgae. Controls
were prepared by adding 4 mL of filtered f/2 medium to 25-mL Erlenmeyer flasks containing 20 mL of cell suspensions of the target microalgae. These tests were conducted in triplicate. In all these experiments, the initial ratio of donor picocyanobacteria to target species in Erlenmeyer flasks was adjusted to 1:1 based on the Chl a content (the initial chlorophyll a concentration in the experimental cultures was 0.8 µg Chl a mL⁻¹ for donor and target species). The experiments lasted 7 days.

Determination of cell abundances

The number of cells (N) in cultures was counted with flow cytometer BD Accuri™ C6 Plus (BD Biosciences, San Jose, CA, USA). Events were recorded in list mode. Samples were run at a flow rate of approximately 14 µl min⁻¹. Flow was daily calibrated with Spherotech 6- and 8- Peak Validation Beads (BD, San Jose, USA). This ensures that the cytometer is working properly before running experimental samples. FITC, PE, and PE-Cy5 detectors were daily calibrated with SPHERO™ Rainbow Calibration Particles (BD, San Jose, USA), and the APC channel was calibrated with SPHERO 6-peaks Allophycocyanin Calibration Particles (APC). Detectors FL1, FL2, and FL3 read fluorescence emissions excited by the blue laser (480 nm), while detector FL4 reads emissions excited by the red laser (640 nm). In the co-cultures, the populations of Synechococcus sp. and the corresponding target microalgae were completely discriminated using a combination of red and orange fluorescences (Fig. 1). N was determined in all the experiments at times 0 (1h) and 1st, 3rd and 7th day of the experiments.

Fig. 1

Statistical analyses

Different ANOVAs were performed in order to test the effect of picocyanobacterial filtrates or co-culture on the growth of the targeted microalgae. A post-hoc Dunnett’s test was used to
determine significant differences between the control and the other treatment levels. These statistical analyses were performed with Statistica® 13.1 software.

**Results**

*Allelopathic effect of cell-free filtrates*

The addition of cell-free filtrate obtained from *Synechococcus* sp. significantly affected the number of cells of *Porphyridium purpureum*, *Stichococcus bacillaris* and *Prymnesium parvum* (ANOVA, $F_{7,16} = 280.3$, $P < 0.001$, ANOVA, $F_{7,16} = 459.5$, $P < 0.001$ and ANOVA, $F_{7,16} = 279.5$, $P < 0.001$, respectively, Fig. 2A, B, C). Considering individual days, the allelopathic effect of the picocyanobacterium significantly reduced the number of cells of *P. purpureum* at day 7 (85% relative to the control; Dunnett, $P < 0.01$), and of *S. bacillaris* at day 3 (87% relative to the control; Dunnett, $P < 0.05$) (Fig. 2A, B). In contrast, the addition of cell-free filtrate obtained from *Synechococcus* sp. had a significantly positive effect on the growth of *P. parvum*, which, at day 7 was higher than the control by 19% (Fig. 2C; Dunnett, $P < 0.001$). The addition of the cell-free filtrate obtained from *Synechococcus* sp. had no effect on the target diatom *Nitzschia dissipata* (ANOVA, $F_{7,16} = 0.5$, $P = 0.7$, Fig 2D).

*Fig. 2*

*Allelopathic effects in co-cultures*

After the addition of *Synechococcus* sp. cells, it was observed not only a reduction, but also a decline with time in the number of cells of *Porphyridium purpureum* (ANOVA, $F_{7,16} = 1526.6$, $P < 0.001$, Fig. 3A). Significant differences were found on the first, third and seventh day of the experiment, when cell numbers of *P. purpureum* constituted 38% (Dunnett, $P < 0.001$), 17% (Dunnett, $P < 0.001$) and 1% (Dunnett, $P < 0.001$), of control. For *Stichococcus*
bacillaris, it was observed a reduction in the number of cells (ANOVA, \( F_{7,16} = 599.8, P < 0.001 \), Fig. 3B). By the first, third and seventh day of the experiment the growths of *S. bacillaris* were reduced by 80% (Dunnett, \( P < 0.001 \)), 86% (Dunnett, \( P < 0.01 \)) and 94% (Dunnett, \( P < 0.05 \)), respectively, relative to the control treatment. On the other hand, *Synechococcus* sp. cells had significantly positive effect on the growth of *Prymnesium parvum* (ANOVA, \( F_{7,16} = 491.1, P < 0.001 \), Fig. 3C). The number of cells of *P. parvum* significantly increased after third (Dunnett, \( P < 0.01 \)) and seventh (Dunnett, \( P < 0.001 \)) day of the experiment and constituted 114% and 131%, respectively, of control (Fig. 3C). No effects were detected in *Nitzschia dissipata* cultures (ANOVA, \( F_{7,16} = 1.2, P = 0.3 \), Fig. 3D).

![Fig. 3](image-url)

**Discussion**

There are very few reports of allelopathic compounds from phytoplankton that affect red algae. Just very recently, García-Espín *et al.* (2017) showed that both cyanobacteria extracts obtained from *Rivularia haematites* and *Rivularia biasolettiana* as well as pure microcystin affected the photosynthetic activity of *Chroothecia richteriana*. In this sense, our report of the strong negative effect of *Synechococcus* sp. on *P. purpureum*, particularly evident in the co-culture, is also a novel finding. *P. purpureum* was more strongly inhibited in co-culture compared to cell-free filtrate addition. This could be explained simply by the renewal of allelochemical compounds in the presence of cells.

Some studies have shown that green algae are a phytoplankton group that are particularly sensitivity to allelopathic compounds from cyanobacteria (Schlegel *et al.* 1999; Schagerl *et al.* 2002; Žak *et al.* 2012). The data presented here constitutes, to our knowledge, the first report of an allelopathic effect of a picocyanobacteria against a specific species of
chlorophyte. These inhibitory effects on *S. bacillaris* in monoculture and co-culture were stronger during the first days of exposition both with cell-free filtrates and co-culture. This suggests that this species may become resistant to these compounds through adaptation.

Previous studies did not find allelopathic effects from different cyanobacteria (*Nodularia spumigena*, *Aphanizomenon flos-aquae* and *Anabaena lemmermannii*) against *Prymnesium parvum* (Suikkanen et al. 2004). Our study shows that *Synechococcus* sp. affects *P. parvum* positively both through cell-free filtrates and co-culture. Suikkanen et al. (2005) also noted that cyanobacterial filtrates could promote the growth of some microalgae due to stimulatory allelochemicals. This could explain our results in the cell-free filtrate treatment. Also, osmotrophic feeding by *P. parvum* on exudates from *Synechococcus* sp. could be an explanation. In the co-culture treatment, the stronger positive effect could be explained by the higher concentration of exudates in the presence of *Synechococcus* sp. cells, but, it is also possible the occurrence of phagotrophic feeding by *P. parvum*, (Tillmann 1998).

Many authors showed different sensitivity of diatoms for the allelopathic compounds produced by cyanobacteria. In this work, the diatom *Nitzschia dissipata* was found to be tolerant to *Synechococcus* sp. cell-free filtrate and co-culture. Suikkanen et al. (2004) showed that *Thalassiosira weissflogii* was inhibited by cell-free filtrates of three cyanobacteria: *N. spumigena*, *A. flos-aquae* and *A. lemmermannii*. The tolerance of our species could be due to its relatively large cell size, as suggested by Lyczkowski and Karp-Boss (2014).

Our results suggest that allelopathic effects of *Synechococcus* sp. may have a differential effect in phytoplankton communities, due to the contrasting responses found among target species. The effects of picocyanobacteria on coexisting phytoplankton species is very important, since they account for the majority of primary producer biomass in the oceans, and their relative abundance is even expected to increase in scenarios predicted by global change (Dutkiewicz et al. 2015).
Acknowledgments

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References


Fig. 1. Cytograms obtained with a monocultures (a) and co-cultures with *Synechococcus* sp. (b) of four microalgae: *P. purpureum* (A), *S. bacillaris* (B), *P. parvum* (C) and *N. dissipata* (D) analyzed using a BD Accuri™ C6 Plus flow cytometer.
Fig. 2. The number of cells (N) for *P. purpureum* (A), *S. bacillaris* (B), *P. parvum* (C) and *N. dissipata* (D) for controls and experiments with additions of cell-free filtrate obtained from *Synechococcus* sp. cultures after 0 (1 h), 1, 3 and 7 days of exposure. The values refer to means (n = 3, mean ± SD). Asterisk indicates significant difference compared with control. Levels of significance were: *P < 0.05; **P < 0.01; ***P < 0.001.
**Fig. 3.** The number of cells (N) for *P. purpureum* (A), *S. bacillaris* (B), *P. parvum* (C) and *N. dissipata* (D) for controls and experiments with additions of *Synechococcus* sp. cultures after 0 (1 h), 1, 3 and 7 days of exposure. The values refer to means (n = 3, mean ± SD). Asterisk indicates significant difference compared with control. Levels of significance were: * P < 0.05; ** P < 0.01; *** P < 0.001.