Light-dependent cytolysis in the allelopathic interaction between picoplanktic and filamentous cyanobacteria

ALDO BARREIRO FELPETO1, SYLWIA ŚLIWIŃSKA-WILCZEWSKA2*, ILONA ZŁOCH3 AND VITOR VASCONCELOS1,4

1INTERDISCIPLINARY CENTER OF MARINE AND ENVIRONMENTAL RESEARCH—CIMAR/CIMAR, UNIVERSITY OF PORTO, AV. GENERAL NORTON DE MATOS s/n, 4450-208 MATOSINHOS, PORTUGAL, 2DIVISION OF MARINE ECOSYSTEMS FUNCTIONING, INSTITUTE OF OCEANOGRAPHY, UNIVERSITY OF GDANSK, AV. PIESUDSKIEGO 46, 81-378 GDYNIA, POLAND, 3DIVISION OF MARINE BIOLOGY AND ECOLOGY, INSTITUTE OF OCEANOGRAPHY, UNIVERSITY OF GDANSK, AV. PIESUDSKIEGO 46, 81-378 GDYNIA, POLAND AND 4DEPARTMENT OF BIOLOGY, FACULTY OF SCIENCES, PORTO UNIVERSITY, RUA DO CAMPO ALEGRE, 4069-007 PORTO, PORTUGAL

*CORRESPONDING AUTHOR: ocessl@ug.edu.pl

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Allelopathic compounds produced by cyanobacteria may play important roles in the dynamics of several biological systems. The main goal of this work was to investigate reciprocal allelopathic effects between species of two relevant groups of marine cyanobacteria: the picocyanobacterium *Synechococcus* sp. and the filamentous species *Nodularia spumigena*. Our experimental design consisted of cell-free filtrates and co-cultures. We demonstrated that *Synechococcus* sp. had a strong inhibitory effect on *N. spumigena*, and surprisingly, there was no reciprocal effect from the filamentous cyanobacteria. We detected this effect both in co-cultures and cell-free filtrate bioassays. These effects depended on light conditions in the culture of the allelopathic species. This allelopathic effect against *N. spumigena* triggered physiological responses leading to reduced chlorophyll *a* (Chl *a*) and carotenoid (Car) content, cell shape distortions and, often, cell lysis. Surprisingly, no evidence was found of allelopathic effects of our strain of *N. spumigena* (a well-known allelopathic species) against *Synechococcus* sp. These results support the fact that allelopathic interactions between *Synechococcus* sp. and *N. spumigena* may be a factor influencing the formation of massive bloom of the former organisms in many aquatic ecosystems, like the Baltic Sea, where the two species constitute a relevant fraction of phytoplankton biomass.

KEYWORDS: allelopathy; cyanobacteria; bloom; filamentous cyanobacteria; *Nodularia*; picocyanobacteria; *Synechococcus*; cytolysis; chlorophyll; carotenoid
INTRODUCTION

Frequency and strength of cyanobacterial blooms are increasing worldwide, a phenomenon explained by eutrophication and climate change as most important environmental drivers (O’Neil et al., 2013; Visser et al., 2016; Rybak and Gąbka, 2017). However, allelopathic interactions may also have an important role in bloom dynamics (Granéli and Hansen, 2006; Figueredo et al., 2007; Leflaive and Ten-Hage, 2007; Antunes et al., 2012; Rzymski et al., 2014). Cyanobacteria from marine and freshwater habitats are known to produce a diverse array of allelopathic compounds. However, the functional role of these compounds in cell physiology and ecological interactions remains largely unknown. A limited number of studies have suggested that some of these allelopathic compounds may be useful in inhibiting growth of coexisting species, and constitute a potential factor structuring phytoplankton communities (B-Béres et al., 2012; Leão et al., 2012; Almeida et al., 2015; Allen et al., 2017; Barreiro et al., 2017). At the same time, some allelopathic compounds could also affect potential grazers (Engström et al., 2001; Engström-Ost et al., 2011; Weissbach et al., 2011; Van Abstyn et al., 2014). The understanding of allelopathic interactions involving cyanobacteria is, therefore, very important.

Cyanobacteria are the most widely distributed group of oxygenic photosynthetic prokaryotes. They are, quantitatively, among the most important organisms on Earth (Whitton and Potts, 2012). Some species form dense blooms, which frequently occur in eutrophic freshwater bodies, such as lakes, ponds and slow-flowing rivers, but also in marine lagoons, bays and open waters, like the Baltic Sea (Whitton and Potts, 2012; Mazur-Marzec et al., 2013). In the Baltic Sea, the cyanobacterial community is mainly composed of filamentous nitrogen-fixing forms (i.e. the toxic Nodularia spumigena) and single-celled picocyanobacteria, represented by Synechococcus spp. (Mazur-Marzec et al., 2013). In the brackish waters of the Baltic Sea, summer blooms of filamentous cyanobacteria from the order Nostocales are recurrent phenomena. On the other hand, the contribution of picocyanobacteria to total cyanobacterial biomass is usually high during most of the summer period, ranging from 20 to 97% (Mazur-Marzec et al., 2013). The same authors reported that changes in biomass of filamentous cyanobacteria were positively correlated. However, no positive correlation was found between growth of Synechococcus and diazotrophic cyanobacteria, which frequently shift in dominance of the cyanobacterial community. These shifts towards a picocyanobacteria-dominated community might be due to changes in the environmental conditions of the Baltic Sea or the allelopathic activity of Synechococcus sp.

Because picocyanobacteria constitute the major part of marine primary production, there is an increasing concern about their importance in aquatic ecosystems (Callieri, 2010; Sorokin and Zakuskina, 2010; Flombaum et al., 2013; Jasser and Callieri, 2017). This group is also expected to be benefited in future scenarios driven by global change (Dutkiewicz et al., 2015). However, little information is available about their potential bioactivity and allelopathic effects (e.g. Costa et al., 2015; Śliwińska-Wilczewska et al., 2016; 2017). Furthermore, the occurrence of Synechococcus blooms in tropical and sub-tropical coastal systems as well as temperate waters has been rising (Wu, 1991; Philips et al., 1999; Beardall, 2008; Sorokin and Zakuskina, 2010). At the same time, the number of reports of the negative allelopathic effects of related organisms, like other cyanobacteria and microalgae, has been steadily increasing (e.g. Antunes et al., 2012; Barreiro and Hairston, 2013; Barreiro and Vasoncelos, 2014; Zak and Kosakowska, 2015; Wang et al., 2017).

The filamentous nitrogen-fixing cyanobacteria Nodularia spumigena is a relevant species in brackish and hypersaline environments, but also low-salinity seas, like the Baltic Sea. Blooms of this species are frequent, and hepatotoxic cyclic pentapeptides, the nodularins, are the most common cyanobacterial toxins that have been identified in Baltic Nodularia spumigena (Sivonen et al., 1989; Mazur-Marzec et al., 2015). The allelopathic activity of this species against other co-occurring phytoplankton species is also well known (Stuikkanen et al., 2004; 2006; Śliwińska and Latala, 2012; Zak et al., 2012). Several works reported no positive correlation between nodularin production and allelopathic effects of Baltic N. spumigena (Engström-Ost et al., 2002; Stuikkanen et al., 2006). Hepatotoxins produced by cyanobacteria are released into the environment mostly due to cell degradation, while the strongest allelopathic effect of N. spumigena has been reported during the exponential growth phase. The chemical nature of N. spumigena allelochemicals, however, remains unknown.

Irradiance may play a dominant role in the ecosystem of the Baltic Sea during the summer period. Furthermore, irradiance is one of the major factors controlling growth, photosynthetic activity and distribution of picocyanobacteria (Jodłowska and Śliwińska, 2014; Jasser and Callieri, 2017) as well as filamentous cyanobacteria N. spumigena (Jodłowska and Latala, 2010; Mazur-Marzec et al., 2013). Jodłowska and Śliwińska (2014) demonstrated that optimal irradiance for three Baltic Synechococcus sp. strains was relatively low (10 μmol photons m⁻² s⁻¹). Baltic Synechococcus and Nodularia strains, however, showed tolerance to a wide range of irradiances, to which they can acclimate by changing their pigment composition (Jodłowska and Latala, 2010; Jodłowska and Śliwińska, 2014). This explains why Baltic Synechococcus sp. and N. spumigena grow successfully in both well-illuminated surface waters and deeper waters (Stal et al., 2003). The way external factors regulate secondary
metabolite production in cyanobacteria is still unknown. However, some cases showed that irradiance could play an important role in the regulation of allelochemical production in some cyanobacteria (Pedrol et al., 2006; Antunes et al., 2012).

Despite the co-occurrence of these ecologically relevant picoplanktonic and filamentous groups of cyanobacteria in aquatic ecosystems, very little is known about their reciprocal allelopathic interactions. A better understanding of the allelopathic interaction between these cyanobacteria could contribute to explain the taxonomic and functional succession in phytoplankton, and also the rising phenomenon of cyanobacterial blooms.

The aim of this work was to study the reciprocal allelopathic effect between two co-occurring bloom-forming species of cyanobacteria: the picocyanobacterium Synechococcus sp. and the filamentous N. spumigena, and the role of light irradiance in this process. These allelopathic interactions were studied with cell-free filtrates and co-cultures under different light irradiance regimes. We hypothesized that (i) the effect of N. spumigena (a species well known to be allelopathic) would be more pronounced to single-celled picocyanobacterium Synechococcus than vice versa, (ii) the allelopathic effects would be more pronounced in the co-cultures than in the cell-free filtrate experiments and (iii) the mechanism of action of allelopathy would be evident in terms of cell damage and loss of pigments.

**METHOD**

**Cyanobacteria strains and culture conditions**

The strains *Synechococcus* sp. BA-124 and *Nodularia spumigena* BA-15 were isolated from the coastal area of the Gulf of Gdańsk (southern Baltic Sea) and are maintained as monoclonal cultures in the Culture Collection of Baltic Algae (CCBA) at the Institute of Oceanography, University of Gdańsk, Poland (http://ccba.ug.edu.pl). Cultures to be employed in the experiments were grown in f/2 medium (Guillard, 1975) in 25 mL glass Erlenmeyer flasks that were swirled daily during the experiments. Culture media was prepared with Baltic seawater filtered through glass fiber filters (Whatman GF/C) and autoclaved. Salinity was 7 PSU, measured with a salinometer (inoLab Cond Level 1, Weilheim in Oberbayern, Germany).

**Test of the allelopathic effect of cell-free filtrates**

Allelopathic interactions were studied in monocultures based on a modified version of the method of Suikkanen et al. (2004). An amount of 10 mL of cell-free filtrate from donor cyanobacterial cultures (which density was $10^7$ cells mL$^{-1}$ for *Synechococcus* sp. and $10^6$ cells mL$^{-1}$ for *N. spumigena*) was added to the target cyanobacterial cultures (see below and Fig. 1). Donor cyanobacterial cultures were incubated at 18°C under 16:8 h light:dark cycle at two, non-saturating (10 μmol photons m$^{-2}$ s$^{-1}$) and also saturating (190 μmol photons m$^{-2}$ s$^{-1}$) regimes of PAR (irradiances above 200 μmol photons m$^{-2}$ s$^{-1}$ inhibit the growth of these strains). The intensity of PAR was measured using a quantum-meter (LI-COR, Nebraska, USA) with a cosine collector. The cultures were kept growing under these conditions during 7 days. Afterwards, they were gently filtered through 0.45 μm pore size filters (Macherey-Nagel MN GF-5) using a vacuum pump (400 mbar). The filtrates were observed under an epifluorescence microscope (Nikon Eclipse 80i, Nikon, Tokyo, Japan) in order to confirm the absence of cyanobacterial cells. Target cyanobacterial cultures were grown under the same conditions, with a single PAR regime, 10 μmol photons m$^{-2}$ s$^{-1}$. All cultures were in exponential phase at the start of the experiment. Experimental treatments were prepared by adding 10 mL of the cell-free filtrate to 25 mL Erlenmeyer flasks containing 10 mL of cell suspensions of the targeted cyanobacteria. Controls consisted of 10 mL of filtered f/2 medium and 10 mL of cell suspensions of the same cyanobacteria species. Tests were conducted in triplicate. In all experiments, the ratio of donor cyanobacteria to target species was adjusted to 1:1 based on the Chl $a$ content (final Chl $a$ concentration in the experimental cultures was 0.8 μg Chl $a$ mL$^{-1}$). To simulate the effects of continuous release of allelochemicals, an additional experimental set was used. These cultures were performed by the daily removal of 2 mL of culture. The volumes removed were replaced by fresh cell-free filtrate in the case of experimental treatments and filtered f/2 medium in the case of control cultures. Other conditions were the same as above. The experiments were run in triplicate and lasted 7 days.

Prior to the experiments, the concentration of major inorganic nutrients in the cultures (nitrate-nitrogen: N-NO$_3$ and orthophosphate: P-PO$_4$) was measured using spectrophotometric methods described by Grasshoff (1976). Then, the concentrations of N-NO$_3$ and P-PO$_4$ were adjusted to the same level as in the f/2 medium. These nutrient concentrations of the cultures were also measured at the end of the experiment, using the same method. The initial and final concentrations of these nutrients are shown in Table 1. On the first and last day of the experiment, pH was measured in all replicates (pH-meter, Elmetron CP-401, Zabrze, Poland). The pH did not differ between the treatments and the control, and ranged between 8.0 and 8.4.
Test of the allelopathic effect in co-cultures

Allelopathic interactions in co-cultures were studied following a modification of the method proposed by Ji et al. (2011); allelopathic activity was tested by adding donor cyanobacterial culture to target cyanobacterial culture (Fig. 1). Both donor and target species were cultured under the same light conditions for the control and the allelopathy treatment levels (10 and 190 μmol photons m⁻² s⁻¹). Temperature was 18°C and salinity 7 PSU. About 10 mL of culture from the donor cyanobacteria were added to 25 mL Erlenmeyer flasks containing 10 mL of culture from the target cyanobacteria. Experimental controls were prepared by adding 10 mL of filtrated f/2 medium to 25 mL Erlenmeyer flasks containing 10 mL of monocultures of Synechococcus sp. and N. spumigena. All cultures employed in this step were in exponential phase. In all these experiments, the initial ratio of donor cyanobacteria to target species in Erlenmeyer flasks was adjusted to 1:1 in the same way as detailed above for the cell-free filtrate experiments. The experiments were run in triplicate and lasted 7 days.

Prior to the experiments, the concentrations of major inorganic nutrients (nitrate, N-NO₃ and orthophosphates, P-PO₄) was set to that of f/2 medium in the same way as described above. Initial and final nutrient concentrations are shown in Table I.

Calculation of cell densities and relative abundances

Cell densities in the monocultures were estimated with previously determined linear regression models between the cell abundance (N mL⁻¹) and optical density (OD) (Jodłowska and Latałowa, 2010; Jodłowska and Sliwińska, 2014). Synechococcus sp. cells were counted under a light microscope (Nikon Eclipse 80i, Nikon, Tokyo, Japan, at a magnification of 1000×) in a Bürker counting chamber (48 squares per count) following a procedure according to Guillard and Sieracki (2005). Cell abundance of N. spumigena was estimated from filament size data. We considered a filament unit as 100 μm of filament. We counted the number of filament units and later these filament units were converted to cell numbers (1 filament unit = 20 cells). OD was measured spectrophotometrically at 750 nm with a Multiskan GO UV–VIS spectrophotometer (Thermo Scientific, MA, USA). These data were used to fit a linear regression model between the variables cell abundance and OD. For Synechococcus sp. and N. spumigena, the correlation coefficients for their respective linear regression models were r = 0.99, and r = 0.98, respectively, and the model equations: y [N mL⁻¹] = 92.7 × 10⁶ × −4.0 × 10⁵ and y [N mL⁻¹] = 20 × 12.4 × 10⁵ × −6.0 × 10³, respectively, where y = number of cells and x = OD. The change in relative abundances of Synechococcus sp. and N. spumigena in
co-cultures was estimated by directly counting the number of cells (Guillard and Sieracki, 2005). N was estimated in all the experiments performed at times 0 (1 h) and first, third and seventh day of the experiments.

### Study of allelopathy effects on cell status

Cell status (morphology, integrity) of both species was examined under the microscope for the treatment level of 190 μmol photons m⁻² s⁻¹, after seventh day of exposure, both in the single and repeated filtrate addition experiments. We chose only this treatment level because it was the one showing stronger allelopathic effect (see Results section) and our aim was to deepen our knowledge about the mechanism causing allelopathy, in this case, to check whether if there was a mechanism leading to cell lysis. Two categories were established a priori: “healthy cells” (those with undamaged cell walls and keeping 75–100% cytoplasm) and “damaged cells” (those with visible damage in cell wall and keeping less than ~75% cytoplasm). Independent from the cell status, 300 cells were counted in all cultures. We employed a light and epifluorescence microscope (Nikon Eclipse 80i, Nikon, Tokyo, Japan) equipped with a camera Nikon DS U2, Plan Apo VC 100 objective and a epifluorescence module with UV-2A/B-2A/G-2A block filters. This fluorescence is widely used in plant physiology as an indicator of the condition of chloroplasts and algal cells (Maxwell and Johnson, 2000; Boluda et al., 2014).

### Measurements of pigment content

The concentration of photosynthetic pigments was analyzed in both species for the treatment level of 190 μmol photons m⁻² s⁻¹, after the seventh day of exposure, both in the single and repeated filtrate addition experiments. We chose only this treatment level because it was the one showing stronger allelopathic effect (see Results section) and our aim was to deepen our knowledge about the mechanism causing allelopathy, in this case, whether if there is a potential reduction in photosynthetic efficiency. Chl a and carotenoids (Car) were extracted with cold 90% acetone in the dark for 2 hr at −60°C. To remove cell debris and filter out particles, these extracts were centrifuged at 13,000 rpm for 2 min (Sigma 2-16 P, Osterode am Harz, Germany). The extinction values were determined at 480, 664 and 750 nm with a DU530 UV–VIS Life Science spectrophotometer ( Beckman, CA, USA). The concentration of Chl a and Car was calculated according to Strickland and Parsons (1972).
Statistical analyses

In the data from the cell-free filtrate addition and co-culture experiments, a general linear model (GLM) with the independent variables “filtrate addition”, “light intensity”, “time” and their interactions was fitted to the response variable “cell abundance”. Least significant difference post hoc tests were performed to find which individual level combinations of the different factors differed from the control on each day. A Mann–Whitney test was applied to test for differences in cell status between the control and treatment. Differences in pigment content were analyzed with independent variables with P < 0.1.

RESULTS

Growth of cyanobacteria in cell-free filtrates and co-cultures

Both in the case of the single addition and the repeated addition experiments, the “filtrate addition” showed significant negative effect in the daily abundances of N. spumigena (Table II and Fig. 2). In the experiment with a single addition of filtrate, the significant effect was mainly due to the data from the seventh day, when the cell abundance of N. spumigena cultures grown under 10 and 190 μmol photons m−2 s−1, constituted, relative to the control, 75% (post hoc test, P < 0.001) and 65% (post hoc test, P < 0.001), respectively. In the experiment with the repeated addition of filtrate, a significant effect was found only at 190 μmol photons m−2 s−1 on the seventh day, when N. spumigena cell abundance constituted, relative to the control, 70% (post hoc test, P < 0.001). However, light was not found to be significant in these cases (although in the repeated addition experiment, it was close, see Table II). The effect of time, as a numeric covariate, was always significant, and also its interaction with the factor “filtrate addition”, as it could be expected (Table II). The co-culture experiment showed the same negative effect of Synechococcus against N. spumigena, but stronger than both filtrate addition experiments (Table II and Fig. 3). In this case, light also had a significant effect, as well as its second and third order interactions (Table II). Here, the allelopathic effect was stronger with Synechococcus grown at 190 μmol photons m−2 s−1. In the 10 μmol photons m−2 s−1 treatment level, the effect was significant by the seventh day of the experiment, when the cell abundance of N. spumigena constituted 76% (post hoc test, P < 0.001) relative to the control treatment (Fig. 3A). For the 190 μmol photons m−2 s−1 treatment level, significant differences were found on the third and seventh day of the experiment, when cell abundance of N. spumigena constituted 77% (post hoc test, P < 0.001) and 57% (post hoc test, P < 0.001), respectively, of the control (Fig. 3B). On the other hand, no reciprocal effect from N. spumigena against Synechococcus was found in the experiments with addition of filtrate or co-culture (Figs 2 and 3 and Table II). In the coculture experiment, under 190 μmol photons m−2 s−1, it was even possible to detect a weak positive effect of the filamentous cyanobacteria, since the abundance of Synechococcus sp. was a bit larger than the control in the seventh day of the experiment (post hoc test, P < 0.01), constituting 110% of the control (Fig. 3B). In all these experiments, the macronutrients consumed were relatively low (Table I).

Table II: Results of GLMs

<table>
<thead>
<tr>
<th>Response variable (number of cells)</th>
<th>Independent variable</th>
<th>F</th>
<th>df (factor, total)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SYN single</td>
<td>Time</td>
<td>3208</td>
<td>1, 47</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SYN repeated</td>
<td>Time</td>
<td>516</td>
<td>1, 47</td>
<td>&lt;0.001</td>
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<tr>
<td>NOD single</td>
<td>Filtrate</td>
<td>38.33</td>
<td>1, 47</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>734</td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>NOD repeated</td>
<td>Filtrate</td>
<td>30.2</td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Light</td>
<td>3.26</td>
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<td>0.08</td>
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<tr>
<td></td>
<td>Time</td>
<td>111</td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SYN co-culture</td>
<td>Light</td>
<td>3.16</td>
<td>1, 47</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>Time</td>
<td>1337</td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Time x light</td>
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<td></td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>NOD co-culture</td>
<td>Filtrate</td>
<td>38.2</td>
<td>1, 46</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>Light</td>
<td>13.8</td>
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<td>&lt;0.05</td>
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<td></td>
<td>Time</td>
<td>1390</td>
<td></td>
<td>&lt;0.001</td>
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<td></td>
<td>Filtrate x time</td>
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<td>0.15</td>
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<td>Filtrate x light</td>
<td>7.86</td>
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<td>Filtrate x time x light</td>
<td>9.60</td>
<td></td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

SYN, Synechococcus sp.; NOD, Nodularia spumigena; single, single addition of filtrate; repeated, repeated addition of filtrate. Showing only independent variables with P < 0.1.
Effects of cyanobacterial filtrates on cell status

The allelopathic effect from *Synechococcus* sp. had a visible effect in the morphology of *N. spumigena* cells, causing the collapse of large portions of filaments (Fig. 4A). Cell bleaching and deformation could be observed with light microscopy, and, under epifluorescence microscopy, degeneration of thylakoids. In the experiment with a single addition of filtrate, the control contained, on average, 93% of healthy cells, whereas the treatment 86% (*P* < 0.001). In the experiment with repeated addition of filtrate, cell degeneration and lysis were more evident, and the control contained an average of 95% of healthy cells whereas the treatment, 28% (*P* < 0.001). In contrast, no reciprocal effect was observed using cell-free filtrate from *N. spumigena* against the picocyanobacterium *Synechococcus* sp. (*P* > 0.05 for all, Fig. 4B).

**DISCUSSION**

Light-dependent regulation in the allelopathic interaction

The present study highlighted that this strain of the brackish picocyanobacterial *Synechococcus* can inhibit the growth of the co-occurring filamentous cyanobacteria *Nodularia spumigena*. This inhibition was observed both with cell-free extracts and co-cultures. Our results, coincident with field observations from Mazur-Marzec et al. (2013), suggest that picocyanobacterial allelopathic effects play a significant role in structuring phytoplankton communities (Stal et al., 2003).

Other authors also reported negative allelopathic effects of picocyanobacteria. Costa et al. (2015) demonstrated that marine picocyanobacterium *Cyanobium* sp. inhibited *Nannochloropsis* sp. growth. Paz-Yepes et al. (2013) have found a clear effect of growth impairment by two strains of *Synechococcus* sp. when they were cultured in the...
The majority of allelopathic interactions are studied either in co-culture experiments, or more often, through the exposure of target species to cell-free filtrates from the donor species. In the present work, both approaches were employed, showing that the highest degree of inhibition of *N. spumigena* was detected in co-culture. *Suikkanen et al.* (2004) noted that, in the natural environment, phytoplankton are constantly exposed to chemicals released by other species, but in batch culture experiments, involving just an initial filtrate addition, the effect may be lost after some time of exposure, due to allelochemical degradation and recovery of the target species. *Vardi et al.* (2002), *Mohamed* (2008) and *B-Béres et al.* (2012, 2015) also showed that the strongest allelopathic effect was recorded in co-cultures. It seems obvious that when organisms are cultured together, production and release of bioactive compounds to the medium is continuous, causing stronger allelopathic effects. Nevertheless, this simple relation between the presence of "allelopathic cells" and induction of a stronger allelopathic effect is not straightforward. For instance, the well-known allelochemical producer *Phormidium* sp. had no significant allelopathic effect in co-cultures, but the extract of this taxa significantly decreased the diversity of target community (*Dias et al.*, 2017). This simply means that allelochemical production is subjected to regulation by environmental factors, and hence, it could be downregulated under specific circumstances. In our case, *Synechococcus* sp. allelochemicals were effective in all cases, but were was stronger in co-culture, meaning that, whatever factors are controlling their production, they had a positive effect under our experimental conditions.

In the co-culture experiments, however, it is not possible to completely discriminate between allelopathy and competitive interactions for light, nutrients, etc. In these competitive interactions, *Synechococcus* sp. strains would be expected to be, in the majority of cases, a stronger competitor than *Nodularia spumigena*. However, from the nutrient data shown in Table 1, we can conclude that, at least for macronutrients, competition was not strong, since small amounts of nutrients were consumed. This explains the relatively reduced increases in biomass observed during the experiments, which always remained within the same order of magnitude, even in the controls (Figs 2 and 3).

Remarkably, the highest level of irradiance induced a stronger effect, although it was not always significant (Figs 2 and 3). In the experiment with repeated addition of filtrate, a significant effect of *Synechococcus* sp. on *N. spumigena* was found only at the highest light level. In the experiment with single filtrate addition, however, the negative effect was observed in both light levels. This could be explained by the fact that, due to repeated addition of filtrate, the "control" was also diluted daily, and its growth reduced when compared to the "control" from the experiment with single addition (Fig. 2). Then, what happened in the single addition experiment is that there was greater numerical difference between the "control" and the levels with filtrate addition, and statistically significant differences were then easier to find in this case. During the experiment, all the physical and chemical factors were constant while light conditions were set at different levels. This is why it is possible to state that the regulation of allelochemicals produced by *Synechococcus* strain is light-dependent. But it requires further examination. However, other authors also reported that high light positively affects the production of...
Allelopathic compounds by cyanobacteria (Antunes et al., 2012; Śliwińska-Wilczewska et al., 2016). Moreover, Dyble et al. (2006) suggested that light influences production of secondary metabolites and may have an effect in the development of harmful cyanobacterial blooms.

Allelopathic effects on cell status and pigment content

Our results show that allelochemicals from *Synechococcus* sp. can cause not only significant reduction of pigment content of *Nodularia* but finally, total cell lysis of filamentous cyanobacteria can occur due to these allelopathic compounds. This significant increase in cell lysis suggests that the loss of pigments is a secondary effect of cell deterioration, and that *Synechococcus* allelochemicals activate mechanisms leading to cytotoxicity. Other authors have also reported cyanobacterial filtrates causing cell deterioration in competing species (Valdor and Aboal, 2007; Gantar et al., 2008). A specific effect of cyanobacterial allelochemicals on pigment content, which does not seem to be our case, was suggested by Suikkanen et al. (2006).

Allelopathic interactions between picoplanktonic and filamentous cyanobacteria

No inhibitory effect of *N. spumigena* against *Synechococcus* sp. was detected in our experiments. This is remarkable, since this filamentous species is well known to be allelopathic, and this strain in particular was found to be allelopathic against the diatom *Skeletonema marinoi* (Śliwińska and Latala, 2012). More surprisingly, the abundance of picocyanobacterial cells was even a bit greater in the treatment than in the control at the end the co-culture experiment (Fig. 3). This could be simply due to the release of certain nutrients by *N. spumigena*, or to the release of stimulatory allelochemicals. Several authors have previously reported that *N. spumigena* could cause negative allelopathic effects. In previous studies, we have shown that the same *Nodularia* strain had inhibitory allelopathic activity on the diatom *Skeletonema marinoi* and that the production of the unknown allelopathic substances was influenced by the temperature and growth phase (Śliwińska and Latala, 2012). Suikkanen et al. (2004) also found that *N. spumigena* filtrates caused growth inhibition.
owing sensitivity–resistance between phytoplankton species might reflect an effect of group dynamics during succession, and not just adaptation to co-occurring species.

We investigated allelopathic activity using cultures under non-axenic conditions. There is growing evidence that interactions between phytoplankton and associated bacteria or viruses, constitute an important ecological relationship in aquatic environments (Seymour et al., 2017; Schatz and Vardi, 2018). The exchange of metabolites in the microenvironment, known as the phycosphere, drives different kinds of phytoplankton–bacteria relationships; mutualism, commensalism, antagonism, parasitism and competition (Seymour et al., 2017). Given that no chemical measurements of allelochemicals were conducted, it cannot be discarded that our results were influenced by bacteria and/or viruses associated to Synchococcus sp., and not the picocyanobacterium itself. However, in the best studied cases, heterotrophic bacteria do not play any role in production of allelopathic substances (e.g. Tillmann and John, 2002; Suikkanen et al., 2004).

Despite our demonstration of allelopathic effects, ours is just a laboratory experimental study. Laboratory experiments have the main advantage of being able to manipulate variables in order to obtain a clear demonstration of principles. However, the concomitant isolation from the natural environment, where many factors are interacting, makes these kinds of experiments inadequate to straightforward extrapolations. In this sense, before considering how to extrapolate our observations to real conditions in the Baltic Sea, we need to take into account that we are missing, fundamentally, the effects of allelochemical dilution due to lower cell abundances, and the potential effect of environmental factors not tested here (i.e. nutrients, temperature) on the rates of allelochemical production. An interesting prospect for future research would be to extend the present experiments to a real field situation in the Baltic Sea. An ideal pre-requisite would be to identify the chemical nature of Synchococcus sp. allelochemicals. Then, as soon as it is available an efficient method for allelochemical detection, field mesocosm experiments could be performed. These experiments would provide very useful information to determine the relevance of allelopathy interactions in this phytoplankton community.

CONCLUSIONS

A strain of the picocyanobacteria Synchococcus sp. showed an inhibitory allelopathic activity against the co-occurring filamentous cyanobacteria Nodularia spumigena both in cell-free filtrate treated cultures and in co-cultures. Our findings also pointed towards the existence of light-dependent production of allelochemicals in this species. The allelochemicals
from Synechococcus sp. caused significant reduction of pigment content and cell damage of Nodularia spumigena. Conversely, Synechococcus was resistant to this strain of the filamentous cyanobacteria, a well-known allelopathic species. This allelopathic interaction may constitute a mechanism to explain the formation of monospecific picocyanobacterial blooms, since both species are relevant components of phytoplankton communities in many aquatic ecosystems.

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REFERENCES


