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High pH and not allelopathy may be responsible for negative effects of *Nodularia spumigena* on other algae

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ABSTRACT: The bloom forming marine cyanobacterium *Nodularia spumigena* Mertens produces the toxic compound nodularin. The production and excretion of nodularin during growth were measured in a monoculture of *N. spumigena* and in a mixed culture with the cryptophyte *Rhodomonas salina*. Most of the toxin produced was found intracellularly, and extracellular nodularin was not detected until the stationary growth phase. The presence of *R. salina* did not significantly affect the production and excretion of nodularin by *N. spumigena*. Addition of pure nodularin did not affect the growth of a monoculture of *R. salina*. Similarly, no allelopathic effects were found when *N. spumigena* was tested in mixed culture experiments with 6 different phytoplankton species. Instead of being inhibited by an allelopathic compound, the growth of the test algae was controlled by the pH level in the culture media and by the pH tolerance of each species involved. Because *N. spumigena* can elevate pH up to very high levels (10.6), it out-competed 5 of the 6 phytoplankton species. This suggests that the high pH tolerance of *N. spumigena* in natural environments might be important for *N. spumigena* in the competition with other phytoplankton species.

KEY WORDS: Allelopathy · *Nodularia spumigena* · Nodularin · pH · Blooms · Cyanobacteria

INTRODUCTION

The cyanobacterium *Nodularia spumigena* Mertens is a common component of the phytoplankton in brackish and marine waters, like the Baltic Sea and Danish waters, and the cyanobacterium grows well at salinities between 0 and 20 PSU (Kononen et al. 1993, Kankaanpää et al. 2001, Moisander et al. 2002, Henriksen 2005). The blooms of *N. spumigena* are often dense, and only a few other phytoplankton species co-occur in such cases (Wasmund 1997). The water containing the blooms may both appear and smell unpleasant, destroying the recreational use of the water body. In addition, *N. spumigena* strains from various places in the world, including the Baltic Sea, produce a very potent liver toxin, nodularin, with an LD_{50} of 60 to 70 µg kg^{-1} (mouse, intraperitoneal; Carmichael et al. 1988, Runnegar et al. 1988, Laamanen et al. 2001). The toxin inhibits the protein phosphatase of liver cells, causing the collapse of the cytoskeleton and subsequent haemorrhage of the liver of animals (Kuiper-Goodman et al. 1999).

Dense blooms of *Nodularia spumigena* and cyanobacteria in general are associated with a number of different factors. The most important factors are eutrophication, low N:P ratios favouring growth of diazotrophic (N_2-fixing) cyanobacteria, e.g. *N. spumigena* (Mur et al. 1999), periods of warm and calm weather with high irradiance and a stratified water column (Paerl 1988). The stratified water column prevents a fast exchange of nutrients and dissolved inorganic carbon (DIC) between deeper water layers and the surface water, and it also prevents fast exchange of CO_2 between the atmosphere and the surface water. Periods of high photosynthetic activity will eventually lead to a depletion of DIC and thus an elevation of pH

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in the vicinity of the photosynthetic organisms (Paerl 1988). The sensitivity of the pH in the water to changes in DIC concentrations, i.e. the buffering capacity, is determined by the total concentration of DIC in the water. Since seawater generally has a higher concentration of DIC than freshwater, the pH of seawater is less sensitive to changes in DIC (Chen & Durbin 1994). High pH levels in freshwaters have indeed been measured during blooms of cyanobacteria (An & Jones 2000, López-Archilla et al. 2004), but only a few similar recordings of cyanobacteria from marine environments exist (Wasmund & Heerkloss 1993, Wasmund 1996). Recent investigations provide further data supporting the assumption that elevated pH levels occur in marine environments and therefore are important for phytoplankton growth and succession (Hinga 1992, 2002, Hansen 2002, Pedersen & Hansen 2003, Lundholm et al. 2004, 2005).

In addition to the importance of abiotic factors for the success of cyanobacteria, some species have been reported to produce bioactive compounds that affect the growth of other phytoplankton species (Keating 1977, 1978, Mason et al. 1982, Smith & Doan 1999). The phenomenon is termed allelopathy and is defined as the ability of a plant to affect another plant or microorganism in a positive or negative way, by excreting a secondary metabolite (Rice 1974). Studies on allelopathy often focus on the inhibiting effects of allelopathic interactions. An alga with inhibiting allelopathic abilities has an obvious advantage compared to the species it affects, and this might therefore be a mechanism enhancing the proliferation of cyanobacterial blooms in aquatic environments. Among cyanobacteria, species of the genera *Microcystis*, *Fischerella*, *Nodularia*, *Calothrix* and *Nostoc* among others have been reported as allelopathic (Gross et al. 1991, Pushparaj et al. 1999, Schlegel et al. 1999, Sukenik et al. 2002), but the extent to which allelopathy plays a role in succession among algal groups in natural environments is still debated. It has been argued that a massive development of cyanobacteria and a coinciding decline of green algae in an Austrian-Hungarian lake was partly due to allelopathy and partly to eutrophication (Schagerl et al. 2002). Similar shifts in the dominance of phytoplankton communities from green algae to cyanobacteria have, however, also been argued to be a response to elevated pH levels (Shapiro 1990).

A fundamental problem in most reports on allelopathy among cyanobacteria is that they are based on experiments with cell extracts. In such cases the test organisms are affected by the intracellular content of the cyanobacterium, not the extracellular content in the media. The concentration of secondary metabolites in the cell extracts might be higher than in the growth media, and the extract might even contain compounds not found in the media. This makes the extrapolation to natural conditions difficult, since only compounds released into the surroundings by the cyanobacterium can potentially affect the test organisms. Cyanobacteria produce numerous bioactive metabolites, with different modes of action (Patterson et al. 1994, Smith & Doan 1999), and if *Nodularia spumigena* has allelopathic effects on other phytoplankton species, these effects would be caused by secondary metabolites released by the cyanobacterium. Nodularin might be one of these allelopathic substances, but other compounds could also be responsible for potential allelopathic effects of *N. spumigena.*

Several factors must be considered when studying interactions among phytoplankton species in the laboratory. Nutrients and vitamins must, of course, be added in ample supply to the culture media to avoid nutrient limitation. In addition, when DIC is removed from the culture media during photosynthesis, pH in the culture media will increase as mentioned above. This demands a thorough supervision of pH in the cultures, because cyanobacteria can drive the pH up to very high levels. Otherwise, conclusions of allelopathic effects could be based on false assumptions.

The mechanisms involved in the initiation and maintenance of *Nodularia spumigena* blooms are many, and the importance of allelopathy and pH in these blooms is not well described. On that basis, our aims were to examine the potential allelopathic effects of nodularin and any other substances that *N. spumigena* might produce and excrete. Furthermore, we examined the effects of elevated pH during photosynthesis on the interaction between *N. spumigena* and the test algae.

MATERIALS AND METHODS

Organisms and culture conditions. All phytoplankton cultures were non-axenic and clonal. Data on strain designation, place and date of origin as well as sources of strains are listed in Table 1. It is important to stress that all test species come from the Baltic Sea and inner Danish waters with the exception of *Phaeodactylum tricornutum*. However, this species can be found in checklists from the area. All algae were cultured in B1 medium (Hansen 1989) based on pasteurised seawater with a salinity of 15 PSU. The medium was modified using 0.5 ml vitamin stock solution per liter of seawater. In all experiments the DIC concentration was 2 ± 0.03 mM.

The organisms were cultured on a plankton wheel with approximately 2 rotations min^{-1} at 15 ± 2°C with a light:dark cycle of 16:8 h and an irradiance of 65 µmol photons m^{-2} s^{-1}. Cool fluorescent lamps provided the

light, and the irradiance was measured using a Li-1000 Li-Cor sensor equipped with a Li-193SA spherical Quantum probe. A fan, directed towards the plankton wheel, prevented elevation of temperatures in the culture bottles due to heat from the light source. Experiments were started when the batch cultures, acclimated to the experimental conditions, showed exponential growth. The test organisms were chosen based on their ability to tolerate high pH levels. Since *Nodularia spumigena* can elevate pH to 10.6 in monoculture, allelopathic effects would be difficult to detect using test species with a low pH tolerance.

Measuring phytoplankton growth. Phytoplankton growth was determined by enumerating the number of algae in samples fixed in Lugol's solution using a Sedgewick-Rafter counting chamber and an inverted light microscope (Olympus). The concentration of the test algae was defined as cells ml^{-1}, while trichomes ml^{-1} defined the concentration of *Nodularia spumigena*. To determine whether cell number or trichome number per volume should be used for quantification of *N. spumigena*, a pilot study was carried out prior to the experiments. A monoculture of *N. spumigena* was incubated for 32 d. Samples were withdrawn 13 times and the number of cells per trichome was determined in 40 to 70 trichomes. Furthermore, in 1 experiment, growth of *N. spumigena* was measured as fluorescence emitted by the culture as a function of time, using a fluorometer (Turner Designs). A regression analysis was used for comparison between trichomes ml^{-1} and fluorescence.

In experiments on nodularin production and allelopathic effects of *Nodularia spumigena* (see below), trichome and/or cell concentration in the cultures were diluted at every sampling due to the addition of new growth media. To compensate for this, both trichome and cell concentrations as well as nodularin concentrations ($\mu g\ l^{-1}$) were estimated by multiplying the counted/measured concentrations by a dilution factor, powered by the number of samples since start.

Regression analyses were performed on the exponential growth phase of the control and the mixed cultures in a study on nodularin production and in a study on allelopathic effects of *Nodularia spumigena* (see below). The slope of the regression line (ln [nodularin concentration l^{-1} or cells ml^{-1} or trichomes ml^{-1}] vs. time [d]) equals the nodularin production rate or the growth rate of the culture in the exponential growth phase.

Nodularin production and excretion. In the experiment on production and excretion of nodularin, *Nodularia spumigena* was added to 24 Nunc® bottles (265 ml): 12 bottles containing monocultures and 12 containing mixed cultures with *Rhodomonas salina*. *N. spumigena* was added to reach an initial concentration of 4000 trichomes ml^{-1}. Samples of 12.5 ml (4.7 % of the total volume) were taken at 1 to 2 d intervals, and fixed in Lugol's solution. Subsequently, pH in the culture was measured using a pH meter (Radiometer Copenhagen, equipped with a Metro-ohm or Radiometer Copenhagen glass electrode) and the bottles were refilled with fresh media with the same pH as the culture. The experiment was terminated after 3 d of stationary growth. Trichome and/or cell concentrations in all mixed culture replicas and in 3 of the monocultures were enumerated, and fluorescence of the monoculture replica was measured on a fluorometer to monitor the variation between replicates.

Four times during the experiment, 3 bottles from each of the experimental cultures were removed for analysis of nodularin concentration in both cells and media. Bottles were removed twice during the exponential growth phase, once in the transition between exponential- and stationary growth phase and finally

Table 1. Phytoplankton species used in the experiment with details about clone designation, place of origin and date of isolation. KAC: Kalmar Algae Collection; SCCAP: Scandinavian Culture Collection of Algae and Protozoa; PML: Plymouth Marine Laboratory, UK

Phytoplankton species	Clone	Place	Date	Source
Cyanophyceae				
Nodularia spumigena Mertens	KAC 66	Askö, Baltic Sea, Sweden	1996	KAC
Cryptophyceae				
Rhodomonas marina (Dangeard) Lemmermann	K-0435	Kattegat, Denmark	1990	SCCAP
Rhodomonas salina (Wislouch) Hill and Wetherbee	K-0294	Øresund, Denmark	1989	SCCAP
Dinophyceae				
Heterocapsa triquetra Ehrenberg	K-0481	Øresund, Denmark	1988	SCCAP
Prorocentrum micans Ehrenberg	K-0335	Kattegat, Denmark	1989	SCCAP
Prorocentrum minimum (Pavillard) Schiller	K-0295	Kattegat, Denmark	1989	SCCAP
Bacillariophyceae				
Phaeodactylum tricornutum Bohlin	–	Unknown	Unknown	PML

at the termination of the experiment, when cultures had entered the stationary growth phase. Samples from mono- and mixed cultures were not necessarily removed on the same day, but instead at approximately the same pH level.

The nodularin in the media was extracted within 3 h, whereas filters containing the *Nodularia spumigena* trichomes were stored in the freezer for later analysis. The cell bound nodularin was released after continued freeze-thaw cycles, followed by ultrasound sonication in 75% methanol and centrifugation. The supernatant was diluted with Milli-Q water to obtain a methanol concentration in the extract below 15%. The nodularin from both media and cells was concentrated using Isolute C18 solid phase extraction syringes placed on a Vac master chamber connected to a vacuum hose. The nodularin content in each sample was analysed using high performance liquid chromatography (HPLC) on a Shimazu LC 10A system. A Waters Symmetry C18 column with a flow rate of 1 ml min^{-1} and a linear gradient of (1) 10 mM ammonium acetate (pH 5.0; 80%) with acetonitrile (20%), and (2) acetonitrile was used. The column temperature was 40°C.

For further details on the extraction and HPLC analysis of nodularin see Henriksen (2005). Pure nodularin (Calbiochem) was used as standard.

Allelopathic effects of pure nodularin. Pure nodularin was analysed for allelopathic effects on 1 phytoplankton species. Based on the results from Suikkanen et al. (2004), which suggested allelopathic effects of *Nodularia spumigena* towards *Rhodomonas* sp., *R. salina* was chosen as the test alga. *R. salina* was subjected to 2 different concentrations of purified nodularin: 7.5 and 75 µg l^{-1}, and a monoculture of *R. salina* containing no nodularin was used as a control. The concentration of 7.5 µg l^{-1} was chosen based on other authors' measurements of nodularin concentrations in the water of natural *N. spumigena* blooms (Table 2) and on concentrations detected by HPLC in the previous experiment. The possibility that a strain of *N. spumigena* with a larger production of nodularin may exist was taken into account by testing the toxicity of a 10 times larger concentration of nodularin (75 µg l^{-1}).

Triplicate cultures were grown in Nunc® (74 ml) bottles for 5 d. Samples (2 ml) were taken daily and fixed in Lugol's solution for cell counts, and pH in the cultures was measured. The bottles were not refilled with new media. To make sure that the concentration of pure nodularin in the cultures did not change significantly during the experiment, the degradation of nodularin was determined. Three *Rhodomonas salina* cultures, with a nodularin concentration of 75 µg l^{-1}, were removed for nodularin analysis by HPLC at the beginning of the experiment, and 3 cultures, with the same initial concentration of nodularin, were analysed at the end of the experiment.

Allelopathic effects of *Nodularia spumigena*. To study the allelopathic activity of *Nodularia spumigena*, mono- (controls) and mixed cultures containing *N. spumigena* and various test algae were prepared in triplicates. Six phytoplankton species were used in this experiment: the 2 cryptophytes *Rhodomonas marina* and *R. salina*, the 3 dinoflagellates *Heterocapsa triquetra*, *Prorocentrum micans* and *Prorocentrum minimum* and the diatom *Phaeodactylum tricornutum*. *N. spumigena* was added to the mixed cultures at an initial concentration of 4000 trichomes ml^{-1}, and the test algae were added at the following initial concentrations: *R. marina* and *R. salina* (1000 cells ml^{-1}), *H. triquetra* (400 cells ml^{-1}), *P. micans* (300 cells ml^{-1}), *P. minimum* (700 cells ml^{-1}) and *P. tricornutum* (200 cells ml^{-1}). Cultures were grown in 74 ml Nunc® culture bottles filled to capacity. Every 1 to 4 d, 3.5 ml samples (4.7% of the total volume) were fixed in Lugol's solution for cell counts. Subsequently, pH was measured and the bottles were refilled with fresh media with the same pH as in the culture.

RESULTS

Quantification of *Nodularia spumigena*

We used trichomes ml^{-1} as a measure for *Nodularia spumigena* density. Enumeration of single cells was impossible in the inverted microscope as distinction between individual cells was difficult. During the ini-

Table 2. Concentration of dissolved nodularin (µg l^{-1}) in water samples from natural habitats

Locality	Year	Nodularin conc.	Source
Bothnian Sea	1990	0.01–18.70	Kononen et al. (1993)
Gulf of Finland	1990	0.08–1.72	Kononen et al. (1993)
Lake Albert, Australia	1994–1995	0.00–1.00	Heresztyn & Nicholson (1997)
Lake Alexandrina, Australia	1994–1995	0.00–1.60	Heresztyn & Nicholson (1997)
Gulf of Finland	1999	0.50–2.60	Kankaanpää et al. (2001)
Gulf of Gdansk	2001	Traces–18135 (in scums?)	Mazur & Plinski (2003)
Gulf of Gdansk	2002	Traces–12.60	Mazur & Plinski (2003)

tial part of the experiment the variation in the number of cells per trichome was larger than later in the experiment due to the occasional occurrence of very long trichomes (Fig. 1). However, the period used for measuring exponential growth of *N. spumigena*, the first 7 d, showed no significant change in the number of cells per trichome (Kruskal-Wallis 1-way ANOVA on ranks, $p > 0.01$).

As an additional tool for measuring the density of *Nodularia spumigena* in monoculture, fluorescence was used. The fluorescence emitted by the monoculture of *N. spumigena* was significantly correlated to the concentration of trichomes in the culture ($r^2 = 0.957$, $p < 0.001$) (Fig. 2). The correlation was highly significant in the exponential growth phase ($r^2 = 0.984$, $p < 0.001$), but was non-significant in the stationary growth phase ($r^2 = 0.041$, $p > 0.01$).

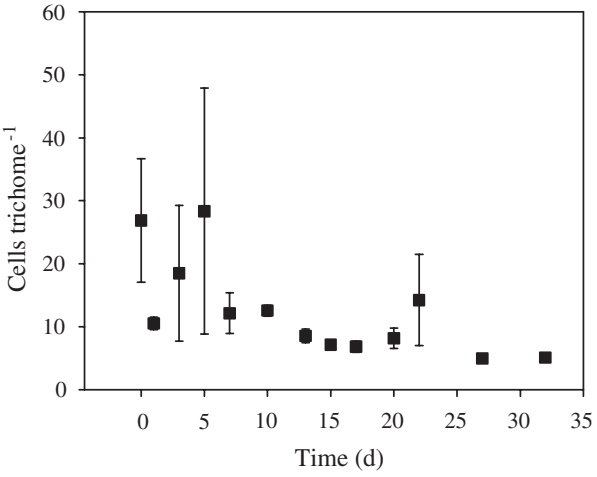


Fig. 1. *Nodularia spumigena.* Number of cells per trichome as a function of incubation time. Data points refer to mean ± 1 SE, n = 40 to 70 trichomes

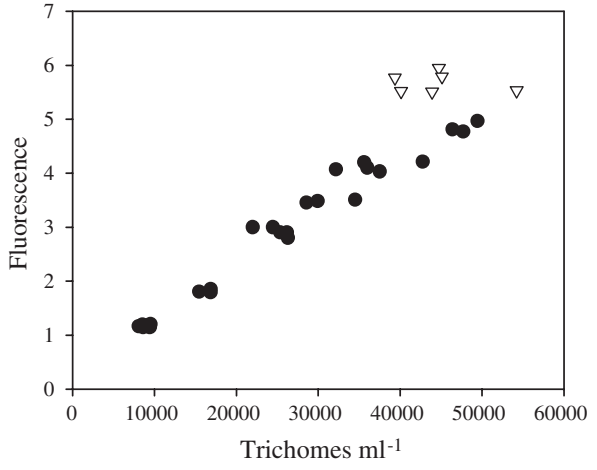


Fig. 2. *Nodularia spumigena.* Amount of fluorescence (relative units) as a function of trichome concentration (monoculture). ●: fluorescence emitted by the culture in exponential growth phase; ∇: fluorescence emitted from cells in stationary growth phase

Nodularin production and excretion

Nodularia spumigena entered the exponential growth phase after an initial lag period of 2 d, and the exponential growth rate was 0.27 d^{-1} in both mono- and mixed cultures (Fig. 3, lag phase not included). The monoculture entered the stationary growth phase after 10 d, and the transition between exponential and stationary growth was sharp. In the mixed culture, the exponential growth stopped after 8 d and the growth rate slowly declined as the stationary growth phase was entered. The pH increased faster in the mixed culture compared to the monoculture (Fig. 3C) and exponential growth of *N. spumigena* stopped at approximately the same pH in the 2 cultures. When the pH level in the cultures increased beyond 10.5, visual observations of *N. spumigena* revealed curly trichomes with a diffuse and rough cell surface. At a lower pH, the trichomes were straight and the cell surface smooth. *Rhodomonas salina* grew exponentially from Days 2 to 5, after which the growth rate slowly decreased (Fig. 3C). Stationary growth phase was entered after 9 d.

During exponential growth of *Nodularia spumigena*, the intracellular nodularin concentration per volume of water increased simultaneously with the concentration of trichomes, indicating that the intracellular nodularin concentration remained fairly constant in the exponential growth phase. Growth rates of *N. spumigena* and production rates of nodularin did not differ significantly from each other, neither within cultures nor between mono- and mixed cultures (*t*-test $p > 0.01$) (Fig. 3). The final concentration of intracellular nodularin per volume of water was larger in the mono- than in the mixed culture due to the higher trichome concentration in the monoculture. Extracellular nodularin remained below the detection limit until the stationary growth phase, where it only amounted to 9% (monoculture) and 12% (mixed culture) of the total nodularin concentration (Fig. 3A,B).

Nodularin concentration per trichome (Fig. 4) did not change significantly in the monoculture during growth (1-way ANOVA, $p > 0.01$). In the mixed culture though, a significant difference was observed between the first and last nodularin extraction (Tukey test, $p = 0.01$). In both cultures, the intracellular nodularin concentration greatly exceeded the extracellular concentration (Tukey test, $p < 0.001$). The presence of *Rhodomonas salina* had no significant effect on the intracellular or on the extracellular concentration of nodularin (1-way ANOVA, $p > 0.01$).

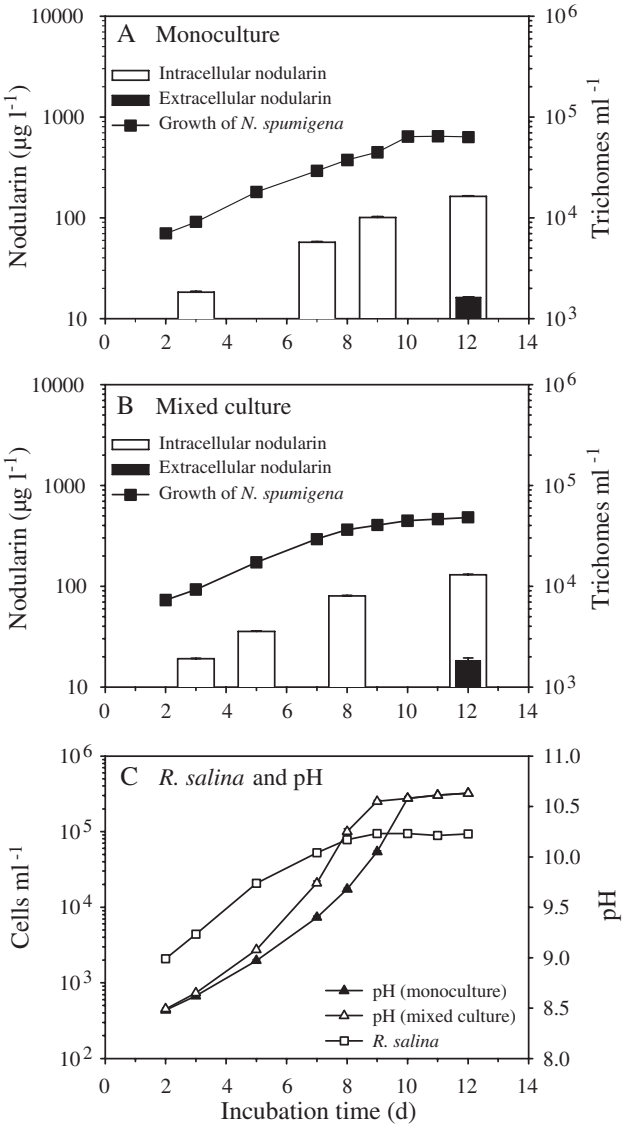


Fig. 3. Nodularin concentration (µg l^{-1} of growth media) in cells (intracellular) and growth media (extracellular) as a function of incubation time in batch cultures of *Nodularia spumigena*, under 2 different growth conditions: (A) monocultures, and (B) mixed cultures with *Rhodomonas salina*. (C) Cell concentrations: *R. salina* in mixed culture with *N. spumigena*, pH in the monocultures, and mixed cultures. Data points refer to mean + 1 SE, n = 3. Error bars difficult to see

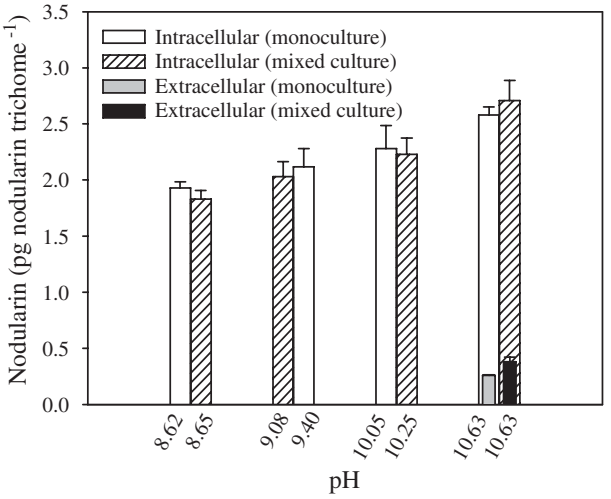


Fig. 4. *Nodularia spumigena*. Nodularin concentrations (pg nodularin $trichome^{-1}$) in cells (intracellular) and growth media (extracellular) in both monoculture and mixed with *Rhodomonas salina* as a function of incubation time. Numbers on the *x*-axis refer to the pH in the cultures at time of sampling. Bars refer to mean + 1 SE, n = 3

Allelopathic effects of pure nodularin

Rhodomonas salina grew exponentially in all cultures during the experimental period, and the pH remained between 8.2 and 8.4 (Fig. 5). Growth of *R. salina* was unaffected by the presence of nodularin (Fig. 5), and no significant differences in growth rates were detected between cultures with different nodularin concentrations (1-way ANOVA, $p > 0.01$). At the end of the experiment, pH levels in the 3 *R. salina* cultures were not significantly different from one another (1-way ANOVA, $p > 0.01$). The nodularin concentration did not change significantly during the experiment (*t*-test, $p > 0.01$) and averaged 74 ± 0.8 µg l^{-1}.

Allelopathic effects of *Nodularia spumigena*

All test species grew exponentially after an initial lag phase of 1 to 2 d in the monocultures. Exponential growth proceeded for varying periods of time until the cultures entered the stationary growth phase (Fig. 6, lag phase not included). The transition between exponential and stationary growth phases was gradual in all monocultures but one (Fig. 6Q), and the growth rates slowly decreased. The cell density in the monocultures did not change considerably when the stationary growth phase was entered, except for *Rhodomonas marina*, which decreased in cell number (Fig. 6B). The maximum pH level measured in the monocultures varied between test species from 9.8 for *Heterocapsa triquetra* to 10.5 for both *Rhodomonas* species (Table 3).

The exponential growth phase of all test species was terminated earlier in the mixed cultures than in the monocultures. At the end of the experiment the cell density was lower in the mixed culture, except in the culture with *Phaeodactylum tricornutum*, which had the same cell density as in the monoculture (Fig. 6Q). After entering the stationary growth phase, the cell density decreased in 4 of the mixed cultures (Fig. 6B,H, K,N). Only *Rhodomonas salina* and *P. tricornutum* (Fig. 6E,Q) maintained approximately the same cell

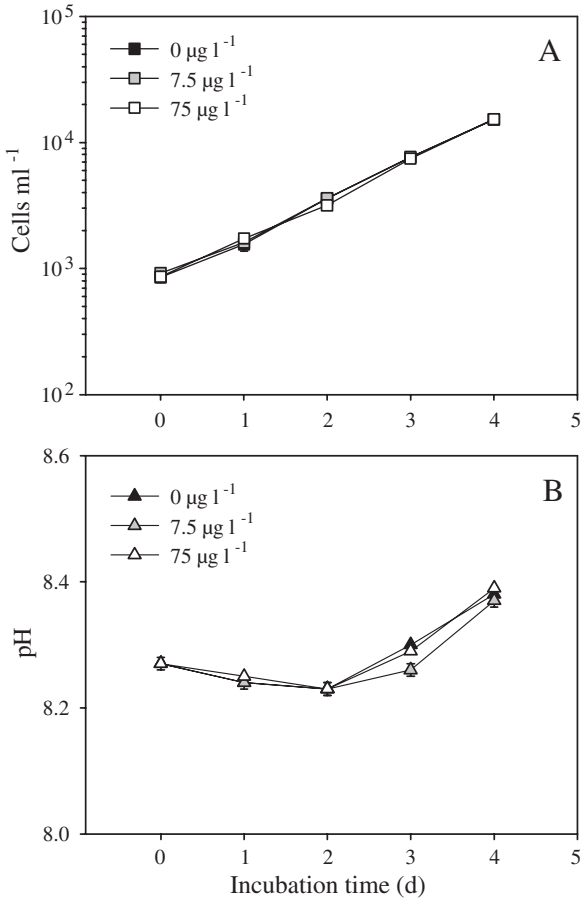


Fig. 5. *Rhodomonas salina*. (A) Cell concentration and (B) pH as a function of incubation time, when incubated at 3 different concentrations of nodularin. Data points refer to mean ± 1 SE, n = 3. Error bars difficult to see

concentration throughout the stationary growth phase. The presence of *N. spumigena* did not significantly affect the exponential growth rates of the test species (*t*-test, $p > 0.01$). At the end of the experiment, the cell density was, however, highly affected by the presence of *Nodularia spumigena* (*t*-test, $p < 0.001$) (Fig. 6), except for *P. tricornutum* (*t*-test, $p > 0.01$) (Fig. 6Q). For all species tested, the termination of the exponential growth phase in the mono- and the mixed cultures coincided with the same pH range (Table 3).

After an initial lag phase of 1 d, *Nodularia spumigena* in monoculture grew exponentially until Day 10, when the stationary growth phase was entered (Fig. 7, lag phase not included). The transition between the exponential and the stationary growth phase was sharp, and the trichome density did not change considerably when the pH exceeded 10.5. The exponential growth rate of *N. spumigena* was not significantly affected by the presence of any of the test species (*t*-test, $p > 0.01$), though the exponential growth phase was terminated 1 to 2 d earlier in all mixed cultures, except for that with *Prorocentrum minimum* (Fig. 6M). In 4 of the mixed cultures (Fig. 6A,D,J,P), the trichome density was significantly affected by the presence of the test alga (*t*-test, $p < 0.001$) due to the faster increase in pH. As in the monoculture, the trichome density did not change considerably in any of the mixed cultures, when pH exceeded 10.5.

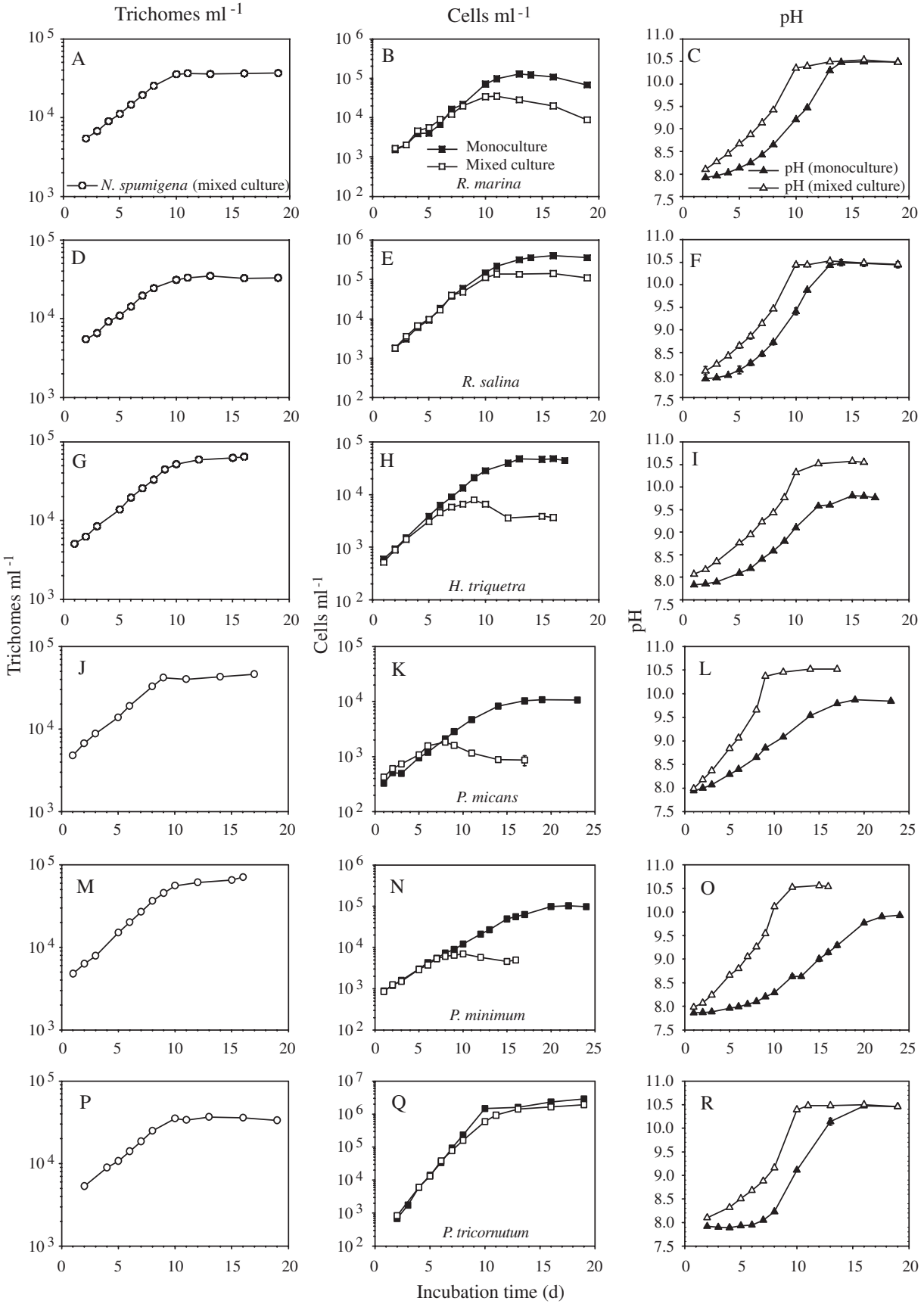
All species used in this study were able to continue positive growth up to quite high pH levels. The maximum pH level measured in the media of the *Nodularia spumigena* monoculture was 10.6, and in the monocultures of the test species (*Rhodomonas marina*, *R. salina* and *Phaeodactylum tricornutum*) the maximum pH levels were almost equally high with values of ca. 10.5. In the remaining species, the maximum pH in the monocultures was less than 10 (Table 3).

DISCUSSION

Nodularin production and excretion

The intra- and extracellular concentrations of nodularin did not deviate significantly between the mono and the mixed culture, indicating that the presence of *Rhodomonas salina* had no impact on the production or excretion of nodularin by *Nodularia spumigena* (Figs. 3 & 4). During exponential growth of *N. spumigena*, nodularin was only detected intracellularly. After *N. spumigena* had entered the stationary growth phase and the pH level was above 10.5, extracellular nodularin was also detected in the growth media (Fig. 3). At this growth phase the trichomes of *N. spumigena* shifted from straight, smooth trichomes to curly, rough trichomes, indicating cell lyses. This is in accordance with previous laboratory studies by Lehtimäki et al. (1997) and Sivonen & Jones (1999), who found that leakage of nodularin to the surrounding water was due to lyses of *Nodularia* cells.

In natural blooms of *Nodularia spumigena* consisting of healthy cells the toxin is intracellular (Hereztyn & Nicholson 1997). However, during dense blooms of *N. spumigena* around 10 to 20% of the toxin concentration can be found dissolved in the water (Mazur & Plinski 2003), which is similar to our results during stationary growth phase. We observed a slightly higher percentage of extracellular nodularin in the mixed culture compared to the monoculture of *N. spumigena*. This is probably because the pH level had been above 10.5 for 4 d in the mixed culture as opposed to only 3 d in the monoculture. In other words, the course of decomposition probably started 1 d earlier in the mixed culture, and more nodularin might have been released into the surrounding medium.


Trichomes ml -1
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N. spumigena (mixed culture)
Monoculture
Mixed culture
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pH (monoculture)
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H. triquetra
P. micans
P. minimum
P. tricornutum
Trichomes ml -1
Cells ml -1
pH
Incubation time (d)

Fig. 6. Left-hand column: Cell concentrations of *Nodularia spumigena* in mixed cultures as a function of incubation time. Middle column: cell concentrations of the test algae in monoculture and in mixed cultures containing *N. spumigena* as a function of incubation time. Right-hand column: pH in the monoculture of the test algae and in the mixed culture. All panels in each row refer to the same experiment. Test alga (A–C) *Rhodomonas marina*; (D–F) *Rhodomonas salina*; (G–I) *Heterocapsa triquetra*; (J–L) *Prorocentrum micans*; (M–O) *Prorocentrum minimum*; (P–R) *Phaeodactylum tricornutum*. Data points refer to mean ± 1 SE, n = 3. Error bars difficult to see

Allelopathic effects of pure nodularin

The pure nodularin added to the cultures was not degraded during the experiment. Thus, the lack of effects of pure nodularin on the growth of *Rhodomonas salina* could not be ascribed to a decrease in the nodularin concentration. In 2 other experiments, Heresztyn & Nicholson (1997) and Twist & Codd (1997) found that degradation of pure nodularin was related to the presence of *Nodularia spumigena*, or filtrates from it, and concluded that the degradation was due to either enzymes or bacteria associated with the cyanobacterium. In the present experiment, *N. spumigena* was absent from the cultures and, hence, so was the bacteria or enzymes associated with *N. spumigena*. This might explain why nodularin was not degraded in the present experiment. Furthermore, our results suggest that microorganisms associated with *R. salina* did not degrade nodularin.

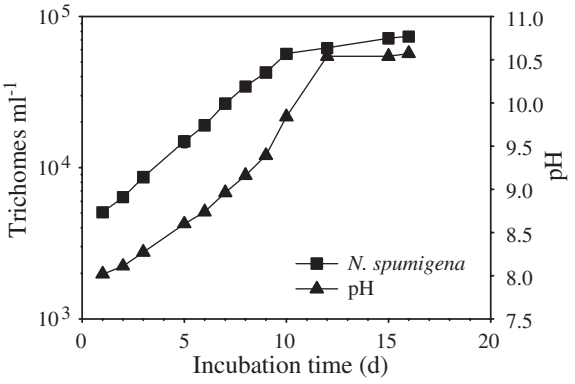

Fig. 7. *Nodularia spumigena*. Growth in monoculture, and pH in the culture. Data points refer to mean ± 1 SE, n = 3. Error bars are very small and difficult to see

Table 3. Exponential growth rates (d^{-1}), the range of pH in which exponential growth was terminated and the maximum pH measured in the cultures. All values, except the pH range, are means ± SE, n = 3

Test species	Growth rate	pH range	Max. pH
Nodularia spumigena			
Control	0.27 ± 0.00	9.84–10.54	10.57 ± 0.00
Mixed culture with:			
Rhodomonas marina	0.26 ± 0.01	9.42–10.35	
Rhodomonas salina	0.25 ± 0.01	9.46–10.44	
Heterocapsa triquetra	0.28 ± 0.00	9.77–10.33	
Prorocentrum micans	0.27 ± 0.00	10.37–10.46	
Prorocentrum minimum	0.28 ± 0.00	10.11–10.52	
Phaeodactylum tricornutum	0.25 ± 0.01	9.16–10.39	
Rhodomonas marina			
Control	0.50 ± 0.02	9.21–9.47	10.49 ± 0.00
Mixed culture	0.47 ± 0.05	8.88–9.13	10.53 ± 0.00
Rhodomonas salina			
Control	0.56 ± 0.01	9.41–9.88	10.49 ± 0.00
Mixed culture	0.58 ± 0.02	9.14–9.46	10.53 ± 0.01
Heterocapsa triquetra			
Control	0.44 ± 0.01	9.10–9.58	9.81 ± 0.00
Mixed culture	0.43 ± 0.01	8.95–9.23	10.57 ± 0.00
Prorocentrum micans			
Control	0.26 ± 0.01	9.08–9.54	9.87 ± 0.01
Mixed culture	0.24 ± 0.02	9.06–9.66	10.52 ± 0.01
Prorocentrum minimum			
Control	0.28 ± 0.00	9.00–9.14	9.93 ± 0.00
Mixed culture	0.30 ± 0.01	9.05–9.26	10.56 ± 0.01
Phaeodactylum tricornutum			
Control	0.96 ± 0.01	9.11–9.64	10.47 ± 0.00
Mixed culture	0.91 ± 0.01	8.88–9.16	10.50 ± 0.00

Pure nodularin had no allelopathic effects on the growth of the cryptophyte *Rhodomonas salina*, even though the concentration of 75 µg nodularin l^{-1} is higher than the concentrations usually found in nature (Kononen et al. 1993, Heresztyn & Nicholson 1997, Kankaanpää et al. 2001). Nodularin was only tested for allelopathic effects on 1 species of phytoplankton; hence it cannot be excluded that the toxin can affect other phytoplankton species. Consistent with the present experiment, Engström-Öst et al. (2002) monitored the effects of a decaying *Nodularia spumigena* bloom and found no harmful effects on other organisms in the vicinity of the cyanobacteria in spite of a soluble nodularin concentration of 19.7 µg l^{-1}. Likewise, Suikkanen et al. (2004) found no allelopathic effects of nodularin-containing media extracted from stationary growing *N. spumigena* on different phytoplankton species. The concentration of nodularin in

the extracts used by Suikkanen et al. (2004) was 10.8 µg nodularin l^{-1} (calculated from information in Suikkanen et al. 2004).

There are, to our knowledge, no published reports on allelopathic effects of nodularin, but inhibitory effects of other cyanotoxins have been observed. In an experiment by Pietsch et al. (2001), natural concentrations of pure microcystins had no significant effect on the photosynthetic oxygen production of a microalga and a macrophyte, but the microcystins elevated the activity of 2 detoxication enzyme systems in the test organisms. Effects of microcystins-LR and anatoxin-a have been observed on aquatic macrophytes and in the case of anatoxin-a also on macroalgae (Pflugmacher 2002, Mitrovic et al. 2004). Microcystin-LR significantly inhibited the growth of the test organisms at environmentally relevant concentrations, but the anatoxin-a data were difficult to extrapolate to natural conditions because the concentrations necessary to cause inhibition of the test organisms were above concentrations usually found in nature.

Allelopathic effects of *Nodularia spumigena*

As previously described, nodularin was not detectable in the media until the stationary growth phase. Hence, any inhibiting effects on the growth of the test species caused by the presence of exponentially growing *Nodularia spumigena* could not be due to the release of nodularin into the growth media. In this study, the presence of *N. spumigena* did not significantly alter the exponential growth rates of the 6 phytoplankton species tested. This indicates that the strain of *N. spumigena* used in this study did not produce any substances that had allelopathic effects on the phytoplankton species tested. In contrast to the present study, Suikkanen et al. (2004) found allelopathic effects of *N. spumigena* on a *Rhodomonas* sp. They supplied the *Rhodomonas* sp. with growth media that had previously contained a monoculture of exponentially growing *N. spumigena*. The discrepancies between the observations in the present experiment and the one by Suikkanen et al. (2004) might be due to several different factors. Most importantly, the strains of *N. spumigena* and *Rhodomonas* sp. were not the same in those 2 studies, and different strains of the same species may have different toxins (Skulberg 2000). Furthermore, the experimental conditions and design are not identical, a factor which might alter the susceptibility of the test algae. Thus, the present study cannot exclude allelopathic abilities of some strains of *N. spumigena*, but the strain used in this experiment was not allelopathic towards the species tested under the given conditions.

We did observe that *Nodularia spumigena* affected the test species for the duration of the exponential growth phase, but the effect was not due to allelopathic substances. Nutrient depletion could also be ruled out as the cause, because nutrients were added in excess (see Hansen et al. 2002, Lundholm et al. 2004). *N. spumigena* grew at pH levels up to 10.6 and our experiments showed that growth of the test algae in the mixed cultures stopped at a pH level coinciding with the maximum pH found in monocultures of these algae. The pH level in the culture media, therefore, appeared to be the controlling factor for growth.

None of the test species affected the exponential growth of *Nodularia spumigena*. The duration of the exponential growth phase was determined by the pH in the culture, and as the pH increased faster in the mixed culture, the duration of the exponential growth phase of *N. spumigena* was reduced in all mixed cultures except the one with *Prorocentrum minimum*. The trichome density at the end of the experiment was highly affected by the presence of the test algae, with the exception of *P. minimum* and *Heterocapsa triquetra.* The lower trichome density in some of the mixed cultures was caused by a faster increase in pH. The trichome concentration of *N. spumigena* remained unchanged after the stationary growth phase was entered, indicating that its pH tolerance was not exceeded. Hence, apparently the maximum pH level reached in the mixed cultures was controlled by *N. spumigena*, except in the culture with *P. tricornutum* where both species had an influence on the maximum pH level.

The test species in our experiments were selected based on their tolerance to high pH. In the natural environment, *Nodularia spumigena* may encounter many species which are much less tolerant to high pH levels than the species used in this experiment, and the pH effect of *N. spumigena* may be much more profound than these experiments indicate. Based on results obtained from a field investigation, Wasmund (1997), proposed that cyanobacteria might have an advantage compared to other algal groups, due to their ability to tolerate high pH.

Data on pH during algal blooms from coastal marine areas are sparse, but pH may increase to values above 9 and become as high as 9.25 and 9.75 in enclosed water bodies (Hinga 1992 and references therein, Macedo et al. 2001, Hansen 2002). Only a few reports from the marine environment relate mass occurrences of cyanobacteria to high pH levels in the seawater. In the Baltic Sea, pH values above 9.3 have been measured during such blooms (Wasmund 1996). However, a database for marine data (MADS 2005), hosted by the National Environmental Research Institute in Denmark, contains observations of pH values from Hjelm

Bugt, opposite the entrance to the Baltic Sea. pH values were often above 9 during summer months and on rare occasions even above 10. Unfortunately, the pH data from MADS (2005) were not accompanied by a survey of the phytoplankton community, but large concentrations of the cyanobacteria *Aphanizomenon flos-aquae* and *Nodularia spumigena* are common in that area during summer months (MADS 2005).

In freshwater environments high pH levels have often been reported during blooms of cyanobacteria. López-Archilla et al. (2004) monitored the phytoplankton diversity in a hypereutrophic, highly alkaline lake in SW Spain, during a period of increasing pH from 7.3 to above 10.5. The authors observed a rapid increase in the pH values due to photosynthesis, reaching a pH level above 9. This resulted in an equally rapid decrease in the phytoplankton diversity, leading to a community almost completely dominated by cyanobacteria (>99%, when pH >10.5). Similarly, An & Jones (2000) found that cyanobacteria were more abundant in a Korean lake during a light than during a heavy monsoon. During the light monsoon, the pH level in the lake water was higher than during the heavy monsoons, where the lake water was 'diluted' and hence the pH was low.

CONCLUSION

Growth of the test species was controlled by the pH in the culture media, not by allelopathic substances produced by *Nodularia spumigena*. Phytoplankton species encountered in nature vary considerably with respect to pH tolerance (e.g. Hinga 2002), and compared to other species *N. spumigena* is tolerant to very high pH levels. This might be a competitive advantage that contributes to the success of this cyanobacterial species. Thus, to understand phytoplankton succession and dynamics of cyanobacterial blooms in particular, the role of pH should be taken into account along with other abiotic and biotic factors.

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