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Toxin-producing cyanobacterium *Nodularia spumigena*, potential competitors and grazers: testing mechanisms of reciprocal interactions

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ABSTRACT: Interactions among toxic cyanobacteria, sympatric algae and planktivorous grazers are key processes governing plankton dynamics and cyanobacterial blooms. We studied interactions between the cyanobacterium *Nodularia spumigena* and microalgae (*Rhodomonas salina* and *Tetraselmis suecica*) as well as effects of zooplankton (copepod *Eurytemora affinis*) grazing on these interactions. *N. spumigena* was incubated without algae or with algae at different concentrations and with or without copepods. Following ~24 h incubation, we assayed changes in *N. spumigena* and algae abundance, concentration of intracellular (IC) and dissolved nodularin (toxin produced by *N. spumigena*) and quantity of *Nodularia* DNA in copepod guts (as a proxy for grazing pressure on the cyanobacterium). In the presence of algae, IC nodularin levels increased in a concentration-dependent manner; however, when copepods were present in the mixtures of algae and cyanobacterium, this increase was significantly less. The presence of *T. suecica* negatively affected the growth rate of *N. spumigena*, whereas the presence of the cyanobacterium strongly impeded growth of *R. salina*, but not of *T. suecica*. The IC nodularin quota correlated negatively with growth of *R. salina*, implicating the toxin's involvement in the observed growth suppression of the eukaryotic alga. Copepods actively ingested *N. spumigena*, even when the alternative food was plentiful, and neither *N. spumigena* quantity nor its toxin concentrations influenced copepod feeding rates and survival. These findings suggest complex allelopathic interactions between the autotrophs, whereas mesozooplankton grazers have an indirect negative effect on the nodularin concentrations by suppressing the competitors. These findings underscore the need to study ecologically important interactions among toxic cyanobacteria, sympatric algae and grazers, if we are to understand mechanisms regulating cyanobacterial blooms.

KEY WORDS: Algae · Allelopathy · Grazers · Molecular diet analysis · Nodularin · Toxic cyanobacteria · Trophic interactions

INTRODUCTION

Grazer effects on phytoplankton stocks and community structure is a major topic in foodweb studies. In particular, toxin production by some algae has received much attention, with 2 main mechanisms being considered: allelopathy and inducible defence. The term allelopathy refers to inhibitory and stimulatory effects of plants and microorganisms on other plant species or microorganisms through the release of organic compounds (Rice 1984). Some authors also consider grazer deterrence as an allelopathic property (Leflaive & Ten-Hage 2007). Inducible defences of phytoplankton against grazing may appear as changes

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in morphology (e.g. thorns, spines, colonies and filaments), as well as production of chemical substances, such as toxins or other bioactive compounds, suppressing growth or activity of grazers (Harvell & Tollrian 1999, Van Donk 2007).

In the Baltic Sea, *Nodularia spumigena*, a toxin-producing filamentous cyanobacterium, is present throughout summer, peaking in July to August. A warmer climate is a potent catalyst for the further expansion of these blooms (Paerl & Huisman 2008). Nodularin is a predominantly intracellular hepatotoxin that inhibits protein phosphatases in eukaryotic cells (MacKintosh et al. 1990). While abiotic environmental factors affecting nodularin production have been studied rather extensively using cultures (Lehtimäki et al. 1994, Jonasson et al. 2008), factors affecting the toxin production and its ecological role are much less understood. Allelopathic responses of phytoplankton to *N. spumigena*, its cell-free filtrate and pure nodularin have been studied experimentally in algal cultures (Suikkanen et al. 2004, 2006, Møgelhøj et al. 2006). In these experiments, *N. spumigena* and its filtrate suppressed algal growth, while no evidence for nodularin operating as the allelopathic agent has been found using pure nodularin (Møgelhøj et al. 2006, Suikkanen et al. 2006). Suikkanen et al. (2006) proposed that the allelopathic effects of *N. spumigena* are most probably due to metabolite(s) other than nodularin. Also, nodularin has been implicated as the cause of harmful effects of *N. spumigena* on various crustaceans (Koski et al. 1999, Kozlowsky-Suzuki et al. 2003) and small fish (Kankaanpää et al. 2001, Pääkkönen et al. 2008). On the other hand, some animals ingest this cyanobacterium with no apparent harm (cladocerans: Sellner et al. 1994, mysids: Engström et al. 2001, ostracods and oligochaetes: Nascimento et al. 2009, copepods: Gorokhova & Engström-Öst 2009) and may actually benefit from its presence (Schmidt & Jónasdóttir 1997, Engström et al. 2001).

Recent reports have indicated that toxin levels decrease in *Nodularia spumigena* exposed to zooplankton grazers (Sopanen et al. 2009, Gorokhova & Engström-Öst 2009), whereas in freshwater *Microcystis* sp., an increased accumulation of microcystin, a closely related toxin (Berry et al. 2008), has been observed in the presence of grazers (Jang et al. 2007). In *Anabaena flos-aquae*, the responses were toxin-specific, with accumulation of anatoxin increasing and that of microcystin decreasing as a result of exposure to a green alga in a laboratory experiment (Kearns & Hunter 2000). These responses are highly relevant for understanding processes behind cyanobacterial bloom formation as well as consequences of the blooms, including effects on aquatic primary producers and grazers, toxin transfer in the food webs and secondary production. The specific objectives of our study were to investigate (1) how grazing by copepods and the presence of competitors (eukaryotic algae) affect intra- and extracellular nodularin concentrations and growth of *N. spumigena*, (2) how *N. spumigena* affects algal growth, and (3) how copepod feeding responds to changes in food composition when offered algae and *N. spumigena* in different ratios. To address these questions, we conducted experiments using mixtures of the most common toxin-producing cyanobacterium *N. spumigena* and the eukaryotic algae *Rhodomonas salina* or *Tetraselmis suecica*. While *R. salina* co-occurs with the cyanobacterium throughout the Baltic Sea, distributions of *T. suecica* and *N. spumigena* overlap only in the Kattegat (Hällfors 2004). As a grazer, the calanoid copepod *Eurytemora affinis*, which is able to graze on *N. spumigena* (Engström et al. 2000), was used.

MATERIALS AND METHODS

Test organisms. The algal culture of *Rhodomonas salina* (Cryptophyceae; TV22; Tvärminne Zoological Station, University of Helsinki) was grown using f/2-Si medium at 18°C and a practical salinity of 6 in aged seawater, and *Tetraselmis suecica* (Prasinophyceae; CCMP908; US National Center for Culture of Marine Phytoplankton) was grown using f/2 medium at 15°C and a practical salinity of 7 in artificial seawater (ASW; Instant Ocean™, Aquarium Systems). The culture of *Nodularia spumigena* (strain AV1, a potent nodularin producer) was obtained from Prof. K. Sivonen, University of Helsinki. The cyanobacterium was grown in a modified Z8 nutrient solution (Sivonen et al. 1989) in a 16:8 h light:dark regime at 15°C. Shortly before the cruise, the cultures were transferred onboard the ship and held in a climate chamber at 15°C with constant light. Cell concentrations in the cultures were determined by microscopy (Leica DM IRB, 10×, 25×, 40×) using a haemocytometer (*R. salina* and *T. suecica*) or Utermöhl chambers (*N. spumigena*). Also, the dry weight (DW, µg l^{-1}) was determined by filtering a known volume of well-mixed cultures on combusted, pre-weighed filters (Whatman GF/F), and drying at 65°C for 24 h.

The copepods were collected and the experiments were conducted on board RV 'Aranda' (Finnish Environment Institute) in August 2008 in the Gulf of Finland, northern Baltic Sea. Zooplankton samples were taken from 15 to 10 m using a closing 200 µm WP2 net. The contents of the tow were diluted with surface water and kept at 16°C with gentle aeration until sorting commenced. Older copepodites (CV–VI) of *Eurytemora affinis* were sorted in Petri dishes filled with 0.2 µm filtered seawater (FSW) using wide-mouthed pipettes.

Experimental setup and sampling. Experimental treatments included mixtures of *Nodularia spumigena* with or without algae (*Rhodomonas salina* or *Tetraselmis suecica*) and with or without copepods (Table 1). Two consecutive experiments were conducted, Expt 1 and Expt 2, using different algal species, *R. salina* and *T. suecica*, respectively. The treatment that included copepods exposed to *N. spumigena* without algae (CN) was run within Expt 2 only. A control treatment (C), where copepods were incubated in FSW, was included in both experiments to monitor background mortality. These individuals were also used as negative controls in quantitative PCR-based diet analysis. In both experiments, 1.2 l bottles were prepared with FSW and dilutions of algal cultures to arrive at nominal concentrations (Table 1). To prevent nutrient limitation during incubation, a surplus of f/2 nutrient media was added into each bottle to arrive at 16 and 1 µM for nitrate and phosphate, respectively. Groups of ~10 individuals of *Eurytemora affinis* (prosome length, PL, 615 ± 15 µm) were placed in each bottle of the copepod treatments. In each experiment, 1 group was sampled initially (start animals) and treated in the same way as described below for the experimental animals. All treatments were carried out in 5 replicates for ~24 h on a rotating plankton wheel (0.5 rpm) at ambient temperature (14 to 18°C) and illumination.

Table 1. Experimental design. C: copepods; N: *Nodularia spumigena*; A_{low}: eukaryotic algae (*Rhodomonas salina* or *Tetraselmis suecica*) at low (50 µg C l^{-1}) concentration; A_{high}: eukaryotic algae (*R. salina* or *T. suecica*) at high (250 µg C l^{-1}) concentration

Treatment	Copepods (ind. l^{-1})	*Nodularia* (µg C l^{-1})	*Rhodomonas* (µg C l^{-1})	*Tetraselmis* (µg C l^{-1})
Expt 1				
CNA_{low}	10	5	50	
CNA_{high}	10	5	250	
NA_{low}		5	50	
NA_{high}		5	250	
A_{low}			50	
A_{high}			250	
N		5		
C	10			
Expt 2				
CNA_{low}	10	5		50
CNA_{high}	10	5		250
NA_{low}		5		50
NA_{high}		5		250
A_{low}				50
A_{high}				250
CN	10	5		
N		5		
C	10			

Upon termination of the experiment, the animals were gently sieved through a partly submerged 90 µm sieve. A total of 50 ml of filtrate was preserved in plastic tubes with acid Lugol's solution for determination of algal concentrations. The remaining volume (1000 to 1140 ml) was filtered on Whatman GF/F filters (47 mm) that were frozen in Eppendorf tubes or wrapped in aluminium foil at –20°C until determination of intracellular nodularin concentrations. A total of 2 ml of the GF/F filtrates were collected in Eppendorf tubes and frozen at –20°C for measuring extracellular nodularin concentrations. The copepods were examined with a dissecting microscope, and dead individuals were noted and removed. In all incubations, survivorship was never below 90%, with no apparent differences between the treatments, including controls (Kruskal-Wallis statistic = 0.89, $p > 0.9$). Live copepods were individually picked with forceps, rinsed twice in FSW, transferred to Eppendorf tubes containing 100 µl of RNA*later* and stored at –20°C for approximately 4 mo (Gorokhova 2005). These animals were used for quantitative PCR (qPCR) analysis.

Microscopic analysis of *Nodularia spumigena*, algae and biomass determination. The abundance of *N. spumigena* in the experiments and in the culture was determined from 50 and 2 ml samples, respectively, preserved with acid Lugol's solution. Filaments were counted as 100 µm filaments (120 to 790 units $sample^{-1}$) in Utermöhl chambers at 125× magnification using a Leica microscope (Utermöhl 1958). The microscopy counts of *N. spumigena* were converted to carbon according to Olenina et al. (2006). The concentrations of *Rhodomonas salina* and *Tetraselmis suecica* (cells ml^{-1}) in the cultures and feeding media were determined using a laser particle counter (Spectrex PC-2000). The concentrations were converted to carbon equivalents using the regression $\log_{10}C = 0.76 \times \log_{10}V - 0.29$ (Mullin et al. 1966), where C is carbon content (pg $cell^{-1}$) and V is cell volume (μm^3) obtained from the particle counter. Growth rates of all phytoplankton were calculated according to Levasseur et al. (1993). The ingestion rates (I, µgC $ind.^{-1}$ d^{-1}) were calculated according to Frost (1972).

Nodularin analysis. The intracellular (IC) and extracellular (EC) nodularin concentrations were analysed by ELISA, using a microcystin plate kit (EnviroLogix) and nodularin standards (0.1 to 1.5 ng ml^{-1}), according to the kit instructions. Nodularin concentrations were analysed from the *Nodularia spumigena* culture (30 ml $sample^{-1}$; IC) and from *N. spumigena* from the experimental incubations collected on the filters (IC) as well as from the filtrates (GF/F filtered seawater, EC). Before the measurement of IC nodularin, filters were freeze-dried (Edwards Freeze Dryer Super Modulyo, GWB) for 48 h, dissolved in 5 ml 100% methanol, soni-

cated (Soniprep 150, MSE, Scientific Instruments) for 5 min and extracted overnight. Subsequently, the samples were filtered through GF/F filters (Whatman, 25 mm) with a syringe-operated device, and filtrates were dried with N_2 gas in glass vials. Then 40 µl of 50% methanol and 280 µl MilliQ water were added to the samples, starting with the methanol. During the following 4 d, 70 µl MilliQ water were added daily, until a final concentration of 6.25% methanol was reached (Metcalf et al. 2000). On the fifth day, the samples were analysed by ELISA. Samples were diluted 1:10 to 1:20 000, depending on the nodularin concentration. The EC nodularin samples (GF/F FSW) were completely melted and properly shaken before analysis, but not treated in any other way. A negative control and a standard curve were measured accordingly. The toxin concentration was determined by dual readings from the absorbance at 450 nm and at 655 nm as a reference (Microplate Reader, Bio-Rad Benchmark).

qPCR analysis. To quantify *Nodularia spumigena* in copepod guts, a real-time qPCR assay was applied using *Nodularia*-specific primers. From each experimental replicate, 3 samples (2 to 3 ind. sample^{-1}) were analysed and a mean amount of *N. spumigena* DNA ind.$^{-1}$ was estimated. To extract DNA, samples were incubated in 30 µl of 10% Chelex for 30 min at 105°C. After spinning in a centrifuge (12 000 × *g*, 2 min), the supernatant (20 µl) was transferred to a clean tube and stored at 4°C for 2 to 3 d. The total DNA yield sample^{-1} was quantified using a PicoGreen TM double-stranded DNA quantification kit (Molecular Probes) and a microplate spectrofluorometer (FLUOstar Optima, BMG Labtechnologies); it ranged from 2 to 4 µg ml^{-1}. The amplifications of a 200 bp fragment of *N. spumigena* 16S rDNA were performed using the *Nodularia*-specific primer NTS (TGT GAT GCA AAT CTC A(C/A)A; Moffitt et al. 2001) and universal 16S rRNA reverse primer 1494Rc (TAC GGC TAC CTT GTT ACG AC; Neilan et al. 1997) with a StepOne real-time cycler (Applied Biosystems). The applicability of these primers to evaluate grazing on *N. spumigena* by mysid shrimps and copepods has been tested using a conventional PCR (Gorokhova 2009, Gorokhova & Engström-Öst 2009). To prepare standards, defined volumes of exponentially growing *N. spumigena* culture were filtered onto GF/F filters, and DNA was extracted according to Becker et al. (2002). qPCR reactions were performed in duplicate using the QuantiTect SYBR Green PCR Kit (QIAGEN). Standard curves were generated using 10-fold dilutions of the extracted DNA; duplicate negative controls (water) were included in all runs. For qPCR of the copepod samples, an intermediate concentration of standard DNA was added to all samples to ensure that sample concentrations occurred in the central portion of the standard curve. The measured concentration of samples was later adjusted to account for this added DNA to determine the concentration of *Nodularia* DNA in the copepods. Amplifications were performed in a 25 µl reaction mixture with an initial denaturing step of 15 min at 95°C, and 40 cycles of 30 s at 94°C, 30 s at 53°C and 30 s at 72°C. An end-point melt-curve analysis was generated after each run and analysed to assure the absence of non-specific PCR products; amplification efficiency was 92 to 95%, with $R^2 > 0.98$. To verify the authenticity of the PCR products obtained from the test samples, a random selection was purified using the Nucleo-Spin® Extract Kit (Macherey-Nagel) according to the manufacturer's instructions, sequenced using ABI 3730 PRISM® DNA Analyzer at KIGene (Karolinska Institutet, Stockholm, Sweden) and aligned with the *N. spumigena* BY1 16S rDNA sequence from GenBank (AY075067).

Determination of non-ingestion contamination by *Nodularia*. The amount of *N. spumigena* that might have been attached to external body parts of copepods, but not ingested, was quantified using qPCR, employing the same *Nodularia*-specific primers as described above. Older copepodites (CIII–IV) of *Pseudocalanus* sp. collected in the northern Baltic proper during a time when no *N. spumigena* occurred (27 March 2008) were exposed to the same analytical procedures as used in the feeding experiments with *Eurytemora affinis*. These animals were killed by preservation in 95% ethanol prior to exposure to *N. spumigena* to prevent ingestion of the cyanobacterium. Replicate groups of 10 individuals were exposed to *N. spumigena* (0.16 mg DW l^{-1}) and washed twice in 7 PSU artificial sea water; these were compared against individuals that were washed in the same manner, but not exposed to *N. spumigena*. All individuals were stored in RNA*later* and analysed using qPCR as described above.

Statistical analyses. To evaluate effects of algal species (*Rhodomonas salina* or *Tetraselmis suecica*) and concentration (low/high) on *Nodularia spumigena* biomass, growth and nodularin concentration, a 2-way analysis of variance (ANOVA) was conducted. This was done using treatments containing cyanobacteria with or without algae in the absence of copepods. When interaction between the factors was significant, an unpaired *t*-test was applied to investigate differences between treatments followed by an *F*-test to compare variances. The treatments in which *N. spumigena* was exposed to the algae with and without copepods were analysed by 3-way ANOVA to evaluate effects of the grazers (presence/absence), algal species and concentration on *N. spumigena* biomass. To evaluate effects of algal species, algal concentration and grazing on nodularin concentration,

analysis of covariance (ANCOVA) was applied using *N. spumigena* biomass (treatments with copepods) or growth rate (treatments without copepods) as the covariate. The biomass or growth correction was necessary because of the differences in N treatments between the experiments and a significant negative correlation between *N. spumigena* biomass/growth and nodularin concentration in the treatments with no grazing (Pearson r = −0.58 and −0.60 for biomass and growth, respectively, $p < 0.05$ in both cases, n = 29). Effects of algal species and concentration on ingestion rate and amount of *Nodularia* DNA in copepod stomachs were evaluated with 2-way ANOVA. When post hoc testing was needed, we used a Tukey HSD test. Prior to correlation analysis, ANCOVA and ANOVA, data were tested for normality and homogeneity of variances using Wilk-Shapiro and Levene's tests. In all cases, EC nodularin concentrations were below the detection limit and were therefore not subjected to statistical comparisons. Algal growth rates and IC nodularin concentrations were log(x+1) transformed to achieve a normal distribution; all other data were used without transformation. All tests were 2-tailed and, unless stated otherwise, data are reported as means and SE.

RESULTS

Nodularia spumigena biomass and growth rate

In the absence of copepods (i.e. N, NA_{low} and NA_{high} treatments), 2-way ANOVA with algal species and concentration as factors, and final *N. spumigena* biomass and growth rate as response variables, revealed significant interactions (biomass: $F_{2,23} = 4.31$, $p < 0.02$; growth rate: $F_{2,23} = 4.77$, $p < 0.03$) indicating that the concentration effect on *N. spumigena* growth was not consistent for the 2 algae (Fig. 1A,B). Specific growth rate of *N. spumigena* varied from 0.01 to 0.02 d^{-1} and from 0.02 to 0.03 d^{-1} in incubations with *Tetraselmis suecica* and alone, respectively. Both biomass and growth rate were negatively affected by the increasing *T. suecica* concentration, with significantly lower values in NA_{high} than in the N treatment (unpaired *t*-test; $t_7 = 2.54$, $p < 0.03$ and $t_7 = 2.44$, $p < 0.05$ for biomass and growth, respectively), while no significant changes were observed when the cyanobacterium was incubated with *Rhodomonas salina* (0.021 to 0.025 d^{-1}, $p > 0.05$ in all cases). When *N. spumigena* was exposed to the algae in the presence or absence of copepods (i.e. NA_{low}, NA_{high}, CNA_{low} and CNA_{high} treatments), the negative effect of copepods on cyanobacterial biomass was significant (3-way ANOVA: $F_{1,32} = 11.35$, $p < 0.002$), whereas no other effects and interactions were significant ($p > 0.3$ in all cases). Without the algae, the negative effect of grazing on *N. spumigena* biomass in the CN treatment compared to the N treatment was also significant (unpaired *t*-test; $t_7 = 3.38$, $p < 0.02$; Fig. 1A,B).

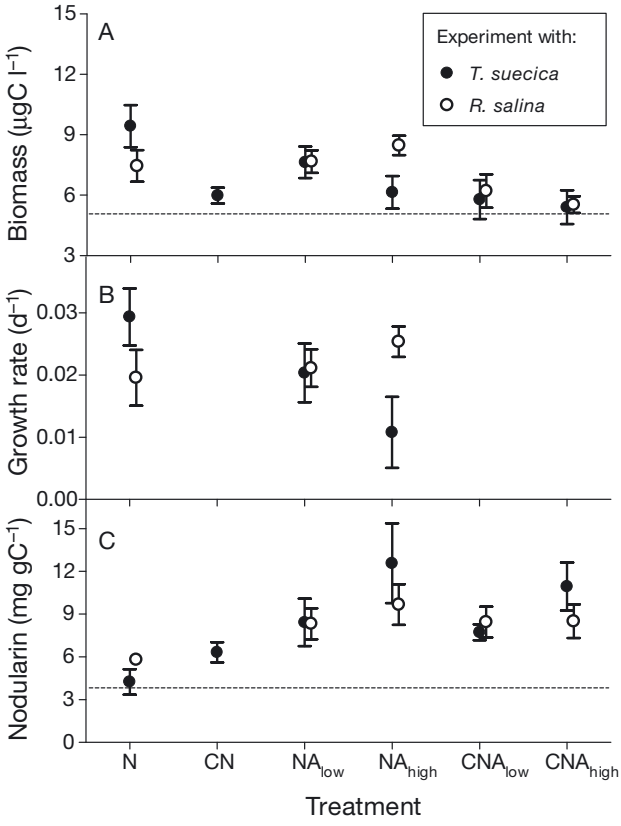

Fig. 1. *Nodularia spumigena.* Changes in (A) biomass (µgC l^{-1}), (B) growth rate (d^{-1}) and (C) intracellular nodularin concentration (mg toxin gC^{-1}) in experiments where the cyanobacterium was incubated with or without copepods *Eurytemora affinis* (10 ind. l^{-1}) and with or without eukaryotic algae (*Tetraselmis suecica* or *Rhodomonas salina*) added at low (50 µgC l^{-1}) or high (250 µgC l^{-1}) concentrations. Treatments: N: *N. spumigena*; C: copepods; A_{low}: algae at low concentration; A_{high}: algae at high concentration. Dashed lines indicate inoculated concentrations of the cyanobacterium (A) and nodularin concentrations (C) in the inocula. Data are given as means ± SE. See also Table 1 for details on the experimental treatments

Intracellular nodularin concentration

When *Nodularia spumigena* was exposed to grazers as the sole food, no significant effect of copepods on nodularin concentration was observed (CN: 6.3 versus N: 4.2 to 5.8 mg toxin g C^{-1}; unpaired *t*-test, $t_7 = 1.86$, $p = 0.11$; Fig. 1C). In the absence of copepods (i.e. in N, NA_{low} and NA_{high} treatments), ANCOVA with algal

species and algal concentration as factors, *N. spumigena* growth rate as the covariate and nodularin concentration as the response variable, indicated a significant positive effect of algal concentration ($F_{2,22}$ = 6.20, p < 0.008) and a negative effect of *N. spumigena* growth ($F_{1,22}$ = 8.56, p < 0.008) on nodularin, whereas the effect of algal species was not significant ($F_{1,22}$ = 0.095, p > 0.7; Fig. 1C). When *N. spumigena* was exposed to the algae in the presence (CNA_{low} and CNA_{high}: 7.7 to 10.9 mg toxin g C^{-1}) or absence (NA_{low} and NA_{high}: 8.3 to 12.6 mg toxin g C^{-1}) of copepods, nodularin concentration was significantly negatively affected by the copepods (ANCOVA: $F_{1,30}$ = 8.08, p < 0.008) and by *N. spumigena* biomass ($F_{1,30}$ = 18.45, p < 0.001), while no other significant effects and interactions were observed (p > 0.1 in all cases).

Growth rates of eukaryotic algae

Growth rates of *Rhodomonas salina* incubated in the presence of *Nodularia spumigena* were negative, with a significant difference between the treatments with and without the cyanobacterium (–0.1 to –0.13 d^{-1} versus 0.74 to 0.8 d^{-1}; 2-way ANOVA, $F_{1,19}$ = 146.5, p < 0.0001), but no significant effect of algal concentration ($F_{1,19}$ = 0.04, p > 0.8). Moreover, there was a significant negative correlation between IC nodularin and *R. salina* growth rate (Pearson r = –0.88, p < 0.0001). Neither presence of *N. spumigena* (2-way ANOVA, $F_{1,19}$ = 0.60, p > 0.4) nor algal density ($F_{1,19}$ =3.40, p > 0.1) had a significant effect on growth rate of *Tetraselmis suecica* (1.12 to 1.36 d^{-1}), and no significant correlation between the algal growth and IC nodularin was observed (p > 0.2). No significant interaction effects for initial algal density and presence of *N. spumigena* were found ($F_{1,19}$ = 0.4 and 0.2 for Expt 1 and Expt 2, respectively, p > 0.6; Fig. 2).

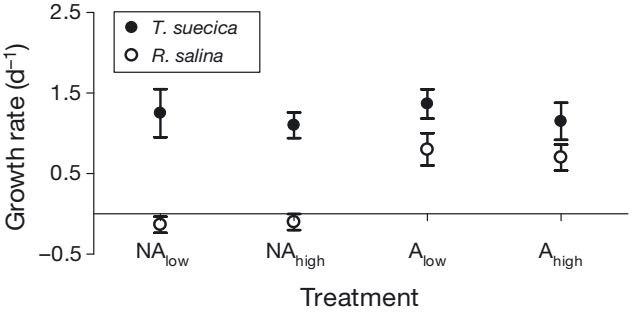

Fig. 2. *Tetraselmis suecica*, *Rhodomonas salina*. Growth rates (d^{-1}) with or without exposure to *Nodularia spumigena*. Abbreviations for the treatments as in Fig. 1. Data are means ± SD. See also Table 1 for details on the experimental treatments

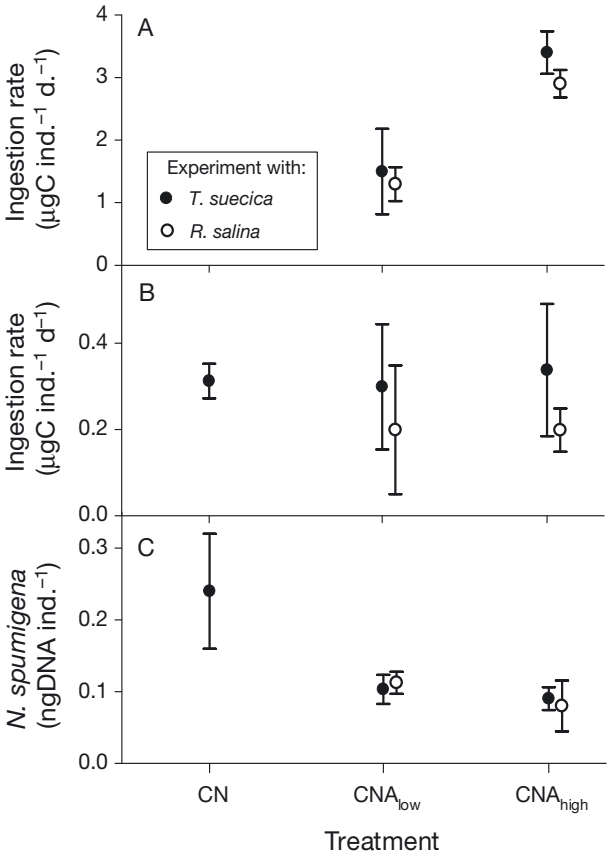

Fig. 3. Ingestion rate (µgC ind.$^{-1}$ d^{-1}) of copepods on (A) *Tetraselmis suecica* and *Rhodomonas salina* and (B) *Nodularia spumigena*, and (C) amount of ingested *N. spumigena* (ng *Nodularia* DNA ind.$^{-1}$) quantified by qPCR in the copepods from different treatments. Abbreviations for the treatments as in Fig. 1. Data are given as means ± SD

Ingestion rates

There were differences between both the algal concentrations and species (Fig. 3A), with feeding rates being significantly higher in the incubations with *Tetraselmis suecica* (2-way ANOVA; $F_{1,19}$ = 4.6, p < 0.05) and at the high algal concentrations ($F_{1,19}$ = 84.4, p < 0.001). No significant correlations between either *Nodularia spumigena* biomass or nodularin concentrations and ingestion of the algae were observed (p > 0.5 in both cases). Feeding rates on the cyanobacterium were significantly positively related to its biomass (ANCOVA, $F_{1,16}$ = 160.8, p < 0.00001) and algal species, with copepods feeding more on *N. spumigena* in the presence of *T. suecica* than when incubated with *Rhodomonas salina* ($F_{1,16}$ = 31.31, p < 0.0001), whereas they were not affected by algal densities ($F_{1,16}$ = 0.18, p > 0.5) and IC nodularin ($F_{1,16}$ = 0.09, p > 0.7).

Quantity of *Nodularia* in individual copepods

In copepods exposed to *N. spumigena* with or without alternative food, the amount of *Nodularia* DNA ind.$^{-1}$ was significantly different from 0 (1-sample *t*-test, p-values varying from < 0.0001 to 0.003; Fig. 3C), while in copepods incubated in FSW (negative controls), it was not (data not shown; $p > 0.05$). In food-containing treatments, the amount of *Nodularia* DNA in copepods varied from 0.038 to 0.34 ng DNA ind.$^{-1}$, with highest mean values in copepods incubated with *N. spumigena* without alternative food, and lowest mean values in those from the treatments with high algal concentrations (Fig. 3C). When treatments with *Rhodomonas salina* and *Tetraselmis suecica* at different concentrations were compared, the effect of algal concentration on *Nodularia* DNA ind.$^{-1}$ was significantly negative (2-way ANOVA, $F_{1,16} = 4.791$, $p < 0.05$), while the effect of algal species was not ($F_{1,16} = 0.002$, $p > 0.8$). No significant correlation between nodularin concentrations and *Nodularia* DNA ind.$^{-1}$ were observed ($p > 0.5$).

Non-ingestion background controls

Trace amounts of *Nodularia* DNA were observed in samples from killed copepods exposed to the experimental conditions (< 5 pg ind.$^{-1}$; data not shown). However, there were no significant differences between individuals exposed and not exposed to *N. spumigena* (*t*-test, assuming unequal variances, $t_5 = 2.571$, $p > 0.4$). In addition, the amount of DNA in these samples was not significantly different from that in the negative controls ($t_4 = 2.776$, $p > 0.8$), indicating that in the experimental animals, the amount of *Nodularia* adhering to the outside of copepods and caused by possible contamination during the sorting procedure (i.e. background contamination) was negligible.

DISCUSSION

The responses observed in our experiments included growth alterations in both *Nodularia spumigena* and eukaryotic algae as well as increased intracellular nodularin concentration in the presence of the potential competitors. However, whereas growth of *Rhodomonas salina* was strongly suppressed by the presence of the cyanobacterium, this was not true for *Tetraselmis suecica*, indicating species-specific responses. Moreover, copepods were actively feeding on both algae and *N. spumigena*, with no sign of suppressed feeding or survival, and the presence of grazers was associated with decreased nodularin–cell quota. Increased toxin production in response to grazing demonstrated for cyanobacteria (Jang et al. 2003, 2007) is an expected inducible defence (Harvell & Tollrian 1999), while negative effects of grazers on toxin levels (Kearns & Hunter 2000, Gorokhova & Engström-Öst 2009, Sopanen et al. 2009) are much less understood. Our findings demonstrate ecologically important interactions among toxic cyanobacteria, non-toxic eukaryote autotrophs and grazers and emphasise that laboratory studies evaluating effects of abiotic factors on growth and toxin production in cyanobacteria may not be representative of the complex interactions in natural plankton assemblages.

One of the most striking effects observed in our experiments was that *Nodularia spumigena* severely inhibited growth rates in *Rhodomonas salina*, whereas those in *Tetraselmis suecica* were unaffected (Fig. 2). Growth inhibition by cyanobacteria has been reported for many heterotrophic bacteria, fungi, cyanobacteria and algae (Jaiswal et al. 2008), including *Rhodomonas* sp. exposed to *N. spumigena* (Suikkanen et al. 2004). Moreover, the general sensitivity of cryptophytes to phytoplankton allelochemicals is well known (Infante & Abella 1985, Rengefors & Legrand 2001, Karjalainen et al. 2007). However, despite its sensitivity to the cyanobacterium, *R. salina* coexists with *N. spumigena* in the Baltic Sea (Sivonen et al. 1989, Hill 1992). The impact of the allelopathy on this co-existence may depend on the genetic diversity of both the alga and the cyanobacterium and their co-adaptation (Leflaive & Ten-Hage 2007). Interestingly, Suikkanen et al. (2007) suggested that a decrease in cryptophyte biomass since the late 1970s in the Gulf of Finland was, at least in part, a result of the increased cyanobacteria and the associated allelopathic interactions. By contrast, *T. suecica,* which has been reported to produce antimicrobial agents (Irianto & Austin 2002), suppressed the growth of *N. spumigena*. The 'exotic' nature of the algae for the AV1 strain of *N. spumigena* that was isolated at the entrance of the Gulf of Finland, where *T. suecica* does not occur (Hällfors 2004), is the most probable cause of this allelopathic response (Leflaive & Ten-Hage 2007).

The difference in algal growth responses suggests that they are caused by the excretion of allelopathic substances by *Nodularia spumigena* rather than unsuccessful competition for nutrients. Both potential competitors and grazers exerted significant effects on intracellular nodularin, with toxin increasing in the presence of algae in a concentration-dependent manner. When grazers were present in the mixtures of algae and cyanobacterium, however, the increase was significantly less pronounced. Moreover, growth of *Rhodomonas salina* correlated negatively with intracellular nodularin. These results suggest that nodu-

larin production is a part of the allelopathic response of *N. spumigena*, a known producer of many potentially linked bioactive compounds (Wiegand & Pflugmacher 2005), towards other phytoplankton, and that this response is not alga-specific. A similar response has been observed in *Anabaena flos-aquae*, which almost doubled microcystin concentration in the presence of green algae (Kearns & Hunter 2000). The difference in growth response between the 2 algae implies that either they differ greatly in their sensitivity to nodularin (which is not unexpected) or the allelopathic interactions include the production of other compounds with inhibitory properties (Berry et al. 2008). The latter is supported by findings of Møgelhøj et al. (2006) and Suikkanen et al. (2006), who found no evidence for pure nodularin having allelopathic properties against *Rhodomonas* sp. Also, Jonasson et al. (2008) suggested that nodularin is not a secondary metabolite, but an important primary intracellular nitrogen compound, because *N. spumigena* cells continuously express the nodularin synthetase genes and maintain a threshold level of nodularin in cells. This view about a physiological role of nodularin is supported by the positive relationship between exponential growth and nodularin cellular concentration in monocultures (Lehtimäki et al. 1994). However, in mixed communities, intracellular nodularin concentrations and cyanobacterial growth could correlate both positively and negatively (Gorokhova & Engström-Öst 2009). When *N. spumigena* was exposed to algae, there were significant negative correlations between IC nodularin and the cyanobacterial biomass or growth rate, whereas this was not the case in the treatments without algae. The precise biological mechanisms by which copepods modulated the cellular nodularin quota are unclear, but the most likely explanation is that they simply decreased abundance of algal competitors by grazing. Alternatively, copepods could have caused changes in nutrient conditions, potentially affecting nodularin production. Indeed, Jonasson et al. (2008) found that the expression of nodularin synthetase genes decreases in the presence of ammonium. In our experiments, all nutrients were supplied at saturating levels at the start of each experiment, which is indicated by the constant growth rate in *Tetraselmis suecica* (Fig. 2). However, when copepods were present, ammonium levels might have increased due to their feeding and excretion; this would lead to the suppressed gene expression and nodularin production. Jointly, these nutrient-mediated mechanisms might explain, at least partly, the observed differences between the treatments. Clearly, further studies are necessary that would include a complex assessment of nodularin synthesis, its turnover and transfer between cells and media under ecologically realistic conditions.

Our results suggest that increased nodularin production in response to mesozooplankton grazing is not an inducible defence in *Nodularia spumigena*, as no relationships between toxin concentrations and copepod feeding, assayed as either ingestion rate by microscopic analysis or as gut content by qPCR, were observed. At *N. spumigena* concentrations commonly observed before the onset of a bloom, copepods feed on the cyanobacterium, regardless of the presence of alternative food (Fig. 3B). Conversely, presence of cyanobacteria did not affect copepod feeding on the alternative food, with copepods having higher feeding rates at higher food concentrations and when offered mixtures containing *Tetraselmis suecica* (Fig. 3A). The latter is expected in view of the higher average food concentrations in incubations with *T. suecica* compared to those with *Rhodomonas salina*, due to the growth inhibition of the latter (Fig. 2). This agrees with previous studies showing that moderate concentrations of the cyanobacterium do not inhibit feeding in *Eurytemora affinis* (Kozlowsky-Suzuki et al. 2003). The feeding rates were comparable to those observed earlier in trials with *N. spumigena* as a sole food (Engström et al. 2000) or in mixtures with similarly sized algae (Kozlowsky-Suzuki et al. 2003). The invariant ingestion rates on *N. spumigena* in the CNA_{low} and CNA_{high} treatments might indicate more efficient detection of large filaments by copepods compared with that of small unicellular *R. salina* and *T. suecica*. Once detected, the cyanobacteria might also be ingested using a raptorial mode of feeding rather than passive filtration of small algae. By contrast, quantities of *Nodularia* DNA measured in copepod guts indicate that its proportion in the total ingested food decreases somewhat at higher concentrations of the alternative food (by 29 and 13% for mixtures with *R. salina* and *T. suecica*, respectively; Fig. 3C). The DNA-based gut content analysis provides an independent and complementary estimate of the cyanobacterium consumption and evidence that copepods actively ingest *N. spumigena* even when an alternative food is plentiful (Fig. 3C); this could be a particularly valuable tool in field studies. It has a greater sensitivity compared to that based on microscopy or gut pigment analysis. However, as with the latter, the kinetics of gut emptying as well as DNA decay rate during digestion must be known to infer feeding rates from the gut content (Gorokhova 2009, Troedsson et al. 2009). The discrepancy in the between-treatment variations observed by microscopy-based ingestion rate versus PCR-based gut content emphasise the importance of studying digestion and assimilation of the cyanobacteria in different grazers. It is likely, for example, that the gut evacuation rate was much lower for the ingested cyanobacterium than for the ingested algae, thus lead-

ing to an overestimated proportion of *N. spumigena* in the diet. Moreover, the gut evacuation rates were likely to be higher in NA_{high} incubations compared to the incubations with *N. spumigena* as a sole food, which could have contributed to the observed differences.

CONCLUSIONS

Our results suggest that in *Nodularia spumigena*, nodularin production is not an inducible defence trait against mesozooplankton grazers, but rather a metabolite involved in allelochemical response. The responses of toxic cyanobacteria to competitors and grazing pressure, in terms of growth and toxin production, are highly relevant in order to understand processes behind bloom formation and its effects on community grazers, water quality and toxin bioaccumulation in the food webs. For example, the bloom-forming cyanobacterium *Anabaena flos-aquae* has been reported to be involved in community-shaping chemical signalling. This cyanobacterium produces anatoxin and microcystin-LR, which respond differently to the presence of eukaryotic algae and contribute to a shift from eukaryote algal dominance to cyanophyte dominance (Kearns & Hunter 2000). With such shifts, changes in grazer communities are also expected. Therefore, community level effects of a cyanobacterial bloom in the pelagic system are a function of (1) the direct effects of cyanotoxins on competitors and grazers, and vectorial transport of toxins in the food web; (2) the direct and indirect effects of increased cyanobacteria standing stock on competitive and trophic interactions; (3) the reciprocal effects on cyanotoxin production and turnover; and (4) the resulting changes in nutrient cycling. To understand the dynamics of toxic cyanobacterial blooms, studies on cyanobacterial toxicity should extend beyond monospecies cultures to include competition, microbial interactions and grazing using naturally co-occurring non-toxic as well as toxic phytoplankton in natural assemblages.

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