

Grazer-induced defense in *Phaeocystis globosa* (Prymnesiophyceae): Influence of different nutrient conditions

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Abstract

We examined the combined effects of grazer infochemicals and nutrient status on colony development of *Phaeocystis globosa* cultures grown under nitrogen and phosphorus (NP)–sufficient, P-deficient, and N-deficient conditions exposed to high and low *Acartia* spp. density filtrates. Changes in colony development relative to controls receiving no grazer signals were estimated. *P. globosa* colony development responded to grazer infochemicals regardless of nutrient status, although the expression of the response varied between nutrients. Significant colony suppression (in terms of percent of cells allocated to colonies) occurred in both NP-sufficient and P-deficient experiments, with the response being dependent on the density of grazers for NP-sufficient cells. The percent of cells in colonial form in N-deficient *P. globosa* decreased in response to low grazer density filtrates but increased in response to high grazer density filtrates. These opposite results for the N-deficient experiment are related to a high mortality of *Acartia* in the high grazer density filtrate treatment, which may affect the infochemicals released from such grazers.

Some infochemicals released when predator and prey interact serve as reliable signals that allow phytoplankton to detect potential predators (Tang 2003; Selander et al. 2006; Long et al. 2007), enabling phytoplankton to adjust their defensive strategies to the risk of predation. Such inducible defenses are opposed to constitutive ones, which are genetically fixed defenses that are always present. Induced defenses save costs because they are only employed when triggered and should therefore be selected in environments where predators vary on temporal and spatial scales (Tollrian and Harvell 1999), which predators of phytoplankton often do (Durbin and Durbin 1981; Cervetto et al. 1993).

Much research on chemically induced defenses in phytoplankton has concerned freshwater species, especially the chlorophyte genus *Scenedesmus*, which forms a large number of four- to eight-celled coenobia when it is exposed to infochemicals released during grazing activities of the cladoceran *Daphnia* (Lüring and van Donk 1996, 1997). Such responses result in reduced grazing pressure due to size mismatch (Hessen and van Donk 1993). Chemical communication between phytoplankton and zooplankton in marine ecosystems may have indirect and cascading effects on the evolution and ecology of entire communities and ecosystems (Hay and Kubanek 2002).

Phaeocystis globosa is a marine species that can change biovolume by more than three orders of magnitude by alternating between various morphotypes. These morphotypes include small solitary cells (3–9.3 μm ; Rousseau et al. 2007) and larger colonies (8000–9000 μm ; Jahnke and Baumann 1987; Rousseau et al. 1990). Size and shape are first-order determinants of prey suitability at the predator species level (Tillmann 2004). The wide span of sizes observed among solitary cells and colonies of *P. globosa* could therefore offer protection against a broad range of

predators. *P. globosa* is consequently a model candidate for the study of morphological defense induced by grazer infochemicals in marine waters. The ability of *P. globosa* to effectively escape grazing by alternating between various morphotypes and sizes has also been identified as one factor responsible for the global success of this species (Turner et al. 2002; Tang 2003). Studies of nitrogen (N) and phosphorus (P) sufficient *P. globosa* have shown that infochemicals associated with various predator activities influence the formation and the development of colonies. Tang (2003) reported colony enlargement of *P. globosa* but no alterations in the rates of colony formation when these were exposed to infochemicals from heterotrophic dinoflagellates and calanoid copepods. A similar mechanism of colony enlargement was reported for the closely related *Phaeocystis antarctica* in response to infochemicals released from natural zooplankton assemblages dominated by small copepods such as *Oithona similis* (Tang et al. 2008). Long et al. (2007) found consumer-specific but opposing morphological transformations in *P. globosa*. Copepod-associated infochemicals suppressed colony formation in terms of percent of cells in colonies, whereas ciliate-associated infochemicals enhanced colony formation. These differing responses were adaptive since the copepods significantly grazed more on colonies than on solitary cells, whereas the ciliates grew much faster when fed solitary cells than when fed colonies (Long et al. 2007).

Grazer-mediated infochemicals thus play a significant role in the alternation between solitary cells and colonies. In addition, various abiotic factors, such as light regimes, small-scale turbulence, and nutrient conditions, also influence transitions between morphotypes of *P. globosa* (Rousseau et al. 2007). Thus, the transition between solitary cells and colonies is complicated. We investigated the influence of a combination of grazer infochemicals and various nutrient regimes on the transition from solitary cells to colonies of *P. globosa*.

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P. globosa (CCMP 1528) grown under nitrogen and phosphorus (NP)-sufficient and P- and N-deficient conditions were exposed to filtrate from the copepod *Acartia* spp. Percentages of cells in colonies, colony densities, and colony sizes were assessed and compared to grazer-free control filtrate treatments. Based on a small-scale preliminary experiment with NP-sufficient *P. globosa*, in which we observed a decrease in the percentage of cells in colonies in response to *Acartia* infochemicals, we hypothesized that exposure to such infochemicals would result in a decreased proportion of colonial cells. Solitary cells approach the lower limit of effective retention of adult suspension-feeding copepods (Nival and Nival 1976). *P. globosa* could therefore gain the ecological advantage of decreased feeding rates of *Acartia* by decreasing the proportion of cells in colonial form (Long et al. 2007). We further hypothesized that the decreased proportion of cells in colonial form would be even stronger if the cells were N and P deficient, because the lower growth rate of nutrient-deficient cells might not be able to compensate for grazing losses, which could occur at unlimited nutrient availability (van Donk and Hessen 1993).

Methods

Experimental design—Three sets of *P. globosa* batch cultures with different nutrient regimes (NP sufficient [NP suff], P deficient [P def], and N deficient [N def]) were prepared in triplicate (Fig. 1, part I). As grazers we used adult females of *Acartia* spp. raised from eggs originating from wild-caught females (Fig. 1, part II) that were cultured in the laboratory on a diet of *Rhodomonas salina*. We had previously used *R. salina* successfully to breed copepods and therefore decided to use this species as an initial food source. A second generation of copepods provided adult females of approximately the same age throughout the experiments.

NP-sufficient, P-deficient, and N-deficient *P. globosa* were exposed to direct grazing by *Acartia* spp. (Fig. 1, part III). Each experiment consisted of three treatments, one control treatment (C) with no copepods and a low-density (LD) and a high-density (HD) grazer treatment, as described in Table 1. One hundred thousand solitary *P. globosa* cells per milliliter were inoculated in the LD and the HD grazer treatments and the control replicates. In the LD and HD treatments, adult female *Acartia* spp. were added at densities of 16 and 160 copepods L⁻¹, respectively. All treatments within each experiment were replicated three times, and 250-mL tissue culture flasks were used as experimental containers. After 3 d, filtrates were collected by passing the contents through a GF/F filter (low vacuum, ≤ 20 kPa). Before filtration, the contents of the grazing flasks were examined in a petri dish with a binocular microscope to quantify the mortality of copepods.

The combined effects of *Acartia* infochemicals and nutrient availability on colony formation of *P. globosa* were evaluated in the final step of each nutrient experiment. The cell-free filtrates, which contained released infochemicals, were used to inoculate new solitary cells at a concentration of 100,000 cells mL⁻¹, obtained from the

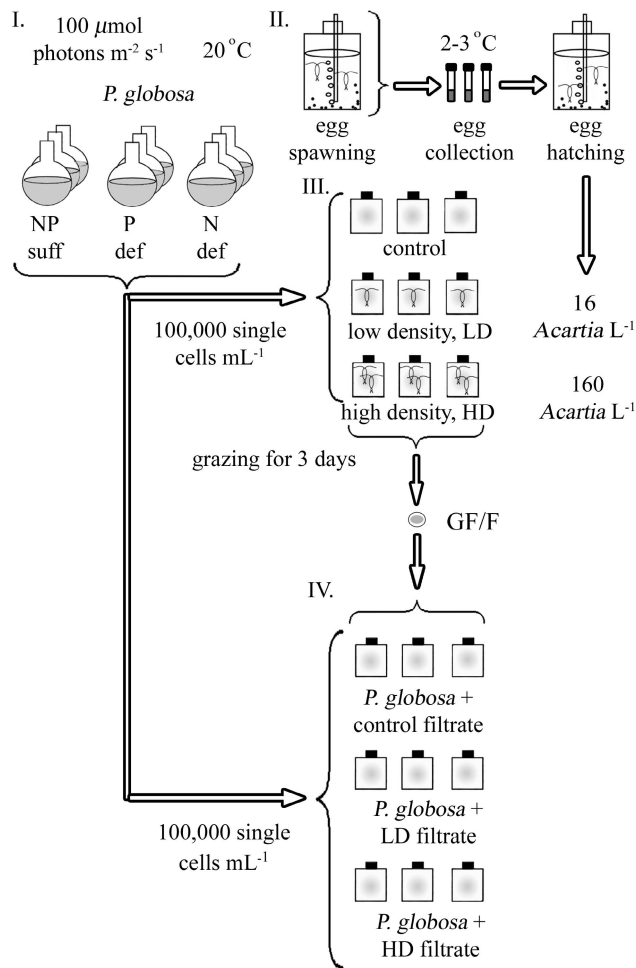


Fig. 1. Schematic representation of the experimental set-up used to test the effect of *Acartia* spp. infochemicals on colony formation and colony size of *Phaeocystis globosa* growing in NP-sufficient (NP suff), P-deficient (P def), and N-deficient (N def) conditions.

different cultures of *P. globosa* (Fig. 1, part IV). Filtrate incubation lasted for 3 d, after which samples for cell counts, pH, and dissolved inorganic nutrient analyses were taken. Samples for pH and inorganic nutrients were taken to ensure that differences in colony formation were caused by grazer infochemicals and not by alterations of these parameters that could have occurred between the grazer treatments and the controls of each experiment.

Phytoplankton and grazers—The non-axenic *P. globosa* strain used in this study (CCMP 1528, which originates from the Galapagos Islands, Ecuador) was obtained from the Bigelow Laboratory (West Boothbay Harbor, Massachusetts). The cryptophyte *R. salina* (KAC 30) was obtained from Kalmar Algal Collection (KAC, Linnaeus University, Kalmar, Sweden).

The medium used to grow and maintain phytoplankton cultures during the experiment was prepared with filtered (Munktell glass-fiber filter, 1.2- μ m mesh size) and autoclaved natural seawater from the Swedish west coast, with a salinity of 31 g kg⁻¹, adjusted to 26 g kg⁻¹ by adding Milli-

Table 1. Each experiment (NP sufficient, P deficient, and N deficient) with its three respective treatments, including abbreviations used throughout the study.

Experiment	Treatment		
	Control	16 Copepods L ⁻¹ (low density, LD)	160 Copepods L ⁻¹ (high density, HD)
NP sufficient	C-NP suff	LD-NP suff	HD-NP suff
P deficient	C-P def	LD-P def	HD-P def
N deficient	C-N def	LD-N def	HD-N def

Q water. The salinity of the *P. globosa* culture was adjusted because *R. salina* had been growing for many years at a salinity of 26 g kg⁻¹, and this salinity had previously been used to grow copepod cultures.

NP-sufficient, P-deficient, and N-deficient batch cultures (3 × 1.5 liters for each nutrient regime) were grown in 2-liter Schott Duran bottles at 20°C ± 1°C with 100 μmol photons m⁻² s⁻¹ in a 14:10-h light:dark cycle. NP-sufficient cultures were grown in modified F/2 medium without silicon (Si; 580 μmol L⁻¹ NO₃⁻; 36.6 μmol L⁻¹ PO₄³⁻; N:P, 16.0:1) (Guillard 1975). In the N-deficient cultures, nitrate concentrations were adjusted to 94 μmol L⁻¹, while phosphate concentrations were kept as in the F/2 (N:P, 2.6:1). In P-deficient cultures, phosphate concentrations were adjusted to 8.7 μmol L⁻¹ (N:P, 66.7:1), and nitrate concentrations were kept at F/2 levels. Growth of *P. globosa* batch cultures was monitored daily by measuring chlorophyll *a* concentrations with a Turner Designs 10-AU fluorometer. The N- and P-deficient cultures grew to a stationary phase before they were used in the experiments, while cells for the NP-sufficient experiment were harvested in the mid-exponential phase. Experiments were started at different times; the NP-sufficient experiment started on day 6, whereas the P-deficient and N-deficient experiments started at days 15 and 16, respectively. All *P. globosa* cultures were sampled for dissolved inorganic as well as particulate organic nutrient analyses, before grazer incubations on days 6, 15, and 16 for NP-sufficient, P-deficient, and N-deficient cultures, respectively, and before filtrate incubations on days 9, 18, and 19 for NP-sufficient, P-deficient, and N-deficient cultures, respectively, to examine if the cultures actually were NP sufficient, P deficient, and N deficient at these times. Cell count samples were taken from all cultures before grazer incubations to estimate the effect of varying nutrient status of *P. globosa* on colony development.

Copepods were collected with a 450-μm plankton net off the Swedish west coast, placed in a 10-liter thermos bottle, and transported to Kristineberg Marine Research Station, where the thermos bottle was kept in a temperature-controlled room at 4°C and gently bubbled with air overnight. The following morning, the thermos was transported to the laboratory within 8 h. Adult individuals of *Acartia* spp. were extracted from this water sample, and mature females were allowed to produce eggs in the laboratory while feeding on *R. salina* at 16°C. *R. salina* were grown in F/2-Si medium (Guillard 1975) at 16°C under the same light conditions as the *P. globosa* cultures and were allowed to grow to a high density in late-

exponential phase, after which 10% of the culture volume was removed and fed to the copepods everyday and replaced with F/2-Si medium. Eggs were collected after 14 d and stored in a refrigerator at 2–3°C. These eggs were then used to raise a new generation of *Acartia* spp. on a diet of *R. salina*. Two days prior to egg hatching the *R. salina* culture was adapted from 16°C to 20°C, which was the temperature at which egg hatching was initiated. The NP-sufficient experiment started when the eggs had developed to adults, which in this case took 17 d.

To empty their guts, adult females were extracted and placed in filtered seawater for 12 h before the start of each nutrient experiment. Solitary *P. globosa* cells for grazer and filtrate incubations were collected by gravity filtering part of the different *P. globosa* cultures through a 10-μm net. After inoculation of these solitary *P. globosa* cells and the addition of copepods, experimental containers were mounted on a rotating plankton wheel rotating at 1 revolution per minute (2.80 × 10⁻⁴ g) under the same temperature and light conditions in which the *P. globosa* cultures were grown.

Cell counts—Cell counts were carried out according to the method described in Long et al. (2007). A total of three samples per replicate were preserved in acidic Lugol's solution. To count solitary cells, colonies were removed from one sample per replicate by filtering through a 10-μm net before preservation. Samples for total cell counts were shaken to disrupt colonies, diluted, settled in Palmer-Maloney chambers (0.1 mL), and counted at 400× magnification. At least 600 cells were counted per replicate. To determine how many cells were in colonial form, solitary cell counts were subtracted from total cell counts; this difference was expressed as a percentage of cells in colonies. Colony density and size were determined from the third sample from each replicate. These samples were settled for 15 h in 2.65-mL Utermöhl chambers. After enumeration, colony size was assessed by measuring the diameter of the mucus envelope at 200× magnification with a calibrated ocular meter. We measured the diameter of each colony along one chamber diameter until 50 colonies from each sample had been measured. All samples taken for colony density and size were analyzed within 2 d (Jakobsen and Tang 2002).

Nutrient analyses—Analyses of inorganic nitrogen (NO₃⁻ and NH₄⁺) and phosphorus (PO₄³⁻) were carried out using colorimetric methods, in accordance with the methodology described by Valderrama (1995). Samples for

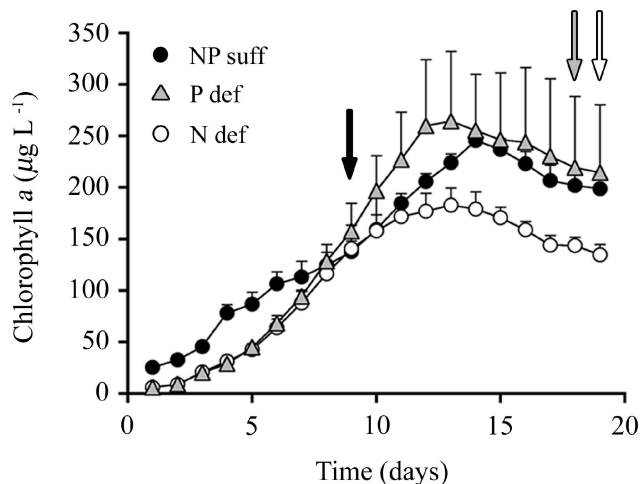


Fig. 2. Growth of *Phaeocystis globosa* in NP-sufficient (NP suff), P-deficient (P def), and N-deficient (N def) conditions. Arrows indicate time of filtrate incubation (mean \pm SD; SD bars sometimes hidden behind symbol, $n = 3$).

particulate organic C (POC), particulate organic N (PON), and particulate organic P (POP) were taken by filtering experimental water onto pre-combusted (450°C, 2 h) Whatman GF/C glass-fiber filters. Prior to analysis the filters were dried at 60°C for 24 h and stored in a desiccator. POC and PON samples were analyzed in a Fisons NA 1500 CHN analyzer, while POP was measured using high-temperature combustion of dried samples, treated with magnesium sulfate, according to the procedure outlined by Solorzano and Sharp (1980).

Data analysis—All statistical analyses were performed with SPSS 13.0. The Shapiro–Wilks test was used to check data for normal distribution, while the Levens test was used to test homogeneity of variances. ANOVA and Tukey's post hoc test were used to test differences between means when the Shapiro–Wilks and Levens tests demonstrated no significant differences. Percentage of cells in colonies and percentage mortality data were arcsine transformed. For the other data, if the Levens test demonstrated significant differences, these data were log transformed before the ANOVA and Tukey's post hoc test were run.

Results

Growth and nutrient analyses of *P. globosa* cultures—*P. globosa* grew well in all nutrient conditions; the lowest biomass occurred in N-deficient cultures. P-deficient batch culture replicates of *P. globosa* showed a large variation in biomass (Fig. 2). No significant differences were found between NP-sufficient, P-deficient, and N-deficient cultures in terms of percentage of cells in colonies ($F_{2,6} = 2.43$, $p = 0.168$; Table 2). N-deficient cultures contained 328.0 ± 107.7 colonies mL^{-1} , which is significantly higher than the value identified in P-deficient cultures, which contained 127.7 ± 25.9 colonies mL^{-1} ($F_{2,6} = 9.70$, $p = 0.010$; Table 2). No such differences were found between NP-sufficient and P- or N-deficient cultures. P- and N-deficient

Table 2. Percentage of cells in colonies, colony concentration, and colony size of NP-sufficient, P-deficient, and N-deficient batch cultures (mean \pm SD, $n = 3$).

	NP sufficient	P deficient	N deficient
% of cells in colonies	40.8 \pm 4.3	23.1 \pm 13.1	28 \pm 10.2
Colonies mL^{-1}	206.3 \pm 21.6	127.7 \pm 25.9	328 \pm 107.7
Colony size (μm)	59.7 \pm 1.2	78.6 \pm 5.1	79.9 \pm 1.3

colonies averaged $78.6 \pm 5.1 \mu\text{m}$ and $79.9 \pm 1.3 \mu\text{m}$, respectively, and were significantly larger ($F_{2,6} = 152.33$, $p < 0.001$) than NP-sufficient colonies, which averaged $59.7 \pm 1.2 \mu\text{m}$. There was no difference in colony size between P- and N-deficient colonies (Table 2).

In the NP-sufficient cultures both NO_3^- and PO_4^{3-} were in excess at the time of both grazer and filtrate incubations. PO_4^{3-} was below the analytical detection limit in the P-deficient cultures, and NO_3^- was low in the N-deficient cultures. The concentration of NH_4^+ was low in all cultures at all times (Table 3).

Nutrient deficiency affected the cellular concentration of C, N, and P and the atomic ratios of these nutrients in *P. globosa* cells (Table 4). P-deficient cultures contained less P than did NP-sufficient cultures ($F_{2,6} = 9.67$, $p = 0.018$ prior to grazer incubations and $F_{2,6} = 13.41$, $p = 0.005$ prior to filtrate incubations) and N-deficient cultures ($p = 0.024$ prior to grazer incubations and $p = 0.036$ prior to filtrate incubations). N was significantly lower in N-deficient cultures than in NP-sufficient cultures ($F_{2,6} = 6.47$, $p = 0.045$ prior to grazer incubations and $F_{2,6} = 6.81$, $p = 0.039$ prior to filtrate incubations) and P-deficient cultures ($p = 0.049$ prior to grazer incubations and $p = 0.047$ prior to filtrate incubations). There was no significant accumulation of the nutrient in excess ($p > 0.05$ for all cultures at all times). Cellular concentrations of C often increase as a result of decreased cellular division under periods of nutrient deficiency (Cembella et al. 1984; Latasa and Berdalet 1994). However, in our experiments there were no significant differences in the amount of C among NP-sufficient, P-deficient, and N-deficient cultures at any time ($F_{2,6} = 1.14$, $p > 0.05$ prior to grazer incubations and $F_{2,6} = 1.50$, $p > 0.05$ prior to filtrate incubations). Atomic nutrient ratios in cells from the different cultures indicated nutrient limitation of both P- and N-deficient cultures. C:N ratios increased more in N-deficient cultures than in NP-sufficient cultures ($F_{2,6} = 17.01$, $p = 0.003$ prior to grazer incubations and $F_{2,6} = 15.06$, $p = 0.004$ prior to filtrate incubations) and P-deficient cultures ($p = 0.018$ prior to grazer incubations and $p = 0.020$ prior to filtrate incubations). Prior to grazer incubations, N:P ratios decreased more in N-deficient cultures than in NP-sufficient cultures ($F_{2,6} = 44.38$, $p = 0.048$) and P-deficient cultures ($p < 0.001$), but only compared to P-deficient cultures prior to filtrate incubations ($F_{2,6} = 10.42$, $p = 0.010$). C:P ratios of P-deficient cultures increased more than did those of NP-sufficient cultures ($F_{2,6} = 11.95$, $p = 0.009$ prior to grazer incubations and $F_{2,6} = 6.76$, $p = 0.041$ prior to filtrate incubations) and N-deficient cultures ($p = 0.020$ prior to grazer incubations and $p = 0.046$ prior to filtrate incubations). N:P ratios increased more in P-

Table 3. Inorganic nutrient concentrations ($\mu\text{mol L}^{-1}$) of NP-sufficient, P-deficient, and N-deficient *P. globosa* batch cultures at times of grazer (days 6, 15, and 16) and filtrate (days 9, 18, and 19) incubations (mean \pm SD, $n = 3$). u.d.l., under detection limit.

	NP sufficient		P deficient		N deficient	
	Day 6	Day 9	Day 15	Day 18	Day 16	Day 19
NO_3^-	448.9 \pm 9.8	428.0 \pm 7.7	480.7 \pm 5.7	475.7 \pm 4	1.9 \pm 2.6	0.7 \pm 0.6
PO_4^{3-}	27.7 \pm 1.4	26.4 \pm 0.8	u.d.l.	u.d.l.	11.6 \pm 0.9	37.6 \pm 1.2*
NH_4^+	0.2 \pm 0.0	0.3 \pm 0.0	0.3 \pm 0.0	0.3 \pm 0.0	0.3 \pm 0.0	0.3 \pm 0.0

* N-deficient batch cultures of *P. globosa* were spiked with $36.3 \mu\text{mol L}^{-1} \text{PO}_4^{3-}$ at day 16, hence the big difference between day 16 and day 19.

deficient cultures than in NP-sufficient cultures ($F_{2,6} = 44.40, p = 0.002$ prior to grazer incubations and $F_{2,6} = 10.42, p = 0.048$ prior to filtrate incubations) and N-deficient cultures ($p < 0.001$ prior to grazer incubations and $p = 0.010$ prior to filtrate incubations).

Combined effects of nutrient limitation and Acartia spp. infochemicals on colony development of P. globosa—Colony formation: Higher grazer density was significantly correlated with suppression of colonies in the NP-sufficient experiment, in which $53\% \pm 3.3\%$ of all cells in C-NP suff (which received no grazer infochemicals) formed colonies. The percentage of cells forming colonies decreased to $16\% \pm 2.4\%$ in LD NP suff ($F_{2,6} = 190.77, p < 0.001$) and to $9.8\% \pm 2.1\%$ in HD-NP suff ($p < 0.001$). LD-NP suff had significantly more cells in colonial form than did HD-NP suff ($p = 0.028$) (Fig. 3A).

In addition, there were significant differences in colony density among treatments in the NP-sufficient experiment. The mean number of colonies per milliliter decreased from $192 \pm 7 \text{mL}^{-1}$ in C-NP suff to $148 \pm 12 \text{mL}^{-1}$ in LD-NP suff ($F_{2,6} = 105.47, p = 0.002$) and to $87 \pm 7 \text{mL}^{-1}$ in HD-NP suff ($p < 0.001$) (Fig. 3D). LD-NP suff differed significantly from HD-NP suff ($p < 0.001$). This indicates that the development of colony density is dependent on grazer density in NP-sufficient *P. globosa*.

P-deficient *P. globosa* responded to grazer-associated infochemicals in a similar manner. The percentage of cells forming colonies in C-P def was $36.2\% \pm 1.8\%$. Significant colony suppression occurred in LD-P def, with $19.3\% \pm 3.8\%$ of cells forming colonies ($F_{2,6} = 12.33, p = 0.010$), and in HD-P def, with $20.4\% \pm 6.3\%$ of cells in colonies ($p = 0.014$). However, the response was not dependent on grazer density: LD-P def and HD-P def did not differ ($p =$

0.960) (Fig. 3B). Furthermore, colony densities did not differ between the control and any grazer treatment for P-deficient *P. globosa* ($F_{2,6} = 4.20, p > 0.05$). Colony density per milliliter in C-P def was $267.0 \pm 21.7 \text{mL}^{-1}$, while the corresponding numbers were $199.3 \pm 42.9 \text{mL}^{-1}$ in LD-P def and $240.0 \pm 13.5 \text{mL}^{-1}$ in HD-P def (Fig. 3E).

A different scenario developed for N-deficient *P. globosa*. Significant colony suppression occurred in LD-N def ($F_{2,6} = 193.86, p < 0.001$), in which only $11.3\% \pm 1.7\%$ of all cells developed into colonial form. This compares with $41.4\% \pm 4.1\%$ in C-N def or in HD-N def, with $49.6\% \pm 0.9\%$ of all cells in colonies. This was significantly higher than that in C-N def ($p = 0.030$) and higher than that in LD-N def ($p < 0.001$) (Fig. 3C). *Acartia* infochemicals did not have any effect on colony density of N-deficient *P. globosa* ($F_{2,6} = 0.27, p > 0.05$). The colony density in LD-N def was similar to that of HD-N def, namely $229.7 \pm 43.7 \text{mL}^{-1}$ and $244 \pm 36 \text{mL}^{-1}$, and was also similar to that of C-N def, with a density of $257 \pm 55.8 \text{colonies mL}^{-1}$ (Fig. 3F).

Colony size: Treatment LD or HD, receiving infochemicals, did not show *P. globosa* colony enlargement in any of the three tested nutrient conditions (Fig. 3G–I). The largest colonies were in the N-deficient treatments, while the smallest colonies were in the NP-sufficient treatments, with mean colony diameters of $80 \pm 0 \mu\text{m}$ and $63 \pm 2 \mu\text{m}$, respectively. Mean colony diameter for NP-sufficient *P. globosa* was $62 \pm 1 \mu\text{m}$ in C-NP suff, $62 \pm 1 \mu\text{m}$ in LD-NP suff, and $65 \pm 3 \mu\text{m}$ in HD-NP suff ($F_{2,6} = 1.99, p = 0.21$) (Fig. 3G).

The mean colony diameter in C-P def was $71 \pm 5 \mu\text{m}$, while the corresponding diameters were $79 \pm 11 \mu\text{m}$ in LD-P def and $70 \pm 3 \mu\text{m}$ in HD-P def ($F_{2,6} = 1.27, p = 0.35$) (Fig. 3H). The mean colony diameter in N-deficient *P. globosa* controls, C-N def, was $80 \pm 5 \mu\text{m}$, which was similar

Table 4. Cellular C, N, and P content and N:P, C:P, and C:N ratios of *P. globosa* batch cultures varying in nutrient supply at times of grazer (days 6, 15, and 16) and filtrate (days 9, 18, and 19) incubations (mean \pm SD, $n = 3$).

	NP sufficient		P deficient		N deficient	
	Day 6	Day 9	Day 15	Day 18	Day 16	Day 19
POC (pg C cell ⁻¹)	13.1 \pm 0.8	14.7 \pm 1.7	15.6 \pm 3.5	16.6 \pm 4.2	15.1 \pm 0.4	12.7 \pm 1.6
PON (pg N cell ⁻¹)	2.6 \pm 0.2	2.6 \pm 0.5	2.6 \pm 0.5	2.8 \pm 0.6	1.7 \pm 0.04	1.6 \pm 0.04
POP (pg P cell ⁻¹)	0.5 \pm 0.08	0.5 \pm 0.03	0.3 \pm 0.04	0.3 \pm 0.02	0.5 \pm 0.09	0.4 \pm 0.08
N:P (atomic)	11.6 \pm 0.7	12.9 \pm 1.6	21.1 \pm 4.1	22.1 \pm 5.9	8.6 \pm 0.4	8.9 \pm 1.6
C:P (atomic)	69.5 \pm 10.6	78.3 \pm 4.2	150.0 \pm 33.8	154.2 \pm 47.8	82.2 \pm 12.4	80.3 \pm 13.9
C:N (atomic)	6.0 \pm 0.6	6.1 \pm 0.5	7.1 \pm 0.2	6.9 \pm 0.3	9.5 \pm 1.2	9.1 \pm 1.0

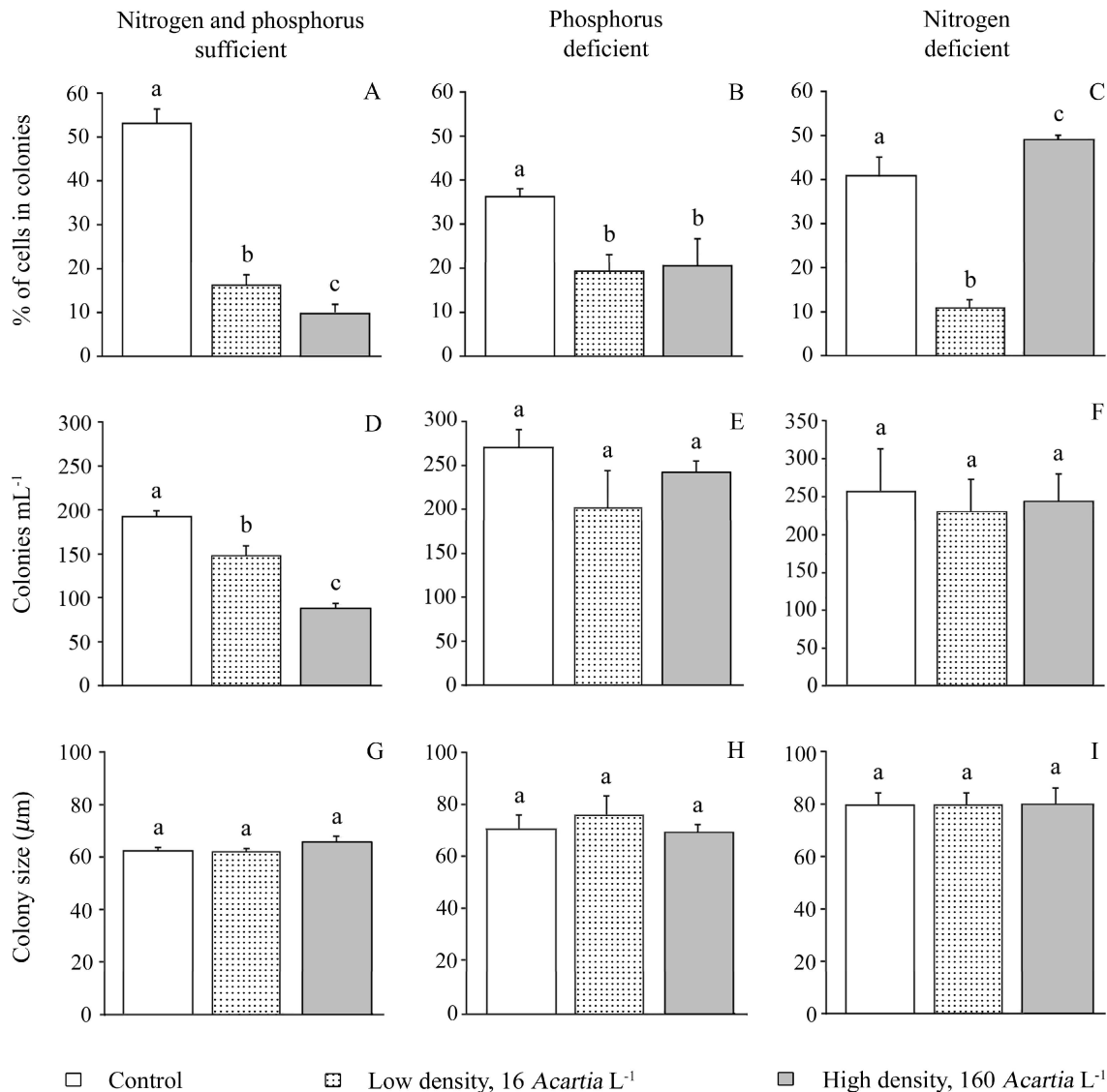


Fig. 3. Percentage of total *P. globosa* cells in colonial form, colony density, and colony size after exposure to infochemicals from *P. globosa* cultures containing no copepods (control, open bars) and low (dotted bars) and high copepod density (gray bars) filtrates under (A, D, G) NP-sufficient, (B, E, H) P-deficient, and (C, F, I) N-deficient conditions. Letters above bars indicate significant (Tukey, $p < 0.05$) differences among treatments (mean \pm SD, $n = 3$).

to the value for LD-N def, $80 \pm 4 \mu\text{m}$, and for HD-N def, $80 \pm 6 \mu\text{m}$ ($F_{2,6} = 0.01$, $p = 0.989$) (Fig. 3I). These effects are in accordance with results from a different *P. globosa* strain, CCMP 627 (Long et al. 2007), but differ from those in which strain CCMP 1528 was used (Jakobsen and Tang 2002; Tang 2003), which is the same strain that was used in this study.

Copepod mortality: The number of dead *Acartia* spp. after 3 d of grazing was counted, and mortality was expressed in percent (Table 5). Lowest mortality occurred in the NP-sufficient experiment, for which the percentages of dead copepods in LD-NP suff and HD-NP suff were $8.3\% \pm 14.4\%$ and $2.5\% \pm 2.5\%$, respectively. Significantly higher mortality, $19.2\% \pm 3.8\%$, occurred in HD-P def than in HD-NP suff ($F_{2,6} = 39.10$, $p = 0.008$). N-deficient *P. globosa* was associated with even more severe death of

Acartia spp. in the high grazer density treatment. LD-N def had a mean mortality of $8.3\% \pm 14.4\%$, which increased to $45.8\% \pm 7.6\%$ in HD-N def. Furthermore, the mortality observed in HD-N def was significantly higher than in HD-NP suff ($F_{2,6} = 39.10$, $p < 0.001$) and in HD-P def ($p = 0.014$) (Table 5).

Discussion

NP-sufficient *P. globosa* responded to grazer infochemicals by suppressing the proportion of cells allocated to colonial form and by reducing the density of colonies. Such responses have previously not been shown for this *P. globosa* strain (CCMP 1528). Responses induced by grazer infochemicals occurred at naturally occurring copepod densities. These inducible reactions also occurred at lower

Table 5. Percentage mortality of *Acartia* spp. in the low-density (LD, 16 *Acartia* L⁻¹) and high-density (HD, 160 *Acartia* L⁻¹) treatments of the NP-sufficient, P-deficient, and N-deficient experiments (mean \pm SD, $n = 3$).

	NP sufficient	P deficient	N deficient
Treatment low density, LD	8.3 \pm 14.4	8.3 \pm 14.4	8.3 \pm 14.4
Treatment high density, HD	2.5 \pm 2.5	19.2 \pm 3.8	45.8 \pm 7.6

and realistic nutrient concentrations, such as P deficiency or N deficiency, although the response is more complex in these situations. This could possibly be an effect of lower food quality of nutrient-stressed algae, which in turn could affect the grazers and the infochemicals released from them and, consequently, the response in *P. globosa*. The mortality of copepods did increase significantly in deficient experiments, especially in HD-N def, which could explain the different responses detected between nutrient experiments. No support was found for any effect of grazer infochemicals on colony size of *P. globosa* in any tested nutrient condition.

Nutrient limitation and colony development of P. globosa cultures—During initiation of *Phaeocystis* spp. blooms in the eutrophicated coastal parts of the southern North Sea, concentrations of NO₃⁻ are high, varying from 50 μ mol L⁻¹ (Cadée and Hegeman 2002) to 27 μ mol L⁻¹ (Veldhuis et al. 1986). NO₃⁻ is lower as the bloom proceeds, and at the peak of the bloom concentrations range from 1.8 μ mol L⁻¹ (van Boekel et al. 1992) to 4.0 μ mol L⁻¹ (Veldhuis et al. 1986), the former being comparable to the NO₃⁻ concentrations in the N-deficient cultures (Table 3). Some authors (Riegman et al. 1990; van Boekel et al. 1992) have postulated that *Phaeocystis* spp. at times could be N limited in the southern North Sea. Riegman et al. (1992) reported a decrease in N:P ratios in the southern North Sea from > 30 in the 1970s to < 15 in the 1980s and concluded that N limitation had increased. However, at the end of the 1980s, N:P ratios increased so that phytoplankton in this area are now believed to again be P controlled (Philippart et al. 2000). Some authors have reported on P limitation of *Phaeocystis* spp. blooms (Veldhuis et al. 1986). Thus, *Phaeocystis* spp. blooms have occurred and might occur in contrasting nutrient environments. The undetected levels of PO₄³⁻ in the P-deficient cultures strongly indicate that P was limiting in this experiment. However, with regard to the N-deficient experiment, NO₃⁻ concentrations above 1 μ mol L⁻¹ were detected in N-deficient cultures (Table 3), which are not accepted as being N deficient in the field (Paasche and Erga 1988; Justic et al. 1995). Still, the low concentrations reported here were evidently in the range that could be encountered by *Phaeocystis* spp. blooms, even those suspected to be N limited (van Boekel et al. 1992). In addition, P-deficient cells displayed significantly lower cellular contents of P, and N-deficient cells had lower

cellular contents of N, compared to NP-sufficient cells, at times of grazer and filtrate incubations (Table 4). Such responses are in accordance with limitation by P and N (Cembella et al. 1984; Sakshaug and Olsen 1986). A further indicator of nutrient limitation is changes in nutrient ratios. C:P and N:P ratios usually increase under conditions of P-limited growth, whereas N-limited growth causes the C:N ratio to increase and the N:P ratio to decrease (Sakshaug and Holm-Hansen 1977; Goldman et al. 1979; Healey and Hendzel 1980). N:P ratios above 20, as found for the P-deficient cultures in this study, would indicate severe P limitation, whereas the N:P ratios of 8.6 and 8.9 found for N-deficient cultures indicate strong N limitation (Paasche and Erga 1988). Additionally, C:P ratios of P-deficient and C:N ratios of N-deficient cultures increased significantly more than did those of NP-sufficient cultures: this also indicates nutrient limitation (Table 4).

Previous reports on the role of nutrients in colony formation of *Phaeocystis* are contradictory: while Veldhuis and Admiraal (1987) suggested that P depletion was a trigger for colony formation from free-living solitary cells, Riegman et al. (1992) found that colonies of *P. globosa* were dominant under NO₃⁻ limitation and absent under both P limitation and NH₄⁺ limitation. Cariou et al. (1994) reported that very low concentrations of PO₄³⁻ (0.23 μ mol L⁻¹) completely prevented colony formation and that nutrient deprivation can also induce lysis and subsequent release of free-living *Phaeocystis* cells (Verity et al. 1991; van Boekel et al. 1992), indicating that low nutrient concentrations would favor solitary cells over colonies. However, an observation that is in opposition to this has been observed (Verity et al. 1991). Thus, from these studies it is hard to discern any unequivocal trend on the role of nutrients with regard to colony development in *Phaeocystis*.

In this study, *Phaeocystis* colonies were observed in P-deficient, N-deficient, and NP-sufficient stationary-phase cultures. Colony density increased significantly in N-deficient cultures compared to P-deficient cultures, but not compared to NP-sufficient cultures. P-deficient and NP-sufficient cultures did not differ in this respect (Table 2). Because of the large variation within each nutrient condition, no significant changes in percentage of cells in colonies were detected (Table 2). It is therefore hard to draw any tenable conclusions as to which nutrient regime causes a dominance of either single cells or colonies. Colonies were significantly larger in both P-deficient and N-deficient cultures when compared with NP-sufficient ones (Table 2), perhaps because the colonies present in deficient cultures during the stationary phase grew for a longer period than did colonies in the NP-sufficient cultures, which were harvested during the exponential phase.

Combined effects of nutrient limitation and Acartia spp. infochemicals on colony development—NP-sufficient *P. globosa* responded to infochemicals released during grazing activities of the copepod *Acartia* sp. by decreasing its proportion of cells in colonial form. This response also depended on grazer density, since exposure to signals from

Table 6. Inorganic nutrient concentrations ($\mu\text{mol L}^{-1}$) and pH values of the control and the two grazer treatments, for each nutrient condition, after 3 d of filtrate incubation. F and p -values are also given (mean \pm SD, $n = 3$). u.d.l., under detection limit.

	Control	Treatment low density, LD	Treatment high density, HD	$F_{2,6}$	p -value
NP sufficient					
NO_3^-	1143.8 \pm 22.6*	1122.8 \pm 22.7	1151.2 \pm 2.7	4.00	0.08
PO_4^{3-}	67.8 \pm 0.6*	67.8 \pm 1.5	68.7 \pm 0.3	2.64	0.15
NH_4^+	0.4 \pm 0.1	0.5 \pm 0.2	0.4 \pm 0.2	0.16	0.86
pH	8.6 \pm 0.0	8.6 \pm 0.0	8.6 \pm 0.0	0.01	0.99
P deficient					
NO_3^-	463.3 \pm 21.8	447.6 \pm 45.6	513.0 \pm 17.8	3.67	0.09
PO_4^{3-}	u.d.l.	u.d.l.	u.d.l.	—	—
NH_4^+	0.3 \pm 0.1	0.5 \pm 0.1	0.4 \pm 0.1	1.48	0.30
pH	8.5 \pm 0.0	8.6 \pm 0.0	8.6 \pm 0.1	0.74	0.52
N deficient					
NO_3^-	0.1 \pm 0.0	0.1 \pm 0.0	0.2 \pm 0.0	3.25	0.11
PO_4^{3-}	42.0 \pm 5.0	44.2 \pm 2.1	46.4 \pm 2.2	1.26	0.34
NH_4^+	0.4 \pm 0.2	0.2 \pm 0.2	0.4 \pm 0.0	0.68	0.54
pH	8.5 \pm 0.1	8.6 \pm 0.1	8.6 \pm 0.1	0.32	0.74

* NP-sufficient experimental containers were spiked with F2 nutrients at the time of grazer incubation, hence the big difference between NP-sufficient and P-deficient treatments in terms of NO_3^- and between NP-sufficient and N-deficient treatments in terms of PO_4^{3-} .

16 *Acartia* spp. L^{-1} (LD-NP suff) resulted in colony formation being suppressed by about 70%, whereas HD-NP suff, in which *P. globosa* received signals from 10 times the grazer density (160 *Acartia* spp. L^{-1}), resulted in a significantly higher suppression (81%) when compared with the control. Increasing concentration of chemicals from *Acartia* grazing on NP-sufficient *P. globosa* also resulted in declining colony density.

The outcome of the NP-sufficient experiment is in good agreement with the findings of Long et al. (2007), who found that signals from grazing *Acartia tonsa* suppressed colony formation in *P. globosa*, both in terms of colony density and in the relative amounts of cells allocated to colonial form; they interpreted this as being an adaptive mechanism, since the copepods grazed significantly more on colonies than on solitary cells. Given the similar responses of decreased percentages of cells in colony form and decreased colony densities in the present study and that of Long et al. (2007), the mechanisms underlying such responses seen in the NP-sufficient experiment would be adaptive for *P. globosa*, at least in the present context, although grazing responses of *Acartia* were not measured. Long et al. (2007) included assays with copepod densities that could occur in natural conditions (i.e., up to 20 L^{-1} ; Weisse 1983) and found that the response was rapid and strong, even at such copepod densities. This finding, in conjunction with our results from the LD treatments, supports the conclusion that these induced responses are ecologically relevant, at least as they concern copepod density.

A decrease in the percentage of cells allocated to colonial form was also seen for P-deficient *P. globosa*, although this decrease was not as marked as in the NP-sufficient experiment: compared to the control, LD-P def resulted

in colony suppression of 47% and HD-P def in colony suppression of 44%. In contrast to the NP-sufficient experiment, these results also show that the response was not grazer density dependent, as the two grazer treatments did not differ significantly from each other.

P. globosa limited by N showed remarkably different responses: even though colony suppression comparable to that seen in the NP-sufficient experiment occurred in LD-N def (71% compared to the control), this response was absent in HD-N def. In addition, the significant decrease in absolute colony density observed in the NP-sufficient experiment was absent in both the P-deficient experiment and N-deficient experiments. This implies that the nutrient status of *P. globosa* has a complex effect on grazer-induced responses. However, these results do not support the hypothesis that there is a stronger response in either P- or N-deficient experiments, as compared with the NP-sufficient experiment. Therefore, the initial hypothesis is not supported.

Abiotic factors such as pH and nutrient levels also affect colony formation in *Phaeocystis* (Cariou et al. 1994; Peperzak 2002). In the present study, however, neither pH nor inorganic concentrations of nitrate, phosphate, or ammonium differed between grazer treatments and the controls in any of the tested nutrient conditions (ANOVA, $p > 0.05$ for all; Table 6). These factors therefore cannot account for the changes in colony formation reported above.

The food quality of *P. globosa* could change as a result of nutrient deficiency. There could, therefore, be an effect on the infochemicals released from grazing *Acartia* and, consequently, the response seen in *P. globosa*. It appeared that N-deficient *P. globosa* cells had a greater effect on the mortality of grazers than did P-deficient or NP-sufficient cells. The mortality of *Acartia* in LD and HD of the

different nutrient experiments supports this: HD-N def resulted in a significantly higher mortality (46%) than did HD-P def and HD-NP suff (with mortalities of 19% and 2.5%, respectively; Table 5). Of all the factors measured, such as inorganic nutrients and pH, copepod mortality was the only one differing significantly between grazer treatments in the N-deficient experiment. The high mortality observed in HD-N def might be one explanation for the differing responses, both when comparing the different nutrient experiments and when comparing grazer treatments within the N-deficient experiment. An explanation for this is that infochemicals released from dying or dead copepods might have confounded the effects of infochemicals released from living ones. The mortality of *Acartia* in HD-P def was also significantly higher than in HD-NP suff, which could explain the weaker response detected in this treatment.

There could be several reasons for the higher mortalities observed in nutrient-deficient grazer treatments, especially in HD-N def. Since copepods such as *Acartia* contain more N than P (Pertola et al. 2002), the amount of the former in a given phytoplankton cell might be more important than P in determining food quality for these copepods. The exceptionally high mortality in HD-N def could have been caused by a combined and synergistic effect of crowding (160 *Acartia* L⁻¹ in HD, compared to 16 *Acartia* L⁻¹ in LD) and a low N content of food.

Some studies have also reported dramatic enlargements of *P. globosa* colonies as a result of both direct (Jakobsen and Tang 2002) and indirect (Tang 2003) grazing activity of a variety of predators. Tang et al. (2008) showed that *P. antarctica* responded to infochemicals released during grazing activities of natural zooplankton assemblages by increasing the size of its colonies, which is in contrast to our results, in which colony size did not differ significantly between control and grazer treatments in any nutrient experiment. Similarly, Long et al. (2007) detected no changes in colony size in response to infochemicals released from copepods or microzooplankton. Various hypotheses have been put forward to explain the discrepancies between these different studies, including interstrain differences, variations in experimental design, and differences in grazer death (Long et al. 2007). Our results do not indicate that interstrain differences are important because the same *P. globosa* strain (CCMP 1528) that we used was also used by Jakobsen and Tang (2002) and Tang (2003), but we obtained results similar to those of Long et al. (2007), who used another strain (CCMP 627). However, grazer death might be an important factor. Our results indicate that grazer death has a substantial role in the responses seen in *P. globosa*, at least when nutrient-deficient cells are offered as a food source. Differences in experimental design could also influence the results. Whereas Tang (2003) used diffusion incubators, allowing a continuous signal production, we used filtrate incubations, as did Long et al. (2007).

Although the results presented here give some new insights into the ability of *P. globosa* to sense and respond to grazer infochemicals under varying nutrient supply, the present study has some limitations and drawbacks. Our *P. globosa* strain has been growing in laboratory conditions

for almost 20 yr, since it was isolated from the Galapagos Islands in 1991. As is discussed in Nejstgaard et al. (2007), this could have implications for the interplay between *Phaeocystis* and its predators. The chemical grazing signals and the possible grazing deterrents released from laboratory cultures might differ from those displayed by *Phaeocystis* growing in natural conditions (Nejstgaard et al. 2007). Consequently, this could have an effect on the expression of *Phaeocystis* defense strategies induced by the chemical presence of predators. On the other hand, similar responses of increased colony sizes were found using natural *P. antarctica* and zooplankton assemblages (Tang et al. 2008) and when using cultures of *P. globosa* (Tang 2003), indicating that chemically induced defenses of phytoplankton do not necessarily solely occur as a result of a long-term confinement to laboratory conditions.

Another potential problem is that the *Phaeocystis* culture and the copepods originated from different locations. However, the *P. globosa* cultures and the copepods were grown, and all the experiments were conducted, in the same water, which originated from the west coast of Sweden, where the copepods were collected. *P. globosa* had grown well in this water for at least 6 months before the experiments were started; it is therefore not likely that differences in origin had an effect on the results.

Our cultures were started at 100,000 solitary cells mL⁻¹, which is considerably higher than most peak values reported for *Phaeocystis* spp. blooms; total cell abundances usually lie within a range of 20,000 to 67,000 cells mL⁻¹ (Veldhuis et al. 1986, 2005; van Boekel et al. 1992). However, peak values of as much as 177,000 total cells mL⁻¹ (Cadée 1996) and 190,000 total cells mL⁻¹ (Cadée and Hegeman 1986) have been observed. Solitary cells have peak values of around 50,000 cells mL⁻¹ (Weisse and Scheffel-Möser 1990; Cadée 1996). Other studies regarding chemically induced morphological changes in *P. globosa* have used lower solitary cell densities (20,000 cells mL⁻¹; Tang 2003; Long et al. 2007) than we used. The ratio of released infochemicals to cell density might have an effect on infochemically induced responses, depending on the nature of the chemical signal (i.e., more infochemicals could be required to induce a response at higher cell densities). However, this is not likely given the similar responses of decreased percentages of cells in colonial form seen in our study and in that of Long et al. (2007), which used lower cell densities but similar concentrations of copepods.

It should also be stressed that, even if the within-nutrient experiment comparisons should not be affected, all comparisons between nutrient experiments are confounded by time, as the different nutrient experiments were conducted on different occasions. Furthermore, *P. globosa* cells for the NP-sufficient experiment were harvested at the mid-exponential phase, while cells for P-deficient and N-deficient experiments were harvested at the stationary phase. It is therefore hard to disentangle the effects of different growth phases from the effects of different nutrient statuses of *P. globosa* cells on the results presented in Table 2 and Fig. 3. This study does nevertheless provide information on how *P. globosa* responds to *Acartia*

infochemicals during periods of non-limited nutrient conditions and exponential growth in the NP-sufficient experiment, which occurs during the build-up of a *P. globosa* bloom (Veldhuis et al. 1986). This study also provides information on how *P. globosa* responds to *Acartia* infochemicals during periods of stationary growth caused by limitation of N in the N-deficient experiment and of P in the P-deficient experiment, which could occur after the peak of a *P. globosa* bloom (Veldhuis et al. 1986; van Boekel et al. 1992). In conclusion, despite the limitations of the present study, we believe that the experimental set-up was adequate to test the initial hypothesis.

In a natural context, *P. globosa* is exposed to a variety of grazers, phytoplankton, viruses, bacteria, parasites, and anthropogenically derived chemical substances; how chemically induced anti-predator defenses have evolved and how they are expressed during such in situ conditions are still largely unknown. Although we used a single prey-predator set-up, the results of the present study could reflect the behavior of *P. globosa* in natural waters. The succession of the ratio of solitary to colonial *P. globosa* cells and the abundance of copepods in natural conditions seem to corroborate the results of decreased percentages of cells in colonial form in response to copepod infochemicals that were found in the NP-sufficient experiment. *P. globosa* occur mainly as solitary cells during pre-bloom conditions (Schoemann et al. 2005), when copepod abundances are high. As copepod abundance starts to decrease, possibly as a result of the preceding diatom bloom diminishing or as a result of deleterious effects of *P. globosa* itself, *P. globosa* starts to develop large numbers of colonies. Copepod abundances, and therefore the quantity of infochemicals suppressing colony formation, remain low during the rest of colonial *P. globosa* blooms (Bautista et al. 1992). In addition, the number of solitary cells increases after the peak of *P. globosa* blooms, probably as a result of nutrient depletion, thereby causing colonies to release solitary cells (Verity et al. 1991; Davies et al. 1992; van Boekel et al. 1992). At the same time, copepod abundances start to increase again (Bautista et al. 1992), which, as indicated by our P-deficient and N-deficient experiments, could be another factor contributing to the increase in the relative number of solitary cells observed at conditions of nutrient limitation, at least as long as the copepods remain healthy and alive.

To the best of our knowledge, this study is the first to examine the effects of grazer infochemicals and of various nutrient conditions that could occur in natural environments on colony development of the cosmopolitan phytoplankton *P. globosa*. The results demonstrate that the strain, previously shown to increase in colony size (Tang 2003), also responds to grazer infochemicals by suppressing the proportion of cells in colonies. Further, grazer death, which increased in N- and P-deficient experiments, had a substantial influence on the responses detected. Grazer infochemically induced responses have a demonstrable effect on whether *P. globosa* is in solitary or colonial form. This could affect both food web efficiency and the structure of pelagic ecosystems dominated by this enigmatic species.

Acknowledgments

We thank Bengt Lundve and co-workers at the Kristineberg Marine Research Station for sampling and transport of zooplankton samples. We also thank Christina Esplund-Lindquist for performing the particulate carbon, hydrogen, and nitrogen analyses. Two anonymous reviewers are acknowledged for valuable and constructive criticism on an earlier version of the manuscript. We are grateful to the Linnaeus University (formerly University of Kalmar) for financial support.

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Associate editor: Everett Fee

Received: 13 October 2009

Accepted: 24 March 2010

Amended: 20 May 2010