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Phagotrophy and toxicity variation in the mixotrophic *Prymnesium patelliferum* (Haptophyceae)

Abstract—Phagotrophy was investigated in the photosynthetic and ichthyotoxic *Prymnesium patelliferum* (Haptophyceae) using fluorescent microspheres, fluorescently labeled bacteria, and live bacteria cells. Ingestion rates were estimated both from prey uptake and disappearance experiments in phosphorus (P)-limited and -replete algal cultures. *Prymnesium patelliferum* was feeding preferentially on bacteria (bact) compared to fluorescent microspheres (FM). Large fluorescent microspheres (1.6 and 3 μm) were ingested at very low rates ($<$0.1 FM alga^{-1} h^{-1}), and small microspheres (0.5 μm) were not ingested. Ingestion of bacteria (mean size 2 μm) was highest in P-limited *P. patelliferum* cultures (up to four bact alga^{-1} h^{-1}) compared to P-replete cultures (0–1.2 bact alga^{-1} h^{-1}). In addition, cellular P content of P-limited cells fed with bacteria became similar to those of P-replete cultures after 48 h, indicating a close relation between cellular P content and feeding behavior. Hemolytic activity of *P. patelliferum* was up to four times higher in P-limited cultures compared to P-replete cultures. During the transition from P-limiting to P-replete conditions, the addition of bacteria and/or the corresponding bacterial filtrate ($<$0.2 μm) and/or PO_4^{3-} to P-limited cultures resulted in a decrease (50%) of the hemolytic activity after 24 h in relation to controls (no addition of bacteria, filtrate, or P). No PO_4^{3-} was detectable as a result of enriching cultures with bacterial cells or bacterial filtrates. These results indicate that *P. patelliferum* can use different sources of P (inorganic and dissolved, organic and particulate) and adapt its mode of nutrition in a short time. Furthermore, the decrease of hemolytic activity in the highly toxic P-limited cells also occurred rapidly following a recovery in cellular P status through mixotrophic feeding or uptake of inorganic phosphate, suggesting that toxicity in *P. patelliferum* cells can be minimized by nutrient manipulation.

Blooms of toxic haptophytes, mainly consisting of *Chrysochromulina polylepis, C. leadbeateri, Prymnesium parvum,* and *P. patelliferum*, have occurred in Scandinavia for the last 10 yr (Edvardsen and Paasche 1998). These blooms, mostly *C. polylepis,* have simultaneously killed hundreds of tons of farmed fish, resulting in large economic losses in this region. The toxic substances of the genera *Chrysochromulina* and *Prymnesium* have been largely studied in terms of their physiological effects (i.e., ichthyotoxic, cytotoxic, neurotoxic) and their responses to environmental conditions. Toxicity can be greatly affected by the growth phase and stress conditions of the alga (i.e., light, salinity, nutrients; Edvardsen and Paasche 1998). Both nitrogen and phosphorus limitation have been shown to increase toxicity in *P. parvum* compared to nutrient repletion (Johansson and Granéli 1999).

Phagotrophy in photosynthetic haptophytes (i.e., mixotrophy) is well described in the genus *Chrysochromulina* (Jones et al. 1993; Nygaard and Tobiesen 1993) and to a lesser extent in the genus *Prymnesium* (Nygaard and Tobiesen 1993; Tillmann 1998). Most attention has been directed toward the physiology of these mixotrophs (i.e., the influence of biotic/abiotic factors on their heterotrophic capabilities). Nutrient limitation has been shown to have different effects on grazing activity in various mixotrophs (i.e., stimulation) (Caron et al. 1993; Legrand et al. 1998) or no effect (Skovgaard 1996). Phagotrophic nutrition may in turn affect cellular chemical composition and, thus, the synthesis of toxic substances. The percentage of naturally occurring *Dinophysis acuminata* cells containing food vacuoles has been shown to be related to the content of okadaic acid or dinophysis toxin (DTX) per cell and seemed to be dependent on the nutrient conditions in the environment (*see* fig. 8 in Granéli and Carlsson 1998). However, the interaction between mixotrophic nutrition and toxicity in toxic phytoplankton has not been investigated.

In this study, we present data on bacterivory in *Prymnesium patelliferum,* showing that the ingestion of bacteria provided an immediate source of phosphorus, which enhanced the growth of phosphorus (P)-limited *P. patelliferum* cells and, in turn, decreased the hemolytic activity in previously P-limited cells.

Culture and growth conditions—A nonaxenic strain of *P. patelliferum* (Rhpat89 obtained from the Oslo Algal collection, Norway) was grown in triplicates in modified f/10 medium (Guillard 1995) corresponding to two different N : P ratios (160 : 1, 16 : 1), giving a limited P supply and no nutrient limitation with the following concentrations (N : P = 160 : 1: NO_3 = 120 μM, PO_4^{3-} = 0.75 μM; N : P = 16 : 1: NO_3 = 120 μM, PO_4^{3-} = 7.5 μM). The abundance of background bacteria (bact, mainly small cocci) ranged from 0.02 to 0.05 $\times$ 10^6 bact ml^{-1} in the cultures used in this study.

The cultures were grown at a temperature of 17 ± 1°C, salinity 33‰, and irradiance of 100 μmol photons m^{-2} s^{-1} under a 16 : 8 h light : dark cycle. Bacterivory experiments were performed 4–5 d after phosphorus was exhausted ($PO_4^{3-} < 0.05$ μM) from the P-deficient (N : P = 160 : 1) cultures. To determine the nutrient status of the cells (before addition of bacteria), particulate organic carbon (POC) and nitrogen (PON), and phosphorus (POP) were measured in 100 and 20 ml of culture (time [t] = 0), respectively.

The strain of bacteria used in this study was isolated from a culture of the diatom *Chaetoceros affinis* (courtesy of S. Myklestad). Stock cultures were grown in Marine Broth Media ($PO_4^{3-} = 258$ μM) at 17°C, salinity 33‰. The bacteria cells were rod shaped, and cell volumes were calculated as $W^2(L - W/3)/4$, where L and W are the measured length and width of a cell. An average volume of 0.39 μm^3 was determined for exponentially growing cells used in the bacterivory experiments. These bacteria showed no hemolytic activity.

Prey ingestion rate—Phagotrophy in *P. patelliferum* was estimated by the ingestion of fluorescent microspheres (FM) of different size (Fluoresbrite 0.5, 1.6, 3 μm) and fluorescently labeled bacteria (FLB) stained with 5-(4,6-dichlorotriazin-2-yl)aminofluorescein (DTAF) according to Sherr et al. (1987). The FLBs were kept in 1-ml Eppendorf centrifuge tubes (2×10^9 FLB ml^{-1}) at −20°C and sonicated after thawing prior to addition to *P. patelliferum* cultures. Subsamples (20 ml, 2×10^5 cells ml^{-1}) were withdrawn from *P. patelliferum* cultures grown under P-replete and P-limiting conditions ($PO_4^{3-} < 0.05$ μM) and placed in scintillation vials. Fluorescent microspheres and FLB were added separately to the *P. patelliferum* cultures (background bacteria abundance: 0.05×10^6 bact ml^{-1}) to a final concentration of 1×10^7 FM ml^{-1} and 5×10^6 FLB ml^{-1}, respectively. Controls were fixed with 2% (v/v) formaldehyde and exposed to the same concentrations of FM and FLB. Prey uptake was monitored for 3 h, with samples being withdrawn at 0, 10, 30, 60, 120, and 180 min, and fixed with Lugol's solution and formaldehyde in order to minimize prey egestion. During this short-term incubation, ingestion rate (prey algal $cell^{-1}$ h^{-1}) was calculated from the slope of the number of prey ingested per *P. patelliferum* versus time, with values being corrected for the number of prey ''ingested'' in killed controls.

Prey disappearance and prey density—To estimate feeding on live bacteria and determine the importance of prey density in *P. patelliferum* feeding, aliquots of bacterial cultures (20–400 μl, corresponding to a gradient of $1–21 \times 10^6$ bact ml^{-1}) were added to P-limited *P. patelliferum* cultures ($n = 6$) and *P. patelliferum* filtrates in borosilicate tubes (15 ml). The abundance of background bacteria was 0.05×10^6 bact ml^{-1} in the *P. patelliferum* cultures before addition of the cultured bacteria or bacterial filtrates. Prior to addition of bacteria into the algal cultures, bacteria were centrifuged two times (3,500 × g, 6 min), and the supernatant was replaced with filtered seawater in order to limit the addition of nutrients and organic substrates from the marine broth. Two sets of replicates were incubated in light. Controls consisted of tubes with the corresponding volume of bacterial culture filtrate (<0.2 μm) in order to determine whether the initial bacterial inoculum affected *P. patelliferum* growth. Algal and bacterial cells were counted initially (t = 0) and after 24 h. The ingestion of bacteria was calculated using Frost's equations (Frost 1972) corrected for growth of *P. patelliferum* (Heinbokel 1978).

Bacterivory and toxicity in P. patelliferum—To investigate the relation between bacterivory and toxicity, P-limited cultures of *P. patelliferum* ($n = 3$, 15 ml) were incubated for 24 h with PO_4^{3-}, with bacteria (bacteria : *P. patelliferum* = 20 : 1) or without bacteria and with the corresponding volume of bacterial culture filtrate (<0.2 μm). P-replete *P. patelliferum* cultures were used as the control. The abundance of background bacteria was 0.02 and 0.04×10^6 bact ml^{-1}, respectively. All cultures were incubated as described above. Samples for POP (20 ml), and POC and PON (100 ml), and cell counts were taken initially (t = 0), both from the P-replete and the P-limited cultures. Hemolytic activity of the algal cells was measured initially (t = 0) and after 24 h in all treatments.

Analytical procedures—Algal samples were preserved with acid Lugol's solution and cells were counted after sedimentation of 1-ml samples using an inverted microscope. Bacteria samples were fixed with 2% (v/v) formaldehyde and were counted using flow cytometry according to Del Giorgio et al. (1996).

To determine the ingestion of fluorescently labeled microspheres and bacteria by *P. patelliferum*, algal cells were preserved with 5% (v/v) Lugol's solution, bleached with sodium thiosulfate (3%), and centrifuged (5 min at 6,000 rpm). The supernatant was removed and cells were resuspended in saline phosphate buffer (pH 10). Resuspended cells (20 μl) were mounted on a glass slide with a coverslip sealed with nail polish. Cells were observed in epifluorescence (Nikon TMS) and at least 100–200 cells were counted on each slide.

Particulate analyses of cultures for POC and PON (100 ml) and POP (20 ml) were filtered onto precombusted glass fiber filters (Whatman GF/F) and dried at 60°C for 24 h. POC and PON were analyzed with a CHN autoanalyser, and POP was analyzed according to the method of Solorzano and Sharp (1980).

To extract hemolytic substances in *P. patelliferum* cells, aliquots of cultures (20–25 ml, pooled replicates) were filtered onto precombusted glass fiber filters (Whatman GF/F). Cells retained on the filter were immediately extracted in a chloroform-methanol-water (13 : 7 : 5) phase system, which allowed the recovery of the hemolytic substances in the chloroform phase after separation of the different fractions. The chloroform was then evaporated to dryness under nitrogen, and residue were dissolved in methanol (70%). Final extracts were stored at −20°C prior to analysis of hemolytic activity. Algal extract (1 ml) was added to 4 ml of 2.5% horse blood in an isotonic phosphate buffer. After 30 min of incubation at 37°C, the mixture was centrifuged for 15 min at 530 × g, and hemolytic activity was determined spectrophotometrically by measuring the absorbance of the supernatant at 540 nm. The test was done in triplicates, and meth-

Table 1. Carbon, nitrogen, phosphorus, and hemolytic activity of *Prymnesium patelliferum* in P-deplete and P-replete cultures (mean $\pm$ SD, $n = 3$).

	Activity (48-h incubation)		
Cell status	P-deplete	P-deplete + bacteria	P-replete
Carbon (fg cell^{-1})	33,000 $\pm$ 500	—	32,000 $\pm$ 520
Nitrogen (fg cell^{-1})	2,800 $\pm$ 320	—	3,500 $\pm$ 800
Phosphorus (fg cell^{-1})	300 $\pm$ 100	400 $\pm$ 100	700 $\pm$ 200
Toxicity (HA $\times$ 10^{-5} SnEq cell^{-1})	13.2 $\pm$ 1.5	—	3.14 $\pm$ 0.6

anol was used as an optical blank. A standard hemolytic curve based on concentrations of saponin (SIGMA S-2149) in an isotonic buffer was used as a reference, and the hemolytic activity of the cells was determined as saponin equivalents (SnEq). The results are expressed as saponin equivalents per cell (SnEq cell^{-1}).

Results—The cellular P content of *P. patelliferum* was reduced in P-limited cultures in comparison to P-replete cultures (Table 1), but there was no significant difference in cellular C and N. The C : P (286 : 1) and N : P (21 : 1) ratios in the P-limited cultures were in excess of Redfield proportions.

Few fluorescent microspheres (FM) were ingested by *Prymnesium patelliferum*, irrespective of cellular nutrient status (P-limited or P-replete, Table 2). Although, the uptake rates of 1.6 and 3 μm FM were significantly different from zero (slope variability, $P < 0.05$), there was no difference in the uptake rates of 1.6 and 3 μm FM between P-limited and P-replete *P. patelliferum* cultures. The ingestion of FLB was higher than the uptake of FM (Table 2). Values were higher (up to 4.57 $\pm$ 0.29 bact alga^{-1} h^{-1}; slope $\pm$ SE) in P-limited cultures in comparison to P-replete cultures ($\approx$1 bact alga^{-1} h^{-1}). Although, the standard deviation was relatively small in each single experiment, the variation of the ingestion rate obtained between two independent experiments with P-limited cultures (Fig. 1) was as high as 45%.

The cell number of *P. patelliferum* increased significantly from 365 to 408–460 $\times$ 10^3 cells ml^{-1} (t-test < 0.05) 24 h after the addition of live bacteria (bacteria : algae = 5–28 : 1) to P-limited cultures. Bacteria present in the control (0.05 $\times$ 10^6 bact ml^{-1}) were those associated with *P. patelliferum* cells in the cultured inoculum (P-replete) and not to the addition of bacteria (isolated from *Chaetoceros affinis*). In the control and the P-limited cultures with 1 $\times$ 10^6 live bact ml^{-1}, no significant increase of cell number was observed (Fig. 2).

Final bacterial densities were lower in cultures with added bacteria compared to controls (filtrates of *P. patelliferum* only) (Fig. 3). The ingestion rate of bacteria (0.2–2 bact alga^{-1} h^{-1}) from the suspension was higher in all P-limited *P. patelliferum* cultures, irrespective of the amount of bacteria added, compared to the control cultures with only background bacteria (Fig. 4). The filtrates contained $>2 \times 10^7$ bact ml^{-1}; therefore, a large standard deviation of the ingestion rates calculated from cultures with the addition of $>4 \times 10^6$ bacteria ml^{-1} is expected.

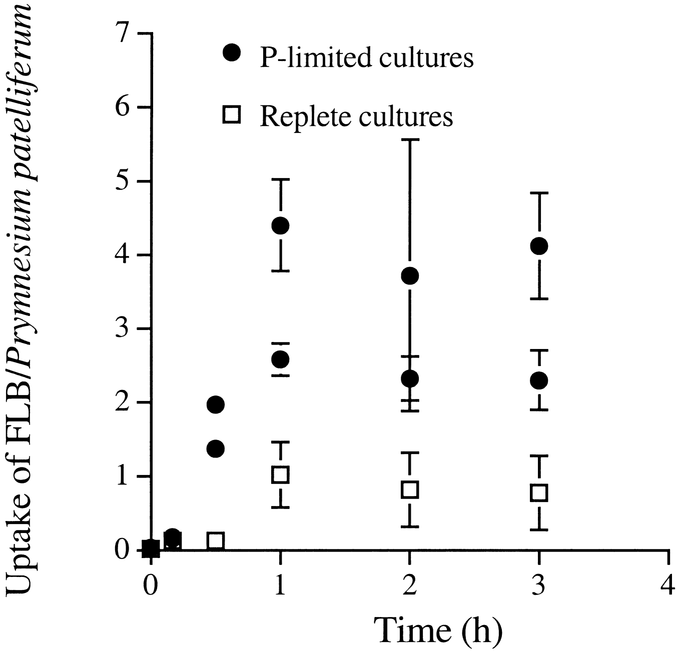

Fig. 1. Uptake of fluorescently labeled bacteria by P-limited and P-replete *Prymnesium patelliferum* cells (mean $\pm$ SD, $n = 3$). The abundance of background bacteria in the *P. patelliferum* cultures ranged from 0.02 to 0.04 $\times$ 10^6 bact ml^{-1}.

Hemolytic activity was higher (HA = 13.2 $\pm$ 1.5 $\times$ 10^{-5} SnEq cell^{-1}) in P-limited cultures compared to P-replete cultures (HA = 3.1 $\pm$ 0.6 $\times$ 10^{-5} SnEq cell^{-1}) (Fig 5). The addition of bacteria (ratio 20 : 1), bacterial filtrate, and PO_4^{3-} decreased significantly the toxicity of P-limited cultures after 24 h (t-test < 0.05), whereas no significant difference was found between the control (P-limited and no addition) initially and after 24 h. In the cultures with addition of bacterial filtrates, PO_4^{3-} was below detection (<0.05 μM). The reduction of the toxicity was highest with the addition of PO_4^{3-}. The *P. patelliferum* cultures with added bacteria were run for 48 h and showed no significant increase of cellular P.

Table 2. Ingestion rate of fluorescent microspheres (FM) and fluorescently labeled bacteria (FLB) by *Prymnesium patelliferum* under P-deplete and P-replete conditions (slope $\pm$ SE).

	Ingestion rate (prey alga^{-1} h^{-1})	
Prey	P-deplete	P-replete
FM		
0.5 μm	0	0
1.6 μm (n = 16)	0.033 $\pm$ 0.005*	0.023 $\pm$ 0.003*
3 μm (n = 16)	0.01 $\pm$ 0.003*	0.028 $\pm$ 0.004*
FLB (2-μm rod, n = 12)†	2.67 $\pm$ 0.13 4.57 $\pm$ 0.29	0.97 $\pm$ 0.20

* $P < 0.05$.

† Calculated from two independent experiments, as shown in Fig. 1.

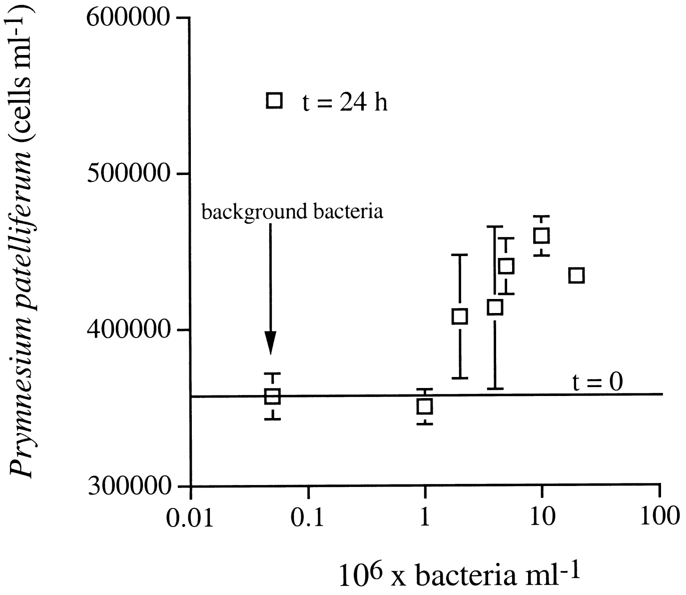

Fig. 2. Cell numbers of P-deplete *Prymnesium patelliferum* cells after 24 h with bacteria (mean ± SD, *n* = 3). Bacteria were added to a final concentration of 1 × 10^6 to 2 × 10^7 bact ml^{-1}. The abundance of background bacteria in the *P. patelliferum* cultures was 0.05 × 10^6 bact ml^{-1}. The straight line represents the initial *P. patelliferum* abundance (350 × 10^3 cells ml^{-1}) in the cultures before the addition of bacteria (t = 0).

Discussion—This study confirmed the hypothesis that the ingestion of bacteria contributes to the mixotrophic growth of P-limited *P. patelliferum* cells compared to phototrophically grown cells. Furthermore, ingestion of bacteria decreased the toxicity of the flagellate by decreasing cellular physiological stress due to P-limitation.

Fluorescent microspheres have often been used as prey analogs to estimate phagotrophy in flagellates and various protists (Sherr et al. 1987 and references therein). Discrimination of FM over natural (e.g., live or heat-killed prey) by nanoflagellates has, however, been reported. For example, Dolan and Simek (1998) reported low ingestion rates of FM by the heterotrophic flagellate *Bodo saltans* in the presence of *Synechococcus* sp. In our study, the ingestion of FM by *P. patelliferum* was very low compared to FLB (direct estimation) or live bacteria (indirect estimation), confirming that *P. patelliferum* has a preference for natural rather than

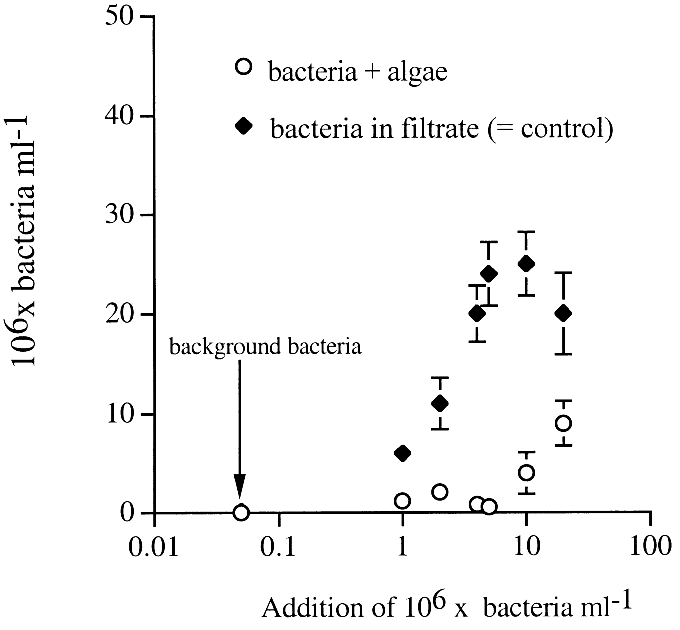

Fig. 3. Bacterial cell numbers in P-deplete *Prymnesium patelliferum* cultures and filtrates (bacteria only, no *Prymnesium*) after 24 h incubation (mean ± SD, *n* = 3). The abundance of background bacteria in the *P. patelliferum* cultures was 0.05 × 10^6 bact ml^{-1}.

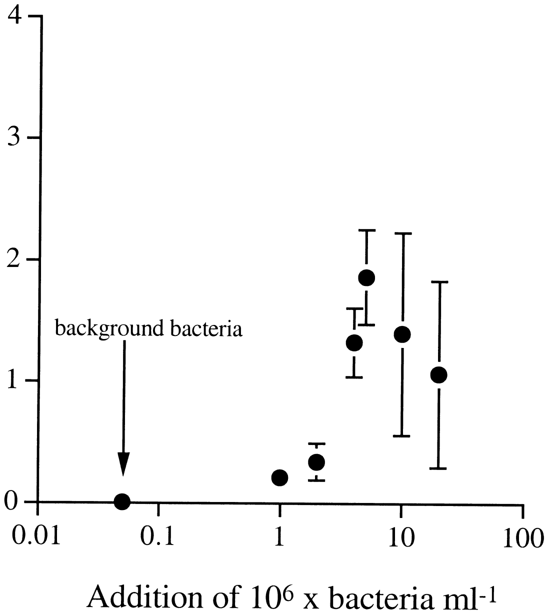

Fig. 4. Ingestion rate of bacteria in P-deplete *Prymnesium patelliferum* cultures (calculated from Figs. 2 and 3). Mean ± SD, *n* = 3.

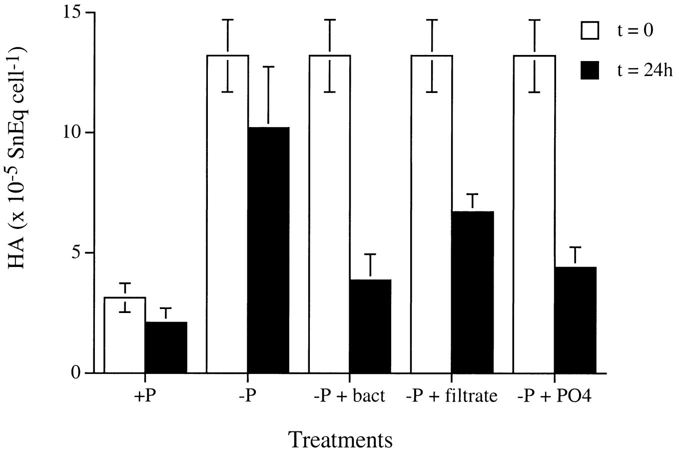

Fig. 5. Hemolytic activity in *Prymnesium patelliferum* cells. +P, P-replete cultures; −P, P-deplete cultures with addition of bacteria (+bact), bacteria filtrates (+filtrate), and inorganic phosphorus (+PO_4). Mean ± SD, *n* = 3.

inert prey. This preference might be related to the size and shape of FM (spherical, 1–3 μm) compared to bacteria (rod shaped, 2–3 μm).

Factors controlling bacterivory in P. patelliferum—In nutrient-depleted environments, primarily phototrophic algae may derive nutrients from organic food sources (i.e., become mixotrophic). It has been hypothesized that they feed in response to low inorganic nutrients and are described as specific physiological group type II mixotrophs (Stoecker 1998). A clear relationship between nutrient limitation and increased phagotrophy has been found in dinoflagellates (Bockstahler and Coats 1993; Stoecker et al. 1997; Legrand et al. 1998; Li et al. 1999), chrysophytes (Caron et al. 1993), and prymnesiophytes (Jones et al. 1993; Nygaard and Tobiesen 1993). Similar results were obtained in our study because the mixotrophic *P. patelliferum* was able to sustain or increase its biomass through the ingestion of bacteria in P-deplete cultures. Furthermore, the ingestion rates of bacteria were lower in P-replete cultures (1 bact cell^{-1} h^{-1}) compared to P-limited cultures (2–4 bact cell^{-1} h^{-1}). However, this is not the case for all mixotrophic flagellates (e.g., *Poterioochromonas malhamensis* [Caron et al. 1990], *Ochromonas* spp. [Andersson et al. 1989], *Fragilidium subglobosum* [Skovgaard 1996], and *Chrysochromulina polylepis* [Legrand et al. unpubl. data]), where phagotrophy also occurs at high nutrient (N, P) concentrations.

Prey density is also important in determining the phagotrophic capacity of primarily heterotrophic mixotrophic flagellates (type III mixotroph, Stoecker 1998). *Prymnesium patelliferum* is not a type III mixotroph because it can grow in the presence of high nutrients and without bacteria in axenic cultures (Larsen et al. 1993). However, our data show that the ingestion of bacteria (calculated from the disappearance of bacteria from the suspension) by P-deplete *P. patelliferum* cells increased significantly when bacteria numbers were between 1×10^6 and 10^7 bact ml^{-1} in the cultures. This threshold (1×10^6 bact ml^{-1}) for feeding is in agreement with other studies (e.g., *P. malhamensis* [Caron et al. 1990], *C. polylepis* [Legrand et al. unpubl. data]). We assume that the presence of background bacteria did not interfere with the determination of ingestion rates of added rod-shaped bacteria by *P. patelliferum* because background bacteria were small cocci (0.5 μm), and their densities were $<1 \times 10^6$ bact ml^{-1}. We hypothesize that previously reported growth rates of *P. patelliferum* in axenic cultures and cultures with associated bacteria are similar because background bacterial densities in these cultures were below this threshold ($<1 \times 10^6$ bact ml^{-1}) or because associated bacteria may be resistant to grazing.

Contribution of bacterivory to P. patelliferum *growth*—The competitive advantage of mixotrophs over obligate autotrophs or heterotrophs has been hypothesized to exist only when nutrients or light or prey are limiting (Raven 1997). The maximal growth rate ($\mu = 0.48 \pm 0.1$ d^{-1}, $n = 7$) of phototrophic *P. patelliferum* cells was estimated from several independent batch cultures experiments (data not shown). This growth rate is comparable to previous values reported for the same species ($\mu = 0.36$–0.57, Larsen and Bryant 1998). However, based on the increase in cell numbers of P-deplete cultures in our experiments, the mixotrophic growth rate of *P. patelliferum* can be estimated at $\mu = 0.11$–0.23 d^{-1}, indicating that the mixotrophic growth of *P. patelliferum* may be less efficient than phototrophic growth, at least on a short time scale (24 h). Nevertheless, bacterivory support approximately 30% of *P. patelliferum* maximal growth rate.

Bacteria are rich in both N and P, and the ingestion of bacteria by mixotrophic haptophytes has been reported to constitute a significant P source (Nygaard and Tobiesen 1993). In our study, assuming an average bacterial P content of 50 fg P bact^{-1} (Vadstein and Olsen 1989) and an ingestion rate of 2.5–5 bact alga^{-1} h^{-1}, the potential uptake of P was 120–240 fg P alga^{-1} d^{-1}. The average cellular P content in P-depleted cells was 300 fg P cell^{-1}. These results indicate that bacterial P could contribute up to 40 to 80% of the total P requirements of *P. patelliferum* cells in 24 h. The increase of cellular P content from 300 to 400 fg P cell^{-1} in P-deplete cells with bacteria for 48 h is consistent with these calculations.

Relation between mixotrophy and toxicity in P. patelliferum—Several factors (e.g., limiting nutrient conditions, salinity, light, and growth phase) have been shown to influence the toxicity of *Prymnesium* spp. (Edvardsen and Paasche 1998). Nutrient availability seems to be an important regulation factor for the production of different types of algal toxins (Anderson et al. 1990; Bates et al. 1991; McLachlan et al. 1994; Meldahl et al. 1994; Johansson and Granéli 1999). In *Prymnesium* spp., laboratory experiments indicate that toxin production is enhanced under nutrient stress irrespective of which nutrient (N, P) is limiting growth (Johansson and Granéli 1999). Phosphorus limitation stimulates the toxicity of *P. patelliferum* compared to nutrient-replete conditions (Larsen et al. 1993; Meldahl et al. 1994; this study). Because N and P are minor elements in the composition of the toxin complex (e.g., prymnesins) found in ichthyotoxic haptophytes (Igarashi et al. 1996), it has been hypothesized that the toxicity in haptophytes such as *Chrysochromulina* and *Prymnesium* spp. is not directly coupled to the cellular N or P content but to the physiological status of the cell (i.e., stress, low growth) (Johansson and Granéli 1999). Tillmann (1998) reported the ingestion of prey of various size (5–45 μm) by senescent-stage cells (i.e., physiologically stressed) of *P. patelliferum.* He hypothesized that *P. patelliferum* may secrete a toxin that would immobilize and perhaps kill the prey before ingestion.

Most of the physiological studies on toxic marine algae are focused on the abiotic and biotic factors leading to high toxin production/content or to induction of toxicity. The ultimate aim of physiological studies is to effectively manage or prevent harmful algal blooms (HABs). However, successful prevention of HABs in coastal waters will require more scientific and policy efforts (e.g., nutrient management policies, EUROHAB 1999). At present, it is also relevant to consider means of minimizing toxin production when a toxic bloom is already established in risk areas (e.g., fish farms). There is, to our knowledge, no information about the reduction (both quantitative and qualitative) of toxicity in *P. pa-*

telliferum. In our study, the hemolytic activity (i.e., toxicity) decreased by 40% when diverse sources of P were added to P-deplete cultures. The separate addition of bacteria and inorganic phosphorus led to increased cell numbers compared to the control, whereas the addition of bacterial filtrate reduced the hemolytic activity but had no effect on cell numbers. The production of toxic substances expressed on a per-cell basis confirm that the addition of bacteria and inorganic phosphorus led to a larger decrease of hemolytic activity than the sole addition of bacterial filtrates. These data show that P-deplete *P. patelliferum* cells could utilize inorganic and organic P for their cellular P requirements, thereby decreasing cellular physiological stress (e.g., increase of cellular P content) and toxin production in a rather short time scale (24 h). Similar data were obtained when the toxicity of P-deplete *P. patelliferum* cells was tested against brine shrimp *Artemia salina* 48 h after the separate addition of PO_4^{3-} and bacteria (Legrand, data not shown). In this study, we used nonaxenic cultures of *P. patelliferum,* and we can speculate that nonaxenic conditions may influence the speed of the toxicity response if algal cells are adapted to feed on bacteria. However, background bacterial numbers in nonaxenic cultures were below the observed threshold for *P. patelliferum* to feed on bacteria. Moreover, the ingestion rates of background bacteria by *P. patelliferum* were not different from zero. Our data confirm that minimizing toxin production involves decreasing physiological stress (e.g., toxicity decrease) after the addition of various P sources to P-limited cells.

Toxin production by the mixotrophic *P. patelliferum* increases because of cellular physiological stress (e.g., nutrient limitation). Eutrophication and unbalanced N : P ratios (which deviate from the Redfield ratio) occur in most of the coastal areas where ichthyotoxic haptophytes bloom (Edvardsen and Paasche 1998), and it is possible that these unbalanced nutrient conditions enhance cellular stress in these species, stimulating the cells to become more toxic. The extracellular release of toxic substances by *P. patelliferum* may serve to immobilize or kill a prey and incorporate nutrients from organic origins via phagotrophy. This would result in a decrease of nutrient stress, as well as a decrease of toxin production.

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A test of the ash-free dry weight technique on the developmental stages of *Patiriella* spp. (Echinodermata: Asteroidea)

Abstract—Determination of the ash-free dry weight (AFDW) of marine specimens requires samples to be rinsed, soaked, and centrifuged. Problems associated with this technique were examined with the developmental stages of seastar species (*Patiriella*) with different modes of development. The influence of three rinsing solutions (ammonium formate [AF], filtered seawater [FSW], and reverse osmosis water [RO]) was assessed. The hypothesis that the AFDW technique is a measure of organic material was addressed by drying inorganic salts. Developmental stages of *Patiriella calcar* rinsed in FSW were twice as heavy as those rinsed in RO or AF, indicating that samples should be rinsed in RO or AF before weighing. Soaking treatments had a significant effect on the AFDW of samples of *P. calcar* (planktonic developer), indicating that the rinsing period should be brief. Zygotes of *Patiriella regularis* (planktonic developer) were significantly heavier than ova or gastrulae, regardless of treatment. In contrast, there were no significant differences in the AFDW of any stages or treatments of *Patiriella exigua* (benthic developer). This may be due to the presence of a modified fertilization envelope, which protects these benthic embryos. Inorganic salts with water of crystallization and FSW lost 20–75% and 14% of their dry weight, respectively, after ashing. We propose that salt ions may retain water, which does not evaporate during drying but is lost during ashing, resulting in the overestimation of sample AFDW. If a similar process occurs in the developmen-