Vol. 29: 97–105, 2002 | AQUATIC MICROBIAL ECOLOGY
Aquat Microb Ecol | Published September 3

Utilization efficiency of nitrogen associated with riverine dissolved organic carbon (>1 kDa) by two toxin-producing phytoplankton species

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ABSTRACT: Riverine high molecular weight dissolved organic material (HMWDOM) >1 kDa contains varying amounts of nitrogen. This nitrogen is partly available for growth of phytoplankton and can therefore contribute to algal bloom formation. However, there is a lack of knowledge on species-specific utilization efficiency of this HMWDOM-bound nitrogen. A specific question is whether this nitrogen can be used by toxic or otherwise harmful species and, thus, contribute to unwanted eutrophication effects in coastal waters. In this context, 2 phytoplankton species and known toxic bloom-formers in marine coastal waters were cultured in nitrogen-limited semi-continuous cultures with river extracted HMWDOM and nitrate as nitrogen source in the following proportions (%): 0:100, 25:75, 50:50, 75:25 and 100:0 (HMWDOM-bound nitrogen). *Prymnesium parvum* f. *patelliferum* (Green, Hibberd et Pienaar) A. Larsen, an ichthyotoxic prymnesiophyte, was not able to utilize the nitrogen bound to HMWDOM. The toxicity of this species, measured as hemolytic activity, did not show any significant correlation with HMWDOM concentration. In contrast, *Alexandrium tamarense*, a toxin-producing dinoflagellate responsible for paralytic shellfish poisoning (PSP), was able to use nitrogen from riverine HMWDOM as efficiently as nitrate, when the HMWDOM fraction was up to 75% of the total dissolved nitrogen. When HMWDOM was supplied as the sole nitrogen source, the growth efficiency was reduced by approximately half. At steady state, the total cellular toxin content and toxicity of *A. tamarense* was negatively correlated with the fraction of HMWDOM. The use of organic nitrogen sources by marine dinoflagellates is well known. Here, we show that nitrogen from a terrestrial origin can be very efficiently used by toxic *A. tamarense* and could therefore contribute to unwanted eutrophication effects.

KEY WORDS: Utilisation efficiency · HMWDOM · Phytoplankton

INTRODUCTION

Toxic marine phytoplankton blooms in coastal waters are a globally recurring phenomenon. Although the occurrence of toxic blooms preceded anthropogenic eutrophication, enhanced human-induced discharge of inorganic nutrients into coastal seawater has been suggested to increase the frequency and magnitude of these events (Smayda 1990). In some coastal areas, the loading of river-transported high molecular weight dissolved organic matter (HMWDOM) represents a significant proportion of the total nutrient input. For example, organic nitrogen bound to humic material contributes significantly (up to 50%) to the total nitrogen influx into large parts of the Baltic Sea (Wulff et al. 1996). Furthermore, the concentration of humic substances in fresh water in this area has been found to show an increasing temporal trend (Andersson et al 1991). Nitrogen bound to this humic material can directly and/or indirectly stimulate phytoplankton growth (Carlsson & Granéli 1993), especially dinoflagellates (Granéli & Moreira 1990, Carlsson et al. 1995). However, there is a lack of

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knowledge of how efficiently humic-bound nitrogen can be used by different species of phytoplankton. Toxic species are of special interest due to the need to understand if and to what extent observed increases of humic HMWDOM discharge could affect the formation of harmful algal blooms.

Blooms of the dinoflagellate *Alexandrium tamarense* are mostly restricted to coastal areas, under influence of river plumes (Anderson 1997, Ichimi 2001). Bloom dynamics research has mostly concentrated on the encystment and germination of cysts, however, nitrogen, including organically bound nitrogen from land run-off, might contribute to growth of the usually nitrogen-limited coastal phytoplankton populations. In culture, *A. catenella* was capable of taking up large, organic molecules, in particular high molecular weight (>2000 kDa) dextrans (Legrand & Carlsson 1998). Moreover, the ingestion of these dextrans increased in the presence of humic material. Nitrogen, originating from the humic material, contributed to the growth of this species in axenic culture experiments (Carlsson et al. 1998). In those experiments, the abundance of bacteria did not increase the efficiency of nitrogen utilization. In general, utilization of organic nitrogen (Carlsson et al. 1995, Yamaguchi & Itakura 1999) and/or stimulation of growth by riverine HMWDOM (Granéli et al. 1985, Doblin et al. 1999) by dinoflagellates is commonly reported.

The haptophytes *Prymnesium parvum* f. *patelliferum* and *P. parvum* f. *parvum* which now are considered to be one and the same species (Larsen 1999) are known to form toxic blooms in brackish coastal waters or high mineral freshwater lakes (Guo et al. 1996). In such environments, the influence of riverine HMWDOM can be high and contribute to bloom formation. Therefore, HMWDOM may be a potentially important source of nutrients for these species. Although no direct uptake of HMW organically bound nitrogen has been described for *P. parvum* f. *patelliferum*, osmotrophy as well as phagotrophy has been found among different *Prymnesium* spp. (Rahat & Jahn 1965, Green 1991) including *P. parvum* f. *patelliferum* (Tillmann 1998, Legrand et al. 2001). Moreover, stimulation of bacterial growth by the addition of riverine HMWDOM, could indirectly sustain the growth of *P. parvum* f. *patelliferum*.

The aim of the current study was to assess the utilization efficiency of nitrogen bound to riverine dissolved organic material by 2 bloom-forming toxic phytoplankton species, *Prymnesium parvum* f. *patelliferum* and *Alexandrium tamarense.* This research was performed in semi-continuous cultures at different HMWDOM/nitrate supply ratios. The results were analyzed in terms of steady state cell density and cellular toxicity.

MATERIALS AND METHODS

The cultures. *Prymnesium parvum* f. *patelliferum* (KAC 39), isolated from southern Norwegian coastal waters, and *Alexandrium tamarense* (KAC 2), originating from the Swedish west-coast, are maintained in the Kalmar Algal Collection, Marine Science division, University of Kalmar, Sweden. Stock cultures of these species were kept in F/2 medium based on aged Baltic seawater, at original salinity of 6.2 psu for *P. parvum* f. *patelliferum*, and at salinity adjusted to 26 psu with NaCl for *A. tamarense.* Prior to the experiment, attempts were made to culture both strains axenically. For *P. parvum* f. *patelliferum*, an antibiotic treatment with ampicillin (10 mg ml^{-1}) was used in combination with serial dilution in multi-well plates to statistically less than 1 cell per well. From the highest dilutions where growth could be detected, new cultures were started. The presence or absence of bacteria was determined by epifluorescence microscopy after staining with DAPI, and flow cytometry (Beckton-Dickinson FACS-Calibur) after staining with SYTO13 (Molecular Probes) according to del Giorgio et al. (1996). Three cultures of *P. parvum* f. *patelliferum* were obtained where no bacteria could be detected, one of which was used as an inoculum for 1 experiment (initially axenic series). The other experiment was started with the original strain, containing bacteria. During the experiment, bacteria re-appeared in the initially axenic cultures. Because the stock cultures also showed bacterial re-appearance during the experiment, we conclude that bacterial growth was probably not due to contamination during experimental handling.

For *Alexandrium tamarense*, different methods were applied in an attempt to obtain an axenic strain. Antibiotic treatment (12, 24, 48 and 72 h) using gentamicin (50 to 400 µg ml^{-1}), dihydrostreptomycin-sulfate and ampicilin (both 12.5 to 100 µg ml^{-1}), and a combination of washing and antibiotic treatment were applied to exponentially growing cells. Washing of the cultures was done by centrifuging (3 min at 100× *g*) 10 ml of *A. tamarense* culture and resuspending the algae in fresh medium. The antibiotic treatment was then applied as described above. From the samples where cells of *A. tamarense* survived and divided, subsamples were taken for inspection for bacteria contamination by epifluorescence microscopy. However, none of these cultures were axenic, and all experiments were thus done with unialgal cultures.

Experimental set-up and HMWDOM extraction. Riverine HMWDOM (>1000 Da) was isolated from the river Ljungbyån in southern Sweden by tangential flow filtration using a Millipore Pelican™ system. Approximately 150 l of river water was concentrated to a 3 l extract, which was frozen until use. After thawing,

the extract was sonicated and filtered through sterile 0.2 µm filters (Gelman AcroDisk) before adding to the cultures. Total dry matter in the filtered extract was 1.00 g l^{-1} of which 35.8 ± 0.3% (w/w) was carbon and 1.14 ± 0.01% (w/w) was nitrogen, measured using a CHN-analyzer (see next section) after drying at 70°C.

Except for the salinity of the medium, the experimental set-up and medium preparation was the same for both species. Medium for all experiments was prepared from aged Baltic seawater ([PO_4^{3-}] < 0.02 µM, [NO_3^-] < 0.1 µM). Prior to sterilization, the seawater was acidified with 0.4 ml 1.2 N HCl l^{-1} to prevent precipitation of carbonates. After sterilization, sterile trace elements, FeCl/EDTA and NaH_2PO_4 solutions were added according to F/2 medium definition (Guillard 1975). Finally, 2 mM $NaHCO_3$ was added aseptically and the pH of the medium was adjusted to 8.0 ± 0.1. Prior to the start of the semi-continuous culture experiments, nitrogen was added to the medium in different proportions of nitrate and riverine HMWDOM to a final concentration of 20 µM total nitrogen. Nitrate and HMWDOM additions did not change pH more than 0.1 pH units. Five different fractions of HMWDOM to total available nitrogen were applied (0, 25, 50, 75 and 100%), resulting in final concentrations of HMWDOM/nitrate-nitrogen in the 5 treatments (2 replicates each) of: 0/20, 5/15, 10/10, 15/5 and 20/0 µM. Cultures were grown at 15°C and an incident photon flux density of 100 µmol photons m^{-2} s^{-1} at a 16:8 h day:night cycle. In the bottles with highest HMWDOM concentrations, irradiance was not reduced more than 10% as compared to the cultures with only nitrate.

All cultures were started from batch cultures with a working volume of 1 l, grown on 20 µM nitrate (the ones that were going to be diluted with 50% or less HMWDOM-bound nitrogen), or 10 µM nitrate and 10 µM HMWDOM-bound nitrogen (cultures that were going to be diluted with 75% or more HMWDOM-bound nitrogen) to allow for pre-adaptation to the most abundant nitrogen source. When the specific growth rate in the batch cultures started to decrease, as judged from cell counts, the cultures were diluted. Daily dilution of the *Prymnesium parvum* f. *patelliferum* cultures was 26% of the culture volume per day, resulting in an average dilution rate of 0.3 d^{-1}. *Alexandrium tamarense* was diluted only once every 4 d, because this species is known to be negatively affected by turbulence introduced by mixing and pouring. Every fourth day, 32% of the culture volume was replaced, resulting in an average dilution rate of 0.1 d^{-1}. In both experiments, subsamples for determination of cell abundance (immediately Lugol-fixed) were collected from the culture volume that was withdrawn at every dilution, in order to monitor biomass development.

After 11 d for *Prymnesium parvum* f. *patelliferum* and 33 d for *Alexandrium tamarense*, the semi-continuous cultures were ended, and samples were taken for analyses of cell number, bacterial abundance, toxin and chlorophyll (chl *a*) concentration, and concentration of intra- and extracellular nitrogen and carbon.

Analytical methods. Algal cell counting was done using a Nikon Diaphot inverted microscope. For *Prymnesium parvum* f. *patelliferum*, 1 to 250 µl subsamples, previously fixed with Lugol's solution [the subsample volume depending on expected cell concentration] were counted in duplicate in flatbottom 96-well multiplates. *Alexandrium tamarense* was counted in 2.5 to 5 ml volume sedimentation chambers. Bacterial number was determined using flow –cytometry, counting at least 45000 cells per sample (del Giorgio et al. 1996), calibrated with TruCount beads (ca. 50000 ml^{-1}; Becton-Dickinson). Chl *a* was determined fluorometrically (Turner TD-700) after filtration of samples (20 ml) through Gelman AE glass fiber filters and ethanol extraction of the filter for at least 8 h at room temperature (Jespersen & Christoffersen 1988).

For the determination carbon and nitrogen content of the extracted HMWDOM, 25 ml (triplicate) of the extract was dried at 70°C. The dry matter (0.025 g) was analyzed using a Fisons NA 1500 CN analyzer using a certified (OAS & IAS) analytical standard acetanilide (71.09% C and 10.36% N) (EMAL technology) for calibration. Particulate carbon and nitrogen determinations on algal material in the cultures was determined after filtration on Whatman GF/C filters. The measurements were hampered by the HMWDOM, which visibly adhered to the glass fiber filters we used. Therefore, particulate carbon and nitrogen values could not be determined in the treatment with HMWDOM, and no correlation with the HMWDOM/NO_3 supply ratio could be obtained. Dissolved nitrate and ammonium were quantified using the methods described in Valderrama (1995).

The toxicity of *Prymnesium parvum* f. *patelliferum* cells was determined as hemolytic activity of a cell methanol extract (Igarashi et al. 1998). After centrifugation of 15 ml of culture (10 min at 500× *g*), the overlying medium was carefully removed with a pipet, and the toxin in the pellet was extracted for 30 min in the dark with 2 ml of methanol. Extracellular toxin concentration was measured directly on the filtered medium. The methanol extract or filtered medium was then mixed in different ratios (0 to 50%) with isotonic phosphate buffer in microplates (Corning Costar 3603 Black) (total volume 50 µl). After which, 200 µl of 5% horse blood suspension (Swedish Veterinary Institute) in isotonic phosphate buffer was added. The total methanol concentration in the reaction mixture was thus never higher than 10% of the final volume. After

testing, pure methanol did not show any hemolytic effect itself up to a final concentration of 15% v/v of the total test volume, from which we conclude that methanol did not interfere with the hemolytic assay. Microplates were gently mixed every 15 min and incubated for 1 h in the dark at room temperature, then the whole plate was centrifuged (5 min, 200× *g*). From each well, 100 µl of the blood solution was transferred to a new plate and the absorbance at 540 nm was measured with a plate reader (BMG FLUOstar). Saponin was used as a standard. The standard curve was fitted with a sigmoidal curve according to the Eq. (1), using Statistica for Windows (Fig. 1):

$$A_{540} = A_{\max} - \frac{A_{\max} - A_{\min}}{1 + \left(\frac{[S]}{HC_{50}}\right)^{h}} \quad (1)$$

where $A_{\max}$ is the theoretical maximal absorbance when all the red blood cells have lysed, $A_{\min}$ is the minimum absorbance when no red blood cells have lysed, [*S*] is the concentration of saponin in the test solution, and HC_{50} is the concentration of saponin where 50% of the blood cells have lysed. The toxicity of the culture extract was then estimated from the standard curve as mg l^{-1} saponin equivalents, using only data where hemolysis was between 15 and 85% of the hemolysis obtained at the highest standard concentration (Fig. 1). Measurements of at least 4 replicates were averaged.

The content of paralytic shellfish poisoning (PSP) toxins in *Alexandrium tamarense* cultures was measured in cells collected on glass fiber filters after filtration of 400 ml culture. Filters containing *A. tamarense* cells were placed in glass vials with 1 ml of 0.03 M acetic acid on ice. Then, cells were sonicated 3 times for 1 min using a Misonix XL sonicator with microtip (level 3), and filtered (PTFE, 0.45 µm pore size) before injection into the HPLC-system (Merck-Hitachi 7000 series with fluorescence detection, Phenomenex C18 column 5 × 300 mm). Separation and analysis of gonyautoxins (GTX) and saxitoxins (STX) were done using post-column oxidation (Yu et al. 1998) and compared with suitable standards for quantification (NRC Institute for Marine Biosciences).

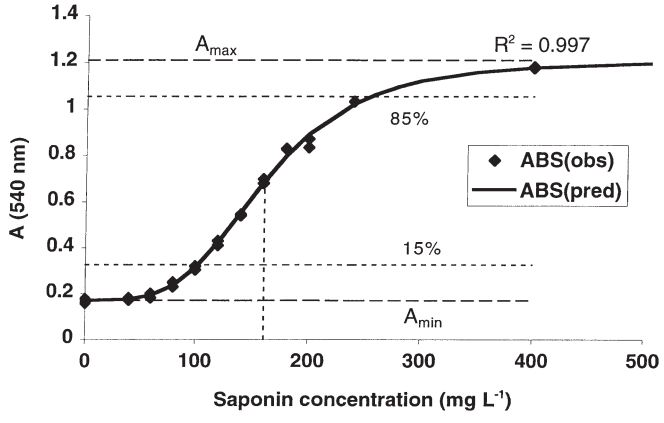

Fig. 1. Calibration curve of the hemolytic effect of saponin on horse red blood cells. The saponin concentration causing 50% lysis of the red blood cells (EC_{50}) was 161 mg saponin l^{-1}. For toxicity measurements, only data between 15 and 85% of total hemolysis were used as indicated in the figure

Growth rate calculation and statistical analyses. The increase of cell concentrations during batch culturing were fitted with exponential functions, in order to estimate the specific growth rates. Cell concentrations during the time that cultures were diluted were fitted assuming a continuous increase or decrease towards the steady state value by assuming first order kinetics, see Eq. (2).

$$N_t = N_\infty + (N_0 - N_\infty)e^{-D(t-t_0)} \quad (2)$$

where N_∞ is the theoretical steady state cell concentration (ml^{-1}) after infinite time. N_0 is the cell concentration at t_0 (ml^{-1}), D is the dilution rate of the culture (0.1 d^{-1} for *Alexandrium tamarense* and 0.3 d^{-1} for *Prymnesium parvum* f. *patelliferum*), and t_0 is the time at which dilution of the cultures started. The theoretical steady state cell concentration, N_∞, and the accompanying asymptotic standard error was estimated by applying Eq. (2) to the cell concentration data of all cultures using the 'non-linear estimation' module of STATISTICA for Windows (StatSoft 1998).

RESULTS

Prymnesium parvum f. *patelliferum*

During exponential growth, the initially axenic cultures of *Prymnesium parvum* f. *patelliferum* had a significantly (ANOVA, $p < 0.05$) higher specific growth rate (0.62 ± 0.04 d^{-1}) than the cultures that were started with the accompanying original bacterial community (0.46 ± 0.03 d^{-1}). There was no effect of HMWDOM on the specific growth rate during exponential growth. During steady state, cell numbers in all *P. parvum* f. *patelliferum* cultures receiving HMWDOM were lower than the expected values if HMWDOM would not have been used at all (Fig. 2A). This indicates that nitrogen from HMWDOM was neither directly nor indirectly used by *P. parvum* f. *patelliferum*, and even that HMWDOM has a negative effect on nitrate utilization by this species. Reduced light quantity and quality in the treatments receiving HMWDOM may have resulted in lower efficiency of the energy demanding nitrate and nitrite reduction by *P. parvum* f. *patelliferum*. Bacterial numbers were only recorded at the end of the experiment during steady state. Bacterial density in both –B (initially axenic) and +B (non-axenic

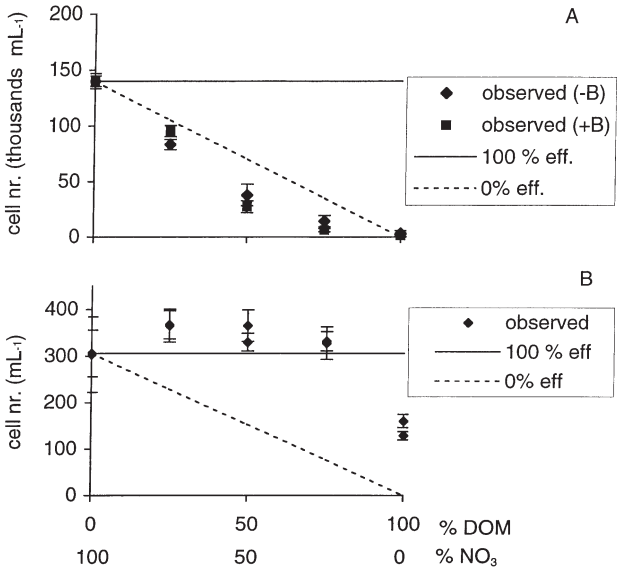

Fig. 2. Steady state cell concentration of (A) *Prymnesium patelliferum* and (B) *Alexandrium tamarense* grown as semi-continuous cultures at different supply ratios of HMWDOM-bound nitrogen (*x*-axis). For *P. patelliferum*, –B refers to the initially axenic cultures and +B to cultures with original bacterial population. The dotted line refers to expected cell numbers if HMWDOM-bound nitrogen was used (0% efficiency), while the solid line is the expected trend when the utilization efficiency of HMWDOM-bound nitrogen is equal to nitrate (100% efficiency)

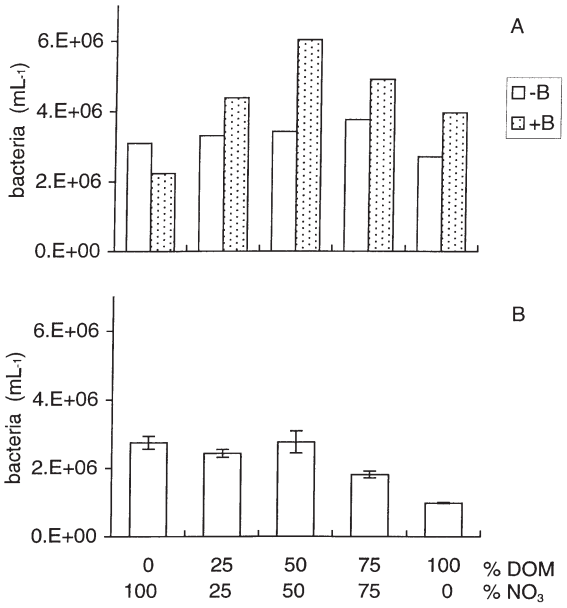

Fig. 3. Steady state bacterial concentration in semi-continuous cultures of (A) *Prymnesium patelliferum* and (B) *Alexandrium tamarense* at different supply ratios of HMWDOM-bound nitrogen and nitrate (*x*-axis). Symbols as in Fig. 2

from the start of the experiment) series appeared to be highest at HMWDOM fractions of 50 to 75% (Fig. 3), but no significant trend was observed (regression analysis).

In the cultures with more than 50% HMWDOM (and thus less than 50% nitrate), the toxicity of *Prymnesium parvum* f. *patelliferum*, measured as hemolytic activity, could not be detected due to too low biomass. For the other treatments, cellular toxicity did not correlate with the fraction of HMWDOM, and was at average between 1 and 1.6 ng saponin equivalents cell^{-1} (Fig. 4A). Toxicity in the filtered medium was below the detection limit (80 mg saponin equivalents l^{-1}).

The addition of HMWDOM had a significant negative effect ($p < 0.05$, linear regression) on the chl *a* content of this species (Fig. 5A). This effect was proportional to the fraction of HMWDOM as compared to inorganic nitrogen. Chl *a* varied from 0.2 pg cell^{-1} in the treatment with only nitrate to less than 0.1 pg cell^{-1} in the 100% HMWDOM treatment.

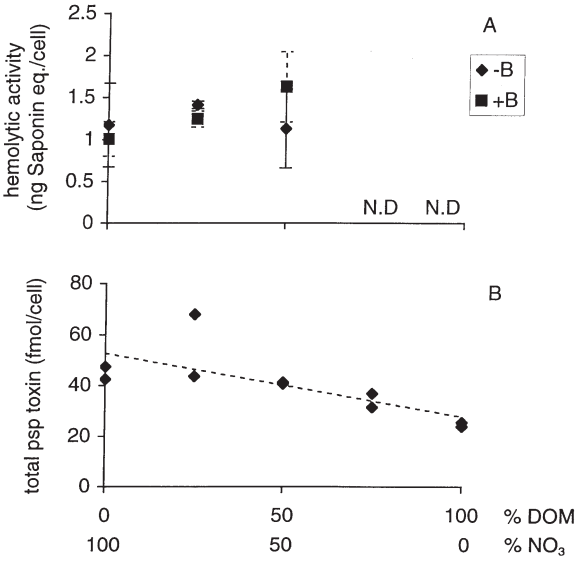

Fig. 4. Hemolytic activity of (A) *Prymnesium patelliferum* and (B) total cellular PSP toxin content of *Alexandrium tamarense* during steady state in semi-continuous cultures at different supply ratios of HMWDOM-bound nitrogen and nitrate (*x*-axis). The dotted line indicates a significant ($p < 0.05$) linear relationship. N.D.: not detectable. Symbols as in Fig. 2

Alexandrium tamarense

Because all efforts to clean *Alexandrium tamarense* cultures from bacteria failed (see 'Materials and methods'), all presented results originate from non-axenic uni-algal cultures. The maximum specific growth rate of *A. tamarense* in exponential phase was on average 0.23 d^{-1} (SD ± 0.05 between cultures) and cell concentration increased to 600–1000 ml^{-1}. After dilution

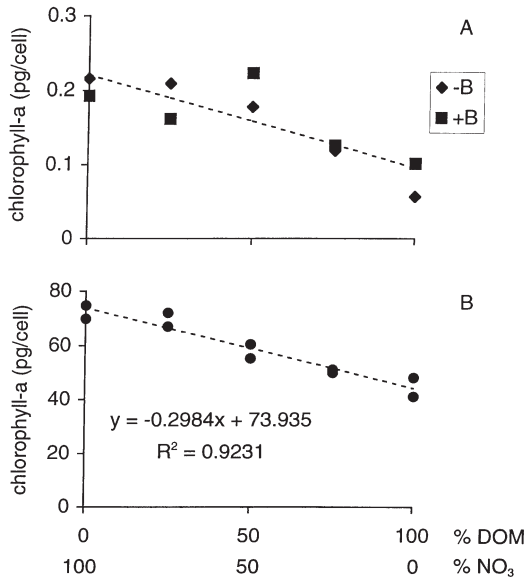

Fig. 5. Cellular chlorophyll *a* content during steady state in semi-continuous cultures of (A) *Prymnesium patelliferum* and (B) *Alexandrium tamarense* at different supply ratios of HMWDOM-bound nitrogen and nitrate (*x*-axis). The dotted lines indicate a significant ($p < 0.05$) linear relationship. Symbols as in Fig. 2

started, the cell concentration decreased in all treatments. After 9 dilutions (36 d), the experiment was ended, and cell concentration had stabilized at around 300 to 370 cells ml^{-1} in all treatments except where DON was the only nitrogen source (100% HMWDOM), when cell numbers decreased and stabilized at approximately 150 cells ml^{-1} (Fig. 2B).

Bacterial numbers during steady state in the *Alexandrium tamarense* cultures were lowest at the 2 highest HMWDOM concentrations and varied from $2.9 \pm 0.2 \times 10^6$ ml^{-1} at 0% HMWDOM to $1.2 \pm 0.2 \times 10^6$ ml^{-1} at 100% HMWDOM (Fig. 3B). However, the observed trend was not significant over the whole range of HMWDOM:nitrate ratios (regression analysis).

Toxin results are summarized in Table 1. Total cellular PSP toxin content was around 45 fmol $cell^{-1}$ in the treatment receiving 100% nitrate (0% HMWDOM) and approximately 25 fmol $cell^{-1}$ in the treatment where HMWDOM was the sole source of nitrogen (Fig. 4B). N-sulfocarbamoyl-11-hydroxysulfate toxins (C-1 to C-4) contributed on average 64 ± 3% to the total toxin content, but were only responsible for 9 ± 1% of the total toxicity in mouse units (MU, which is defined as the amount of toxin that kills a mouse in 24 h.). In one 25% HMWDOM treatment, the contents of GTX-4 and GTX-3, neosaxitoxin (NEO) and STX were 2 times higher that in the duplicate treatment. This replicate was therefore treated as an outlier and not taken into account in the regression analyses. Total cellular toxin content, toxicity as MU, and the cellular contents of STX, GTX-2, C-1 and C-2 were significantly negatively correlated with HMWDOM (linear regression, $p < 0.05$; Fig. 4). All other toxins did not vary significantly with HMWDOM.

The content of chl *a* per cell was significantly (linear regression $p < 0.05$) correlated with the proportion of HMWDOM in *Alexandrium tamarense*. When supplied with only nitrate, cells contained 72 pg chl *a* $cell^{-1}$, which was reduced to 45 pg chl *a* $cell^{-1}$ when HMWDOM was the only nitrogen source (Fig. 5B). Dis-

Table 1. Cellular content (in fmol $cell^{-1}$) of different PSP toxins in *Alexandrium tamarensis* cells and factors used to calculate toxicity in mouse units (MU). nd = not detectable

HMWDOM-N (%)	GTX-4	GTX-1	GTX-2	GTX-3	NEO	STX	C-4	C-3	C-1	C-2	Total	$\times 10^{-3}$ MU $cell^{-1}$
0	0.31	0.81	6.47	2.18	3.80	3.07	0.26	0.01	17.69	7.99	42.6	0.030
0	0.25	0.97	6.91	2.13	2.79	3.59	nd	nd.	22.12	9.02	47.5	0.030
25	0.72	1.26	6.73	4.87	5.65	8.83	0.75	0.73	22.66	15.71	67.9	0.057
25	0.42	1.26	5.37	2.58	2.80	3.73	0.64	0.46	17.51	8.82	43.6	0.031
50	0.31	0.66	4.88	2.03	3.39	3.12	0.99	0.40	16.54	8.97	41.3	0.028
50	0.38	0.95	5.12	1.90	3.73	2.56	0.42	nd	16.84	8.95	40.7	0.028
75	0.30	0.61	3.74	2.15	2.55	2.39	0.50	nd	12.11	7.31	31.5	0.023
75	0.37	0.74	4.74	2.59	2.84	2.76	0.50	0.04	13.93	8.26	36.8	0.026
100	N.D.	1.62	2.65	1.15	1.16	1.19	1.03	0.08	9.94	5.02	23.8	0.015
100	0.46	0.41	2.99	1.19	1.56	1.26	N.D.	2.12	10.74	5.12	25.4	0.014

Calculation factors used (Oshima 1995)

	GTX-4	GTX-1	GTX-2	GTX-3	NEO	STX	C-4	C-3	C-1	C-2
Specific toxicity MU µmol^{-1}	1803	2468	892	1584	2295	2483	143	33	15	239
MW (free base)	411.4	411.4	395.4	395.4	315.3	299.3	491.4	491.4	475.4	475.4

solved nitrate during steady state was only detectable in the cultures receiving 0% HMWDOM (2.5 µM) and 25% HMWDOM (0.5 µM). Dissolved ammonium was between 0.5 and 1 µM and did not vary significantly with the HMWDOM supply ratio.

DISCUSSION

The ability of phytoplankton to use organically bound nitrogen is widespread (Antia et al. 1991). However, there are few studies that show utilization of riverine HMWDOM-bound nitrogen by phytoplankton species. Here, we present quantitative data on the efficiency of HMWDOM-bound nitrogen utilization by phytoplankton. The semi-continuous culturing method we used facilitated a rather long adaptation time for the algal cells. The algae were cultured under continuous nutrient limitation at ecologically relevant growth rates.

Between the 2 species tested in the current study, there was an apparent difference in the utilization of HMWDOM-bound nitrogen. The prymnesiophyte *Prymnesium parvum* f. *patelliferum* could not use this nitrogen source for growth, while HMWDOM-bound nitrogen was efficiently used for growth by *Alexandrium tamarense* at different ratios of organic to inorganic nitrogen. Only at the highest concentration of HMWDOM-bound nitrogen, when no nitrate was present at all, did the biomass production decrease.

Blooms of *Prymnesium parvum* f. *patelliferum* have been associated with high inorganic nutrient availability, moderate salinities (Komarovsky 1951) and relatively high temperatures (Hickel 1976). Small organic solutes can contribute to their energy and nitrogen requirements (Rahat & Jahn 1965), but direct uptake of larger molecules is not documented. This is consistent with our finding that *P. parvum* f. *patelliferum* reached highest biomass with only nitrate as a nitrogen source. *P. parvum* f. *patelliferum* is known to ingest particles (Tillmann 1998) and bacterial numbers seemed to be highest in the treatments receiving 50 to 75% HMWDOM. Possibly, bacterial growth was stimulated by both HMWDOM and algal exudates, explaining lower bacterial numbers in the 100% HMWDOM treatment. However, the higher bacterial numbers at these intermediate HMWDOM availability did not result in increased cell numbers of *P. parvum* f. *patelliferum*, indicating that although *P. parvum* f. *patelliferum* is able to ingest bacteria, the abundant bacterial population did not sustain algal growth under nitrogen limitation. In contrast, phosphorus-limited cells of *P. parvum* f. *patelliferum* were recently shown to use different sources of phosphorus, such as particles (bacteria) and dissolved organic compounds (Legrand et al. 2001).

In contrast, the dinoflagellate *Alexandrium tamarense* was able to utilize HMWDOM as a nitrogen source. Although we did not study the mechanism in detail, this may well be due to phagocytotic ingestion of colloids as has been observed for *A. catanella* (Legrand & Carlsson 1998). At the highest HMWDOM fraction, *A. tamarense* was still able to grow, but the cell yield was reduced as compared to the other treatments. Possibly, the cells were not able to utilize HMWDOM-bound nitrogen efficiently in the total absence of nitrate. We can however not exclude that the highest concentration of HMWDOM had a direct negative effect on *A. tamarense*, reducing their nitrogen use efficiency. The highest concentration of HMWDOM in terms of nitrogen in our experiments was twice as high as in another study where *A. catenatum* was able to grow on HMWDOM as the only nitrogen source (Doblin et al. 1999).

Although no bacteria could be detected in the *Prymnesium parvum* f. *patelliferum* culture at the start of the experiment, neither of the species used in this study was cultured axenically. Therefore, we could not define the role of bacteria in the availability of the HMWDOM-bound nitrogen. However, utilization of HMWDOM-bound nitrogen by another dinoflagellate, *Alexandrium catenella*, did not show any positive effect of bacteria as opposed to axenic cultures (Carlsson et al. 1998). It is likely that the utilization of HMWDOM-bound nitrogen by *A. tamarense* in our study was not mediated by bacteria. However, only experiments with axenic cultures of the same strains will provide a definitive answer.

The toxicity of *Prymesium parvum* f. *patelliferum* was not detectable in all treatments due to poor growth in the treatments receiving more than 50% HMWDOM. For this reason, conclusions regarding the influence of HMWDOM on the toxicity of *P. parvum* f. *patelliferum* based on our experiments are limited to the treatments where toxicity was measurable (0 to 50% HMWDOM). Over this range, no effect of HMWDOM on toxicity of *P. parvum* f. *patelliferum* could be observed. In the dinoflagellate *Alexandrium tamarense*, toxicity showed a negative correlation with the fraction of HMWDOM-bound nitrogen. In an earlier study, no effect of HMWDOM on the toxicity or toxin profile in a related species, *A. catenella* was found (Doblin et al. 2000). Possibly, the longer adaptation time in our semi-continuous culture experiments allowed for adaptations of the toxin profile that would not be noticed in batch culture. The significant correlation of total PSP toxins with % HMWDOM was mostly caused by the variation in GTX 2 and C-1 content, since these 2 toxins corresponded to 53 ± 5% of the total toxin content. Nevertheless, total toxicity in mouse units (MU) was mostly contributed by STX

(28%) and NEO (25 %) and also showed a significant negative correlation with HMWDOM. An explanation for the significant negative correlation between toxin content and HMWDOM could be that the cells were relatively more nitrogen limited in the treatments receiving HMWDOM. Although the specific growth rate in steady state equaled the dilution rate, and thus was equal in all treatments, growth on HMWDOM-bound nitrogen could enhance the costs associated with assimilation of this nitrogen into new cell material. This explanation for the inverse relation between toxin content and the HMWDOM proportion is consistent with the fact that PSP toxins are nitrogen-rich toxins as well as their synthesis is therefore dependent on a high cellular nitrogen content (Flynn et al. 1994). However, although cells grown on HMWDOM-bound nitrogen were less toxic than those growth on nitrate, HMWDOM could contribute to dinoflagellate growth and toxicity by supplying nitrogen when no alternative sources are available. Alternatively, reduced light availability in the cultures receiving HMWDOM could have contributed to a lower energy availability for toxin synthesis. Although the total light availability was not reduced more than 10% due to HMWDOM additions, HMWDOM has a pronounced effect on the light quality, absorbing especially short wavelength light. This change in light quality may have affected toxin production more than the reduction of total light availability.

In nitrogen-limited marine coastal systems, the growth of dinoflagellates could be selectively promoted by an extra input of nitrogen bound to riverine HMWDOM or humic material (Carlsson et al. 1999), implying that dinoflagellates can successfully compete with other phytoplankton for this nitrogen source. The current study shows that the utilization efficiency can be up to 100%. This efficient use of HMWDOM by *Alexandrium tamarense* is consistent with the observation that most blooms of this species are found within estuaries (Cembella & Therriault 1989, Larocque & Cembella 1990) or largely confined within coastal zones under the influence of river plumes (e.g. Franks & Anderson 1992, Ichimi et al 2001). Human induced increase of humic substances discharge contributes to enhanced nitrogen input into marine systems, contributing to selection for phytoplankton species that can directly access this nitrogen source. In areas where humic material plays a significant role in the nitrogen budget, unwanted blooms of toxic dinoflagellates might be promoted due to their apparent utilization capacity of nitrogen in the high-molecular fraction.

Overall, land-originating HMWDOM has proven to be a potentially important nitrogen source to *Alexandrium tamarense*. Land-derived HMWDOM may therefore contribute to bloom formation of this species in inorganic nitrogen-depleted waters influenced by river discharge. In contrast, *Prymesium parvum* f. *patelliferum* was not able to utilize the nitrogen associated with HMWDOM. Under nitrogen-limiting conditions, blooms of this species will therefore not be stimulated by HMWDOM from rivers, but will be more dependent on inorganic and perhaps more easily accessible organic nitrogen.

Acknowledgements. We acknowledge the support from the European commission, Marine Science and Technology program under contract MAS3-CT97-0149 (DOMTOX), and STINT (Swedish Foundation for International Cooperation in Research and Higher Education), contract holder Wilhelm Granéli, project 9694(97). Per Carlsson is acknowledged for his expertise and help with the HMWDOM extraction and Jorge Hernandez for technical assistance.

LITERATURE CITED

Anderson DM (1997) Bloom dynamics of toxic *Alexandrium* species in the northeastern U.S. Limnol Oceanogr 42(5/2): 1009–1022

Andersson T, Nilsson Å, Jansson M (1991) Coloured substances in Swedish lakes and rivers — temporal variation and regulating factors. In: Allard (ed) Humic substances in the aquatic and terrestrial environment. Springer-Verlag, Berlin, p 243–253

Antia NJ, Harrison PJ, Oliveira L (1991) The role of dissolved organic nitrogen in phytoplankton nutrition, cell biology and ecology. Phycologia 30:1–89

Carlsson P, Granéli E (1993) Availability of humic bound nitrogne for coastal phytoplankton. Estuar Coast Shelf Sci 36:433–447

Carlsson P, Granéli E, Tester P, Boni L (1995). Influences of riverine humic substances on bacteria, protozoa, phytoplankton, and copepods in a coastal plankton community. Mar Ecol Prog Ser 127:213–321

Carlsson P, Edling H, Béchemin C (1998) Interactions between a marine dinoflagellate (*Alexandrium catenella*) and a bacterial community utilizing riverine humic substances. Aquat Microb Ecol 16:65–80

Carlsson P, Granéli E, Segatto AZ (1999) Cycling of biologically available nitrogen in riverine humic substances between marine bacteria, a heterotrophic nanoflagellate and a photosynthetic dinoflagellate. Aquat Microb Ecol 18:23–36

Cembella AD, Therriault JC (1989) Population dynamics and toxin composition of *Protogonyaulax tamarensis* from the St. Lawrence estuary. In: Okaichi T, Anderson DM, Nemoto T (eds) Red tides — biology and toxicology. Elsevier Science Publishers, New York, p 81–85

del Giorgio PA, Bird DF, Prairie YT, Planas D (1996) Flow cytometric determination of bacterial abundance in lake plankton with green nucleic stain SYTO 13. Limnol Oceanogr 41(4):783–789

Doblin MA, Blackburn SI, Hallegraeff GM (1999) Growth and biomass stimulation of the toxic dinoflagellate *Gymnodinium catenatum* (Graham) by dissolved organic substances. J Exp Mar Biol Ecol 236:33–47

Doblin MA, Legrand C, Carlsson P, Hummert C, Granéli E, Hallegraeff G (2002) Uptake of humic substances by the toxic dinoflagellate *Alexandrium catenella*. In: Hallegraeff GM, Blackburn SI, Bolch CJ, Lewis RJ (eds) 9th Int Conf Harmful

Algal Blooms, Hobart, Tasmania, February 2000. Intergov Oceanogr Comm Tech Ser UNESCO, Paris, p 336–339
Flynn K, Franco JM, Fernandez P, Reguera B, Zapata M, Wood G, Flynn KJ (1994) Changes in toxin content, biomass and pigments of the dinoflagellate *Alexandrium minutum* during nitrogen refeeding and growth into nitrogen or phosphorus stress. Mar Ecol Prog Ser 111:99–109
Franks PJS, Anderson DM (1992) Alongshore transport of a toxic phytoplankton bloom in a buoyancy current: *Alexandrium tamarense* in the Gulf of Maine. Mar Biol 112: 153–164
Granéli E, Moreira MO (1990) Effects of river water of different origin on the growth of marine dinoflagellates and diatoms in laboratory cultures. J Exp Mar Biol Ecol 136: 89–106
Granéli E, Edler L, Gedziorowska D, Nyman U (1985) Influence of humic and fulvic acids on *Prorocentrum minimum* (Pav.) J. Schiller. In: Anderson DM, White AW, Baden DG (eds) Toxic dinoflagellates. Elsevier Science Publishing, Amsterdam, p 201–206
Green JC (1991) Phagotrophy in prymnesiophyte flagellates. In: Patterson GW (ed) The biology of free-living heterotrophic flagellates. Clarendon Press, Oxford, p 401–414
Guillard RRL (1975) Culture of phytoplankton for feeding marine invertebrates. In: Smith WL, Chanley MH (eds) Culture of marine invertebrate animals. Plenum Press, New York, p 26–60
Guo M, Harrison PJ, Taylor FJR (1996) Fish kills related to *Prymnesium parvum* N. Carter (Haptophyta) in the People's Republic of China. J Appl Phycol 8:111–117
Hickel B (1976) Fish mortality in a carp pond associated with mass development of the toxic phytoflagellate *Prymnesium parvum* Carter (Haptophyceae). Arch Fischwiss 27: 143–148
Ichimi K, Yamasaki M, Okumura Y, Suzuki T (2001) The growth and cyst formation of a toxic dinoflagellate, *Alexandrium tamarense*, at low water temperatures in northeastern Japan. J Exp Mar Biol Ecol 261:17–29
Igarashi T, Aritake S, Yasumoto T (1998) Biological activities of prymnesin-2 isolated from a red tide alga *Prymnesium parvum*. Nat Toxins 6:35–41
Jespersen AM, Christoffersen K (1987) Measurements of chlorophyll-a from phytoplankton using ethanol as extraction solvent. Arch Hydrobiol 109:445–454
Komarovsky B (1951) Some characteristic water-blooms in Lake Tiberias and fish ponds in the Jordan Valley. Proc Int Assoc Theor Appl Limnol 11:219–223
Larocque R, Cembella AD (1990) Ecological parameters associated with the seasonal occurence of *Alexandrium* spp. and consequent shellfish toxicity in the lower St. Lawrence estuary (Eastern Canada). In: Granéli E, Sundstrom B, Edler L, Anderson DM (eds) Toxic marine phytoplankton. Elsevier Science Publishers, New York, p 368–373
Larsen A (1999) *Prymnesium parvum* and *P. patelliferum* (Haptophyta)—one species. Phycologia 38(6):541–543
Legrand C, Carlsson P (1998) Uptake of high molecular weight dextran by the dinoflagellate *Alexandrium catenella*. Aquat Microb Ecol 16:81–86
Legrand C, Johansson N, Johnsen G, Borsheim KY, Granéli E (2001) Phagotrophy and toxicity variation in the mixotrophic *Prymnesium patelliferum* (Haptophyceae). Limnol Oceanogr 46(5):1208–1214
Oshima Y (1995) Post-column derivatization HPLC methods for paralytic shellfish poisons. In: Hallegraeff GM (ed) Manual on harmful marine microalgae. UNESCO, Paris, p 81–94
Rahat M, Jahn TL (1965) Growth of *Prymnesium parvum* in the dark; note on the ichthyotoxin formation. J Protozool 12:246–250
Smayda T (1990) Novel and nuisance phytoplankton blooms in the sea: evidence for a global epidemic. In: Granéli E (ed) Toxic marine phytoplankton. Lund, Sweden, p 29–40
Tillmann U (1998) Phagotrophy by a plastidic haptophyte, *Prymnesium patelliferum*. Aquat Microb Ecol 14:155–160
Valderrama JC (1995) Methods of nutrient analysis. In: Hallegraeff GM, Anderson DM, Cembella AD (eds) Manual of harmful marine microalgae, IOC Manuals and Guides, No. 33. UNESCO, Paris, p 251–268
Wulff F, Perttilä M, Rahm L (1996) Monitoring, mass balance calculation of nutrients and the future of the Gulf of Bothnia. AMBIO 8:28–35
Yamaguchi M, Itakura S (1999) Nutrition and growth kinetics in nitrogen- or phophorus-limited cultures of the noxious red tide dinoflagellate *Gymnodinium mikimotoi*. Fish Sci 65:367–373
Yu RC, Hummert C, Luckas B, Qian PY, Li J, Zhou MJ (1998) A modified HPLC method for analysis of PSP toxins in algae and shellfish from China. Chromatographia 48(9/10):671–676

Editorial responsibility: David Caron, Los Angeles, California, USA

Submitted: December 19, 2001; Accepted: June 17, 2002
Proofs received from author(s): August 16, 2002