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# Ecto-cellular enzyme activity associated with filamentous cyanobacteria

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ABSTRACT: Extra-cellular enzyme activity was investigated during a cyanobacterial bloom dominated by *Nodularia spumigena* on the SW coast of Finland, Baltic Sea. When filaments of *N. spumigena* were abundant in surface waters, alkaline phophatase (AP) activity was elevated, and ~37% of the total activity was associated with the >2 µm fraction. About 30% of the total leucine aminopeptidase (LAP) activity was also associated with the >2 µm size fraction. When spherical aggregates containing a mixture of moribund and decaying *N. spumigena* strands mixed with other cells were abundant in surface waters, ~50% of the total AP activity and ~22% or less of the total LAP activity was associated with the >2 µm fraction. Both total LAP and AP activity were positively correlated with chlorophyll *a*. LAP activity in the >2 µm fraction was positively correlated with thymidine uptake, suggesting that much of the observed LAP activity in the >2 µm fraction was due to heterotrophic bacteria associated with cyanobacteria. In contrast, AP activity in this size fraction was not significantly associated with bacterial numbers or activity. Experiments with isolated cyanobacterial aggregates demonstrated that they were sites of enhanced enzymatic activity. Cultured, axenic strands of *N. spumigena* displayed LAP and AP activity. Our data indicate that colonies and aggregates of cyanobacteria in the Baltic Sea can be important sites of hydrolytic activity and therefore of nutrient regeneration.

KEY WORDS: Baltic Sea · *Nodularia spumigena* · Leucine aminopeptidase · Alkaline phosphatase



## INTRODUCTION

Blooms of filamentous cyanobacteria have been recognised as common, rather regular and even annual events in the Baltic Sea (Finni et al. 2001). The blooms occur in late summer, usually in mid-July through August, and consist of various cyanobacteria species. Typical bloom-forming species are the filamentous and heterocystous *Nodularia spumigena* and *Aphanizomenon flos-aquae*. Since the 1990s these blooms have become more intense and have spread throughout the whole of the Gulf of Finland in addition to their previous occurrence in the Baltic Proper and at the entrance to the Gulf (Kahru et al. 1995, Kononen et al. 1996). This spread is thought to be a consequence of eutrophication due to human activities in the watershed, leading to internal phosphorus loading (Pitkänen et al. 2001). In contrast, paleolimnological studies suggest that cyanobacterial blooms are natural phenomena in the Baltic and can even occur in relatively pristine environments (McGowan et al. 1999, Bianchi et al. 2000, Poutanen & Nikkilä 2000).

In summer 2002, there was a prominent cyanobacterial bloom in the Gulf of Finland and in the Baltic Proper (Autio unpubl. data). The bloom was initiated in the second week of July and became more intense in the following weeks. During the bloom the phytoplankton biomass was dominated by filamentous cyanobacteria, the most important species being *Nodularia spumigena*, *Aphanizomenon flos-aquae* and *Anabaena lemmermannii*, which bloomed together forming surface scums. *N. spumigena* dominated, but at the beginning and end of the bloom *A. flos-aquae* contributed ~30 to 50% of the cyanobacterial biomass. *A. lemmermannii*

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contributed about 20% of the biomass throughout the bloom (M. Huttunen, Finnish Institute of Marine Research, pers. comm.). Although cyanobacteria dominated, the bloom plankton community also included bacteria, flagellates, diatoms (*Cylindrotheca closterium* and *Nitzschia paleacea*) and zooplankton.

Natural cyanobacterial colonies have associated heterotrophic bacteria and often small protists as well, and thus the filaments, colonies and aggregates can be important sites of microbial activity and enzymatic degradation of organic matter in the ocean (Paerl et al. 1989, Smith et al. 1992, Sellner 1997). Coupled hydrolysis of organic compounds and uptake of small organic compounds, ammonium and phosphate at the surface of cyanobacterial colonies may contribute to the intensity, size and persistence of blooms. However, investigations of the enzymatic activity and hydrolysis of organic substrates in the water column usually ascribe the activity to heterotrophic bacteria and ignore the contribution of larger organisms, as it has been thought to be minor (Rosso & Azam 1987, Sinsabaugh et al. 1997). Enzymes that hydrolyze organic substrates in the water column may be extracellular (exo-enzymes) that occur free in the water or ecto-enzymes on the surfaces of cells or in the periplasmic space of bacteria (Chróst 1991).

Leucine aminopeptidase (LAP) is commonly used as a measure of exo- and ecto-proteolytic activity in plankton (Hoppe 1983, Rosso & Azam 1987, Crottereau & Delmas 1998, Patel et al. 2000). LAP hydrolyses a broad spectrum of substrates, including polypeptides, with a free amino group, but has a preference for N-terminal leucine and related amino acids (Mahler & Cordes 1966). LAP activity in seawater is usually related to the abundance and activity of heterotrophic bacteria (Rosso & Azam 1987) but this ecto- or exo-enzyme is also found in some phytoplankton species (Martinez & Azam 1993, Langheinrich 1995, Berg et al. 2002, Stoecker & Gustafson 2003). The function of cell-surface-associated LAP in phytoplankton is controversial, but amino acids and peptide products may be assimilated by the phytoplankton as a source of C and N (Mulholland et al. 2002). Some eukaryotic phytoplankton have cell-surface amino acid oxidases that release ammonium from amino acids and are thought to be involved in N acquisition (Palenik & Morel 1990, Mulholland et al. 2002). Small organic compounds can also be used as a source of C by many phytoplankton (Lewitus & Kana 1994).

An important extracellular enzyme in the water column that is known to be associated with eukaryotic phytoplankton is alkaline phosphatase (AP), which releases inorganic phosphate from organic phosphorus compounds (Perry 1972, Ammerman 1991, 1993). AP activity is often used as an indicator of P limitation (Perry 1972, Ammerman 1991, 1993, Dyhrman & Palenik 2001), which is commonly encountered in the Gulf of Finland in late summer (Grönlund et al. 1996). AP activity and inorganic P availability are often, however, not linked, perhaps due to variations in the availability of dissolved organic P (Boström et al. 1988, Kononen & Nõmmann 1992, Grönlund et al. 1996).

Little is known about the exo- or ectocellular enzyme activity of cyanobacteria. LAP activity has been reported in coccoid cyanobacteria (Martinez & Azam 1993), but there are no reports of it in filamentous cyanobacteria. Although many cyanobacteria, including the genera *Nodularia*, *Aphanizomenon* and *Anabaena*, are heterocystous and can fix nitrogen, nitrogen fixation is costly and 12 mol of ATP are needed to for each mole of $N_2$ fixed (Voet & Voet 1995, Stahl et al. 2003). Thus, utilisation of dissolved organic N should be advantageous even in N-fixing taxa. At least some cyanobacteria can be grown in the dark on organic substrates (Vonshak et al. 2000), suggesting that organic compounds might be utilised as a supplemental source of fixed C as well, under some circumstances, as N. Some cyanobacteria, including *Nodularia* spp., are able to incorporate the amino acid leucine (Kamjunke & Jähnichen 2000, Hietanen et al. 2002). The oceanic cyanobacteria *Trichodesmium* spp. have been shown to concurrently utilise combined N (mostly $NH_4^+$ and dissolved organic nitrogen) and fix $N_2$ at high rates (Mulholland & Capone 1999). High amino acid oxidase activity has also been associated with *Trichodesmium* spp. blooms, which suggests that cyanobacteria have the capacity to obtain $NH_4^+$ from the oxidation of amino acids. Uptake of N from dissolved organic compounds has been associated with cyanobacterial blooms in the Baltic and elsewhere (Berman 2001, Berg et al. 2003, Glibert et al. 2004). Thus, evidence suggests that LAP activity could be advantageous in both N and C acquisition by cyanobacteria.

AP activity would be an advantage to nitrogen-fixing cyanobacteria since their growth is often P-limited. In the Baltic Sea during mid-summer stratification, inorganic phosphate concentration approaches the detection limit and >70% of the total phosphorus may be in the form of dissolved organic phosphorus (Kononen & Nõmmann 1992). In lakes, AP activity with a low half-saturation constant is thought to be associated with blooms of the cyanobacteria *Aphanizomenon flos-aquae* and *Gloetrichia echinulata* (Pettersson 1980). AP activity has been associated with blooms of the cyanobacteria *Trichodesmium* spp. in the Red Sea (Stihl et al. 2001) and with cyanobacterial blooms in the southern Baltic Sea (Hoppe 2003).

We conducted field studies at a coastal location (Tvärminne area) near the Hanko Peninsula, SW coast

of Finland, to assess the relative importance of the cyanobacterial contribution to AP and LAP activity in surface waters during the late summer. We estimated the wet weight of filamentous cyanobacteria and other phytoplankton and the abundance of heterotrophic bacteria, and measured chlorophyll *a* (chl *a*) (as an indicator of phytoplankton biomass) in order to investigate the relationships of these groups to enzymatic activity in size-fractionated water. We also measured thymidine uptake as an indicator of bacterial activity. If filamentous cyanobacteria are important sites of ecto-enzymatic activity, LAP and AP in the >2 µm fraction but not the bacterial fraction should be positively correlated with abundance of filamentous cyanobacteria. If nano- and microphytoplankton are important sources of activity, the correlation should be better with other phytoplankton than with cyanobacterial abundance. Conversely, if only heterotrophic bacteria are important, enzymatic activity in both size fractions should be correlated with bacterial biomass and thymidine uptake. Enzymatic activity associated with cyanobacterial filaments could be due to the cyanobacteria themselves, to associated bacteria or, most likely, to both. To determine if filamentous cyanobacteria have their own ecto-enzymes, enzyme assays were done with axenic cultures of *Nodularia spumigena*.

In addition to typical filaments of cyanobacteria which were included in the water sampling, we observed unusual spherical cyanobacterial aggregates during our field work. We collected these by scooping them from surface patches and describe their species composition and associated AP and LAP activity.

## MATERIALS AND METHODS

**Sampling.** Field studies on cyanobacterial blooms were conducted at a coastal location near the Hanko Peninsula, SW coast of Finland (Fig. 1). The area was sampled on 4 subsequent days during the second week of August 2002. Surface water was collected along four 1 to 2 km horizontal transects and at some separate stations; altogether 22 stations were sampled. *In situ* temperature and salinity were measured with a YSI 63 (Yellow Springs Instruments). Samples were collected for inorganic nutrient and chl *a* determinations. Water samples were collected for enumeration of bacteria and phytoplankton and for measurement of thymidine uptake and exo- or ecto-enzymatic activity (LAP, AP). In addition to the collection of surface-water samples at the stations, cyanobacterial aggregates were collected when patches of these were observed along transects. Water from the patches was scooped up with a bucket and stored in 1 l containers. Water and aggregate samples were kept in a cooler and returned to the laboratory within 1 h of collection. Water subsamples were immediately fixed for microscopy, filtered for chl *a* determinations, and incubated for thymidine uptake measurements and enzymatic activity assays. Aggregate subsamples were fixed for light and electron microscopy and individual aggregates were removed for rate measurements and chlorophyll determinations.

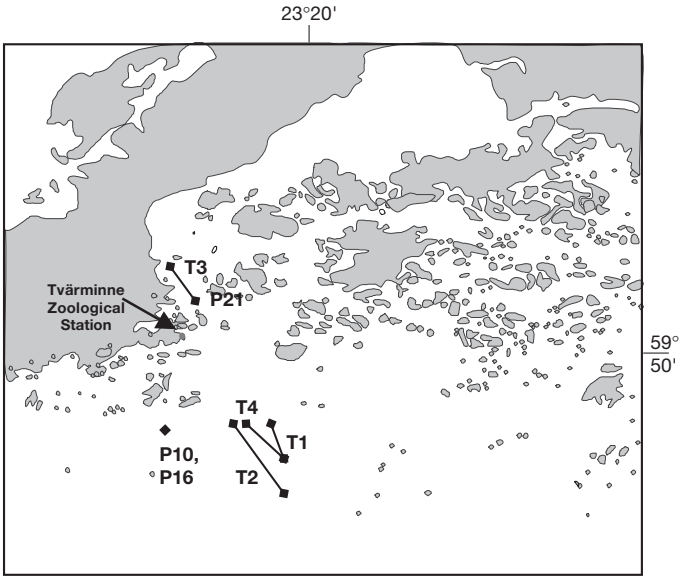


Fig. 1. Study area near the Hanko Peninsula, SW coast of Finland, Baltic Sea. T1, T2, T3, T4: transects marked by end points; P10, P16, P21: stations sampled separately from transects

**Chlorophyll *a*.** Chl *a* was measured from duplicate 50 ml subsamples (for both total and <2 µm) from each station. The subsamples were filtered on GF/F (Whatman) glass-fibre filters and extracted into ethanol in darkness overnight. The extract was filtered again through a Whatman GF/F filter and the chl *a* fluorescence was measured with a Jasco FP-750 spectrofluorometer calibrated with pure chl *a*. Chl *a* concentrations were calculated according to HELCOM (1988).

Chlorophyll determinations were made on individual aggregates by picking them with a micropipette and transferring individual aggregates to a glass-fibre filter. The filters were extracted as described above.

**Inorganic nutrients.** Concentrations of inorganic nutrients ($NO_3$-N, $NH_4$-N, $PO_4$-P) in the whole-water samples were determined applying standard seawater analysis methods in accordance with Grasshoff (1976).

**Thymidine uptake and bacterial cell numbers.** Bacterial cell numbers and activity as thymidine incorporation rate were determined from both whole-water samples and the <2 µm size fraction. For bacterial cell counts, 20 ml samples were fixed with glutaraldehyde (25% EM grade, 1.25% final conc.) and stored refrigerated. Subsamples of 1 ml were stained with particle-

free Acridine Orange (0.01% final conc.) and collected on 0.2 µm pore-sized black polycarbonate filters (Poretics). The filters were examined with a Leitz Aristoplan microscope under blue excitation light (1000× magnification, I3 filter block).

Thymidine uptake by bacteria was measured basically according to Fuhrman & Azam (1980, 1982). *Nodularia* spp. (and presumably other filamentous cyanobacteria) do not incorporate thymidine and thus their presence does not interfere with bacterial production measurements based on thymidine uptake (Hietanen et al. 2002). Duplicate 5 ml subsamples and a formalin-killed adsorption control were incubated with [methyl-$^3$H]-thymidine (42 Ci mmol$^{-1}$) for 54 to 149 min. Labelled thymidine was added at saturation level (ca. 14 nM). The incubations were stopped by formalin addition and the samples were stored cooled until further treatment. The ice-cold samples were extracted within 2 d with TCA (5% final conc.) and filtered onto 0.2 µm mixed cellulose ester filters (Advantec MSF) in ice-cold conditions. The filters were washed with 5% TCA and then soaked in scintillation cocktail (InstaGel, Packard Instrument) for at least 24 h. The incorporated radioactive thymidine was assayed with liquid scintillation techniques (1414 WinSpectral, Wallac LKB) using external standards.

**LAP assays.** L-leucine-7-amido-4-methyl-coumarin (Leu-AMC) (Sigma Chemical) was used as the substrate in LAP activity measurements (Sarath et al. 1989). Water samples were fractionated using a 2 µm pore-size polycarbonate filter. Duplicate whole-water and <2 µm fraction samples were incubated along with control cuvettes. We added 20 µl of the substrate stock (5 mM) to 1 ml of samples in semi-UV cuvettes (Fisherbrand, 98 µM final conc.). This concentration of substrate is saturating. The samples were incubated for up to 30 min in the dark at 20°C (Stoecker & Gustafson 2003). Reactions were terminated by adding 100 µl of 10% dodecyl (lauryl) sulfate and then the cuvettes were stored frozen (–20°C) until fluorescence was determined (Crottereau & Delmas 1998).

Readings were made with a Jasco FP-750 spectrofluorometer (excitation 380 nm with 10 nm slit, emission 440 nm with a 1.5 nm slit width). A standard curve was used with concentrations of the product; 7-amino-4-methyl-coumarin (AMC) (Sigma Chemical), ranging from 0 to 2.5 µM in sterile filtered (Sartobran 300, Sterile Capsule) seawater. Whole-water samples without added substrate were used to control for natural fluorescence. Autoclaved seawater, to which substrate was added, was incubated in cuvettes along with the experimental samples, and was used to control for fluorescence of the substrate and for bacterial contamination of the substrate which could result in product formation. LAP activity in the >2 µm fraction was estimated by subtracting activity in the <2 µm fraction from activity in the whole-water sample. If negative values were obtained, they were converted to zero. In coastal waters, negative values for the >2 µm fraction, when obtained, are equivalent to <5% of total activity.

To determine the LAP activity of *Nodularia spumigena* aggregates, aggregates up to 4 mm in diameter were detached from natural samples using a Pasteur pipette, and washed in sterile seawater; 1 aggregate was added to a cuvette containing 1 ml sterile seawater, and the fluorogenic substrate was added. The cuvettes were incubated as described above, except that the incubation was subsampled at 5, 10, 20 and 30 min. At each time point, 100 µl samples were withdrawn from each tube with a micropipette and added to 1.9 ml sterile seawater containing 200 µl dodecyl sulfate. At the end of the incubation, each aggregate was removed with a micropipette from the incubation cuvette and transferred to a glass-fibre filter and frozen for chl *a* determination. LAP activity was calculated per aggregate and per unit chlorophyll.

**AP assays.** The total AP activity was measured in accordance with Pettersson (1980), with modifications (Kononen et al. 1993, Grönlund et al. 1996). Duplicate 8 ml subsamples of the total and <2 µm water were collected in acid-washed glass test tubes and put into a temperature controlled water bath at +20°C. The substrate 4-methylumbelliferyl-phosphate (MUP) (Sigma Chemical) was added to obtain a final concentration of 0.11 mM. The zero reading was obtained with a Jasco FP-750 spectrofluorometer equipped with a sipper Jasco SHP 292, using excitation at 365 nm with a 10 nm slit and 460 nm emission filter with a 5 nm slit. The reaction was followed throughout the 75 min incubation, with measurements every 15 min. A standard curve was used with the product, 4-methylumbelliferone (MU) (Sigma Chemical). AP measurements were not made on aggregates because they would have clogged the sipper.

**Wet weight (biomass) of filamentous cyanobacteria and other phytoplankton.** Phytoplankton biomass and species composition in the surface water were analysed by settling 10 ml of acid Lugol's solution preserved samples in accordance with Utermöhl (1958). A Leitz inverted microscope with phase contrast was used. Filamentous cyanobacteria were counted from the whole chamber area at 125×, and smaller more numerous phytoplankton species from 10 to 40 random fields at 500×. Standard biovolumes for phytoplankton species in the Baltic Sea were used to convert abundance data to wet weight, a measure of biomass (Finnish Institute of Marine Research unpubl. data).

**Microscopy of aggregates.** Subsamples of the aggregates for light microscopy and transmission electron microscopy (TEM) were fixed with 2% (final

conc.) glutaraldehyde for 4 h, after which they were preserved in phosphate buffer solution for 2 d. A Leitz inverted microscope equipped with phase contrast and epifluorescence was used for light microscopy. For TEM, after 4 h fixation the aggregates were transferred to a phosphate buffer solution for 2 d. The aggregates were then dehydrated in series of 96% ethanol and post-fixed with osmium and embedded in plastic according to standard methods (Unit of Electron Microscopy, Institute of Biotechnology, University of Helsinki). The thin sections were cut with an ultramicrotome and observed with a TEM JEOL 1200ex transmission electron microscope at 60 kV tension.

**Biomass, chl *a* content and enzyme activity of axenic *Nodularia spumigena* cultures.** An axenic strand of *Nodularia spumigena* (Strain up16f) was obtained from the research group of Professor Kaarina Sivonen (Viikki Biocenter, University of Helsinki). We grew 3 separate batch cultures of the same strain in sterile Greiner bio-one GmbH Cellstar® tissue culture flasks in Z8XS culture medium (Sivonen et al. 1989) at 6 psu on a 12:12 light:dark cycle at ca. 80 µE $m^{-2}$ $s^{-1}$ at room temperature (ca. +20°C). Z8XS is used to grow N-fixing cyanobacteria and is low in fixed N (estimated 0.16 µM fixed N) but is rich in phosphorous (200 µM phosphate). The chl *a* and carbon contents for each batch culture were determined simultaneously in order to achieve empirically valid chl *a* to carbon ratios for axenic *N. spumigena*. The carbon content was determined from triplicate 1, 2 and 4 ml samples of each batch culture that were collected on precombusted GF/F filters (Whatman) and analysed with a mass spectrometer (Europa Scientific, ANCA-MS Tracer Mass & RoboPrep). LAP and AP activities of each batch were measured as described above and activity was calculated per unit chlorophyll and per unit carbon.

**Statistical analysis.** Correlations between different parameters were analysed applying parametric testing (STATGRAPHICS Plus 5.1, Statistical Graphics). There was 1 outlier in the data set (Sample 10, see Tables 1 & 2), which contained several times more chl *a* than the other samples. It was removed to ensure normal distribution of the data.

## RESULTS

### Occurrence of filmentous cyanobacteria

On 6 August, *Nodularia spumigena* filaments of the 'pea soup' type were observed along Transect 1 (Table 1, Fig. 1). The filaments resembled small flakes, typical of actively growing colonies. Average surface water chl *a* concentration was relatively high, with about 61% of the chlorophyll in the >2 µm fraction (Table 1). On Transect 2, taken the next day adjacent to Transect 1 (Fig. 1), spherical yellowish green aggregates up to 4 mm in diameter (Fig. 2A) were found floating in uneven patches 1 to 10 m in extent. The aggregates did not break up easily (typically aggregates formed by *N. spumigena* break up easily; Stahl et al. 2003), but were easily picked out of the water with forceps. They had a solid structure that was quite hard in the centre and difficult to break apart. The aggregates were particularly prevalent at Stn 10, which had a very high chl *a* concentration in the >2 µm fraction (Table 1). A third transect was sampled the same day (Fig. 1); average chl *a* levels were lower along this transect and *N. spumigena* was not abundant (Table 1).

The next day (8 August), 2 surface-water samples (Fig. 1, Stns 16 and 21) were collected in the vicinity of the stations where *Nodularia spumigena* had been collected on 6 and 7 August; however, filaments or aggregates were not abundant in these samples (Table 1). *N. spumigena* aggregates were again observed in samples on 9 August from Transect 4, which was in the same location as Transect 2 (Fig. 1). However, the aggregates were less abundant than 2 d earlier.

Table 1. Sampling stations in Tvärminne area, SW coast of Finland, August 2002; physical, chemical and biological (mean ± SD) parameters for surface samples. nd: no data

| Transect | Date | Stns | Temp. (°C) | Salinity | Chlorophyll *a* (µg $l^{-1}$) | >2 µm (%) | Inorg. N (µg $l^{-1}$) | Inorg. P (µg $l^{-1}$) | Biomass (wet wt) of: Colonial cyanobacteria (µg $l^{-1}$) | Other phytoplankton (µg $l^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 6 | 1–5 | 19 | 5.8 | 6.9 ± 0.77 | 61 ± 30 | 2.8 ± 1.34 | 0.8 ± 0.45 | 356 ± 139.8 | 361 ± 91.9 |
| 2 | 7 | 6–9 | 20 | 5.8 | 5.5 ± 1.87 | 67 ± 79 | 1.08 ± 0.50 | 0.25 ± 0.50 | 528 ± 208.9 | 379 ± 103 |
| | 7 | 10 | 20 | 5.8 | 15.75 | 78 | 10.9 | 5 | nd | nd |
| 3 | 7 | 11–15 | 21 | 5.4 | 3.1 ± 0.64 | 46 ± 59 | 3.6 ± 3.49 | 3.4 ± 0.89 | 104 ± 103.1 | 314.2 ± 64.9 |
| | 8 | 16 | 21 | 5.8 | 4.5 | 56 | 2.2 | 2.0 | 157 | 111 |
| | 8 | 21 | 21 | 5.4 | 2.3 | 29 | 1.2 | 3.0 | 56 | 296 |
| 4 | 9 | 26–30 | 20 | 5.8 | nd | nd | 2.0 ± 0.47 | 1.0 ± 1.22 | 707 ± 306.6 | 86.8 ± 30.2 |

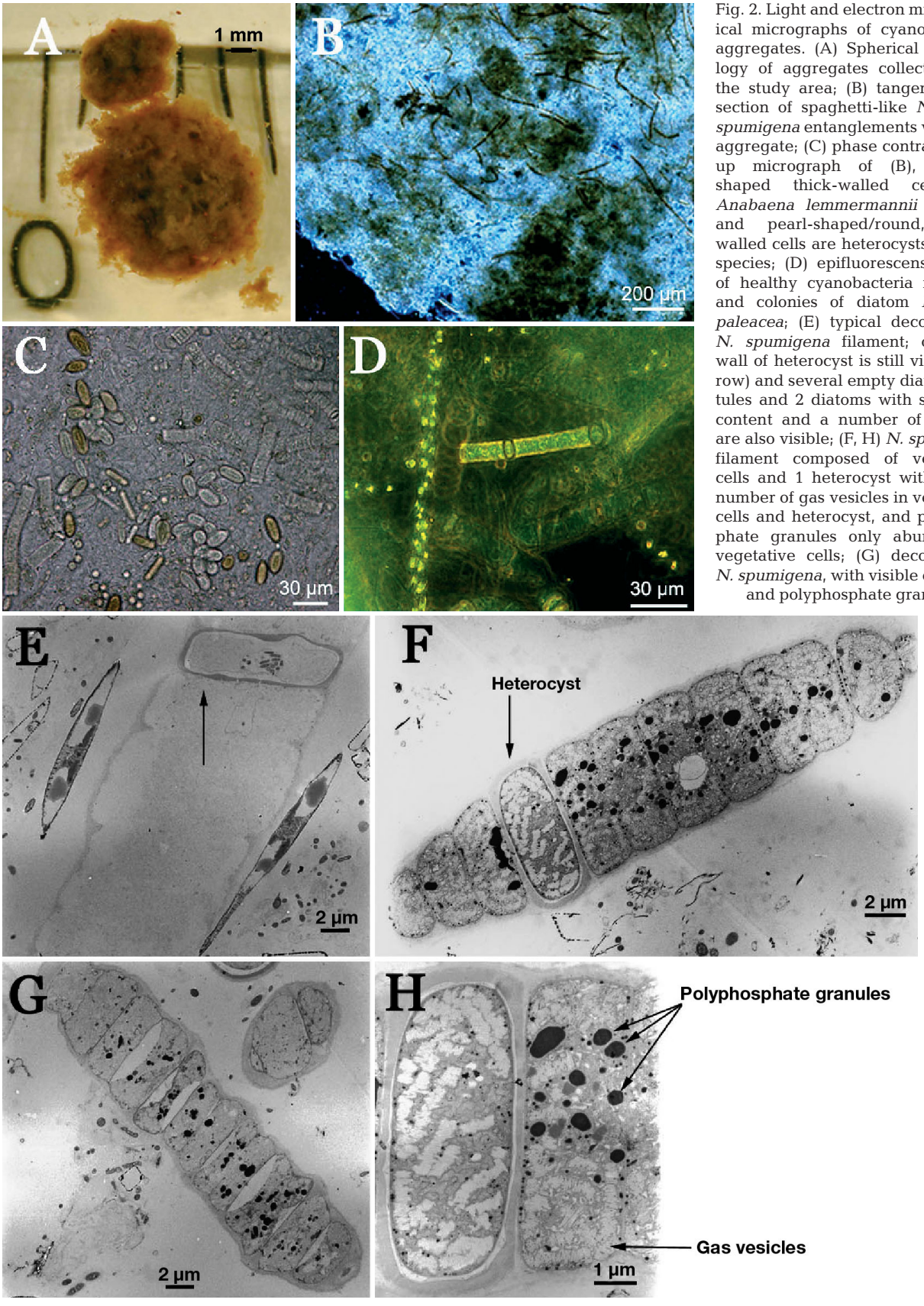


Fig. 2. Light and electron microscopical micrographs of cyanobacterial aggregates. (A) Spherical morphology of aggregates collected from the study area; (B) tangential thin section of spaghetti-like *Nodularia spumigena* entanglements within an aggregate; (C) phase contrast close-up micrograph of (B), kidney-shaped thick-walled cells are *Anabaena lemmermannii* akinetes and pearl-shaped/round, thick-walled cells are heterocysts of same species; (D) epifluorescense image of healthy cyanobacteria filaments and colonies of diatom *Nitzschia paleacea*; (E) typical decomposing *N. spumigena* filament; only cell wall of heterocyst is still visible (arrow) and several empty diatom frustules and 2 diatoms with some cell content and a number of bacteria are also visible; (F, H) *N. spumigena* filament composed of vegetative cells and 1 heterocyst with a high number of gas vesicles in vegetative cells and heterocyst, and polyphosphate granules only abundant in vegetative cells; (G) decomposing *N. spumigena*, with visible cell walls and polyphosphate granules

### Composition of spherical aggregates

The aggregates were mainly composed of the cyanobacteria *Nodularia spumigena*. The trichomes showed different degrees of decomposition (Fig. 2A,E,G) and it was estimated that >20% of the cells were in good condition and appeared to be alive (Fig. 2B,C,F,H). The yellowish surface of the aggregates was formed mainly of decomposing *N. spumigena* (Fig. 2A). A variety of algal cells were imbedded in the aggregates. A prominent part of the mixed community was akinetes and heterocysts of another cyanobacteria, *Anabaena lemmermannii* (Fig. 2C). Picoplanktonic cyanobacterial cells and colonies of small cyanobacteria (*Merismopedia* sp. and *Aphanocapsa* sp.), trichomes of a small *Pseudanabaena* sp. (Cyanophyceae) as well as a number of eukaryotic algae were also encountered. A small diatom of the genus *Nitzschia*, probably *N. paleacea* (Fig. 2D,E), was abundant, as was a small *Amphora* species, but most cells of both species appeared to be moribund. In addition, *Oocystis* spp. colonies (Chlorophyceae) and colonies composed of small cells most probably belonging to golden algae (Chrysophyceae) were also encountered in the aggregates.

### Enzymatic activity in surface waters and aggregates

Average total LAP activity ranged from 0.44 to 0.94 µmol AMC $l^{-1}$ $h^{-1}$ (Stn 10 excluded as an outlier) and the distribution of activity between size fractions was much more variable than total activity (Table 2). LAP activity:chl *a* ratios were relatively constant, ranging from 0.11 to 0.14 µmol AMC $h^{-1}$ (µg chl *a*)$^{-1}$ for Transects 1 to 3 and Stn 10, but a higher ratio (0.21 to 0.22) was found for Stns 16 and 21 sampled on 8 August (calculated from data in Tables 1 & 2). LAP activity was not significantly correlated with inorganic nitrogen concentrations (Table 3). In Transects 1 and 2, along which *Nodularia spumigena* was abundant, an average of 22 to 30% of the activity was in the >2 µm fraction (Tables 1 & 2). Total LAP activity was positively correlated with chl *a* and with filamentous cyanobacterial wet weight but not with wet weight of other phytoplankton (Table 3). LAP activity in the <2 µm but not the >2 µm fraction was positively correlated with wet weight of cyanobacteria. Interestingly, there was an inverse relationship between LAP in the <2 µm fraction and biomass of other phytoplankton. LAP activity in the >2 µm fraction was correlated with bacterial thymidine uptake (Table 3). However, LAP activity in the <2 µm fraction was positively associated with bacterial abundance but not with thymidine uptake (Table 3).

Total AP activity ranged from an average of 15 to 97 µmol MU $l^{-1}$ $h^{-1}$ (Stn 10 excluded as an outlier) and was much more variable than LAP activity (Table 2). The average AP activity in samples from Transects 1, 2 and 4, where *Nodularia spumigena* densities were high, was clearly higher than in the samples from the other sampling areas, and 37% or more of the activity was associated with the >2 µm fraction in these samples (Table 2). AP activity:chl *a* ratios varied from 6.5 to 14.1 µmol MU $h^{-1}$ (µg chl *a*)$^{-1}$ (Stn 10 excluded as an outlier, with a ratio of 35.7) (calculated from data in Tables 1 & 2). The highest ratios (14.1 and 9.8) were associated with transects in which filamentous cyanobacteria were abundant (Transects 1 and 2, respectively). AP:chl *a* ratios were between 6 and 7 for the transect (Transect 3) and stations (Stns 16 and 21) in which cyanobacteria were not abundant (calculated from data in Tables 1 and 2). Although total AP activity and AP activity in the <2 µm fraction were not associated with cyanobacterial wet weight, AP activity in the >2 µm fraction was positively associated with wet weight of filamentous cyanobacteria.

Total AP activity was positively correlated with chl *a*. AP activity in the >2 µm fraction was correlated with chl *a* in this size fraction, but not with bacteria or thymidine uptake (Table 4). In contrast to the >2 µm fraction, AP activity in the <2 µm fraction was correlated with thymidine uptake, a measure of bacterial activity (Table 4). Although there appeared to be an inverse relationship between AP activity and inorganic

Table 2. Mean ± SD bacterial abundance, thymidine uptake, and enzymatic activities. LAP: leucine aminopeptidase; AP: alkaline phosphatase; AMC: 7-amino-4-methyl-coumarin; MU: 4-methylumbelliferone; –: stations sampled separately from transects

| Transect (Stns) | Bacteria abund. free cells (×$10^6$ $ml^{-1}$) | Bacteria abund. >2 µm (%) | Thymidine uptake pmol $l^{-1}$ $h^{-1}$ | Thymidine uptake >2 µm (%) | LAP activity µmol AMC $l^{-1}$ $h^{-1}$ | LAP activity >2 µm (%) | AP activity µmol MU $l^{-1}$ $h^{-1}$ | AP activity >2 µm (%) |
|---|---|---|---|---|---|---|---|---|
| 1 (1–5) | 7.8 ± 1.79 | 2 ± 14.1 | 82.6 ± 4.87 | 76 ± 8.2 | 0.73 ± 0.175 | 30 ± 13.0 | 96.6 ± 10.89 | 37 ± 14.6 |
| 2 (6–9) | 9.0 ± 0.46 | 23.9 ± 2.3 | 82.2 ± 2.9 | 11.7 ± 2.2 | 0.65 ± 0.03 | 22 ± 1.5 | 53.6 ± 22.82 | 51 ± 6.89 |
| – (10) | 9.8 | 7.4 | 98.3 | 14.1 | 2.14 | 60 | 560.2 | 74.4 |
| 3 (11–15) | 8.92 ± 1.07 | 5 ± 24.3 | 84.4 ± 4.20 | 8 ± 10.2 | 0.44 ± 0.015 | 0 ± 8.0 | 21.0 ± 11.66 | 18 ± 25.5 |
| – (16) | 11.5 | 25 | 83.4 | 11 | 0.94 | 24 | 30.24 | 50 |
| – (21) | 9.5 | 7 | 64.16 | 27 | 0.50 | 4 | 14.98 | 4 |
| 4 (26–30) | 12.43 ± 1.00 | 0 ± 10.1 | 86.4 ± 2.43 | 14.0 ± 3.8 | 0.82 ± 0.098 | 4 ± 8.8 | 58.6 ± 36.17 | 48 ± 29.5 |

Table 3. Correlation of LAP activity with biomass of filamentous cyanobacteria, biomass of other phytoplankton, chlorophyll *a*, bacterial abundance, thymidine uptake, and inorganic N (n = 21, except for chl *a*, for which n = 16; r = product-moment correlation coefficient). ns: not significant

| Parameter | r | p |
|---|---|---|
| **Total LAP activity vs. total** | | |
| Filamentous cyanobacteria biomass | 0.553 | <0.01 |
| Other phytoplankton biomass | −0.402 | ns |
| Chl *a* | 0.560 | <0.05 |
| Bacteria ml$^{-1}$ | 0.430 | ns |
| Thymidine uptake | 0.245 | ns |
| **LAP activity in <2 µm fraction vs.** | | |
| Filamentous cyanobacteria biomass | 0.654 | <0.01 |
| Other phytoplankton biomass | −0.797 | <0.01 |
| Chl *a* in <2 µm fraction | 0.117 | ns |
| Bacteria ml$^{-1}$ in <2 µm fraction | 0.774 | <0.01 |
| Thymidine uptake in <2 µm fraction | 0.255 | ns |
| Inorganic N | −0.169 | ns |
| **LAP activity in >2 µm fraction vs.** | | |
| Filamentous cyanobacteria biomass | 0.021 | ns |
| Other phytoplankton biomass | 0.330 | ns |
| Chl *a* in >2 µm fraction | 0.452 | ns |
| Bacteria ml$^{-1}$ in >2 µm fraction | 0.038 | ns |
| Thymidine uptake in >2 µm fraction | 0.572 | <0.01 |
| Inorganic N | −0.097 | ns |

Table 4. Correlation of AP activity with biomass of filamentous cyanobacteria, biomass of other phytoplankton, chlorophyll *a*, bacterial abundance, thymidine uptake, and inorganic P and LAP. (n = 21, except for chl *a*, for which n = 16; r = product-moment correlation coefficient)

| Parameter | r | p |
|---|---|---|
| **Total AP activity vs. total** | | |
| Filamentous cyanobacteria biomass | 0.368 | ns |
| Other phytoplankton biomass | 0.089 | ns |
| Chl *a* | 0.906 | <0.01 |
| Bacteria ml$^{-1}$ | −0.242 | ns |
| Thymidine uptake | 0.012 | ns |
| Total LAP | 0.552 | <0.01 |
| **AP activity in <2 µm fraction vs.** | | |
| Filamentous cyanobacteria biomass | 0.121 | ns |
| Other phytoplankton biomass | 0.312 | ns |
| Chl *a* in <2 µm fraction | 0.831 | <0.01 |
| Bacteria ml$^{-1}$ in <2 µm fraction | −0.273 | ns |
| Thymidine uptake in <2 µm fraction | −0.866 | <0.01 |
| Inorganic P | −0.400 | ns |
| LAP activity in <2 µm fraction | −0.155 | ns |
| **AP activity in >2 µm fraction vs.** | | |
| Filamentous cyanobacteria biomass | 0.459 | <0.05 |
| Other phytoplankton biomass | −0.123 | ns |
| Chl *a* in >2 µm fraction | 0.816 | <0.01 |
| Bacteria ml$^{-1}$ in >2 µm fraction | −0.210 | ns |
| Thymidine uptake in >2 µm fraction | 0.299 | ns |
| Inorganic P | −0.423 | ns |
| LAP activity in >2 µm fraction | 0.437 | <0.05 |

phosphate, the correlation coefficient was not significant (Table 4). AP and LAP activity were positively correlated in the whole-water sample and in the >2 µm fraction (Table 4). In contrast, AP and LAP activity were not correlated in the <2 µm fraction (Table 4).

Unfortunately, we did not observe typical 'pea soup' *Nodularia spumigena* colonies after the first day of sampling and do not have colony-specific measurements of activity. LAP activity was measured for *N. spumigena* aggregates collected on 7 August from Stn 10, and on 9 August (when aggregate densities were lower) from pooled surface samples collected along Transect 4 (Table 5). Average LAP activity of the aggregates was 0.051 µmol AMC aggregate$^{-1}$ h$^{-1}$ (Table 5). Chl *a*-specific activity was relatively constant, with 0.18 µmol AMC produced (µg chl *a*)$^{-1}$ h$^{-1}$ (Table 5). This chl *a*-specific rate is about 4.5× the chl *a*-specific rate for the >2 µm fraction: average 0.04 µmol AMC produced (µg chl *a*)$^{-1}$ h$^{-1}$, Transects 1, 2 and 3 (data from Tables 1 & 2).

### Enzymatic activity of axenic *Nodularia spumigena*

Both LAP and AP activity was found in axenic cultures of *N. spumigena* grown in phosphate-replete medium (Table 6). Chlorophyll-specific rates of LAP activity in the cultures were ca. 0.003 µmol AMC (µg chl *a*)$^{-1}$ h$^{-1}$. Chlorophyll-specific rates of AP activity in the cultures were ca. 0.13 µmol MU (µg chl *a*)$^{-1}$ h$^{-1}$. Carbon-specific rates were calculated using an empirically derived chl *a*:carbon ratio of 6.7 for the axenic culture of *N. spumigena* (Table 6).

Table 5. *Nodularia spumigena*. Enzymatic activity (mean ± SD) associated with aggregates (aggr.) collected from natural assemblages in August 2002 at +20°C

| Date | n | µg chl *a* aggr.$^{-1}$ | µmol AMC (aggr. h)$^{-1}$ h$^{-1}$ | µmol AMC (µg chl *a*)$^{-1}$ h$^{-1}$ |
|---|---|---|---|---|
| Aug 7 | 8 | 0.28 ± 0.118 | 0.051 ± 0.0295 | 0.182 |
| Aug 9 | 10 | 0.29 ± 0.115 | 0.053 ± 0.0146 | 0.183 |

Table 6. *Nodularia spumigena*. Enzymatic activity (mean ± SD) associated with axenic, cultured *N. spumigena* at +20°C (n = 3). LAP data: µmol AMC produced (µg chl *a*)$^{-1}$ h$^{-1}$ and (µg C)$^{-1}$ h$^{-1}$; AP data: µmol MU produced (µg chl *a*)$^{-1}$ h$^{-1}$ and (µg C)$^{-1}$ h$^{-1}$

| | Enzymatic activity (µg chl *a*)$^{-1}$ h$^{-1}$ | (µg C)$^{-1}$ h$^{-1}$ |
|---|---|---|
| LAP | 0.003 ± 0.0002 | 0.0004 ± 0.0001 |
| AP | 0.130 ± 0.038 | 0.020 ± 0.007 |

## DISCUSSION

We conducted field studies to assess the importance of the cyanobacterial contribution to LAP and AP activity in surface waters of the Baltic Sea. We obtained surface water samples with and without high biomass (wet weight) of filamentous cyanobacteria (mostly *Nodularia spumigena*). The pattern of correlations among enzyme activity, abundance and activity of bacteria and the biomass of cyanobacteria and other phytoplankters suggests that the presence of filamentous cyanobacteria is associated with enhanced enzymatic hydrolysis in surface waters. However, the relationships of LAP and AP activities to cyanobacteria, other phytoplankton and bacterial biomass differ.

LAP appears to be a constitutive enzyme in phytoplankton and perhaps also in filamentous cyanobacteria. We observed carbon-specific rates of ca. 0.0004 µmol AMC (µg C)$^{-1}$ h$^{-1}$ in axenic *Nodularia spumigena*, which are comparable to rates for cultured dinoflagellates at a similar temperature (Stoecker & Gustafson 2003). Chl *a*-specific LAP activity of the axenic cultures was approximately 1 order of magnitude lower than chl *a*-specific rates for the >2 µm fraction measured in surface water and about 2 orders of magnitude lower than chl *a*-specific rates measured on field-collected, decomposing *N. spumigena*-dominated aggregates at the same temperature. These data suggest that although cyanobacteria have constitutive LAP activities, most of the LAP activity associated with filamentous cyanobacteria or cyanobacterial aggregates in natural assemblages is due to associated bacteria. However, physiological differences in the magnitude of LAP activity between cultured and natural filaments cannot be ruled out.

The pattern of correlation between LAP activity and other parameters in size-fractionated water is consistent with the interpretation that most of the LAP activity associated with cyanobacterial abundance in our field investigation was due to bacteria. Total LAP activity and LAP activity in the <2 µm fraction but not in the >2 µm fraction were associated with abundance of filamentous cyanobacteria. The relationship of LAP with the abundance of other phytoplankton was negative or non-significant. LAP activity in size-fractionated samples was positively associated with bacterial abundance or activity. This suggests that bacteria with LAP were positively associated with filamentous cyanobacteria; this association could arise because both the bacteria and the cyanobacteria are favoured by the same environmental parameters or because the growth of cyanobacteria promotes the growth of bacteria.

AP activity was detected in axenically grown *Nodularia spumigena*, demonstrating that filamentous cyanobacteria can have their own AP. AP, although found at low levels in many cell types, is often inducible by phosphate limitation in phytoplankton (Dyhrman & Palenik 2001). The low base level of AP activity in the *N. spumigena* cultures grown in high phosphate medium (200 µM) compared to natural assemblages exposed to dissolved phosphate levels orders of magnitude lower is compatible with phosphate limitation inducing or stimulating AP. Similarily, Stihl et al. (2001) reported chlorophyll-specific AP activity for *Trichodesmium* spp. colonies from open waters of the Red Sea that was 1 order of magnitude higher than that of colonies from coastal waters or cultured filaments. In our investigation, the correlations of AP activity with phosphate concentrations *in situ*, although negative, were not significiant. We suspect that this was due to the low *in situ* phosphate concentrations, which were at or near detection limits; it is possible that at most or all of the stations phosphate was limiting and AP stimulated. Data on the effect of phosphate limitation on AP levels in axenic cultures of cyanobacteria would answer the question of AP inducibility in filamentous cyanobacteria.

In our field study, the pattern of associations between AP activity and other parameters in the size-fractionated surface waters suggest that cyanobacteria are more important as a source of AP than LAP activity. Total AP activity was positively correlated with chl *a*, but not with bacteria or their activity. In many samples, as much or more of the AP activity was found in the >2 µm size fraction as in the bacterial size fraction. In the >2 µm size fraction, AP activity was positively associated with cyanobacterial biomass and chl *a*, but not with bacterial abundance or activity.

The observation of dense patches of unusual spherical cyanobacterial aggregates at our study site was interesting. Cyanobacterial aggregates or dense surface patches of cyanobacterial filaments are difficult to sample quantitatively, and often are not represented in routine sampling. However these patches have the potential to be 'hot spots' of microbial activity and to make significant contributions to microbial processes in surface waters. Cyanobacterial colonies are known to be sites of enhanced primary production and microbial activity in the open ocean (Borstad & Borstad 1976, Paerl et al. 1989, Smith et al. 1992). Our data on *Nodularia spumigena* aggregates demonstrate that they can also be important sites of microbial activity and ectoenzymatic activity. For example, the presence of 1 *N. spumigena* aggregate l$^{-1}$ could account for 1.8 to 12.4 % of total chl *a* and 2.4 to 20 % chl *a* in the >2 µm fraction in surface water samples (Tables 1 & 5), and 1 *N. spumigena* aggregate l$^{-1}$ could account for 2.4 to 11.8 % of the total and 22 to 100 % of the >2 µm fraction LAP activity in the same waters (Tables 2 & 5). Because the aggregates were a mixture of organisms it is not

possible to separate the activity of cyanobacteria themselves from that of other organisms in the aggregates.

The significance of LAP and AP activity to the physiological ecology and growth of filamentous cyanobacteria in coastal waters is not known. Cyanobacteria may profit from their own ecto-enzymatic activity and perhaps that of their microbial associates if amino acids or inorganic phosphate is released at their cell surface. *Nodularia* spp. are known to assimilate amino acids at high rates (Hietanen et al. 2002). Some cyanobacteria have cell surface amino acid oxidases which break down amino acids, releasing ammonium, and ammonium is readily assimilated by cyanobacteria as a source of nitrogen (Mulholland et al. 1998, 2001, Moore et al. 2002, Berg et al. 2003). Inorganic phosphate released by cell surface AP should also be readily assimilated by cyanobacteria (Stihl et al. 2001). The relatively higher levels of AP activity than LAP activity, the common phosphate limitation of cyanobacterial blooms, and the ability of many filamentous cyanobacteria to fix nitrogen suggest that AP may be more important in nutrient acquisition than LAP in filamentous cyanobacteria. However, quantitative data on the role of ecto-enzymatic activity in nutrient acquisition by cyanobacteria and other phytoplankton are largely lacking.

At the community or ecosystem level, cyanobacteria filaments, colonies and aggregates, besides contributing to nitrogen fixation, primary production and phytoplankton biomass (Sivonen et al. 1990, Sellner 1997, Mulholland & Capone 1999), may be important sites of enzymatic hydrolysis of organic matter. Enzymatic activities are probably due to both the cyanobacteria themselves and their associated microbes. Remineralisation rates of C, N and P may be enhanced during and immediately after cyanobacterial blooms.

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