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Competition for inorganic and organic forms of nitrogen and phosphorous between phytoplankton and bacteria during an *Emiliania huxleyi* spring bloom

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Abstract. Using ^{15}N and ^{33}P, we measured the turnover of organic and inorganic nitrogen (N) and phosphorus (P) substrates, and the partitioning of N and P from these sources into two size fractions of marine osmotrophs during the course of a phytoplankton bloom in a nutrient manipulated mesocosm. The larger size fraction ($>0.8\,\mu$m), mainly consisting of the coccolithophorid *Emiliania huxleyi*, but also including an increasing amount of large particle-associated bacteria as the bloom proceeded, dominated uptake of the inorganic forms NH_4^+, NO_3^-, and PO_4^{3-}. The uptake of N from leucine, and P from ATP and dissolved DNA, was initially dominated by the 0.8–0.2 μm size fraction, but shifted towards dominance by the $>0.8\,\mu$m size fraction as the system turned to an increasing degree of N-deficiency. Normalizing uptake to biomass of phytoplankton and heterotrophic bacteria revealed that organisms in the 0.8–0.2 μm size fraction had higher specific affinity for leucine-N than those in the $>0.8\,\mu$m size fraction when N was deficient, whereas the opposite was the case for NH_4^+. There was no such difference regarding the specific affinity for P substrates. Since heterotrophic bacteria seem to acquire N from organic compounds like leucine more efficiently than phytoplankton, our results suggest different structuring of the microbial food chain in N-limited relative to P-limited environments.

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1 Introduction

Under conditions of mineral nutrient limitation, heterotrophic bacteria may compete with phytoplankton for inorganic nutrients (e.g. orthophosphate (PO_4^{3-}), ammonium (NH_4^+), and nitrate (NO_3^-)). The outcome of this competition potentially influences the carbon cycle both by heterotrophic microbes indirectly limiting primary production by depriving phytoplankton of nutrients (Joint et al., 2002), and by phytoplankton indirectly limiting the extent of heterotrophic degradation of organic material (Havskum et al., 2003). Understanding how competition, predation, and other trophic interactions structure the flows of C, N, P, and other elements through the microbial food web is central to our understanding of the role and function of this part of the pelagic ecosystem, both in a biological and in a biogeochemical context.

Competition between phytoplankton and bacteria potentially influences the species composition of the phytoplankton and bacterial communities (Jacobsen et al., 1995; Samuelsson et al., 2002). It may also affect the fundamental functioning of the microbial ecosystem by shifting the balance between phytoplankton and bacteria (Bratbak and Thingstad, 1985), as well as the bacterial degradation of organic matter (Pengerud et al., 1987; Grossart et al., 2006b). In principle, the competition between heterotrophic bacteria and phytoplankton for N and P may be different for the organic forms than it is for the inorganic ones. In addition,

the case of N- and P-limitation may be different. Bacteria have traditionally been expected to be more superior in competition for dissolved organic N (DON) (reviewed by Antia et al., 1991), than they are for dissolved organic P (DOP), where both phytoplankton and bacteria are known to produce enzymes, such as alkaline phosphatase, splitting orthophosphate off from the organic part before uptake (reviewed by Chróst, 1990). If this is correct, simple mathematical models suggest that the microbial part of the C-cycle may differ substantially between N- and P-limited systems (Thingstad, 2000).

Although heterotrophic uptake by bacteria has long been recognized as the major process removing DON (e.g. as amino acids (Paul, 1983; Billen, 1984)), uptake of amino acids and other DON-compounds by phytoplankton can occur under several environmental conditions (Pantoja and Lee, 1994; Palenik and Henson, 1997). Proteolytic activity has been found in association with eukaryote algae (Berges and Falkowski, 1996) and cyanobacteria (Martinez and Azam, 1993). Laboratory experiments leave no doubt that the majority of aquatic algal species are able to utilize common organic compounds as N sources for growth if sufficient substrate concentration is provided and enough time is allowed for metabolic adaptation (reviewed by Berman and Bronk, 2003). Most of these studies, however, used axenic laboratory batch cultures growing on high initial substrate concentrations, thus the ability of organisms to exploit the much lower concentrations of these compounds encountered in situ still remains unclear (Paul, 1983; Berman and Bronk, 2003).

This paper focuses on algal and bacterial uptake of different N- and P-substrates during an artificially induced phytoplankton bloom dominated by coccolithophorids, mainly *Emiliania huxleyi*. The experiment was done in a mesocosm set-up with a time dependent change from presumably C- or P-stressed bacteria and P-stressed phytoplankton (i.e.; non-N-limited), to N-limitation, at least of autotrophic processes. Bacterial and algal uptake of inorganic N (NH_4^+, NO_3^-) and of leucine as a model substrate for DON, as well as inorganic P (PO_4^{3-}) and the two organic forms ATP and dissolved DNA (dDNA) as model substrates for DOP was measured. Analyses of ^{15}N and ^{33}P uptake from these sources in algal and bacterial size fractions were performed in order to compare algal vs. bacterial competition for organic and inorganic dissolved N and P by means of their biomass-specific affinities. For a detailed description of the concept of biomass-specific affinity, and how it relates to the more familiar Michaelis-Menten parameters, see Thingstad and Rassoulzadegan (1999) and Løvdal et al. (2007).

2 Materials and methods

2.1 Experiment

An outdoor mesocosm experiment was carried out at the Marine Biological Field Station, Espeland, 20 km south of Bergen, western Norway from 4 to 24 May, 2003. The study was part of the Pelagic Ecosystem CO_2 Enrichment Study II (PeECE II). Experimental setup and sampling procedures are described elsewhere (Engel et al., 2005; Grossart et al., 2006a). The data referred to here were obtained from enclosure number 4 referred to as "present" in the paper of Grossart et al. (2006a) because it represented the present-day level of 370 ppmV atmospheric CO_2 concentration. The enclosure (volume $\sim$20 m^3) was filled with unfiltered, nutrient-poor, post-spring bloom sea water from the fjord and supplemented with 9 μmol L^{-1} NO_3^-, 0.5 μmol L^{-1} PO_4^{3-}, and 12 μmol L^{-1} $Si(OH)_4$ on day 0 of the experiment to induce a phytoplankton bloom.

Stormy weather from day 4 led eventually to a collapse of the enclosure on day 6. The enclosure was restored to an upright position, and 705 mg of KH_2PO_4 was added to the enclosure on day 7 to maintain the phytoplankton bloom. The addition of KH_2PO_4 would ideally correspond to a PO_4^{3-} enrichment corresponding to a concentration of 0.3 μmol L^{-1} PO_4^{3-}, but was in fact slightly higher since the enclosure was not entirely unfolded at the time. From day 10 onwards, the weather calmed down, and the enclosure was underlayered with an unknown volume of deep, high salinity water (with a soluble reactive phosphorus (SRP) concentration of 0.77 μmol L^{-1}) in order to unfold the enclosure after the storm.

2.2 Chemical analysis

SRP was measured using the molybdate blue method (Koroleff, 1983). Nitrate and nitrite (NO_x) were determined with a Skalar AutoAnalyser, based on the sulphanilamide colorimetric method (Grasshoff, 1983). Ammonium (NH_4^+) was analysed by the indophenol blue method according to Koroleff (1969). Dissolved free amino acids (DFAA) were analyzed by High Pressure Liquid Chromatography (HPLC) after ortho-phtaldialdehyde derivatization as described elsewhere (Grossart et al., 2006a).

2.3 Biomass estimations

Free and particle-associated bacteria were enumerated and their volumes calculated as described elsewhere (Grossart et al., 2006a). C biomass of bacteria was calculated assuming a ratio of C content to volume (V) of 397 fg C μm^{-3} for cells $<$0.1 μm^3 (Loferer-Krößbacher et al., 1998). For larger bacteria, an allometric conversion factor between V (in cubic micrometers) and C content (in femtograms) of $C=220\times V^{0.9}$ was applied. This factor is comparable to a

factor (C=218$\times V^{0.86}$) for both cultures and bacterioplankton from lakes of different trophic status (Loferer-Krößbacher et al., 1998); it is also similar to a factor (C=224$\times V^{0.89}$) for *Vibrio splendidus* grown along a gradient from C to P limitation (Løvdal et al., 2008).

Samples of phytoplankton were fixed with Lugol and cell counts were made under inverted light microscope (Utermöhl, 1958). Cell size was measured under the microscope, and cell volume was calculated using morphological-specific equations (Hillebrand et al., 1999). The C content (in picograms) of each cell was determined from V (in cubic micrometers) based on the equation (Menden-Deuer and Lessard, 2000):

$$\log\ \mathrm{C\ cell}^{-1} = \log a + (b \times \log V), \quad (1)$$

whith values for a and b (y-intercept and slope, respectively, for model I least-squares regression of $\log_{10}$-transformed data) in diatoms and dinoflagellates according to Table 4 in Menden-Deuer and Lessard (2000). Mean values from the literature were used as cellular C for cryptophyceae and coccolithophorids (Montagnes et al., 1994) and uncharacterized nanoflagellates (Moal et al., 1987). Phytoplankton were counted every second day, as were bacteria, but the two samplings were not synchronized. Thus, phytoplankton C biomass estimates were linearly interpolated.

2.4 Alkaline phosphatase activity (APA)

APA was measured fluorometrically using 3-0-methylfluorescein-phosphate (MFP) as substrate (Perry, 1972). Samples were mixed with MFP solution in 0.1 mol L^{-1} Trizma-HCl pH 8.3 (final concentration 0.1 μmol L^{-1}). Fluorescence was measured directly after the addition of the reagent and at two subsequent times according to the expected activity using a Perkin Elmer fluorometer LS50B. After correcting fluorescence values of samples to those of autoclaved samples used as blanks, APA (nmol-P L^{-1} h^{-1}) was calculated using linear regression of fluorescence values versus incubation time.

2.5 Uptake of ^{15}N-compounds

Water for use in ^{15}N uptake studies was collected with a tube covered with 18 μm mesh under stirring into thoroughly cleaned 1000 mL septum glass bottles. ^{15}N enriched NH_4Cl, $NaNO_3$, or L-leucine (>98 atom% ^{15}N; Larodan Fine Chemicals AB), respectively, in a concentration of about 10% of the ambient concentrations was added. Due to very low NH_4^+ concentrations, unlabelled NH_4Cl was routinely added to a final concentration of 0.5 μmol L^{-1} 1 to 4 h before tracer addition in NH_4^+ uptake studies, in order to get reliable time series. The precise portion of tracer added was calculated after determination of the ambient NH_4^+, NO_3^-, or DFAA (Grossart et al., 2006a) concentrations. The bottles were closed gas-tight and were incubated at in situ temperature and light. Four time points were taken within 3 to 5 h for each measurement. The reaction was terminated by filtering the samples through silver-membrane filters (Osmonics; pre treated 500°C for 1 h) with pore sizes of 0.2 and 0.8 μm. The filtration volume was 400 to 500 mL. Filters were flushed with 0.2 μm filtered sea water to remove adherent tracer-containing water and stored frozen until analysis.

After drying in a drying chamber, the filters were wrapped in silver cups (6×6×12 mm; Elementar Analysensysteme) and formed into pellets with a laboratory press. As the amount of N on the 0.2 μm filters was under the measurement limit, δ^{15}N values were measured together with a well defined N isotope standard (peptone; Merck). 1 μmol L^{-1} peptone was pipetted onto the 0.2 μm filters before packing and N isotopes were measured by continuous flow isotope ratio mass spectrometry (CF-IRMS) on a Finnigan MAT Delta *plus* coupled with a Thermo NA 2500 CHN analyser. The N content was calculated using acetanilide (Fisons) as a standard. The standard deviation of the δ^{15}N values measured for the standards was on average 0.15‰. The stable N isotope ratio was calculated in terms of δ^{15}N-values as follows:

$$\delta^{15}\mathrm{N} = \left(\frac{R_{\mathrm{sample}} - R_{\mathrm{standard}}}{R_{\mathrm{standard}}}\right) \times 1000\ \ [‰] \quad (2)$$

where $R = \frac{^{15}N}{^{14}N}$. The measured δ^{15}N values were converted to atom percent ^{15}N following the formula (Montoya et al., 1996):

$$\mathrm{atom\%\ ^{15}N} = 100 \times \left[\frac{(10^{-3} \times \delta^{15}\mathrm{N} + 1) \times (^{15}\mathrm{N}/^{14}\mathrm{N})_{\mathrm{atmosphere}}}{1 + (10^{-3} \times \delta^{15}\mathrm{N} + 1) \times (^{15}\mathrm{N}/^{14}\mathrm{N})_{\mathrm{atmosphere}}}\right] \quad (3)$$

with $\left(\frac{^{15}\mathrm{N}}{^{14}\mathrm{N}}\right)_{\mathrm{atmosphere}}$=0.003676 (Junk and Svec, 1958). Turnover times (T; see below) were estimated from the regression relationship between uptake of added ^{15}N and time.

2.6 ^{33}P-labelling of DNA

Radio-labelling of DNA was performed by random oligonucleotide primed synthesis (ROPS) with the DecaLabel DNA labeling kit (Fermentas K0621) in accordance with the manufacturer's instructions. [^{33}P]DNA was prepared for use in uptake studies as described by Løvdal et al. (2007). This procedure yields labelled DNA products with an average length of 0.5 kilobase pairs (kb) which is at the lower end of the range for naturally occurring dDNA (0.12–35.2 kb) in aquatic environments (DeFlaun et al., 1987). We did not correct for potential shortening of the DNA chain length by radiochemical decay, but the radio-labelled DNA was used well within one half life of the radioactive precursor in order to avoid significant shortening. The final product had a specific activity of approximately 10^8 counts per minute (cpm) μg^{-1} with 97.0±0.6% (mean ±SD of 3 replicates) incorporation of label and a concentration of 12.6±0.1 ng μL^{-1} (mean ±SD of 3 replicates) as calculated by the DE-81 filter-binding assay (Sambrook and Russell, 2001).

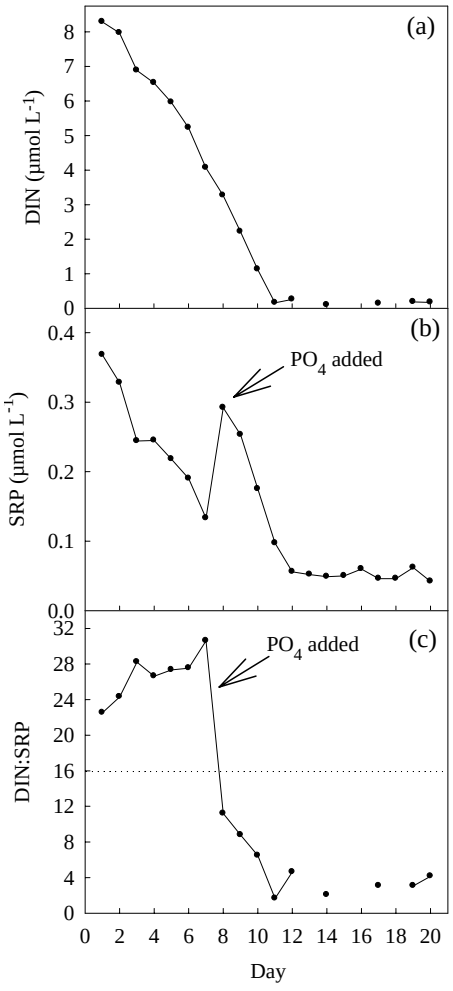

Fig. 1. Ambient concentrations of DIN **(a)**, SRP **(b)** and the DIN:SRP molar ratio **(c)** through the course of the experiment.

2.7 Uptake of ^{33}P-compounds

Uptake of $^{33}PO_4^{3-}$, AT^{33}P and [^{33}P]DNA was measured according to Thingstad et al. (1993), modified as described by Løvdal et al. (2007), except that the samples were not assayed for inorganic P released from organic substrates which was not taken up by the organisms. All incubations were done in 10 mL subsamples in 15 mL Falcon tubes at subdued light and 15.5±1.0°C. Samples were incubated according to the expected turnover time; for samples incubated with $^{33}PO_4^{3-}$, AT^{33}P and [^{33}P]DNA, the respective incubation times varied between 15 min and 1 h, 1–2 h, and 2–5 h, respectively. Incubations were stopped by cold chase (Løvdal et al., 2007). Bacteria and phytoplankton were separated into different size fractions by filtration onto polycarbonate filters (Poretics) with pore sizes of 0.2 and 0.8 μm according to Løvdal et al. (2007). Subsamples and filters were radioassayed by liquid scintillation counting (Løvdal et al., 2007).

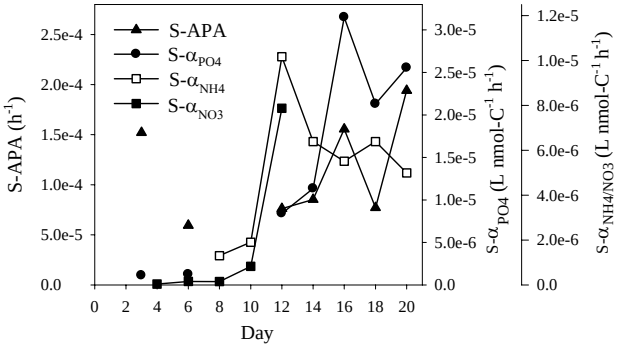

Fig. 2. APA and affinity for PO_4^{3-}, NO_3^- and NH_4^+ uptake, normalized for the summed C-biomass of phytoplankton and bacteria.

2.8 Estimation of turnover times and biomass-specific affinity

Turnover times (T; h) were calculated by the equation (Thingstad et al., 1993):

$$T = \frac{t}{-\ln(1-R)} \tag{4}$$

where R is the consumed fraction of added label. Hence, T represents here the turnover of substrates into particulate matter and does not include the release of hydrolysis products to the water phase. For samples incubated with ^{33}P compounds, t is the incubation time. For samples incubated with ^{15}N compounds, t is the incubation time + half the filtration time. This distinction was made because the uptake was not stopped with cold chase in samples incubated with ^{15}N compounds, but terminated by filtration. Thus, uptake will proceed during the filtration procedure until the whole sample volume has passed the filter (15–45 min). T for ^{33}P substrates was calculated from single time-point measurements (Thingstad et al., 1993), whereas T for ^{15}N substrates was calculated from the regression line between four time points.

Biomass-specific affinity for N (α_N) and P (α_P) uptake (L nmol-C^{-1} h^{-1}) was estimated according to the procedure proposed by Thingstad and Rassoulzadegan (1999):

$$\alpha = f/(TB) \tag{5}$$

where f is the fraction of uptake in the respective size fraction and B is the biomass-C. The biomass of particle-associated bacteria was attributed to the $>0.8\,\mu$m size fraction because they could be trapped on a 5 μm filter (Grossart et al., 2006a). Hence, due to the particle-associated bacteria, the mechanical separation of bacteria and phytoplankton by size fractionation was not entirely successful. In our estimates of biomass-specific affinity, this was corrected for by assuming that particle-associated bacteria had the same affinity for uptake as free bacteria. From this assumption, the

fraction of uptake by particle-associated bacteria was calculated by rearranging Eq. (5) to

$$f = \alpha T B \quad (6)$$

inserting the estimated biomass of particle associated bacteria. Corrected estimates corresponding to algae and bacteria were then calculated respectively by subtracting this value from the $>0.8\,\mu$m size fraction, and by attributing it to the 0.8–0.2 μm size fraction.

2.9 Statistical analysis

Statistical analysis was performed by student's t-tests according to Sokal and Rohlf (1995). The confidence level of all analyses was set at 95%.

3 Results

3.1 Nutrients, APA, and bloom development

Dissolved inorganic N (DIN), represented as the sum of NH_4^++NO_x was dominated by NO_x concentrations ranging from $8\,\mu$mol L^{-1} on day 1, to $4\,\mu$mol L^{-1} on day 7, before it rapidly declined to under the detection limit ($0.05\,\mu$mol L^{-1}) on day 13 (Fig. 1a), whereas NH_4^+ concentrations were always close to or below the detection limit ($0.1\,\mu$mol L^{-1}). SRP concentrations declined from approximately $0.4\,\mu$mol L^{-1} on day 1 to $\sim$0.04 μmol L^{-1} on day 20 (detection limit; $0.01\,\mu$mol L^{-1}) (Fig. 1b). Thus, the DIN:SRP ratio was well above the Redfield ratio of 16 prior to the second addition of PO_4^{3-} on day 7 before it rapidly declined (Fig. 1c). DFAA concentrations ranged between 0.4 and $1.6\,\mu$mol L^{-1}, with the lowest concentrations after the peak of the bloom (Grossart et al., 2006a).

APA ranged from 1 nmol-P L^{-1} h^{-1} on day 2 of the experiment, to 5 nmol-P L^{-1} h^{-1} on day 20. Specific values for APA (S-APA) and for phosphate affinity (S-α_{PO_4}) normalized with respect to the summed C-biomass of phytoplankton and bacteria are shown in Fig. 2. Both values increased after Day 8, but remained below 0.0002 h^{-1} and 0.00004 L nmol-C^{-1} h^{-1}, respectively. S-APA and S-α_{PO_4} above 0.2 h^{-1} and 0.02 L nmol-P^{-1} h^{-1} (i.e., normalized to P-biomass) may be used as indicators for P-limitation (Tanaka et al., 2006). Assuming, as a high estimate, a C:P molar ratio of 200 in algal + bacterial biomass, our data on S-APA and S-α_{PO_4} (Fig. 2) do not indicate severe P-limitation during any parts of the study period according to these criteria.

The algal bloom, almost exclusively dominated by coccolithophorids (mainly *Emiliania huxleyi*), was initiated by the addition of inorganic nutrients on day 0 and reached its maximum on day 11 (Fig. 3a) when the DIN:SRP ratio was $\sim$2. Highest numbers of total as well as free bacteria were recorded on day 8, when the DIN:SRP ratio was $\sim$11 and NO_x accounted for 99% of DIN, whereas highest

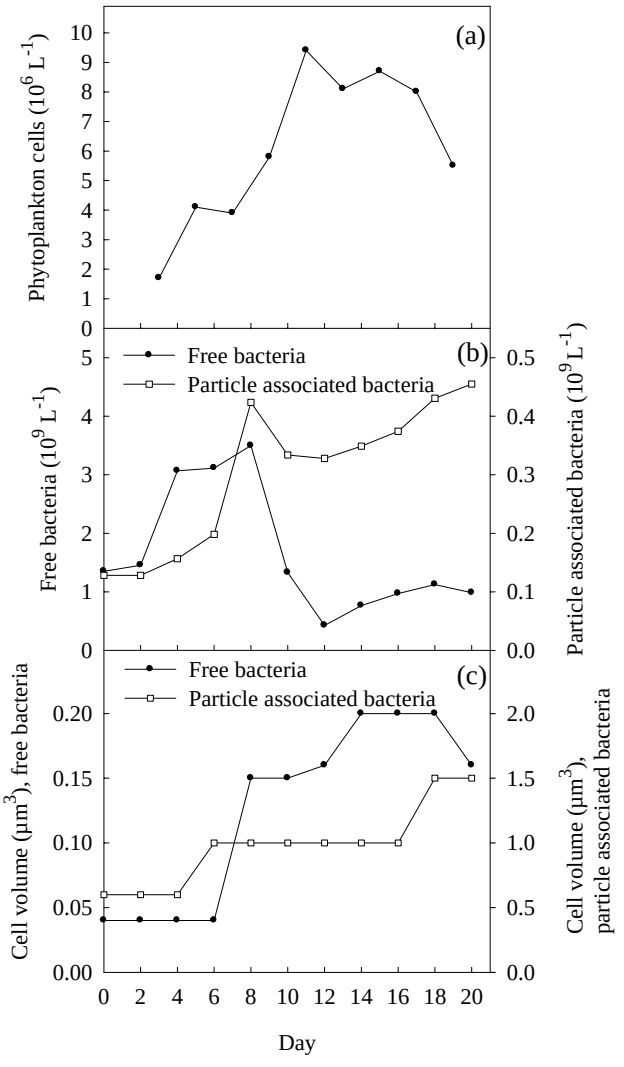

Fig. 3. Abundances of phytoplankton **(a)**. Abundances **(b)** and size **(c)** of free and particle associated bacteria. Data in (b) and (c) are from Grossart et al. (2006a).

numbers of particle associated bacteria were reached at day 20 (Fig. 3b). Particle-associated bacteria were significantly bigger than free bacteria, and increased from 0.6 to 1.5 μm^3 at the end of the experiment (Fig. 3c). Particle-associated bacteria contributed to 40–80% of the total bacterial volume (Grossart et al., 2006a). From our estimates, the contribution of particle-associated bacteria to the total C-biomass of algae and bacteria increased from $\sim$15% on day 0 to $\sim$50% on day 20.

3.2 Turnover times

During the first week of the experiment, T of all substrates were in general long and T of the organic N and P substrates measured were shorter than T of the respective inorganic substrates (Fig. 4). This may indicate C-stress of

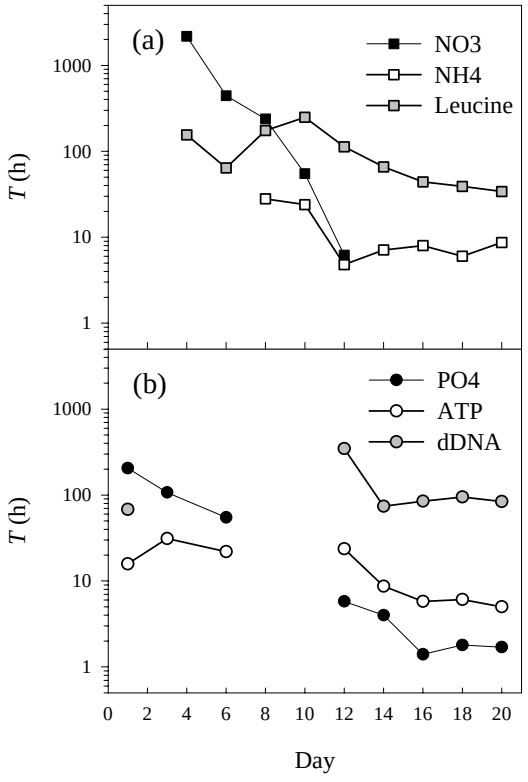

Fig. 4. Turnover times (logarithmic scale) of **(a)** N-substrates and **(b)** P-substrates.

heterotrophic processes in this phase (see discussion). The peak of the phytoplankton bloom (Fig. 3a) coincided with the shortest T_{NH_4} (4.8 h) and T_{NO_3} (6.2 h). From this point on, NH_4^+ and NO_x concentrations were both in the nanomolar range. In contrast to NO_x, which is formed by nitrification of NH_4^+, NH_4^+ is released by mineralization by protozoans, flagellates and zooplankton. As NH_4^+ is the preferentially regenerated form of N and taken up quickly, nitrification is supposed to be low under the given conditions. So, even though NH_4^+ may not be measurable, it can be presumed to be available for uptake. Therefore, T for $NH_4^+$was measured as the only DIN substrate. T_{NH_4} stayed stable below 10 h from day 12 onwards, whereas $T_{Leucine}$ decreased from >100 h to 34 h at the same time (Fig. 4a).

Despite high DIN:SRP ratios (Fig. 1c), which could have been interpreted to indicate phosphate limitation, T_{PO_4} was longer than T_{ATP} at the beginning of the experiment (Fig. 4b). Because P limitation was not expected immediately after the addition of PO_4^{3-} on day 7, T for P substrates was not measured between day 6 and day 12. On day 12, T_{PO_4} (5.8 h) was shorter than T_{ATP} and was comparable to T_{NH_4} and T_{NO_3}, before it reached a minimum of 1.4 h on day 16 (Fig. 4b). T_{ATP} and T_{dDNA} declined from 24 and 346 h, respectively, on day 12 before it stabilized at 6±2 h (mean ±SD, n=4) and 84±8 h (mean ±SD, n=4), respectively, for the rest of the study period (Fig. 4b).

3.3 Uptake distribution of ^{15}N- and ^{33}P-substrates and biomass-specific affinity

The distribution of added activity taken up by the two size fractions is shown in Fig. 5. The 0.8–0.2 μm size fraction took up most of the organic substrates during the first part of the study period, whereas the >0.8 μm size fraction dominated uptake of the inorganic substrates. In our set of data, both T_{NH_4} and T_{PO_4} dropped below 10 h at the peak of the phytoplankton bloom (Fig. 4). There are strong indications for a transition from a non-N-limited to an N-limited system at this point (see Discussion). Hence, we used T_{NH_4} as an indicator to split the dataset into non-N-limitation (T_{NH_4}>10 h) and N-limitation (T_{NH_4}<10 h) which also represents the periods before and after the peak of the phytoplankton bloom, respectively. After the peak of the phytoplankton bloom, the >0.8 μm size fraction dominated uptake of all substrates, and NH_4^+ uptake was almost exclusively by the >0.8 μm size fraction. There were small changes in the distribution of inorganic N and P compared to before the phytoplankton peak, with the >0.8 μm size fraction taking up slightly more after this peak (Fig. 5).

From DON (i.e. leucine-N) and DOP uptake, dominated by the 0.8–0.2 μm size fraction during T_{NH_4}>10 h, there was a shift towards DON and DOP uptake dominated by the >0.8 μm size fraction during T_{NH_4}<10 h (Fig. 5). There were no drastic shifts in the distribution of inorganic substrates, with the >0.8 μm size fraction dominating uptake in both periods, but more so during T_{NH_4}<10 h (Fig. 5).

S-α_{NO_3}, S-α_{NH_4}, and S-α_{PO_4} are specific affinities normalized for the summed C biomass, of algae and bacteria, and are shown in Fig. 2. Affinities for all substrates were low during the first half of the study period. Maximum values of S-α_{NH_4} paralleled the peak of the phytoplankton bloom, whereas S-α_{PO_4} peaked on day 16.

Comparing competitive ability of osmotrophs in the 0.8–0.2 μm and the >0.8 μm size fraction by means of biomass-specific affinity for N-uptake in the two size fractions, focusing on the N-limited phase (T_{NH_4}<10 h), reveals a significant shift depending on whether N is present in organic or inorganic form (Fig. 6). The >0.8 μm size fraction had a significantly higher biomass-specific affinity for NH_4^+ than the 0.8–0.2 μm size fraction (p=0.0005), and a significantly lower biomass-specific affinity for leucine-N than the 0.8–0.2 μm size fraction (p=0.038) (Fig. 6a, b). No such shift between the competition for DIP and DOP were observed; no significant differences were found between biomass-specific affinity in the two size fractions for PO_4^{3-} (p=0.314), ATP-P (p=0.429), or dDNA-P (p=0.245) (Fig. 6c–e).

From Eq. (6), it was estimated that much of the uptake in the >0.8 μm size fraction was by particle-associated bacteria. Particle-associated bacteria took up larger shares of DON compared to DIN (Table 1). In fact, it was estimated that the majority (63±19%, n=5) of total DON uptake was by particle-associated bacteria during N-limitation

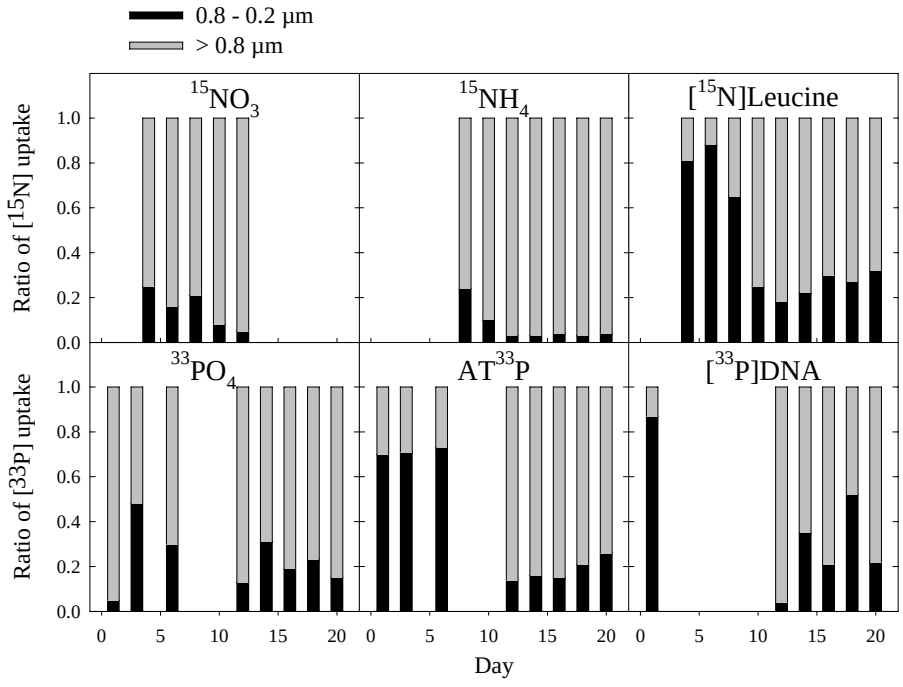

Fig. 5. Relative proportions of incorporated label in the two size fractions.

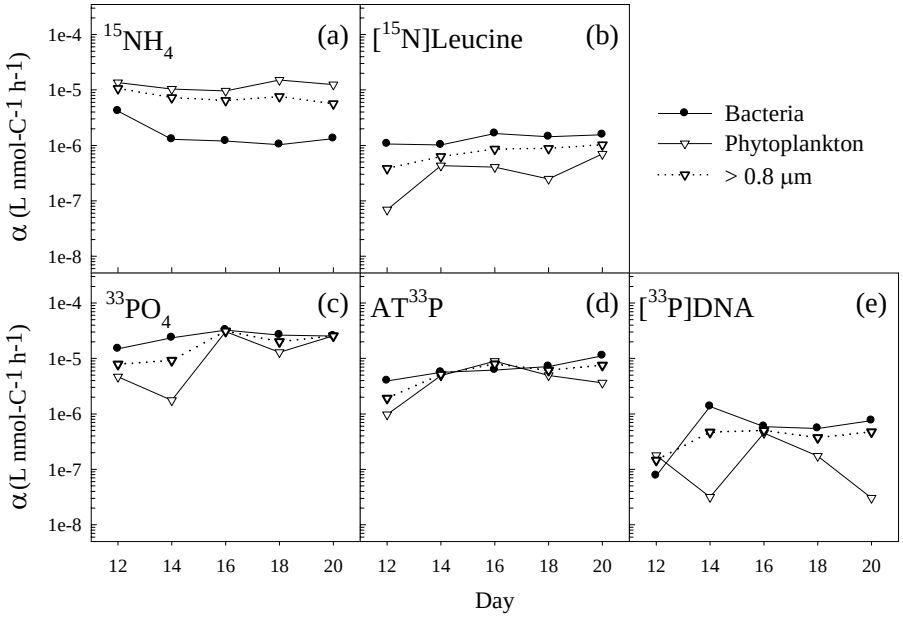

Fig. 6. Biomass-specific affinity (logarithmic scale) for the uptake of NH_4^+ **(a)**, Leucine-N **(b)**, PO_4^{3-} **(c)**, ATP-P **(d)**, and dDNA-P **(e)** in the $>0.8\,\mu$m size fraction (dotted line), and in bacteria and phytoplankton after correcting for particle-associated bacteria (solid lines) during the N-limited phase. Note that corrected "bacteria" equal the 0.8–0.2 μm size fraction. See text for details.

($T_{NH_4}<10$ h), leaving phytoplankton with ~20% (Table 1), and thus free-living bacteria with approximately 15% of total DON uptake. Hence, from the corrected estimates, phytoplankton had a significantly lower biomass-specific affinity for DON than heterotrophic bacteria (p=0.0003) (Fig. 6b) but a significantly higher biomass-specific affinity for NH_4^+ (p=0.0002) (Fig. 6a). No shift between the competition for DIP and DOP were observed; no significant differences were

Table 1. Uptake of labelled substrates by particle-associated bacteria and phytoplankton, given as percent of total uptake. Estimates from Eq. (6) assuming similar affinity in particle-associated bacteria as in free-living bacteria (see text). Pooled data for inorganic and organic N and P substrates, grouped in samples with T_{NH_4} longer than and shorter than 10 h. Means with standard deviation.

Substrate	$T_{NH_4}>10$ h		$T_{NH_4}<10$ h	
	particle-associated bacteria	phytoplankton	particle-associated bacteria	phytoplankton
DIN	13±2	72±9	11±5	85±6
DON	37±4	21±28	63±19	19±10
DIP	19±13	53±34	50±11	30±15
DOP	42±5	1±1	50±24	33±25

found between biomass-specific affinity in algae and bacteria for PO_4^{3-} (p=0.175), ATP-P (p=0.263), or dDNA-P (p=0.056) (Fig. 6c–e).

4 Discussion

It has been hypothesized that phosphate limitation, classically indicated by DIN:DIP ratios >16, is one of the critical factors allowing the coccolithophore *Emiliania huxleyi* to bloom (reviewed by Lessard et al., 2005). This hypothesis is based on physiological studies showing that *E. huxleyi* has an exceptionally high affinity for PO_4^{3-} and is able to use DOP (Kuenzler and Perras, 1965; Riegman et al., 2000). High NO_3^-:PO_4^{3-} ratios, however, appear to be the exception rather than the rule in *E. huxleyi* blooms. In fact, in most blooms studied to date, NO_3^-:PO_4^{3-} ratios were low, and nitrate was low or undetectable (Lessard et al., 2005). In the current experiment, the addition of NO_3^- and PO_4^{3-} in a molar ratio of 18 initiated a bloom of coccolithophorids, dominated by *E. huxleyi*, reaching its maximum after 11 days when the DIN:SRP ratio was ∼2. The first half of the period prior to the peak of the bloom was characterized by high DIN:SRP ratios (Fig. 1c), thus the phytoplankton growth may be suspected to have been restricted by the availability of inorganic P. Low S-APA and low S-α_{PO_4}, S-$\alpha_{NO_3}$and S-α_{NH_4} values (Fig. 2), however, does not indicate severe mineral nutrient limitation, and T of the organic N and P substrates measured where shorter than that of the respective inorganic substrates (Fig. 4), possibly indicating C-stress of heterotrophic processes in this phase. Bacteria hydrolyze specific DON and DOP-compounds in the presence of inorganic mineral nutrients, presumably to obtain other associated mineral nutrients and C (Jørgensen et al., 1993; Benitez-Nelson and Buesseler, 1999). DIN:SRP ratios rapidly declined from day 7 (Fig. 1) due to the PO_4^{3-} enrichment. However, the additional PO_4^{3-} was rapidly consumed followed by increasing values of S-APA and S-α_{PO_4} (Fig. 2). S-α_{NO_3} and S-α_{NH_4} values rapidly increased from day 8 with S-α_{NH_4} peaking at day 12 (Fig. 2). Additionally, T_{NO_3} and T_{NH_4} became shorter as the experiment proceeded, with T_{NH_4} reaching minimum values at day 12 before stabilizing, and $T_{Leucine}$ declining from day 10 to the end (Fig. 4). Conclusively, the splitting of the investigation period into a non-N-limited and an N-limited phase can be justified.

4.1 Methodological considerations

We acknowledge that the biomass-specific affinity estimated for N-substrates is low compared to what one would expect during N-limitation (Fig. 7a, b). The reason for this is unknown. One possibility, contradictory to our previous conclusion, could be that the growth rate of heterotrophic bacteria was limited by C or other factors, rather than N. Another possibility could be artefacts in our estimation procedure. However, the main reason that biomass-specific affinities for N-compounds, both in bacteria and algae, seem unrealistically low for a presumably N-stressed system, compared to that of P-compounds, stems probably from a methodological limitation. Although the continuous flow isotope ratio mass spectrometry (CF-IRMS) is very sensitive, it requires a certain amount of N (about 10 μg). Hence, following long filtration time due to relatively large sample volumes, the time points between two filtrations have to be distinct enough to get over uncertainties due to background noise. Additionally, substrate concentrations must be high enough to avoid exhaustion of substrate in the bottles during the incubation time. Thus, this method is not optimal in environmental labelling studies when the ambient turnover time is extremely short and the substrate concentration is low. In our experiment, usually below 10% of ambient concentrations of tracer ^{15}N isotope was added. In the case of NH_4^+ uptake studies, additional unlabelled NH_4Cl had to be added to the incubation bottles for two reasons. First, it is not possible to calculate the correct tracer amount of label to add when the ambient substrate concentration is unknown (e.g., under the detection limit). Second, NH_4^+ is formed by mineralization. Hence, under very low concentrations, the amount of NH_4^+ regenerated during the experiment could be too high in comparison to the amount of NH_4^+ in the water, and thus, could not be neglected when calculating the amount of tracer to add. During the N-limited phase, the enrichment

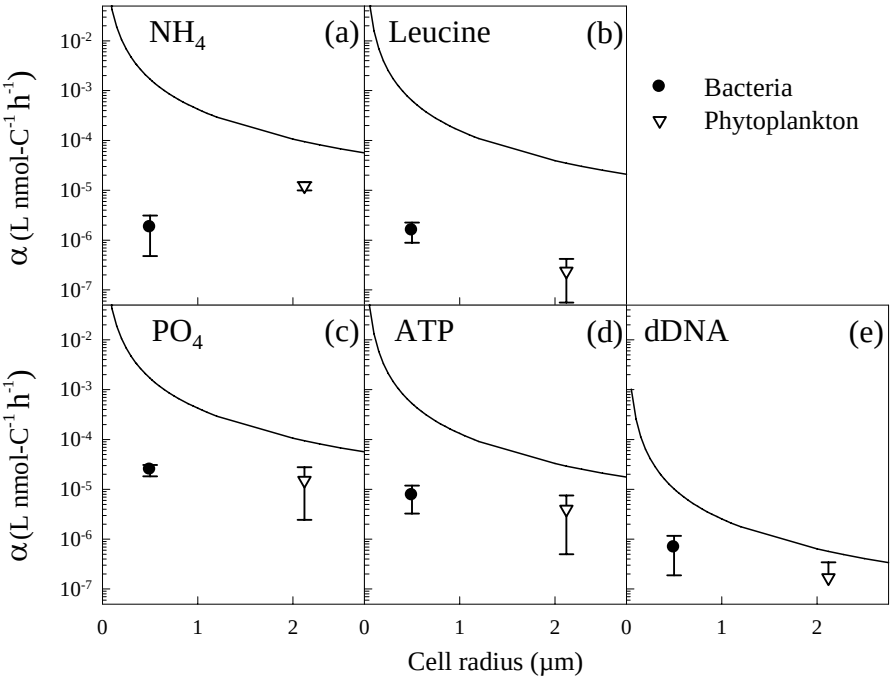

Fig. 7. Comparison of the mean estimated biomass-specific affinity values during the N-limited phase (±SD, n=5) of phytoplankton and bacteria vs. the theoretical maximum affinity by the diffusion model. The assumption for the diffusion model is that the diffusion constant (D) for small molecules like NH_4^+ **(a)** and PO_4^{3-} **(c)** is 10^{-5} cm^2 s^{-1}, D for leucine **(b)** is 3.7×10^{-6} cm^2 s^{-1} (Nimer et al., 2003), D for ATP **(d)** is 3.0×10^{-6} cm^2 s^{-1} (Diehl et al., 1991), and D for DNA **(e)** is 4.9×10^{-6} cm^2 s$^{-1}\times$(basepair size)$^{-0.72}$ (Lukacs et al., 2000). The solid curves denote the theoretical maximum for bacteria and phytoplankton, assuming the cell density is 1.2 g cm^{-3}, dry weight is 50% of wet weight, and C weight is 50% of dry weight.

of unlabelled NH_4^+ could increase the DIN:SRP ratio in the incubation bottles by a factor of up to 10 compared to the ambient concentrations (Fig. 1c), but the DIN:SRP ratio in the incubation bottles was still below 16. We acknowledge that this treatment presumably relaxed the N-limitation in these bottles, which led to significant overestimates of the ambient NH_4^+ turnover times (Fig. 4a). Additions of ^{15}N-leucine were based on measurements of ambient DFAA concentrations. Most likely, the DFAA pool measured chemically is larger than that utilized by osmotrophs since not all parts of the DFAA pool are equally energetically expensive to incorporate. We acknowledge that $T_{[leucine]}$ and affinity$_{[leucine]}$ values reported here are estimates of the ambient pool of enzymatically hydrolysable free leucine, and not all components of the DON pool. Hence, ^{15}N enrichment presumably exceeded that typically regarded as true tracer levels, leading to an overestimate of turnover times, and hence underestimates of biomass-specific affinities for N-compounds (Fig. 6a, b).

The liquid scintillation counting technique on the other hand, applied for ^{33}P uptake studies, is not hampered with this problem because the high specific activity of ^{33}P allows for true tracer level enrichment, e.g. picomolar concentrations.

Although this problem with the CF-IRMS technique can be expected to have given significant underestimates of biomass-specific affinity, we believe that it did not affect the relative distribution of N-substrates in the two size fractions considerably. Thus, qualitative differences between the 0.8–0.2 μm and the $>0.8\,\mu$m size fractions were attributed to the differences between bacterial and algal metabolism.

4.2 Algal – bacterial competition; implications for the microbial food web

From our data, there is evidence for a shift in terms of algal – bacterial competition when N is available in the form of leucine contrary to NH_4^+ (Fig. 6), with organisms in the $>0.8\,\mu$m size fraction having significantly lower biomass-specific affinity for leucine-N and significantly higher biomass-specific affinity for NH_4^+ compared to smaller organisms (0.8–0.2 μm). Correcting for the contribution of particle-associated bacteria in the $>0.8\,\mu$m size fraction by assuming they have similar affinity per biomass as free-living bacteria, probably represents a best estimate for the competition between algae and heterotrophic bacteria. From theoretical arguments linking cell size, shape, and elemental stoichiometry to uptake efficiency (Løvdal et al., 2008), we calculated the biomass-specific affinity in

the large, particle-associated bacteria (assumed to be rod-shaped) to be comparable to that in the smaller, free-living bacteria (assumed to be coccoid), given the bacterial C biomass estimated, and assuming diffusion as the rate limiting step for uptake, and that the active fraction of bacteria is the same in the two compartments. However, since specific aminopeptidase (Karner and Herndl, 1992; Middelboe et al., 1995), protease (Becquevort et al., 1998) and phosphatase (Smith et al., 1992; Simon et al., 2002) activities in particle-associated bacteria are often higher than those characteristic for free-living bacteria, our affinity values for heterotrophic bacteria may have been underestimated, and the values for phytoplankton correspondingly overestimated.

In a previous investigation in P-limited estuarine mesocosms, Løvdal et al. (2007) found no significant shift in algal – bacterial competition for P from ATP and dDNA relative to PO_4^{3-}, in terms of biomass-specific affinity. In the current experiment, using comparable methodology, a shift from bacteria dominating the competition for DON towards algae dominating the competition for DIN was evident (Fig. 6). This does indeed indicate that the structure of the microbial food web in N-limited environments may be different from that in P-limited environments. If amino acids are utilized almost exclusively by bacteria, this changes the food web structure relative to P-limited environments, since there will be a pool of dissolved N for which bacteria do not experience significant competition from phytoplankton to acquire. One effect of this is, theoretically, that the probability for C-limitation of bacteria could be larger in N-deficient regions than in P-deficient regions (Thingstad, 2000).

E. huxleyi has been shown to be a moderate competitor for nitrate and a good competitor for phosphate compared to other algal species (Riegman et al., 1992; Riegman et al., 2000). During natural *E. huxleyi* blooms, *E. huxleyi* primarily use NH_4^+ and urea (Kristiansen et al., 1994; Fernández et al., 1996; Rees et al., 2002). The high P acquisition capacity and ability to use non-nitrate N has been suggested (Lessard et al., 2005), at least in part, to explain the success of *E. huxleyi* in nutrient-depleted waters. This is supported by our data because the biomass-specific affinity for all P-substrates in *E. huxleyi* was comparable to that in heterotrophic bacteria, whereas *E. huxleyi*, although they seemed to have low amino acid-N acquisition capacity, dominated the competition for NH_4^+. Our results also agree with previous findings that DFAA are an important N source to marine bacteria (Hollibaugh and Azam, 1983; Jørgensen et al., 1993). Concentrations of dissolved combined amino acids (DCAA) ranged between 2 and 10 μmol L^{-1} with maxima on day 8 (Grossart et al., 2006a). DCAA are potentially important N sources, and the hydrolysis of the DCAA pool may substantially have increased the bacteria's ability to utilize DFAA (including leucine), and hence, N from the amino acids.

4.3 Experimental affinity estimates compared to the theoretical maxima

The biomass-specific affinity values estimated in the present study can be compared with those given by diffusion limitation of substrate transport to the cell surface – that is, the theoretical maximum. Assuming that the cell is diffusion-limited, i.e., that the cell's uptake system is so efficient (and the bulk nutrient concentration so low) that all substrate molecules hitting the cell surface are captured; it is possible to derive a theoretical expression for maximum biomass-specific affinity (α_{max}) for a spherical cell (Fig. 7; see also Thingstad and Rassoulzadegan, 1999):

$$\alpha_{max} = 3D/(\sigma r^2) \quad (7)$$

where D is the diffusion constant for the substrate molecules and σ is the volume-specific content of the element in question. Assuming free-living and particle-associated bacteria to have volumes of 0.2 and 1.5 μm^3, respectively, and particle-associated bacteria to make up 30% of the total bacterial community (cf. Grossart et al., 2006a), their mean equivalent r can be calculated to be $\sim$0.5 μm. Assuming that the phytoplankton community is dominated by N-limited *E. huxleyi* with volumes of $\sim$40 μm^3 (cf. Riegman et al., 2000), their mean r may be set to 2.1 μm.

The mean biomass-specific affinity for NH_4^+ uptake in phytoplankton was higher than that for bacteria, approaching that predicted by the diffusion model (Fig. 7a), contrary to that estimated for leucine (Fig. 7b). Although *E. huxleyi* is reported to grow fairly on leucine as the sole N-source (Ietswaart et al., 1994), our data suggest that the natural amount of leucine does not serve as an important N-source for *E. huxleyi* in our study. One should however keep in mind that genetic and physiological intraspecific diversity in *E. huxleyi* has been documented, and multiple strains can be present within an environment (Paasche, 2002), complicating comparison between laboratory and field studies.

The model for diffusion limited uptake may serve as a theoretical prediction for algal – bacterial N and P competition as illustrated in Figure 7. The model indicates that bacteria should be superior competitors to phytoplankton because of the difference in size. Changes in the surrounding medium are however accompanied by changes in size and elemental stoichiometry of algal and bacterial biomass, complicating the theoretical prediction (Thingstad et al., 2005; Løvdal et al., 2008).

DNA and ATP contain 16% and 14% N, and 10% and 18% P, respectively. Hence, they contain as much N per dry weight as amino acids of algal proteins (Laws, 1991). Therefore these substrates may serve as potential N and P sources at the same time. The high biomass-specific affinities for dDNA and ATP, considering their diffusion constants, compared to PO_4^{3-} (Fig. 7c–e), may indicate that these substrates were hydrolyzed for their N content rather than their P content. dDNA is mainly utilized as a P source by

marine bacteria, and accounts <10% of their N and C requirements (Jørgensen et al., 1993; Jørgensen and Jacobsen, 1996). The possibility then exists, that organic compounds, such as dDNA, are more bioavailable to phytoplankton than DON-compounds, such as amino acids, and are thus utilized as alternative sources for N by phytoplankton when the ambient DIN-concentrations are too low to support their growth. The potential for dDNA and ATP to also have rapid turnover times in non-P-limited environments, and to substantially support algal N-demand, indicates that phytoplankton may rely on these compounds to support their N-demand, rather than those traditionally looked upon as important DON-sources. Yet, isotope P-labelled substrates may yield limited information about N uptake. Therefore, an N-isotope is preferable in assessing phytoplankton dDNA and ATP utilization.

4.4 Concluding remarks

The main conclusion of our study is that the competitive fitness of bacteria and algae for nutrients (and particularly N) vary dramatically depending on whether the substrates are organic or inorganic. Whereas bacteria appear to be superior competitors for organic forms of N, phytoplankton appear to be superior competitors for inorganic N supplied as NH_4^+. This observation has major implications for our understanding of marine food webs and effects of nutrient status on community structure.

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