

# **Competition between marine osmotrophs for phosphorus and nitrogen;**

*Implications for the microplanktonic food web*

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**Til Ole Mathias**

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## PAPERS



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Trond Løvdal

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## List of papers

This thesis is based on the following papers and referred to in the text by their Roman numerals.

- I. Algal – bacterial competition for phosphorus from dissolved DNA, ATP and orthophosphate in a mesocosm experiment.** Trond Løvdal, Tsuneo Tanaka, and T. Frede Thingstad. (Accepted for publication in *Limnology and Oceanography*, 2006).
  
- II. Competition for inorganic and organic forms of nitrogen and phosphorus between phytoplankton and bacteria during an *Emiliana huxleyi* spring bloom.** Trond Løvdal, Christiane Moros, Hans-Peter Grossart, Vincent Carboneel, Lei Chou, and T. Frede Thingstad. (Manuscript).
  
- III. Use of non-limiting substrates to increase size; a generic strategy to simultaneously optimize uptake and minimize predation in pelagic osmotrophs?** T. Frede Thingstad, Lise Øvreås, Jorun K. Egge, Trond Løvdal, and Mikal Heldal. *Ecology Letters* (2005), 8: 675-682
  
- IV. Changes in morphology and elemental composition of *Vibrio splendidus* along a gradient from carbon-limited to phosphate-limited growth.** Trond Løvdal, Evy F. Skjoldal, Mikal Heldal, Svein Norland, and T. Frede Thingstad. (Submitted to *Microbial Ecology*, 2006).

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## Summary

Understanding how competition for nutrients structures the flows of C, N, P, and other elements through the microbial food web may seem central to our understanding of the role and function of this part of aquatic ecosystems, both in biological and biogeochemical contexts. Competition between phytoplankton and heterotrophic bacteria potentially influences the species composition of the respective communities and the flow of carbon (C) and energy to higher trophic levels. The dominating hypotheses on algal – bacterial competition for inorganic substrates are based on studies from freshwater environments, there is relatively little work on the competition for organic substrates, and previous studies on this topic may seem contradictory. This thesis includes studies performed in nutrient manipulated coastal mesocosms where the algal – bacterial competition for organic and inorganic forms of P and N were assessed. Algal – bacterial competition for these substrates was compared by means of biomass-specific affinity estimates. Biomass-specific affinity is regarded as the best index to measure competitive ability. However, we are not aware of any published papers reporting such data on organic P- and N-compounds. Results from two mesocosm studies, one focusing on P-competition and the other focusing on N-competition, demonstrates a potential for different structuring of the microbial food web in P- versus N-limited environments. One paper considers possible theoretical solutions to the coexistence of organisms competing for the same limiting nutrient. Traditionally, the smallest organisms have been viewed as superior competitors, based on a relatively constant relationship between volume and the intracellular content of the limiting element. However, the paper points out strategies in which organisms can benefit from other resources to change their stoichiometry and thus obtain competitive advantages, as well as predator defense. Experimental evidence to support this theory was obtained from a chemostat experiment. The results demonstrated that *Vibrio splendidus* could use excess organic C (glucose) to increase in size thereby optimizing uptake of the limiting element (P).

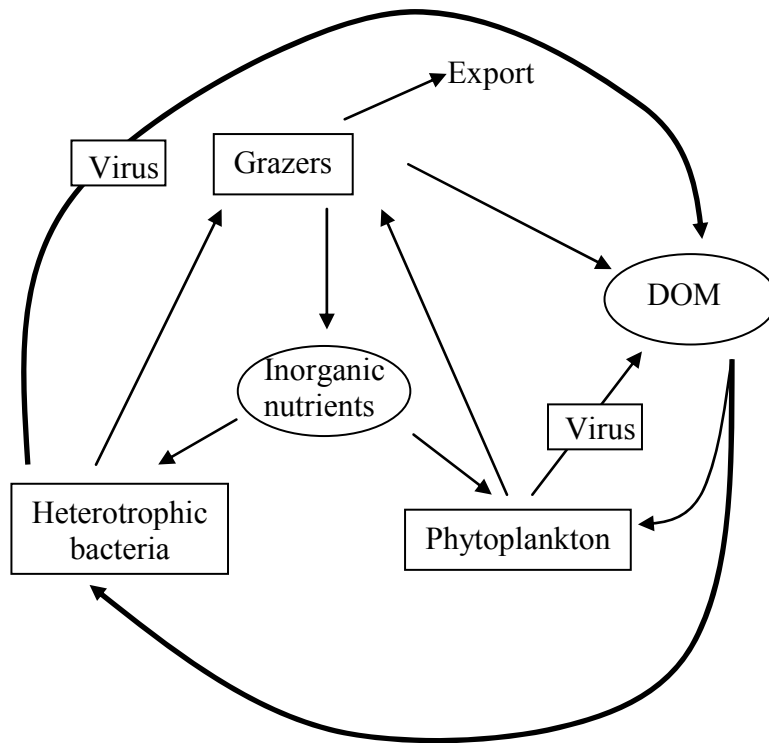


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## Introduction

As osmotrophic organisms, both bacteria and phytoplankton take up mineral nutrients through transport proteins located in their cell walls. Because of restricted permeability of the phytoplankton plasma-membrane and the bacterial cytoplasmic membrane, polymeric or phosphorylated organic compounds must be hydrolyzed before uptake in order to be available for osmotrophic organisms (for reviews, see Paul 1983; Cembella et al. 1984; Nikaido and Vaara 1985; Ammerman 1991; Berman and Bronk 2003; Hoppe 2003). Extracellular enzymes produced by osmotrophs hydrolyze substrates external to the cell, and are either excreted to the water-phase by the organisms or bound to the cell membrane (Wetzel 1991). The released molecule may thus be intermediately mixed into the background ambient pool of free inorganic mineral nutrients, or be physically connected to the cell before its uptake, potentially shifting competition to favour those organisms that possess membrane-bound enzymes. The hydrolysis of inorganic molecules from organic or other complex compounds, soluble or particulate, in which the hydrolyzed inorganic molecule is released outside the cell, is often referred to as regeneration.

Until 1983, when Azam *et al.* formalized the concept of the “microbial loop”, bacteria were regarded as remineralizers, i.e. responsible of transforming organic material to inorganic, thus recycling nutrients to the primary producers. The basis for the “microbial loop” (Figure 1) is that large amounts of dissolved organic extracellular products are produced by prokaryotes and small eukaryotes. The released energy, in the form of dissolved organic matter (DOM), is returned to the main food chain through the microbial loop by bacteria, flagellates and ciliates. Bacteria, excreting minerals and respiring carbon (C), have the advantage of a large surface to volume ratio and compete efficiently with phytoplankton for the mineral nutrients. The competition is influenced by flagellates controlling bacterial abundance. Heterotrophic flagellates and ciliates then remains as remineralizers; bacteria and phytoplankton as consumers (Azam et al. 1983).



**Figure 1:** A simplified diagram of an aquatic food web emphasizing the microbial loop connecting heterotrophic bacteria, phytoplankton, grazers, viruses and DOM. Osmotrophs both take up and excrete inorganic nutrients, thus arrows between these indicate net flow.

The dynamic behaviour of the “microbial loop” depends on the interacting ecological relationships of commensalism, competition, predation (Azam et al. 1983) and parasitism in the sense of bacterial (Bergh et al. 1989) and algal (Bratbak et al. 1993) virus infection. Competition for mineral nutrients is found between phytoplankton and bacteria and is influenced by predation and parasitism. Mineral nutrient limitation seems to stimulate phytoplankton excretion of extracellular organic C (EOC). Bacterial growth on EOC requires additional uptake of mineral nutrients. Commensalism thus occurs in the production of EOC by phytoplankton and utilization by bacteria. This competition – commensalism relationship between algae and bacteria is known as the ‘phytoplankton-bacteria paradox’ (Bratbak and Thingstad 1985) because phytoplankton stimulate their competitors to take up the lacking nutrients. The regeneration of mineral nutrients resulting from predation and

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parasitism will provide a feedback of some of the material flows within the microbial loop. The net effect of the loop is to convert organic material to dissolved inorganic nutrients. However, the flux patterns are a result of intricate interactions of a diverse biota with a complex pool of organic matter. Thus, the classical version of the microbial loop (Figure 1) is a conceptual model simplified to emphasize the major path for DOM in the pelagic food web. But, as pointed out by Azam (1998), this approach should serve as a unifying theme and a framework to understand the maintenance of microbial diversity. Nevertheless, as predicted by Azam in the same paper: “It now seems that things will get even more complicated before they get simpler” .....

While phytoplankton acquires inorganic C while obtaining energy from light, heterotrophic bacteria get these from organic material. However, since the DOM-pool is very complex and poorly characterized, little is known about the utilization of various components of the DOM-pool. In principle, the algal – bacterial competition for major nutrients as dissolved organic phosphorus (DOP) and dissolved organic nitrogen (DON) may be shifted relative to the competition for the inorganic forms. This competition is to a large extent dependant upon the enzyme apparatus of the organisms. The competition for DOP, where both bacteria and algae are known to produce enzymes for the utilization of phosphorus (P) from organic substrates (Chróst 1990; Hoppe 2003), may be shifted relative to the competition for DON, where bacteria are traditionally expected to be more superior in the competition for nitrogen (N) (e.g. amino acid-N; Paul 1983; Berman and Bronk 2003). The principal questions of how uptake of P and N from the DOM-pool (Figure 1) is distributed between bacteria and phytoplankton, and which mechanisms regulate this distribution, is however unresolved (cf. Flaten et al. 2005; Andersson et al. 2006). In order to make reliable conceptual and mathematical models of the marine ecosystem, we need answers to these questions.

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## Scope of the study

The purpose of this study was to find answers to the questions:

- (1) Is the uptake distribution of, and hence the algal – bacterial competition for, organic dissolved P and N between phytoplankton and bacteria different from the distribution of the inorganic dissolved forms?
- (2) Does algal – bacterial competition for P in P-deficient systems differ from the algal – bacterial competition for N in N-deficient systems, i.e. is the answer to (1) different in the P and N cases?

**Paper I** and **Paper II** relate to the algal – bacterial competition for the potentially limiting nutrients P and N. The algal – bacterial competition for dissolved inorganic P (DIP; e.g. orthophosphate ( $\text{PO}_4^{3-}$ )) and DOP (e.g. ATP and dissolved DNA (dDNA)) was studied in mesocosms manipulated to varying degrees of P-deficiency and organic-C status, situated off the southwest coast of Finland in the Baltic Sea (**Paper I**). The algal – bacterial competition for dissolved inorganic N (DIN; e.g. ammonium ( $\text{NH}_4^+$ ) and nitrate ( $\text{NO}_3^-$ )) and DON (e.g. leucine), as well as the competition for DOP and DIP, was studied in a mesocosm manipulated to an increasing degree of N-deficiency, situated in a western Norwegian fjord (**Paper II**).

Two papers address related topics such as life strategies to optimize uptake and minimize predation in pelagic osmotrophs, with particular interest on the effect of morphology and intracellular elemental composition on the uptake of limiting nutrients in heterotrophic bacteria. **Paper III** offers theoretical solutions to the coexistence of osmotrophs competing for one common limiting nutrient, focussing on different life strategies of competition specialized and defence specialized organisms. A laboratory study offering some experimental support for the strategy hypothesized in **Paper III** is presented in **Paper IV**.



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## **Uptake of phosphorus and nitrogen; - algal – bacterial competition**

Through the normal processes of cell growth, exudation, and cell death by the combined contributions of autolysis, viral infection, and grazing, there is a continual production of dissolved nutrients, especially in the euphotic zone of the ocean. P and N are essential mineral nutrients known to limit the growth of osmotrophs in much of the world's oceans and lakes. The inorganic forms, as orthophosphate (Björkman and Karl 1994; **Paper I**) and ammonium (Veuger et al. 2004; **Paper II**), are the preferred sources of P and N, respectively, in marine osmotrophs. The bulk of the organic P (Cembella et al. 1984; Chróst 1990) and N (Paul 1983; Berman and Bronk 2003) resources require enzymatic hydrolysis prior to uptake by osmotrophs. Thus, when the inorganic forms are depleted from the environment, the nutrient acquisition capacity, and hence, the competitive ability of the organisms, is largely determined by their potential of enzymatic dissolution of organic matter. Because substrate molecules in nature generally occur at very low concentrations, the substrate uptake and growth of osmotrophs may also be limited by diffusion transport towards the cell rather than by physiological constraints. Diffusion theory then states that the competitive ability is determined by the size and shape of the cell, and the internal concentration of the limiting element.

### Utilization of dissolved P

Orthophosphate ( $\text{PO}_4^{3-}$ ) represents less than 25% of the total dissolved P-pool in marine surface waters (Karl and Yanagi 1997). The remaining part of the P-pool is not yet extensively chemically characterized, but is dominated by low molecular weight (<10 kilo Dalton (kDa)) DOP in surface waters (Ridal and Moore 1990; Suzumura et al. 1998) and is believed to mainly consist of P esters (C-O-P bond structures including phosphoproteins, sugar phosphates, nucleotide phosphates and nucleic acids), phosphonates, and perhaps smaller amounts of pyrophosphates, inorganic polyphosphates and other inorganic derivatives (Karl and Yanagi 1997).  $\text{PO}_4^{3-}$  is considered the form of P preferentially utilized by both bacteria and algae,

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but the available fraction of the DOP-pool is also actively utilized (Bentzen et al. 1992; **Paper I**).

Regeneration of  $\text{PO}_4^{3-}$  from organic compounds is a result of hydrolytic degradation by free and cell-surface bound enzymes of phytoplankton and bacteria. In the marine environment, alkaline phosphatase (AP) and 5'-nucleotidase (5PN) are the enzymes which contribute most significantly to  $\text{PO}_4^{3-}$  regeneration (Cembella et al. 1984; Ammerman and Azam 1985). AP activity (APA) is sensitive to low  $\text{PO}_4^{3-}$  concentrations, and is therefore often used as an indicator for phosphate limitation (Hoppe 2003). AP, which is specific for the monophosphate ester bond, is found in highly variable amounts, in both bacterial and algal size fractions, as well as in the free dissolved state (Hoppe 2003). Studies in estuarine and marine environments have shown that the activity of the membrane bound 5PN, which hydrolyzes 5'-nucleotides and regenerates  $\text{PO}_4^{3-}$ , is usually concentrated in the bacterial size fraction (Ammerman and Azam 1985; Ammerman and Azam 1991a; Siuda and Güde 1994). This corresponds to the findings that 5'-nucleotides and dissolved DNA (dDNA) are taken up primarily by bacteria in marine (Paul et al. 1987; Turk et al. 1992) and freshwater environments (Siuda and Güde 1996; Siuda et al. 1998). Nevertheless, both AP and 5PN activities have been found in phytoplankton, as well as in bacteria (Cembella et al. 1984).

Traditional methods for the measurement of enzyme activity in the field only allows for assessment of bulk activities. Size fractionation of enzymes is often difficult, and it is not easy to decide the origin of dissolved enzymes. Hence, only the physiology of the general microbial population can be identified. Therefore, the application of a novel method using the ELF-97 phosphate substrate, allowing for direct microscopic detection of enzyme activity, has been widely used to determine cell-specific APA in recent years (González-Gil et al. 1998; Rengefors et al. 2001; Dyhrman et al. 2002; Strojsová et al. 2003; Lomas et al. 2004). This method applied to natural phytoplankton (Rengefors et al. 2001; Strojsová et al. 2003; Lomas et al. 2004) showed differences in the presence and localization, and seasonal variations, of

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APA in the phytoplankton community. It thus provides a promising tool to obtain more detailed information about the ecology of specific organisms in their natural environment. If this method is further developed so that it can also be applied to natural heterotrophic bacteria, one may come closer to an answer to the often asked question (cf. Cotner and Biddanda 2002; Tanaka et al. 2003): how large fraction of total bacteria counts is actually active in uptake?

There is uncertainty as to whether phytoplankton or heterotrophic bacteria is more efficient in sequestering orthophosphate from the environment or in using DOP as a source of P. In general, the experimental results have indicated that heterotrophic bacteria are more competitive in taking up orthophosphate (Currie and Kalff 1984; Berman 1988; Jürgens and Güde 1990), suggesting that algae may rely on DOP as a supplemental source of P (Tarapchak and Moll 1990; Cotner and Wetzel 1992). It has long been known that algae have the ability to utilize DOP in the absence of inorganic P (Kuenzler and Perras 1965), but their efficiency in obtaining P in natural environments in competition with heterotrophic bacteria is unresolved. The existence of high-affinity P transport systems (Chróst and Overbeck 1987; Ammerman and Azam 1991b) and the presence of the enzyme 5PN in bacteria (Ammerman and Azam 1985) together appear to contribute to their competitive advantage. The available data, however, are incomplete and, in part, contradictory. This could be the result of natural variability among the different habitats investigated, or it may reflect actual differences in community composition in these studies. As an example, the dominance of DOP uptake by phytoplankton during warm water conditions in subtropical coastal waters (Huang and Hong 1999) as opposed to bacterial domination in mesocosms experiments outside the coast of Finland (Tamminen 1989; **Paper I**), could be assumed to be an effect of the different water temperatures at the two sites. APA in algae can increase by a factor of two as the ambient temperature doubles, but this does not mean that activity for a particular species is necessarily highest at the time of year when field temperatures are highest (Hernández et al. 2002). Similarly, the notion that phytoplankton appear to be unable to utilize dDNA in P-deficient freshwater systems (Siuda et al. 1998) as opposed to significant uptake by coastal phytoplankton (**Paper I**; **Paper II**), may be an effect of

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differences in water chemistry, as well as in species composition. Several enzymes involved in the degradation of nucleic acids require the metal ions and salts of sea water to function optimally (Rangarajan and Shankar 2001).

In the mesocosm experiment presented in **Paper I**, we demonstrated tight coupling between DOP hydrolysis and uptake in P-starved mesocosms. When the algal – bacterial competition for inorganic and organic P was assessed by means of their respective biomass-specific affinities, we found that bacteria dominated the competition for all P substrates, although phytoplankton could take up significant proportions. No statistically significant shift in algal – bacterial competition for DOP relative to orthophosphate was found. Our data strongly suggest that competition should be assessed by means of specific affinity and not only as relative uptake in different size fractions or by kinetic parameters. In the mesocosm receiving organic C, a subpopulation of large, filamentous bacteria developed. We estimated this population to have high biomass-specific affinity for P-uptake. Our data from **Paper I** thus shows that the P competition between compartments of marine osmotrophs is influenced by the availability of labile organic C. Additionally, the data corroborate that both phytoplankton and bacteria possess enzymes for the utilization of DOP (Ammerman 1991), and challenge the traditional view that bacteria must be small in order to compete efficiently for limiting nutrients. Since the affinity data for P-uptake in the large bacteria fraction were obtained based on conventional conversion factors for biomass estimates and separation of algal and bacterial processes calculated from simple principles (**Paper I**), some uncertainty is associated with these values. However, several lines of evidence, both theoretical (**Paper III**) and experimental (**Paper IV**), show that large cells with small surface to volume ratios, contrary to the traditional view, can take up limiting nutrients very effectively and thus dominate competition, provided that they keep the intracellular content of the limiting element low in proportion to their size.

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## Utilization of dissolved N

$\text{NH}_4^+$  is considered the form of N preferentially utilized by marine osmotrophs but  $\text{NO}_3^-$  and the available fraction of the DON-pool is also actively utilized (Veuger et al. 2004; Jørgensen 2006).  $\text{NH}_4^+$  uptake by bacteria varies strongly in different regimes and may effect the phytoplankton dynamics in N-limited as well as in non N-limited systems (Kirchman and Wheeler 1998).  $\text{NO}_3^-$  is generally not thought to be important as an N-source for heterotrophic bacteria because they are often energy limited, hence the energy consuming reduction of  $\text{NO}_3^-$  is not favourable (Kirchman 1994). Many studies have concluded that  $\text{NO}_3^-$  uptake by bacteria is negligible (Eppley et al. 1977; Wheeler and Kirchman 1986; Kirchman 1994). Nevertheless,  $\text{NO}_3^-$  taken up by bacteria can account for a fraction as high as, or higher than,  $\text{NH}_4^+$  uptake (Kirchman and Wheeler 1998; Allen et al. 2002).

The DON fraction is dominated by proteins, nucleic acids and humic-like substances, and lower molecular weight compounds (<10 kDa) as peptides, urea, dissolved free amino acids (DFAA), purines, pyrimidines, pteridines and amides. Even though there has been progress understanding the DON-pool recently, much of it remains chemically uncharacterised, and the exact composition of the pool is unknown. The interaction of DON in the N-cycle is very complex and so far poorly understood (reviewed by Paul 1983; Berman and Bronk 2003). The former view of DON being an inert pool has been reversed in recent years. High turnover rates suggest that many DON-compounds are cycled very rapidly, and evidence for the biological cycling of DON has been given by stable isotope measurements (Benner et al. 1997; Veuger et al. 2004). The concentration of DON often exceeds that of DIN and may account for as much as 30-50% of daily phytoplankton N-demand (Benner et al. 1997).

Peptides and proteins make up the majority of the organic N-pool (Berman and Bronk 2003). Free amino acids are meant to be among the main sources of organic N to bacteria and phytoplankton, and therefore constitute a major link in the marine

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food web. Amino acids are rapidly taken up by heterotrophic organisms for protein synthesis and bacterial growth (Hollibaugh and Azam 1983). Although heterotrophic uptake by bacteria has long been recognized as the major process removing amino acids from sea water (Paul 1983; Billen 1984), it has also been shown that algae can grow on some amino acids (Ietswaart et al. 1994; Pantoja and Lee 1994; Palenik and Henson 1997). Laboratory experiments leave no doubt that the majority of aquatic algal species are able to utilize common organic N-compounds as N sources for growth if sufficient substrate concentration is provided and enough time is allowed for metabolic adaptation (e.g. Antia et al. 1975; Berland et al. 1979). These studies, however, used axenic batch cultures growing on high initial concentrations of organic N substrates, thus the ability of organisms to exploit the much lower concentrations encountered in the environment *in situ* is unclear. Nevertheless, it seems reasonable that the utilization of DON by different populations of bacteria and algae can vary considerably (Berman and Bronk 2003).

Bacteria have been viewed as the main consumers of DON, whereas phytoplankton are, in general, thought to require mainly mineral N (and urea) regenerated by bacteria or from remineralization processes in the subphotic ('new production') or the photic layer (Eppley et al. 1973; Paul 1983; Billen 1984). Phytoplankton may utilize urea produced by bacteria during degradation of purines and other DON-compounds (Vogels and van Der Drift 1976). When available, bacteria prefer  $\text{NH}_4^+$  and amino acids to urea as a source of N, despite all compounds contain reduced N (Jørgensen 2006). In 1983, Hollibaugh and Azam concluded, contrary to the then present view, that dissolved enzymes were not important in protein degradation in natural sea water. They showed that proteins were hydrolyzed rapidly by bacteria, with exoproteases structurally bound to their cell membranes, and that only minor fractions of the amino acids hydrolyzed from proteins were mixed with the bulk phase (Hollibaugh and Azam 1983). Consistent with this view, proteolytic activity is often associated with the bacterial size fraction (Billen 1984; Rego et al. 1985; Rosso and Azam 1987). Nevertheless, a recent study using a wide array of peptide analogous substrates (Obayashi and Suzuki 2005) indicates that most

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previous studies focused on degradation of proteins and peptides have significantly underestimated proteolytic enzyme activity.

Although bacteria are still assumed to be the major clients for utilizing proteins and amino acids in aquatic environments, it is now apparent that many phytoplankton species possess enzymes to use these as N-sources. Proteolytic activity has been found in association with eukaryotic algae (Berges and Falkowski 1996) and non-nitrogen-fixing cyanobacteria (Martinez and Azam 1993; Zubkov and Tarran 2005), but there has been little work to quantify its importance to the nutrition of these organisms. One important question is whether cyanobacteria, being a diverse group of prokaryotic algae with cell envelope characteristics similar to those of their eubacterial counterparts (reviewed by Hoiczyk and Hansel 2000), are more like heterotrophic bacteria or eukaryotic algae in their uptake of mineral nutrients.

Another way of amino acid utilization in bacteria and phytoplankton is by virtue of possessing cell surface amino-oxidases (Palenik and Morel 1990; Pantoja and Lee 1994; Mulholland et al. 1998). This extracellular process involves the cell-surface oxidation of L-amino acids or other primary amines to produce an oxidized organic product, ( $\alpha$ -keto acid or aldehyde, respectively), peroxide, and  $\text{NH}_4^+$ . The ammonium produced is assimilated by the cells, whereas the organic product and peroxide remains in solution. The use of this mechanism should be energetically preferred when the concentration of  $\text{NH}_4^+$  is low because it does not require synthesis of different transport enzymes necessary for processing amino acids (Palenik and Morel 1990). Pantoja and Lee (1994) showed that amino-oxidation rates can constitute as much as 40% of the total DFAA removal rate and can be done by phytoplankton at rates similar as those of bacteria. However, in the field, cell-surface oxidative deamination has only been detected at significant rates when the water temperature exceeds 20°C (Pantoja and Lee 1994; Mulholland et al. 1998), possibly explaining some of the variability in DON-uptake among the different habitats investigated.

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In **Paper II**, we explored the algal – bacterial competition in an N-deficient mesocosm and found that the algal community, dominated by *Emiliana huxleyi*, had significantly lower biomass-specific affinity for N derived from leucine, and significantly higher affinity for DIN, than heterotrophic bacteria. This contrasted the P-competition situation (**Paper I**), where no significant shift in algal – bacterial competition for DOP relative to orthophosphate was found. Because of the different community composition in the two investigations, (the phytoplankton community described in **Paper I** was dominated by N<sub>2</sub>-fixing cyanobacteria (Olli et al. 2005)), one should be careful to conclude that the competition for N is shifted compared to the competition for P.

Is the microbial food web differently structured in P-limited vs. N-limited systems?

We do not know if the dominating algal species in **Paper I** (N<sub>2</sub>-fixing cyanobacteria) and **Paper II** (*E. huxleyi*) represents average competitors for P and N, respectively. Thus, a direct comparison of the competition situation in the two systems does not allow firm conclusions whether the algal – bacterial competition shifts between P-limited and N-limited systems. N<sub>2</sub>-fixing cyanobacteria (eg. *Trichodesmium* spp.) are considered poor competitors for DIP, but with a high potential for utilizing DOP (McCarthy and Carpenter 1979; Moutin et al. 2005). However, at higher DIP concentrations, *Trichodesmium* spp. can compete efficiently with other algal species (Fu et al. 2005). Compared to other algal species, *E. huxleyi* compete poorly to moderately for DIN (Riegman et al. 1992; Riegman et al. 2000), but rather good for neutral amino acids (Ietswaart et al. 1994; Palenik and Henson 1997). There are however a great variation in uptake efficiencies between strains in both *Trichodesmium* (Fu et al. 2005) and *E. huxleyi* (Paasche 2002).

Anyway, the results from studies such as those mentioned above, could support theories that (1) phytoplankton use DOP as an alternative source for P in phosphate depleted environments (cf. Cotner and Wetzel 1992), and DON as an alternative source of N in DIN-poor parts of the ocean (cf. Paasche 2002), hence,



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phytoplankton rely on uptake from organic substrates in both P limited and N limited environments, thus the algal – bacterial competition may not be shifted between the two systems. Alternatively, as already mentioned, (2) in P-limited environments, where algae and bacteria both are believed to harbour enzymes for the utilization of DOP, competition is shifted compared to N-limited environments where bacteria dominate uptake of N from DON.

In **Paper I** we found no shift in the algal – bacterial competition for DOP relative to DIP. From (1), assuming N<sub>2</sub>-fixing cyanobacteria to compete good for DOP and poorly for DIP compared to other algae, one may expect the algal community to compete relatively better against bacteria for DOP than for DIP, but they did not. In **Paper II**, phytoplankton were superior in the competition for DIN, as were bacteria in the competition for DON, although *E. huxleyi* is regarded a fairly good algal competitor for DON but a poor competitor for DIN. Data from our experiments presented in **Paper I** and **Paper II**, thus supports the latter hypothesis (2); that bacteria and algae both utilize, and compete against each other, for DOP in P-limited systems, but in N-limited systems, bacteria do not experience significant competition from algae for DON.

If the competition for DON is indeed entirely dominated by bacteria, this changes the food web structure, since there will be a pool of dissolved N restricted to bacterial utilization. One effect of this would be that the probability of C limitation of bacterial growth could be larger in N-deficient regions than in P-deficient regions (Thingstad 2000a). Under such conditions, bacteria and phytoplankton thus not function as competitors for N, but rather that bacteria are remineralizers of N, supplying inorganic N to phytoplankton (Thingstad 2000a), adding another aspect of competition and commensalism to the concept of the microbial loop (Figure 1). This may also have implications for the climate through the global C-cycle, because DOC will be expected to accumulate faster in P-deficient compared to N-deficient systems.

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## **Nutrient limitation and affinity**

The term nutrient limitation is not easy to define, but has been used rather loosely to indicate a state where a lack of one or more nutrients restricts osmotroph growth rate. There is an important difference between physiological limitation and systemic limitation, although both are closely linked (Paasche and Erga 1988; Thingstad and Rassoulzadegan 1995). Physiological nutrient limitation refers to a situation where the nutrient supply is too low to support essential metabolic processes, thus the growth rate of individual cells is reduced. Systemic nutrient limitation would be the limitation on total biomass in the system caused by the restricted amounts of nucleic acids, proteins or other essential cell components which can be formed on the restricted amount of the nutrient available (Thingstad and Rassoulzadegan 1995). This type of limitation would be demonstrated by an increase in biomass following an addition of the limiting nutrient to the system, and thus reflects the ability of the system to convert additional nutrients to new biomass. The coupling between physiological and systemic limitation may be illustrated by simple food chain models of the type analyzed by Thingstad and Sakshaug (1990) who suggested a model in which low total concentration (sum in all biological and biologically available pools) of the limiting nutrient in the photic zone would correspond to a food chain based on primary nutrient uptake in small-sized organisms, few predator levels with inefficient recycling, and a strong physiological limitation of the organisms doing the primary uptake. Increased total concentration of available nutrients would shift the equilibrium towards more predator steps with more recycling and less physiological nutrient limitation at the bottom of the food chain and, at sufficiently high total nutrient concentration, to the introduction of larger primary producers.

Hence, using P as an example; with most of the total P-pool ( $P_T$ ) in the orthophosphate pool, physiological limitation will be small or absent. With most of  $P_T$  immobilized in osmotrophs biomass, there will be severe competition and high physiological limitation. In a system dominated by phagotrophs, however, the biomass of competitors will be small, the recycling rapid, and the physiological limitation reduced.

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Because the natural osmotroph community consists of species and groups with different nutrient demands and uptake kinetics, some osmotrophs may be under physiological limitation but others are not. In **Paper I**, we therefore use the less strict term ‘deficiency’ to illustrate that nutrient supply (e.g. P) is suboptimal for growth, while the term ‘limitation’ is restricted to a situation where most osmotrophs can be assumed to be under physiological limitation. However, the definition of limitation and deficiency for a mixed community is not as absolute as for a single species.

### Detection and diagnosis of nutrient limitation

Dissolved nutrient concentrations were the earliest data used to indicate the trophic status of waters, and their ratios to infer nutrient limitation. It has later been acknowledged that nutrient limitation can not be assessed from dissolved nutrient data alone because of insufficient analytical capacity of the techniques applied (see Dodds 2003), and such data should not be used uncritically to calculate fluxes of nutrients in aquatic ecosystems. Hence, several researchers choose to base the bioavailable nutrient concentrations on kinetic experiments (Rigler 1966), methods that take advantage of the physiological response of inducible/ repressible enzymes (Thingstad and Mantoura 2005), or turnover time and rate (Thingstad et al. 1996; Moutin et al. 2002). One experimental basis for the latter is that the ambient  $\text{PO}_4^{3-}$  concentrations can be estimated by multiplying  $\text{PO}_4^{3-}$  turnover times by  $\text{PO}_4^{3-}$  uptake rate derived from stoichiometric conversion of carbon based primary and bacterial production (Moutin et al. 2002). The rationale of this estimation is explained in detail elsewhere (Flaten et al. 2005; Tanaka et al. in press), but is briefly that  $\text{PO}_4^{3-}$  uptake by osmotrophs is proportional to their biomass and the ambient  $\text{PO}_4^{3-}$  concentration. Hence, assuming P-limitation of growth, the specific P-requirement calculated from production can be set to equal the *in situ* P uptake rates. This value multiplied by the  $\text{PO}_4^{3-}$  turnover time then corresponds to the concentration of bioavailable  $\text{PO}_4^{3-}$ . Data in **Paper I** suggest that the molybdenum blue method to estimate soluble reactive P (SRP; see Karl and Yanagi 1997; Tanaka et al. in press) may overestimate the actual

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bioavailable  $\text{PO}_4^{3-}$  concentration ten fold compared to values estimated by this approach. In theory, this method can also be applied to estimate bioavailable DIN in N-limited waters; the methodology is however limited by the difficulties in obtaining reliable DIN turnover times because the stable  $^{15}\text{N}$ -isotope technique does not allow for true tracer  $^{15}\text{N}$  enrichment when ambient concentrations are low, typically below the detection limit of chemical methods (**Paper II**).

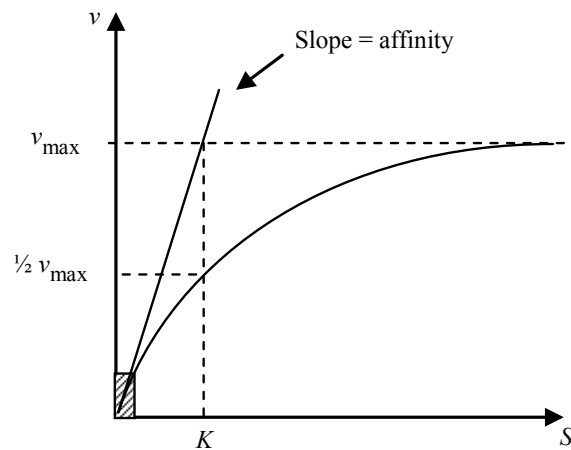
Chemical composition and physiological measurements have been used to determine if osmotrophs are nutrient limited (Sakshaug and Olsen 1986; Vadstein et al. 1988). The experimental basis for such measurements is the Droop model, in which the internal stores of nutrients determine nutrient uptake and growth rates. Given the cellular quotas of limiting nutrients, one can examine the nutritional status of osmotrophs by simple models. The results presented in **Paper IV** demonstrate, however, that in methods applying elemental biomass not based on direct measurements, conversion factors should be locally derived and connected to the size of the organisms and the dominating species, at least factors used to calculate bacterial biomass. This is discussed in more detail later.

Nutrient enrichment bioassays have been widely applied for experimentally assessment of nutrient limitation. Based on the assumption that enrichment of potentially limiting nutrients will be followed by a measurable response, these experiments are often simple to carry out in small scale experiments (**Paper IV**), but may be difficult to interpret in high level systems (i.e., mesocosms; **Paper I, Paper II**) and the natural situation. Such studies will, however, be hampered by the questions of potential effects from water manipulation and confinement. Enzyme activity (Hollibaugh and Azam 1983; Sala et al. 2001; Hoppe 2003) and the velocity of uptake of added tracers (Bentzen et al. 1992; Andersson et al. 2006) are often used as indicators for nutrient status of natural systems. Related to osmotroph biomass, these parameters can serve as diagnostic tools for the detection of limitation (Nausch 1998; Tanaka et al. in press; **Paper I; Paper II**).

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## Biomass-specific affinity

Biomass-specific affinity is defined as the volume of water cleared for substrates per unit biomass per unit time. Biomass-specific affinity is thus analogous to the clearance rate of a phagotrophic organism. The relation between maximum biomass-specific affinity ( $\alpha_{\max}$ ) and the Michaelis-Menten parameters maximum biomass-specific uptake rate ( $v_{\max}$ ) and half saturation constant ( $K$ ) is illustrated in Figure 2. Biomass-specific uptake rate ( $v$ ) is here  $V/B$ , where  $B$  is the biomass; hence,  $v_{\max}$  is  $V_{\max}/B$ .



**Figure 2.** Relationship between the Michaelis-Menten parameters and affinity. At substrate concentrations to the right of the line denoted the affinity constant the enzymatic apparatus of the organisms define their uptake capacity. Maximum biomass-specific affinity corresponds to the constant part of the slope of the Michaelis-Menten-curve at origo where the substrate concentration ( $S$ ) approaches zero (hatched area). The affinity constant defines the maximum uptake capacity for the organisms; thus assuming that all molecules hitting the cell surface are taken up.

The theoretical maximum biomass-specific affinity, denoted  $\alpha_{\max}$  in this thesis, can be described through the equation  $\alpha_{\max} = V_{\max}/KB$ .  $V_{\max}$  and  $\alpha_{\max}$  describes how efficient organisms take up substrates at high and low substrate concentrations, respectively.  $K$ , however, has no such clear function. Biomass-specific affinity is thus regarded as the best index to measure competitive ability.

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Throughout the papers presented in this thesis, the term ‘theoretical maximum affinity’ has been used in stead of ‘affinity constant’. When normalizing affinity to the biomass of algae *or* bacteria, this is termed biomass specific affinity ( $\alpha$ ) (in algae/phytoplankton or bacteria, respectively). When normalizing affinity to the summed biomass of algae *and* bacteria, this is termed S-affinity (S- $\alpha$ ).

In **Paper I**, **II**, and **IV** we applied the procedure to estimate biomass-specific affinity from experimental data as proposed by Thingstad and Rassoulzadegan (1999). This procedure relies on accurate measurement of turnover time for the substrate in question, which necessitates addition of tracer amounts of the respective substrate. This is generally not a problem when radiolabelled  $^{33}\text{P}$ -compounds are applied because of its high specific affinity. However, the  $^{15}\text{N}$  mass spectrometry technique applied in **Paper II** did not allow this, thus our turnover time values for N uptake are overestimates, and hence the biomass-specific affinities are underestimates. Other sources of artefact are discussed in **Paper II**; these are the uncertainty associated with the use of conversion factors, the active fraction of bacteria, and the limitations posed by mechanical separation of algae and bacteria.

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## The concepts of diffusion limitation and maximum affinity

At low ambient substrate concentrations, the uptake efficiency and competitive ability of the organism is characterized by the biomass-specific affinity. In **Paper I** and **Paper II** we compared biomass-specific affinity values derived from experimental data, and the theoretical maximum, to assess limitation and competitive ability.

### Diffusion theory

A well-established biophysical theory, derived from Fick's first law and based on the geometrical features of the simplest case of a spherical osmotroph organism, describes the size dependence of resource acquisition under nutrient limited conditions. Here, nutrient uptake ( $U$ ) per unit cell volume ( $V$ ) depends on nutrient diffusion to the cell surface:

$$\frac{U}{V} = 4\pi r D \Delta C \left( \frac{4}{3} \pi r^3 \right)^{-1} \quad (1)$$

where  $D$  is the substrate diffusion coefficient,  $r$  is cell radius and  $\Delta C$  is the concentration gradient of nutrient from the cell surface to the concentration in the bulk media. 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 ( $\alpha_{\max}$ ) from Equation 1 (**Paper III**):

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

$\sigma$  is here the intracellular concentration of the element in question. Since  $\alpha_{\max}$  decreases with the inverse square of cell radius, small cells should be superior to larger ones in the competition for nutrients when nutrient diffusion transport towards the cell, - and not hydrolysis, is the limiting step. This has led to one of the central

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dogmas within modern microbiology, which is now textbook knowledge; that being small, with a large surface to volume ratio, permits the cell to take up limiting nutrients more efficiently than larger cells. This is based on the principle that the cellular content of the required element is roughly proportional to cell volume, i.e., that  $\sigma$  is constant. However, this is not entirely true, as repeatedly demonstrated (see references in **Paper III**). As discussed in **Paper III**, any strategy to increase size without a proportional increase of the intracellular content of the limiting element will give a competitive advantage.

Equations 1 and 2 are however only valid for spherical cells. To apply for non-spherical cells, one may introduce the term conductance ( $G$ ), which is determined by the shape of the cell. Nutrient uptake can then be expressed as  $U = GD\Delta C$  and Equation 2 can be rewritten as (**Paper IV**):

$$\alpha_{\max} = GD/V\sigma \quad (3)$$

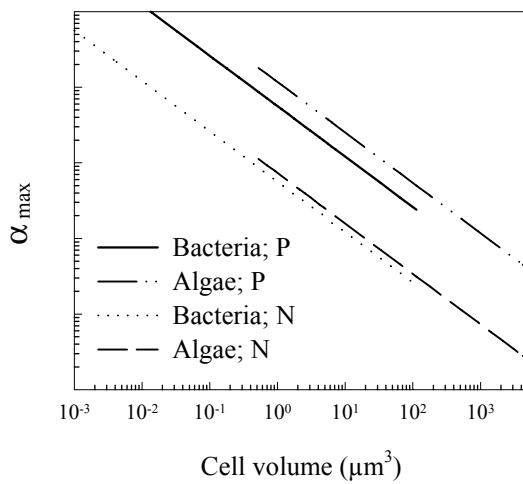
Since  $G$  of a non-spherical cell is always larger than that of a spherical cell of the same volume (Clift et al. 1978), Equation 3 illustrates that, for a given volume, non-spherical cells will have a competitive advantage compared to spherical cells with the same internal cell concentration ( $\sigma$ ). In **Paper IV**, we used the heterotrophic bacterium *Vibrio splendidus* as a model organism to document how a reduction of the internal concentration of the limiting nutrient and a transition from coccoid to rod-shaped cells is used to optimize uptake.

### Optimum stoichiometry

The internal cell concentration ( $\sigma$ ) for N and P in marine osmotrophs may be derived from the assumption that bacteria and phytoplankton cells have a density of  $1.1 \text{ g cm}^{-3}$ , 50% dry weight of wet weight, 50% C of dry weight, and that the C:N:P ratio (mol:mol) is 106:16:1 for algae (Redfield et al. 1963) and 50:10:1 for bacteria (Goldman et al. 1987; Fagerbakke et al. 1996). Thus, algae have less N and P per volume compared to bacteria, and may benefit from a lower  $\sigma$ , in particular when



competing for P. From theoretical arguments (*see also* Thingstad and Rassoulzadegan 1999; Tanaka et al. 2003; **Paper I**; **Paper II**), the model for diffusion limited uptake thus predicts algal – bacterial N and P competition as illustrated in Figure 3. Changes in the chemistry of the surrounding medium is however accompanied by changes in size and elemental stoichiometry of algal and bacterial biomass (Sakshaug and Olsen 1986; Vrede et al. 2002; La Ferla and Leonardi 2005) complicating the theoretical prediction of Figure 3 (**Paper IV**).



**Figure 3:** Log-log plot of the theoretical reduction in maximum biomass-specific affinity for N ( $\alpha_{\max}$ ; Volume water cleared per unit biomass-N per unit time) and P (Volume water cleared per unit biomass-P per unit time) with cell size for bacteria and algae, calculated from Equation 2. The assumption for the diffusion model is that cell density is  $1.1 \text{ g cm}^{-3}$ , dry weight is 50% of wet weight, carbon weight is 50% of dry weight, and the molar C:N:P ratio is 106:16:1 for algae and 50:10:1 for bacteria. Note that the graphs apply to N and P containing substrates, inorganic or organic, of comparable  $D$ , hence the dimensionless y-axis.

If growth is regulated by the single nutrient in shortest supply, the species specific intracellular optimum nutrient ratios may be a basis for exclusion or coexistence of competing species (Rhee and Gotham 1980). The optimum nutrient ratio (Rhee and Gotham 1980) is the ratio at which a transition from one nutrient limitation to another takes place. The determination of optimum N:P ratio is based on the cell quotas ( $q$ ), of N and P to the respective minimum intracellular subsistence quota ( $q_0$ ) ratio. From the Droop model, a limiting nutrient can be defined as the one with the smallest  $q:q_0$

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ratio; a transition between P and N limitation in individual cells thus occurs when (Rhee and Gotham 1980):

$$\frac{q_N}{q_{oN}} = \frac{q_P}{q_{oP}} \quad (4)$$

The optimum N:P ratio by definition is this transition point at low growth rates when the proportion of the storage fraction in the cell quota is minimal. In Rhee and Gotham (1980), this concept is exemplified with two organisms, A and B, with optimum N:P ratios of 10 and 30, respectively. Both organisms are P-limited when N:P ratios in their habitat result in a cellular ratio greater than 30. Organism A, however, will be more P-limited than organism B because of its lower optimum N:P ratio. If their maximum growth rates are similar, B will competitively eliminate A. The reverse will take place, due to the differences in the degree of N limitation, when the N:P ratios in the water results in cellular ratios of less than 10. At N:P cellular ratios between 10 and 30 the two organisms will be limited by different nutrients: A by phosphate, B by nitrogen. Thus they can coexist according to Rhee and Gotham (1980), but the model does not explain coexistence of organisms competing for the *same* nutrient.

Relating this to algal – bacterial competition, means that bacteria, believed to have generally lower N:P ratios than algae, will have higher P requirements and thus be subject to P-limitation at higher ambient P concentrations than algae. Non-nitrogen fixing phytoplankton, on the other hand, may be more subject to N-limitation than bacteria.

### Structural stoichiometry

Laboratory studies show that phytoplankton (Rhee and Gotham 1980; Klausmeier et al. 2004b) and bacteria (Bratbak 1985; Tezuka 1990) are flexible in their overall stoichiometry, often matching their nutrient supply at low growth rates. Although there is a large variability in phytoplankton N:P ratios, the average for the species is

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remarkably close to the Redfield ratio of 16. However, some of the variability is due to stored nutrients: underneath these variable pools is the relative constant composition of the cell's functional machinery, termed the structural stoichiometry by Klausmeier *et al.* (2004a). This structural stoichiometry determines the nutrient requirement of the cells. Building on the chemical composition of cellular machinery, the variability in the species specific N:P structural stoichiometry may be explained by considering two strategies for survival under nutrient limitation (Klausmeier *et al.* 2004a). One strategy would be to put more resources into assembly machinery to maximize growth rates. Assembly machinery corresponds to nucleic acids and ribosomes containing N and P. Organisms with higher growth rates require a larger allocation to ribosomal RNA to satisfy higher rates of protein synthesis. This interpretation is called the 'growth rate hypothesis' (GRH) (Makino *et al.* 2003). Another strategy would be to put more resources into resource acquisition machinery, favouring competitive ability. Resource acquisition machinery corresponds to nutrient-uptake proteins and chloroplasts, which contain N and C, but little or no P. The optimal structural stoichiometry depends on which nutrient is limiting, but the resource acquisition strategy will always result in higher N:P ratios than the assembly machinery strategy. Light harvesting machinery in algae contains N-rich chloroplasts and ribosomes, favouring higher N:P ratios at light limitation. The optimal strategy at equilibrium depends on the mortality rate; so that intense grazing lead to greater allocation to assembly machinery and therefore a lower N:P ratio. In all cases, the optimal strategy balances the conflicting needs for resource acquisition and cellular assembly.

The models of Klausmeier *et al.* (2004a; 2004b) are based on the work of Droop (1974) on phytoplankton growth, they are however particularly relevant to the case of *V. splendidus* in **Paper IV**. In this chemostat experiment (**Paper IV**) the N:P ratio of the reservoirs was kept constant, while the glucose concentration was increased along the gradient; shifting the limiting factor from C to P. Cell size increased along this gradient. As the reservoir C:P ratio increased 150 fold, the cellular C:P ratio increased only two fold and was accompanied by an almost similar increase in the cellular N:P ratio. Thus, the increase in size in P-limited *V. splendidus*

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may not be an effect of stored C alone (cf. **Paper III**), but perhaps also that the cells put more resources into C- and N-rich structures for their resource acquisition machinery than into P-rich nucleic acids for their assembly machinery. The theoretical benefits calculated from diffusion theory (**Paper III, Paper IV**), however, will be the same either the non-limiting element is stored as inclusion bodies or assimilated into the cell's functional machinery.

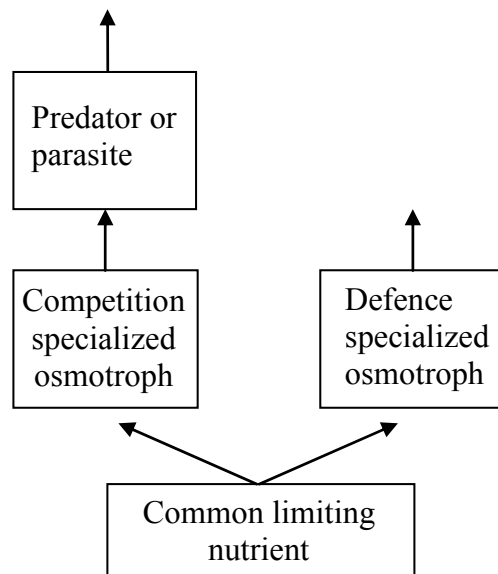
As shown by Makino *et al.* (2003), the GRH alone is not sufficient to explain the large variation in bacterial biomass stoichiometry in nature. In accordance with our results (**Paper IV**), they suggested that single bacterial strains are homeostatic in their C:N:P stoichiometry (or at least more homeostatic than autotrophs), and that the variability observed in *in situ* bacteria are generated by shifts in the dominant species in the environment.

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## **On the theory of uptake specialized and defence specialized osmotrophs**

The success of osmotrophs is often described as the balance between nutrient availability and predation pressure. These factors also influence the morphological structure of algal and bacterial assemblages. Marine bacteria can survive extended periods of starvation (Morita 1997); they do not die predominantly from the lack of resources. The main sources of bacterial mortality in the water column are currently considered to be viral-mediated lysis and grazing by protists (Thingstad 2000b). Several strategies to defend against grazers are proposed, but reduced vulnerability towards one type of grazer can imply enhanced vulnerability towards other predators as exemplified in a review by Jürgens and Güde (1994). Predator and prey size is generally an important variable for feeding ecology (Jürgens and Güde 1994; Boenigk et al. 2004). Both large (Hahn et al. 1999; Matz et al. 2002) and sufficiently small (Chrzanowski and Simek 1990; Boenigk et al. 2004) size can protect bacteria from their predators, or at least make them less vulnerable.

Coexistence of two organisms competing for the same nutrient is possible if one is an ‘uptake’ specialist, and the other a ‘predation defence’ specialist as illustrated in Figure 4. Small, spherical cells are traditionally believed to be more efficient in their uptake of nutrients because of their large surface to volume ratio. From the traditional view, small bacteria then represents the competition specialists in Figure 4, and larger osmotrophs represents the defence specialists, assuming an increase in size is a means to reduce predation. As we prove in **Paper IV**, however, large size does not necessarily represent a trade-off in resource competition. One may also hypothesize situations in which silicate, or light (for photosynthesis) can select for species (e.g. diatoms and *Synechococcus*, respectively) within the osmotroph community that can benefit from these resources, to change their stoichiometry and morphology to increase their nutrient uptake efficiencies (**Paper III**). An increase in size could thus represent a win-win situation, simultaneously increasing competitive ability and reducing predator vulnerability. Unfortunately, we were not able to fully explore this latter element of the proposed ‘Winnie-the-Pooh strategy’ (cf. **Paper III**).



**Figure 4:** Generic 3-member food web allowing co-existence of two competing osmotrophs with different life strategies on one common limiting substrate (redrawn from **Paper III**). The community structure of the microbial loop of Figure 1 is dependant upon the dynamics of the competition between osmotrophs. Illustrated here by a food web where the biomass of the competition specialist is kept in check by predator or parasite control (grazers or virus in Figure 1), which allows some of the limiting element to be incorporated into the defence specialist.

### Predation and anti-predator strategies

Unpublished results from grazing experiments (we measured disappearance rate in 48 h incubation experiments as obtained by epi-fluorescence microscopy) conducted with natural sea water, proved inconclusive. We used fluorescence labelled *V. splendidus* (FLB) (Sherr et al. 1987), grown under C- and P-limitation to yield small ( $0.2 \pm 0.1 \mu\text{m}^3$ ) and large ( $1.4 \pm 0.8 \mu\text{m}^3$ ) size, respectively, inoculated to  $<10\%$  of the ambient bacterial concentration in 500 ml experimental containers. In two experiments conducted on natural sea water there were significant disappearance relative to a control experiment, but no statistically significant difference between the disappearance rates of small and large FLB's. In an experiment conducted on sea water enriched to induce the growth of flagellates, large FLB's disappeared

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significantly faster than small FLB's ( $p < 0.001$ ). After 48 h incubation, the abundance of small protists was always highest in the containers incubated with small FLB, while the final abundance of large protists ( $>10 \mu\text{m}$ ) was approximately the same.

Many factors in antipredator strategies can not be accounted for in experiments such as these (see reviews by Jürgens and Güde 1994; Pernthaler 2005), however, our results indicated that size *alone* did not protect P-limited, large *V. splendidus* from grazing relative to the smaller C-limited *V. splendidus*, given the existing grazer communities. Experiments of longer duration are needed to follow the succession of the grazer community. The effect of physiological change in *V. splendidus* on virus infection and infectivity is unknown. It is previously shown however, that bacterial porins, which are outer membrane transport channels often induced coregulated with substrate binding proteins and enzymes at low substrate concentrations, can serve as phage receptors (Nikaido and Vaara 1985).

The introduction of the bacterivorous flagellate *Ochromonas* into continuous cultures of large, rod-shaped *Flectobacillus* sp. (Hahn et al. 1999) led to an initial decrease in bacterial numbers, but after the mean length of bacteria increased, a steady state was established, in which bacterial length and cell numbers were constant. When *Flectobacillus* sp. was cultured without flagellates, an increase in growth rate (dilution rate) led to an increase in cell length similar to that in the grazing experiment (Hahn et al. 1999). Hahn *et al.* (1999) thus concluded that the formation of grazing resistant forms in bacteria is controlled by predation pressure and growth rate. Strong compensation of bacterial grazing mortality was observed in field mesocosms in which the grazing losses of edible bacteria were completely compensated for by growth of morphologically inedible forms, i.e. aggregated and filamentous bacteria (Jürgens et al. 1994). A clear succession in the grazer community was observed, but the 10 day duration of the experiment was too short to determine any effects of this succession on the bacterial community (Jürgens et al. 1994). Interestingly, there is a relationship between aggregation and filament formation that was not accounted for in our grazing experiments; filamentation

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benefits cells attached to a surface by increasing the specific surface area in direct contact with the solid medium (Young 2006). If an increase in size can be combined with aggregation, either to a solid medium or cell to cell, it may further reduce predation. Pernthaler *et al.* (1997) demonstrated that a slow-growing bacterial community reacted to the addition of bacterivorous flagellates within one day: one group produced filamentous, grazing resistant forms, and another group of bacteria reacted with a massive growth rate increase. Both responses are likely to be favoured by protistan grazing through a recycling of nutrients, and to reflect the different strategies proposed by Klausmeier *et al.* (2004a) and in **Paper III** and **Paper IV**.

#### Excess C; respire or assimilate?

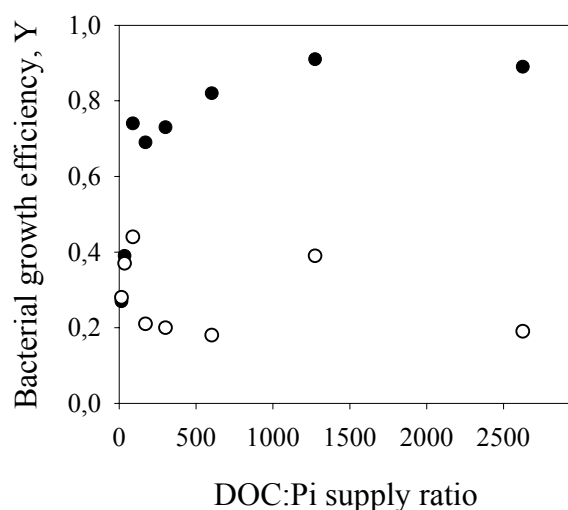
Mesocosm experiments carried out on natural assemblages of freshwater bacteria (Jansson *et al.* 2006) showed that bacterial growth efficiency ( $Y$ ), which can be defined as the organisms' ability to convert organic C into biomass, decreased with increasing DOC:Pi supply ratio, i.e. that P-limited bacteria tended to respire a large portion of assimilated C, while C-limited bacteria used a greater share of DOC for growth. It thus may seem that high respiration was important for growth when growth was restricted by Pi (Jansson *et al.* 2006); i.e. that in this particular case, C was neither stored nor allocated to resource acquisition machinery. The environmental condition for the strategy of increasing cell size as proposed for *V. splendidus* would be access to a pool of assimilable organic C in excess of that required for growth. Growth and respiration must then be balanced so that there is a net intracellular increase of C.

Unpublished results from this experiment showed that, based on DOC-consumption,  $Y$  was stable between 20-40% along the gradient of increasing DOC:Pi supply ratio (Figure 5; open symbols). However, unpublished respiration rate measurements showed that P-limited *V. splendidus* cultures with excess labile DOC had only twice as high respiration rates as C-limited cultures, which was low considering that their total C-biomass was >40 fold higher. Basing  $Y$  on respiration



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measurements thus indicated that  $Y$  increased with increasing DOC:Pi supply ratio (Figure 5; closed symbols), contradictory to the results of Jansson *et al.* (2006). These contrasting results (Figure 5) could indicate that *V. splendidus* assimilated excess labile-DOC (i.e. glucose) that was later released to the cultures as refractory organic C. It is previously shown that bacteria in the natural environment not only take up DOC and convert it to biomass and CO<sub>2</sub>, but also release DOC into the water column as ‘semi-labile’ capsular material (Stoderegger and Herndl 1998). However, our procedure for respiration rate measurements was hampered by the low sensitivity of the Clark electrode. This necessitated incubation of samples in BOD-bottles (30-40 h) prior to measurements, inevitably altering both DOC:Pi ratios and total C-biomass compared to the chemostats. Drift in the electrode sensitivity led to problems in obtaining stable baseline measurements and additional uncertainties. Nevertheless, these preliminary results are interesting in light of the strategies discussed in **Paper III**, and further experiments with more sensitive instruments should be done.



**Figure 5:** Bacterial growth efficiency calculated as increase in C-biomass ( $\Delta C_B$ ) divided by  $\Delta C_B +$  respiration (closed symbols) or  $\Delta C_B$  divided by DOC-consumption (open symbols) as a function of DOC:Pi supply ratios in *V. splendidus*.

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## Limits to size

There are limits to how large a cell can be and still benefit from the proposed strategy of becoming large to optimize affinity for limiting nutrients. The most obvious would be that sinking loss must be balanced by growth. Assuming that the volume-specific nutrient content is *not* constant (**Paper III**; **Paper IV**), lowering intracellular nutrient concentrations would compensate sinking loss, as would active swimming or increased buoyancy (Thingstad 1998). It can be argued that ‘the minimum cell quota’ (cf. **Paper III**; **Paper IV**) will be larger for large cells compared to small cells because, for example, they would need more cell membrane components. Other associated elements are also needed in higher demand. As we see in **Paper IV**, large *V. splendidus* does indeed have higher P and N content than small cells, but when normalized to volume (and C) the concentration is smaller. From a surface to volume perspective, the coccoid forms as observed in the P-sufficient chemostats (**Paper IV**) is less favourable for acquiring resources than the rods observed in the P-limited chemostats, but it is ideal if the aim is to keep what is already taken up and to minimize internal diffusion distances. If more is allocated to assembly under P-sufficient conditions, it may be better to minimize losses rather than to focus on acquisition. As the cell increases, so does also the internal diffusion distances. This can be compensated for by decreasing the internal viscosity. Many of the large bacteria harbour massive cell inclusions of known or unknown function that reduce the volume of metabolically active cytoplasm and, possibly, the internal diffusion limitation (Schulz and Jørgensen 2001). Similarly, the water filled vacuole in diatoms (cf. **Paper III**) may enhance both buoyancy and internal diffusion.

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## Conclusions

In P-limited systems where the algal community was dominated by N<sub>2</sub>-fixing cyanobacteria, heterotrophic bacteria displayed significantly higher biomass-specific affinity for both DOP and DIP uptake than algae. However, the algae could compete well for all P-substrates, and there were no conclusive evidence for a shift in terms of algal – bacterial competition when P was available in the form of either monomeric or polymeric DOP contrary to PO<sub>4</sub><sup>3-</sup>.

In N-limited systems where the algal community was dominated by *E. huxleyi*, heterotrophic bacteria were superior in the competition for DON, and algae were superior in the competition for DIN, and there were conclusive evidence for a shift in terms of algal – bacterial competition when N was available in the form of Leucine contrary to NH<sub>4</sub><sup>+</sup>. This was manifested by poor abilities of *E. huxleyi* to compete against heterotrophic bacteria for DON.

By comparing experimentally derived biomass-specific affinity values to theoretical maximum estimates, it is shown that, diffusion transport to the cells, rather than hydrolysis, can be regarded as the limiting step for utilizing P from dissolved macromolecules like dDNA in severely P-limited marine environments.

Reduced access to bioavailable P may induce a shift from larger organisms dominating the uptake to smaller organisms dominating the uptake that may again be modified in favour of larger organisms by increased availability of labile DOC. This is because subpopulations of heterotrophic bacteria use excess C to increase in size thereby optimizing their competitive ability.

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Using *V. splendidus* as a model organisms, it is shown that, if a large size can be obtained with a less than proportional increase in the cell quota of the limiting element, large size is actually an advantage rather than a disadvantage, also at permanently low substrate concentrations. *V. splendidus* thus may represent an active subgroup within the bacterial community with the ability, under mineral nutrient – excess glucose conditions, to change its stoichiometry and morphology in a manner increasing its nutrient uptake efficiencies and obtaining a competitive advantage.

The putative superiority of small, spherical bacteria in nutrient uptake should be viewed with some caution because the cellular content of the limiting element is not proportional to cell volume; i.e. the intracellular concentration is not constant.

These findings demonstrate the need of using accurate and locally derived cell volume estimates combined with allometric volume to ‘elemental content’ factors in biomass estimates of bacteria.

### **Future perspectives**

More investigation on algal – bacterial competition for organic substrates is needed. Experiments such as those described in **Paper I** and **Paper II** should be performed simultaneously in the same habitat, and competition should also be investigated in the natural environment under differing conditions. As proposed in **Paper II**, a wider range of model substrates should be applied. Additionally, the relationship between nutrient status in the system and predation need more focus. Taken together, this may increase our understanding of how the transport of C and energy is influenced by the P, N, and organic-C status of the environment, and allow for better predictions of the effects on ‘microbial food web dynamics’ and the global C-cycle.

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Results from this work showed that conventional conversion factors must be used with care when estimating osmotroph biomass. The constant factor of 20 fg C bacterial cell<sup>-1</sup> (Lee and Fuhrman 1987) has been widely used to estimate bacterial biomass. Although the authors (Lee and Fuhrman 1987) cautioned against extrapolation of this factor outside a narrow range of relatively small cells, we frequently observe that this is ignored (i.e. that the factor is used without any relation to size). Similarly, we find that C to Chlorophyll *a* conversion factors is sometimes used uncritically; in the Baltic Sea in summer, a factor of 50 (weight: weight) has been applied (Nausch 1998), although the experimentally derived C: Chlorophyll *a* ratio is 8 – 23.5 for the area and season (Gargas et al. 1979). Volume specific scaling factors, shown to correlate well between different habitats (see references in **Paper IV**), is therefore proposed to estimate biomass. The alternative is to directly measure bacterial biomass in the field, preferably by X-ray microanalysis. The combination of two or more of either cell sorting flow cytometry, transmission electron microscopy (TEM), X-ray microanalysis, and X-ray fluorescent (XRF) analysis may in the future help overcome these problems. Additionally, the application of cell specific analysis of enzyme activity may improve calculations of the active fraction of osmotrophs.

The ‘black box’ approach has long served to understand the dynamics within the microbial food web (cf. Azam et al. 1983; Figure 1). Currently, the models arising from this conceptual framework implicitly assume that all heterotrophic bacteria are the same, thus they have been treated as one phylogenetic type. Similarly, phytoplankton has been divided into compartments from simple principles; even sometimes only by their difference in size. With the modern tools mentioned above, and many more novel techniques in the field of molecular biology, we are now able to look inside the boxes, enabling us to understand the phylogenetic diversity inside, - and internal dynamics between, their ecologically functional units. One consequence of this is that bacteria with a low content of DNA, previously believed to represent less active components of related phylogeny and more or less ignored in studying the fate of DOM, are now viewed as superior to other osmotroph groups in the

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competition for P (see Nishimura et al. 2005 and references therein), possibly benefiting from a lower P requirement and higher nutrient uptake efficiencies as discussed in **Paper III** and **Paper IV**. These interpretations are however debatable, and more experiments applying the modern tools are needed to elucidate the mechanisms of mass transfer in aquatic systems. Efforts should be made to include molecular methods in the studies of biogeochemical processes and seasonal dynamics of osmotrophs. Some pioneer work has been done, but as pointed out (Thompson et al. 2004; Allgaier and Grossart 2006), more specific studies are needed.

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