

## Physiological responses of three species of marine pico-phytoplankton to ammonium, phosphate, iron and light limitation

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### Abstract

Experiments were conducted with three species of marine pico-phytoplankton: *Synechococcus* sp. (CCMP 839), *Pelagomonas calceolata* (CCMP 1756) and *Prasinomonas capsulatus* (CCMP 1617) in order to collect physiological parameters for pico-phytoplankton to be utilised in Ocean Biogeochemical Climate Models. The main parameters to follow the effects of ammonium, phosphate, iron and light limitation were cell growth rates ( $\mu$ ), half saturation constants for growth ( $K_m$ ), N, P and Fe quota (per cell or per mol C), and photochemical quantum efficiency ( $F_v/F_m$ ).

The nitrate and phosphate limitation experiments demonstrated that the small phytoplankton species could grow at low N and P concentrations.  $K_m$  values were in the micro-molar ( $\text{NH}_4^+$ ) and sub-micro-molar ( $\text{PO}_4^{3-}$ ) range. N and P quota were in the femto-molar range per cell and varied from nutrient-deplete to nutrient-replete conditions.  $F_v/F_m$  values were only adversely affected at the lowest N and P concentrations in these experiments. In the Fe limitation experiments, it was shown that all three species were adversely affected only at extremely low Fe concentrations. Iron chelating agents had to be added to force the species in Fe limitation till ultimately growth stopped.  $K_m$  values with respect to dissolved Fe were in the femto-molar range. Fe quota were in the low zepto-molar ( $10^{-21}$  M) range per cell, and varied considerably from Fe limiting to Fe replete growth conditions.  $F_v/F_m$  values diminished only at the lowest iron concentrations. In the light limitation experiments, growth rates and photochemical quantum efficiencies were adversely affected only at irradiance levels below  $10 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ . These results indicate that the pico-phytoplankton species will hardly ever be completely stopped in their growth by  $\text{NH}_4^+$ ,  $\text{PO}_4^{3-}$ , Fe or light (separately) under natural conditions.

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**Keywords:** Ammonium limitation; Phosphate limitation; Iron limitation; Light limitation; Pico-phytoplankton; Nutrient quota

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### 1. Introduction

Pico-phytoplankton belongs to the most successful algal group in the marine environment. Their major

distribution is in the open oligotrophic ocean, where they dominate not only in terms of phytoplankton biomass (Partensky et al., 1996), but also in terms of primary production (Bell and Kalff, 2001). Two prokaryotic cyanobacteria, *Prochlorococcus marinus* (Chisholm et al., 1988; Partensky et al., 1999) and *Synechococcus* spp. (Waterbury et al., 1979, 1986; Olson et al., 1990; Li, 1998; Bertilsson et al., 2003; Heldal et al., 2003) belong to the most thoroughly investigated algal species of the pico-phytoplankton community. Only a very limited number of eukaryotic pico-phytoplankton species have been investigated in detail. The paucity of physiological data on the latter group, on for example major- and micro-nutrient usage, hampers the parameterisation of global biogeochemical climate models. Within the IRONAGES project (Iron Resources and Oceanic Nutrients – Advancement of Global Environment Simulations), ecosystem models and Ocean Biogeochemical Climate Models (OBCMs) were developed. In order to obtain physiological parameters to be used in the parameterisation of these OBCMs, the main bloom-forming species of marine phytoplankton such as DMS producing classes (*Phaeocystis* sp. and *Emiliania huxleyi*), diatoms, N<sub>2</sub>-fixing diazotrophs (*Trichodesmium*), and pico-phytoplankton were investigated for their physiological response to ammonium, phosphate, iron and light limitation.

Here, we report on ammonium, phosphate, iron and light limitation experiments using three species of marine pico-phytoplankton: the prokaryote *Synechococcus* sp., and the eukaryotes *Pelagomonas calceolata* and *Prasinomonas capsulatus*. Main parameters to follow the effects of limitation were growth rates ( $\mu$ ), half saturation constants for growth ( $K_m$ ), N, P and Fe quota and the photochemical quantum efficiency ( $F_v/F_m$ ).

## 2. Material and methods

### 2.1. Phytoplankton species and culture conditions

Unialgal, xenic cultures of three species of pico-phytoplankton were used: *Synechococcus* sp. (CCMP 839, Cyanophyceae), spheres with a diameter of slightly less than 3  $\mu\text{m}$ , *Pelagomonas calceolata* (CCMP 1756, Pelagophyceae), spherical cells with a

diameter of 3  $\mu\text{m}$ , and *Prasinomonas capsulatus* (CCMP 1617, Prasinophyceae), slightly elongated cells with an average diameter of 4  $\mu\text{m}$ . With the average sizes of the algae, average carbon concentrations per cell were calculated according to Veldhuis and Kraay (2004): *Synechococcus* sp.: 257 fmol C cell<sup>-1</sup>, *P. calceolata* 300 fmol C cell<sup>-1</sup>, and *P. capsulatus* 725 fmol C cell<sup>-1</sup>. All three species were incubated at 20 °C, and exposed to a light regime of 16 h light, 8 h dark at 120  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , except in the light limitation experiment where the cells were exposed to different irradiance levels (see below). In the NH<sub>4</sub><sup>+</sup> and PO<sub>4</sub><sup>3-</sup> limitation experiments, natural nutrient-poor, 0.2  $\mu\text{m}$  filtered seawater, collected in the Gulf of Biscay, was used as growth medium. The medium was prepared as standard f/2 medium (including trace elements and vitamins), with  $100 \times 10^{-6}$  M EDTA added, but without the increased concentration of N (in the experiments with ammonium as variable) or P (in the experiments with phosphate as variable). In the Fe and light limitation experiments, natural nutrient-rich, Fe-poor 0.2  $\mu\text{m}$  filtered seawater, collected during the ANT18/2 cruise with RV 'Polarstern' at 40°S, 20°E, (November 2000) was used as growth medium. In the Fe limitation experiments, the medium was prepared as standard f/2 medium, with  $5 \times 10^{-6}$  M EDTA added, but without the addition of iron. In the light limitation experiments, the same medium was used as in the Fe limitation experiments, but with  $100 \times 10^{-6}$  M EDTA and  $5 \times 10^{-6}$  M Fe added (final concentrations). In both Fe and light limitation experiments all major and micro nutrients were in ample supply. In all experiments, the cells were grown in diluted cultures, to avoid self-shading. Transfer to fresh medium was done every 14 d, or if cell numbers reached 200 000 cells ml<sup>-1</sup>, but the cultures were always followed till cell growth halted, so as to determine the ultimate yield of cells under the given growth conditions.

Prior to all experiments, filter sterilisation of the medium was applied using 0.2  $\mu\text{m}$  filters. All incubations were performed in acid-washed, microwave-sterilised 60 ml square polycarbonate bottles (NALGENE®). Despite the small volume, no bottle effects were observed. Manipulation and sampling of the incubations took place under class 100 clean air conditions in order to prevent contamination by trace metal and/or micro-organisms.

## 2.2. $\text{NH}_4^+$ and $\text{PO}_4^{3-}$ limitation experiments

Growth rates in relation to ammonium and phosphate were determined in at least three consecutive series of incubations with cultures of *Synechococcus* sp., *P. calceolata* and *P. capsulatus*. The following incubations were grown: +0.0 (blank), +1.0, +1.5, +2.0, +5.0, +10.0  $\times 10^{-6}$  M  $\text{NH}_4^+$  (total background  $\text{N}_{\text{inorg}}$ :  $< 0.1 \times 10^{-6}$  M) and +0.0 (blank), +0.05, +0.1, +0.2, +0.5, +1.0  $\times 10^{-6}$  M  $\text{PO}_4^{3-}$  (total background  $\text{P}_{\text{inorg}}$ :  $< 0.02 \times 10^{-6}$  M).

## 2.3. Iron limitation experiments

Growth rates in relation to varying concentrations of dissolved Fe were determined in at least three consecutive series of incubations with cultures of *Synechococcus* sp., *P. calceolata* and *P. capsulatus*. The background concentration of dissolved Fe in the growth medium was measured using Chemoluminescence Flow Injection Analysis (Croot and Laan, 2002), and was  $1.1 \times 10^{-9}$  M. The background concentration of organic ligands was measured using Cathodic Stripping Voltammetry (Gledhill et al., 1998), and was  $1.68 \times 10^{-9}$  M. From these data it can be calculated that the  $\text{Fe}'$  concentration (all inorganic Fe species) was  $2 \times 10^{-12}$  M. As addition of EDTA did not result in growth-limiting Fe conditions for any of the three species used, additional DFB (Desferrioxamine-B, a fungal siderophore) was added in increasing concentrations. The resulting  $\text{Fe}'$  concentrations were calculated (Timmermans et al., 2001) (Table 1). As check on the effect of DFB just on Fe, and not on any other trace element, and to exclude toxic effects of DFB on the phytoplankton, additional spiking with surplus Fe was done to some incubations.

## 2.4. Light limitation experiments

Growth rates in relation varying irradiance levels were determined in series of incubations with cultures of the three species. At least 25 irradiance levels (lowest 2, highest 120  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) were tested using neutral grey filters. In each of the incubations, the irradiance levels were measured inside the incubation bottles, using a QSL-100 irradiance meter (Biospherical Instruments Inc.).

Table 1

Concentrations of DFB ( $\times 10^{-9}$  M) and resulting  $\text{Fe}'$  ( $\times 10^{-18}$  M) concentrations in the Fe limitation experiments in cultures of *Synechococcus* sp., *P. calceolata* and *P. capsulatus*

<i>Synechococcus</i> sp. [DFB]	<i>P. calceolata</i> [DFB]	<i>P. capsulatus</i> [DFB]	[ $\text{Fe}'$ ]
0.00	0.00	0.00	542 000
0.38	–	–	317 000
0.76	–	–	139 000
1.14	–	–	27 500
1.52	1.52	1.52	7710
1.90	–	–	4200
2.28	–	–	2900
3.04	3.04	3.04	1780
4.56	4.56	4.56	1000
6.08	6.08	6.08	697
7.60	7.60	7.60	534
11.40	11.40	11.40	337
15.20	15.20	15.20	247
–	30.40	30.40	119
–	60.80	60.90	58.3
–	–	91.20	38.6
–	121.60	121.60	28.9
–	182.40	182.40	19.2
–	243.20	243.20	14.4
–	364.80	364.80	9.83
–	483.40	483.40	7.21

## 2.5. Parameters

Cell numbers were quantified on a daily basis using a flowcytometer (Beckman-Coulter XL MCL). A PAM fluorometer (Pulse Amplitude Modulated-CONTROL Universal Control Unit, WATER-mode, Walz, Germany) was used to determine  $F_0$  (chlorophyll-a autofluorescence),  $F_m$  (maximum chlorophyll-a fluorescence) and  $F_v/F_m$  (photochemical quantum efficiency, where  $F_v = F_m - F_0$ ) of fresh samples in relation to the different treatments.  $F_0$  was always measured on samples taken during the light period, but the samples were stored in the dark for 15 min prior to the actual PAM measurements.

## 2.6. Data treatment

Maximum growth rates ( $\mu_{\text{max}}$ ,  $\text{d}^{-1}$ ) and half saturation values ( $K_m$ , M or  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) were determined using a non-linear Monod fit (Monod, 1950) of the changes in cell numbers in time. Growth rates were calculated over at least three consecutive days during exponential growth. The 95%

upper and lower confidence limits of  $\mu_{\max}$  and  $K_m$  were calculated using SYSTAT. Subsequently, N, P and Fe quota (mol cell<sup>-1</sup>, as well as mol mol<sup>-1</sup> C) were calculated based on the total nutrient concentration at the start of the experiment divided by the maximum yield in cell numbers and the carbon content of the cells, respectively.

### 3. Results and discussion

Pico-phytoplankton (prokaryotic and eukaryotic) has a circumglobal distribution, and can be found in relatively high numbers during all seasons (Li, 1995; Agawin et al., 1998), also in oligotrophic waters (Veldhuis et al., 2005). The importance of pico-phytoplankton on a global scale is in sharp contrast to the paucity of basic physiological data on most of the species. Especially for the parameterisation of Ocean Biogeochemical Climate Models (OBCMs), experimental data are lacking for the pico-eukaryotes. The NH<sub>4</sub><sup>+</sup>, PO<sub>4</sub><sup>3-</sup>, Fe and light-limitation experiments that we have performed in this study provide additional data for some representative species. In conclusion, it is difficult to stop growth of these pico-phytoplankton species as result of low NH<sub>4</sub><sup>+</sup>, PO<sub>4</sub><sup>3-</sup> or Fe concentrations or decreased light conditions. Moreover, nutrient quota are, as can be expected for small cells, low, both expressed as mol cell<sup>-1</sup>, and as expressed on C basis (when differences in size are ruled out). The cells appear well adapted to oligotrophic, and low light conditions, in agreement with their dominance in oligotrophic waters and in the deep chlorophyll maximum (Raven, 1994).

#### 3.1. NH<sub>4</sub><sup>+</sup> and PO<sub>4</sub><sup>3-</sup> limitation experiments

Increased NH<sub>4</sub><sup>+</sup> concentration in the medium resulted in stimulation of the growth rates for all three species (Figs. 1A, 2A, 3A). At the lowest NH<sub>4</sub><sup>+</sup> concentrations, growth rates decreased, but did not halt completely. The  $\mu_{\max}$  values for ammonium varied between 0.34 and 0.67 d<sup>-1</sup>, with a considerable range of the upper and lower 95% confidence limits (Table 2). The  $K_m$  values ranged between 1.03 and  $2.64 \times 10^{-6}$  M NH<sub>4</sub><sup>+</sup>, as with the  $\mu_{\max}$  data, with a considerable variance (Table 2).

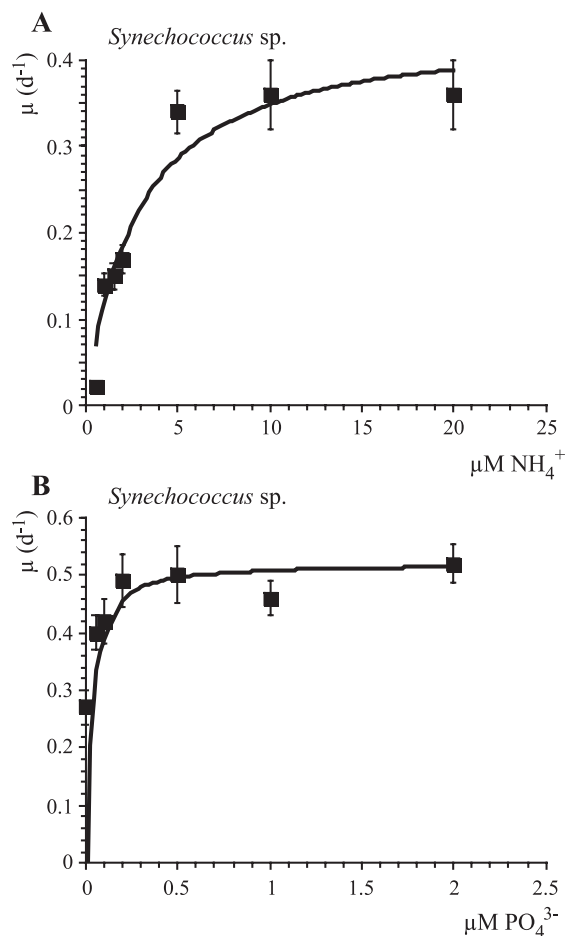


Fig. 1. Growth rates ( $\mu$ , d<sup>-1</sup>) versus (A) concentrations of NH<sub>4</sub><sup>+</sup> and (B) concentrations of PO<sub>4</sub><sup>3-</sup> (both in 10<sup>-6</sup> M) in cultures of *Synechococcus* sp. The data points indicate average growth rates  $\pm$  S.D. (n=3). When no error bars are visible, they are smaller than the symbol. The line is a non-linear Monod fit, used to calculate  $\mu_{\max}$  and  $K_m$  (Table 2).

For the PO<sub>4</sub><sup>3-</sup> limitation experiments (Figs. 1B, 2B, 3B), effects on growth rates were seen only in cultures of *P. capsulatus* and *Synechococcus* sp. Higher growth rates were measured at higher PO<sub>4</sub><sup>3-</sup> concentrations for these species. Of the three species tested, only *P. capsulatus* did not grow when no phosphate was added, indicating that the other two species were able to grow on the very low concentrations present in the filtered seawater. Increased PO<sub>4</sub><sup>3-</sup> concentrations in the medium of cultures of *P. calceolata* did not result in stimulatory effects. The  $\mu_{\max}$  values in these experiments varied between 0.50 and 0.82 d<sup>-1</sup>, with a

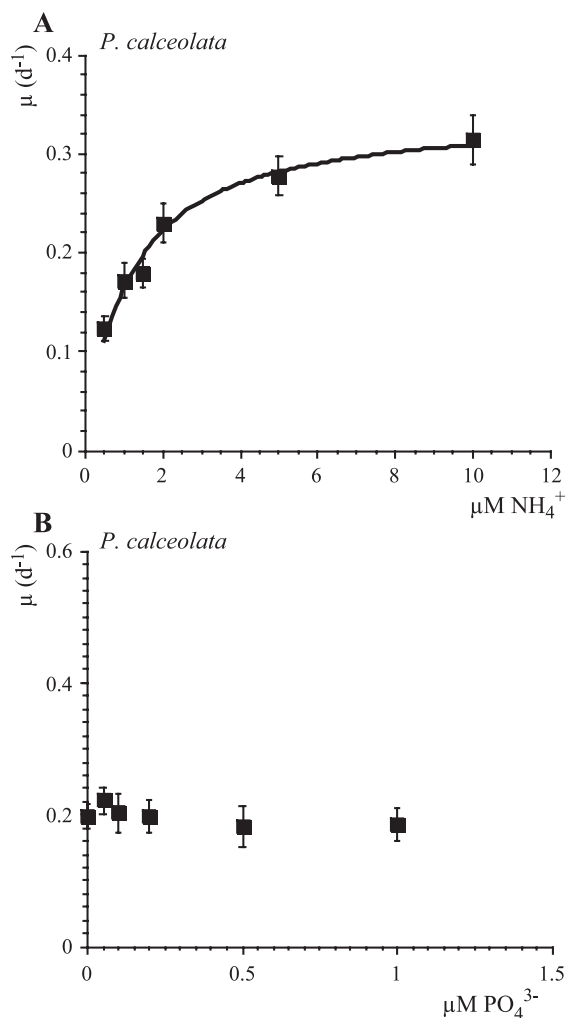


Fig. 2. Growth rates ( $\mu$ ,  $\text{d}^{-1}$ ) versus (A) concentrations of  $\text{NH}_4^+$  and (B) concentrations of  $\text{PO}_4^{3-}$  (both in  $10^{-6}$  M) in cultures of *P. calceolata*. The data points indicate average growth rates  $\pm$  S.D. ( $n=3$ ). When no error bars are visible, they are smaller than the symbol. The line is a non-linear Monod fit, used to calculate  $\mu_{\text{max}}$  and  $K_m$  (Table 2).

considerable range of the upper and lower 95% confidence limits (Table 2). The  $K_m$  values were between  $0.014$  and  $0.094 \times 10^{-6}$  M  $\text{PO}_4^{3-}$  and varied considerably (Table 2).

Maximum growth rates of *Synechococcus* sp. and *P. calceolata* in both  $\text{NH}_4^+$  and  $\text{PO}_4^{3-}$  limitation experiments were relatively low, but they lie in the range of those reported for other pico-phytoplankton in laboratory cultures. Maximum growth rates of 5 species of pico-eukaryotes were reported in the range

of  $0.23$ – $0.91$   $\text{d}^{-1}$  (Jacquet et al., 2001). Similarly, maximum growth rates in the range of  $0.8$ – $2.0$   $\text{d}^{-1}$  were reported for different classes of pico-eukaryotes (Glover et al., 1987). The maximum growth rates for the three species used in our study were lower than those reported in the field studies. For example, maximum in situ growth rates of *Synechococcus* of  $2.1$   $\text{d}^{-1}$  have been reported (Furnas and Crosbie, 1999). For *Synechococcus* sp. in our study, differ-

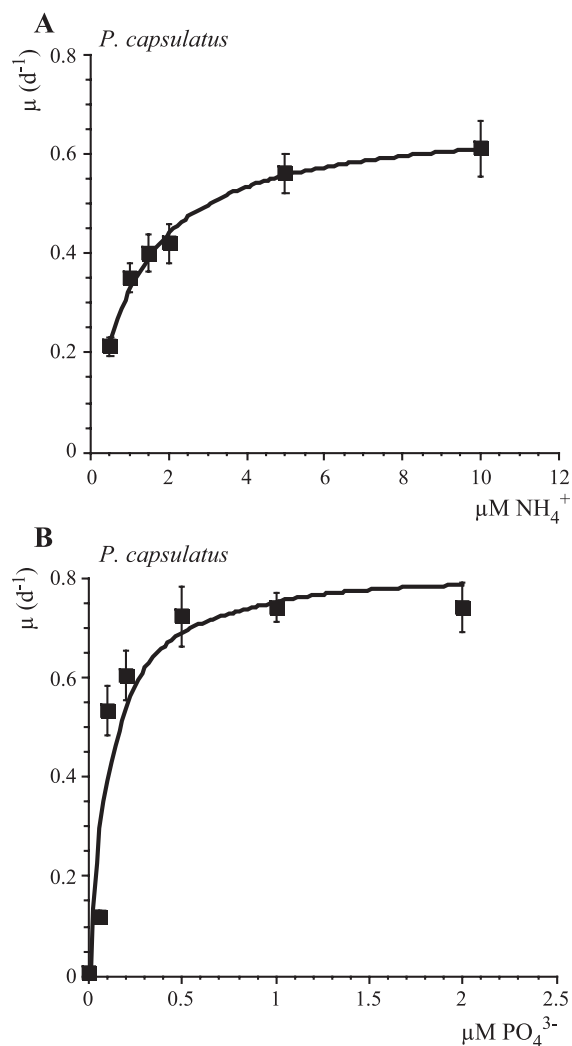


Fig. 3. Growth rates ( $\mu$ ,  $\text{d}^{-1}$ ) versus (A) concentrations of  $\text{NH}_4^+$  and (B) concentrations of  $\text{PO}_4^{3-}$  (both in  $10^{-6}$  M) in cultures of *P. capsulatus*. The data points indicate average growth rates  $\pm$  S.D. ( $n=3$ ). When no error bars are visible, they are smaller than the symbol. The line is a non-linear Monod fit, used to calculate  $\mu_{\text{max}}$  and  $K_m$  (Table 2).

Table 2

Maximum specific growth rates ( $\mu_{\max}$ ,  $\text{d}^{-1}$ ) and half saturation values ( $K_m$ , M) with respect to  $\text{NH}_4^+$ ,  $\text{PO}_4^{3-}$  and  $\text{Fe}'$  in cultures of *Synechococcus* sp., *P. calceolata* and *P. capsulatus*

	$\text{NH}_4^+$	
	$\mu_{\max}$	$K_m$
<i>Synechococcus</i> sp.	0.441 (0.334–0.548)	$2.635 (1.314\text{--}5.286) \times 10^{-6}$ $R^2=0.94$
<i>P. calceolata</i>	0.341 (0.297–0.384)	$1.037 (0.681\text{--}1.580) \times 10^{-6}$ $R^2=0.97$
<i>P. capsulatus</i>	0.672 (0.622–0.722)	$1.036 (0.813\text{--}1.322) \times 10^{-6}$ $R^2=0.99$
	$\text{PO}_4^{3-}$	
	$\mu_{\max}$	$K_m$
<i>Synechococcus</i> sp.	0.503 (0.312–0.695)	$0.014 (0.0003\text{--}0.79) \times 10^{-6}$ $R^2=0.93$
<i>P. calceolata</i>	n.d.	n.d.
<i>P. capsulatus</i>	0.822 (0.634–1.010)	$0.094 (0.0374\text{--}0.231) \times 10^{-6}$ $R^2=0.92$
	$\text{Fe}'$	
	$\mu_{\max}$	$K_m$
<i>Synechococcus</i> sp.	0.551 (0.550–0.581)	$1.39 (1.12\text{--}1.73) \times 10^{-15}$ $R^2=0.98$
<i>P. calceolata</i>	0.903 (0.846–0.961)	$31.0 (21.8\text{--}44.1) \times 10^{-18}$ $R^2=0.95$
<i>P. capsulatus</i>	1.193 (1.038–1.350)	$0.376 (0.242\text{--}0.584) \times 10^{-15}$ $R^2=0.94$

A non-linear Monod fit was used to calculate  $\mu_{\max}$  and  $K_m$ . In parentheses, the 95% upper and lower confidence limits are indicated, as well as  $R^2$  of the fit (all SYSTAT).

ences in maximum growth rates were observed in the  $\text{NH}_4^+$  and  $\text{PO}_4^{3-}$  limitation experiments. The reason for this is yet unknown. When using the same medium, one would expect similar (maximum) growth rates.

All three species tested in our study had low half saturation values with respect to  $\text{NH}_4^+$  and  $\text{PO}_4^{3-}$  concentrations. The experiments could only be done with oligotrophic waters as medium because otherwise the cells would not grow into limitation. The half saturation values that we observed are difficult to compare to results from other studies, given the lack of other data. But our data corroborate findings by others that pico-phytoplankton has the advantage of having a large surface area per unit volume and therefore a low nutrient requirement and/or a very efficient nutrient uptake system (Raven, 1994; Veldhuis et al., 2005). Downscaling the size would be a general ecological advantage for phytoplankton cells (Raven, 1994). As demonstrated in our experimental study, growth rates were affected only at the detection limits of  $\text{NH}_4^+$  and  $\text{PO}_4^{3-}$ .  $K_m$  values were well above ambient levels of inorganic N and/or P.

In a theoretical approach, it was shown that even at nitrate levels in the order of  $10$  to  $20 \times 10^{-9}$  M, typical for open ocean waters, this is sufficient for a phytoplankter with a cell size of  $1 \mu\text{m}$  to grow at a

rate of 1 cell division per day (Chisholm, 1992). With respect to phosphorus limitation, experiments conducted with a marine *Synechococcus* indicated that the ambient inorganic phosphorus concentration in the surface ocean of  $0.5 \times 10^{-9}$  M would be sufficient to maintain a growth rate of  $1.0 \text{ d}^{-1}$  (Ikeya et al., 1997). It has been demonstrated that in addition to inorganic N and P, a variety of organically bound N (Wafar et al., 1995) and P (Bjorkman and Karl, 1994; Li, 1998) is present. For both *Prochlorococcus* (Chisholm et al., 1992) and *Synechococcus* (Donald et al., 1997) there is evidence that growth is optimised by enriching the media with these organic compounds. If we assume that organic nutrients act as an (additional) nutrient source for pico-phytoplankton, the growth of this size class of small algae would not be stopped because of nutrient limitation at all in the surface waters of the oligotrophic oceans.

The N and P quota for the three species tested in our study varied from deplete to replete growth conditions. Under deplete conditions N quota ( $\text{QN}_{\min}$ , cf. Droop, 1973) ranged from  $25.3$  to  $31.1 \times 10^{-15}$  mol cell $^{-1}$  and from  $0.035$  to  $0.158$  mol N mol C $^{-1}$  (Table 3). Under nutrient-replete growth conditions, the N quota were higher and varied from  $45.0$  to  $136.2 \times 10^{-15}$  mol N cell $^{-1}$  and from  $0.062$  to  $0.495$  mol N mol $^{-1}$  C

Table 3

Ranges of N, P (deplete and replete growth conditions) and Fe (only Fe replete growth conditions) quota per cell and standardised per carbon for cultures of *Synechococcus* sp., *P. calceolata* and *P. capsulatus*, in comparison with literature data

This study	N $\times 10^{-15}$ mol cell <sup>-1</sup>	P $\times 10^{-15}$ mol cell <sup>-1</sup>	Fe $\times 10^{-21}$ mol cell <sup>-1</sup>	
<i>Synechococcus</i> sp.	31.1–136.2	1.76–3.83	1.6	
<i>P. calceolata</i>	47.4–112.1	1.93–4.66	0.9	
<i>P. capsulatus</i>	25.3–45.0	1.30–2.93	3.1	
	N mol mol <sup>-1</sup> C	P mmol mol <sup>-1</sup> C	Fe nmol mol <sup>-1</sup> C	
<i>Synechococcus</i> sp.	0.113–0.495	6.36–13.93	5.8	
<i>P. calceolata</i>	0.158–0.408	6.43–15.5	3.0	
<i>P. capsulatus</i>	0.035–0.062	1.80–4.04	4.3	
<i>Synechococcus</i> (Bertilsson et al., 2003)	nutrient replete		P-limited	
	N $\times 10^{-15}$ mol cell <sup>-1</sup>	P $\times 10^{-15}$ mol cell <sup>-1</sup>	N $\times 10^{-15}$ mol cell <sup>-1</sup>	P $\times 10^{-15}$ mol cell <sup>-1</sup>
<i>Synechococcus</i> WH8012	1.4	0.06	1.5	0.02
<i>Synechococcus</i> WH8103	1.5	0.11	2.9	0.03
<i>Synechococcus</i> (Heldal et al., 2003)	N (average) $\times 10^{-15}$ mol cell <sup>-1</sup>		P (average) $\times 10^{-15}$ mol cell <sup>-1</sup>	
<i>Synechococcus</i> WH7803	1.5		0.2	
<i>Synechococcus</i> WH8103	2.1		0.1	

(Table 3). The latter corresponds to C : N ratios ranging from 2.0 (N replete) to 28.6 (N deplete). Under deplete conditions P quota (QP<sub>min</sub>, cf. Droop, 1973) ranged from 1.30 to 1.76  $\times 10^{-15}$  mol cell<sup>-1</sup> and from 1.8 to 6.36 mmol P mol<sup>-1</sup> C (Table 3). Under nutrient-replete growth conditions the P quota ranged from 2.93 to 4.66  $\times 10^{-15}$  mol P cell<sup>-1</sup> and from 4.04 to 15.5 mmol P mol<sup>-1</sup> C (Table 3). The latter corresponds to C : P ratios varying from 65 (P replete) to 555 (P deplete). Standardisation of the N and P quota on the basis of C showed that differences between the three species cannot be explained by cell size. The species with the largest cells, *P. capsulatus*, also have the lowest N and P quota on C basis. However, due to the changes in N and P concentrations, small changes in cellular volumes could have taken place. Using the flowcytometer we could not detect these changes.

The findings on the variations in N and P quota are in agreement with the observations that the cellular composition of pico-phytoplankton is subject to

substantial change, for instance due to changes in the physico-chemical environment (Bertilsson et al., 2003; Heldal et al., 2003; Veldhuis et al., 2005). However, the absolute values that we report were higher than reported in other studies. The N and P quota in *Synechococcus* ranged from 1.4–1.5  $\times 10^{-15}$  mol N cell<sup>-1</sup> and 0.06–0.11  $\times 10^{-15}$  mol P cell<sup>-1</sup> under nutrient-replete conditions to 1.5–2.9  $\times 10^{-15}$  mol N cell<sup>-1</sup> and 0.02–0.03  $\times 10^{-15}$  mol P cell<sup>-1</sup> under P-limited conditions (Table 3). Similarly low N and P quota were measured in two strains of *Synechococcus* (Heldal et al., 2003) (Table 3). These values are typically one to two orders of magnitude lower than those reported in our study.

The F<sub>v</sub>/F<sub>m</sub> values had no clear relation with the concentrations of NH<sub>4</sub><sup>+</sup> or PO<sub>4</sub><sup>3-</sup> in the medium for all three species tested (Table 4). The results of the F<sub>v</sub>/F<sub>m</sub> measurements in our study confirm the above observations on the (limited) effects of NH<sub>4</sub><sup>+</sup> and/or PO<sub>4</sub><sup>3-</sup> limitation on cultures of *Synechococcus* sp., *P. calceolata*

Table 4

Photochemical quantum efficiency in relation to  $\text{NH}_4^+$  or  $\text{PO}_4^{3-}$  (concentrations in  $10^{-6}$  M) for cultures of *Synechococcus* sp., *P. calceolata* and *P. capsulatus*, expressed as  $F_v/F_m$  (arbitrary units)

NH <sub>4</sub> <sup>+</sup> limitation experiment			
concentration	<i>Synechococcus</i> sp.	<i>P. calceolata</i>	<i>P. capsulatus</i>
0.00	0.452	0.348	0.447
1.00	0.546	0.301	0.449
1.50	0.548	0.286	0.501
2.00	0.541	0.256	0.528
5.00	0.562	0.295	0.500
10.0	0.605	0.279	0.520
PO <sub>4</sub> <sup>3-</sup> limitation experiment			
concentration	<i>Synechococcus</i> sp.	<i>P. calceolata</i>	<i>P. capsulatus</i>
0.00	0.506	0.193	0.360
0.05	0.496	0.216	0.479
0.10	0.474	0.199	0.355
0.20	0.539	0.233	0.444
0.50	0.535	0.228	0.408
1.00	0.638	0.162	0.386

*lata* and *P. capsulatus*. The relatively high  $F_v/F_m$  values of *Synechococcus* sp. and *P. capsulatus* may be explained by the observations that these values may restore themselves by adaptation to nutrient stress (Parkhill et al., 2001). It has been demonstrated that especially iron limitation has a strong effect on  $F_v/F_m$  values (Geider and LaRoche, 1994; Schmidt et al., 1999).  $F_v/F_m$  is reported also to be affected by N limitation (Kolber et al., 1990), but this is obviously not confirmed in our experiments. For *P. calceolata*, the  $F_v/F_m$  values were low throughout the experiment, but the reasons for this are unknown.

### 3.2. Fe limitation experiments

The three species tested in this study showed decreased growth rates with low  $\text{Fe}'$  concentrations (Fig. 4A–C). The  $\mu_{\max}$  values for  $\text{Fe}'$  varied between 0.55 and  $1.19 \text{ d}^{-1}$ , with a considerable range of the upper and lower 95% confidence limits (Table 2). The  $K_m$  values ranged from 0.38 to  $31.0 \times 10^{-15} \text{ M Fe}'$ , and varied considerably (Table 2). The deviating data point (high growth rate) at the lowest  $\text{Fe}'$  concentrations is a check on the use of DFB. For these data points, excess Fe was added, and as a result growth rates resumed, indicative of the fact that DFB caused only  $\text{Fe}'$  concentrations to become limiting.

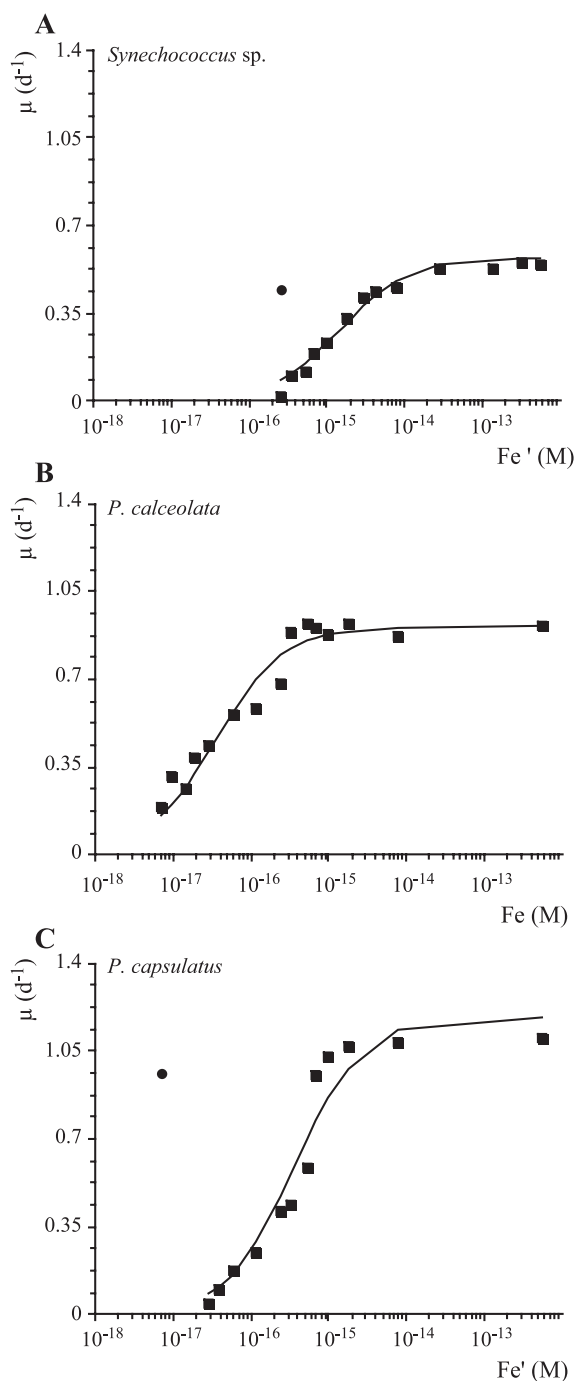


Fig. 4. Growth rates ( $\mu$ ,  $\text{d}^{-1}$ ) versus concentrations of  $\text{Fe}'$  (M) in cultures of (A) *Synechococcus* sp., (B) *P. calceolata*, and (C) *P. capsulatus*. Note the log scale of  $\text{Fe}'$  concentrations. The line is a non-linear Monod fit, used to calculate  $\mu_{\max}$  and  $K_m$  (Table 2). The filled circle indicates the result of addition of excess Fe to the cultures with the lowest growth rate.

Even the cleanest seawater medium, manipulated under clean conditions, contained too high Fe concentrations to induce Fe limitation in any of the three species tested. Only increased concentrations of DFB (and only high concentrations) could halt growth completely. The Fe' concentrations that did affect growth rates in *Synechococcus* sp., *P. calceolata* and *P. capsulatus* were in the range of  $10^{-15}$  to  $10^{-19}$  M. This corresponds to concentrations of dissolved Fe of  $10^{-13}$  to  $10^{-14}$  M, which is much lower than reported for field conditions (De Baar and De Jong, 2000). For *P. calceolata*, only a slight reduction in growth rates was reported at a concentrations of 3 pM Fe' (Sunda and Huntsman, 1995).

Based on the results of growth in relation to iron availability, it is not a realistic scenario that under field conditions growth of these small phytoplankton species will be completely stopped. Our experimental results are supported by numerous field observations. The Southern Ocean, together with the Subarctic Pacific and Equatorial Pacific, belongs to the high nutrient-low chlorophyll (HNLC) regions and it is now well accepted that iron plays a key role in the structure and functioning of these ecosystems (Martin et al., 1990; De Baar and Boyd, 2000). It is generally assumed that the smaller sized phytoplankton species, due to a large surface to volume ratio, possess an ecological advantage in terms of having a smaller nutrient (iron) requirement or a more efficient uptake of iron compared to larger species (Boyd et al., 2000; Timmermans et al., 2001). Despite this apparent ecological advantage, it does not result in a total dominance of the pico-phytoplankton community in these waters. In terms of chlorophyll biomass, the pico-phytoplankton component in the Southern Ocean is much lower than in the tropical oceans. Other factors, such as a high grazing pressure on the smaller sized phytoplankton (Price et al., 1994), must therefore be important. The maximum growth rates as found in the Fe limitation experiments are higher than in the previously described  $\text{NH}_4^+$  and  $\text{PO}_4^{3-}$  limitation experiment. In the Fe limitation experiments, Antarctic seawater was used, which was previously not depleted in major and micro nutrients. This in contrast to the Gulf of Biscay water, which was collected at the end of the growing season of the phytoplankton.

It should be noted that extremely high concentrations of DFB were necessary to induce full Fe

limitation in *Synechococcus* sp., *P. calceolata* and *P. capsulatus*. Up to about  $500 \times 10^{-9}$  M DFB had to be added to seawater with a background concentration of dissolved Fe of  $1.1 \times 10^{-9}$  M. Previously, a more gradual response of the small Antarctic diatom *Chaetoceros brevis* to increased concentrations of DFB was demonstrated (Timmermans et al., 2001). This was interpreted as evidence that, as long as DFB is not in excess, some species of Fe were still available for growth. However, with higher molar Fe : DFB ratios (up to 1 : 10), as used by Timmermans and co-workers (Timmermans et al., 2001), iron obviously became unavailable, thereby totally inhibiting growth for *C. brevis*. It was demonstrated that with molar ratios of Fe to DFB of 1 : 5 to 1 : 10, some Fe was still available for the small diatom *Thalassiosira weissfloggii* (Hutchins et al., 1999). The growth rates of *Synechococcus* were hardly effected, not even in the presence of DFB (Wells et al., 1994). The same can be concluded for the three species of pico-phytoplankton that were tested in this study. However, here the ratios were much more extreme: only with an excess of DFB (ratio Fe : DFB 1 : 500 !) did all Fe become unavailable. This demonstrates that more information is needed on the bio-availability of Fe for phytoplankton, certainly in experiments in which the Fe concentrations are manipulated with a surplus of a complexing agent.

Measurements of Fe iron quota in pico-phytoplankton are scarce and hindered by technical complications, as for example Fe adsorbed externally (Wilhelm et al., 1996), and/or the inability to measure the concentration and kinetics of the bioavailable iron pool. Minimum Fe quota (only for cultures grown at Fe replete conditions) in the three species that we tested during our study were extremely low, in the zepto-mol ( $10^{-21}$  mol)  $\text{cell}^{-1}$  range (Table 3). It is realised that these extremely low values are biased by taking in account only dissolved Fe concentrations, ignoring kinetics of Fe. In the case of *Synechococcus*, cellular iron values were reported to vary over 4 orders of magnitude with changing ambient iron concentration (Wilhelm et al., 1996). A 'true' estimate of the iron content of *Synechococcus* was achieved using continuous cultures resulting in an iron quatum of  $0.42 \times 10^{-18}$  M  $\text{cell}^{-1}$  (Wilhelm and Trick, 1995), about 100 times higher than the value reported for *Synechococcus* in this study. Similarly, Fe quota on C basis of 10–20  $\mu\text{mol mol}^{-1}$  have been reported for *P. calceolata* (Sunda and Huntsman, 1995), about three

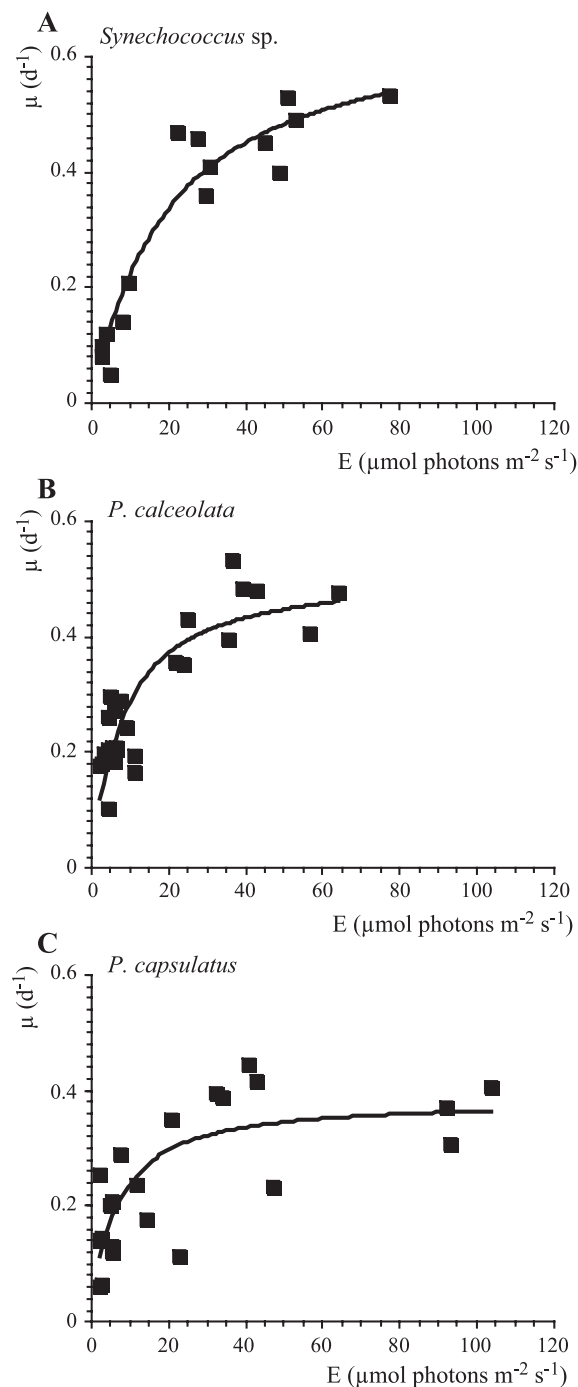


Fig. 5. Growth rates ( $\mu$ ,  $\text{d}^{-1}$ ) versus irradiance ( $E$ ,  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) in cultures of (A) *Synechococcus* sp., (B) *P. calceolata*, and (C) *P. capsulatus*. The line is a non-linear Monod fit, used to calculate  $\mu_{\text{max}}$  and  $K_m$  (Table 5).

orders of magnitude higher than the same quatum reported for this species in our study ( $3.0 \text{ nmol Fe mol}^{-1} \text{ C}$ ).

### 3.3. Light limitation experiments

The lower irradiance levels resulted in decreased growth rates in cultures of all three species (Fig. 5A–C). At the lowest irradiance levels, growth was limited but not completely stopped. The  $\mu_{\text{max}}$  values ranged from 0.39 to  $0.67 \text{ d}^{-1}$ , with a considerable range in upper and lower 95% confidence limits (Table 5). The half saturation values for light ranged from 5.75 to  $19.7 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ , and varied considerably (Table 5).

The maximum growth rates in the light limitation experiments with *Synechococcus* sp., *P. calceolata* and *P. capsulatus* were in the range of those previously described for *Prochlorococcus* sp. and *Synechococcus* sp. (Armbrust et al., 1989; Moore et al., 1995; Moore and Chisholm, 1999), and pico-eukaryotes (Glover et al., 1987; Jacquet et al., 2001). Our experimental findings once more demonstrate that the pico-phytoplankton growth rates will only be decreased at low irradiances ( $<10 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ). This is a good illustration of the fact that the small phytoplankton cells are often found at the deep chlorophyll maximum (Iriarte and Purdie, 1993; Raven, 1994; Veldhuis and Kraay, 2004). The fact that *Synechococcus* sp. had the highest  $K_m$  values in our experiments confirms the apparent advantage of oceanic eukaryotic ultraplankton in low light conditions, possible due to their efficient use of dim blue-violet light (Glover et al., 1987). During our experi-

Table 5

Maximum growth rates ( $\mu_{\text{max}}$ ,  $\text{d}^{-1}$ ) and half saturation values ( $K_m$ ,  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) for cultures of *Synechococcus* sp., *P. calceolata* and *P. capsulatus* with respect to different irradiance levels

	$\mu_{\text{max}}$	$K_m$
<i>Synechococcus</i> sp.	0.674 (0.523–0.826)	19.7 (10.7–36.1) $R^2=0.92$
<i>P. calceolata</i>	0.516 (0.430–0.603)	7.66 (4.71–12.5) $R^2=0.74$
<i>P. capsulatus</i>	0.385 (0.303–0.467)	5.75 (2.64–12.81) $R^2=0.58$

A non-linear Monod fit was used to calculate  $\mu_{\text{max}}$  and  $K_m$ . In parentheses, the 95% upper and lower confidence limits are indicated, as well as  $R^2$  of the fit (all SYSTAT).

ments the highest irradiance levels were such that no photo-inhibition occurred.

#### 4. General conclusions

Our experimental results indicate that the growth of the pico-phytoplankton species as tested here will hardly ever be halted by low  $\text{NH}_4^+$ ,  $\text{PO}_4^{3-}$ , Fe concentrations or low irradiance levels.  $K_m$  values with respect to  $\text{NH}_4^+$ ,  $\text{PO}_4^{3-}$ , Fe concentrations and irradiance levels generally were below ambient levels of these factors. N, P and Fe quota (per cell and per mol C) were low, but for *Synechococcus* higher than reported by other studies. The N and P quota did vary substantially from nutrient-deplete to nutrient-replete conditions. This can have major implications for the assessment and modelling of the role of pico-phytoplankton in the fluxes of N and P (and Fe !) through oceanic foodwebs.

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