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Effect of N:P ratios on response of Mediterranean picophytoplankton to experimental nutrient inputs

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ABSTRACT: The effect of variations in N:P ratios on Mediterranean picoplankton *Synechococcus* sp. was tested with a nutrient enrichment experiment in large-scale mesocosms in a coastal Mediterranean bay community during summer 1998. By adding either N or P in excess of 42 mmol m^{-2} d^{-1} N or 2.1 mmol m^{-2} d^{-1}, the mesocosm units (16 m^3) received nutrient ratios varying between 2.5 to 160, i.e. 8-fold lower to 8-fold higher than background levels (N:P 20). The total phytoplankton had increased significantly after 1 wk of the experiment on mesocosms receiving high N:P ratios (excess N), with diatoms being mainly responsible for this increase. In contrast, *Synechococcus* sp., which along with small flagellates initially dominated the water column, rapidly increased in biomass, abundance and primary production, with higher abundance in mesocosms receiving excess N and P, and lower abundance in mesocosms receiving the background N:P load of 20 that is normal for the Bay of Blanes. Our study indicated that this complex pattern resulted from the response of the grazing community, which was highest at the 'background' ratio. Indeed, the potential grazers of *Synechococcus* sp. (heterotrophic nanoflagellates and phagotrophic ciliates) were highly abundant at the background ratio. Our results also showed that primary production of *Synechococcus* sp. was significantly inversely correlated ($r = -0.98$, $p < 0.05$) with specific grazing rates, suggesting that picophytoplankton responses to N:P loads arose from top-down effects related to the responses of the grazing community to the various N:P levels. The top-down effects were, however, obscured during the early part of the experiment, when there were transient imbalances between growth and loss rates of picophytoplankton owing to time lags between the growth of picophytoplankton and the growth of their grazers (heterotrophic nanoflagellates and phagotrophic ciliates). This resulted in a general net increase in *Synechococcus* sp. abundance in all mesocosms.

KEY WORDS: Picophytoplankton · N:P ratios · Mediterranean Sea · Nutrient

INTRODUCTION

Comparative (Agawin et al. 2000, Bell & Kalff 2001) and experimental (Duarte et al. 2000) analyses have shown that increased nutrient inputs lead to a dominance of microphytoplankton. There are, however, exceptions, and dense blooms of picophytoplankton in P-enriched systems have been reported (Phlips et al. 1999). Picophytoplankton seem to be frequently P-limited (Vaulot et al. 1996, Thingstad et al. 1998), so that a shift from pico- to microphytoplankton dominance with increasing nutrient input may be dependent on the N:P ratio of such input, a possibility that had not yet been tested in marine systems. Shifts from pico- to microphytoplankton dominance have important implications (such as a shift from a microbial to a 'linear' food web: Thingstad & Sakshaug 1990) for carbon flow in the pelagic ecosystem. In freshwater systems, the issue of N:P ratio and picophytoplankton has been quite extensively studied in laboratory and

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field conditions. Suttle & Harrison (1988) reported the dominance of picocyanobacteria *Synechococcus* sp. at a higher N:P ratio (N:P = 45) in laboratory conditions, while diatoms dominated at a lower N:P ratio. Likewise, Takamura & Nojiri (1994) reported a positive correlation of the picophytoplankton contribution to total phytoplankton biomass with the N:P ratio in lake waters.

Contrasting effects of N:P ratios on picophytoplankton and microphytoplankton are plausible, since experimental evidence suggests that the 2 groups have different phosphorus nutrition requirements (e.g. *Synechococcus* sp. vs *Thalassiosira* spp.: Donald et al. 1997) and different nutrient uptake efficiencies, due to their different surface area to volume ratios (Fogg 1986, Raven 1998). Changes in N:P ratios may affect the species composition of an ecosystem at all trophic levels, and may have an impact on competition between different species (Dederen 1992, Egge & Heimdal 1994). Hence, changes in N:P ratios may affect picophytoplankton through the responses of the grazing community, which will lead to changes in top-down control. These responses could alter the paths of carbon and nutrient flow in the food web. There is, therefore, a need to experimentally test the response of the picophytoplanktonic community to varying N:P ratios.

Here, we report the effect of variations in the N:P ratio on the responses of Mediterranean coastal picophytoplankton (population size, primary production, growth rates and grazing losses) in a large-scale nutrient-enrichment experiment in mesocosms in the Bay of Blanes, NW Mediterranean. During summer, the picophytoplankton (mostly composed of *Synechococcus* sp.), provide an important source of organic carbon and nutrients for this coastal Mediterranean food web, contributing >30% of total phytoplankton biomass and production (Agawin et al. 1998).

MATERIALS AND METHODS

Experimental design. The mesocosm experiment was conducted from 1 to 20 July 1998 in the Bay of Blanes, NE Spain, a NW Mediterranean bay (41° 39.90′ N, 2° 48.03′ E). The bay lies off the town of Blanes, and the background stoichiometric ratio of nutrient inputs during summer was 20:7:1 N:Si:P (Duarte et al. 2000). The experimental set-up consisted of 8 mesocosm units (8 m in height, 4.2 m^2 cross-sectional area, with an effective volume of 16 m^3), suspended from a platform moored 1 km offshore in the Bay of Blanes at a depth of 35 m. Based on a previous mesocosm experiment (Duarte et al. 2000), a load of 42 mmol m^{-2} d^{-1} N and 2.1 mmol m^{-2} d^{-1} P (about 4 times the normal nutrient loading rate in the bay), was considered sufficient to induce a clear stimulation of growth in the planktonic community. The experiment tested the response to N:P ratios varying between 2.5 and 160, from 8-fold lower to 8-fold higher than the N:P ratio of the background load, i.e. 20 N:P. The actual amounts of nutrients added to the mesocosm were 42 mmol m^{-2} d^{-1} N, 2.1 mmol m^{-2} d^{-1} P, and 14.7 mmol m^{-2} d^{-1} Si (added to the N:P background load of 20). The N and P loads were then raised by 2-, 4- and 8-fold to achieve the other N:P treatment ratios. Silicon was kept at non-limiting concentrations by scaling the Si:nutrient ratio to the added nutrients. The range of experimental N:P input reflected the range of dissolved inorganic N:P in the Bay of Blanes (A. Lucea et al. unpubl. data). We set up 2 mesocosms receiving an N:P load of 20, i.e. the background nutrient ratio in the Bay of Blanes. The nutrients were added as NH_4Cl (N), KH_2PO_4 (P), and Na_2SiF_6 (Si) every second day by filling a tube extending from the water surface to the bottom of the mesocosms. The tube was slowly withdrawn to ensure homogeneous distribution throughout the water column. To determine whether the responses observed over the first 14 d of the experiment (Phase I, a period considered sufficient for the development of response blooms in this area: Duarte et al. 2000), were due to N or P imbalance, the mesocosms were enriched with N or P, as required, to equilibrate the N:P ratios to 20:1, the background nutrient ratio in the Bay of Blanes (Phase II). Integrated water samples (0 to 6 m depth) of 50 l were taken daily over the first 4 d (to examine the initial response of the picoplankton), and subsequently at 2 d intervals for biological and chemical (dissolved nutrient concentrations) analyses. The use of an integrated sample during the experiment was justified since the seawater inside the mesocosm units, as well as that outside the mesocosms, was well mixed. The walls of the mesocosms were flexible (polyethane), and as they were not filled to capacity, turbulence propagation was possible, resulting in a thorough mixing of the contents within a few minutes, as revealed by dye experiments in a pilot experiment. (The same type of mesocosm units and sampling procedure were used by Agawin et al. 2000, 2002, and Duarte et al. 2000.) A water volume of 200 ml was filtered through Whatman GF/F filters for fluorometric analysis of total chlorophyll concentrations in the water (Parsons et al. 1984). The filters were homogenized and kept refrigerated in the dark, and pigments were extracted in 90% acetone for ca. 6 h. Following extraction, fluorescence was measured in a Turner Designs fluorometer calibrated with pure chlorophyll *a* (Sigma). Dissolved inorganic phosphate, nitrate and nitrite were analyzed following standard methods (Hansen & Koroleff 1999), and ammonium concentrations were measured spectrofluorometrically (Kéruel & Aminot 1997).

Picophytoplankton abundance, primary production, growth rate and losses. The abundance of autotrophic picoplankton, dominated by *Synechococcus* sp. (93% of the population), was estimated using flow cytometry. Fresh subsamples of water from the different mesocosm units were filtered through a 50 µm mesh, maintained in the dark, and analyzed in a FACSCalibur (Becton Dickinson) flow cytometer according to the population fluorescence and light-scatter characteristics reported in Vaulot et al. (1990). Cell size (diameter) of *Synechococcus* sp. was calculated from the forward scatter (FSC) data of the cells, and calibrated using fluorescent beads of various sizes mixed with algal cultures of known sizes. The average cell volume was calculated from the estimated diameter of the cells, based on the coccoid shape of *Synechococcus* sp.

Primary production of *Synechococcus* sp. was measured with water samples from the mesocosm units filtered through 2 µm pore-size polycarbonate filters. Three 125 ml clear Nalgene bottles were dispensed with 120 ml fractionated samples from each mesocosm unit; 2 bottles were used for photosynthesis measurements, 1 was used as a dark control (bottles wrapped in black plastic bags). We added 1 ml of ^{14}C solution in varying concentrations (4.9 to 20 µCi), depending on the increase in phytoplankton biomass and the anticipated uptake of C. The light and dark bottles were incubated for 3 h at 200 µE m^{-2} s^{-1} in an incubator with temperature control (adjusted to *in situ* temperature). After incubation, the samples were filtered through 0.45 µm Millipore filters, and the filters were fumed over concentrated HCl to remove traces of inorganic carbon. Each of the filters was placed in a scintillation vial, 1 to 2 drops of hydrogen peroxide were added to avoid quenching, followed by 12 ml scintillation fluid. Total phytoplankton primary production was measured using the same method but with whole seawater samples.

The contribution of the picophytoplankton fraction to total phytoplankton primary production and biomass was calculated based on the size-fractionated primary production measurements and estimates of picophytoplankton chlorophyll biomass. The fractional picophytoplankton chlorophyll was determined by converting *Synechococcus* sp. biovolume to chlorophyll using a specific chlorophyll *a* concentration of 3645 g chl m^{-3} cell volume (Barlow & Alberte 1985).

The gross specific growth rate (µ) of *Synechococcus* sp. was determined following the method of Welschmeyer & Lorenzen (1984) as:

$$\mu = \frac{-\ln\left[1-\left(P \times t \times C_p^{-1}\right)\right]}{t}$$

where P is the primary production or C uptake rate (mg C m^{-3} d^{-1}), t is the duration of incubation (d), and C_p (mg C m^{-3}) is the cell carbon present at the end of the incubation:

$$C_p = \Delta C + C_0$$

where ΔC is the carbon fixed during the incubation period, equal to $P \times t$. The cell carbon (C_0) of the *Synechococcus* sp. population at the beginning of the incubation was determined as the product of their biovolume (µm^3 m^{-3}) and the cellular C concentration (mg C µm^{-3}). *Synechococcus* sp. biovolume was calculated as the product of abundance and average cell volume determined (1.15 µm^3). A *Synechococcus* sp. C-content of 0.123 pg µm^{-3} was used in the calculations (Waterbury et al. 1986).

Loss rates (m, which would include grazing losses, mortality due to lysis and, sinking losses) of *Synechococcus* sp. were calculated as:

$$\text{Loss rates } (m, d^{-1}) = \mu\ (d^{-1}) - \text{net population growth rate } (d^{-1})$$

where net population growth rate (d^{-1}) =

$$\frac{\ln\left(\frac{\text{abundance}_t}{\text{abundance}_{t-1}}\right)}{\text{time between samples } (=1 \text{ or } 2 \text{ d})}$$

Protist grazing on *Synechococcus* sp. Specific grazing rates on *Synechococcus* sp. in response to changes in N:P ratios were estimated by following the disappearance rate of a fluorescently labeled analog over time in a mesocosm bag receiving nutrient inputs at the background N:P ratio of 20 for the Bay of Blanes, and those receiving nutrient inputs at N:P ratios of 2.5, 10, 40 and 160. The procedure is a modification of that of Sherr & Sherr (1993), involving (1) the preparation of a fluorescently labeled analog of *Synechococcus* sp., by staining cultured *Synechococcus* SYNMED2 (Mediterranean Sea, July 1993, N. Simon pers. obs.) for 24 h with a yellow green fluorescing dye, DTAF-5-(4'6-dichlorotriazin-2-yl) aminofluorescein without heat-killing the population, and (2) long time-course experiments to assess the disappearance of the fluorescent analog. The experiment was performed twice during Phase I of the experiment (2 and 8 July) and once during Phase II (20 July), using duplicate bottles per mesocosm, and with duplicate subsampling of each bottle. A volume of 700 ml sample water each was gently poured into duplicate 1 l bottles and an additional duplicate of 0.2 µm-filtered water from the same sample water were prepared to control for disappearance of the fluorescent analog due to processes other than grazing (possible growth, and loss of the analog through adsorbance to the bottles). The bottles were placed in an incubator at *in situ* temperature, and left undisturbed for at least 30 min to allow the microbial assemblage to recover from handling stress. The fluorescent analog solution was briefly sonicated for

several 2 s bursts, and uniformly vortex-mixed. A subsample was added to each bottle and quickly but gently mixed into the sample to create a uniform suspension. At selected time intervals (initial $t = 0$, final $t =$ 24 h), duplicate 100 ml subsamples were withdrawn from each bag, and immediately preserved with glutaraldehyde to 1% final concentration in individual containers and stored in the dark at 5°C until epifluorescence analysis. The samples were filtered through 0.2 µm black filters for counts using epifluorescence microscopy. Specific grazing (g) rates (d^{-1}) on *Synechococcus* sp. were calculated following the method of Salat & Marrasé (1994) as $g = -1/t \ln(F_t/F_0)$, where t = incubation time; F_t = number of fluorescent tracers at final time; and F_0 = number of fluorescent tracers at initial time.

Table 1. Concentrations of phosphate, nitrite, nitrate, ammonium and total dissolved inorganic nitrogen, DIN (nitrite + nitrate + ammonium) (µM, mean ± SE for each phase) in the various mesocosms (designated by N:P ratios). Phase I: period of initial experimental conditions (first 14 d); Phase II: period of homogeneous nutrient loading. Initial nutrient concentrations are also shown (for N:P 20, standard error is for 2 duplicate mesocosms)

Mesocosm (N:P ratio)	Initial	Phase I	Phase II
Phosphate			
2.5	0.06	0.33 (0.10)	0.04 (0.03)
5	0.05	0.19 (0.05)	0.03 (0.02)
10	0.34	0.12 (0.03)	0.02 (0.01)
20	0.08 (0.02)	0.11 (0.01)	0.03 (0.02)
40	0.04	0.16 (0.03)	0.07 (0.02)
80	0.06	0.11 (0.02)	0.15 (0.11)
160	0.10	0.13 (0.02)	0.05 (0.04)
Nitrite			
2.5	0.15	0.16 (0.00)	0.18 (0.00)
5	0.14	0.16 (0.00)	0.18 (0.00)
10	0.16	0.16 (0.00)	0.18 (0.01)
20	0.14 (0.01)	0.16 (0.00)	0.18 (0.00)
40	0.14	0.19 (0.01)	0.19 (0.01)
80	0.14	0.18 (0.01)	0.18 (0.01)
160	0.13	0.18 (0.01)	0.19 (0.00)
Nitrate			
2.5	0.36	0.63 (0.07)	4.12 (0.33)
5	0.29	0.63 (0.09)	4.46 (0.73)
10	0.49	0.73 (0.10)	4.21 (0.85)
20	0.39 (0.06)	0.64 (0.00)	4.03 (0.73)
40	0.48	0.62 (0.07)	3.62 (0.61)
80	0.44	0.88 (0.14)	3.63 (0.64)
160	0.31	0.71 (0.08)	3.34 (0.56)
Ammonium			
2.5	0.15	0.31 (0.09)	0.70 (0.38)
5	0.14	0.38 (0.10)	0.67 (0.31)
10	0.23	0.34 (0.10)	0.34 (0.01)
20	0.20 (0.05)	0.33 (0.05)	0.53 (0.14)
40	0.24	2.71 (1.00)	0.37 (0.03)
80	0.23	3.51 (1.41)	0.51 (0.16)
160	0.22	9.60 (3.82)	0.42 (0.07)
DIN			
2.5	0.66	1.11 (0.12)	5.00 (0.48)
5	0.57	1.12 (0.16)	5.30 (0.77)
10	0.87	1.23 (0.14)	4.73 (0.87)
20	0.73 (0.10)	1.13 (0.10)	4.75 (0.58)
40	0.85	3.51 (1.06)	4.18 (0.63)
80	0.80	4.57 (1.42)	4.33 (0.80)
160	0.66	10.48 (3.82)	3.95 (0.63)

Abundance of protists. Water samples of 100 ml were preserved with glutaraldehyde (1% final conc.) and stored from a few to 48 h at 4°C until staining and filtration. Subsamples of 30 to 40 ml (for nanoflagellate counts) were stained with DAPI for 5 min (Porter & Feig 1980; final conc. 1 µg ml^{-1}) and filtered through 0.6 µm black-stained polycarbonate filters to collect nanoflagellates. The filters were then mounted on a slide with a drop of immersion oil and frozen at –20°C until examination under the microscope. The abundance of these microorganisms was determined by epifluorescence microscopy at 1250× (Nikon Optiphot). Ciliate abundance was examined in 1 l samples preserved in acid Lugol's solution (1% final conc.), and settled in 100 ml sedimentation chambers for at least 48 h before enumeration. Samples were examined at 200× or 400× magnification using an inverted microscope (Axiovert 35, Zeiss); 1 replicate was counted per sample. Ciliates were identified to genus level when possible (Lee et al. 1985), and ciliates were grouped into naked oligotrichs (species of *Halteria*, *Strombidium*, *Strobilidium*, *Laboea* and *Tontonia*), loricate oligotrichs (e.g. tintinnids), and scuticociliates and euplotids. The results are given for total phagotrophic (heterotrophic + mixotrophic) ciliates, with data for scuticociliates + euplotids (which are also known bacterivores: Christaki et al. 1998) also given separately.

RESULTS

Variations in the N:P load in the mesocosm units resulted in nutrient imbalance and accumulation in the mesocosms. In mesocosms receiving nutrients with N:P loads >40, dissolved inorganic nitrogen (DIN), primarily in the NH_4^+ form, rapidly increased during the first 4 d of nutrient addition from <0.05 µM at the start of the experiment to 35 µM at the highest N:P load used. After 1 wk, however, the DIN concentrations decreased and leveled off until Phase II of the experiment. On average, DIN concentrations increased up to 16-fold higher than the initial value during Phase I of the experiment in the mesocosm with the highest N:P load used (Table 1). Inorganic phosphorus concentrations followed the same pattern as that of dissolved inorganic nitrogen, except that accumulation of inorganic phosphorus was also observed in mesocosm units receiving N:P loads <10. In the mesocosm receiv-

ing an N:P of 2.5 for example, inorganic phosphate increased from 0.01 to 0.9 µM. Phosphate concentrations increased up to 6-fold higher than the initial value during Phase I of the experiment in the mesocosm at the lowest N:P load (Table 1).

Total phytoplankton biomass measured as chlorophyll concentration significantly increased after 1 wk of the experiment, with the highest value of 10.2 µg l^{-1} at a high N:P load (N:P = 40) (Fig. 1a). Total phytoplankton biomass was also high at N:P loads of 80 and 160, but with lower values than at N:P 40 (Fig. 1a). Pigment signature and direct microscopic counts (S. Agustí unpubl. data) revealed that the diatoms were the main group responsible for this increase. On the other hand, *Synechococcus* sp., which along with small flagellates dominated the water column initially, showed a rapid increase in chlorophyll 3 d after initiation of the experiment at N:P ratios higher and lower than the normal load, but the increase was not sustained during the latter part of the experiment (Fig. 1b).

Abundance of *Synechococcus* sp. increased rapidly (an average of 280% more than initial abundance in all mesocosms) 3 d after the onset of nutrient addition in all mesocosms (Fig. 2a), but the rate of increase tended to vary as a function of N:P load (Fig. 2b). The net rates of population growth during the first 3 d of the experiment were higher in mesosocosms receiving excess N and P than in those receiving the normal N:P load (20) in the Bay of Blanes (Fig. 2b). As the experiment progressed, abundance generally levelled off in all mesocosm units (Fig. 2a), with very low mean (±SE) net population growth rates of 0.015 (±0.011) d^{-1}.

Average picophytoplankton primary production tended to increase with both excess N (at an N:P of 160) and with slightly excess P (at an N:P of 10) compared to the normal load in the Bay of Blanes (Table 2). After nutrient re-equilibration, the resulting picophytoplankton primary production rates were similar, independent of the N:P load previously applied (Table 2). Gross specific growth rates of *Synechococcus* sp. at the onset of the experiment averaged 1.2 (±0.15) d^{-1} among the mesocosm units. During the first 3 d of the experiment, the gross specific growth rates of *Synechococcus* sp. tended to be higher in the mesocosm receiving the normal N:P load for the Bay of Blanes compared to the remaining mesocosms (Table 3). This pattern, however, was not apparent in the mean gross specific growth rates of *Synechococcus* sp. for the 14 d of the experiment, which showed that gross specific growth tended to increase at an N:P load of 10 and at the highest N:P load of 160 (Table 3). The loss rates of *Synechococcus* sp. in response to nutrient ratio manipulation followed the same pattern as that of the growth responses during the 14 d of the experiment (Table 3). However, during the first 3 d of the experiment, gross specific growth rates exceeded loss rates (Fig. 3) resulting in a net increase in the *Synechococcus* sp. population during the early part of the nutrient ratio manipulation (Fig. 2).

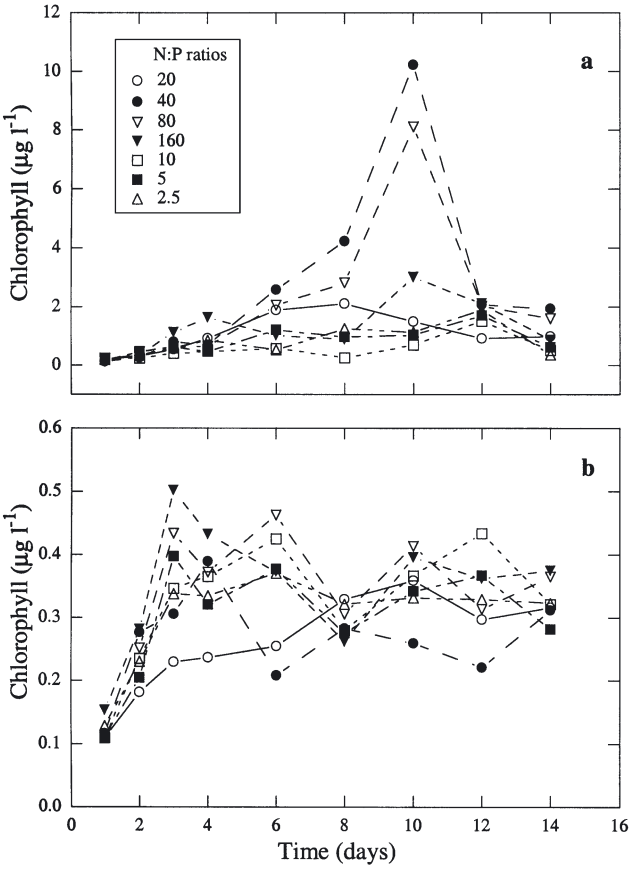

Fig. 1. Temporal evolution of chlorophyll *a* biomass in mesocosms in the Bay of Blanes receiving different N:P loads. (a) Total phytoplankton; (b) *Synechococcus* sp. community

Based on the disappearance of fluorescently labeled analogs of *Synechococcus* sp., loss rates (presumably due to grazing) were high in a mesocosm receiving the background N:P of 20 and at the N:P of 10 at the onset of the experiment (Fig. 4a). After a week of nutrient manipulation, specific grazing rates were lower, although the rate for the mesocosm receiving the background N:P (20) remained high compared with the remaining mesocosms (Fig. 4a). Specific grazing rates on *Synechococcus* sp. after nutrient re-equilibration were similar in all mesocosms tested (Fig. 4b). The average specific grazing rates on *Synechococcus* sp. during Phase I of the experiment was inversely correlated (r = –0.72), albeit not significantly (due to the few data points), with the net growth rates of *Synechococcus* sp. during the first 3 d of the experiment (Fig. 2b). The average primary production of *Synechococcus* sp.

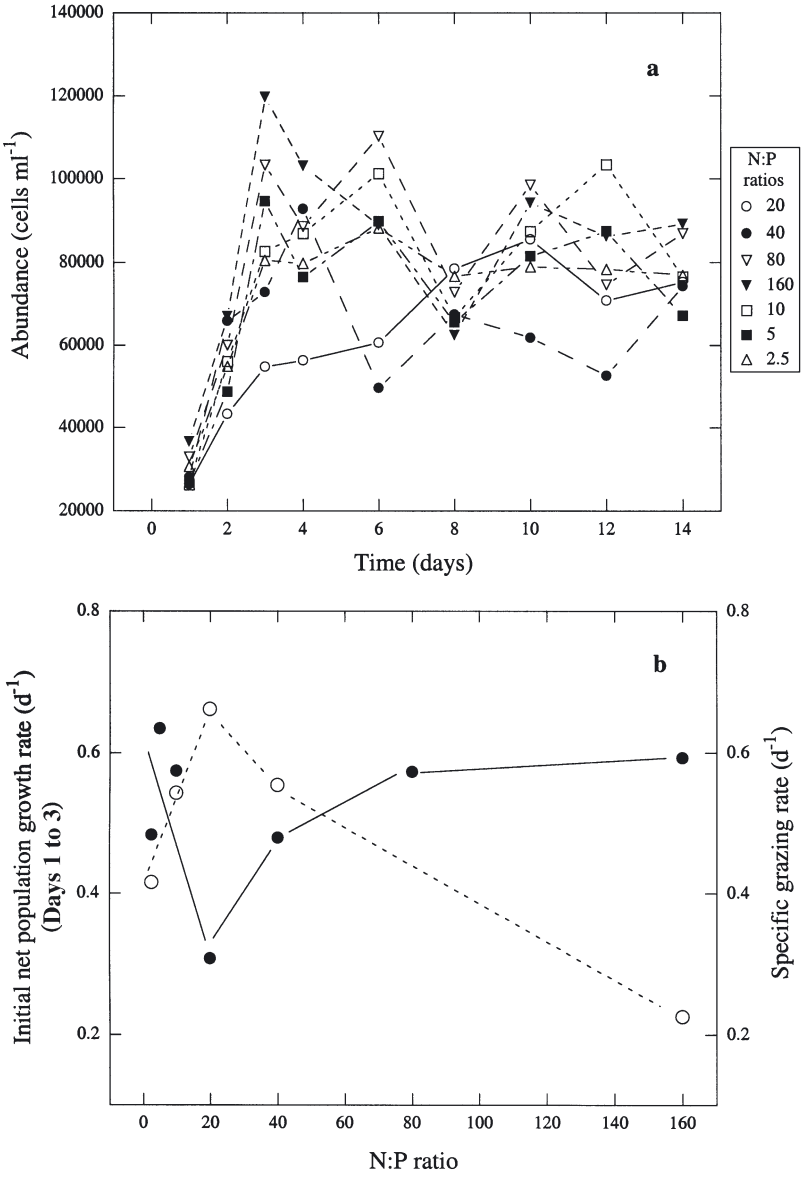

Fig. 2. *Synechococcus* sp. (a) Temporal evolution in abundance in mesocosms receiving different N:P loads in the Bay of Blanes; (b) average net population growth rate (●) during the first 3 d of the experiment and average specific grazing rates (○) during the first week of the experiment, along the gradient of N:P ratios. Lines indicate trends

during the first week of the experiment was significantly negatively correlated with the specific grazing rate after 1 wk of N:P manipulation ($r = -0.98$, $p < 0.05$; Fig. 5).

The abundance of heterotrophic nanoflagellates (HNF), known grazers of *Synechococcus* sp., also increased during the first 3 d after initiation of nutrient ratio manipulation (Fig. 6) but not as fast as that of *Synechococcus* sp. (Fig. 2a). The net growth rates of these protists was 70% of the average gross growth rates of *Synechococcus* sp. Peak abundance of HNF was high at the normal ratio load (N:P = 20) and higher, while lower abundances were observed at lower N:P loads (Figs. 6 & 7). After reaching their peak abundance on the third day of the experiment, abundance of HNF generally dropped (Fig. 6) (as also observed for *Synechococcus* sp., Fig. 2a), coinciding with the increased abundance of total phagotrophic ciliates (mostly naked oligotrichs). These ciliates can prey on bacterial-sized organisms as well as HNFs in the size range 2 to 5 µm (these small HNFs are also abundant in the samples). The abundance of total phagotrophic ciliates (mostly naked oligotrichs) reached highest values at the normal N:P load of 20 (Figs. 6 & 7). The drop in *Synechococcus* sp. and HNF abundance on the 4th day of the experiment also coincided with an increased abundance of scuticociliates + euplotids which can graze on *Synechococcus* sp. (Fig. 6). These ciliates also increased during the latter part of the experiment (Fig. 6), when they contributed substantially to total ciliate abundance. There was a somewhat inverse relationship between the abundance of HNF and that of scuticociliates + euplotids over the range of N:P loads (Fig. 7).

The contribution of picophytoplankton to total phytoplankton production at the start of the experiment averaged 44% in the mesocosms. Upon nutrient addition, the contribution of picophytoplankton to total phytoplankton primary production decreased down to only 6% after 2 wk of the experiment. During the early part of the experiment, the contribution of the picophytoplankton fraction to total phytoplankton production was generally higher in mesocosms receiving excess N and P, compared to the contribution of picophytoplankton in the 2 mesocosms receiving the background nutrient load ratio for the Bay of Blanes (N:P 20; Fig. 8a). The contribution of picophytoplankton to total phytoplankton chlorophyll biomass at the start of the experiment averaged 76% for all mesocosms, and decreased upon nutrient addition. The picophytoplankton contribution to chlorophyll biomass was generally higher in N-deficient mesocosms (units receiving N:P ratios of 2.5, 5 and 10) and in the extremely P-deficient mesocosm (unit receiving an N:P of 160) compared to the mesocosms receiving the background nutrient load ratio for the Bay of Blanes (N:P 20; Fig. 8b).

Table 2. *Synechococcus* sp. Average primary production (mg C m^{-3} d^{-1}, mean ± SE) in the different mesocosm units. Phase I: period of initial experimental conditions (first 14 d); Phase II: period of homogeneous nutrient loading. N:P 20 is normal N:P load in study area

N:P	Phase I	Phase II
2.5	5.35 (1.19)	3.48 (1.94)
5	5.38 (1.02)	
10	6.36 (1.16)	
20	3.37 (0.92)	4.62 (2.79)
40	4.39 (1.70)	
80	5.44 (1.17)	
160	6.90 (1.94)	3.11 (2.06)

Table 3. *Synechococcus* sp. Average gross specific growth rates (μ, d^{-1}, mean ± SE) in the different mesocosm units during first 3 d of experiment and during entire Phase I (period of initial experimental conditions, the first 14 d). Average loss rate (d^{-1}, mean ± SE) is also shown for Phase I

N:P	First 3 d	Phase I	Loss for Phase I
2.5	0.76 (0.13)	0.43 (0.10)	0.26 (0.10)
5	0.68 (0.25)	0.43 (0.09)	0.24 (0.19)
10	0.54 (0.18)	0.48 (0.06)	0.27 (0.17)
20	0.90 (0.33)	0.40 (0.13)	0.27 (0.09)
40	0.62 (0.25)	0.32 (0.13)	0.14 (0.17)
80	0.64 (0.48)	0.30 (0.07)	0.08 (0.18)
160	0.51 (0.11)	0.47 (0.13)	0.27 (0.19)

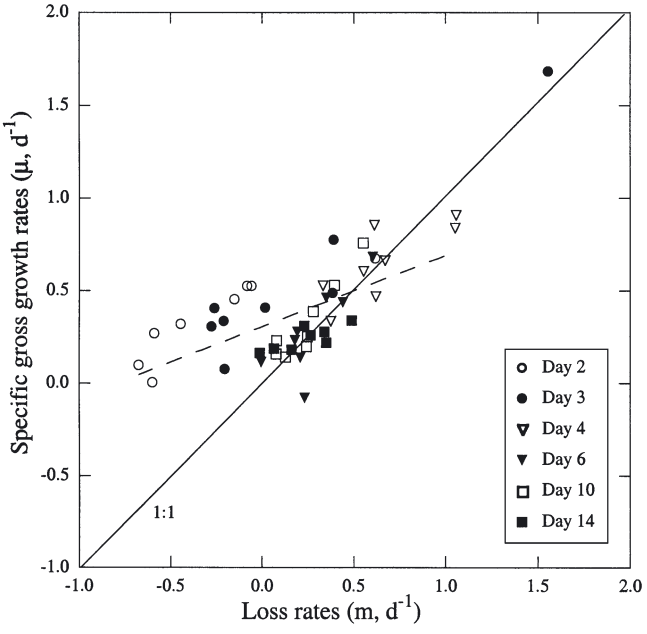

Fig. 3. *Synechococcus*. Relationship between gross specific growth and loss rates during mesocosm experiments in the Bay of Blanes. Continuous line: 1:1 correspondence line; dashed line: fitted linear regression line described by $y = 0.31 (\pm 0.03) + 0.38 (\pm 0.07) \times x$ ($r^2 = 0.41$, $p < 0.05$). Loss rates, *m*, include grazing losses, mortality due to lysis and sinking losses

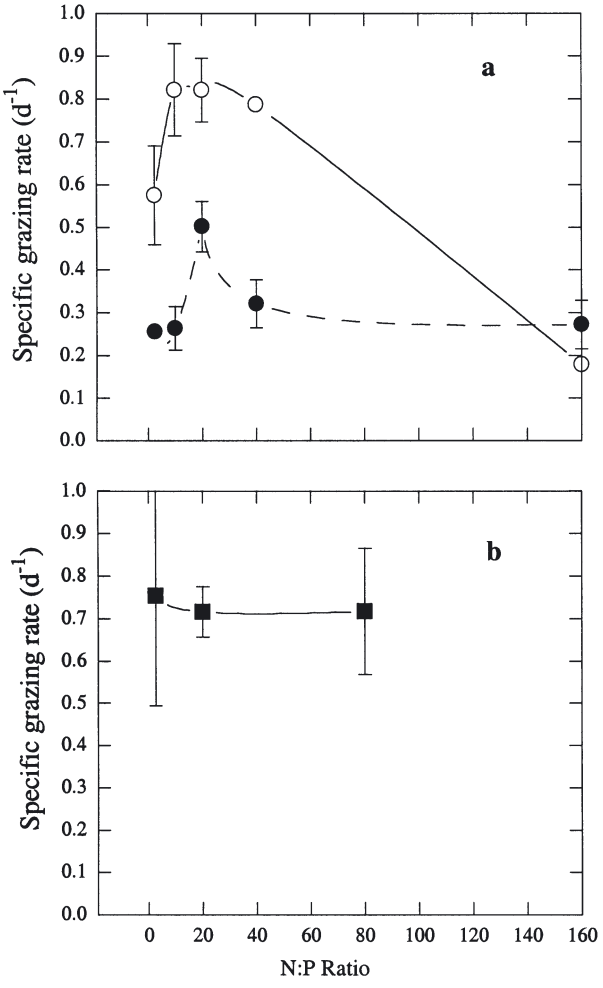

Fig. 4. Specific grazing rates on *Synechococcus* sp. measured as disappearance rate of their fluorescently labeled analog in waters receiving different N:P loads during (a) 1 d (○) and 7 d (●) from the start of the experiment (Phase I), and (b) 6 d from the start of nutrient equilibration (Phase II) in the Bay of Blanes. Lines indicate trends

DISCUSSION

The rapid (1 to 3 d) response of *Synechococcus* sp. abundance to increased nutrient loads (in all mesocosms), is consistent with previous results (Agawin et al. 2000, Duarte et al. 2000). As population size is a result of the interplay between growth and loss rates (Lehman 1991), the early response of an overall increase in population size of *Synechococcus* sp. with increased nutrient load in the mesocosm units suggests an imbalance of the 2 rates during the early part of the experiment. Indeed, the gross specific growth rates of *Synechococcus* sp. were relatively higher than the loss rates during the early part of the experiment (Fig. 3). This imbalance allowed a population increase and may be attributable to time lags between the growth of the

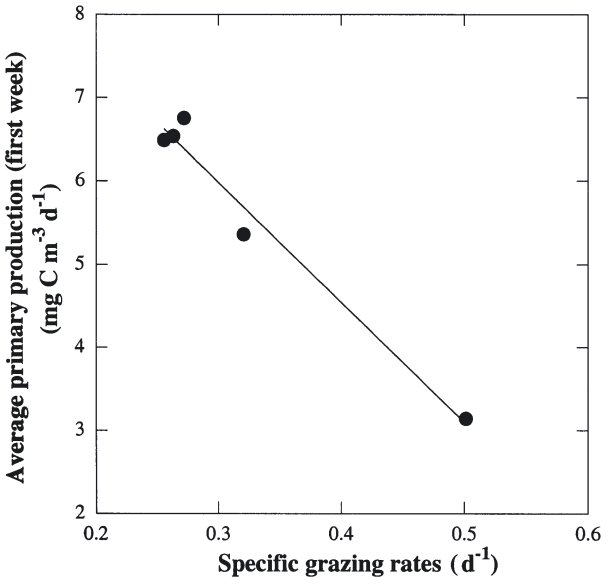

Fig. 5. Significant negative correlation between the specific grazing rates on *Synechococcus* (data from 8 July grazing experiment) and their average primary production during the first week of the experiment ($r = -0.98$, $p < 0.05$)

picophytoplankton and that of their protist grazers in response to an increased nutrient load, resulting in a transient proliferation of the picophytoplankton population. The population of heterotrophic nanoflagellates (HNF), considered to be the main grazers of picoplankton (Stockner & Antia 1986, Šimek et al. 1997, Christaki et al. 2001), also increased in abundance during the first 3 d of the experiment, but with net growth rates lower than those of *Synechococcus* sp. (HNF net growth rate was 70% of the average gross growth rate of *Synechococcus* sp.). Moreover, other potential *Synechococcus* sp. phagotroph predators such as heterotrophic ciliates (Sherr et al. 1989), naked oligotrichs and scuticociliates + euplotids exhibited a time-lag of ~2 to 3 d in their response.

The early increase in gross specific growth and abundance of *Synechococcus* sp. as a response to nutrient addition suggests strong nutrient limitation of the natural population in the Bay of Blanes. The higher gross specific growth rates of *Synechococcus* sp. at the normal balanced N:P load of 20 during the first 3 to 4 d of the experiment could indicate co-limitation of both nitrogen and phosphorus to *Synechococcus* sp. growth, and supports the observation in freshwater studies that N:P ratios >25 are unfavorable to growth of cyanobacteria (Wilcox & De Costa 1990). The gross specific growth rates of *Synechococcus* sp. during the first 3 d of the experiment in all mesocosms were generally higher than later in the experiment (Table 3). The higher gross specific growth rates of *Synechococcus* sp. during the first 3 to 4 d coincided with increased DIN and phosphate concentrations in the mesocosm units, whereas as the experiment progressed, the nutrient concentrations decreased due to rapid uptake by the larger phytoplankton, and nutrient concentrations leveled off. This may have caused the decrease and leveling off in the gross specific growth

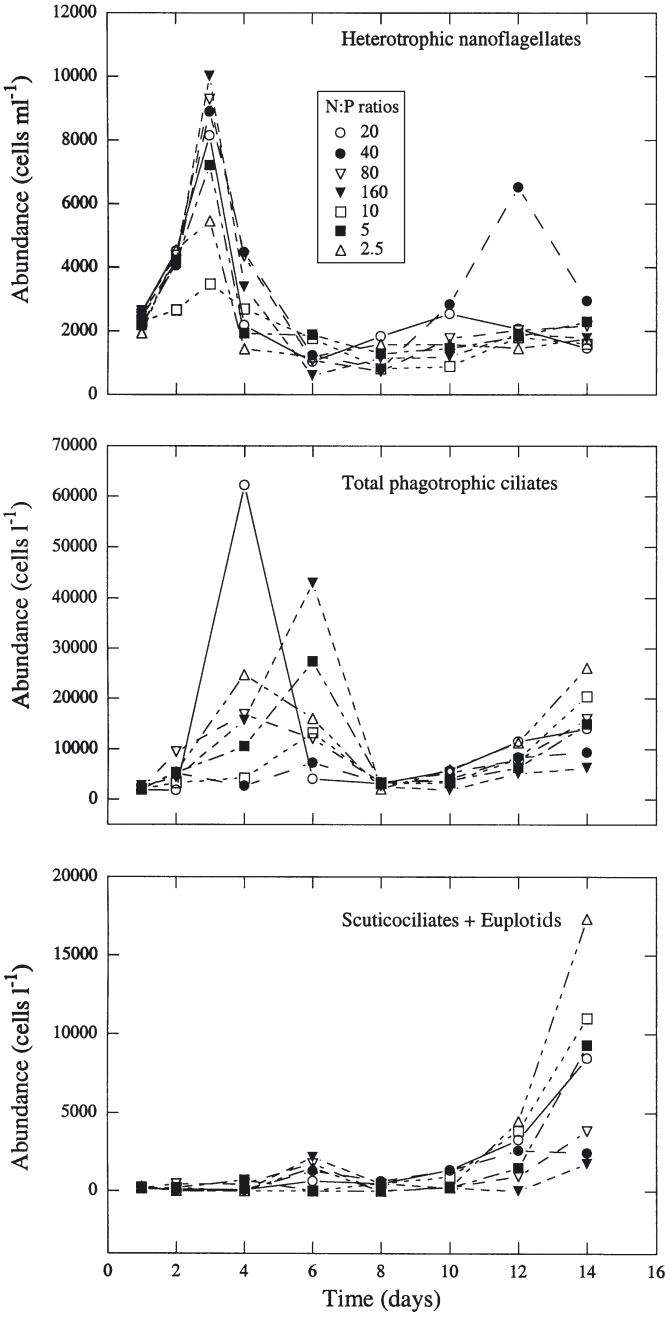

Fig. 6. Temporal evolution in abundance of protists (heterotrophic nanoflagellates, total phagotrophic ciliates and ciliates belonging to scuticociliates + euplotids) in mesocosms receiving different N:P loads in the Bay of Blanes

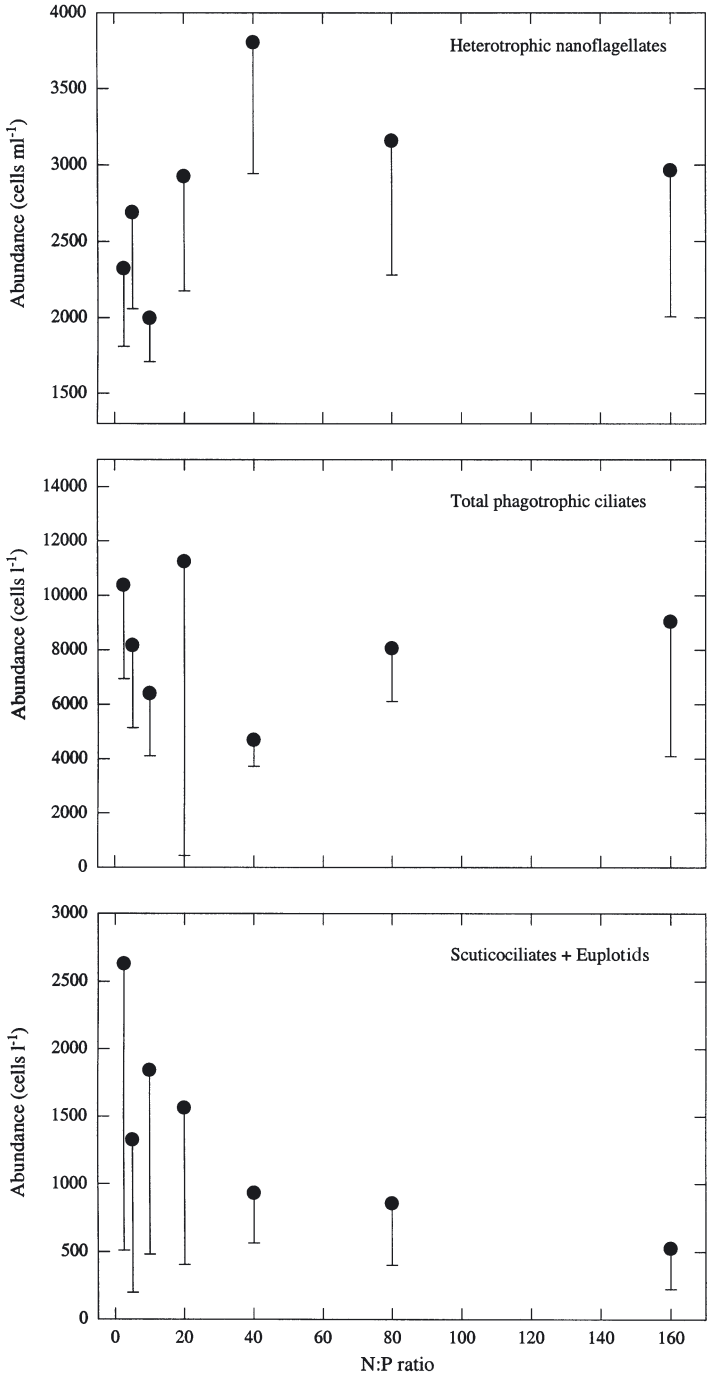

Fig. 7. Average (±SE) abundance of protists (heterotrophic nanoflagellates, total phagotrophic ciliates and ciliates belonging to scuticociliates + euplotids) along the gradient of N:P loads during Phase I of the experiment in the Bay of Blanes values

rates of *Synechococcus* sp. This overall leveling off in abundance with time further suggests a strong top-down control; this is supported by the balanced growth and loss rates of picophytoplankton later in the experiment.

The early response of *Synechococcus* sp. abundance to varying N:P loads was complex, involving a decreased average population size of *Synechococcus* sp. in those mesocosms receiving an N:P load of 20, i.e. the normal load for the Bay of Blanes (Fig. 2b). This decrease did not result from a depressed gross specific growth rate of *Synechococcus* sp. at the background N:P ratio, since the initial gross growth rates of *Synechococcus* sp. tended to be higher in the mesocosm receiving the normal loading ratio in the Bay of Blanes compared to the remaining mesocosms (Table 3). Instead, the depressed net growth rate of *Synechococcus* sp. in the mesocosms receiving a balanced N:P input was a function of grazing rates. These were highest in those mesocosms receiving the normal N:P load of 20, and declined with either excess N or P. The specific grazing rates observed were positively correlated with the abundance of HNF ($r = 0.81$, $p < 0.05$) except at the highest N:P load of 160. At N:P 160 during the first week of the experiment, there was a high abundance of HNF, but their grazing rate on *Synechococcus* sp. was low, as also observed for heterotrophic bacteria (D. Vaque unpubl. data). Although it is possible that protists were ingesting both bacteria and *Synechococcus* sp. at N:P 160, thereby decreasing their grazing rate on *Synechococcus* sp., this explanation may not be valid, since protists could also have been ingesting both bacteria and *Synechococcus* sp. at the other N:P loads. Indeed, HNF have been reported to show no negative selection against heterotrophic bacteria or *Synechococcus* sp. and both picoplankton groups are grazed in the oligotrophic Mediterranean Sea (Christaki et al. 2001). A plausible explanation for HNFs displaying their highest abundance at N:P 160 during the first week of the experiment is that HNFs prey on their own smaller members (Sherr & Sherr 2002). HNF average cell volume and cell biovolume were higher at N:P 160 than at the other ratios (30 µm^3 per cell and 3.0×10^5 µm^3 ml^{-1} for N:P 160 and < 30 µm^3 per cell and $< 3.0 \times 10^5$ µm^3 ml^{-1} for the other N:P ratios), indicating a reduction in smaller-sized HNFs through predation by larger HNFs. Furthermore, at N:P 160, phototrophic nanoflagellates (which were, on average, smaller than the HNFs in this mesocosm) had higher average abundance and biovolume than at the other nutrient loads (D. Vaque unpubl. data), indicating that they possibly contributed to the diet of the HNFs also. Generally, the primary production of *Synechococcus* sp. was significantly inversely correlated ($r = -0.98$, $p < 0.05$; Fig. 5) with specific grazing rates, suggesting that the apparently complex picophytoplankton responses to different N:P loads resulted from top-down effects originating from the response of the grazing community to the vari-

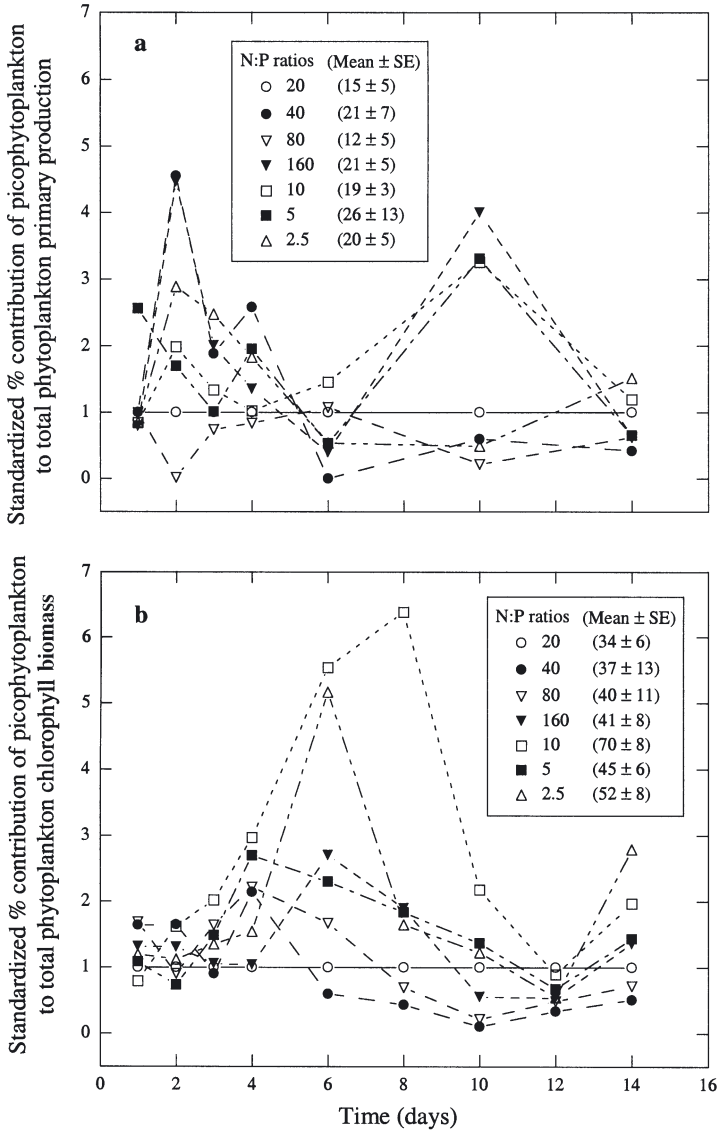

Fig. 8. Average standardized (value for each mesocosm unit divided by that for N:P = 20) contribution of picophytoplankton to (a) total phytoplankton primary production, and (b) total phytoplankton chlorophyll biomass in mesocosms receiving different N:P loads in the Bay of Blanes. Mean (±SE) percent contribution of picophytoplankton during the experiment are indicated in key

ous N:P loads. The dependence of the specific grazing rate on *Synechococcus* sp. on the N:P ratio was further confirmed during the nutrient re-equilibration phase of the experiment, when grazing rates became similar in all mesocosms. The high grazing rates on *Synechococcus* sp. in the mesocosms receiving the normal N:P load of 20 is consistent with the greater abundance of HNF and also of phagotrophic ciliates at this normal load during the first week of the experiment. During the first week of the experiment, phagotrophic ciliates seem to control the high abundance of both HNF and *Synechococcus* sp., as evidenced by the simultaneous drop in both HNF and *Synechococcus* sp. abundance and the increased abundance of the phagotrophic ciliates.

The increase in total phytoplankton (mainly diatoms) biomass after 1 wk of the experiment at high N:P loads suggests that excess nitrogen favors the growth of larger phytoplankton (mainly diatoms) in this NW Mediterranean bay. This is consistent with the depletion in DIN after 1 wk of the experiment. On the other hand, *Synechococcus* sp., which, along with small flagellates, initially dominated the water column, showed a rapid increase in biomass 3 d after the initiation of the experiment at high and low N:P loads compared to the normal N:P load, but the increase was not sustained during the latter part of the experiment. This suggests that variations in the N:P load in the mesocosms resulted in changes in the species composition of the community originally dominated by the picophytoplankton fraction (average = 76% of total phytoplankton biomass). Except in those mesocosms submitted to N-deficiency (N:P loads ≤10) and extreme P-deficiency (N:P load = 160), where the picophytoplankton fraction continued to dominate (>40% of total phytoplankton biomass), the picophytoplankton fraction decreased in the remaining mesocosms with balanced N:P loads and N-replete conditions. This indicates that shifts in the phytoplankton species composition due to increased nutrient loads depend on the N:P load. These shifts may result from differences in nutritional requirements (e.g. ratio of minimum cell requirement for N and P) and nutrient uptake efficiencies, as it had been suggested that *Synechococcus* sp. are better competitors in low nutrient conditions, particularly low P concentrations (Wehr 1993). However, changes in the grazing community as a function of variation in the N:P load, that are propagated to picophytoplankton through top-down control, may affect the response of *Synechococcus* sp. abundance to different nutrient ratios.

In summary, we have provided evidence that the complex response of *Synechococcus* sp. to N:P loads results from a simple response of the protist community grazing on the *Synechococcus* sp. population; this response was highest at the balanced nutrient load prevalent in the Mediterranean bay studied, suggesting that the response of *Synechococcus* sp. is forced by strong top-down effects. This top-down effect was, however, not present at the start of the experiment, when time lags between the growth of the picophytoplankton and their protist grazers (heterotrophic nanoflagellates and phagotrophic ciliates) led to transient imbalances between growth and loss rates of the picophytoplankton, resulting in a transient general net increase in *Synechococcus* sp. abundance in the mesocosms.

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