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Determining the availability of phosphate and glucose for bacteria in P-limited mesocosms of NW Mediterranean surface waters

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ABSTRACT: Availability of phosphate for phytoplankton and bacteria and of glucose for bacteria was studied in mesocosms using NW Mediterranean coastal water with added phosphate, glucose, or a combination of both. We observed an initial increase of bacterial production in all mesocosms, a continuous increase of particulate P only in the P-enriched mesocosms, and a greater accumulation of dissolved organic C in the glucose-enriched mesocosms compared to those enriched by both glucose and P. This suggests that the water used was initially P-starved with a certain pool of degradable organic C available for bacteria. Specific phosphate affinities indicated a P limitation for the bacterial community in all mesocosms. Specific glucose affinities were different between the mesocosms but much lower than the theoretical maximum predicted from the diffusion-limited model. This suggests that the glucose pool was not a strong controlling factor of bacterial growth. In the P-enriched mesocosms, it is indicated that the extent of P limitation shifted from highest to lowest, while the available pool of glucose steadily decreased during the experiment. The explanation suggested for these observations is that phosphate regeneration was enhanced in the plankton food web, by which bacterial carbon demand became higher than the degradable organic C produced in the P-enriched mesocosms.

KEY WORDS: Specific affinity · Nutrients · P limitation · Degradable organic carbon · Mesocosm · Blanes Bay · NW Mediterranean

INTRODUCTION

Heterotrophic prokaryotes (hereafter 'bacteria') are an important component in pelagic systems. For example, a significant fraction of primary production can pass through bacteria in pelagic systems (Cole et al. 1988). Bacterial production is closely linked to remineralization of organic matter that would otherwise be transported to deeper waters. In order to better understand the biogeochemical cycling of organic matter, it is important to deepen our knowledge of how bacterial production is controlled. Overall, it has been assumed that bacterial growth rate is generally organic C-limited. However, experimental evidence that bacterial growth rate is limited by availability of P has been reported from some systems subject to freshwater input such as the Chesapeake Bay (Fisher et al. 1992), Baltic Sea (Lignell et al. 1992), and Norwegian fjords (Thingstad et al. 1993), as well as from some open oceanic systems (Karl et al. 1995, Cotner et al. 1997, Ammerman et al. 2003).

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Mediterranean waters are generally P-starved because of N:P ratios far in excess of the Redfield ratio of 16 (e.g. Krom et al. 1991, Béthoux et al. 1992). During the stratified period, nutrient-addition bioassay experiments show that P is the most important limiting element for the growth of phytoplankton and bacteria (Fiala et al. 1976, Zweifel et al. 1993, Vaulot et al. 1996, Thingstad et al. 1998, Zohary & Robarts 1998, Sala et al. 2002), and dissolved organic carbon (DOC) accumulation in surface mixed layers is commonly observed (Copin-Montégut & Avril 1993, Cauwet et al. 1997, Avril 2002). It has been suggested that this DOC accumulation (i.e. the restricted DOC consumption by bacteria) in surface mixed layers can be explained by (1) assuming that DOC is less degradable (e.g. Legendre & Fèvre 1995) and (2) assuming that bacterial growth is controlled by bacteria–phytoplankton competition for other substrates (e.g. inorganic nutrients) and that biomass is controlled by bacterial predators (Thingstad et al. 1997).

In a previous microcosm study, in which natural microbial communities from Villefranche Bay (NW Mediterranean) received daily additions of phosphate and glucose in a factorial design, there was no effect of glucose addition on bacterial carbon production except when combined with phosphate (Thingstad et al. 1999). A comparison of these results with a simple steady-state model based on P-limited bacterial growth and predator-controlled bacterial biomass suggested that bacterial growth rate was initially P-limited and shifted to C limitation once the accumulated pool of degradable DOC had been consumed because of enhanced bacterial carbon demand (Thingstad et al. 1999). However, the timing at which bacterial growth rate limitation changed was not examined in detail in that study.

For osmotrophs such as bacteria and phytoplankton, the specific affinity is the slope of the curve between specific uptake rate versus substrate concentration, and is analogous to a specific clearance rate for filter feeders: the volume cleared of food (substrate) per unit biomass and time (Thingstad & Rassoulzadegan 1999). Maximum specific affinities for a substrate are expected as substrate concentrations approach zero. The maximum specific affinity can be predicted by assuming molecular diffusion toward the cell is the rate-limiting step (Thingstad & Rassoulzadegan 1999). Recent field studies in Mediterranean surface waters show that the specific affinities for phosphate uptake by bacteria, and pico- and nanophytoplankton, respectively, are close to their respective theoretical maxima during the stratified period (Moutin et al. 2002, Tanaka et al. 2003, 2004, Flaten et al. 2005). These results suggest that the biologically available phosphate pool in Mediterranean surface waters can be reduced to a level so low that diffusive transport toward cells becomes the most limiting step for substrate uptake. Moreover, based on the dataset compiled from different marine waters, Tanaka et al. (2006) has proposed that specific phosphate affinity can be a useful tool to examine the extent of phosphate availability for the bacterial and phytoplankton community in different P-starved marine systems. Similarly, specific glucose affinity has been used to examine the glucose availability for bacteria in laboratory studies (reviewed by Koch 1971) and also in a pCO_2-manipulated mesocosm study (Tanaka et al. 2008).

The objective of this study is to examine how the availability of phosphate for phytoplankton and bacteria, and of glucose for bacteria, is affected by different enrichments of phosphate and glucose to P-starved waters. Our hypotheses were that (1) if bacterial growth is initially limited by P, bacterial growth rate and plankton P-biomass will increase only when P is added; and (2) even with P enrichment, if bacterial carbon demand becomes higher than the production of degradable carbon in the system, the limiting element for bacterial growth rate would switch from P to degradable organic C. To test our hypotheses, we performed a mesocosm experiment manipulating availability of P and degradable organic C arranged in a factorial design, using waters from Blanes Bay, Catalan coast (NW Mediterranean). We analyzed the extent of phosphate and glucose availability by determining the specific affinities for phosphate and glucose, together with other key parameters during the experiment.

MATERIALS AND METHODS

Mesocosm setup and sampling. Surface water was collected from the Blanes Bay Microbial Observatory, Catalan coast, NW Mediterranean (40° 40′ N, 2° 48′ E) on 19 October 2004, transported to the laboratory (Institut de Ciències del Mar, CSIC, Barcelona), and distributed into 200 l tanks. The tanks were maintained at *in situ* temperature (20°C) under relatively low light (average 100 µE m^{-2} s^{-1}) with a 12:12 h light:dark cycle for 8 d. Four different treatments were designed to evoke different phosphate and organic C availabilities for the phytoplankton and bacterial community. The treatments in duplicate tanks consisted of Control, C enrichment (79.5 µmol C l^{-1} d^{-1}, hereafter '+G treatment'), P enrichment (0.05 µmol P l^{-1} d^{-1}, hereafter '+P treatment'), and C and P enrichment (79.5 µmol C l^{-1} d^{-1}, 0.05 µmol P l^{-1} d^{-1}, hereafter '+GP treatment'). To drive all mesocosms to P limitation with respect to the Redfield ratio (C:N:P = 106:16:1), ammonium (2 µmol N l^{-1} d^{-1}) was added to all mesocosms so that the C:N:P ratio of the nutrient enrichment was

1590:40:1. C, P, and N were added as glucose, KH_2PO_4, and NH_4Cl, respectively. Every morning, samples were taken using an acid-cleaned plastic jar and then the nutrients were added. Twice per day water was mixed with a soft flat plastic pole, once before sampling and again in the afternoon.

Concentrations of inorganic nutrients, particulate P, and DOC. Soluble reactive phosphorus (SRP), ammonium, and nitrate were measured spectrophotometrically with an Alliance Evolution II autoanalyzer (detection limit: 20, 40, 10, and 50 nmol l^{-1} for PO_4, NH_4, NO_2, and NO_3, respectively) following a standard procedure (Hansen & Koroleff 1999). Particulate P concentrations were measured using the molybdenum blue reaction method (Koroleff 1983). Particulate P samples (50 to 100 ml) were collected on 0.2 µm polycarbonate filters. Filters were transferred to polycarbonate test tubes with 5 ml of Milli-Q water and oxidized by acid persulphate at 121°C. For each sample, liberated P was measured by 3 parallel determinations using a spectrophotometer with 5 cm cell.

Samples for DOC measurements were filtered through Whatman GF/F filters using a glass filtration system and 8 ml of filtered samples were transferred to glass ampoules. The Whatman GF/F filters and all glassware were pre-combusted (450°C for 4 h). The ampoules were sealed after adding 50 µl of 40% phosphoric acid to each sample, and stored at 4°C in the dark until analysis. DOC concentrations were determined in triplicate using a Shimadzu TOC-5000 analyzer (Benner & Strom 1993).

Abundance and production of microbial components. Bacteria and picophytoplankton (*Synechococcus*, *Prochlorococcus*, and picoeukaryotes) were counted using a FACSCalibur flow cytometer (BD Bioscience) with a laser emitting at 488 nm following the methods of Olson et al. (1993) and Gasol & del Giorgio (2000). Samples (1.8 ml) were fixed with paraformaldehyde plus glutaraldehyde (final concentration, 1% and 0.05% respectively), left 10 min in the dark, deep-frozen in liquid N_2, and stored at −80°C until analysis. Subsamples (200 µl) for bacterial counting were stained for 10 min with a DMSO-diluted SYBR Green I (Molecular Probes) (final concentration, 1:1000 [v:v] of the initial stock), and those for phytoplankton counting were not stained. Flow rates of the flow cytometer were checked every 10 samples by determining sample volume before and after a 10 min run. In all cases 10 µl sample^{-1} of a suspension of yellow-green 1 µm Polysciences latex beads (10^6 beads ml^{-1}) were added as an internal standard.

Autotrophic nanoflagellates (ANF) were fixed with glutaraldehyde (final concentration, 1.5%), stained with 4′,6′diamidino-2-phenylindole (DAPI, final concentration, 5 µg ml^{-1}; Porter & Feig 1980), collected onto black polycarbonate filters of 0.8 µm pore size (Millipore), and counted using epifluorescence microscopy. At least 300 ANF cells of 3 to 20 µm were counted at 1250× magnification. Microscopic samples were mostly analyzed during the experiment, and the few unfinished were done within 3 mo.

In order to estimate biomass of microbial components, we chose the following conversion factors: 20 fg C $cell^{-1}$ for bacteria (Lee & Fuhrman 1987), 50 fg C $cell^{-1}$ for *Prochlorococcus* (Campbell et al. 1994), 200 fg C $cell^{-1}$ for cyanobacteria, and 183 fg C $µm^{-3}$ for autotrophic flagellates (AF; Caron et al. 1995). With regard to AF, a constant volume was assumed for each size class: 4.2 $µm^3$ $cell^{-1}$ for picoeukaryotes and 35 $µm^3$ $cell^{-1}$ for ANF. We used a C:P ratio of 50 for bacteria (Fagerbakke et al. 1996) and 106 for the other groups (Redfield et al. 1963). C:P ratios are variable for both phytoplankton and bacteria (Fagerbakke et al. 1996, Geider & La Roche 2002), but since direct measurement of osmotrophs' P biomass was not done in this study, we applied the literature average C:P ratios for phytoplankton and bacteria (see 'Discussion' for potential biases by these fixed ratios).

Primary and bacterial production. Primary production was measured as particulate organic ^{14}C using the ^{14}C method following standard procedures. $NaH^{14}CO_3$ was added to samples in 70 ml bottles (Corning) (10 µCi per bottle). Thirteen light bottles and 1 dark bottle were incubated in a water bath at *in situ* temperature for 2 h in a gradient of light irradiance (ca 10 to 1000 µmol photons m^{-2} h^{-1}). Light was measured with a small spherical light meter (Illuminova AB). After the incubation, the samples were filtered at low vacuum pressure through cellulose ester filters (Millipore 0.22 µm), and the filters were subsequently exposed overnight to concentrated HCl fumes. After addition of scintillation cocktail (Optiphase Hisafe 2), the filters were radioassayed by a Beckman LS6000 scintillation counter. Total *in situ* primary production was determined from the *P-E* curve and the *in situ* irradiance in the tanks.

Bacterial production was estimated using the ^3H-thymidine incorporation method (Fuhrman & Azam 1982). For each sample, triplicate or quadruplicate aliquots (1.2 ml) and 1 or 2 trichloro-acetic acid (TCA) killed controls were incubated with 20 nmol l^{-1} of ^3H-thymidine for about 2 h at *in situ* temperature in the dark. The incorporation was stopped with the addition of 120 µl of cold TCA 50% to the vials and samples were kept frozen at −20°C until processing, which was carried out by the centrifugation method. Finally, after addition of scintillation cocktail, the samples were radioassayed by a Beckman scintillation counter.

Uptake of $^{33}PO_4$ and ^{14}C-glucose. Uptake rate of orthophosphate was measured using ^{33}P-orthophos-

phate (Thingstad et al. 1993). Carrier-free ^{33}P-orthophosphate (Amersham, 370 MBq ml^{-1}) was added to samples at a final concentration of 125 pmol l^{-1}. Samples to determine background levels were fixed with 100% TCA before isotope addition. Samples were incubated under subdued (laboratory) illumination at *in situ* temperature. The incubation time varied between 30 s and 20 min — short enough to assure a linear relationship between the fraction of isotope adsorbed vs. the incubation time but long enough to reliably detect isotope uptake above background levels. Incubation was stopped by a cold chase of 100 mmol l^{-1} KH_2PO_4 (final concentration: 1 mmol l^{-1}). Subsamples were filtered in parallel onto 25 mm polycarbonate filters with 0.2 µm pore sizes, which were placed on a Millipore 12 place manifold with Whatman GF/Cs saturated with 100 mmol l^{-1} KH_2PO_4 as support. After filtration, filters were placed in polyethylene scintillation vials with Ultima Gold (Packard), and radio-assayed. After the radioactivities of the filter were corrected for those of the blank filter obtained from fixed samples, $T_{[PO4]}$ (h) was calculated as $T_{[PO4]} = -t / \ln(1 - f)$ where f is the fraction (no dimension) of added isotope collected on the 0.2 µm filter after the incubation time (t: h).

The uptake rates of glucose, as a model of labile DOC compound, were measured using ^{14}C-glucose (Hobbie & Crawford 1969 as modified by Havskum et al. 2003). D-[U-^{14}C]-glucose (Amersham, 7.4 MBq ml^{-1}) was added to samples at a final concentration of 100 nmol l^{-1}. After 1 h of incubation under subdued (laboratory) illumination at *in situ* temperature, the sample was partitioned. Particulate ^{14}C (>0.2 µm) uptake was measured on 10 ml samples filtered on 0.2 µm pore size cellulose nitrate filters, and $^{14}CO_2$ was absorbed on 25 mm Whatman GF/Fs with 250 µl phenethylamine fixed inside the cap of 20 ml polyethylene scintillation vials containing 10 ml. Filters were placed in polyethylene scintillation vials with Ultima Gold (Packard) and radio-assayed. Turnover time of glucose was calculated as the inverse of the fraction of added isotope consumed per hour.

The specific affinity for phosphate uptake was calculated by normalizing phosphate uptake rates (inverse of phosphate turnover times) to the summed P biomass of phytoplankton and bacteria (the volume of cleared phosphate per unit P biomass and time or l nmol P^{-1} h^{-1}; Tanaka et al. 2006). Similarly, the specific affinity for glucose uptake was calculated by normalizing glucose uptake rates (inverse of glucose turnover times) to the bacterial C biomass (the volume of cleared glucose C per unit C biomass and time or l nmol C^{-1} h^{-1}; Tanaka et al. 2008).

Statistical analysis. The correlation analysis between parameters (turnover times of phosphate and glucose, specific glucose affinity, SRP and DOC concentration, and bacterial biomass and production) and the simple linear regression to determine net increase rate of particulate P, NH_4, and DOC were done using the R software (www.r-project.org).

RESULTS

Concentrations of inorganic nutrients, particulate P, and DOC

SRP concentrations peaked on Day 2 in Control tanks and on Day 3 in +G, although these mesocosms had not received phosphate additions (Fig. 1). In contrast, in P-enriched mesocosms, SRP concentrations decreased to 56% on Day 1 in +P and 29% on Day 2 in +GP compared to the initial SRP concentration. From Day 4 onward, the concentrations varied between 100 and 150 nmol l^{-1} in Control and +G but decreased to below the analytical detection limit on Day 8 in +P and after Day 6 in +GP. Particulate P (>0.2 µm) concentration was 50 nmol l^{-1} at the start of the experiment, increased in +P and +GP, but decreased slightly in Control and +G. Net increase of particulate P was ca. 100 nmol l^{-1} between Days 0 and 4 with a rate of 28 nmol l^{-1} d^{-1} in +P (r^2 = 0.948, p =

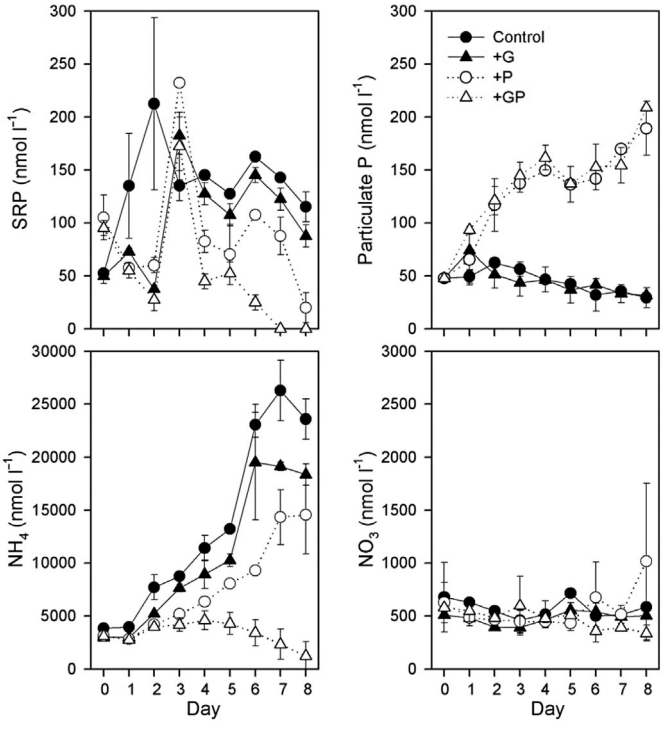

Fig. 1. Temporal changes in concentration of soluble reactive phosphorus (SRP), particulate P, ammonium (NH_4), and nitrate (NO_3) (nmol l^{-1}), following addition of glucose (+G), phosphate (+P) or both (+GP). Data are mean ± SD of 2 replicates

0.0051) and +GP (r^2 = 0.965, p = 0.0028). This corresponds to 56% of the daily phosphate addition (50 nmol l^{-1} d^{-1}). After a slight decrease between Days 4 and 5, particulate P again increased until Day 8 in +P and +GP. At the end of the experiment, particulate P concentrations were ca. 30 nmol l^{-1} in Control and +G, 189 nmol l^{-1} in +P, and 209 nmol l^{-1} in +GP. NH_4 concentrations in Control, +G, and +P increased throughout the experiment. Net increase of NH_4 between Days 0 and 5 was highest in Control (2004 nmol l^{-1} d^{-1}, r^2 = 0.966, p = 0.0004) and similar to the daily NH_4 addition rate. It was about half of the daily addition rate in +P (1036 nmol l^{-1} d^{-1}, r^2 = 0.934, p = 0.0017) and lowest in +GP (333 nmol l^{-1} d^{-1}, r^2 = 0.742, p = 0.028). NH_4 concentration increased more than the daily addition between Days 5 and 6 in Control and +G (9300 to 9800 nmol l^{-1}). In +GP, the concentration decreased from Day 4 or 5 onward. Temporal changes of NO_3 concentration were limited to a range of 340 to 720 nmol l^{-1}, except in +P on Day 8.

DOC concentrations on Day 0 were not measured (Fig. 2). The *in situ* DOC concentration was 96 µmol l^{-1} on Day 1 at the site where the water samples were collected (Herndl & Sintes unpubl. data), thus the initial concentration in the mesocosms was assumed to be around 96 µmol l^{-1}. DOC concentrations increased in all mesocosms during the experiment. The extent of DOC increase was greatest in +G, with the mean rate of 119.3 µmol l^{-1} d^{-1} between Days 1 and 8 (r^2 = 0.997, p < 0.0001), which was ca. 1.5 times higher than the daily C addition as glucose (79.5 µmol C l^{-1} d^{-1}). In +GP the increase of DOC leveled off somewhat after Day 3 and the concentration on Day 8 was 2 times lower than in +G. The net DOC increase in Control and +P was ca. 100 µmol l^{-1} during the experiment.

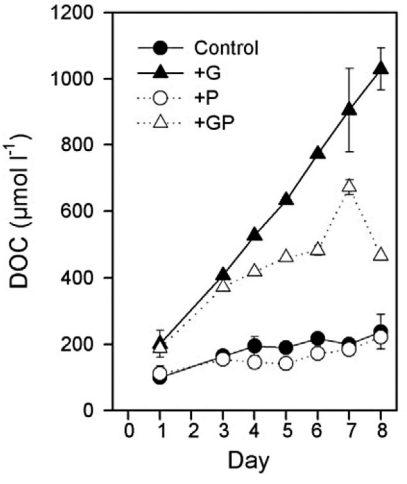

Fig. 2. Temporal changes in concentration of dissolved organic carbon (DOC, µmol l^{-1}). Data are mean ± SD of 2 replicates

Biomass and C production of bacteria and phytoplankton

Bacterial abundance ranged from 3.7 × 10^8 to 4.1 × 10^9 cells l^{-1} (details not shown) and bacterial biomass ranged from 612 to 6751 nmol C l^{-1} (Fig. 3). Bacterial biomass peaked on Day 2 in all mesocosms and decreased afterwards in Control and +G, but increased again towards the end of the experiment in +P and +GP. Bacterial production in all mesocosms increased between Days 0 and 2 with a range of 9 to 24 times in Control and +G and of 86 to 104 times in +P and +GP. Temporal changes of bacterial production were similar between Control and +G (range: 9.6 to 258 nmol C l^{-1} h^{-1}) and between +P and +GP (range: 9.6 to 1270 nmol C l^{-1} h^{-1}), and the production was consistently much higher in the latter.

Total chlorophyll *a* (chl *a*) concentrations showed pronounced maxima in +P and +GP (1.3 and 1.0 µg l^{-1}, respectively) significantly greater than in Control and +G (Allers et al. 2007). On average, 45% of total chl *a* was in the <3 µm fraction (range: 9 to 80%, n = 62, data not shown). Small phytoplankton (pico + nanophytoplankton) biomass ranged from 184 to 1965 nmol C l^{-1} (Fig. 3). Phytoplankton biomass decreased from Day 1 or 2 in all mesocosms. *Prochlorococcus* and *Syne-*

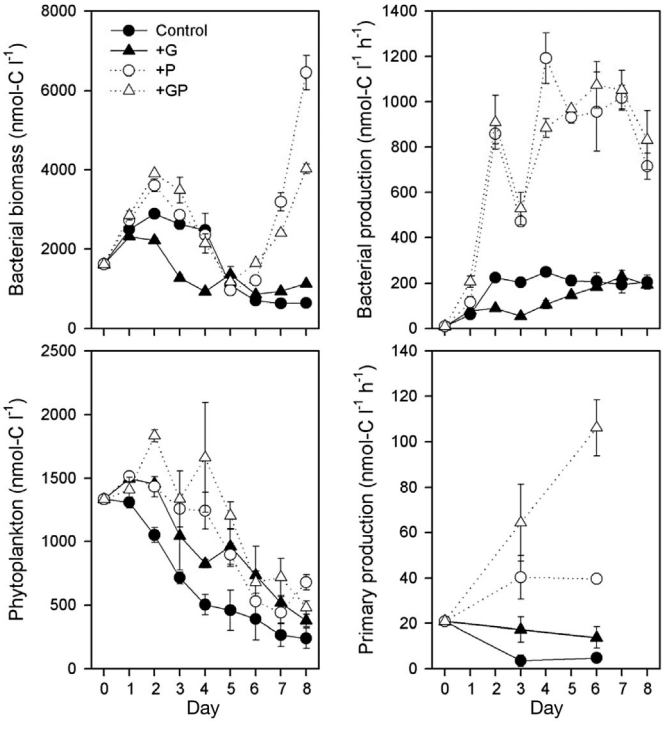

Fig. 3. Temporal changes of biomass (nmol C l^{-1}) and production (nmol C l^{-1} h^{-1}) of bacteria and phytoplankton. Phytoplankton biomass consists of pico- and nanophytoplankton (see 'Materials and methods'). Data are mean ± SD of 2 replicates

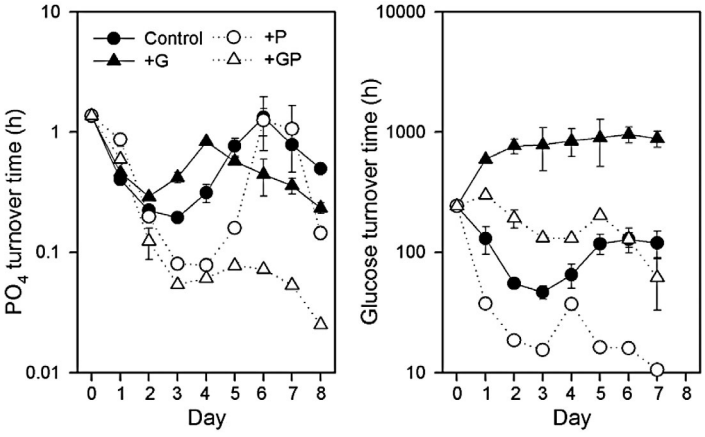

Fig. 4. Temporal changes of phosphate and glucose turnover time. Data are mean ± SD of 2 replicates

chococcus rapidly decreased from Day 0 and were below the detection limit and 1.6 to 20% of the initial abundance, respectively, from Day 5 onward in all mesocosms (data not shown). While temporal changes of autotrophic pico and nanoflagellates were similar to those of the other phytoplankton groups, the biomass of ANF became dominant between Days 5 and 8 (mean: 58%, range: 27 to 89%, n = 32) (details not shown). Biomass ratios of bacteria to phytoplankton were 1.2 at the start of the experiment and increased in all mesocosms during the experiment: from 2.1 to 3.6 in Control and +G and from 7.6 to 10.6 in +P and +GP on Day 8. Primary production ranged from 3.5 to 106 nmol C l^{-1} h^{-1}. It decreased in Control, changed little in +G, and increased in +P and +GP. Ratios of bacterial to primary production were 0.5 on Day 0 and increased with a range from 10 (+GP) to 44 (Control) on Day 6.

Turnover times of phosphate and glucose

Phosphate turnover time was relatively short (1.4 h) on Day 0 and ranged from 0.02 to 1.8 h during the experiment (Fig. 4). No significant correlation was detected between turnover time and SRP concentration (r = 0.102, p = 0.39, n = 72). In all treatments, there seemed to be an oscillatory pattern in phosphate turnover time, with the shortest around Days 2 to 4 and the longest around Days 4 to 6, to some extent resembling a mirror image of the oscillations in bacterial biomass (Fig. 3). The minimum on Days 2 to 4 was lower in the treatments receiving phosphate (+P and +GP) while the maximum on Days 4 to 6 was lower in glucose treatments (+G relative to Control, and +GP relative to +P).

Glucose turnover time ranged from 10 to 1165 h (Fig. 4). It was shorter in the treatments receiving phosphate (+P and +GP compared to Control and +G, respectively), while it increased in the treatments receiving glucose (+G and +GP compared to Control and +P, respectively). Tendencies of oscillatory behavior resembling those for phosphate turnover time could be seen also here, but not to the same extent. Glucose turnover time was significantly correlated with DOC concentration (r = 0.681, p < 0.0001, n = 46), bacterial biomass (r = −0.425, p < 0.0005, n = 64), and bacterial production (r = −0.450, p < 0.0002, n = 64).

Specific affinity for phosphate and glucose

The specific affinity for phosphate uptake by the phytoplankton and bacterial community increased in all mesocosms during the experiment, with a range of 0.0095 to 0.4788 l nmol P^{-1} h^{-1} (Fig. 5). Specific phosphate affinity similarly increased in +P and +GP until Day 5. Thereafter, the specific affinities in +GP continued to increase onward, but those in +P drastically decreased and became as low on Day 7 as on Day 0. While phosphate turnover times in +P were similar to those in Control between Days 6 and 7 and to those in Control on Day 8 (Fig. 4), specific phosphate affinities were lower in +P than in Control and +G during these days because of higher P biomass in +P (Figs. 3 & 5). Specific phosphate affinity in Control and +G varied similarly, being lower than +P and +GP from Days 3 to 5 and higher than +P after Day 6.

Responses of specific affinity for glucose uptake by bacteria mirrored those of glucose turnover time (Fig. 4), with a range of 5.4×10^{-7} to 6.4×10^{-5} l nmol-C^{-1} h^{-1} (Fig. 5). From Day 1 onward, specific glucose affinity in +G was consistently lowest with few temporal variations. Specific glucose affinity gradually increased in Control, +P, and +GP and was consistently

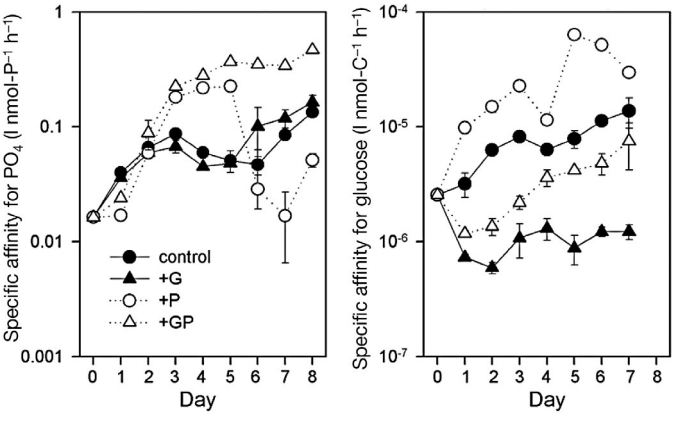

Fig. 5. Temporal changes of specific affinity for phosphate by phytoplankton and bacteria (l nmol P^{-1} h^{-1}) and specific affinity for glucose by bacteria (l nmol C^{-1} h^{-1}). Data are mean ± SD of 2 replicates

highest in +P, followed by Control and by +GP. While glucose turnover times in Control were shorter between Days 1 and 5, and longer on Day 8 than those in +GP (Fig. 4), specific affinities were consistently greater in Control than in +GP because of higher bacterial biomass in +GP (Figs. 3 & 5). All data on DOC concentration and specific glucose affinity showed a significant negative but weak relationship ($r = -0.397$, $p = 0.0030$, $n = 54$).

DISCUSSION

A seasonal study at the Blanes Bay sampling site, where the waters for this study were collected, reports that the maximal concentration of dissolved inorganic nitrogen, SRP, and DOC was 5700 nmol l^{-1}, 220 nmol l^{-1}, and 177 µmol l^{-1}, respectively, between January 2003 and July 2004 (Pinhassi et al. 2006). In contrast, the maximal concentration of NH_4 and DOC, respectively, were 4.6 and 5.8 times higher in the mesocosms than the *in situ* concentrations (Figs. 1 & 2). This might cause differences in the physiological response of the plankton community in the mesocosms as compared to that in natural seawaters. However, the C:N:P ratios used in this study (higher than 1590:40:1) are indeed sometimes observed at the sampling site (Pinhassi et al. 2006). Our objective was to investigate how the availability of phosphate for phytoplankton and bacteria, and of glucose for bacteria, is affected by the different nutrient enrichments; therefore we added nutrients to make all mesocosms P limited with respect to the Redfield ratio, rather than to strictly reproduce *in situ* conditions.

Lack of correlation between SRP concentration and phosphate turnover time suggests that temporal variations of SRP did not significantly reflect those of the phosphate pool in this study. Note that while the measurement of dissolved inorganic phosphorus concentration is usually based on the molybdenum blue reaction method (e.g. Koroleff 1983), this method measures not only phosphate but also has a potential 'background,' such as from acid-labile dissolved organic phosphorus (DOP) and arsenate. This is why the measured phosphorus is defined as SRP (reviewed by Karl & Björkman 2002). SRP concentrations peaked on Day 2 or 3 in all mesocosms despite the different phosphate treatments (Fig. 1). The initial increase of SRP (133 to 173 nmol l^{-1}) was not accounted for by the corresponding change of particulate P or the daily PO_4 addition. Phosphate regeneration from dissolved organic matter was suggested from high alkaline phosphatase activity in all mesocosms (M. M. Sala unpubl. data). However DOP concentrations were not measured in this study. The biomass oscillations of bacteria (Fig. 3) were followed by those of heterotrophic nanoflagellates (HNF), with a lag of 1 to 3 d (K. Šimek, R. Massana, F. Unrein, J. Jezbera unpubl. data). This suggests that the PO_4 regeneration by HNF predation on bacteria was another source of PO_4. The NH_4 addition was planned to be in excess, however NH_4 concentration slightly decreased towards the end of the experiment in +GP. The +GP treatments may thus not have been N-replete towards the end of the experiment, or the NH_4 could have been transformed to dissolved organic nitrogen more in +GP.

Particulate P concentrations increased only in the P enriched mesocosms (+P and +GP) (Fig. 1). The continuous accumulation of DOC in +G with a rate twice as high as in +GP suggests that consumption of degradable DOC was largely controlled by PO_4 availability (i.e. addition of P to the system). The same response of DOC concentration was observed in a microcosm experiment with a similar nutrient manipulation in Villefranche Bay (NW Mediterranean) (Thingstad et al. 1999). The linear DOC increase in +G throughout the experiment suggests that most of the added glucose was accumulated. The DOC accumulation rate was, however, higher than the daily glucose-C addition. In Control and +P, the DOC accumulation during the experiment was much greater than primary production. However we could not identify other sources of DOC. At the start of the experiment, bacterial biomass was similar to phytoplankton biomass and bacterial production was half of phytoplankton production (Fig. 3). Bacterial production immediately increased in all mesocosms, but the extent in increase of bacterial production was much greater in the P-enriched mesocosms (+P and +GP). During the first half of the experiment, a phylogenetic group of bacteria *Alteromonadaceae* formed >50% of total cells and decreased as HNF increased in all mesocosms, indicating selective removal of these phylotypes by the bacterivores (Allers et al. 2007). Allers et al. (2007) interpret the dominance of this group as a response to the manipulations of the mesocosm set-up during this period. Further shifts observed in bacterial community composition could be related to different responses between distinct bacterial phylotypes to the changing pool of available P (cf. Šimek et al. 2006, Allers et al. 2007).

Because of a decline in phytoplankton biomass and an increase in bacterial production, both biomass and production shifted to a marked dominance by bacteria during the experiment. That is, the plankton system in all mesocosms shifted to the bacteria-dominated system during the experiment. One may speculate that the relatively low artificial illumination limited phytoplankton growth. The extent of bacterial dominance was greatly enhanced by P addition. These results suggest that the initial status of the water used in this

study was P-starved with presence of a degradable DOC, and thus bacterial growth or production was controlled by P availability at the start of the experiment. This is supported by the marked differences in net accumulation rate of NH_4 between Control and +P and between +G and +GP between Days 0 and 5 (Fig. 1). This agrees with a previous study that showed bacterial growth in Blanes Bay waters to be limited by P throughout the year, and by C at times during the non-stratified period (Pinhassi et al. 2006). The presence of a degradable DOC pool may be explained by the competition between bacteria and phytoplankton for P and the top-down control on bacteria (cf. the 'malfunctioning' microbial loop: Thingstad et al. 1997) and by allochthonous DOC input to the coastal waters.

The osmotroph-specific phosphate affinities measured in this study (range: 0.0095 to 0.4788 l nmol P^{-1} h^{-1}, n = 72, Fig. 4) were similar to previous field studies in coastal NW Mediterranean waters (0.0021 to 0.0510 l nmol P^{-1} h^{-1}, Tanaka et al. 2003, 2004) and in off-shore Eastern Mediterranean waters (0.0280 to 0.1614 l nmol P^{-1} h^{-1}, Tanaka et al. 2006 using the data from Flaten et al. 2005). The bacterial dominance in the osmotroph community in this study (Fig. 3) suggests that the specific phosphate affinities mainly reflect those of bacteria. Tanaka et al. (2006) have proposed that a specific phosphate affinity >0.02 l nmol P^{-1} h^{-1} indicates P limitation, i.e. the growth rate of the existing organisms is reduced due to the reduced P availability (cf. Thingstad & Rassoulzadegan 1995). Osmotrophs obtain P through the cell membrane, mainly in the form of orthophosphate (Cembella et al. 1982). When free PO_4 is depleted from the ambient pool, many osmotrophs produce extracellular enzymes to help them to obtain PO_4 from DOP (Chróst 1990). While a large pool of DOP appeared to build up in +P and +GP, and high alkaline phosphatase activity was observed in all mesocosms (M. M. Sala unpubl. data), the DOP hydrolysis rate could thus limit P utilization by bacteria. The specific PO_4 affinity should be regarded as an indicator of the ambient pool of PO_4 available for osmotroph community (Tanaka et al. 2006). According to the above criterion, these specific phosphate affinities suggest that the bacterial communities were P-limited in all mesocosms except in +P on Day 7 throughout the experiment.

C:P ratios in phytoplankton and bacteria are variable (Fagerbakke et al. 1996, Geider & La Roche 2002). The different nutrient enrichments could result in different temporal variations of C:P ratio of the osmotrophs between the mesocosms. Because the initially P-starved waters were forced to be more P-starved compared to N and organic C in this study, it would not be unexpected that C:P ratios increased in all mesocosms during the experiment. A chemostat experiment with the marine heterotrophic bacterium *Vibrio splendidus* grown in various glucose-C to P ratios (range: 17 to 2666) showed a relatively limited change in mean cellular P compared to mean cellular C (4.7 vs. 9.7 times, Løvdal et al. 2008). Although we do not have data on cellular elements in this study, we believe that the potential differences in cellular P content do not significantly change our results about the temporal changes of specific phosphate affinity.

A significant positive correlation between glucose turnover time and bulk DOC concentration can be explained by the excess glucose enrichment in +G and +GP, and therefore by the dominance of glucose-C in the bulk DOC. The significant negative correlation between glucose turnover time and bacterial biomass, and between glucose turnover time and bacterial production, suggests that glucose was an important component in the degradable DOC pool for bacteria. Since the ^{14}C-glucose concentration (final conc. 100 nmol l^{-1}) might not always have been at tracer level, this could to some extent have caused an overestimation of glucose turnover time, especially in Control and +P. Responses of specific glucose affinity fitted well to the pattern and extent of glucose availability expected in this study: highest in +P and lowest in +G (Fig. 5). The specific glucose affinities measured in all mesocosms except +G were similar to those in the SW Norwegian coastal waters (Tanaka et al. 2008). A higher cellular C in +G and +GP would give lower specific glucose affinities but would not significantly change our results about the temporal changes of specific glucose affinity. The experimentally determined specific affinity (α) can be compared with the theoretical maximum (α_{max}) in order to examine the substrate availability for osmotrophs. A theoretical expression for the maximum specific affinity for a spherical cell of radius r can be derived by:

$$\alpha^{max} = \frac{4\pi Dr}{m} \quad (1)$$

where D is the diffusion constant for the substrate molecules and m is the amount of the limiting element required to produce a new cell (Thingstad et al. 2005). For carbon, the amount required for a cell is determined by both cell quota and respiration. Taking the respiration coefficient (R) into account for cell quota (Q) in units of C, m is described as $Q/1 - R$. If we assume that bacterial cells have a density of 1.2 g cm^{-3}, 50% dry weight:wet weight, 50% carbon:dry weight, a cell radius of 0.25 µm, and R of 0.5 and that the diffusion constant for glucose is 6×10^{-6} cm^2 s^{-1} (e.g. Koch 1971), the maximum specific affinity given by the diffusion limitation of substrate transport is calculated to be 2.1×10^{-3} l nmol C^{-1} h^{-1}. This theoretical maximum is 2 orders of magnitude higher than the highest value

observed in this study (6.4×10^{-5} l nmol C^{-1} h^{-1} in +P on Day 5). This suggests that, while the size of the glucose pool was very different between the mesocosms, bacterial growth was not severely limited by the glucose availability in any mesocosms throughout the experiment. Note that an increase of bacterial *R* from 0.1 to 0.9 results in a decrease of the theoretical maximum from 3.7×10^{-3} to 4.1×10^{-4} l nmol C^{-1} h^{-1}. If glucose-C assimilation by bacteria were very low (*R* = 0.9), the glucose availability for bacteria in +P might have been limited by molecular diffusion. An overestimation of the number of active bacteria (here assumed to be 100%) would lead to underestimation of the specific glucose affinity. If the active fraction were 10%, bacterial growth in all but +G might have been limited by the glucose availability. However, if this active fraction contributed to bacterial carbon production, the generation time of the active bacteria would have to be as short as 4 min, which seems unrealistically short.

Temporal variations in availability of phosphate and glucose for bacteria during the experiment can be plotted on a scatter plot of the specific affinity for glucose and phosphate (Fig. 6). Compared to the points of the Control, the points in a region of higher specific glucose affinity indicate less glucose availability and those in a region of higher specific phosphate affinity indicate less phosphate availability, and vice versa. Responses in 4 different treatments were summarized as follows: (1) slight decrease in availability of both substrates in Control; (2) in +G a slight decrease of phosphate availability, which was similar to Control, and the greatest glucose availability; (3) in +P, the lowest glucose availability and the highest temporal variability of phosphate availability (from the lowest to the highest values registered); and (4) the lowest phosphate availability among the mesocosms and its continuous decrease, and a slight decrease of glucose availability in +GP. It should be noted that, in +P, specific glucose affinity increased between Days 4 and 7, while specific phosphate affinity greatly decreased between Days 5 and 8. This suggests a shift of the substrate availability for bacteria towards a reduced extent of P limitation and a reduced glucose pool. Specific bacterial growth rates in +P, which were calculated by dividing bacterial C production by C biomass (Fig. 3), peaked on Day 5 and decreased thereafter. This period corresponded to the period of enhanced bacterial biomass and production compared to Control and +G (Fig. 3), an increase of viral abundance (Sandaa et al. 2009), and high ciliate biomass (J. Masmitjà & D. Vaqué unpubl. data) in +P. The explanation suggested for these observations in +P is that phosphate regeneration was enhanced through either ciliate ingestion or viral infection of bacterial cells followed by enzymatic degradation of dissolved organic P or both, and stimulated bacterial carbon demand, which became larger than the degradable organic C produced in the system through processes such as exudation from phytoplankton, sloppy feeding, or viral infection.

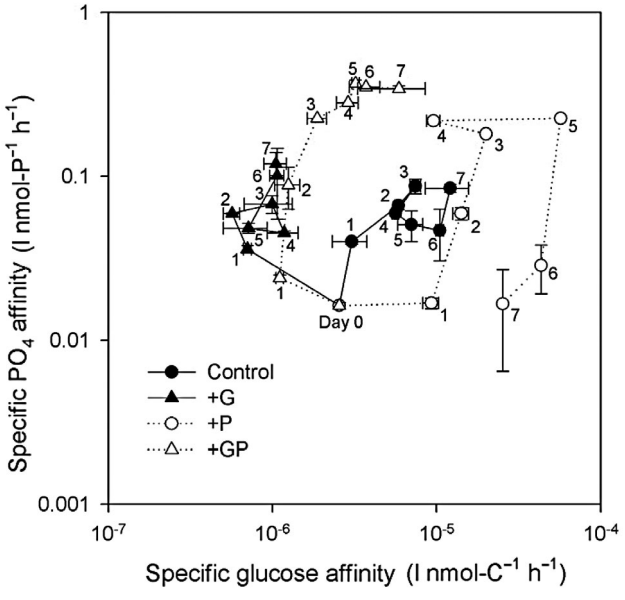

Fig. 6. Relationships between the specific affinities for phosphate and for glucose. Data are mean ± SD of 2 replicates. Each point represents a dataset of 1 d and lines connect points of consecutive days for each treatment (e.g. '1' denotes Day 1). Points with high values on the *y*-axis (upper part of figure) are considered to represent P limitation of the phytoplankton and bacterial community, while points with high values on the *x*-axis (right part of figure) represent C limitation of bacteria

We conclude that the Mediterranean coastal waters used in this study were initially P-starved and contained a certain pool of degradable DOC. The addition of either inorganic N or glucose, or both, in excess of P, to the mesocosms led to large dominance in biomass and production by bacteria, highest P limitation in +GP, and lowest C availability in +P. In an accompanying study, bacteria affiliated with *Rhodobacteriaceae* were dominant (>50%) in the P enriched mesocosms, while the persistence of *Alteromonadaceae* was related to the glucose enrichment during the second half of the experiment (Allers et al. 2007). Moreover, Sandaa et al. (2009) found that the changes in substrate availability in the same mesocosm study resulted in a shift of dominant members of the bacterial community and the corresponding viral community without dramatic changes in the number of co-existing host-virus pairs. A shift from P limitation to a reduced P limitation and a reduced pool of glucose for bacteria in +P during the experiment appeared to be coupled to the occurrence of 3 phylotypes (Mes8/9/49), belonging to the genus *Roseobacter* (Sandaa et al. 2009). The availability of and competition for a limiting substrate

within and between bacterial and phytoplankton communities have an important consequence for the structure and function of the plankton food web (Thingstad 2000). We demonstrated that the specific affinity for uptake of phosphate and glucose allowed us to determine the temporal changes in availability of phosphate and glucose for the bacterial community in different nutrient manipulations.

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