Changes in microbial communities in response to submarine groundwater input

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ABSTRACT: The effects of submarine groundwater on the native plankton populations of a coastal area were examined through a series of *in situ* groundwater addition experiments carried out during the summer in a Mediterranean embayment. Different percentages (4, 8, 10 and 12%) of groundwater extracted from 2 intertidal coastal localities subjected to different land uses were added to picoplankton-dominated natural populations. The responses of the phytoplankton and bacterioplankton biomass were analyzed. The biomass of the phytoplankton community increased by as much as 96% above the mean initial value in ammonium-enriched groundwater and by a maximum of 400% in groundwater enriched in nitrate. Groundwater additions were followed by an enhancement in the biomass of all major autotrophic groups, with the most notable response in picophytoplankton, whereas bacterioplankton abundance increased only slightly. The abundance of diatoms was initially low although their growth rates increased faster than those of dinoflagellates, thus shifting the community composition towards a higher relative microphytoplankton proportion. An initial dinoflagellate community dominated by small naked dinoflagellates shifted to one characterized by a high abundance of *Prorocentrum minimum*. Our results demonstrate that, even in areas with low anthropogenic activity, groundwater discharges to the coast can effectively stimulate autotrophic plankton growth, thereby producing shifts in the microbial food-web structure of coastal waters. This, in turn, increases the possibility of outbreaks of opportunistic species, which can eventually result in harmful algal bloom episodes.

KEY WORDS: Submarine groundwater discharge · Coastal eutrophication · Phytoplankton biomass · Noxious bloom · Opportunistic algae · Nutrient input · Mediterranean Sea

INTRODUCTION

The Mediterranean Sea is among the most oligotrophic areas in the world (Redfield et al. 1963, Bethoux et al. 1992). Low nutrient availability and, particularly, inorganic phosphorus concentrations limit primary production (Thingstad & Rassoulzadegan 1995, Thingstad et al. 1998). Consequently, chlorophyll concentrations in the sea's surface waters are low over large areas for most of the year (<0.2 mg chlorophyll *a* m^{-3}). However, although the waters of the open Mediterranean Sea are charac-

terized by low phytoplankton biomass, higher nutrient availability and phytoplankton abundance are observed in the nearshore areas, where episodes of harmful algal blooms (HABs) are frequent (Garcés & Camp 2011). The enrichment of coastal waters occurs through inputs from rivers but also from continental runoff, groundwater discharges, aerosol deposition and anthropogenic inputs, the latter including wastewater discharges in populated coastal areas. Therefore, biological nearshore production is highly responsive to processes that deliver nutrients to surface waters, thus causing

wide variability both across and within ecosystems (Cloern & Jassby 2010).

Submarine groundwater discharge (SGD) along the coast serves as a major delivery pathway for nutrients and other chemical constituents. SGD, defined as the discharge of fresh and recirculated saline groundwater along the coastline at the land–sea interface (Burnett et al. 2006), is highly enriched in nutrients, metals, organic and inorganic carbon, and other compounds of natural or anthropogenic origin (Johannes 1980, Moore 1999). These terrestrially derived elements may be present in concentrations that are much higher than those typical of the receiving surface water bodies (Burnett et al. 2001, Taniguchi 2002, Kim & Swarzenski 2005) and, therefore, their potential contribution to coastal budgets may be significant (Li et al. 1999), especially in oligotrophic areas where other external nutrient sources are few (Shellenbarger et al. 2006). This is the case in the arid and semi-arid coasts of the Mediterranean region, where overland flow is low and often restricted to the wet season (Peixoto et al. 1982).

In nearshore coastal waters, the role of SGD in the transport of new nutrients is of particular importance because nearshore phytoplankton dynamics and, more specifically, some types of HABs require, among other factors, a significant source of nutrients (Paerl 1997). A growing body of evidence suggests that there is a relationship between SGD and algal blooms in coastal regions. Indirect measurements, for example, have shown that SGD affects coastal biological communities through different pathways: varying nutrient cycling in the coastal zone, changing the character of nutrient limitation by altering stoichiometric relationships, increasing primary production and altering phytoplankton community composition (Gobler & Sanudo-Wilhelmy 2001, Hu et al. 2006, Lee & Kim 2007, Liefer et al. 2009, Lee et al. 2010). However, obtaining direct evidence of the effects of SGD in coastal communities has been more challenging. de Sieyes et al. (2008) reported increases in chlorophyll *a* (chl *a*) in response to the addition of nutrient-rich groundwater to natural phytoplankton populations grown under laboratory conditions (constant light). Also, Gobler & Boneillo (2003) found that groundwater discharge may result in long-term eutrophication, with impacts on microphytoplankton communities. Effects of the groundwater in the microphytobenthos and higher trophic levels have also been reported (Waska & Kim 2010). However, because system-specific attributes (biotic and abiotic) synergistically act to modulate phytoplankton responses to enrichment, depending on the loads and characteristics of the receiving ecosystem, the responses of the coastal communities can vary markedly (Cloern 2001). The timing, amount, residence time, circulation and internal transformation of nutrient input determine a broad spectrum of phytoplankton community growth and bloom responses (Paerl 1997). Biological interactions and their role in the cycling of elements throughout the different compartments of the pelagic food web also influence the responses to groundwater enrichment.

Nutrient additions and altered nutrient ratios may play an important role in the aquatic environment by altering the existing balance among microorganisms that compete for nutrients, consequently shaping community structure by selecting for particular groups (Tilman et al. 1986, Berdalet et al. 1996). For example, studies have shown that under conditions in which algae and bacteria compete (unlimited phosphorus or nitrogen), diatom growth is favoured in the presence of excess silicate, and bacterial growth is favoured in the presence of excess organic carbon but not silicate (Thingstad et al. 1999, Thingstad & Rassoulzadegan 1999, Zohary et al. 2005), although algal–bacterial interactions might be more intricate (Havskum et al. 2003). Structural changes can also occur within the autotrophic community. In mesocosm studies, Duarte et al. (2000) reported long-term shifts in the structure of nearshore Mediterranean summer communities, with dominance shifting from picophytoplankton to microphytoplankton, when nutrients were added (N, P and Si). Thus, both algal–bacterial and algal–algal competition for nutrients are a key elements among the mechanisms controlling the structure of the pelagic food web.

The supply of nutrients and other elements from adjoining aquifers via groundwater diffuse discharges is potentially of primary relevance in the sustenance of coastal phytoplankton in the Mediterranean Sea (Basterretxea et al. 2010); yet, the potential impact on the whole planktonic community level is not well understood. The aim of the present study was to evaluate the response of a coastal microbial food web to the input of enriched groundwater obtained from 2 localities, one low-impacted and the other anthropogenically influenced. The short-term (days) responses of the coastal plankton communities were quantified and then compared with the responses to individual additions of inorganic phosphate, iron or both, based on the hypothesis that these macronutrients and micronutrients have a positive effect on microbial growth. Nutrient concentrations found to stimulate the growth of nearshore Mediterranean summer communities were also quantified.

MATERIALS AND METHODS

Study area

Groundwater addition experiments were performed at the port of Cabrera, an inlet in the National Park of the Archipelago of Cabrera. Located 9 km southeast of the island of Mallorca, the Archipelago of Cabrera comprises 2 major islands and a number of small islets. The port of Cabrera is a sheltered and elongated (~1 km) natural harbour with a maximum depth of 50 m at its offshore opening. Access to the National Park is restricted and land-based activities are reduced to maintenance operations. The prevailing summer meteorological conditions in Cabrera are characterized by low precipitation (~12 mm mo^{-1}) and persistent sea breezes blowing from the SSW, which are notably reduced by the shelter provided by the steep hills around the harbour. The absence of rivers and significant point-source anthropogenic discharges make this bay a relatively simple system. In order to characterize nearshore and offshore Cabrera waters, samples were collected from a boat during the summer of 2008 at 29 stations distributed inside the port and in open waters around the archipelago, referred to in the text as nearshore and offshore stations, respectively. Nutrients, metals and chl *a* samples were analyzed as explained below.

Groundwater and nutrient addition experiments

In situ growth experiments were performed to test the response of coastal planktonic communities to the addition of groundwater and of inorganic nutrients. Surface seawater was collected in 50 l acid-washed plastic carboys at 2 stations in the bay (Fig. 1) and prefiltered through a 60 μm mesh to exclude large grazers. Seawater from Stn 1 was used in the 2008 experiments and from Stn 2 in the 2009 experiments. Incubations for each treatment were performed in triplicate 8 l acid-washed PE bottles. The bottles were submerged 1 m below the sea surface in 3 nearby moorings located at approximately 2.5 m depth and incubated for 3 d to obtain growth rate estimates of the phytoplankton and bacterioplankton communities. Water temperature at the incubation depth was monitored with HOBO temperature loggers, and underwater irradiance was measured with a LI-193 quantum sensor. The bottles were sampled every 12 h for chl *a*, bacterioplankton and phytoplankton, and inorganic nutrients. No more than 10% of a bottle's volume was removed over the course of the experiment. All experiments were carried out under clear skies (noon PAR = 2033 ± 41 μmol m^{-2} s^{-1}). Mean diel irradiance at the incubation depth was 683 ± 185 μmol m^{-2} s^{-1}. Incubation temperatures generally oscillated between 24.5°C at night and 26.5°C at noon. Exceptionally, on
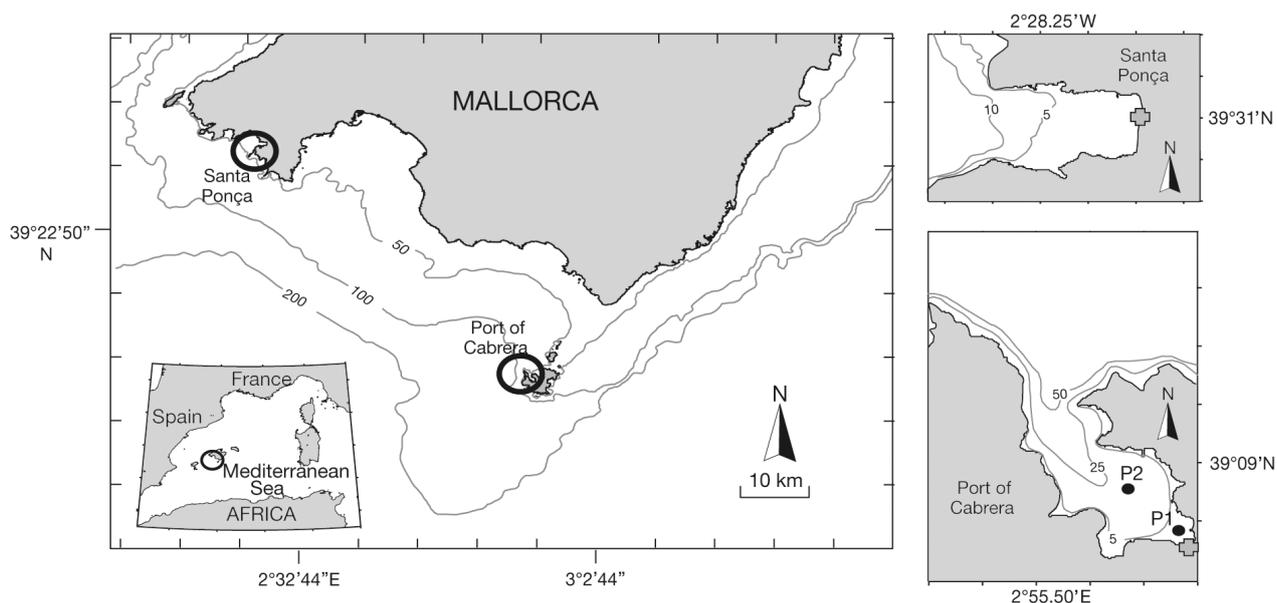

Fig. 1. Sampling stations. Grey crosses (⊡): groundwater collection sites: Site 1, Santa Ponça; Site 2, Cabrera. P1 and P2: sea water collection sites for enrichment experiments (2008 and 2009)

3 July 2008, the seawater temperature reached 27.5°C.

Submarine groundwater for inoculation was obtained from 2 sites: Cabrera harbour and Santa Ponça beach (Fig. 1). Cabrera is considered a relatively pristine location within a national park, where the anthropogenic impact is low and groundwater enrichment is mainly from natural sources. By contrast, Santa Ponça is an intensively urbanised tourism-dedicated beach with groundwater enrichment from urban and agro-gardening sources. The beach is located in a small bay on the southern coast of Mallorca and has an average depth of 5.5 m. Tourism resorts concentrate around the bay, with golf courses and agricultural land extending further inland. Detailed descriptions of land uses, significant anthropogenic pressures and impacted coastal areas are published in the administrative document (CAIB 2011). Moreover, Balearic nearshore waters have undergone eutrophication, as shown by significant nutrient-rich groundwater discharges along the shoreline and the recurrent summertime dinoflagellate outbreaks along the beach (Basterretxea et al. 2007). In Cabrera, the origin of the submarine groundwater was considered as low-impacted (LGW) whereas in Santa Ponça it was anthropogenically influenced (AGW1 and AGW2), consistent in each case with the type of land use (see CAIB 2011) and the level of inorganic nutrient concentration, specifically nitrate and ammonium, which are used as tracers of anthropogenic activities (Gobler & Boneillo, 2003). Groundwater for the study was extracted in the intertidal zone from an approximate depth of 0.5 m below the seafloor using a stainless steel, shielded-screen drive-point piezometer (Retract-a-Tip, AMS; Charette & Allen 2006) and then filtered through GF/F fiberglass filters to remove particles. Prior to the inoculation, salinity was equilibrated to that in seawater by adding NaCl (Merck, reagent grade quality).

In situ growth experiments were designed as follows. Three experiments consisted of the graduated addition of groundwater from the 3 extractions (AGW1, LGW and AGW2; Fig. 3) to seawater to yield final concentrations (v/v) of 4, 8, 10 and 12%

(Expt 1 with AGW1, Expt 2 with LGW and Expt 3 with AGW2). In 3 other independent experiments, the inorganic nutrients phosphate, iron or both (P, Fe or P+Fe, respectively) were added to seawater using $1\ \mu mol\ l^{-1}\ NaH_2PO_4.H_2O$ and $10\ \mu mol\ l^{-1}\ FeCl_3.6H_2O$.

Chl *a*, inorganic nutrients and metals analysis

Chl *a* samples were collected in all experiments on GF/F filters and stored frozen. The samples were filter-extracted in acetone for 48 h and analyzed in a Turner Designs fluorometer (Yentsch & Menzel 1963). Samples for the estimation of inorganic nutrients (ammonium, nitrate, phosphate and silicate) were prefiltered through Whatman GF/F filters, stored at −20°C and further determined with an autoanalyzer (Alliance Evolution II) using standard colorimetric techniques (Grasshoff et al. 1983). Samples for trace metals (Cu, Fe, Mo, Ni, Pb and Zn) in offshore and nearshore waters and groundwater for
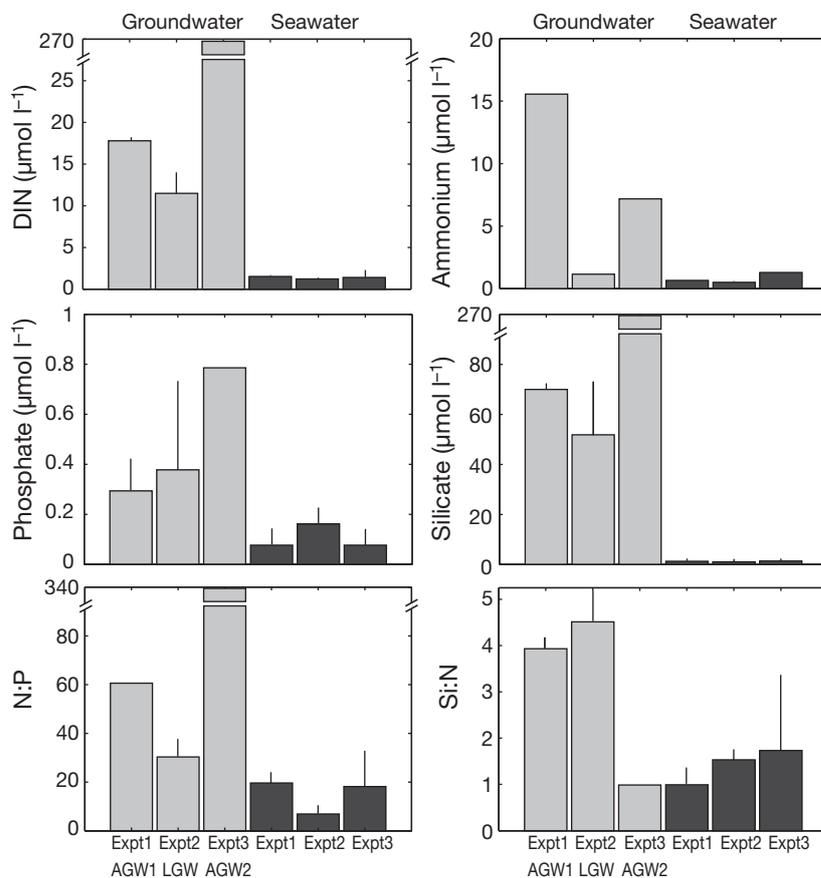

Fig. 2. Dissolved inorganic nitrogen (DIN), ammonium, phosphate, silicate, and N:P and Si:N ratios in low-impacted (LGW) and anthropogenically influenced (AGW1 and AGW2) groundwater (grey) and seawater (black) used for the experiments

Expt 3 were collected using clean protocols (Montluçon & Sañudo-Wilhelmy 2001). Water was collected in acid-washed Teflon tubing connected to a peristaltic pump, with acid-washed C-flex tubing (Cole Parmer) in the pump head. Samples were filtered through an acid-washed 0.22 μm polypropylene calyx capsule filter and collected in acid-cleaned 0.5 l LDPE bottles. Trace-metal samples were acidified to pH<2 with ultrapure grade HCl (Merck) in a class-100 HEPA laminar flow hood and stored for at least 1 mo before extraction. Metals were preconcentrated by the APDC/DDDC organic extraction method of Bruland et al. (1979), and analyzed by ICP-AES (Perkin Elmer Optima 5300 DV).

Microplankton, nanoplankton and bacterioplankton

In Expt 3, samples were collected for the identification and quantification of microplankton assemblages and were maintained in Lugol's–iodine solution (experimental samples), sedimented (24 h), and a suitable area was quantified using a Leica-Leitz DM-IL inverted microscope (Andersen & Throndsen 2003). For flow-cytometric quantification of bacterioplankton, *Prochlorococcus* spp., *Synechoccocus* spp. and picoeukaryotes, 2 ml of the samples were fixed in the dark for 30 min with paraformaldehyde plus glutaraldehyde (1 and 0.05 % final concentrations, respectively), stored in liquid nitrogen *in situ*, and transferred after arrival in the laboratory to a −80°C freezer. The samples were analyzed with standard protocols on a Becton-Dickinson FACScalibur flow cytometer following the recommendations of Gasol & Del Giorgio (2000). Briefly, unfrozen samples were stained with Syto 13 (Molecular Probes), mixed with yellow-green latex beads (Polyscience), and run at low speed until 10 000 events had been registered in a right-angle side scatter (SSC) vs. green fluorescence (FL1, at 530 ± 30 nm) plot. Orange and red fluorescence were used to discriminate among the populations. The presence of nanoflagellates was detected by the observation of cytometric plots and identified as autotrophic nanoflagellates according to their flow cytometric fluorescence characteristics and further epifluorescence microscopy. Discrete samples were filtered through 0.6 μm Nucleopore black polycarbonate filters and stained with DAPI at a final concentration of 5 μg ml^{-1}. The filters were then placed on glass slides with a drop of fluorescent oil and observed by Nikon epifluorescence microscopy under UV radiation and blue light. Autotrophic nano-

flagellates were confirmed by their red-orange fluorescence and plastidic structures and quantified by flow cytometry as described for bacteria.

Growth rates

Species group-specific net growth rates (d^{-1}) were calculated using the algorithm $\mu = (\ln N_0/\ln N_t)/t$, where N_0 and N_t are initial and final cell densities, respectively, and t is the time interval in days (Guillard 1973).

RESULTS

Cabrera nearshore and offshore waters

Offshore values of dissolved inorganic nutrients (DIN) and chl *a* were low, in the range of those reported for the area during the summer period (Vives 1993). Except for phosphate concentrations, significant differences in DIN and chl *a* concentrations (*t*- test, p <0.0001) were determined between nearshore and offshore stations, suggesting nearshore enrichment sources and a phytoplankton biomass response to this enrichment (Fig. 2). Regarding micronutrient trace metals, similar concentrations of Mo and Ni were measured nearshore (Mo: 122.7 nmol l^{-1}; Ni: 2.7 nmol l^{-1}) and offshore (Mo: 127.1 nmol l^{-1}; Ni: 3.8 nmol l^{-1}); however, the concentrations of the other measured trace metals, i.e. Cu, Fe, Pb and Zn, were 2, 1.4, 9.6 and 3.8 times higher, respectively, at nearshore than at offshore stations (Table 1).

Groundwater and seawater macronutrients and micronutrients in the nutrient addition experiments

The groundwater used for the addition experiments was rich in several inorganic nutrients and trace metals but its composition notably varied depending on the source, either low-impacted (LGW) or anthropogenically influenced (AGW; Fig. 3, Table 1). The extent of the anthropogenic influence on the obtained groundwater was determined based on both the ammonium concentration, which was high in the groundwater examined in Expts 1 and 3 (15 and 7 μmol l^{-1}, respectively), and the low nitrate/nitrite ratio (14) of the groundwater from Expt 3 (Expt3-AGW2), which was the lowest in all groundwater samples tested. Both LGW and AGW were par-
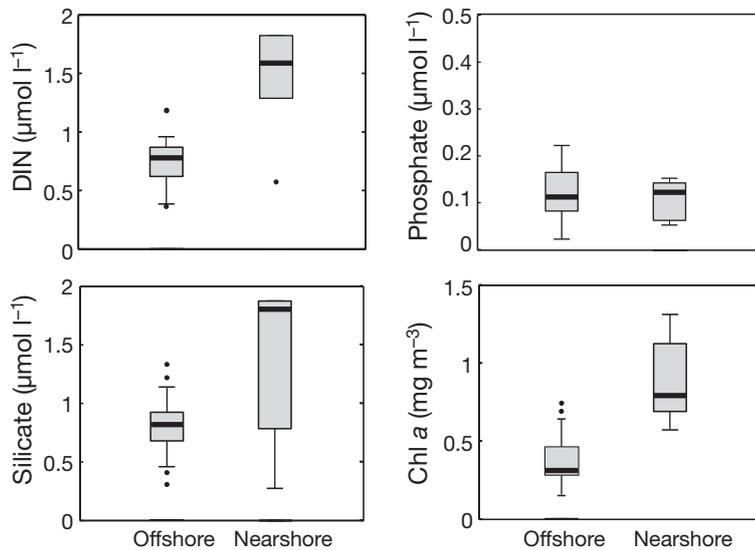
Fig. 3. Box-whisker plot of nearshore and offshore dissolved inorganic nitrogen (DIN), phosphate, silicate and chl *a* concentrations during the summer in Cabrera (n = 29). Box plots show the median values (line), 25 and 75% quantiles (box), 5 and 95% quantiles (whiskers), and outliers (dots)

ticularly highly enriched in Si (from 52 to 264 µmol l⁻¹), with concentrations 1 to 2 orders of magnitude greater than in seawater. Similarly, the concentrations of P, Fe, Pb, Zn and Ni also markedly ex-

Table 1. Mean (±SD) trace metals in the offshore (n = 18), nearshore (n = 2) waters of Cabrera during 2008 and in the anthropogenically influenced groundwater used in Expt 3 (Expt3-AGW2)

Metal	Offshore	Nearshore	Expt3-AGW2
Cu (nmol l⁻¹)	11.3±5.2	23.0±18.1	28.7
Fe (nmol l⁻¹)	10.0±4.6	14.2±2.1	87.7
Pb (pmol l⁻¹)	123.9±13.6	1191.2±162.0	2949.6
Zn (nmol l⁻¹)	5.8±2.2	22.0±4.3	114.4
Mo (nmol l⁻¹)	127.1±10.3	122.7±0.1	44.3
Ni (nmol l⁻¹)	3.8±0.2	2.7±0.9	35

ceeded those in seawater (Table 1). In contrast, inorganic nutrient concentrations in the natural seawater used for the incubations were modest and similar in all 3 experiments.

Differences in the inorganic nitrogen and phosphorous concentrations in the groundwater resulted in N:P ratios higher (ratios from 30 to 340) than the Redfield N:P ratio in the experiments (Fig. 3). The low inorganic phosphorus levels measured in the seawater used for the addition experiments contributed to high inorganic N:P ratios and a possibly P limitation. High Si concentration in groundwater resulted in high Si:N ratios. Although a balanced Si:N ratio was observed in the seawater used for the addition experiments, groundwater addition probably contributed to high silicate availability. In the case of Fe, P and P+Fe addition, the low inorganic phosphorus and nitrogen levels present in the seawater used for the addition experiments led to a high inorganic N:P ratio of 22. After P and P+Fe additions, the inorganic N:P ratio decreased by approximately 1 (data not shown).

Growth rates of phytoplankton biomass and group abundances

Initial phytoplankton (chl *a*) biomass was 0.84, 1.26 and 0.3 mg chl *a* m⁻³ in graduated groundwater addition Expts 1, 2 and 3, respectively. With some exceptions (i.e. 4% in Expts 1 and 2), groundwater additions significantly increased phytoplankton growth rates, as determined based on chl *a* variation relative to control treatment (*post hoc* Tukey's test after a sig-
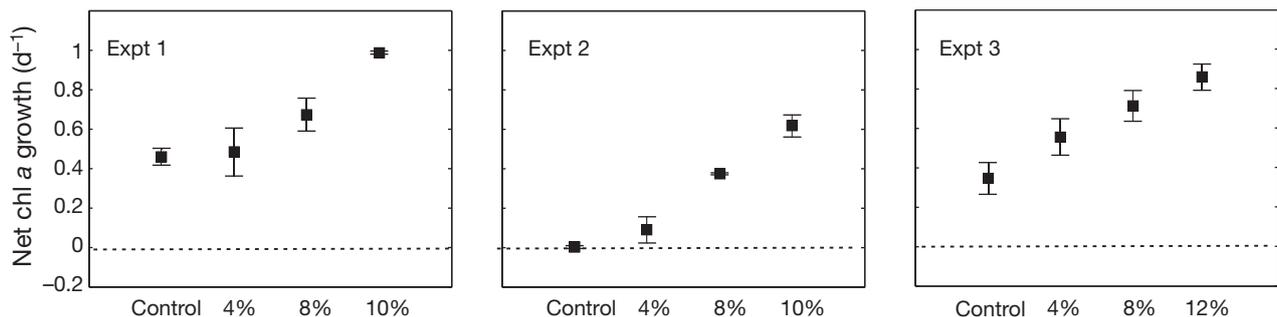

Fig. 4. Mean (±SD) net growth rates, measured as chl *a*, following either the addition of different percentages of groundwater or no addition (control) in 3 experiments. Expts 1 and 3: addition of anthropogenically influenced groundwater extracted from 2 different sites (AGW1 and AGW2); Expt 2: addition of low-impacted groundwater (LGW)

nificant ANOVA, p < 0.001; Fig. 4). Phytoplankton biomass reached maximum values ranging from 96 to 400% above the mean initial value. Maximum increases occurred in the inoculation of 12% groundwater in Expt3-AGW2, which was characterized by an initially low biomass inoculum and groundwater enriched in nitrate. Chl a growth was saturated at the 12% groundwater addition in this experiment. The addition of the AGW orLGW yielded similar responses with respect to chl a growth, as the differences between the 3 slopes obtained from a plot (Fig. 3). The addition of the AGW or LGW yielded similar responses with respect to chl a growth, as the differences between the 3 slopes obtained from the plot of chl a growth-rate response versus percentage of groundwater addition (Fig. 3) were not significant (ANCOVA, $F = 0.5633$, p = 0.5759). The remarkable increase in the chl a growth rate that occurred in the groundwater gradient corresponds to a wide range of measured nutrients: 2.2 to 10 µmol DIN l^{-1}, 0.07 to 0.19 µmol P l^{-1} and 5 to 9.9 µmol Si l^{-1}.

The additions of P and P+Fe increased phytoplankton growth rates (based on chl a) relative to the control treatment, but no change was observed following the addition of only Fe (p < 0.001; Fig. 5). Although the phosphate concentration in the 12% groundwater treatment was 1 order of magnitude lower, phytoplankton growth rates were higher than in the P and P+Fe additions (400% increase above the mean initial value in 12% AGW vs. a 46% increase in the P and P+Fe additions).

Fig. 5. Mean (±SD) net growth rates, measured as chl a, following the addition of 12% groundwater, no addition (control), and P, Fe and P+Fe nutrient additions

higher than those in the seawater control (*post hoc* Tukey's test, p < 0.001). Similar trends were observed for *Prochlorococcus* spp. (data not shown). Groundwater additions increased the growth rates of high-DNA bacterioplankton relative to the control treatment (Fig. 6), in which total bacterioplankton increased as well (data not shown) but with a lower slope than that of picoplankton (ANCOVA, p < 0.0001). In the P, P+Fe and Fe additions, bacterioplankton growth rates relative to the control treatment were increased by the P and P+Fe additions, but no change was observed following the addition of only Fe (p < 0.001; Fig. 7). Bacterioplankton growth rates in P+Fe additions were comparable to those in the 12% groundwater treatment AGW2. P, Fe, and P+Fe additions raised *Synechoccocus* spp.

Bacterioplankton and phytoplankton dynamics

The initial bacterioplankton, picoplankton, nanoplankton and microplankton abundances in Expt 3 are listed in Table 2. Picoplankton and nanoplankton represented approximately 98% of the total population of autotrophic phytoplankton, with *Synechoccocus* spp. accounting for 90% of picoplankton abundance. The increased biomass of the autotrophic community (chl a growth) was largely attributable to that of the picoplankton community in response to the nutrient enrichment provided by anthropogenic groundwater (AGW2). The growth rates of *Synechoccocus* spp. in AGW2 were significantly
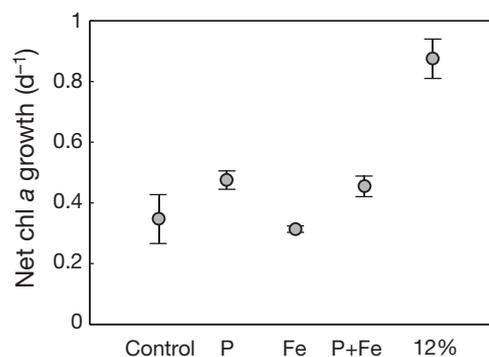
Table 2. Summary of initial values of chlorophyll a concentration (mg chl a m^{-3}) and cell abundance of bacterioplankton, picoplankton, nanoplankton (2 to 20 µm) (cells ml^{-1}) and microplankton (>20 µm) (cells l^{-1}) during nutrient addition Expt 3. Picoplankton comprises prochlorophytes and *Synechoccocus* spp., both measured by flow cytometry. Diatom and dinoflagellate taxa are included within the microplankton, and nauplii, ciliate and heterotrophic dinoflagellate are included within the heterotrophs

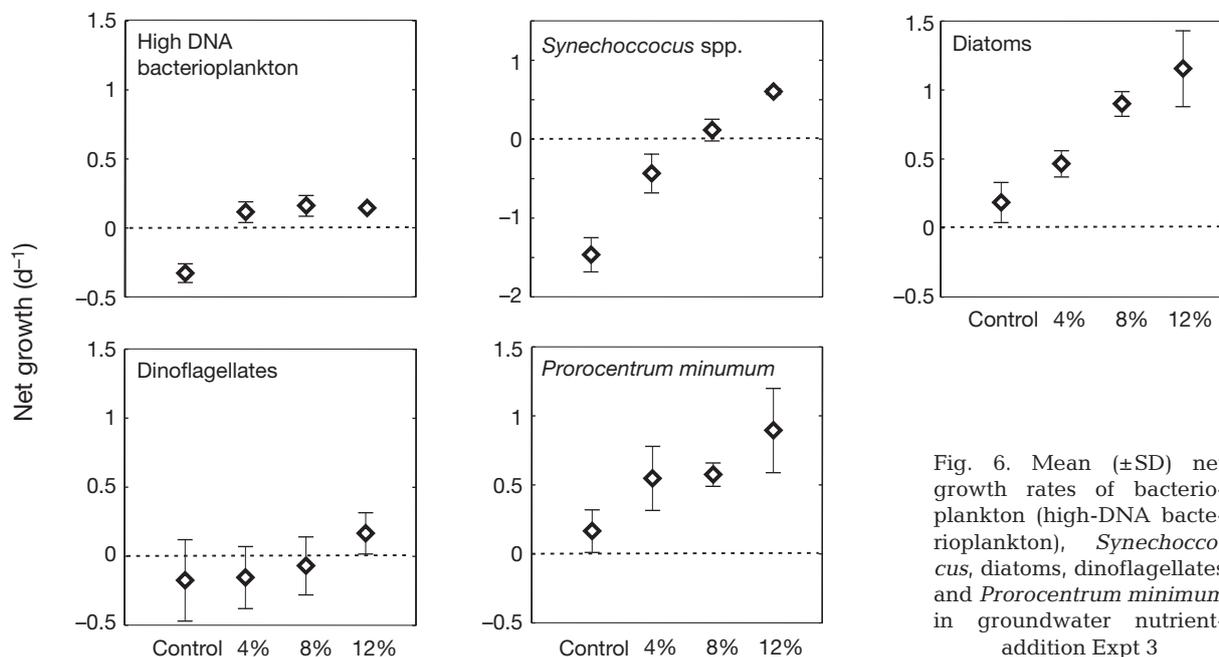| Functional group		Mean	SD
–	Chlorophyll a	0.30	0.02
Bacterioplankton	High DNA	1.46×10^5	1.20×10^4
	Total	3.01×10^5	2.49×10^4
Autotrophic picoplankton	*Synechoccocus*	1.50×10^4	0.38×10^3
	Prochlorococcus	1502	130
	Other	167	32
Nanoplankton (2 to 20 µm)	Autotrophs	65	24
Microplankton (>20 µm)	Diatoms	150	71
	Dinoflagellates	2390	240
	Other autotrophs	160	60
	Heterotrophs	270	99

Fig. 6. Mean (±SD) net growth rates of bacterioplankton (high-DNA bacterioplankton), *Synechoccocus*, diatoms, dinoflagellates and *Prorocentrum minimum* in groundwater nutrient-addition Expt 3

growth rates relative to the control treatment, but with high variability in the P and P+Fe treatment. P, P+Fe and Fe additions were significantly lower (p < 0.001) than the 12% groundwater treatment.

The microplankton component mainly consisted of dinoflagellates (94%), mostly naked species, small flagellates (generally <5 μm) and small solitary pennate diatom species. Microplankton components of the community responded differently to the graduated addition of nutrients of anthropogenic groundwater (AGW2). Although the growth rates of diatoms significantly increased in response to AGW2 (Fig. 6), this was not the case in the dinoflagellate group (p = 0.0785). Nevertheless, among the latter, *Prorocentrum minimum* exhibited a significant (p < 0.001) response relative to the seawater control. The initial dinoflagellate community was dominated by small naked dinoflagellates but finally

changed to a high abundance of *P. minimum* (46% dinoflagellate community).

Overall, the graduated addition of nutrients of anthropogenic groundwater (AGW2; Fig. 6) yielded different growth responses in the bacterioplankton and phytoplankton groups. The differences between the slopes obtained in plots of growth-rate response vs. percent groundwater addition were highly significant (ANCOVA, F = 18.3619, p < 0.0001).

DISCUSSION

The present study confirms that coastal enrichment through groundwater favours autotrophic plankton growth and drives short-term changes in the microbial food-web structure. Increased nutrient availability fuelled the growth of the original summer picoplankton population and other microphytoplankton groups such as diatoms. Diatoms are not characteristic of summer phytoplankton assemblages in the Mediterranean, but nonetheless promptly responded to enhance nutrient availability in our experiments. Autotrophic plankton growth, favoured by increased nutrient availability, partially explains the shoreward biomass gradients and differentiated phytoplankton communities existing in the nearshore waters of
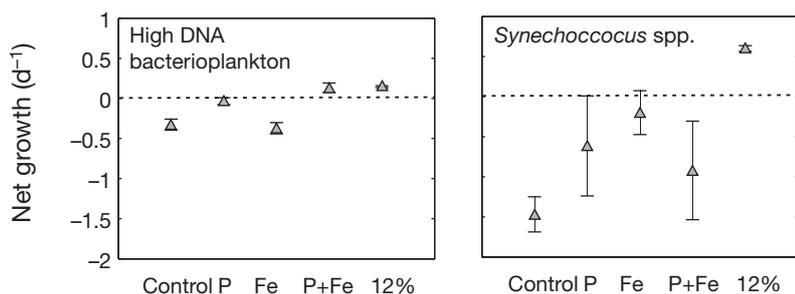

Fig. 7. Mean (±SD) net growth rates of high-DNA bacterioplankton and *Synechoccocus* spp. following the addition of 12% groundwater, no addition (control), and P, Fe and P+Fe in Expt 3

the Mediterranean Sea where groundwater exchanges are enhanced (e.g. the nearshore waters of beaches and sedimentary environments). This is exemplified by our study area, where the coastal area is non-homogeneous as demonstrated by significantly higher concentrations of dissolved inorganic nutrients and chl *a* inshore than offshore.

As in other coastal areas, nitrate and ammonium were the dominant forms of inorganic nitrogen found in the groundwater of the study area (Valiela & Costa 1988, Gobler & Sañudo-Wilhemy 2001). Nitrate was predominant in the groundwater of Cabrera (LGW) whereas high ammonium concentrations were present in groundwater from Santa Ponça (AGW1 and AGW2). The contrasting behaviours of nitrate and phosphate in the respective groundwater systems, mainly caused by the different labilities of N and P in sandy substrates, typically result in a strong increase in the ratio of dissolved inorganic N:P (see Slomp & Van Cappellen 2004 for a compilation of measured groundwater fluxes), as has been measured in our groundwater samples. Groundwater is known to also be naturally rich in silicate and other bioactive elements, such as Fe, Ni and Zn. This could be especially relevant for changes in Fe concentrations in the seawater in the study area, because the area was a carbonate-sediment region where inputs of Fe are limited, and Fe limitation has been suggested for marine organisms such as *Posidonia oceanica* (Marbà et. al (2007). Results from nutrient addition in Expt 3 indicated not only an Fe limitation in the *Synechoccocus* population, but also a co-limitation due to macronutrient and micronutrient shortage. In fact, picoplankton growth was found to be promoted by the mixture of dissolved constituents in groundwater rather than by a single macronutrient or micronutrient. Efforts to analyze the components of the groundwater were insufficient to identify the components that promote higher autotrophic growth in the groundwater addition than the simple addition of P and P+Fe. Other biogenic components such as organic compounds, vitamins (e.g. B12 or B1) or other trace elements (e.g. Co) also need to be investigated.

The increase in chl *a* biomass that occurred in the groundwater gradients represents a wide range of nutrients but with realistic concentrations of nitrate and phosphate for coastal waters. Such nutrient concentrations, shown to boost phytoplankton biomass, are quite frequent in Mediterranean beaches of the Balearic Islands (Basterretxea et al. 2007, Garcia-Solsona et al. 2010). Although our results showed that nutrient enrichment through groundwater favoured autotrophic plankton growth in the area of

study, we are aware that ecological responses can differ depending on groundwater composition (and land uses), flow and discharge conditions (e.g. diffusive, continuous and seasonal), and the characteristics of the receiving waters. The initial characterisation of the added groundwater revealed differences in the forms of specific inorganic nutrients, such as nitrate and ammonium. However, variations in the available nitrate source did not notably influence the chl *a* specific growth rate. This finding has important implications in the context of the environmental management of coastal zones (i.e. European Water Framework Directive, EC, 2000). In assessments of eutrophication, there are tools such as biological elements to be evaluated. One of the most recognised eutrophication assessment methods is using chl *a* as a proxy for phytoplankton biomass. As eutrophication can be natural, human-driven or both, it is necessary for assessment purposes to associate variations in chl *a* concentrations with the driving forces. Here we showed that chl *a* can be formed in response to groundwater inputs, independently of groundwater origin and land use in the catchment area. Therefore, human pressures on the marine environmental can lead to similar changes and impacts such as natural nutrient enrichment, which further complicate management measures.

The increase in biomass of all major autotrophic groups in the groundwater addition experiments was largely attributable to an increase in picoplankton biomass. In contrast to the autotrophic biomass, there was a slight increase in bacterioplankton biomass. As in most oligotrophic oceanographic regions, biomass levels in the Mediterranean Sea are generally associated with the dominance of cyanobacteria, prochlorophytes and tiny flagellates. In our experiment, among the picoplankton, *Synechoccocus* reached high abundances and thus a major increase in biomass. As previously reported, *Synechoccocus* can use different N sources from the marine environment, with the adaptations of the different ecotypes optimized for the environment in which these sources are available (Glibert & Ray 1990, Moore et al. 2002). A similar increase of picophytoplankton was described in mesocosm studies of summer coastal Mediterranean planktonic populations (Duarte et al. 2000). These authors showed that, at the highest nutrient loadings, the structure of the phytoplankton community shifted from an initial dominance of picophytoplankton to one of microphytoplankton in the long term. Our study confirms the response of summer picophytoplankton to the natural nutrient concentrations provided by groundwater sources. The

difference among previous experiments and ours is that we performed short-term incubations, which, while minimizing bottle effects, do not allow assessment of the full potential of the natural microbial community. Nevertheless, it is plausible that, over a longer period, continuous groundwater inputs could lead to new equilibrium states in nearshore communities, resulting in alternative trophic interactions and differentiated phytoplankton assemblages.

The changes in microalgal community composition due to increased diatom growth observed during this study may have been a response to the ratios of dissolved inorganic nitrogen and dissolved silicate in groundwater. Silicate was probably the controlling nutrient in the community shift from diatoms to flagellates, whereas a similar increase in bacterioplankton with respect to diatoms was not observed. This situation could have been caused by competition between these 2 groups for available nutrients. Deficiencies in mineral nutrients limit the growth rate of heterotrophic bacteria, at least in P-deficient regions. In our study, silicate addition (and probably dissolved organic carbon limitation) was such that in the competition between bacteria and phytoplankton, the latter group was favoured. Competition is therefore one of several mechanisms that control the structure of the pelagic food web. The interactions between bacteria and photosynthetic organisms have not only been reported in the Mediterranean Sea but also in other marine pelagic environments. The strategy evolved by diatoms is particularly well suited for the rapid exploitation of short-term situations in which nutrient enrichment allows larger phytoplankton species to become established. For example, groundwater inputs may favour the growth of toxic diatoms, such as those of the *Pseudo-nitzchia* genus (Liefer et al. 2009), blooms of which have been associated with large coastal runoff or discharge events characterized by nutrient pulses. In the specific case of the Mediterranean Sea, the input of silicate through groundwater discharges could be relevant for diatom growth. Terrestrial sources reaching Mediterranean coastal areas have led to a general increase of N over the last several decades but, contrastingly, sources of silicon have remained relatively constant or have even decreased (see the review of Ludwig et al. 2009). The resulting changes in N:Si ratios have been related to shifts in species dominance within the phytoplankton assemblage and food-web structure (Smayda 1990). The input of silicate through groundwater discharges can temporarily and locally alleviate this shift, as has been demonstrated in our area (present study).

Other microphytoplankton groups in the natural sample, such as dinoflagellates, which are less abundant than nanoflagellates but more important in terms of carbon biomass due to their larger size, do not respond significantly to groundwater input. Although dinoflagellates are generally assumed to have low maximum growth rates (although not always; Smayda 1997) and therefore require more time to increase their biomass, our experiments showed that one species of dinoflagellate, *Prorocentrum minimum*, became dominant. *P. minimum* is an HAB species that has long been associated with eutrophication (Heil et al. 2005). Although the species can be mixotrophic (Stoecker et al. 1997), it is able to use ammonium as a source of N (Fan et al. 2003) and blooms of *P. minimum* have been linked to regions with relatively high anthropogenic contributions to dissolved organic N and P exports (such as fertilizers and manures in the case of DIN) (San Diego-McGlone et al. 2008, Glibert et al. 2008).

To conclude, we showed that groundwater inputs favour the growth of particular coastal marine groups or species, producing imbalances in the plankton community that could increase the occurrence of opportunistic species and the probability of HAB episodes. More specifically, this was the case at Santa Ponça, where the increased levels of DIN are induced by anthropogenically enriched groundwater discharges and blooms of phytoplankton are recurrent. However, even naturally enriched groundwater can fuel algal growth and promote the proliferation of HABs in nearshore Mediterranean coastal areas.

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