ICES Journal of Marine Science

ICES Journal of Marine Science (2016), 73(3), 670–679. doi:10.1093/icesjms/fsv130

Contribution to Special Issue: 'Towards a Broader Perspective on Ocean Acidification Research'

Original Article

Contrasting effects of ocean acidification on the microbial food web under different trophic conditions

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Sala, M. M., Aparicio, F. L., Balagué, V., Boras, J. A., Borrull, E., Cardelús, C., Cros, L., Gomes, A., López-Sanz, A., Malits, A., Martínez, R. A., Mestre, M., Movilla, J., Sarmento, H., Vázquez-Domínguez, E., Vaqué, D., Pinhassi, J., Calbet, A., Calvo, E., Gasol, J. M., Pelejero, C., and Marrasé, C. Contrasting effects of ocean acidification on the microbial food web under different trophic conditions. – ICES Journal of Marine Science, 73: 670–679.

Received 24 December 2014; revised 30 June 2015; accepted 1 July 2015; advance access publication 7 August 2015.

We investigated the effects of an increase in dissolved CO_2 on the microbial communities of the Mediterranean Sea during two mesocosm experiments in two contrasting seasons: winter, at the peak of the annual phytoplankton bloom, and summer, under low nutrient conditions. The experiments included treatments with acidification and nutrient addition, and combinations of the two. We followed the effects of ocean acidification (OA) on the abundance of the main groups of microorganisms (diatoms, dinoflagellates, nanoeukaryotes, picoeukaryotes, cyanobacteria, and heterotrophic bacteria) and on bacterial activity, leucine incorporation, and extracellular enzyme activity. Our results showed a clear stimulation effect of OA on the abundance of small phytoplankton (pico- and nanoeukaryotes), independently of the season and nutrient availability. A large number of the measured variables showed significant positive effects of acidification in summer compared with winter, when the effects were sometimes negative. Effects of OA were more conspicuous when nutrient concentrations were low. Our results therefore suggest that microbial communities in oligotrophic waters are considerably affected by OA, whereas microbes in more productive waters are less affected. The overall enhancing effect of acidification on eukaryotic pico- and nanophytoplankton, in comparison with the non-significant or even negative response to nutrient-rich conditions of larger groups and autotrophic prokaryotes, suggests a shift towards medium-sized producers in a future acidified ocean.

Keywords: acidification, eutrophication, Mediterranean sea, mesocosm, microorganisms.

Introduction

Human activity is currently emitting ~10 Pg C in the form of CO_2 into the atmosphere every year (Peters *et al.*, 2012). About 30% of this CO_2 dissolves in the upper layers of the oceans, leading to a lowering of pH and, consequently, to fundamental changes in the chemistry of seawater (Doney *et al.*, 2009; Pelejero *et al.*, 2010). Marine organisms show a wide range of responses to this ocean acidification (OA) (e.g. Kroeker *et al.*, 2010; Harvey *et al.*, 2013).

Many of the studies performed to understand OA effects on picoplankton have used cultures (see Liu *et al.*, 2010 for a review). However, culture-based experiments, which are necessary to understand important physiological aspects, might not reflect the indirect effects that occur through biological interactions. Assays with natural communities in containers that are as large as possible would be an appropriate tool, and mesocosm experiments have been identified as a good approach to ecosystem studies (Lawton, 1995; Duarte *et al.*, 2000; Thingstad *et al.*, 2007).

Table 1. Selection of representative published mesocosm experiments testing the effect of OA on microbes.

Site	Season	pCO_2 or pH levels assayed	Nutrients added	pH manipulation	References
Coastal, North Sea	June 2011	Decrease of 0.4 pH units	NO_3+PO_4+Si	CO_2	Calbet *et al.* (2014)
Coastal, Greenland Sea	May 2010	145–1050 ppm	NO_3+PO_4+Si	CO_2	Motegi *et al.* (2013), Roy *et al.* (2013), Piontek *et al.* (2013), Schulz *et al.* (2013), Brussaard *et al.* (2013)
Open ocean, Greenland Sea	June 2009	250–400 ppm	NO_3+PO_4+Si	Acid	Ray *et al.* (2012)
Coastal, East China Sea	Jan. 2009	800 and 1200 ppm	NO_3+PO_4+Si	CO_2	Hama *et al.* (2012)
Coastal, East China Sea	Nov. 2008	400 and 900 ppm	NO_3+NO_2+PO_4+Si	CO_2	Kim *et al.* (2013)
Coastal, Baltic Sea	Mar. 2008	Decrease of 0.4 pH units	None	CO_2	Lindh *et al.* (2013)
Coastal, North Sea	May 2006	750 ppm	NO_3+PO_4	CO_2	Newbold *et al.* (2012), Meakin and Wyman (2011)
Coastal, North Sea	May 2005	350–1050 ppm	NO_3+PO_4	CO_2	Allgaier *et al.* (2008), Paulino *et al.* (2008)
Coastal, North Sea	Spring 2003	190–750 ppm	NO_3+PO_4+Si	CO_2	Grossart *et al.* (2006), Engel *et al.* (2008), Arnosti *et al.* (2011)
Coastal, NW Mediterranean	Feb. 2010 & July 2011	7.5–8.3 units in pH, variable pH, see Figure 1	None/ NO_3+PO_4+Si	CO_2	This study

Until now, few such mesocosm experiments have addressed OA effects on picoplankton (Table 1). Although comparative analyses are difficult because of differences between experimental set-ups, in general these experiments show a stimulatory effect of OA on photosynthetic picoeukaryotes and nanoeukaryotes (Paulino *et al.*, 2008, Newbold *et al.*, 2012, Brussaard *et al.*, 2013) and complex responses of heterotrophic prokaryotes, which often follow the dynamics of large phytoplankton (Grossart *et al.*, 2006, Allgaier *et al.*, 2008, Arnosti *et al.*, 2011). However, most of these experiments were conducted under high nutrient concentrations, either because they were high in the initial water (Lindh *et al.*, 2013) or because nutrient was added to produce a phytoplankton bloom (e.g. Riebesell *et al.*, 2013). Therefore, these experiments have provided important information on the effects of OA under eutrophic conditions, but not under oligotrophic conditions, which are those that prevail throughout the year in most open-ocean surface waters. Furthermore, mesocosm experiments to test the effects of OA on natural microbial planktonic communities have mostly been conducted at high latitudes [e.g. in the Arctic (Ray *et al.*, 2012) and in the Baltic Sea (Lindh *et al.*, 2013)], while at lower latitudes only two experiments have been published (Hama *et al.*, 2012; Kim *et al.*, 2013) (Table 1). Our knowledge of the responses of microbes to acidification in medium- or low-latitude oligotrophic oceans is still very poor (Maugendre *et al.*, 2015).

The NW Mediterranean is an oligotrophic temperate sea that exhibits contrasting seasonal nutrient and phytoplankton levels in winter vs. summer (e.g. Gasol *et al.*, 2012). The planktonic phytoplankton communities also show contrasting composition, while in winter microphytoplankton are dominant, in summer nano- and picoplankton phototrophs are dominant. The high alkalinity and active overturning circulation of this area compared with the global oceans must involve higher absorption and penetration of anthropogenic CO_2, although it is not clear whether this high penetration translates into a pH decrease (Palmiéri *et al.*, 2014). These characteristics (oligotrophy, seasonality, and high alkalinity) might very well be fundamental in determining the effects of acidification on microbes, but no results have been presented on the variability of the response of the Mediterranean microbial communities to OA in different seasons.

To gain insight into the consequences of acidification of marine ecosystems, we evaluated the effects of OA on microbial dynamics experimentally in NW Mediterranean waters, and determined the importance of contrasting community composition (i.e. winter vs. summer communities) and different levels of nutrient concentrations on the observed responses. We will use the term OA throughout the manuscript to refer to a high pCO_2 level.

Material and methods

Experimental setup

We conducted two experiments: one in winter 2010, during the period of maximum chlorophyll *a* concentration in the area, and one in summer 2011, when chlorophyll *a* is at its annual low in the area (Gasol *et al.*, 2012). Common features of the two experiments were the following: Eight 200-L polyethylene tanks were filled with coastal surface water from the Blanes Bay Microbial Observatory (BBMO), NW Mediterranean, 1 km offshore (41°40′N 2°48′E). The experiments were conducted in a temperature-controlled chamber, set at *in situ* temperature and with a light : dark cycle of 12:12 h. The light conditions were set by a combination of cool-white and GRO-LUX lamps, which mimic the quality of natural light. The pH manipulation was performed by bubbling very small amounts of CO_2 (99.9% purity) directly into the mesocosms. This addition was performed manually every morning, in order to maintain the levels of pH in the acidified tanks ~0.25–0.30 pH units lower than the controls. This pH lowering is equivalent to that expected by the end of the century with atmospheric CO_2 concentrations of 750–800 ppm (Joos *et al.*, 2011). The lowering of pH was closely monitored using glass electrodes (LL Ecotrode plus—Metrohm), which were calibrated on a daily basis with a Tris buffer, following standard procedures (SOP6a of Dickson *et al.*, 2007). The pH in the tanks was continuously recorded by a D130 data logger (Consort, Belgium). In order to mimic the physical perturbation associated with CO_2 bubbling, the control tanks were also bubbled with similar small amounts of compressed air at current atmospheric CO_2 concentrations.

Four experimental conditions were randomly assigned to duplicated tanks: KB_1 and KB_2 (controls), KA_1 and KA_2 (pH-modified), NB_1 and NB_2 (nutrient amended), and NA_1 and NA_2 (nutrient

Table 2. Summary of the *in situ* and experimental conditions.

	Winter		Summer	
In situ conditions				
Temperature (°C)	13		22	
Light intensity (μmol m^{-2} s^{-1})	110		1001	
pH	8.045		8.069	
Salinity	37.92		37.83	
Total alkalinity (μmol kg^{-1})	2533		2540	
Chlorophyll *a* (μg l^{-1})	0.96		0.20	
Experimental conditions				
Temperature (°C)	14		22	
Light intensity (μmol m^{-2} s^{-1})	121		217–261	
	K	*N*	*K*	*N*
Average pH lowering	0.18	0.13	0.28	0.28
NO_3+NO_2 concentration (μM)	3.11	16.8	0.39	4.69
PO_4 concentration (μM)	0.14	1.14	0.02	0.24
SiO_4 concentration (μM)	2.01	31.0	0.34	6.51

Initial conditions are variables measured in Blanes Bay at the time of sampling on the dates of the experiments: winter (17 February 2010) and summer (6 July 2011). Experimental conditions are the temperature of the incubation chamber, the light intensity measured inside the microcosms, nutrient concentrations added in the *N* treatments, and the average lowering of pH in the acidified treatments vs. controls (days 2–9).

amended and pH modified). We gradually modified the pH (in the A treatments) starting immediately after mesocosm filling, and the following day we added inorganic nutrients (to the N treatments) to reach a final molar ratio of 1 : 16 : 30 and 0.25 : 4 : 8 (P : N : Si) in the winter and summer experiments, respectively (Table 2). In both cases, the nitrogen enrichment was close to 8× the monthly average concentration measured in the BBMO during the last 10 years. Nitrogen and phosphorus were added at the Redfield ratio, whereas silicate was added in excess to assure no limitation for diatoms.

In the winter experiment (see initial conditions of the experiments in Table 2), the temperature of the chamber was set to 14 ± 1°C, and the measured light intensity inside of the containers was 121.3 ± 3.5 μmol m^{-2} s^{-1}. In the summer experiment, temperature was set to 22 ± 1°C, and the actual light intensity in the tanks ranged from 217 to 261 μmol m^{-2} s^{-1}.

The pH in the acidified treatments was lowered initially to values ~7.81 units in the winter experiment, which were achieved in the third day, and later they were manipulated to mimic the evolution of the corresponding control (with and without nutrients), but ~0.2 pH units lower. In the summer experiment, pH was progressively lowered in the acidified treatments with the aim of maintaining a relatively constant offset of ~0.2–0.3 units in pH in comparison with the controls.

Chemical parameters

Prior to each morning's controlled addition of CO_2, we measured pH and alkalinity precisely in all the mesocosms. pH was measured by spectrophotometry after the addition of m-cresol purple (Clayton and Byrne, 1993) and alkalinity was determined with a fast, single-point potentiometric titration (Perez *et al.*, 2000). Samples for inorganic nutrients were kept frozen at −20°C until analysis, which was performed using a CFA Bran + Luebbe autoanalyser following the methods described by Hansen and Koroleff (1999).

Chlorophyll *a* was measured according to the procedure of Yentsch and Menzel (1963). Briefly, 50 ml were filtered through Whatman GF/F glass fibre filters. The filters were placed in 90% acetone at 4°C for 24 h and the fluorescence of the extract was measured using a Turner Designs fluorometer.

Microbial abundances

Pico- and nanophytoplankton and bacterial abundance were determined by flow cytometry (Gasol and del Giorgio, 2000). For pico- and nanophytoplankton, the samples were analysed without addition of fixative and run at high speed (ca. 100 μl min^{-1}). Phototrophic populations (*Prochlorococcus*, *Synechococcus*, two groups of picoeukaryotes [small and large] and nanoeukaryotes) were discriminated according to their scatter and fluorescence signals. For heterotrophic bacteria, abundances were estimated after fixing 1.2-ml samples with a 1% paraformaldehyde + 0.05% glutaraldehyde solution, and deep-freezing in liquid N_2. Afterwards, the samples were unfrozen, stained with SybrGreen at a 10× dilution and run at low speed (ca. 15 μl min^{-1}). Cells were identified in plots of side scatter vs. green fluorescence and green vs. red fluorescence.

Microphytoplankton counts were performed after fixing the sample with formalin–hexamine (0.4% final concentration). Afterwards, 50 ml of each sample was placed in sedimentation columns for 24 h. The sedimentation chamber was then scanned in an inverted microscope at ×100 and ×400 magnification.

Bacterial activity

Bacterial heterotrophic activity was estimated using the ^{3}H-leucine incorporation method (Kirchman *et al.*, 1985). Quadruplicate aliquots of 1.2 ml and two trichloroacetic acid (TCA)-killed controls were taken immediately after sample collection. The samples were incubated with 40 nM ^{3}H-leucine (final concentration) for ~1.5 h in the dark in a temperature-regulated room. The incorporation was stopped with the addition of 120 μl of cold TCA 50% to each replicate and the samples were kept frozen at −20°C until processing, which was carried out by the centrifugation method described by Smith and Azam (1992).

Extracellular enzyme activities

The activity of four hydrolytic extracellular enzymes was assayed using fluorogenic substrates that are molecules linked to 4-methylumbelliferone (MUF) or 4-methylcoumarinyl-7-amide, following the method described in Sala and Gude (1999) and adapted to the use of microplates. The enzymes studied were α-glucosidase, β-glucosidase, chitobiase, and leu-aminopeptidase; and the substrates used were 4-methylumbelliferyl α-D-glucopyranoside, 4-methylumbelliferyl β-D-glucopyranoside, 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide, and L-leucine-7-amido-4-methyl coumarin. The assays were arrayed in black, 96-well microplates using a protocol similar to that in Sala *et al.* (2010). For each enzyme, 350 μl of the sample was added to a well together with 50 μl of substrate solution. Each assay was replicated in four wells. The substrate solutions were prepared in sterile deionized water and methanol 1% at a final concentration of 1 mM, yielding a final substrate concentration of 125 μM in the assay wells. Fluorescence was measured regularly at different times for 5 h using a Modulus microplate reader set to an excitation wavelength of 365 nm and an emission wavelength of 450 nm. Fluorescence units were transformed into activity using a standard curve prepared with the end product of the reactions: 7-amido-4-methylcoumarin for leu-aminopeptidase and MUF for the rest of the enzymes.

Statistical analysis

We used STATISTICA version 8.0 (StatSoft, Inc., 2007) for the statistical analyses. To test the hypothesis that there were no effects of acidification within treatments of the same experiment and nutrient addition, we used independent two-tailed *t*-tests. The overall differences between acidified and control treatments were tested with sign tests (Figure 2). Data from day 3 to 9 were chosen for both analyses because it was during this period that the effects of acidification were more conspicuous.

Results

A difference in pH in the range of 0.1–0.5 was maintained between the acidified and control mesocosms in both experiments (Figure 1a and b). In summer, pH varied only slightly between the nutrient-enriched and non-enriched conditions. However, in winter, the intense phytoplankton bloom in the nutrient-enriched treatments (up to 29.6 μg chlorophyll *a* l^{-1}, Figure 1) induced an increase of pH on day 5, reaching levels of up to 8.3 pH units in the non-acidified enriched treatment.

We examined the effects of pH in our set of measured parameters with independent *t*-tests (Table 3). Chlorophyll *a* concentration was positively affected by acidification (Figure 1c and d) except in the nutrient-enriched treatment of the winter experiment, in which no effects were observed (Table 3). The composition of the winter and summer blooms also differed considerably: diatoms of the genus *Thalasssiosira*, *Chaetoceros*, and *Pseudo-nitzschia* dominated the winter bloom (80% of the abundance of the phytoplankton community), whereas the summer community was mostly composed of small pico- and nanoflagellates. No effect of acidification on diatoms was observed, and both positive and negative effects on dinoflagellates were observed (Table 3). The abundances of phototrophic nanoeukaryotes and of large picoeukaryotes were consistently positively affected by acidification (Table 3), with significant differences in both experiments.

Although the initial abundances of heterotrophic bacteria were similar in both seasons ($5–8 \times 10^5$ cells ml^{-1}), their concentration during the experiment increased more in winter than in summer, to maximum values of 4.5×10^6 cells ml^{-1} (Figure 1). The abundance of the different groups of prokaryotes (heterotrophic bacteria, *Synecchococcus*, and *Prochlorococcus*) and leucine incorporation showed a stimulation effect in non-enriched conditions in summer. However, we also found a negative effect of acidification in winter with the nutrient-enriched treatment for *Synechococcus* (Table 3).

Among the four bacterial extracellular enzyme activities tested, α- and β-glucosidase were affected by acidification in summer (Table 3). The other enzyme activities, leu-aminopeptidase, and chitobiase were not affected by acidification under any of the conditions tested.

To summarize the main results obtained, Figure 2 shows a comparison of the abundances of the main groups of organisms between the control and acidified pairs of mesocosms in the two experiments (filled circle, winter; empty circle, summer). To compare the general trends of the effects of acidification on the measured variables, we performed a sign test, i.e. a non-parametric test aimed at comparing pairs of data of acidified vs. non-acidified treatments. The sign test revealed no significant global differences between treatments for heterotrophic bacteria and *Synechococcus* (a negative sign of acidification effect in winter neutralized the positive sign in summer). In contrast, significantly higher concentrations were found in acidified treatments for nanoeukaryotes ($n = 56$; $p < 0.001$), large picoeukaryotes ($n = 56$; $p < 0.001$), and chlorophyll *a* concentrations ($n = 54$; $p < 0.001$) (Figure 2).

Discussion

We assessed the response of the microbial community to a decrease in pH in the Mediterranean. Because in the likely future ocean the response of the microbial planktonic community to OA will interact with other stressors linked to global change, such as eutrophication and increased temperature, we tried to deepen our understanding of possible OA-eutrophication interactions. The major findings of this study are (i) phototrophic pico- and nanoeukaryotes were consistently stimulated by acidification; (ii) prokaryotic picoplankton and phototrophic microplankton exhibited different responses to acidification in winter and summer; and (iii) overall, the magnitude of the acidification effects was lower under nutrient-rich conditions.

Effects of acidification on phototrophic pico- and nanoeukaryotes

We observed a consistent stimulation of the large pico- and nanoeukaryotic phototrophic communities in both winter and summer and in conditions with high and low nutrient levels (i.e. treatments). Stimulation of pico- or nanoeukaryotes by acidification has previously been reported in some mesocosm studies (Engel *et al.*, 2008, Newbold *et al.*, 2012, Brussaard *et al.*, 2013, Calbet *et al.*, 2014), although no effect or a negative effect has also been observed (Paulino *et al.*, 2008, Calbet *et al.*, 2014). Here, the positive effect found in most of the experimental conditions contrasts with the negative effect observed in dinoflagellates in the winter, nutrient-enriched conditions. These divergent results might be due to differences in the species-specific sensitivities to CO_2, in accordance with different carbon concentration mechanisms (CCMs; Rost *et al.*, 2003). Eutrophication and pH decreases are predicted in coastal waters for the near future (IPCC, 2013). In such scenarios, our results suggest that small phototrophic organisms will show a relative increase in comparison with large ones. Temperature rises will also promote small-sized organisms (Morán *et al.*, 2009). These changes in size distribution will affect food web interactions and sedimentation processes (Legendre and Le Fèvre, 1991), which should be considered when it is attempted to predict the likely communities of a future ocean.

Effects of acidification in winter vs. summer

The number of variables affected by acidification in the controls was lower in winter than in summer, and the sign of the responses was always positive in summer, whereas in winter it was negative for *Synechococcus* and dinoflagellates (Table 3). This differential response to acidification in the two experiments suggests a clear role of the initial community composition and environmental trophic conditions. Blanes Bay surface waters exhibit a temperate seasonal cycle with a winter phytoplankton bloom driven in part by elevated nutrient concentrations, typically with diatom dominance, and a summer period dominated by picophytoplankton, microheterotrophs, and low inorganic nutrient concentrations (Alonso-Sáez *et al.*, 2008; Gasol *et al.*, 2012). Contrasting with phototrophic pico- and nanoeukaryotes, which showed a similar number of positive responses in both seasons, all prokaryotes were affected positively in summer and no effects, or negative effects, were detected in winter (Table 3). The only study that evaluated the planktonic community response to OA in different seasons, conducted in 1-l bottles in the Baltic Sea, showed no clear differences in acidification effects on bacterial abundance and diversity during

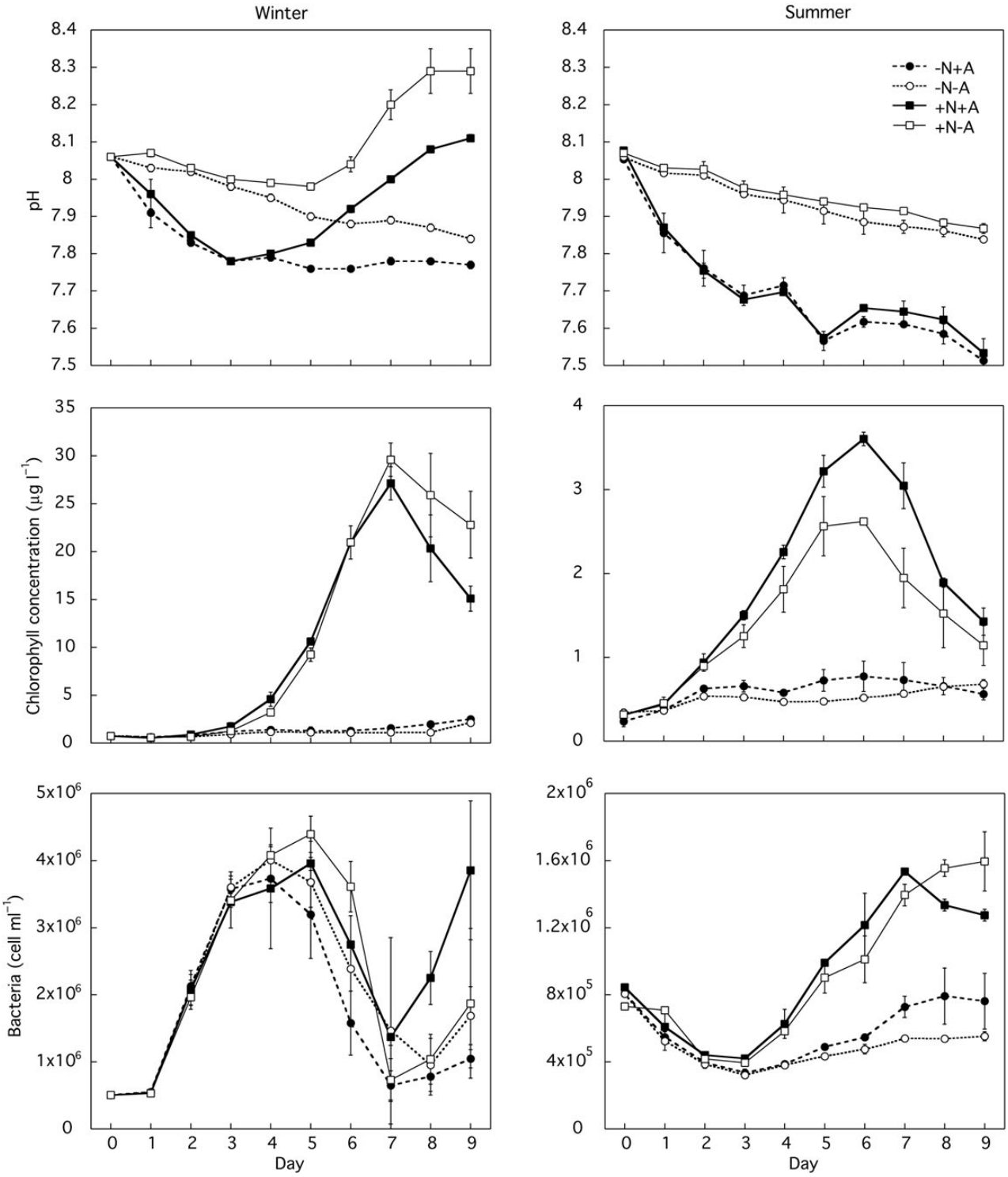

Figure 1. Evolution of pH, chlorophyll concentration, and bacterial abundance in the winter (left panels) and summer (right panels) experiments. Squares, nutrient-amended treatments; circles, controls without nutrient additions; full symbols, acidified treatments; empty symbols, and non-acidified treatments. Error bars denote standard deviations for duplicate treatments.

the year (Krause *et al.*, 2012). Ours is the first study to show differences between winter and summer in the prokaryote response to OA. The higher number of effects of acidification in summer than in winter may be related to differences in initial species composition, but also to the different nutrient levels or to a combination of both factors (see next section).

Cyanobacteria are known to use CCMs to actively transport inorganic C species and maintain their growth even at low external dissolved inorganic carbon concentrations (Badger and Price, 2003). Therefore, it is reasonable to assume that, at increased CO_2, the need for CCMs would decrease, and this would result in energy savings that could be allocated to growth. Indeed, several

Table 3. Results of independent *t*-tests for various abundance and microbial activity data.

	Winter		Summer	
	−Nutrients	+Nutrients	−Nutrients	+Nutrients
Parameter				
Chlorophyll *a* ($\mu g\ l^{-1}$)	0.041		0.007	0.046
Chlorophyll *a* < 3 μm ($\mu g\ l^{-1}$)				
Microplankton (cell l^{-1})				
Diatoms				
Dinoflagellates		0.031	0.004	
Picoplankton (cell ml^{-1})				
Nanoeukaryotes	<0.001	0.014		
Large picoeukaryotes			0.002	0.049
Small picoeukaryotes				
Prokaryotes (cell ml^{-1})				
Synechococcus		<0.001	<0.001	
Prochlorococcus			0.020	
Heterotrophic bacteria			0.050	
Prokaryotic activity				
Leucine incorporation			0.001	
Leu-aminopeptidase				
Chitobiase				
α-Glucosidase			0.050	
β-Glucosidase			0.010	<0.000

Comparison between acidified and control duplicate treatments after day 3. Numbers correspond to *p*-values and only those <0.05 are shown. Light grey denotes the positive effect and dark grey denotes the negative effect of acidification.

culture-based studies have found a stimulation of the growth rate or CO_2 fixation in cyanobacteria with acidification (Fu *et al.*, 2007; Hutchins *et al.*, 2009). However, negative responses to acidification were found in our winter experiment and also in some previous mesocosm studies (e.g. Paulino *et al.*, 2008).

The recent literature tends to suggest that the impact of OA on bacterioplankton community composition is negligible (Ray *et al.*, 2012, , 2013), but compositional shifts (Lindh *et al.*, 2013; Krause *et al.*, 2012) and strain-dependent effects (Teira *et al.*, 2012) have also been encountered. Studies in nutrient-enriched mesocosms have reported decreases (Rochelle-Newall *et al.*, 2004; Grossart *et al.*, 2006) or lack of effects (Allgaier *et al.*, 2008) in bacterial abundance in acidified conditions. In addition, contrasting results were also found in the magnitude and direction of the effect of OA on bacterial activity (Grossart *et al.*, 2006; Allgaier *et al.*, 2008; Motegi *et al.*, 2013).

We also looked at specific functions mediated by heterotrophic bacteria, such as the extracellular enzymatic activities which mediate important biogeochemical processes such as the decomposition and transformation of organic carbon and the release of nutrients (Hoppe, 1983). Still very little is known about the direct effects of OA on extracellular enzyme activities, but effects could be expected since changes in pH have direct effects on the functioning of enzymes in bacterial cultures (Page *et al.*, 1988). However, the results to date have been controversial (see Cunha *et al.*, 2010, for a review). Some authors have found that OA stimulated the activity of a set of extracellular enzymes in different environments (Grossart *et al.*, 2006; Piontek *et al.*, 2010, 2013; Maas *et al.*, 2013), but others reported a lack of stimulation (Arnosti *et al.*, 2011; Engel *et al.*, 2014). In our study, the only enzyme activities for which we found some effect were α- and β-glucosidases, which mediate the final step of the hydrolysis of polysaccharides. Similar to the bulk heterotrophic activity, both glucosidase activities were higher in summer under acidified treatments; Piontek *et al.* (2010) and Maas *et al.* (2013) similarly observed increased β-glucosidase activities under OA. These results suggest higher hydrolysis of carbohydrates. Since carbohydrates represent a large fraction in the composition of organic aggregates of marine ecosystems, an increase in glucosidase activities might contribute to changes in the degradation patterns of different types of organic matter, which could eventually lead to a reduction of the strength of the biological pump in the future ocean.

Effects of nutrient addition on OA

In general, effects of OA on plankton communities have been studied under high nutrient concentrations, either induced or natural (Table 1), and the combined effects of extra inorganic carbon and nutrients promoted phytoplankton growth on most occasions. It is noteworthy that in our experiments the effects of acidification on bacterial abundance and production were more evident in the treatments with the lowest nutrient concentrations. Moreover, the amendment with nutrients altered the responses of phototrophic picoeukaryotes and microplankton. Overall, the compilation of variables that were significantly affected by acidification in our experiment (Table 3) shows that the treatment with the clearly lowest nutrient concentrations (summer non-enriched, see Table 2) had a higher number of variables with significant responses to acidification ($n = 9$) than the other treatments ($n = 2–3$).

The most similar approximation to our study in terms of nutrient dynamics is the EPOCA mesocosm experiment, in which a set of very large enclosures manipulated with CO_2 were enriched in nutrients only after day 14 (Riebesell *et al.*, 2013). In that study, unlike in ours, no significant effects on the abundance of any of the analysed planktonic groups were observed before nutrient addition. However, contrasting responses of phytoplankton abundance and activity to OA (from positive to negative) were observed between the first and the second bloom phases of their study. In the EPOCA experiment, nutrients were added at a point when treatments might have evolved differently during the first few days of the experiment. Although their results cannot be directly compared

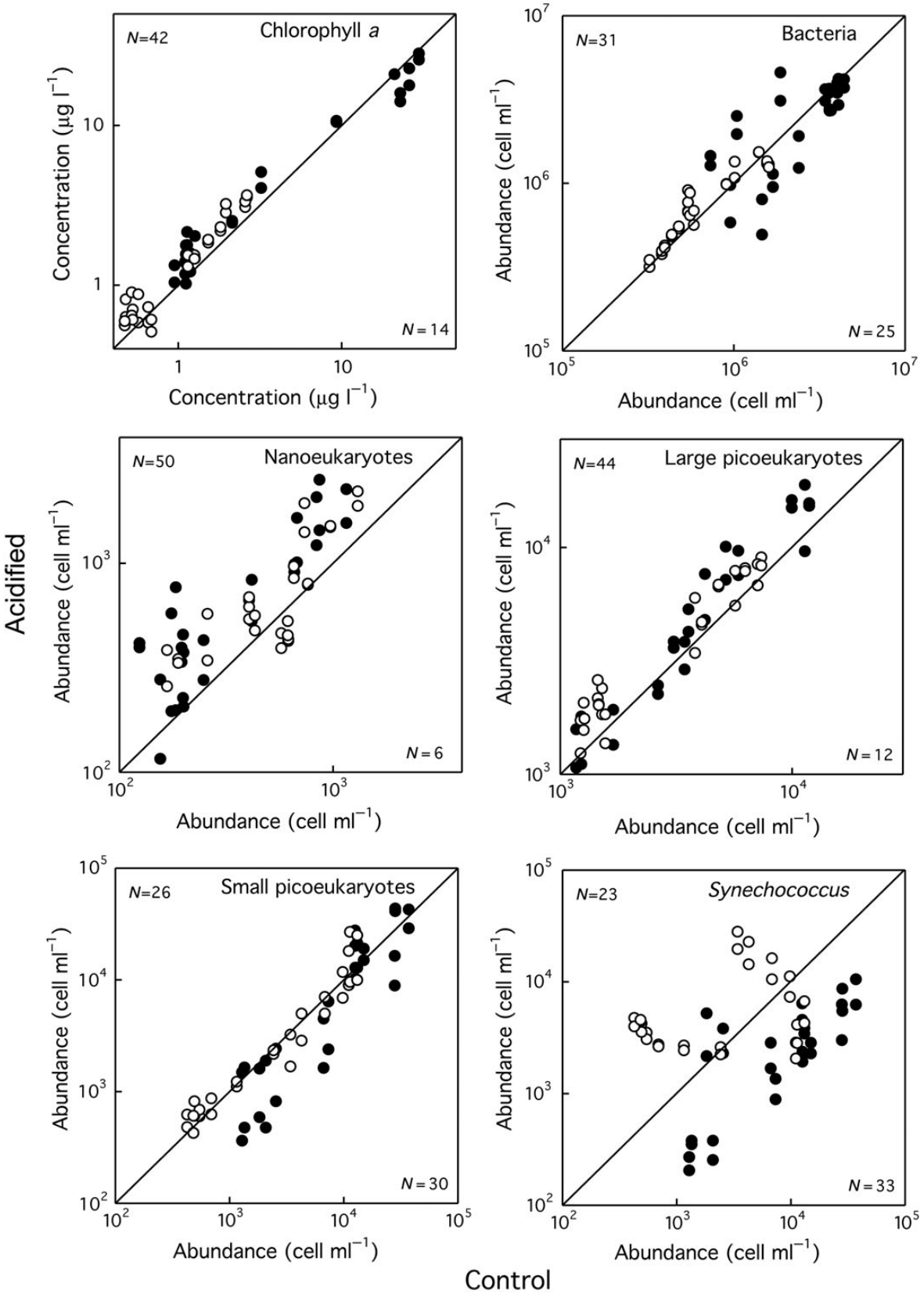

Figure 2. Concentration of chlorophyll *a*, heterotrophic bacteria, and photosynthetic picoplankton groups in acidified vs. non-acidified treatments. Empty symbols, summer experiment; full symbols, winter experiment. The *N* in the upper left corner corresponds to the number of cases in which the value of the acidified treatment is higher than that of the non-acidified treatment. The *N* in the lower right corner is the number of cases with values lower in the non-acidified than in the acidified treatment.

with ours, the present findings corroborate their conclusions about the importance of examining the effect of acidification in contrasting stages of plankton community succession. They evaluated different phases of succession by conducting longer experiments than ours (1 month), whereas we collected natural water in two different seasons with divergent community structures. The initial percentage of diatoms with respect to the total phytoplankton cells was 22% in winter and 2% in summer, a finding that could explain the clear positive response of chlorophyll in summer in contrast with the low response in winter. This result agrees with those of Riebesell *et al.* (2013), in which diatoms responded negatively to acidification. Different photosynthetic groups reacting in opposite ways and indirect effects mediated by changes in prey biochemical composition (i.e. changing the food quality of prey; Schoo *et al.*, 2012) would induce changes in the community structure of grazers, which in turn would feed back on their prey. For instance, if different predators are favoured, then a different nutrient competition could be established between prokaryotes and microphytoplankton, etc. The existence of these complex interdependences requires further research on multiple trophic interactions when ecosystem responses to acidification are evaluated.

At a smaller scale (4-l bottles), a recent study in the Mediterranean reported a very limited impact of OA on the planktonic communities of nutrient-depleted waters (Maugendre *et al.*, 2015). This experiment could be closely related to our work because of the area of study, although the original community may have been different. The species-specific responses to nutrient additions and acidification, usually veiled by bulk analyses (chlorophyll, cytometry, etc.), may be the key to explaining the discrepancies between that study and ours. As seen above, many groups benefit from nutrient additions and OA. Others, however, may be outcompeted by faster-growing species with better capabilities of incorporating and accumulating CO_2.

The fact that we observed a larger number of effects of acidification under low nutrient concentrations emphasizes the importance of running experiments under natural conditions. When the effects of OA on very productive communities need to be investigated, it would be advisable to conduct the experiments during natural bloom situations, rather than generating an artificial one. In addition, to better represent the natural environmental conditions, we advise that research should focus on communities of areas of great importance that remain mostly unexplored so far, such as the oligotrophic ocean.

The duration of the mesocosm experiment is an additional important variable to consider. Normally, mesocosm studies on planktonic communities have been conducted over timescales ranging from days to one month. Experimentation over longer periods of time would be interesting to address issues related to acclimation and adaptation. However, prolonging mesocosm experiments for too long is not trivial, and there is an inherent danger of driving the community away from the real world over time (i.e. due to wall effects, unreal water mixing, etc.). In our case, though the 9-d experiment is too short to be meaningful for assessing potential acclimation/adaptation, it is set up over a temporal pH variability that parallels the real world, particularly in the coastal areas. These environments often exhibit natural short-term changes in seawater at scales of days or even hours of similar magnitude to that induced in our experiments. Furthermore, progressive OA due to anthropogenic CO_2 emissions superimposed on these short-term pH changes will induce similar changes to those experienced by our mesocosm communities.

Further understanding of OA effects on plankton communities will come from the integration of single-species studies, short mesocosm experiments such as the present one, field observations in naturally or artificially acidified environments and ecosystem modelling, together with data on potential acclimation and adaptation through multi-generational studies.

As a general trend, in our study we observed a stimulatory effect of OA on the abundance of small phytoplankton (pico- and nanoeukaryotes) independently of the nutrients added. Considering the effects of combining acidification and eutrophication, our observations point towards a lower sensitivity of the microbial community to OA under eutrophic conditions, which may have implications on the interpretation of the effects of OA in coastal and more nutrient-rich systems compared with oligotrophic open-ocean environments. They also highlight the need for comparative experimental studies during different periods of the year and with different levels of nutrient concentrations to provide a broader assessment of the effects of acidification on marine ecosystems

Supplementary data

Supplementary material is available at the *ICESJMS* online version of the manuscript.

Acknowledgements

The authors thank Despoina Sousoni, Berta de la Vega and Anderson Cabral for research assistance. Financial support from the Spanish Ministry of Economy and Competitiveness is acknowledged, specifically through the projects STORM (CTM2009–09352), DOREMI (CTM2012-34294), MANIFEST (CTM2012-32017), and PROTOS (CTM2009-08783) of the Spanish Ministry of Economy and Competitiveness. We are also grateful for the financial support of the Generalitat de Catalunya to the "Grup de Diversitat Microbiana en Ecosistemes Acuàtics" (2014SGR/1591) and the "Grup d'Estructura i Funció de Xarxes Tròfiques Microbianes Planctòniques" (2014SGR/1179).

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Handling editor: Howard Browman