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Original Article

Effect of ocean warming and acidification on a plankton community in the NW Mediterranean Sea

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The effect of ocean warming and acidification was investigated on a natural plankton assemblage from an oligotrophic area, the bay of Villefranche (NW Mediterranean Sea). The assemblage was sampled in March 2012 and exposed to the following four treatments for 12 days: control (~360 µatm, 14°C), elevated pCO_2 (~610 µatm, 14°C), elevated temperature (~410 µatm, 17°C), and elevated pCO_2 and temperature (~690 µatm, 17°C). Nutrients were already depleted at the beginning of the experiment and the concentrations of chlorophyll *a* (chl *a*), heterotrophic prokaryotes and viruses decreased, under all treatments, throughout the experiment. There were no statistically significant effects of ocean warming and acidification, whether in isolation or combined, on the concentrations of nutrients, particulate organic matter, chl *a* and most of the photosynthetic pigments. Furthermore, ^{13}C labelling showed that the carbon transfer rates from ^{13}C-sodium bicarbonate into particulate organic carbon were not affected by seawater warming nor acidification. Rates of gross primary production followed the general decreasing trend of chl *a* concentrations and were significantly higher under elevated temperature, an effect exacerbated when combined to elevated pCO_2 level. In contrast to the other algal groups, the picophytoplankton population (cyanobacteria, mostly *Synechococcus*) increased throughout the experiment and was more abundant in the warmer treatment though to a lesser extent when combined to high pCO_2 level. These results suggest that under nutrient-depleted conditions in the Mediterranean Sea, ocean acidification has a very limited impact on the plankton community and that small species will benefit from warming with a potential decrease of the export and energy transfer to higher trophic levels.

Keywords: climate change, ocean acidification, ocean warming, oligotrophic area, plankton community, primary production.

Introduction

Anthropogenic carbon dioxide (CO_2) emissions are responsible for an important increase in atmospheric CO_2 partial pressure (pCO_2). The consequences of CO_2 emissions are an increase of surface ocean temperature expected to rise by 2–4°C by the end of this century based on the current emission rates (IPCC, 2013). About 25% of anthropogenic CO_2 emissions are absorbed by the ocean (Le Quéré *et al.*, 2013), generating profound modifications of the ocean carbonate chemistry and referred to as "ocean acidification". The pH of the surface ocean has decreased by 0.1 units since the beginning of the industrial era and is projected to decrease by an extra 0.3–0.4 units by the end of the present century (Orr, 2011). Seawater warming and acidification are expected to significantly affect the carbon cycle through the changes in the functioning of marine organisms and communities.

CO_2 fluxes between the atmosphere and the ocean are partly driven by biological activity. In the surface mixed layer, the balance between the autotrophic fixation of CO_2 by primary producers and the consumption/mineralization of organic matter by the whole plankton community is referred to as the net community production (NCP). A system is referred to as autotrophic when production exceeds consumption and heterotrophic when consumption is higher than production. Depending on the atmospheric pCO_2 and sea surface temperature, the surface ocean potentially acts

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as a sink of CO_2 for the atmosphere (Gattuso *et al.*, 1998) when the surface mixed layer exports organic matter to the deep ocean. Primary producers have then a key role on carbon cycle and climate regulation.

In many marine plants and algae, RuBisCO, a key enzyme involved in CO_2 fixation, is generally limited at environmental CO_2 concentrations. An increase in CO_2 could therefore enhance phytoplankton photosynthesis and growth (Riebesell *et al.*, 2007). However, experimental studies reported contrasting effects of elevated CO_2 on photosynthesis: stimulating, neutral or even inhibitory effects were found (see review by Riebesell and Tortell, 2011 and references therein). Such different responses could be due to species-specific differences in the efficiency of carbon concentrating mechanisms (CCMs; e.g. Giordano *et al.*, 2005). Depending on the efficiency of their CCM, some species will benefit from elevated CO_2 conditions while others will not, provoking changes in community composition. Shifts toward smaller (e.g. Yoshimura *et al.*, 2010) or larger phytoplankton cells (Tortell *et al.*, 2002) have been observed while no change in the taxonomic composition have been reported for communities acclimated to large seasonal pH changes (Nielsen *et al.*, 2012). Changes in community composition could have consequences on ecological processes (such as modifications of energy transfer to higher trophic levels) and biogeochemical cycling (i.e. modifications of the export to the deep ocean; Riebesell *et al.*, 2007). With respect to ecosystem function, several studies have shown enhanced carbon fixation and an increase in the organic carbon to nitrogen ratio (e.g. Riebesell *et al.*, 2007), while others reported limited or no effect (Feng *et al.*, 2009).

Projected warming is also expected to significantly affect marine organisms and communities. Culture experiments and *in situ* sampling have shown increased metabolic rates as temperature increases (e.g. Eppley, 1972; Regaudie-de-Gioux and Duarte, 2012). However, as thermal tolerance greatly differs between species, some will face conditions outside of their tolerance range and will be forced to move their ecological niches (Gao *et al.*, 2012 and references therein). Several experiments have shown that warming could induce a shift towards smaller phytoplankton species (Sommer and Lengfellner, 2008) as well as a tighter coupling between phytoplankton and bacteria with possible consequences on remineralization and carbon export (Hoppe *et al.*, 2008). Furthermore, phytoplankton exhibits higher nitrogen to phosphate requirements in warmer conditions (Toseland *et al.*, 2013), which might also impact biogeochemistry.

In recent years, an experimental effort has been initiated to investigate the effect of both drivers at the community level. Elevated temperature combined or not with elevated pCO_2 has been shown to enhance photosynthetic rates (Hare *et al.*, 2007; Feng *et al.*, 2009) as well as enhanced dissolved organic carbon relative to particulate organic production (Kim *et al.*, 2011). A recent study focusing on the short- (2 weeks) and long-term (1 year) response of a diatom community showed that elevated pCO_2 and temperature, whether combined or taken in isolation, had an effect on the community structure, with a stronger influence of warming which induces a loss in species richness (Tatters *et al.*, 2013). Another study supported the predominant effect of warming compared with acidification on bacterial phylogenetic composition (Lindh *et al.*, 2013).

Nutrient availability is suspected to also have strong effects on the community response to ocean warming and acidification (Hare *et al.*, 2007) and the great majority of past experiments have been performed under nutrient replete conditions. However, a large part (>60%) of the open ocean is characterized by oligotrophic conditions with very low nutrient concentrations and rates of primary production (Dodds and Cole, 2007). Although the metabolic status (auto- vs. heterotrophic) of these areas is still under debate (Duarte *et al.*, 2013; Williams *et al.*, 2013) oligotrophic provinces represent ~30% of global oceanic primary production (Longhurst *et al.*, 1995). Therefore, changes in the community composition and functioning in these regions could lead to significant changes in the global oceanic CO_2 sink. The Mediterranean Sea is a largely enclosed sea, presenting trophic status varying from mesotrophic in the Northwestern region to extremely oligotrophic in the Eastern basin. Despite these environmental constraints, the Mediterranean Sea hosts from 4 to 18% of the Earth's marine biodiversity (Bianchi and Morri, 2000) with a high percentage of endemic species. There is a growing concern on the effects of climate change and ocean acidification in this area, although, to the best of our knowledge, no experiment on the effect of elevated temperature and pCO_2 on natural plankton communities have been conducted to date.

In the present study, a Mediterranean plankton community sampled in winter was exposed to elevated temperature and pCO_2 as projected for the end of the century (respectively, +3°C and ×2 pCO_2). During 12 days, experimental bottles were placed in a control and a temperature-regulated outdoor tank. Parameters and processes such as carbonate chemistry, nutrients, particulate organic matter, pigments, cells abundance, and primary production were monitored regularly. Stable carbon isotope tracers (^{13}C) were also used to measure carbon fixation.

Material and methods

Experimental setup

A volume of 300 l of seawater was sampled in the bay of Villefranche (France; 43°40′N, 7°18′E) at 5 m on 14 March 2012. Pumping was performed by a trace-metal clean pump activated by pressurized air from a diving tank, preventing any damage on the organisms. Seawater was sieved onto a 200-μm mesh to remove large organisms.

In the laboratory, seawater was transferred to an acid-cleaned 300 l tank. Labelled ^{13}C-sodium bicarbonate was added to a final concentration of 19 μmol l^{-1} corresponding to 0.83% of total dissolved inorganic carbon (DIC) concentration and increasing $\delta^{13}C$-DIC by 760‰. A first set of 4 l acid-cleaned polycarbonate (PC) bottles ($n = 24$) was filled and hermetically sealed. CO_2-saturated filtered seawater was gently mixed with the remaining water to reach a calculated pCO_2 of ~750 μatm. This elevated pCO_2 seawater was then distributed to 4 l acid-cleaned polycarbonate bottles ($n = 24$). Half of the ambient and elevated pCO_2 bottles were placed in a 2 m^3 tank installed on the pier of the Laboratoire d'Océanographie de Villefranche with a continuous flow of *in situ* seawater (20 l min^{-1}). The other half was placed in another identical tank in which temperature was maintained at ca. 3°C above *in situ* temperature. The four treatments, including three replicates bottles per sampling day, were: Control (C; ambient pCO_2 and temperature), Ocean Warming (OW; ambient pCO_2 and elevated temperature), Ocean Acidification (OA; elevated pCO_2 and ambient temperature), and Greenhouse (G; elevated pCO_2 and temperature). The bottles were gently stirred every day to keep particles suspended. Light (natural sunlight) conditions were similar between the different treatments and were representative of surface conditions (~1 m depth). On several occasions, photosynthetic active radiation (PAR) was measured in the tanks with a spherical sensor

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connected to an LICOR data logger, and daily (sunrise–sunset) averages ranged between 1025 and 1213 μmol photons m^{-2} s^{-1}. Temperature was measured with a Seabird SBE37 temperature sensor and temperature in the elevated temperature tank was regulated using a COREMA© controller. Temperature regulation was performed throughout the experiment but, due to a technical problem, data were not logged on the first day.

Sampling and analyses

After 2, 4, 8, and 12 days (thereafter referred to as d2, d4, d8, and d12), three bottles of each treatment were removed from the tanks. Samples for DIC were stored in 60 ml brown borosilicate bottles and poisoned with 10 μl saturated solution of mercuric chloride ($HgCl_2$). For total alkalinity (A_T) determination, 500 ml was filtered through 47 mm GF/F filter, poisoned with $HgCl_2$ and preserved at 4°C pending analysis. Aliquots of 20 ml for the determination of inorganic phosphate (PO_4^{3-}), nitrate and nitrite ($NO_x = NO_3^- + NO_2^-$) were filtered through 0.2 μm polycarbonate filters cleaned with Merck Suprapur hydrochloric acid and rinsed with 18.2 MΩ cm^{-1} ultra pure water. Samples were stored in 125 ml polyethylene (PE-HD) flasks, cleaned with Merck Suprapur hydrochloric acid, and acidified with Merck Ultrapur HCl, and finally processed using a spectrophotometric method with a Liquid Waveguide Capillary Cell optic fibre, allowing the determination of nanomolar concentrations phosphate and NO_x with detection limits of 1 and 9 nmol l^{-1}, respectively, and a precision (coefficient of variation) of ~7% for both parameters (Adornato *et al.*, 2007; Zimmer and Cutter, 2012). Samples for the determination of silicate were stored in acid-cleaned PE vials, poisoned with a saturated solution of $HgCl_2$ and kept at 4°C pending analysis using an AXFLOW AA3 auto–analyzer. A 2 ml aliquot of seawater was added to 80 μl of glutaraldehyde for the analysis of community composition. Samples were snap-frozen in liquid nitrogen and kept at −80°C pending analysis on a FACSCalibur flow cytometer. For pigment analyses, 0.5–1 l of seawater were filtered on 25 mm GF/F membranes which were stored at −80°C pending extraction and analysis on an Agilent Technologies 1200 series following the protocol of Ras *et al.* (2008). For particulate organic carbon (POC) concentration and its isotopic signature (δ^{13}C-POC), 1 l of seawater was filtered through pre-combusted and preweighted 25 mm GF/F filters under low pressure. Filters were dried at 60°C for 24 h then stored at room temperature in the dark. For measurement of δ^{13}C-DIC, samples (20 ml) were poisoned with 10 μl of $HgCl_2$ and stored at room temperature in the dark. The remaining seawater in each 4 l polycarbonate bottle was used for measurements of community metabolism (see below).

DIC was determined immediately after opening the bottles on triplicate 1.2 ml subsamples using an inorganic carbon analyser (AIRICA, Marianda, Kiel, Germany) coupled to an infrared gas analyser (LI-COR 6262). This instrument was calibrated before sample analysis against a certified reference material provided by A. Dickson (Scripps Institution of Oceanography, San Diego, CA, USA; batch 114). The average precision (*SD*) of all measurements ($n = 52$; ran in triplicates) was 0.7 μmol kg^{-1}. A_T was determined on triplicate 50 ml subsamples by potentiometric titration on a Metrohm Titrando 80 titrator coupled to a glass electrode (Metrohm, electrode plus) and a thermometer. The pH electrode was calibrated daily on the total scale using TRIS buffers of salinity 35 provided by A. Dickson. Measurements were carried out at 25°C and A_T was calculated as described by Dickson *et al.* (2007). Along the experiment, standards provided by A. Dickson (batch 108) were used to check precision and accuracy ($n = 18$; 2.9 and 3.7 μmol kg^{-1}, respectively). The parameters of the carbonate system were determined from DIC, A_T, temperature, and salinity using the R package seacarb (Lavigne *et al.*, 2014). To take into account the uncertainty of the measured input parameters during the calculation of the carbonate chemistry parameters, a Monte-Carlo procedure was applied. One thousand values were randomly chosen between the mean ± *SD* of each measured parameter and mean ± *SD* of each computed parameters is reported.

POC samples were analysed for organic carbon content and isotope ratios on an elemental analyser (Thermo Electron Flash EA 1112) coupled to a Delta V isotope ratio mass spectrometer (IRMS). For DIC isotope analyses, a 2 ml helium headspace was created in the vials and samples were acidified with 2 μl of phosphoric acid (H_3PO_4; 99%). After equilibration for 30 min, the CO_2 concentration and its isotopic composition in the headspace were measured on the EA-IRMS.

Data of the ^{13}C-labelling study were expressed in the delta notation (δ) relative to Vienna Pee Dee Belemnite standard. The carbon isotope ratio was calculated as

$$R_{\text{sample}} = \left(\frac{\delta^{13}C_{\text{sample}}}{1000 + 1}\right) \times R_{\text{VPDB}}, \quad \text{with } R_{\text{VPDB}} = 0.0111797.$$

The ^{13}C fraction was calculated as:

$$^{13}\text{F} = \frac{^{13}C}{^{13}C + ^{12}C} = \frac{R}{R + 1},$$

where $R = {}^{13}\text{C}/{}^{12}\text{C}$.

The excess ^{13}C was obtained as $\Delta^{13}\text{F} = {}^{13}\text{F}_{\text{sample}} - {}^{13}\text{F}_{\text{background}}$. Absolute incorporation rates were calculated as ^{13}C-POC = $\Delta^{13}\text{F} \times [\text{POC}]_{\text{sample}}$ (μmol C l^{-1}; De Kluijver *et al.*, 2010).

Finally, ^{13}C-concentrations were converted to total fresh POC:

$$\text{New - POC} = \left(\frac{\Delta^{13}\text{F}_{\text{POC}}}{\Delta^{13}\text{F}_{\text{DIC}}}\right) \times [\text{POC}],$$

where $\Delta^{13}\text{F}_{\text{POC}}$ and $\Delta^{13}\text{F}_{\text{DIC}}$ are the excess values, [POC] is a concentration in μmol C l^{-1}.

NCP is then calculated as:

$$\text{NCP}-{}^{13}\text{C} = \frac{\Delta\text{New - POC}}{\Delta t},$$

where ΔNew-POC is the differences between two consecutive sampling days.

Primary production and community respiration

NCP (NCP-O_2) and community respiration (CR-O_2) were measured using the oxygen light–dark technique. Gross community production was measured using the ^{18}O-labelling method (gross primary production, GPP-^{18}O).

Before sunrise, three 60 ml biological oxygen demand bottles were sampled from each PC bottle. One bottle was immediately fixed with Winkler reagents to determine the initial O_2 concentration. A transparent and a dark bottle were incubated in the outdoor tanks for 24 h for estimating NCP-O_2 and CR-O_2, respectively. O_2 concentrations were measured using an automated Winkler titration technique with potentiometric endpoint detection. Analyses were performed with a Metrohm Titrando 888 with

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a Metrohm ion electrode. Reagents and standardizations were similar to those described by Knap *et al.* (1996). NCP-O_2 and CR-O_2were estimated by regressing O_2 values against time, and CR was expressed as negative values. GPP (GPP-O_2) was calculated as the difference between NCP-O_2 and CR-O_2. The combined errors were calculated as:

$$SE_{x-y} = \sqrt{(SE_x^2) + SE_y^2}.$$

For the ^{18}O-labelling technique, samples were transferred from each PC bottle to two 60 ml transparent glass bottles and sealed. One bottle was directly poisoned with 10 µl saturated solution of $HgCl_2$ to estimate the natural isotopic composition and the other bottle was spiked, with 50 µl of 97% $H_2^{18}O$ to reach a final isotopic composition δ^{18}O-H_2O of 335‰. After 12 h incubations in the outdoor tank (from sunrise to sunset), samples were poisoned using $HgCl_2$ and stored upside down in the dark at room temperature pending analysis. Measurements were performed at KU Leuven (Belgium). A headspace of 3 ml was created with helium and allowed to equilibrate for 30 min (^{18}O-O_2 measurements). The extracted water was injected into helium-flushed vials (^{18}O-H_2O measurements). Pure CO_2 (100 µl) was then added and samples were allowed to equilibrate for 24 h. δ^{18}O-H_2O was therefore measured as δ^{18}O-CO_2. Determinations of δ^{18}O-O_2 and δ^{18}O-CO_2 were accomplished using an elemental analyzer (Flash HT/EA) coupled to a Delta V IRMS. An overflow technique was used to limit air contamination of the needle. For δ^{18}O-O_2, the internal standard used to correct the data and survey instrumental deviation was air from the outside. For δ^{18}O-CO_2, a calibration was performed with a VSMOW standard. GPP rates (µmol O_2 l^{-1} d^{-1}) were calculated using the following equation (Kiddon *et al.*, 1995):

$$GPP\text{-}^{18}O = \left[\frac{\delta^{18}O\text{-}O_{2\ final} - \delta^{18}O\text{-}O_{2\ init}}{\delta^{18}O\text{-}H_2O\text{-}\delta^{18}O\text{-}O_{2\ init}}\right] \times [O_2]_{init},$$

where δ^{18}O-$O_{2\ init}$ and δ^{18}O-$O_{2\ final}$ are measured δ^{18}O-O_2 before and after incubation (‰), respectively, δ^{18}O-H_2O is the final isotopic composition of the labelled water (‰), and $[O_2]_{init}$ is the O_2 concentration before incubations (µmol l^{-1}). The overall error was estimated using a Monte-Carlo procedure as described earlier.

Statistics and data availability

Data are presented as averages ± *SD* (or ± SE for metabolic rates). Due to the small number of replicates (×3), PERMANOVA analyses were performed using the R package RVAideMemoire (Hervé, 2013) to test for differences in parameters/processes between the four different treatments. These analyses were performed considering two interacting factors (pCO_2 and temperature) and one blocking factor (time) over 1000 permutations and a significant effect was considered when $p < 0.05$. Cumulative metabolic rates were calculated for the whole experimental period. Values for days when no incubations were performed were obtained by linear interpolation and the cumulative values were then summed up for the experimental period. The data reported here as well as complementary parameters are freely available in Pangaea: http://doi.pangaea.de/10.1594/PANGAEA.834159

Results

In the two tanks, temperature naturally varied by ~2°C between day and night (Figure 1). The natural average temperature was 14.3 ± 0.3°C while it was on average 17.2 ± 0.7°C in the elevated temperature tank (average difference: 2.8°C). In treatments C and OW, pCO_2 was on average 364 ± 14 µatm (pH_T 8.12 ± 0.02) and 414 ± 12 µatm (pH_T 8.07 ± 0.01), respectively. Elevated pCO_2 conditions were on average 613 ± 22 µatm (pH_T 7.92 ± 0.01) and 690 ± 28 µatm (pH_T 7.88 ± 0.02) for treatments OA and G, respectively. The targeted pCO_2 levels were not reached most likely as a consequence of significant outgassing while bottles were filled. A_T averaged for all treatments was 2568 ± 4 µmol kg^{-1} (Table 1) and did not vary significantly between treatments and sampling days (Table 2).

All dissolved inorganic nutrients were close to the detection limit. The concentration of NO_x and silicate did not vary between treatments (Table 2; Figure 2a and b) but the phosphate concentration was significantly lower under elevated pCO_2 ($F = 13.19$, $p < 0.05$; Table 2, Figure 2c). Phosphate was on average 13 ± 1 nmol P l^{-1} and NO_x ($NO_3^- + NO_2^-$) concentrations remained constant (75 ± 20 nmol N l^{-1}), after an initial decrease between d0 and d2 (Figure 2b). Silicate concentrations did not vary with time and averaged 1.0 ± 0.1 µmol Si l^{-1} (Figure 2a).

The concentration of POC did not significantly differ between treatments (Table 2) with an overall mean of 11 ± 1 µmol C l^{-1} (Figure 3a). PON concentrations were low (mean: 0.8 ± 0.1 µmol N l^{-1}), except on d2 in the OW and OA treatments where concentrations reached 1.9 ± 0.4 µmol N l^{-1} (Figure 3b). The particulate organic C : N ratio was high with a global average of 15 ± 1 (6 ± 1 for OW and OA on d2) and was not different between treatments (Table 2). As for POC and PON, the concentration of chlorophyll *a* (chl *a*) did not differ between treatments (Table 2) but varied significantly throughout the experiment (Figure 3c). It increased from an overall mean of 0.9 ± 0.1 µg l^{-1} on d0 to 1.1 ± 0.0 µg l^{-1} on d2. After d2, it decreased in all treatments to reach an average final concentration of 0.3 < 0.1 µg l^{-1}.

In terms of phytoplankton group succession during the experiment, diatoms (as represented by fucoxanthin pigments), prymnesiophytes (19′-hexanoyloxyfucoxanthin), and cyanobacteria (zeaxanthin) were detected in the samples with a large dominance

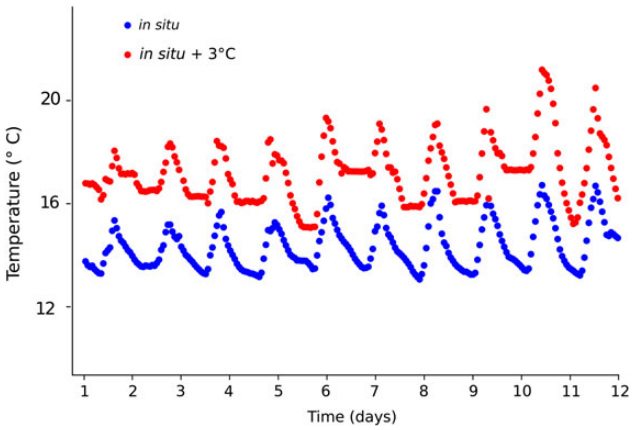

Figure 1. Temperature during the experiment. Blue: *in situ* temperature (Control and Ocean Acidification treatments). Red: *in situ* + 3°C (Ocean Warming and Greenhouse treatments).

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Table 1. Carbonate chemistry parameters in the control (C), ocean warming (OW), ocean acidification (OA), and greenhouse (G) treatments (average ± *SD*).

	Alkalinity (μmol kg^{-1})	DIC (μmol kg^{-1})	pCO_2 (μatm)	pH_T
Day 0				
C	2566 ± 1	2278 ± 0	366 ± 2	8.11 < 0.01
OW	2567 ± 2	2286 ± 0	428 ± 1	8.05 < 0.01
OA	2570 ± 2	2386 ± 0	622 ± 3	7.91 < 0.01
G	2565 ± 1	2352 ± 0	618 ± 2	7.92 < 0.01
Day 2				
C	2566 ± 2	2283 ± 2	377 ± 2	8.10 < 0.01
OW	2563 ± 0	2284 ± 2	431 ± 5	8.05 < 0.01
OA	2565 ± 1	2371 ± 6	586 ± 18	7.94 ± 0.01
G	2566 ± 4	2374 ± 2	666 ± 2	7.89 < 0.01
Day 4				
C	2566 ± 2	2270 ± 3	356 ± 6	8.12 < 0.01
OW	2566 ± 2	2273 ± 1	405 ± 1	8.07 < 0.01
OA	2568 ± 2	2383 ± 3	620 ± 17	7.92 ± 0.01
G	2566 ± 3	2386 ± 3	716 ± 22	7.86 ± 0.01
Day 8				
C	2568 ± 5	2277 ± 4	363 ± 12	8.12 ± 0.01
OW	2567 ± 1	2279 ± 1	415 ± 1	8.06 < 0.01
OA	2567 ± 1	2385 ± 4	629 ± 13	7.91 < 0.01
G	2573 ± 3	2380 ± 2	669 ± 14	7.89 < 0.01
Day 12				
C	2577 ± 11	2281 ± 6	359 ± 23	8.12 ± 0.02
OW	2569 ± 1	2275 ± 3	404 ± 5	8.07 < 0.01
OA	2568 ± 0	2383 ± 2	617 ± 4	7.92 < .01
G	2569 ± 5	2387 ± 8	708 ± 22	7.87 ± 0.01
Average				
C	2569 ± 5	2278 ± 5	364 ± 14	8.12 ± 0.02
OW	2566 ± 2	2279 ± 6	414 ± 12	8.07 ± 0.01
OA	2568 ± 2	2382 ± 6	613 ± 22	7.92 ± 0.01
G	2568 ± 3	2377 ± 12	690 ± 28	7.88 ± 0.02

Total alkalinity (A_T) and dissolved inorganic carbon (DIC) were measured, while the partial pressure of CO_2 (pCO_2) and pH_T were estimated based on DIC and A_T using seacarb (see the Material and method for more details).

of prymnesiophytes (28% on average; Figure 4a–c). Variations in the concentration of diatoms and prymnesiophytes were similar to chl *a* variations, i.e. an increase during the first 2 days followed by a general decrease. Variations in zeaxanthin concentrations indicate that the abundance of cyanobacteria increased from d0 to d8 then declined at the end of the experiment. This is consistent with flow cytometer data which show the same dynamics for the cyanobacteria *Synechococcus* (Figure 4f) with a significant correlation between the two techniques (HPLC and flow cytometry; $r = 0.86$, $p < 0.01$, $n = 52$). The abundance of *Prochlorococcus*, another cyanobacteria, increased until d4 then decreased (data not shown). Pigments and flow cytometry data showed a specific response to elevated temperature alone for cyanobacteria (zeaxanthin: $F = 6.98, p < 0.05$; *Synechococcus* by flow cytometry: $F = 6.11, p < 0.05$; Table 2, Figure 4c and f). The abundance of pico-eukaryotes was significantly different at elevated pCO_2 ($F = 8.69$, $p < 0.05$; Table 2), a difference that could be attributed to a transient higher abundance of this group in the OA treatment on d2 (data not shown). The abundance of viruses and heterotrophic prokaryotes decreased with time (Figure 4d and e). There was no difference between treatments for heterotrophic prokaryotes (Table 2, Figure 4d) and a significant temperature effect was found for viruses ($F = 5.57$, $p < 0.05$; Table 2) due to two very high values in the C and OA treatments on d8 (Figure 4e), this effect disappeared when these two values were omitted.

Based on the O_2 light–dark technique, no significant difference between treatments was observed for the considered metabolic processes (NCP-O_2, GPP-O_2, and CR-O_2; Table 2). NCP-O_2 ranged from -1.6 ± 0.8 to 2.8 ± 0.2 μmol O_2 l^{-1} d^{-1} while CR-O_2 ranged from -4.3 ± 0.8 to -0.8 ± 1.3 μmol O_2 l^{-1} d^{-1} (Figure 5a and b). GPP-O_2 varied from -0.15 ± 1.5 to 5.74 ± 3.1 μmol O_2 l^{-1} d^{-1} (Figure 5c). NCP-O_2 did not show a clear temporal trend except for G treatment for which NCP-O_2 decreased from autotrophic to heterotrophic conditions throughout the experiment and in general, very large variations were observed for all treatments (Figure 5a). GPP-^{18}O followed the decreasing trend of chl *a* (Figure 5d) and was significantly increased under elevated temperature ($F = 15.82$, $p < 0.01$; Table 2) with a significant interaction with pCO_2 ($F = 7.28$, $p < 0.05$). No significant correlation was found between GPP estimated by the two methods (GPP-^{18}O and GPP-O_2; $r = 0.26$, $p > 0.05$, $n = 48$). The cumulative GPP-^{18}O was 33.0 ± 3.4 and 29.0 ± 2.3 μmol O_2 l^{-1} for C and OA treatments, respectively. OW and G treatments presented higher cumulative values with 34.8 ± 2.3 and 38.5 ± 2.4 μmol O_2 l^{-1}, respectively.

δ^{13}C-DIC in natural sample was ~3‰ (data not shown) and reached, as expected, 759 ± 18‰ after addition of labelled ^{13}C-sodium bicarbonate. ^{13}C-DIC did not significantly change during the course of the experiment and did not differ between treatments (Figure 6a, Table 2). The transfer from labelled DIC to POC was very rapid and efficient, allowing the detection of ^{13}C-POC enrichment on the first sampling day (d2; Figure 6b), and saturation was achieved already at d4. ^{13}C-POC enrichment did not differ between treatments (Table 2). $\Delta\delta^{13}$C-POC increased to a final enrichment of 501 ± 23‰. NCP-^{13}C was lower than NCP-O_2 exhibiting a decreasing trend (Table 3) and did not significantly differ between treatments (Table 2). Cumulative NCP-^{13}C was 11.8 ± 0.6 and 11.4 ± 0.2 μmol C l^{-1} for C and OA, respectively. The warmer treatments had slightly higher values of 12.3 ± 0.3 and 12.1 ± 0.5 μmol C l^{-1} for OW and G, respectively.

Discussion

This experiment was designed to study the effects of ocean warming and acidification on the composition and functioning of an oligotrophic plankton community in the coastal NW Mediterranean Sea. The elevated temperature condition was very well controlled with an average offset between ambient and elevated temperature of 2.8 ± 0.4°C. The current rate of warming in the coastal NW Mediterranean Sea has been estimated to range from 0.026 to 0.033°C yr^{-1} (Bensoussan *et al.*, 2009), although temperature projections are difficult to obtain due to large regional differences. Given these rates, the average 2.8°C temperature increase as applied in our study was representative for the end of the century. Although pCO_2 was lower than targeted and therefore lower than commonly used in similar perturbation studies, high-pCO_2 values of ~610–690 µatm correspond to the level of atmospheric CO_2 projected for 2060 according to the RCP 8.5 scenario (Meinshausen *et al.*, 2011).

The *in situ* sampling was performed 1 day after the maximum surface chl *a* concentration was measured in the Bay of Villefranche (data not shown; but see http://somlit-db.epoc.u-bordeaux1.fr/bdd.php). Consequently, nutrients were depleted with levels very close to detection limits. NO_x was consumed rapidly in all treatments during the first 2 days of the experiment (from d0 to d2) and its

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Table 2. Results of the permutational analysis of variance for selected parameters and processes.

	pCO_2		temperature		pCO_2: temperature	
	F	***p***	***F***	***p***	***F***	***p***
Carbonate chemistry						
Alkalinity	0.029	0.874	0.974	0.362	0.686	0.458
DIC	696.193	0.001*	0.171	0.685	0.656	0.443
pCO_2	555.319	0.001*	0.175	0.688	1.000	0.341
pH	678.988	0.001*	0.048	0.831	1.191	0.2917
Nutrients						
NO_x	4.017	0.078	0.551	0.482	0.792	0.387
PO_4^{3-}	13.187	0.005*	0.038	0.836	2.589	0.152
Silicate	0.969	0.376	0.350	0.587	1.652	0.222
Particulate organic matter						
POC	0.039	0.837	1.956	0.184	0.875	0.377
PON	0.000	0.990	0.135	0.729	3.240	0.093
POC : PON	0.050	0.855	0.030	0.876	3.713	0.079
Pigments						
Chl *a*	1.832	0.216	1.146	0.303	0.006	0.934
Fucoxanthin	1.254	0.285	0.548	0.479	0.004	0.966
19′-Hexafucoxanthin	0.384	0.537	2.570	0.125	0.210	0.656
Zeaxanthin	0.017	0.902	6.983	0.027*	0.037	0.842
Flow cytometry						
Synechococcus	0.332	0.566	6.106	0.028*	0.084	0.781
Pico-eukaryotes	8.694	0.016*	3.094	0.114	1.615	0.235
Nano-prokaryotes	0.057	0.816	0.093	0.788	0.042	0.837
Heterotrophic prokaryotes	0.145	0.729	1.392	0.268	0.002	0.974
Viruses	0.656	0.465	5.571	0.040*	0.291	0.588
^{13}C labelling						
^{13}C-DIC	1.746	0.204	0.214	0.642	4.592	0.057
^{13}C-POC	2.124	0.165	2.105	0.052	0.120	0.738
Metabolic rates						
GPP-^{18}O	0.003	0.956	15.824	0.006*	7.283	0.021*
GPP-O_2	0.218	0.642	1.257	0.324	0.179	0.668
NCP-O_2	0.074	0.776	769.0	0.769	0.539	0.483
CR-O_2	0.139	0.728	1.683	0.240	0.035	0.849
NCP-^{13}C	0.867	0.368	2.526	0.155	0.020	0.901

The "*" indicate significant effect ($p < 0.05$).

concentration significantly decreased while chl *a* concentration increased rapidly to a maximum on d2. This important chl *a* increase after enclosure, followed by decline owing to the depletion of inorganic nutrient, was also reported in other bottle experiments (De Madariaga and Fernandez, 1990; Scarratt *et al.*, 2006) and can be due to a containment effect. In addition, it must be stressed that sieving onto 200 μm most likely limited the abundance of large zooplankton releasing predation pressure during the first days but favouring growth of micro-heterotrophs that in turn grazed on small phytoplankton. Although degradation pigments representative for grazer faecal pellets (phaeophytin *a* and phaeophorbid *a*) were close or even below detection limit throughout the experiment (data not shown), suggesting that there were no or very few zooplanktonic organisms, as the available seawater volume for sampling was not sufficient, zooplankton abundances were not estimated during this study. Based on pigment concentrations, prymnesiophytes were the dominant species and all phytoplankton groups decreased in abundance along the experiment, except for cyanobacteria. The dominance of small phytoplankton and the general decrease in biomass are consistent with post-bloom, nutrient-depleted conditions. Cyanobacteria and prymnesiophytes present a higher surface vs. volume ratio and have a higher affinity for nutrients than larger phytoplankton, cyanobacteria being even more competitive and able to grow under these very low nutrient conditions (e.g. Eppley *et al.*, 1969; Irwin *et al.*, 2006). The dominance of pico- and nanoplanktonic species at the end of the bloom period has already been observed in the Bay of Villefranche (Sheldon *et al.*, 1992). While chl *a* concentrations showed very clear variations during the experiment, it was not the case for POC. These values presented large variations in between replicates and could explain the fact that we did not observe any temporal variation in POC.

The rates of community metabolism are comparable with those measured during a previous experiment performed in the bay of Villefranche in March 2003 (González *et al.*, 2008). However, in contrast to this study, GPP-^{18}O and GPP-O_2 were not correlated during our experiment. This can be explained by the relatively small range of values measured during our study in contrast to the large range of values (different seasons, depth, and sites) presented by González *et al.* (2008). Due to the limited amount of water available in the 4 l bottles, NCP-O_2 and CR-O_2 rates have been measured without replication. This led to relatively large uncertainties in the determinations of these rates and, even more important, in the estimates of GPP-O_2. Although the same number of samples were available to estimate GPP-^{18}O rates, the errors associated with this method were much smaller than with the O_2 light–dark techniques as GPP-O_2 is estimated based on two values (NCP- and CR-O_2) associated to large uncertainties. Therefore, the ^{18}O-labelling approach appears much more reliable for estimating GPP rates than the classical

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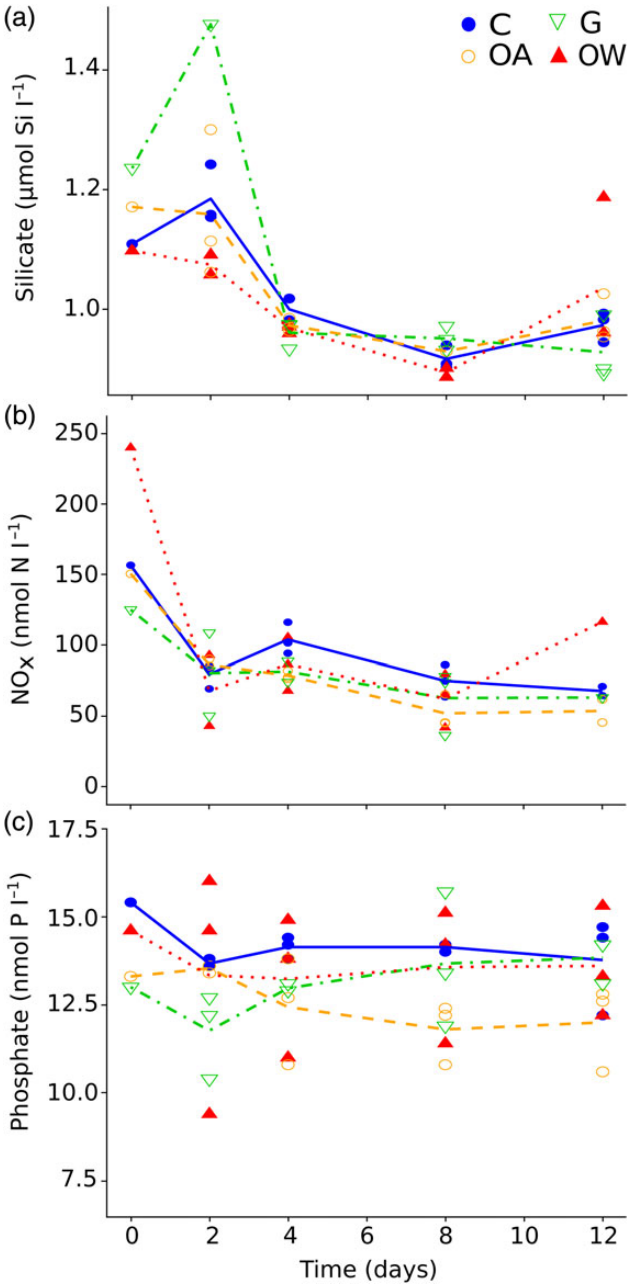

Figure 2. Concentration of inorganic nutrients as a function of time: (a) silicate, (b) NO_x ($NO_3^- + NO_2^-$), and (c) phosphate. Control (C), ocean warming (OW), ocean acidification (OA), and greenhouse (G) treatments. Symbols are for the three replicates of each treatment. Lines: solid (C), dashed (OA), dotted (OW), and dotted–dashed (G).

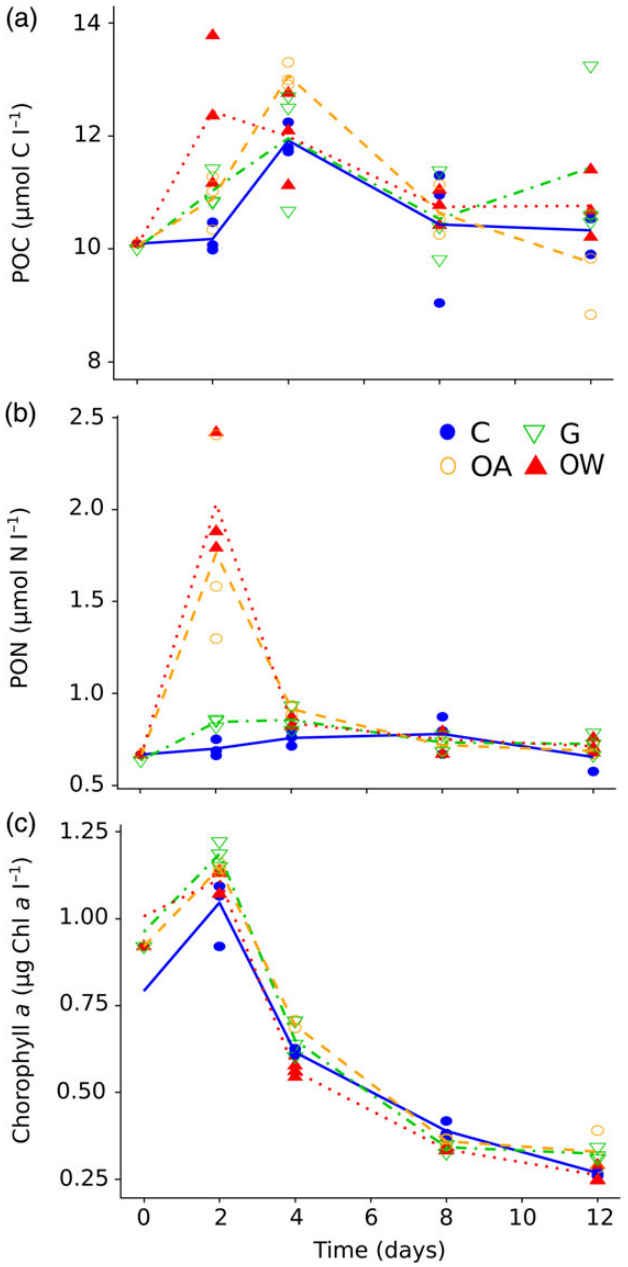

Figure 3. Particulate organic matter and chl *a* as a function of time: (a) POC, (b) particulate organic nitrogen (PON), and (c) chl *a* measured by HPLC. Control (C), ocean warming (OW), ocean acidification (OA), and greenhouse (G) treatments. Symbols are for the three replicates of each treatment. Lines: solid (C), dashed (OA), dotted (OW), and dotted–dashed (G).

light–dark technique when rates are low and only a small sample volume is available. However, it must be stressed that the ^{18}O-labelling techniques does not allow estimating CR, which is critical for determining the autotrophic vs. heterotrophic behaviour of the community. Nevertheless, the observed decrease in GPP-^{18}O during the experiment was consistent with the decrease in the phytoplankton biomass indicated by the pigments analyses.

The ^{13}C enrichment was successful and showed decreasing primary production rates along the experiment which is consistent with the observed decrease in phytoplankton biomass and GPP measured by the ^{18}O method. However, the NCP estimated based on the ^{13}C method (NCP-^{13}C), representing the freshly labelled material produced by phytoplankton, was lower than NCP estimated by the light–dark method (NCP-O_2). The NCP-^{13}C and NCP-O_2 were measured from incubation in different volumes (60 ml vs. 4 l) and over different periods (24 h vs. 2 or more days), which might explain part of this difference. Moreover, it is likely that, a significant part of the organic material produced was released in the DOC pool (Wood and Van Valen, 1990; Lopez-Sandoval *et al.*, 2011) which was not measured during our experiment.

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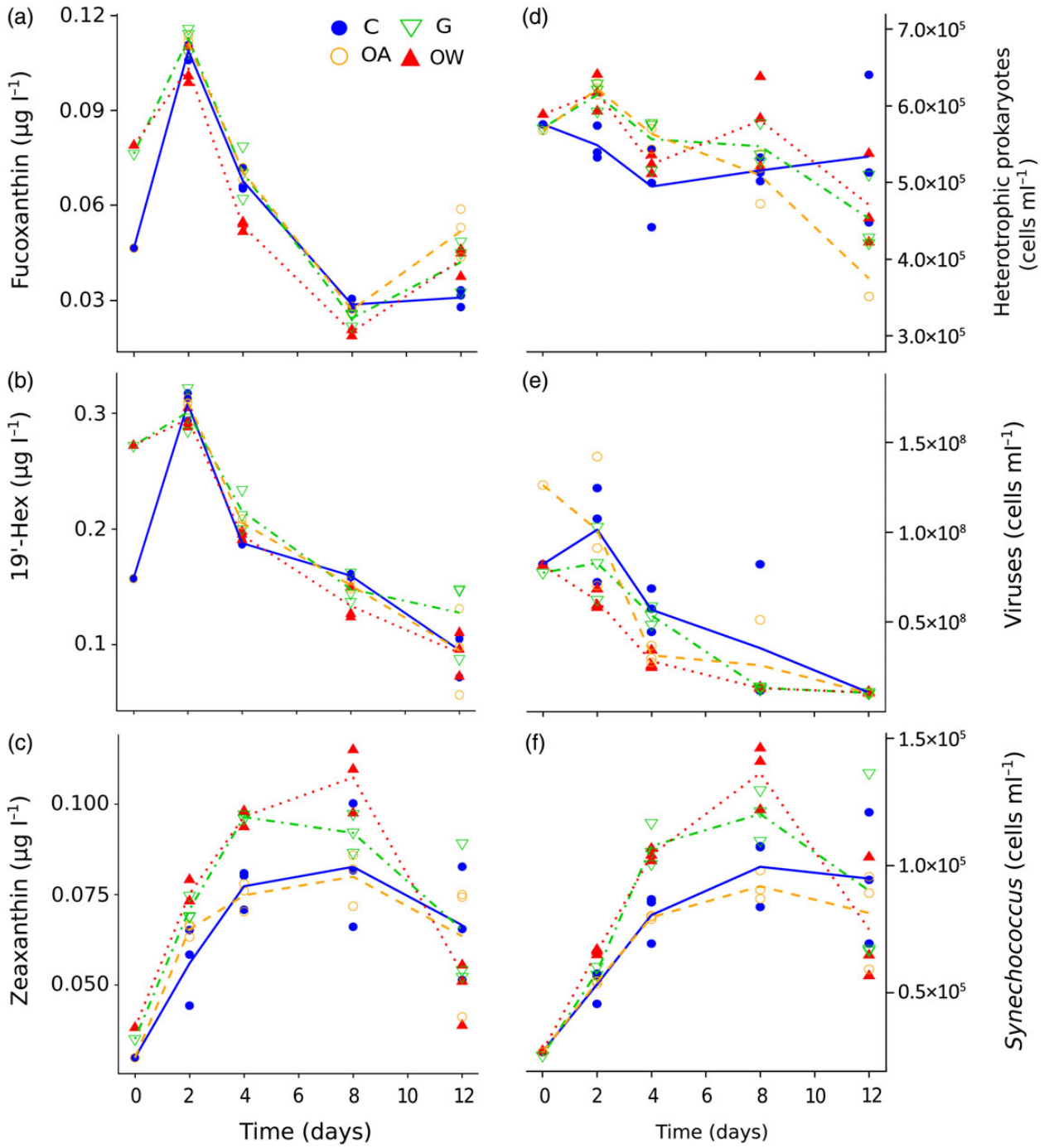

Figure 4. Pigments (left panels) and flow cytometer counts (right panels) as a function of time: (a) fucoxanthin (typically diatoms), (b) 19′-hexanoyloxyfucoxanthin (19′-hex; prymnesiophytes), (c) zeaxanthin (cyanobacteria), (d) abundance of heterotrophic prokaryotes, (e) abundance of viruses, and (f) abundance of *Synechococcus*. Control (C), ocean warming (OW), ocean acidification (OA), and greenhouse (G) treatments. Symbols are for the three replicates of each treatment. Lines: solid (C), dashed (OA), dotted (OW), and dotted – dashed (G).

During the recent years, an experimental effort to study ocean warming and acidification effects at the plankton community level has been initiated; however, there is still a clear lack of information for low productive oceanic regions (oligotrophic) that represent an important, and expanding surface of the global ocean (e.g. Irwin and Oliver, 2009), having then a significant role in carbon cycling. The present experiment was designed to investigate the effect of both ocean acidification and warming under conditions that prevail for most of the year in the oligotrophic Mediterranean Sea, i.e. very low nutrient availability and a community dominated by small phytoplankton species.

In our study, no significant effects of elevated temperature and/or CO_2 were found for most parameters and processes (Table 2). As reported in other experiments (e.g. Feng *et al.*, 2009), C:N ratio was not affected either by temperature or CO_2. The pCO_2 effect detected on the phosphorus concentration is most likely due to sampling

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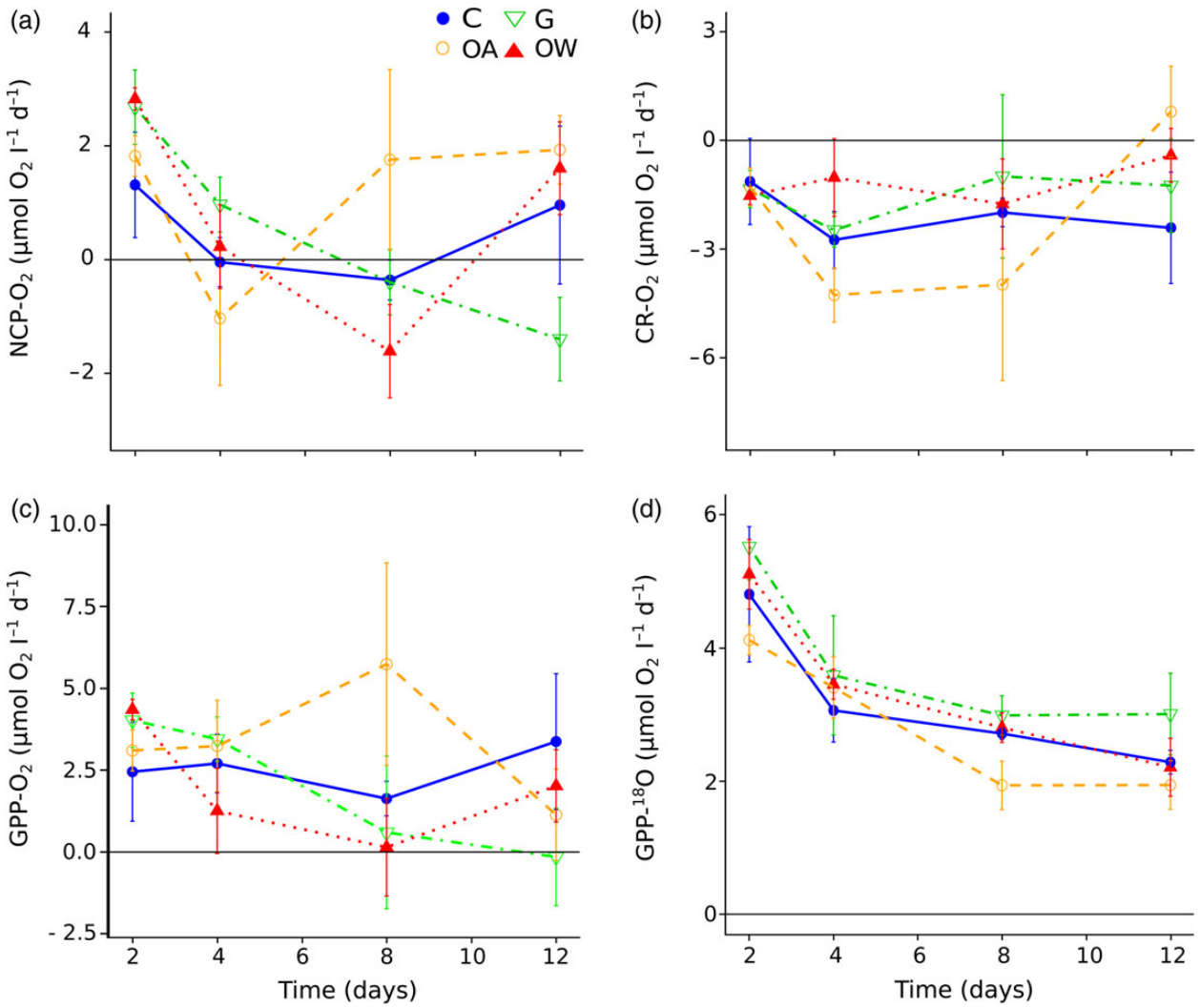

Figure 5. Community metabolism as a function of time: (a) NCP (NCP-O_2), (b) CR (CR-O_2), (c) gross primary production using the O_2 techniques (GPP-O_2), and (d) gross primary production using the ^{18}O-labelling technique (GPP-^{18}O). Control (C), ocean warming (OW), ocean acidification (OA), and greenhouse (G) treatments. Symbols are for the three replicates of each treatment. Lines: solid (C), dashed (OA), dotted (OW), and dotted–dashed (G).

and/or analytical uncertainty. Indeed, during the whole experiment, concentrations varied within a very small range of ± 5 nmol l^{-1} and for most of the sampling days, differences between replicates were larger than between treatments. The GPP-^{18}O rates were higher in warmer treatment and even more enhanced when combined with high CO_2 conditions. This is in agreement with observed stimulations of the metabolism by temperature (Eppley, 1972; Toseland *et al.*, 2013). Our data also support the previous findings of studies with higher nutrient levels in which elevated pCO_2 exerts a moderate effect on primary production rates unless it is combined with an increase in temperature (bottles incubation <3 l; Hare *et al.*, 2007; Feng *et al.*, 2009). An experiment performed in larger volumes (3000 l) has shown an increase in DOC production as well as photosynthetic activity and decrease in POC production in warmer and high CO_2 level conditions while gross community production remained unchanged (Kim *et al.*, 2011, 2013).

It must be mentioned that although no study combining both drivers were conducted in oligotrophic conditions, ocean acidification alone has been reported to decrease DOC production in the nutrient-depleted Okhotsk Sea at high CO_2 level while POC production was unchanged (Yoshimura *et al.*, 2010). Furthermore, in iron-limited areas (Bering Sea and North Pacific) presenting similar chl *a* level than in our study, effects of ocean acidification were investigated and have shown different effect on POC and DOC accumulation that have been related to differences in community structure (Yoshimura *et al.*, 2013). Indeed at the site where small phytoplankton species (70% picoaukaryotes and 20% *Synechococcus*) were dominant, no effect was detected, as in our study, while at the site where diatoms were dominating (75%), POC accumulation was smaller at high CO_2 levels. In our study, cyanobacteria (comprising *Synechococcus* and *Prochlorococccus* spp.) is the only taxonomic group that has shown enhanced abundance in warmer conditions though to a lesser extent when combined with high CO_2 level. Indeed, while the increasing tendency in both warming and greenhouse treatments was similar during the first sampling days, elevated pCO_2 appeared as unfavourable to this population after day 8 of our experiment. This is partially consistent with single-cell experiments that have shown no effect of increased CO_2 alone on *Synechococcus*

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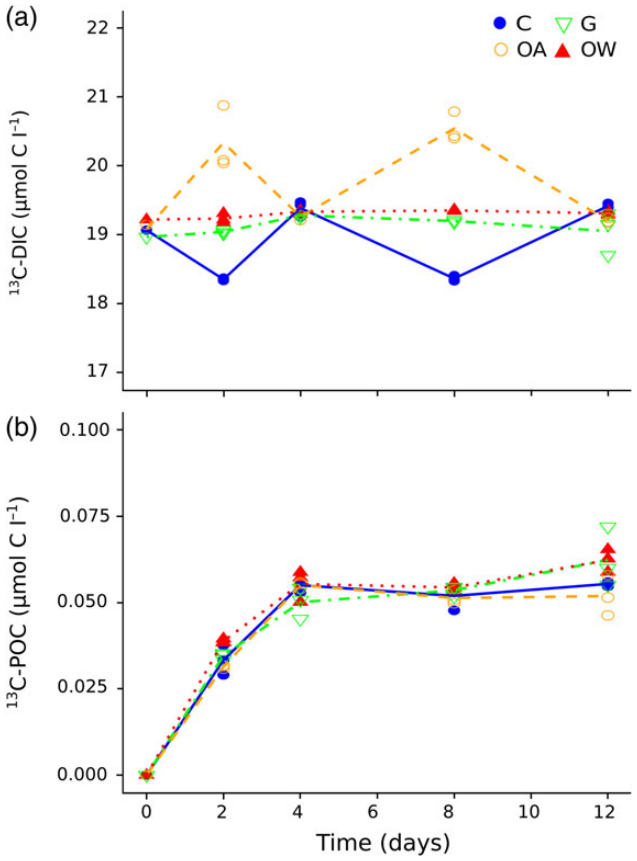

Figure 6. Concentrations of labelled (a) dissolved inorganic carbon (DIC) and (b) POC expressed in µmol C l^{-1}. Control (C), ocean warming (OW), ocean acidification (OA), and greenhouse (G) treatments. Symbols are for the three replicates of each treatment. Lines: solid (C), dashed (OA), dotted (OW), and dotted–dashed (G).

Table 3. NCP based on ^{13}C enrichment in the control (C), ocean warming (OW), ocean acidification (OA), and greenhouse (G) treatments (average ± *SD*).

	NCP-^{13}C (µmol C l^{-1} d^{-1})			
Day	**C**	**OW**	**OA**	**G**
2	1.93 ± 0.26	2.14 ± 0.05	1.69 ± 0.06	2.00 ± 0.06
4	1.52 ± 0.05	1.54 ± 0.13	1.60 ± 0.03	1.46 ± 0.13
8	0.76 ± 0.05	0.76 ± 0.02	0.71 ± 0.03	0.79 ± 0.02
12	0.52 ± 0.01	0.58 ± 0.03	0.51 ± 0.06	0.62 ± 0.08

growth rates and significant effects of temperature alone or in combination with acidification (greenhouse; Fu *et al.*, 2007). However, in contrast to our results, growth rates were not significantly different between the elevated temperature and the greenhouse treatments. Furthermore, the same study reported no effect on *Prochlorococcus*, while increased abundances in the warmer treatment were observed in our study (data not shown). Finally, in a natural community of *Synechococcus* and *Prochlorococcus* spp. carbon fixation rates were also not affected by acidification under both nutrient replete and P-limited conditions (Lomas *et al.*, 2012).

Extrapolating our findings to *in situ* oceanic conditions and facilitating potential future comparison studies necessitates a discussion on the limits of the experimental design considered in the present study. Indeed, it must be stressed that our experiment was performed in relatively small-enclosed bottles (4 l) that undoubtedly led to some confinement effects which constrained the plankton community response (i.e. no turbulence, no nutrient resupply, no movement through the euphotic zone, etc.; Scarratt *et al.*, 2006). In our study, cyanobacteria species observed during the incubations were not N_2 fixers; however, it must be stressed that some strains have been shown to increase their N_2 fixation rates under high CO_2 conditions (e.g. Hutchins *et al.*, 2009). The presence of N_2 fixers species in the community could have had influenced the results reported here. In addition, seawater was sieved through 200 µm to remove large organisms meaning that we did not take into account the complete community and therefore these findings are valid only when top-down control is negligible (Kim *et al.*, 2013). Any potential change in the top-down pressure, due to change in macrozooplankton enhanced grazing in warmer and/or acidified condition, was therefore not investigated in this study. To assess the potential effect of climate change on communities of two or more trophic levels and trophic interactions (e.g. from phytoplankton to macrozooplankton), large mesocosm (~50 m^3) studies are more appropriate as whole communities can be trapped and *in situ* temperature, irradiance and water masses are maintained close to "real world" conditions (Riebesell *et al.*, 2010, 2013).

It must be stressed that a rise in primary production with elevated temperature is not foreseen on a global scale as many studies report a decrease of primary production as a consequence of a stronger stratification of the water column which limits nutrient supply to the surface mixed layer (Bopp *et al.*, 2005). The observed increase in abundance of cyanobacteria in the warmer treatments in our study is in accordance with *in situ* observations, as it is recognized that these species have a wider temperature range than other phytoplankton species. Cyanobacteria will probably benefit from climate change as long as the temperature does not exceed their thermal tolerance (Morán *et al.*, 2010; O'Neil *et al.*, 2012). A potential shift toward small species could lead to diminished energy transfer to microzooplankton as their lipid content is much lower than it is for larger phytoplankton species (e.g. Von Elert and Wolffrom, 2001). Furthermore, the carbon export efficiency depends on the community structure and a shift to smaller species will probably lead to a less efficient carbon export under elevated temperature (Bopp *et al.*, 2005). As nutrients (*N* and *P*) were highly limiting, the absence of effect by ocean acidification alone does not appear as a surprise. Unfortunately, no data on dissolved organic nutrient concentrations are available and we could not precisely investigate any modifications in nutrient acquisition mechanisms (inorganic vs. organic) under the different treatments. Nevertheless, as no change of plankton community composition and functioning were shown at elevated pCO_2, it appears that the community did not take advantage of the increased CO_2 availability. To conclude, this study on the combined effect of both drivers has shown that ocean acidification and warming in isolation do not have the same effect on cells abundances and production rates than when combined, emphasizing the need to study these two drivers synergistically.

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