Ocean acidification effect on prokaryotic metabolism tested in two diverse trophic regimes in the Mediterranean Sea

Mauro Celussi [a, *], Francesca Malfatti [a], Franzo Annalisa [a], Frédéric Gazeau [b, c], Antonia Giannakourou [d], Paraskevi Pitta [e], Anastasia Tsiola [e], Paola Del Negro [a]

[a] Oceanography Division, OGS (Istituto Nazionale di Oceanografia e di Geofisica Sperimentale), v. A. Piccard 54, I-34151, Trieste, Italy
[b] Sorbonne Universités, UPMC Univ Paris 06, UMR7093, LOV, Observatoire océanologique, F-06230, Villefranche-sur-mer, France
[c] CNRS, UMR 7093, LOV, Observatoire océanologique, F-06230, Villefranche-sur-mer, France
[d] Hellenic Centre for Marine Research, Institute of Oceanography, Anavyssos, Athens, Greece
[e] Hellenic Centre for Marine Research, Institute of Oceanography, Ex American Base, Gournes, PO Box 2214, 73001, Heraklion, Crete, Greece

ARTICLE INFO

Keywords:
Ocean acidification
Exoenzymatic activities
Prokaryotes
Heterotrophic production
Organic matter
Mesocosm

ABSTRACT

Notwithstanding the increasing amount of researches on the effect of ocean acidification (OA) on marine ecosystems, no consent has emerged on its consequences on many prokaryote-mediated processes. Two mesocosm experiments were performed in coastal Mediterranean areas with different trophic status: the summer oligotrophic Bay of Calvi (BC, Corsica, France) and the winter mesotrophic Bay of Villefranche (BV, France). During these experiments, nine enclosures (~54 m^3) were deployed: 3 unamended controls and 6 elevated CO_2, following a gradient up to 1250 μatm. We present results involving free-living viral and prokaryotic standing stocks, bacterial carbon production, abundance of highly active cells (CTC+), and degradation processes (beta-glucosidase, chitinase, leucine-aminopeptidase, lipase and alkaline phosphatase activities).

The experiments revealed clear differences in the response of the two prokaryotic communities to CO_2 manipulation. Only abundances of heterotrophic prokaryotes, viruses and lipase activity were not affected by CO_2 manipulation at both locations. On the contrary, the percent of CTC+ was positively correlated to CO_2 only in BC, concomitantly to a bulk reduction of [^{3}H]-leucine uptake. The other tested parameters showed a different response at the two sites suggesting that the trophic regime of the systems plays a fundamental role on the effect of OA on prokaryotes through indirect modifications of the available substrate.

Modified degradation rates may affect considerably the export of organic matter to the seafloor and thus ecosystem functioning within the water column. Our results highlight the need to further analyse the consequences of OA in oligotrophic ecosystems with particular focus on dissolved organic matter.

1. Introduction

Microbes play a major role within biogeochemical cycles in the ocean (Azam, 1998). Heterotrophic and mixotrophic prokaryotes are responsible for more than half of the primary production processing through the modification and utilization of organic macromolecules (Ducklow and Carlson, 1992). Natural and human-induced changes to the environment can result into alteration of microbial activities and consequently of biogeochemical cycling (e.g. Nogales et al., 2011). Nowadays, ocean acidification (OA) is an ongoing process with potential harmful consequences to the marine ecosystem and its resources (e.g. Cerrano et al., 2013; Gazeau et al., 2013). OA is defined as the ocean pH decrease due to the dissolution of atmospheric carbon dioxide. pH is a key parameter for life, especially for aquatic organisms. Cell metabolism is mediated by a wide range of enzymatic reactions that require an optimum pH range. Intracellular pH is maintained near neutrality in order to prevent the damage of acid- or alkali-labile molecules; on the other hand, in a pH-changing environment extracellular enzymes are potentially affected by OA (Piontek et al., 2010 and references therein).

Aquatic prokaryotes utilize exoenzymes to hydrolyse organic matter in order to obtain smaller molecules to be taken up and used in several metabolic pathways (Chróst, 1992). This ability to 'digest'

* Corresponding author.
E-mail address: mcelussi@ogs.trieste.it (M. Celussi).

a wide array of organic material, from simple dimers to complex particles, has led to the definition of bacteria as 'swimming stomachs' (as referenced in Azam, 1998). Expression of glucosidases, proteases, lipases, phosphatases and other hydrolytic enzymes, in fact, allows the breakdown of organic matter and the ultimate assimilation of monosaccharides, amino acids, orthophosphates and other simple compounds into the cell. The hydrolysis-uptake coupling determines the efficiency of microbial communities and their role of master recycler (Buchan et al., 2014) in ecosystem functioning. Prokaryotes rework, utilize and produce dissolved organic matter; under strong predation by bacterivores, prokaryotic biomass is channelled to higher trophic levels. When grazing pressure is limited, their standing stocks are mainly controlled by viral lysis and dissolved organic matter (DOM) availability where DOM consumption and production are maintained in the so called 'microbial loop' (Azam et al., 1983). Therefore, the mineralization of organic material in the surface ocean influences the carbon fluxes in the ecosystem i.e. sinking to the deep ocean vs. transfer to higher trophic levels (Chisholm, 2000).

The effect of OA on marine prokaryotes has been studied in the last decade using several approaches, considering different acidification protocols, different volumes of experimental enclosures and different secondary amendments (e.g. nutrient addition). Researches highlighted a general lack of consensus among the obtained results; in fact, disentangling the role of lowered pH, of increased dissolved inorganic carbon, and indirect modifications of the organic matter pool is challenging. The set-up of experiments in systems with different trophic status further complicates this picture. In the first reported study on this topic, Grossart et al (2006) observed modifications in exoenzymatic activities (increase of protease, and, to a lesser extent, α- and β-glucosidase activities) in relation to OA and interpreted these results as an indirect consequence of enhanced particles production by phytoplankton. Proteolytic activities were found to be either enhanced (Piontek et al., 2013; Endres et al., 2014), unaffected (Maas et al., 2013) or inhibited (Yamada and Suzumura, 2010) by increased partial pressure of CO_2 (pCO_2)/decreased pH, and similar results have been obtained when testing lipase activity (Yamada and Suzumura, 2010; Maas et al., 2013). Limited variations have been reported for alkaline phosphatase (Tanaka et al., 2008; Yamada and Suzumura, 2010; Maas et al., 2013) and chitinase activities (Maas et al., 2013). Polysaccharide degradation was generally enhanced by acidification (Piontek et al., 2010, 2013; Maas et al., 2013) and the tendency is to link such result to phytoplankton and organic matter dynamics (e.g. Arnosti et al., 2011).

Since degradation processes increase the availability of utilizable organic matter, bacterial heterotrophic carbon production (uptake of monomers) might be affected positively or negatively by an increased CO_2 level. The general findings of increased primary production under elevated CO_2 conditions suggest that the highest amount of organic carbon (in the form of dissolved organic carbon, transparent exopolymeric particles, biomass and particulate detritus) in CO_2-treated enclosures would fuel heterotrophic production and prokaryotic growth (Grossart et al., 2006; Piontek et al., 2013). However, different scenarios have been depicted by other researchers. Motegi et al. (2013) reported a decrease in thymidine uptake rates under nutrient enrichment at different pCO_2 levels, suggesting the potential role of several parameters other than CO_2, such as dissolved organic matter composition.

Furthermore, the fate of viruses in an acidified ocean is not clearly unveiled as well. Rochelle-Newall et al. (2004), found no difference in viral abundances between controls and CO_2-amended mesocosms, whereas other authors detected a pCO_2 impact on viruses infecting some phytoplankton species (Larsen et al., 2008; Brussaard et al., 2013). Notably, such findings have to be related to the mutual dependence of viruses on their hosts (metabolism and diversity).

Most of the mesocosms studies aimed at investigating the impact of increased CO_2 on marine plankton have been carried out in mesotrophic or eutrophic waters (naturally or after nutrient addition), and there currently is a lack of information concerning oligotrophic (low-nutrient low-chlorophyll) environments that represent a very large portion of the ocean.

To date, no report on Mediterranean microbial community response to ocean acidification is available. For this reason, two mesocosm experiments have been performed in coastal waters located in the western basin of the Mediterranean Sea in summer 2012 (Bay of Calvi, Corsica, France) and late winter 2013 (Bay of Villefranche, France). The goal of these comparative studies was to understand the response of planktonic communities to increased pCO_2 in low nutrient-low chlorophyll areas. We present our results in the light of the differences emerging from seasonality and site-specific trophic status. The present study focuses on the effect of OA on free-living prokaryotic and viral standing stocks, organic matter degradation processes (beta-glucosidase, alkaline phosphatase, lipase, chitinase and leucine aminopeptidase) and bacterial carbon production. Our aim was to answer the following questions: 1) Are prokaryotic growth and viral dynamics affected by high-pCO_2 in low nutrient-low chlorophyll areas? 2) Is organic matter degradation and utilization altered by ocean acidification in oligotrophic coastal Mediterranean areas?

2. Materials and methods

2.1. Experimental set-up

Two mesocosm experiments were setup in the Northwestern Mediterranean Sea. The first experiment took place in the Bay of Calvi (BC; NW Corsican coast, France) in summer 2012 and the second in the Bay of Villefranche (BV; South-Eastern France) in late winter 2013. Both locations had a water column depth of 25 m. Briefly, nine ~50,000 L-mesocosms (2.3 m in diameter and 12 m in height), made of 500 μm thick films of polyethylene mixed with vinyl acetate and covered with UV-transparent ETFE roofs, were deployed on June 17th 2012 and on February 14th 2013 in BC and BV respectively (for a detailed description of mesocosm materials, setup, acidification and experimental timelines see Gazeau et al., 2017a). Briefly, on June 20th 2012 (BC) and February 17th 2013 (BV), after the closing of the bags, six mesocosms were amended with CO_2-saturated seawater over 4 days in order to obtain a concentration range from ambient level to 1250 μatm (P1−P6), thus covering the projected range of pCO_2 for the end of the century following different emission scenarios (RCP2.6−RCP8.5; IPCC 2013). Three unamended mesocosm were used as controls (C1−C3) with pCO_2 levels of 450 and 350 μatm in BC and BV, respectively. In BC, the six targeted elevated pCO_2 levels were P1: 550, P2: 650, P3: 750, P4: 850, P5: 1000 and P6: 1250 μatm. In BV, the levels were P1: 450, P2: 550, P3: 750, P4: 850, P5: 1000 and P6: 1250 μatm. Mesocosms were anchored in clusters of 3, each cluster containing one control mesocosm accompanied by a medium and a high pCO_2 level (cluster 1: C1, P1, P4; cluster 2: C2, P2, P5 and cluster 3: C3, P3, P6). On June 24th 2012 and February 22nd 2013 in BC and BV, respectively, once targeted pCO_2 levels were reached, the experiment started (day 0). The experiment lasted 20 days in BC, but as a storm caused non-repairable damages to the bags on March 7th in BV, this latter experiment had to be terminated after 12 days.

Before sunrise (4.00 a.m. in BC and 5.00 a.m. in BV, local times), depth-integrated samples (from surface to 10 m) were collected in each mesocosm by means of 5 L Hydro-Bios integrated bottle samplers and processed within 1 h. Samples for heterotrophic

prokaryotes, viruses, bacterial carbon production and exoenzymatic activities were collected every second day (in BC, chitinolytic activity was measured every fourth day from June 28th to July 14th). The abundance of highly active prokaryotes (%CTC+) in BC was estimated on samples collected every four days. In BV, CTC + cells were enumerated on day 1, 9 and 12.

2.2. Physical and chemical parameters

Weather permitting, conductivity-temperature-depth casts (0–10 m) were performed in each mesocosm by means of a SBE 19plus V2 multiparametric probe as detailed in Gazeau et al. 2017a. Daily pH (on the total scale) and pCO_2 levels were determined in each mesocosm from total alkalinity and total inorganic carbon values, as reported in Gazeau et al. 2017a.

Chlorophyll *a* concentration was measured daily by high performance liquid chromatography (HPLC) with a complete Agilent Technologies system after collecting cells onto GF/F membranes (Whatman) from a sample volume of 2 L (Gazeau et al., 2017a). The detection limit for the pigment concentration was 0.3 ng L^{-1}.

A Liquid Waveguide Capillary Cell connected to a spectrophotometer was used to determine orthophosphate concentration from daily samples (detection limit = 1 nM; Louis et al., 2017).

2.3. Heterotrophic prokaryotes and viruses

The abundance of free-living heterotrophic prokaryotes (HP) and viruses (Virus-Like-Particle, VLP) was estimated by flow cytometry. A FACS Calibur (Becton Dickinson) instrument was used, equipped with an air-cooled laser at 488 nm and standard filter setup. Samples were fixed with 0.2 μm pre-filtered 25% glutaraldehyde (0.5% final concentration), kept at 4 °C for approximately 30 min, deep frozen in liquid nitrogen and stored at −80 °C until enumeration. Cryotubes were thawed at room temperature and were then stained with SYBR® Green I nucleic acid dye (Life Technologies), according to Marie et al. (1999) for heterotrophic prokaryotes and Brussaard (2004) for VLP. Briefly, heterotrophic prokaryotes were stained (4×10^{-4} dilution of the commercial stock) and incubated for 10 min in the dark, while VLP were stained (5×10^{-5}, final concentration) and incubated for 20 min in the dark at 80 °C. Samples were diluted in Tris–EDTA buffer (TE, 10 mM Tris and 1 mM EDTA, pH = 8), in order to achieve a rate between 100 and 700 events s^{-1}, only when necessary. TE buffer was autoclaved and 0.2 μm-filtered immediately prior to dilution. Flow cytometry data were acquired and processed with the Cell Quest Pro software (Becton Dickinson). The flow rate was determined on a daily basis, by weighing samples before and after the run. Abundances were then calculated using the acquired cell counts and the respective flow rate.

Virus-to-Prokaryote ratio (VPR) was calculated as a proxy for viral infection (Wilkox and Fuhrman, 1994).

2.4. Highly active prokaryotes

The abundance of metabolically active prokaryotes was determined according to Choi et al. (1996) with modifications by Paoli et al. (2006). A solution of 5-cyano-2,3-ditolyl tetrazolim chloride (CTC – Polysciences) was added to 6 mL samples with a 3 mM final concentration. This concentration was used in order to constrain CTC cellular toxicity (del Giorgio and Gasol, 2008 and references therein). Incubation time was tested before the experiments and the highest % of positive cells was found after 2 h at both sites. Samples amended with CTC were incubated in the dark at *in situ* temperature for 2 h and stopped by adding dolomite-buffered formalin (pre-filtered through 0.2 μm Acrodisc filters) at 5% final concentration. Two-mL aliquots were filtered in triplicates onto 0.2 μm black polycarbonate membranes (Whatman) within 8 h after fixation. The membranes were then placed on a drop (50 μL) of DAPI (4,6-diamidino-2-phenyl-indole, Sigma Aldrich®, 30 μg mL^{-1} final concentration in an autoclaved 3.7% NaCl solution) for 15 min in the dark. The backs of the filters were then gently dried onto a kimwipe tissue, mounted between layers of immersion oil (type A, Cargille) and stored at −20 °C. Cells were counted by epifluorescence microscopy (Olympus BX 60 F5) at 1000 × magnification under a UV (BP 330–385 nm, BA 420 nm) and a green (BP 480–550 nm, BA 590 nm) filter sets. Cells presenting red-fluorescing formazan crystals under the green filter set were counted as positive (CTC+). DAPI-stained cells were also counted in order to calculate the % of CTC + cells. A minimum of 300 cells was counted for each membrane in at least 20 randomly selected fields.

2.5. Exoenzymatic activity

Extracellular enzymatic activities were assayed using fluorogenic substrate analogues (Hoppe, 1993) derived from 7-amino-4-methyl-coumarin (AMC) and 4-methyl-umbelliferone (MUF). Protease activity (leucine-aminopeptidase activity – AMA) was assayed as the hydrolysis rate of leucine-AMC. β-glucosidase (BGLU), lipase (LIP), chitinase (CHIT) and alkaline phosphatase activities (APA) were assayed using MUF-β-D-glucoside, MUF-oleate, MUF-*N*-acetyl-β-D-glucosaminide and MUF-phosphate (Sigma Aldrich®), respectively. Enzyme activities were expressed in terms of the rate of MUF or AMC production. Substrates' saturating concentrations were evaluated in samples collected nearby the mesocosms 1 and 2 days before the beginning of the experiments at both sites. During the experiment in BC, hydrolysis was measured by incubating 2 mL subsamples with 25 μM MUF-β-D-glucoside, 400 μM leucine-AMC, 400 μM MUF-oleate, 800 μM MUF-*N*-acetyl-β-D-glucosaminide and 100 μM MUF-phosphate for 2 h in the dark at *in situ* temperature (max. dev. ± 2 °C). During the experiment in BV, subsamples (2 mL) were amended with 50 μM MUF-β-D-glucoside, 200 μM leucine-AMC, 12.5 μM MUF-oleate, 100 μM MUF-*N*-acetyl-β-D-glucosaminide and 25 μM MUF-phosphate and incubated for 2 h in the dark at *in situ* temperature (±0.5 °C). All samples were run in three replicates, and values represent mean ± standard deviation, which was <10%. Fluorescence increase due to MUF and AMC hydrolysed from the model substrates was measured using a Jasco FP-6500 spectrofluorometer (MUF = 365 nm excitation and 455 nm emission; AMC = 380 nm excitation and 440 nm emission). Calibration curves were performed at each experimental pH utilising 0.2 μm-filtered seawater from the mesocosms and standard solutions of MUF and AMC (5 μM).

2.6. Bacterial carbon production

Bacterial carbon production (BCP), meant as the uptake of organic molecules by prokaryotes for heterotrophic metabolism, was measured using the [^{3}H]-leucine method by Kirchman et al. (1986), as modified by Smith and Azam (1992). Duplicate water samples (1.5 mL) and one trichloracetic acid (TCA) killed control (5.6% final concentration) were incubated in 2 mL-Eppedorf tubes with a mixture of [4,5–3H] leucine (Perkin Elmer, specific activity 115 Ci $mmol^{-1}$) and nonradioactive leucine at final concentrations of 16 and 7 nM, respectively. Samples were incubated for 2 h in the dark at *in situ* temperature. Incubations were stopped with 90 μL of 100% TCA and the samples were stored at 4 °C in the dark until further processing (within 24 h). Centrifugation was carried out at 16,000 *g* for 10 min. After discarding the supernatant, 1.5 mL of 5% TCA was added, the samples were vigorously shaken using a vortex and then centrifuged again. After discarding the supernatant,

1.5 mL of 80% ethanol was added, samples were shaken again and centrifuged. The supernatant was then discarded, and 1.5 mL scintillation liquid was added. The radioactivity incorporated into the pellet was counted using a Packard LS 1600 Liquid Scintillation Counter.

BCP was calculated according to Kirchman (1993), from [^{3}H]-leucine incorporation rates. Time series experiments were carried out to test for linear incorporation of leucine. Furthermore, two concentration kinetic experiments were performed to verify that the concentration of leucine added (20 nM) was sufficient to saturate incorporation (range of concentrations 3–50 nM; Van Wambeke et al., 1997).

2.7. Statistical analysis

All statistical analyses were performed with the software STATISTICA (StatSoft Inc).

The pCO_2 gradient approach utilized in the experiments involves the use of regression statistics for the assessment of possible CO_2 effects (Riebesell et al., 2013).

In order to determine the potential pCO_2 effect on microbial parameters (HP, % of CTC + cells, viruses, VPR, BCP, β-glucosidase, chitinase, alkaline phosphatase, lipase and leucine aminopeptidase activities), the daily deviation (AD_i) of each mesocosm was calculated by subtracting each data (X_i) from the mean of all mesocosms ($\overline{X}$) on the specific day ($AD_i = X_i - \overline{X}$). Since sampling was carried out every two days, linear interpolation was used to calculate values for missing days. These daily deviations were checked for normality (Shapiro–Wilk test) and averaged over time according to $1/N\sum_{i=1}^{N}(ADi)$ with N being the number of sampling days, in order to get the mean deviation (MD) of each mesocosm regarding a particular parameter (Endres et al., 2014). Mean deviations were then tested against mean pCO_2 (data from Gazeau et al., 2017a) in the different enclosures by simple linear regression.

A stepwise multiple regression analysis was performed in order to establish the relationship between microbial (total heterotrophic prokaryote abundance, BCP, β-glucosidase, chitinase, alkaline phosphatase, lipase and leucine aminopeptidase activities) and environmental variables: temperature, pCO_2, chlorophyll a (Chl a), total particulate carbon, nitrogen and phosphorus (TPC, TPN, TPP – data from Gazeau et al., 2015), gross primary production (GPP – Maugendre et al., 2017b), and viruses. In the case of exoenzymatic activities and BCP, heterotrophic prokaryote abundance was added as an independent variable. All non-normally distributed data series were log(10)-transformed before running the analysis.

3. Results

An overview of environmental and experimental data (pCO_2, temperature, salinity, chlorophyll a and orthophosphate concentrations) within mesocosms during both experiments is provided in Table 1, Figs S1 and S2 (Supplement). In BC, low nutrient concentrations (PO_4^{3-} average = 9 ± 4 nmol L^{-1}; Louis et al., 2017) likely prevented from the development of phytoplankton proliferation within mesocosms, yielding to a mean chlorophyll a concentration of 70 ± 10 ng L^{-1}. In this experiment, microbial plankton metabolism was highly heterotrophic and based on regenerated production, as estimated by ^{13}C-labelling analyses (Maugendre et al., 2017a). On the contrary, in BV, photoautotrophic activity was clearly dominating (average chlorophyll a concentration = 987 ± 147 ng L^{-1}) even though orthophosphate concentration was similar to the one measured in BC (average = 9 ± 1 nmol L^{-1}). Total phytoplankton biomass was not affected by CO_2 and displayed a stationary trend over time in BC and a slightly decreasing one in BV (Gazeau et al., 2017b).

3.1. Microbial dynamics: summer conditions (Bay of Calvi)

Abundances of free-living heterotrophic prokaryotes at initial conditions (day 0) were on average in all mesocosms (±SD) 5.00 ± 2.77 × 10^5 cells mL^{-1} (Fig. 1). Over the experimental period, their abundance exhibited small variability and ranged between 3.98 and 6.14 × 10^5 cells mL^{-1}, with the lowest values observed on days 14, 16 and 20 in P3 and the highest ones on the same days in P5.

The percentage of highly active prokaryotes (CTC+) displayed a general decreasing trend along with time, showing an average value of 5.1% at the beginning and 1.2% at the end of the experiment. The highest percentages were reached in P6 at day 4 and 8, being 11.9 and 9.7 respectively (Fig. 1).

Table 1
Time-averaged (±SD) values of environmental conditions in all mesocosms during the experiments in the Bay of Calvi (21 days) and in the Bay of Villefranche (12 days). Sampling and analytical protocols for pCO_2, temperature and salinity can be found in Gazeau et al. (sbm, this issue-b); analytical procedures used to determine chlorophyll a and orthophosphate concentrations are available in Gazeau et al. (sbm, this issue-c) and Louis et al. (sbm, this issue).

		pCO_2 (µatm)	Temperature (°C)	Salinity	Chlorophyll a (ng L^{-1})	PO_4^{3-} (nmol L^{-1})
		Mean ± st. dev.	Mean ± st. dev.	Mean ± st. dev.	Mean ± st. dev.	Mean ± st. dev.
Bay of Calvi	C1	466.79 ± 20.08	23.63 ± 0.86	38.04 ± 0.05	66.76 ± 9.03	7.50 ± 2.22
	C2	464.28 ± 22.67	23.61 ± 0.85	38.06 ± 0.06	68.76 ± 9.71	10.55 ± 5.20
	C3	466.50 ± 22.54	23.63 ± 0.86	38.03 ± 0.04	67.15 ± 8.56	9.36 ± 4.08
	P1	554.29 ± 24.35	23.62 ± 0.85	38.06 ± 0.06	71.67 ± 12.87	8.82 ± 2.32
	P2	640.03 ± 29.78	23.63 ± 0.85	38.07 ± 0.06	69.68 ± 12.54	9.83 ± 4.84
	P3	720.02 ± 30.00	23.62 ± 0.85	38.05 ± 0.05	67.53 ± 8.97	8.58 ± 4.27
	P4	813.82 ± 41.81	23.63 ± 0.85	38.06 ± 0.06	70.27 ± 7.19	8.91 ± 3.59
	P5	903.79 ± 113.59	23.63 ± 0.86	38.04 ± 0.04	71.32 ± 7.91	11.00 ± 6.62
	P6	1087.79 ± 129.02	23.63 ± 0.86	38.06 ± 0.05	73.93 ± 8.47	8.00 ± 1.61
Bay of Villefranche	C1	359.54 ± 14.412	13.14 ± 0.13	38.16 ± 0.01	1023.45 ± 186.51	9.27 ± 1.49
	C2	355.41 ± 13.411	13.15 ± 0.12	38.15 ± 0.01	979.59 ± 121.12	9.00 ± 1.65
	C3	354.07 ± 14.317	13.15 ± 0.13	38.17 ± 0.02	1002.62 ± 115.60	8.75 ± 1.14
	P1	453.89 ± 27.12	13.15 ± 0.13	38.16 ± 0.02	979.94 ± 184.87	9.00 ± 1.26
	P2	485.68 ± 70.585	13.16 ± 0.13	38.15 ± 0.01	989.67 ± 112.72	9.18 ± 1.33
	P3	535.91 ± 96.616	13.17 ± 0.13	38.16 ± 0.01	990.16 ± 110.24	8.27 ± 1.19
	P4	535.49 ± 132.53	13.17 ± 0.13	38.16 ± 0.01	920.05 ± 217.69	9.42 ± 1.68
	P5	700.64 ± 172.19	13.18 ± 0.12	38.16 ± 0.01	994.55 ± 158.93	9.58 ± 2.27
	P6	956.05 ± 235.59	13.17 ± 0.13	38.16 ± 0.01	1003.32 ± 101.40	8.60 ± 1.26

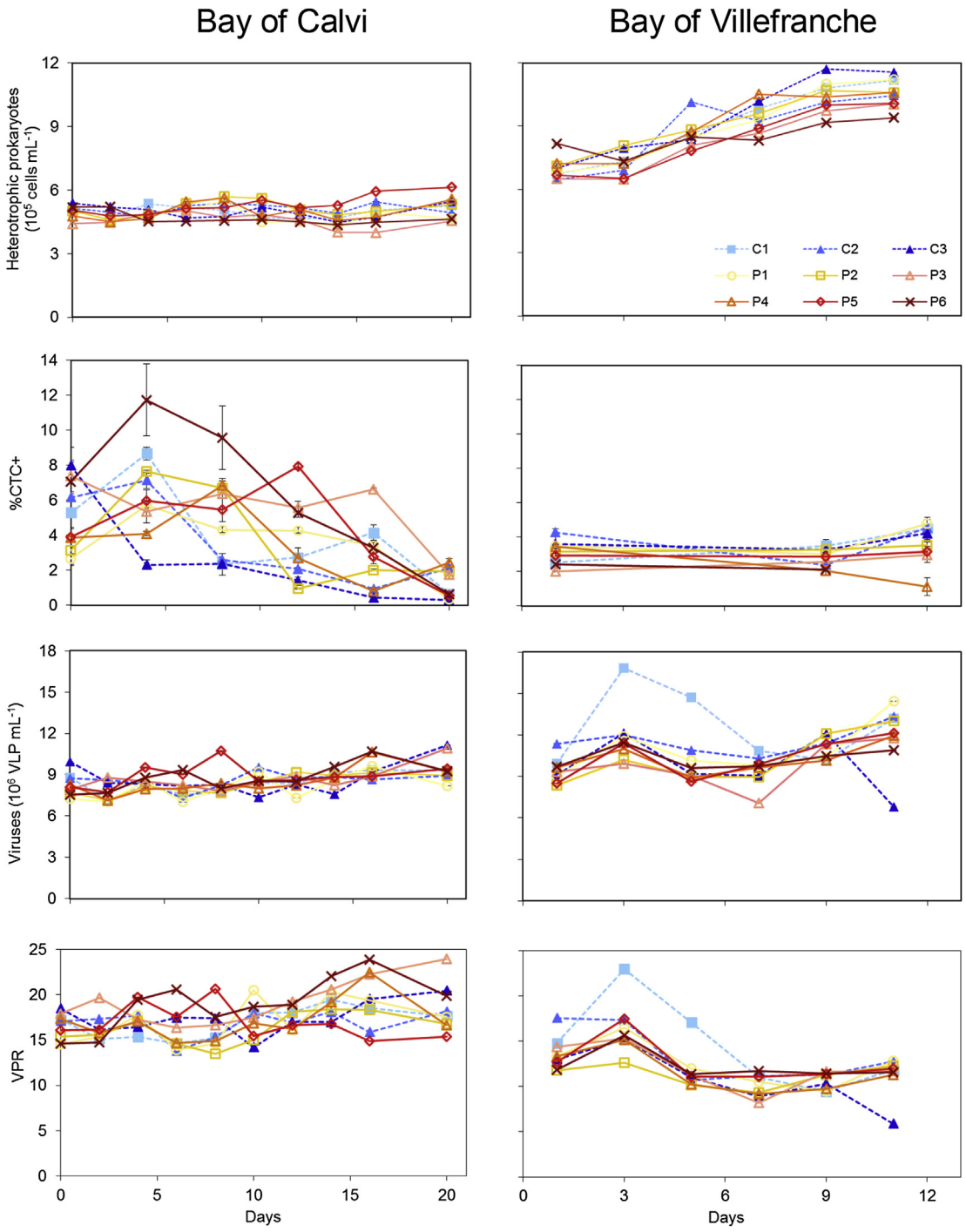
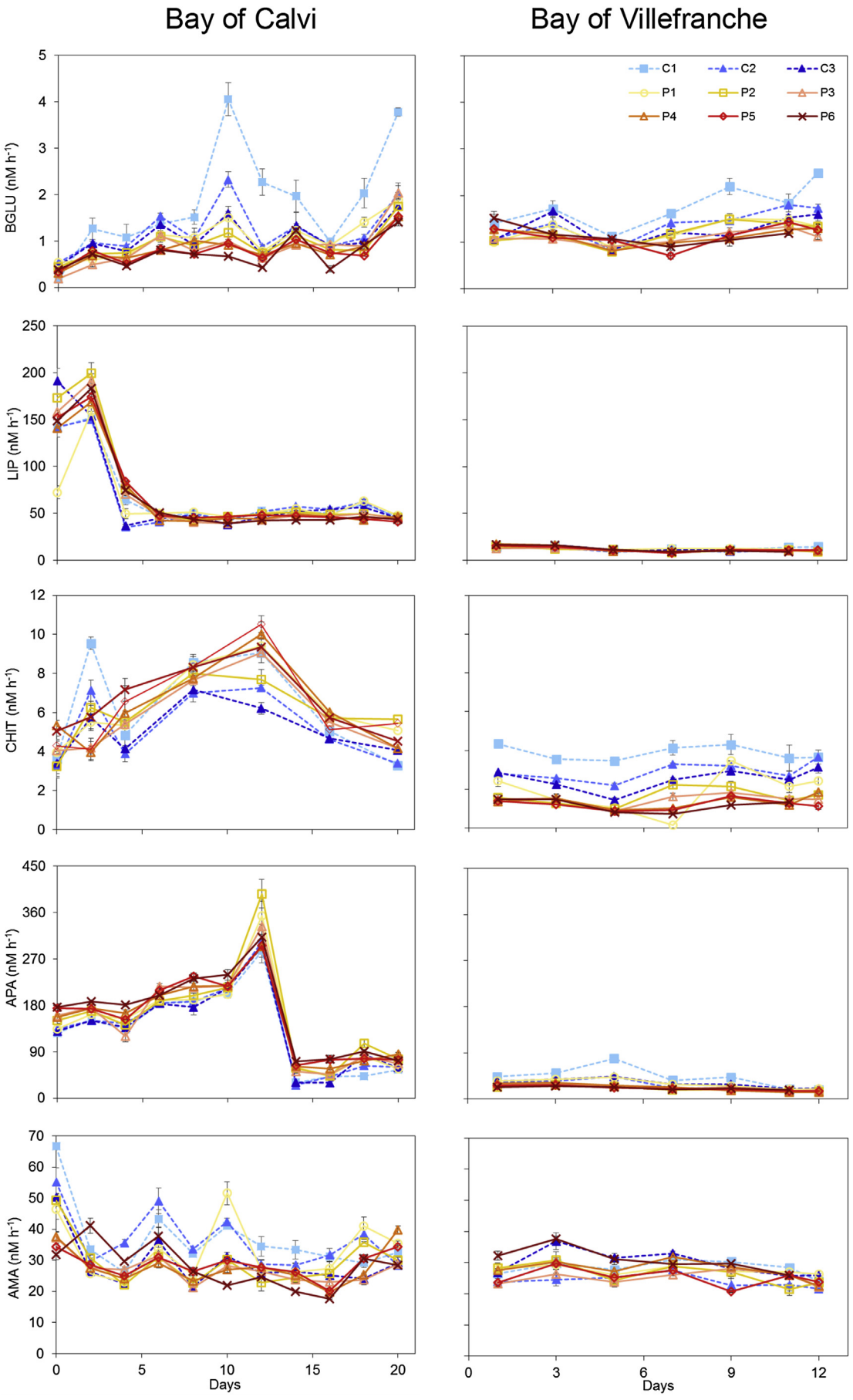


Fig. 1. Abundance of heterotrophic prokaryotes, percent of highly active prokaryotes (%CTC+), viruses and virus-to-prokaryote ratio (VPR) in all mesocosms as a function of time during the experiments in the Bay of Calvi (left) and in the Bay of Villefranche (right). Initial pCO_2 (µatm) in the BC mesocosms were: C1 = 474, C2 = 465, C3 = 462, P1 = 609, P2 = 731, P3 = 790, P4 = 920, P5 = 1198, P6 = 1353; initial pCO_2 (µatm) in the BC mesocosms were: C1 = 378, C2 = 347, C3 = 350, P1 = 494, P2 = 622, P3 = 691, P4 = 744, P5 = 932, P6 = 1250.

The abundance of free viruses ranged between 7.28 and 9.97 × 10^6 VLP mL^{-1} on day 0 (Fig. 1). During the whole experiment, VLP exhibited minor fluctuations with no consistent trend among mesocosms. The lowest VLP were recorded on day 2 in C1, P1, P2 and P4 and day 6 in P1. VLP exceeded 1.00 × 10^7 VLP mL^{-1} on days 8 (P5), 16 (P4 and P6) and 20 (C3 and P3).

The virus-to-prokaryote ratio was generally comprised between 15 and 20 without any detectable difference among mesocosms. After day 12, higher values characterised P3, P4 and P6 with the maximum being 23.9 (P3, day 20).

β-glucosidase activity rates were rather stable in the treated mesocosms along the whole experiment, averaging 0.87 (±0.36 SD) nM h^{-1} (Fig. 2). Slightly faster hydrolysis rates were measured in the controls, especially in C1 where the highest value was found on day 10 (4.05 ± 0.36 nM h^{-1}). Lipolytic activity ranged between 72.42 and 199.15 nM h^{-1} during the first 2 days after which it


Bay of Calvi
Bay of Villefranche
C1
C2
C3
P1
P2
P3
P4
P5
P6
BGLU (nM h-1)
LIP (nM h-1)
CHIT (nM h-1)
APA (nM h-1)
AMA (nM h-1)
Days
Days

decreased considerably, showing unvaried rates until the end of measurements (mean ± SD = 49.17 ± 9.10 nM h^{-1}). Chitinase activity rates resulted rather similar in both controls and treatments over the entire experiment. A slight increase was in fact measured until day 12 in almost all mesocosms. After this sampling time, rates decreased reaching values close to those measured at the beginning of the experiment (mean ± SD = 4.42 ± 0.84 nM h^{-1}). Alkaline phosphatase activity showed a gradual increase from the beginning of the experiment until day 12, when the highest values were measured in all mesocosms. Subsequently, rates considerably decreased, showing unvaried values from day 14 until the end of the experiment (mean ± SD = 61.44 ± 18.86 nM h^{-1}). Polypeptides' degradation rates revealed slightly higher rates in control enclosures and did not vary pronouncedly along with time (mean ± SD = 30.70 ± 8.28 nM h^{-1}).

Excluding the highest value measured in P1 on day 10 (62.34 ± 0.14 ng C L^{-1} h^{-1}), BCP rates were rather stable in all mesocosms over the whole experiment and did not exceed 40 ng C L^{-1} h^{-1} (Fig. 3).

Plots of mean data for all parameters as a function of mean pCO_2 in the mesocosms are reported as Supplementary Information (Figs S3, S4 and S5).

3.2. Microbial dynamics: winter conditions (Bay of Villefranche)

On day 1, free-living heterotrophic prokaryotes ranged between 6.36 and 8.17 × 10^5 cells mL^{-1} in all mesocosms (Fig. 1). All control mesocosms, as well as P1 and P2 followed a constantly increasing trend until the end of the experiment, reaching almost 2 × higher abundances. P3, P4 and P5 displayed a similar increasing trend, with a one-day time lag, reaching over 1 × 10^6 cells mL^{-1}. P6 exhibited a decrease from day 1 to day 3, which was followed by a less pronounced increase − when compared to the rest of the mesocosms − until the end of the experiment.

The percentage of CTC+ was very stable during this experiment (Fig. 1), being comprised between 2.0 (P3, day 1) and 4.7% (P1, day 12). Slightly lower values characterized P6 where highly active prokaryotes never exceeded 2.4%.

Viral abundance had the same temporal pattern in all mesocosms, characterized by a peak on day 3, a drop on day 5 and a second increase until the end of the experiment (except for C3 − Fig. 1). Exceptionally low VLP were recorded in C3 on day 11 (6.82 × 10^6 VLP mL^{-1}). Equally high VLP were found on days 3 and 9, ranging between 1.09 and 1.16 × 10^6 VLP mL^{-1} in all mesocosms.

VPR followed viral abundance trends. At the beginning of the experiment VPR was on average 13.6 and after day 5 it set around 11. A different pattern was observed in C1 where the highest values were computed at days 3 and 5 (22.9 and 17.1, respectively). The lowest value (5.9) was found in C3 at the end of the experiment.

Polysaccharide degradation was rather stable in treatments over the entire experiment, averaging 1.16 (±0.20 SD) nM h^{-1} (Fig. 2). Slightly faster hydrolysis rates were measured in C1, especially during the second half of the experiment and the highest value was measured on day 12 (2.49 ± 0.07 nM h^{-1}). Lipase activity was constant in all mesocosms along the whole experiment and values ranged between 7.86 ± 0.74 nM h^{-1} (P5, day 7) and 17.01 ± 0.27 nM h^{-1} (P4, day 1). Chitin degradation was faster in the controls (mean ± SD = 3.10 ± 0.74 nM h^{-1}) than in the CO_2-enriched mesocoms (mean ± SD = 1.36 ± 0.36 nM h^{-1}) with the exception of P1 where data showed some variability.

Although alkaline phosphatase rates did not vary noticeably among mesocosms, controls, together with P1, showed higher values than those measured in treatments on day 5. Average rates resulted equal to 52.44 ± 17.77 nM h^{-1} in the controls and P1, while they accounted for 24.12 ± 1.69 nM h^{-1} in the other mesocosms. Ranging from 20.71 ± 0.37 (P5, day 9) to 37.70 ± 0.80 nM h^{-1} (P6, day 3), protease rates were rather stable during the experiment and similar trends were observed in all treatments.

BCP was rather constant until day 9, after which leucine uptake decreased considerably in all mesocosms reaching the lowest value of 2.59 ng C L^{-1} h^{-1} (C3, day 11), and increased again at the final sampling (Fig. 3).

Plots of mean data for all parameter as a function of mean pCO_2 in the mesocosms are reported as Supplementary Information (Figs S3, S4 and S5).

3.3. Relationship between microbial parameters and CO_2 during both experiments

In order to determine the potential pCO_2 effect on microbial parameters the mean deviation (*MD*) approach was used (Figs. 4–6). Data treatment based on this approach highlighted that, in BC, β-glucosidase and leucine aminopeptidase activities (Fig. 5) as well as bacterial carbon production (Fig. 6) were negatively related to pCO_2; a significant opposite outcome was found for alkaline phosphatase activity and %CTC+ (Fig. 5). On the other hand, in BV the only activity which was significantly related to acidification (negatively related to pCO_2) was chitinase activity (Fig. 5). During this experiment also %CTC+ were negatively correlated to increasing OA (Fig. 4).

An additional stepwise multiple regression analysis was used to determine the relationship between microbial and environmental parameters (Table 2). No correlation was found between pCO_2 and the abundance of free-living heterotrophic prokaryotes (Fig. 4), the variability of which was not explained by any of the tested environmental parameters in BC (Table 2). On the contrary, in BV, 66% of cell counts variation was explained by Chl *a*, TPC, TPP and TPN, with no significant contribution by pCO_2. In BC, 52% of the variation of β-glucosidase was explained by temperature, Chl *a*, pCO_2, GPP, TPC, TPN and prokaryotic abundance (HP); in BV, only 19% of rates' variability was explained by HP. In BC, a large part (74%) of the variability in lipolytic activity was determined by temperature, Chl *a*, GPP and viruses, while in BV, HP and GPP explained 37% of their variations. Variability in chitinase hydrolysis rates were explained by TPP, viruses, GPP, temperature and prokaryotic abundance (30%) in BC and by pCO_2, TPP, GPP, temperature, HP, TPC (45%) in BV. During the summer experiment in BC, 48% of APA variations were explained by Chl *a*, pCO_2, TPP, TPN, temperature, viral abundance, TPC and a similar variability (52%) was attributable to pCO_2, temperature, HP, TPC and TPP in the late winter experiment in BV. AMA variability was explained for 30% in BC and 32% in BV, the former being determined by temperature, pCO_2, TPN, viruses and HP, and the latter by GPP, pCO_2, HP, temperature and TPP. The stepwise multiple regression analysis could explain only a minor fraction of BCP variability in BC (22%), as explainable by TCP, pCO_2, TPP and temperature, whereas in BV temperature, GPP, pCO_2 and TPP could explain 69% of its patterns.

Fig. 2. β-glucosidase (BGLU), lipase (LIP), chitinase (CHIT), alkaline phosphatase (APA) and leucine aminopeptidase (AMA) activities in all mesocosms as a function of time during the experiments in the Bay of Calvi (left) and in the Bay of Villefranche (right). Dots represent the mean of 3 replicates ± standard deviation. Initial pCO_2 (µatm) in the BC mesocosms were: C1 = 474, C2 = 465, C3 = 462, P1 = 609, P2 = 731, P3 = 790, P4 = 920, P5 = 1198, P6 = 1353; initial pCO_2 (µatm) in the BC mesocosms were: C1 = 378, C2 = 347, C3 = 350, P1 = 494, P2 = 622, P3 = 691, P4 = 744, P5 = 932, P6 = 1250.

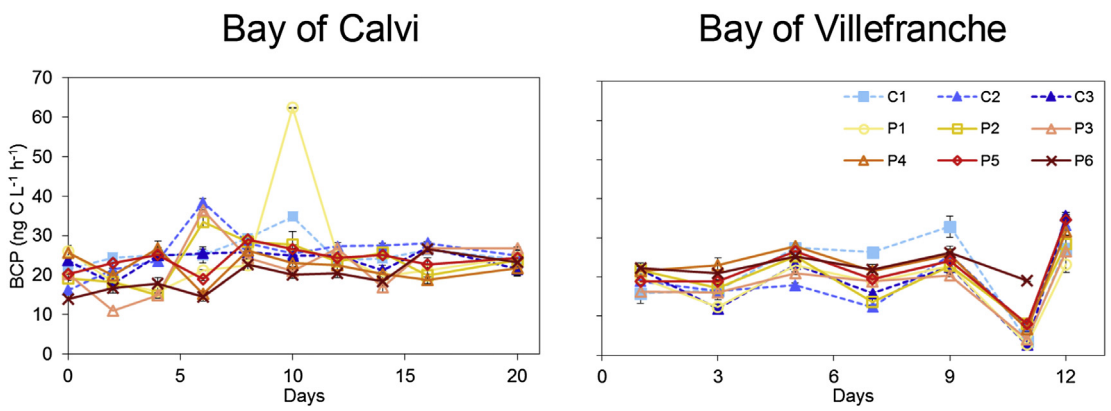


Fig. 3. Bacterial carbon production rates (BCP) in all mesocosms as a function of time during the experiments in the Bay of Calvi (left) and in the Bay of Villefranche (right). Dots represent the mean of 2 replicates ± standard deviation. Initial pCO_2 (μatm) in the BC mesocosms were: C1 = 474, C2 = 465, C3 = 462, P1 = 609, P2 = 731, P3 = 790, P4 = 920, P5 = 1198, P6 = 1353; initial pCO_2 (μatm) in the BC mesocosms were: C1 = 378, C2 = 347, C3 = 350, P1 = 494, P2 = 622, P3 = 691, P4 = 744, P5 = 932, P6 = 1250.

4. Discussion

4.1. Trophic and microbial dynamics in the two experiments

In our experimental systems (BV and BC), no clear phytoplankton bloom occurred (Gazeau et al., 2017a) and primary production (both net and gross – Maugendre et al., 2017b), chlorophyll *a* concentrations, microalgal assemblage composition (Gazeau et al., 2017b), transparent exopolymeric particles' concentration (Bourdin et al., 2015, in BV) and particulate organic matter load (Gazeau et al., 2015) did not change in response to the CO_2 amendments. Furthermore, all these parameters showed little variations over time. However, notwithstanding the common low nutrient – low chlorophyll status, some differences between the two experiments emerged, being related to seasonal and trophic ecosystem features. In particular, while in BC the overall metabolism was directed to heterotrophy and regenerated production, net autotrophy characterised BV mesocosms (Maugendre et al., 2017a). Higher phytoplankton biomass in BV was potentially supported by higher concentrations of NO_x, since $[PO_4^{3-}]$ was similar at the two sites (Louis et al., 2017), and resulted in enhanced particulate organic carbon loads (Gazeau et al., 2015). While chlorophyll *a* concentrations were stable during the BC study (on average 60 ng L^{-1} at day 0 and 80 ng L^{-1} at day 20), in BV they slightly decreased along with time (from – on average – 1126 ng L^{-1} at day 0 to 910 ng L^{-1} at day 12; Gazeau et al., 2017b).

In BC, the heterotrophic metabolism sustained by regeneration processes was also highlighted by faster exoenzymatic activities than in BV (with the only exception of β-glucosidase). This would be the consequence of minor nutrient (inorganic and organic) availability which can stimulate enzyme expression (Chróst, 1992; Baltar et al., 2009), implying that the lower the trophic status is, the more energy needs to be spent to acquire the available resources. In such a system, it would be favourable being able to quickly modify the expressed enzymes via up- or down-regulation. Therefore, in BC, the enzymatic activity temporal dynamics were more variable than in BV, where degradation processes were constant along with time (Fig. 2). The fast decrease of lipolytic activity observed after day 4 was simultaneous to the fast assimilation of dissolved P which characterised all mesocosms (Louis et al., 2017) at the very beginning of the experiment. It has been hypothesised that lipase-driven degradation of phospholipids (among the most abundant P-bearing molecules in the dissolved organic P pool) could provide an extra source of P in P-limited environments (Celussi and Del Negro, 2012). After the decrease in dissolved P concentration, lipase activity stabilised to relatively slow rates. On the contrary, APA (which to a large extend may stem from phytoplankton, especially in P-limited systems; Malfatti et al., 2014) gradually increased until day 12, as a direct consequence of the severe P-limitation within mesocosms (Ammerman and Azam, 1991).

The differences in degradation rates in BC and BV could also be ascribable to different prokaryotic assemblage composition (not analysed here), that typically follows seasonal successions (Celussi and Cataletto, 2007; Cram et al., 2015). Furthermore, since BCP did not differ between sites and also significantly higher prokaryotic abundances were found in BV (Figs. 1 and 3), it is very likely that loss processes driven by heteroflagellates and viruses were more pronounced in BC. During summer, in fact, the eukaryotic community was mainly composed by mixotrophic and heterotrophic organisms, as revealed by fatty acids analysis (Maugendre et al., 2017a).

In BV, lysogeny was a predominant viral life strategy, while lytic infection represented a minor (sometimes undetectable) process (Tsiola et al., 2017). During this experiment, virus-to-prokaryote ratios, often utilised as a proxy for lytic strategies (Wilkox and Fuhrman, 1994), were relatively low, being on average equal to 11.9 ± 3.2 (SD). On the other hand, in BC, where experiments aimed at unveiling viral dynamics were not performed, VPR was higher (on average 16.6 ± SD 2.1), suggesting a more pronounced pressure of phages on prokaryotes. However, it must be taken into account that we did not analyse particle-attached viruses and prokaryotes (that can contribute significantly to degradation and production processes; Grossart et al., 2006), and therefore speculations based on VPR are relative only to free living cells and VLP.

4.2. Effect of ocean acidification on prokaryotic metabolism

The most striking outcome of our experiments is the difference in the response of the two prokaryotic communities to pCO_2 manipulation. We interpret this result as a strong dependence of OA effect on prokaryotes upon the trophic regime (intended as the structure of plankton communities and their related metabolism) of a given environment.

In fact, on a general basis, our results are in contrast to what has been reported in previous mesocosms experiments performed in the Norwegian Sea, in the North Sea and in an Arctic fjord, (see Grossart et al., 2006; Riebesell et al., 2007; Piontek et al., 2013). Contrarily to these studies, in our Mediterranean Sea mesocosms, most prokaryotic activities were negatively correlated to increasing

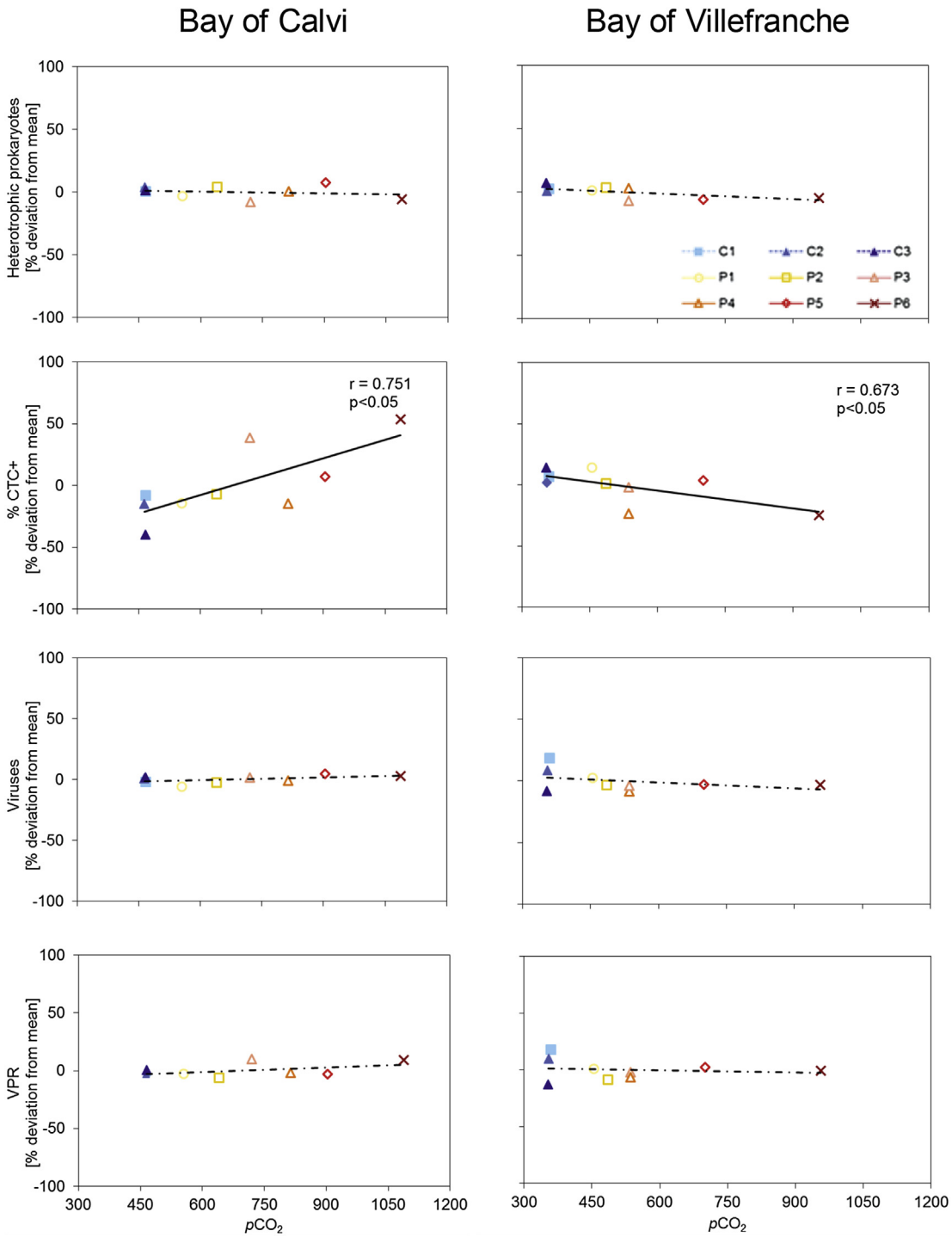


Fig. 4. Heterotrophic prokaryotes, percent of highly active prokaryotes (%CTC+), viruses and virus-to-prokaryote ratio (VPR) as a function of mean pCO_2 measured in the 9 mesocosms in the Bay of Calvi (left) and in the Bay of Villefranche (right). Dots indicate the percentage deviation from the mean of all mesocosms over time of microbial/viral abundance and VPR. Solid lines indicate significant correlation; dashed lines denote non-significant correlation.

pCO_2, as confirmed by the mean deviation analysis (Figs. 5 and 6). In particular, the degradation of polysaccharides (by β-glucosidase activity) and polypeptides (by leucine aminopeptidase activity) was slowed down by acidification in BC, whereas chitinase was inhibited in BV.

Extracellular (both cell-bound and released in the environment) hydrolytic enzymes can be constitutive or subjected to the specific cell metabolic state which can up- or down-regulate their synthesis. It follows that enzyme activity is dependent on many environmental factors, i.e. temperature, substrate concentration and structure, pH as well as catalytic elements' availability (Fukuda et al., 2000; Pomeroy and Wiebe, 2001; Arnosti, 2011).

In discussing our results, we will consider the above-mentioned forcing factors on microbial degradation rates. The range of integrated temperature values recorded during the experiments (21.5–24.7 °C in BC and 12.9–13.4 °C in BV) could, to some extent,

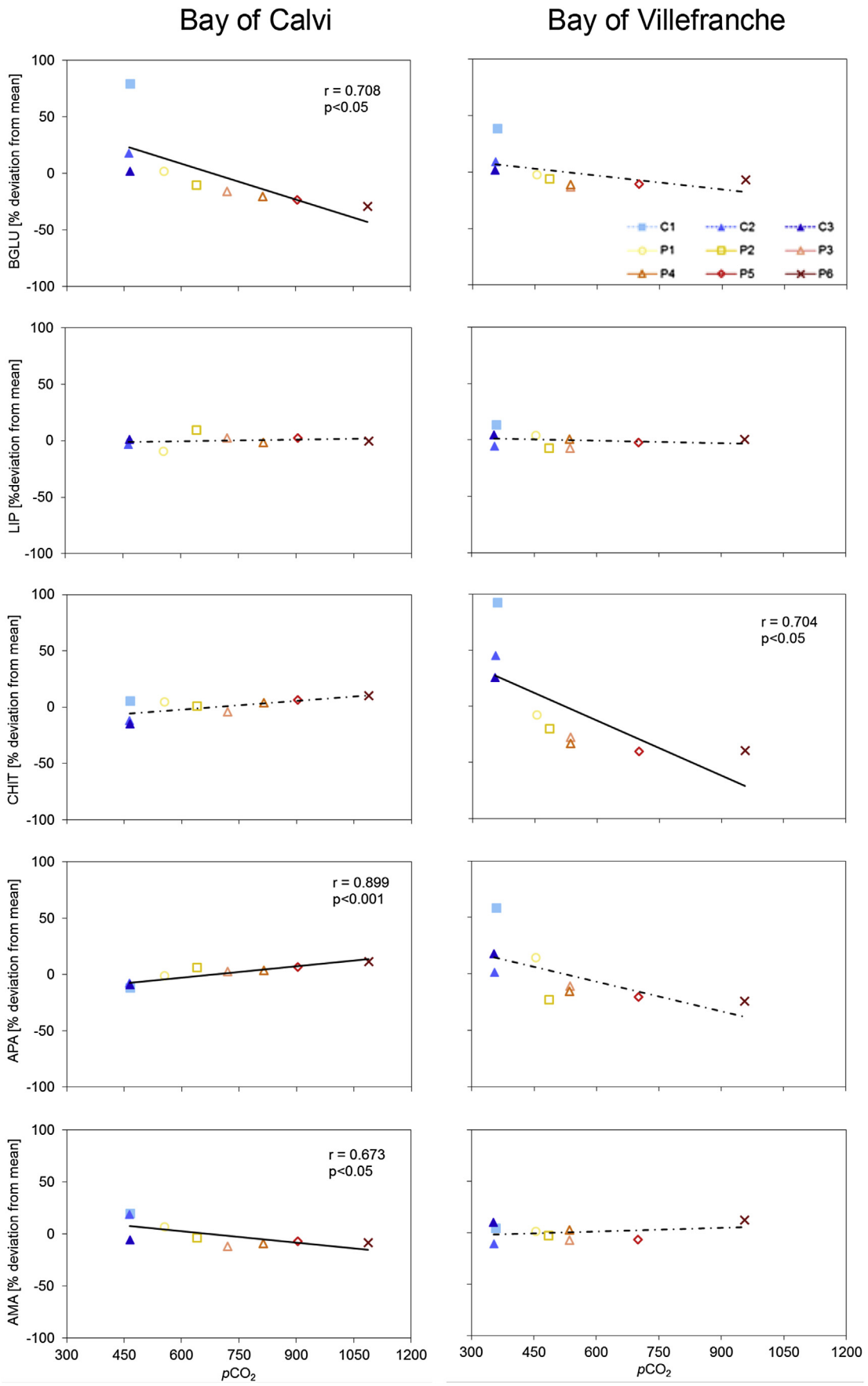


Fig. 5. β-glucosidase (BGLU), lipase (LIP), chitinase (CHIT), alkaline phosphatase (APA) and leucine aminopeptidase (AMA) activities as a function of mean pCO_2 measured in the 9 mesocosms in the Bay of Calvi (left) and in the Bay of Villefranche (right). Dots indicate the percentage deviation from the mean of all mesocosms over time of exoenzymatic activities. Solid lines indicate significant correlation; dashed lines denote non-significant correlation.

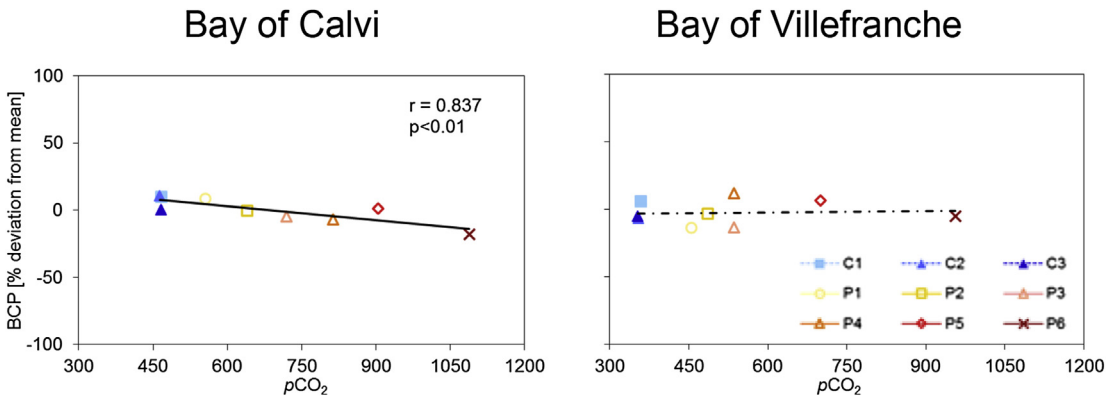


Fig. 6. Bacterial carbon production rates (BCP) activities as a function of mean pCO_2 measured in the 9 mesocosms in the Bay of Calvi (left) and in the Bay of Villefranche (right). Dots indicate the percentage deviation from the mean of all mesocosms over time of BCP. Solid lines indicate significant correlation; dashed lines denote non-significant correlation.

be responsible for temporal variations, but since temperature did not differ within the 9 mesocosms (Gazeau et al., 2017a), it could not be the cause of the observed differential hydrolysis rates in the enclosures. The direct effect of pH on extracellular enzymes has been hypothesised as the cause for organic matter reworking alterations in previous OA mesocosm experiments (Piontek et al., 2010, 2013). pH also determines the redox state of cofactors, thus influencing enzymatic reactions (Gottschalk, 1979). However, this direct influence is still a matter of debate since it is hardly possible to separate the effect of hydrogen ion concentrations from other coexisting forcing factors (Piontek et al., 2010, 2013). Taking these considerations into account, two more possible aspects need to be considered in discussing the degradation rate differences in the treated mesocosms. The first explanation is linked to the

Table 2
Stepwise multiple regression analysis between microbial parameters and environmental variables measured during the experiments in the Bay of Calvi and in the Bay of Villefranche ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). APA = alkaline phosphatase activity; AMA = leucine aminopeptidase activity; BCP = Bacterial carbon production; Temp = temperature; Chl *a* = chlorophyll *a* concentration; TPC = total particulate carbon concentration; TPN = total particulate nitrogen concentration; TPP = total particulate phosphorus concentration; GPP = gross primary production; ns = not significant ($p > 0.05$).

	Bay of Calvi				Bay of Villefranche			
	F	Adj r^2	n	Variables	F	Adj r^2	n	Variables
Het. prokaryotes	ns				16.86***	0.66	41	Chl *a***
								TPC*
								TPP*
								pCO_2
								TPN
β-glucosidase	13.81***	0.52	73	Temp***	10.45**	0.19	41	Het. prokaryotes**
				pCO_2***				
				GPP**				
				TPC*				
				TPN*				
				Het. prokaryotes				
Lipase	52.79***	0.74	73	Temp***	12.81**	0.37	41	Het. prokaryotes***
				Chl *a***				GPP
				GPP				
				Virus				
Chitinase	4.95***	0.30	48	TPP***	6.47***	0.45	41	pCO_2***
				Virus**				TPP
				GPP**				GPP
				Temp				Temp
				Het. prokaryotes				Het. prokaryotes
								TPC
APA	10.34***	0.48	73	Chl *a***	9.63***	0.52	41	pCO_2***
				pCO_2**				Temp**
				TPP*				Het. prokaryotes
				TPN*				TPC
				Temp				TPP
				Virus				
				TPC				
AMA	7.03***	0.30	73	Temp***	4.81**	0.32	41	GPP**
				pCO_2***				pCO_2*
				TPN*				Het. prokaryotes
				Virus				Temp
				Het. prokaryotes				TPN
BCP	6.18***	0.22	73	TPC**	23.15***	0.69	41	Temp***
				pCO_2*				GPP**
				TPP*				pCO_2*
				Temp				TPP

prokaryotic community structure and dynamics. It is known that phylogenetically distinct assemblages tend to have different organic matter degradation patterns (e.g. Pinhassi et al., 1999; Celussi et al., 2011; Arnosti, 2014). Population-level gene activations and regulation cannot be ruled out in explaining the difference in hydrolysis rate of β-glucosidase, chitinase and leucine aminopeptidase due to a change in the physiology of the community and/or a change in the bacterial community structure. Unfortunately, our suite of analyses did not include metagenomic nor metatranscriptomic approaches. As for many other microbial parameters, different experiments have highlighted different responses of prokaryotic assemblages to CO_2 amendments, reporting both modified (Krause et al., 2012) and unvaried (Newbold et al., 2012) communities.

The second explanation is related to the quality of the organic matter produced by phototrophs, which is the natural substrate for extracellular enzymes. The substrate's molecular composition and 3D structure greatly affect its potential cleavage by exoenzymes (Arnosti, 2011). Previous studies have highlighted a general stoichiometric increase in the carbon to nitrogen ratio of phototrophic organisms when subjected to elevated dissolved inorganic carbon concentrations, even if differences may occur in different taxa (reviewed by Riebesell and Tortell, 2011). An increased C:N ratio in the cell is likely to be transferred to the exudation products, thus producing an elemental alteration of the dissolved primary production pool (Kähler and Koeve, 2001). This might influence organic matter lability or recalcitrance thus having an indirect effect on degradation processes. Furthermore, there is a growing body of knowledge about the influence of pH on dissolved organic matter transition phases. At lower pH, dissolved organic molecules are more condensed, whereas at higher pH values, molecules and colloids tend to expand (e.g. Chin et al., 1998). pH-induced differences in substrate structure can therefore influence the rate of hydrolysis, as evidenced for carbohydrates and peptides (Arnosti, 2011 and references therein).

Also APA was influenced by CO_2 perturbation in BC. Alkaline phosphatase is produced both by bacteria (autotrophic and heterotrophic) and eukaryotic microalgae (Nausch, 1998) to compensate P limitation in the environment. PO_4^{3-} concentrations were extremely low at both sites (average = 9 nmol L^{-1} in both experiments; Louis et al., 2017) and it is hard to understand the different responses obtained during the two experiments, where the abundance of bacteria and phytoplankton might only partially be determining factors (lower abundance of both in BC, higher in BV). Additionally, dissolved organic P (DOP) concentration strongly differed in the two experiments (5–30 nM in BC and 40–80 nM in BV; Louis et al., 2017) and this differential substrate availability is one more possible explanation for diverse APA response in the two sites.

The concomitant effect of pCO_2 perturbation and substrate on β-glucosidase, chitinase, leucine aminopeptidase and alkaline phosphatase activities was confirmed by the multiple regression analysis (Table 2). In fact, when significant relationships with pCO_2 were found, they were linked to particulate elements (C, N, P), gross primary production and/or chlorophyll *a* concentration.

Even the uptake of dissolved organic material (as revealed by [^{3}H]-leucine incorporation) was unaffected (in BV) or lowered by acidification (in BC). The slower bacterial carbon production (BCP) in acidified mesocosms during the summer experiment in BC (Fig. 6) could be considered as a result of reduced monomers' supply by hydrolytic enzyme (β-glucosidase and leucine aminopeptidase) activities. This is in accordance to Piontek et al. (2013) who interpreted CO_2-enhanced bacterial production as a consequence of enhanced degradation processes on the time scale of their experiment, but with the opposite net effect. In BC, where plankton was present in lower abundance, slower BCP in treated mesocosms must have been counterbalanced by a minor top-down control on prokaryotes, since their abundance over time did not display any difference among the enclosures. It is worth noting that neither virus (Fig. 1) nor bacterivores (Maugendre et al., 2017a) numbers were related to acidification during both experiments. Surprisingly, a slower uptake of dissolved organic matter in high CO_2 mesocosms was coupled to a larger fraction of highly active (highly respiring, CTC+) prokaryotes. In order to maintain intracytoplasmatic pH levels, prokaryotes decrease membrane permeability to H^+, buffering their cytoplasm and equilibrating the external pH via catabolism. The evacuation of protons (out of the cells) is achieved by enhancing cellular oxidation-reduction systems and accelerating electron-transferring reactions (respiration in aerobic bacteria; Lehninger et al., 1993). The opposite outcome in the BV experiment (lowered %CTC + by acidification) is difficult to be interpreted and is possibly biased by the relatively low sampling frequency (3 samplings in 13 days).

A different interpretation of the strong CO_2-induced inhibition of chitinase activity observed in BV can be provided, being related to the load of available substrate. At this site, in fact, the number of copepods was approximately 8 times higher (on average 6482 ind m^{-3}; F. Gazeau, unpublished data) than in BC (827 ind m^{-3}; Zervoudaki et al., 2017), thus providing a higher amount of chitin to chitin-degrading prokaryotes. Although chitin per se is water insoluble, it has been shown that chitinaceous compounds can become partly soluble as a consequence of lowered pH (Rinaudo, 2006). It is therefore plausible that exoskeletons of dead crustaceans (and/or exuviae) partly underwent an abiotic dissolution process in the most acidified mesocosms, providing prokaryotes with N-acetyl-D-glucosamine which did not need enzymatic cleavage. Such phenomenon would be responsible for the lowered chitinase hydrolysis rates measured in the treated enclosures. On the contrary, in BC, low zooplankton individuals likely did not make enough substrate available for making such differences detectable.

The general inconsistency of our results with previous works where exoenzymatic activities were enhanced by increasing pCO_2 should be discussed in the context of the lower trophic status of our experimental sites. In particular, the enhanced polysaccharide degradation measured by Grossart et al. (2006), Piontek et al. (2010, 2013) and Maas et al. (2013) was found in areas with higher nutrient (with or without experimental nutrients' amendments) and/or higher chlorophyll *a* concentrations. The other published experiments carried out in low-nutrient low-chlorophyll areas did not investigate prokaryotic activities. However, Yoshimura et al. (2010) in the oligotrophic Okhotsk Sea did observe a decreased organic carbon production under elevated pCO_2, and Richier et al. (2014) reported a pCO_2-induced inhibition of small phototrophs' growth in several locations in the NW Europe continental shelf. The trophic status should therefore be regarded as a pivotal factor in determining the fate of carbon in a high CO_2 ocean, since enhanced metabolism (primary productivity) increases the amount of inorganic C which is channelled through the biological system (Riebesell et al., 2007) and is ultimately made available in its organic form to prokaryotes.

The trophic regime-dependent effect of OA on prokaryotes was also clearly pointed out by the experiments presented here.

Although many fundamental processes such as primary production and respiration did not show clear trends after pCO_2 manipulations (Maugendre et al., 2017b), modifications in degradation rates may affect considerably the pathway of organic matter e.g. its export to the seafloor, its recirculation within the microbial loop and its channelling through the trophic webs. Our results highlight the need to further analyse the consequences of OA in oligotrophic ecosystems in order to predict more precisely the potential damage

of anthropogenic CO_2 emissions on marine resources and ecosystem functioning. As a next step, it will also be important to determine changes in prokaryotic communities, in gene expressions (transcriptomics) and organic matter features following the combined effect of several anthropogenic pressures such as OA, seawater warming and eutrophication. Taking into account that oligotrophic systems are the most widespread in the surface ocean it is mandatory to fully understand the modifications in prokaryote-mediated biogeochemical cycles in a high(-er) CO_2 world.

Acknowledgements

This work was funded by the EC FP7 project 'Mediterranean Sea Acidification in a changing climate' (MedSeA; grant agreement 265103), the project European Free Ocean Carbon Enrichment (eFOCE; BNP-Paribas foundation), the MISTRALS-MERMEX program (Institut des Sciences de l'Univers, INSU), the Corsican local authorities and the Rhone-Mediterranean and Corsica Water Agency (http://www.eaurmc.fr). It is a contribution to the Surface Ocean – Lower Atmosphere Study (SOLAS) and Integrated Marine Biogeochemistry and Ecosystem Research (IMBER) projects. The STARESO marine station in Corsica is gratefully acknowledged for its assistance and boat support carried out within the framework of the STARECAPMED project funded by the Rhone-Mediterranean and Corsica Water Agency. J.-M. Grisoni, A. Sallon, G. Obolensky, S. Alliouane, B. Hesse, D. Luquet and D. Robin are acknowledged for assistance with diving operations. We are indebted to A. Konstadinopoulou for on-site BCP analyses. The valuable comments of two anonymous reviewers on earlier versions of the manuscript helped to improve the article.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ecss.2015.08.015.

References

Ammerman, J.W., Azam, F., 1991. Bacterial 5′-nucleotidase activity in estuarine and coastal marine waters: characterization of enzyme activity. Limnol. Oceanogr. 36, 1427–1436. http://dx.doi.org/10.4319/lo.1991.36.7.1427.

Arnosti, C., 2011. Microbial extracellular enzymes and the marine carbon cycle. Annu. Rev. Mar. Sci. 3, 401–425. http://dx.doi.org/10.1146/annurev-marine-120709-142731.

Arnosti, C., 2014. Patterns of microbially driven carbon cycling in the ocean: links between extracellular enzymes and microbial communities. Adv. Oceanogr. http://dx.doi.org/10.1155/2014/706082 article ID 706082.

Arnosti, C., Grossart, H.-P., Mühling, M., Joint, I., Passow, U., 2011. Dynamics of extracellular enzyme activities in seawater under changed atmospheric pCO_2: a mesocosm investigation. Aquat. Microb. Ecol. 64, 285–298. http://dx.doi.org/10.3354/ame01522.

Azam, F., 1998. Microbial control of oceanic carbon flux: the plot thickens. Science 280, 694–696. http://dx.doi.org/10.1126/science.280.5364.694.

Azam, F., Fenchel, T., Field, J.G., Gray, J.S., Meyer-Reil, L.A., Thingstad, F., 1983. The eological role of water-column microbes in the sea. Mar. Ecol. Prog. Ser. 10, 257–263.

Baltar, F., Arístegui, J., Sintes, E., van Aken, H.M., Gasol, J.M., Herndl, G.J., 2009. Prokaryotic extracellular enzymatic activity in relation to biomass production and respiration in the meso- and bathypelagic waters of the (sub)tropical Atlantic. Environ. Microbiol. 11, 1998–2014. http://dx.doi.org/10.1111/j.1462-2920.2009.01922.x.

Bourdin, G., Kerros, M.E., Sallon, A., Gazeau, F., Pedrotti, M.-L., 2015. Effect of acidification on the production and the aggregation of the transparent exopolymeric particles (TEP) during a phytoplankton bloom in the Mediterranean Sea: a mesocosm experiment. Estuar. Coast. Shelf Sci.

Brussaard, C.P.D., 2004. Optimization of procedures for counting viruses by flow cytometry. Appl. Environ. Microbiol. 70, 1506–1513. http://dx.doi.org/10.1128/AEM.70.3.1506-1513.2004.

Brussaard, C.P.D., Noordeloos, A.A.M., Witte, H., Collenteur, M.C.J., Schulz, K., Ludwig, A., Riebesell, U., 2013. Arctic microbial community dynamics influenced by elevated CO_2 levels. Biogeosciences 10, 719–731. http://dx.doi.org/10.5194/bg-10-719-2013.

Buchan, A., LeCleir, G.R., Gulvik, C.A., González, J.M., 2014. Master recyclers: features and functions of bacteria associated with phytoplankton blooms. Nat. Rev. Microbiol. 12, 686–698. http://dx.doi.org/10.1038/nrmicro3326.

Celussi, M., Cataletto, B., 2007. Annual dynamics of bacterioplankton assemblages in the Gulf of Trieste (Northern Adriatic Sea). Gene 406, 113–123. http://dx.doi.org/10.1016/j.gene.2007.07.010.

Celussi, M., Del Negro, P., 2012. Microbial degradation at a shallow coastal site: long-term spectra and rates of exoenzymatic activities in the NE Adriatic Sea. Estuar. Coast. Shelf Sci. 115, 75–86. http://dx.doi.org/10.1016/j.ecss.2012.02.002.

Celussi, M., Bussani, A., Cataletto, B., Del Negro, P., 2011. Assemblages' structure and activity of bacterioplankton in northern Adriatic Sea surface waters: a 3-year case study. FEMS Microbiol. Ecol. 75, 77–88. http://dx.doi.org/10.1111/j.1574-6941.2010.00997.x.

Cerrano, C., Cardini, U., Bianchelli, S., Corinaldesi, C., Pusceddu, A., Danovaro, R., 2013. Red coral extinction risk enhanced by ocean acidification. Sci. Rep. 3, 1457. http://dx.doi.org/10.1038/srep01457.

Chin, W.-C., Orellana, M.V., Verdugo, P., 1998. Spontaneous assembly of marine dissolved organic matter into polymer gels. Nature 391, 568–572. http://dx.doi.org/10.1038/35345.

Chisholm, S.W., 2000. Oceanography: stirring times in the Southern Ocean. Nature 407, 685–687. http://dx.doi.org/10.1038/35037696.

Choi, J.W., Sherr, E.B., Sherr, B.F., 1996. Relation between presence-absence of a visible nucleoid and metabolic activity in bacterioplankton cells. Limonol. Oceanogr. 41, 1161–1168. http://dx.doi.org/10.4319/lo.1996.41.6.1161.

Chróst, R.J., 1992. Significance of bacterial ectoenzymes in aquatic environments. Hydrobiologia 243–244, 61–70. http://dx.doi.org/10.1007/978-94-011-2745-5_5.

Cram, J.A., Chow, C.-E.T., Sachdeva, R., Needham, D.M., Parada, A.E., Steele, J.A., Fuhrman, J.A., 2015. Seasonal and interannual variability of the marine bacterioplankton community throughout the water column over ten years. ISME J. 9, 563–580. http://dx.doi.org/10.1038/ismej.2014.153.

del Giorgio, P.A., Gasol, J.M., 2008. Physiological structure and single-cell activity in marine bacterioplankton. In: Kirchman, D.L. (Ed.), Microbial Ecology of the Oceans, second ed. John Wiley and sons, Hoboken, NJ, pp. 243–298.

Ducklow, H., Carlson, C.A., 1992. Oceanic bacterial production. Adv. Microb. Ecol. 12, 113–181.

Endres, S., Galgani, L., Riebesell, U., Shulz, K.-G., Engel, A., 2014. Stimulated bacterial growth under elevated pCO_2: results from an off-shore mesocosm study. PLos ONE 9 (6), e99228. http://dx.doi.org/10.1371/journal.pone.0099228.

Fukuda, R., Sohrin, Y., Saotome, N., Fukuda, H., Nagata, T., Koike, I., 2000. East-west gradient in ectoenzyme activities in the subarctic Pacific: possible regulation by zinc. Limnol. Oceanogr. 45, 930–939. http://dx.doi.org/10.4319/lo.2000.45.4.0930.

Gazeau, F., Parker, L.M., Comeau, S., Gattuso, J.-P., O'Connor, W.A., Martin, S., Pörtner, H.-O., Ross, P.M., 2013. Impact of ocean acidification on marine shelled molluscs. Mar. Biol. 160, 2207–2245. http://dx.doi.org/10.1007/s00227-013-2219-3.

Gazeau, F., Sallon, A., Maugendre, L., Giani, M., Celussi, M., Michel, L., Gobert, S., Borges, A.V., 2015. Impact of elevated CO_2 on pelagic production and elemental budgets in a Mediterranean mesocosm study. Estuar. Coast. Shelf Sci.

Gazeau, F., Sallon, A., Maugendre, L., Louis, J., Dellisanti, W., Gaubert, M., Lejeune, P., Gobert, S., Alliouane, S., Taillandier, V., Louis, F., Obolensky, G., Grisoni, J.-M., Guieu, C., 2017a. First mesocosm experiments to study the impacts of ocean acidification on plankton communities in the NW Mediterranean Sea (MedSeA project). Estuar. Coast. Shelf Sci. 186, 11–29.

Gazeau, F., Sallon, A., Pitta, P., Tsiola, A., Pedrotti, M.-L., Marro, S., Guieu, C., 2017b. Limited impact of ocean acidification on phytoplankton community structure in an oligotrophic environment: results from two mesocosm studies in the Mediterranean Sea. Estuar. Coast. Shelf Sci. 186, 72–88.

Gottschalk, G., 1979. Nutrition of bacteria. In: Bacterial Metabolism. Springer-Verlag, New York, pp. 1–11. http://dx.doi.org/10.1007/978-1-4684-0465-4_1.

Grossart, H.-P., Allgaier, M., Passow, U., Riebesell, U., 2006. Testing the effect of CO_2 concentration on the dynamics of marine heterotrophic bacterioplankton. Limnol. Oceanogr. 51, 1–11. http://dx.doi.org/10.4319/lo.2006.51.1.0001.

Hoppe, H.G., 1993. Use of fluorogenic model substrates for extracellular enzyme activity (eea) measurement of bacteria. In: Kemp, P.F., Sherr, B.F., Sherr, E.B., Cole, J.J. (Eds.), Handbook of Methods in Aquatic Microbial Ecology. CRC Press, Boca Raton, pp. 423–431.

Kähler, P., Koeve, W., 2001. Marine dissolved organic matter: can its C:N ratio explain carbon overconsumption? Deep Sea Res. I 48, 49–62. http://dx.doi.org/10.1016/S0967-0637(00)00034-0.

Kirchman, D.L., 1993. Leucine incorporation as a measure of biomass production by heterotrophic bacteria. In: Kemp, P.F., Sherr, B.F., Sherr, E.B., Cole, J.J. (Eds.), Current Methods in Aquatic Microbial Ecology. CRC Press, Boca Raton, pp. 509–512.

Kirchman, D.L., Newell, S.Y., Hodson, R.E., 1986. Incorporation versus biosynthesis of leucine: implications for measuring rates of protein synthesis and biomass production by bacteria in marine systems. Mar. Ecol. Prog. Ser. 32, 47–59.

Krause, E., Wichels, A., Giménez, L., Lunau, M., Schilhabel, M.B., Gerdts, G., 2012. Small changes in pH have direct effects on marine bacterial community composition: a microcosm approach. PloS ONE 7 (10), e47035. http://dx.doi.org/10.1371/journal.pone.0047035.

Larsen, J.B., Larsen, A., Thyrhaug, R., Bratbak, G., Sandaa, R.-A., 2008. Response of marine viral populations to a nutrient induced phytoplankton bloom at different pCO_2 levels. Biogeosciences 5, 523–533. http://dx.doi.org/10.5194/bg-

5-523-2008.
Lehninger, A.L., Nelson, D.L., Cox, M.M., 1993. Principles of Biochemistry, second ed. Worth, New York.
Louis, J., Guieu, C., Gazeau, F., 2017. Is nutrients dynamic affected by ocean acidification? Results from two mesocosms experiments in the Mediterranean Sea. Estuar. Coast. Shelf Sci. 186, 30–44.
Maas, E.W., Law, C.S., Hall, J.A., Pickmere, S., Currie, K.I., Chang, F.H., Voyles, K.M., Caird, D., 2013. Effect of ocean acidification on bacterial abundance, activity and diversity in the Ross Sea, Antarctica. Aquat. Microb. Ecol. 70, 1–15. http://dx.doi.org/10.3354/ame01633.
Malfatti, F., Turk, V., Tinta, T., Mozetič, P., Manganelli, M., Samo, T.J., Ugalde, J.A., Kovač, N., Stefanelli, M., Antonioli, M., Fonda Umani, S., Del Negro, P., Cataletto, B., Hozić, A., Ivošević DeNardis, N., Žutić, V., Svetličić, V., Mišić Radić, T., Radić, T., Fuks, D., Azam, F., 2014. Microbial mechanisms coupling carbon and phosphorus cycles in phosphorus-limited northern Adriatic Sea. Sci. Total Environ. 470–471, 1173–1183. http://dx.doi.org/10.1016/j.scitotenv.2013.10.040.
Marie, D., Brussaard, C.P.D., Thyrhaug, R., Bratbak, G., Vaulot, D., 1999. Enumeration of marine viruses in culture and natural samples by flow cytometry. Appl. Environ. Microbiol. 65 (1), 45–52.
Maugendre, L., Gattuso, J.-P., de Kluijver, A., Soetaert, K., van Oevelen, D., Middelburg, J.J., Gazeau, F., 2017a. Carbon-13 labelling studies show no effect of ocean acidification on carbon transfer in Mediterranean plankton communities. Estuar. Coast. Shelf Sci. 186, 100–111.
Maugendre, L., Gattuso, J.-P., Poulton, A.J., Dellisanti, W., Gaubert, M., Guieu, C., Gazeau, F., 2017b. No detectable effect of ocean acidification on plankton metabolism in the NW oligotrophic Mediterranean Sea: results from two mesocosm studies. Estuar. Coast. Shelf Sci. 186, 89–99. http://dx.doi.org/10.1016/j.ecss.2015.03.009.
Motegi, C., Tanaka, T., Piontek, J., Brussaard, C.P.D., Gattuso, J.-P., Weinbauer, M.G., 2013. Effect of CO_2 enrichment on bacterial metabolism in an Arctic fjord. Biogeosciences 10, 3285–3296. http://dx.doi.org/10.5194/bg-10-3285-2013.
Nausch, M., 1998. Alkaline phosphatase activities and the relationship to inorganic phosphate in the Pomeranian Bight (southern Baltic Sea). Aquat. Microb. Ecol. 16, 87–94. http://dx.doi.org/10.3354/ame016087.
Newbold, L.K., Oliver, A.E., Booth, T., Tiwari, B., DeSantis, T., Maguire, M., Andersen, G., van der Gast, C.J., Whiteley, A.S., 2012. The response of marine picoplankton to ocean acidification. Environ. Microbiol. 14, 2293–2307. http://dx.doi.org/10.1111/j.1462-2920.2012.02762.x.
Nogales, B., Lanfranconi, M.P., Piña-Villalonga, J.M., Bosh, R., 2011. Anthropogenic perturbations in marine microbial communities. FEMS Microbiol. Rev. 35, 275–298. http://dx.doi.org/10.1111/j.1574-6976.2010.00248.x.
Paoli, A., Karuza, A., De Vittor, A., Del Negro, A., Fonda Umani, S., 2006. Daily variations of highly active bacteria in the Northern Adriatic Sea. J. Plankton Res. 28, 325–335. http://dx.doi.org/10.1093/plankt/fbi116.
Piontek, J., Lunau, M., Händel, Brochard, C., Wurst, M., Engel, A., 2010. Acidification increases polysaccharide degradation in the ocean. Biogeosciences 7, 1615–1624. http://dx.doi.org/10.5194/bg-7-1615-2010.
Piontek, J., Borchard, C., Sperling, M., Shulz, K.-G., Riebesell, U., Engel, A., 2013. Response of bacterioplankton activity in an Arctic fjord system to elevated pCO_2: results from a mesocosm perturbation study. Biogeosciences 10, 297–314. http://dx.doi.org/10.5194/bg-10-297-2013.
Pinhassi, J., Azam, F., Hemphälä, J., Long, R.A., Martinez, J., Zweifel, U.L., Hagström, Å., 1999. Coupling between bacterioplankton species composition, population dynamics, and organic matter degradation. Aquat. Microb. Ecol. 17, 13–26. http://dx.doi.org/10.3354/ame017013.
Pomeroy, L.R., Wiebe, W.J., 2001. Temperature and substrate as interactive limiting factors for marine heterotrophic bacteria. Aquat. Microb. Ecol. 23, 187–204. http://dx.doi.org/10.3354/ame023187.
Richier, S., Achterberg, E.P., Dumousseaud, C., Poulton, A.J., Suggett, D.J., Tyrrell, T., Zubkov, M.V., Moore, C.M., 2014. Carbon cycling and phytoplankton responses within highly-replicated shipboard carbonate chemistry manipulation experiments conducted around Northwest European Shelf Seas. Biogeosciences 11, 4733–4752. http://dx.doi.org/10.5194/bg-11-4733-2014.
Riebesell, U., Tortell, P.D., 2011. Effects of ocean acidification on pelagic organisms and ecosystems. In: Gattuso, J.-P., Hansson, L. (Eds.), Ocean Acidification. Oxford University Press, pp. 99–121.
Riebesell, U., Schulz, K.G., Bellerby, R.G.J., Botros, M., Fritsche, P., Meyerhöfer, M., Neill, C., Nondal, G., Oschlies, A., Wohlers, J., Zöllner, E., 2007. Enhanced biological carbon consumption in a high CO_2 ocean. Nature 450, 545–549. http://dx.doi.org/10.1038/nature06267.
Riebesell, U., Czerny, J., von Bröckel, K., Boxhammer, T., Büdenbender, J., Deckelnick, M., Fischer, M., Hoffman, D., Krug, S.A., Lentz, U., Ludwig, A., Muche, R., Schulz, K.G., 2013. Technical note: a mobile sea-going mesocosm system – new opportunities for ocean change research. Biogeosciences 10, 1835–1847. http://dx.doi.org/10.5194/bg-10-1835-2013.
Rinaudo, M., 2006. Chitin and chitosan: properties and applications. Prog. Polym. Sci. 31, 603–632. http://dx.doi.org/10.1016/j.progpolymsci.2006.06.001.
Rochelle-Newall, E., Delille, B., Frankignoulle, M., Gattuso, J.-P., Jacquet, S., Riebesell, U., Terbuggen, A., Zondervan, I., 2004. Chromophoric dissolved organic matter in experimental mesocosms maintained under different pCO_2 levels. Mar. Ecol. Prog. Ser. 272, 25–31. http://dx.doi.org/10.3354/meps272025.
Smith, D.C., Azam, F., 1992. A simple, economical method for measuring bacterial protein synthesis rates in sea water using ^{3}H-leucine. Mar. Microb. Food Webs 6, 107–114.
Tanaka, T., Thingstad, T.F., Løvdal, T., Grossart, H.-P., Larsen, A., Allgaier, M., Meyerhöfer, M., Shulz, K.-G., Wohlers, J., Zöllner, E., Riebesell, U., 2008. Availability of phosphate for phytoplankton and bacteria and of glucose for bacteria at different pCO_2 levels in a mesocosm study. Biogeosciences 5, 669–678. http://dx.doi.org/10.5194/bg-5-669-2008.
Tsiola, A., Pitta, P., Giannakourou, A., Bourdin, G., Maugendre, L., Pedrotti, M.L., Gazeau, F., 2017. Ocean acidification and viral replication cycles: frequency of lytically infected and lysogenic cells during a mesocosm experiment in the NW Mediterranean Sea. Estuar. Coast. Shelf Sci. 186, 139–151.
Van Wambeke, F., Christaki, U., Bianchi, M., Psarra, S., Tselepidis, A., 1997. Heterotrophic bacterial production in the Cretan Sea (NE) Mediterranean. Prog. Oceanogr. 46, 205–216. http://dx.doi.org/10.1016/S0079-6611(00)00019-7.
Wilkox, R.M., Fuhrman, J.A., 1994. Bacterial viruses in coastal seawater: lytic rather than lysogenic production. Mar. Ecol. Prog. Ser. 114, 35–45.
Yamada, N., Suzumura, M., 2010. Effects of seawater acidification on hydrolytic enzyme activities. J. Oceanogr. 66, 233–241.
Yoshimura, T., Nishioka, J., Suzuki, K., Hattori, H., Kiyosawa, H., Watanabe, Y.W., 2010. Impacts of elevated CO_2 on organic carbon dynamics in nutrient depleted Okhotsk Sea surface waters. J. Exp. Mar. Biol. Ecol. 395, 191–198. http://dx.doi.org/10.1016/j.jembe.2010.09.001.
Zervoudaki, S., Krasakopoulou, E., Moutsopoulos, T., Protopapa, M., Marro, S., Gazeau, F., 2017. Copepod response to ocean acidification in a low nutrient-low chlorophyll environment in the NW Mediterranean Sea. Estuar. Coast. Shelf Sci. 186, 152–162.