

doi: 10.1093/femsec/fiv058 Advance Access Publication Date: 1 June 2015 Research Article

## RESEARCH ARTICLE

# Response of rare, common and abundant bacterioplankton to anthropogenic perturbations in a Mediterranean coastal site

Federico Baltar<sup>1,2,\*</sup>, Joakim Palovaara<sup>1</sup>, Maria Vila-Costa<sup>3,4</sup>, Guillem Salazar<sup>5</sup>, Eva Calvo<sup>5</sup>, Carles Pelejero<sup>5,6</sup>, Cèlia Marrasé<sup>5</sup>, Josep M. Gasol<sup>5</sup> and Jarone Pinhassi<sup>1</sup>

<sup>1</sup>Centre for Ecology and Evolution in Microbial Model Systems, EEMiS, Linnaeus University, Barlastgatan 11, SE-391 82 Kalmar, Sweden, <sup>2</sup>Department of Marine Sciences, University of Otago, PO Box 56, Dunedin 9054, New Zealand, <sup>3</sup>Limnological Observatory of the Pyrenees (LOOP)—Department of Ecology, University of Barcelona Av. Diagonal 643, 08028 Barcelona, Spain, <sup>4</sup>Department of Environmental Chemistry, IDAEA-CSIC, C/Jordi Girona 18–26, 08034, Barcelona, Catalonia, Spain, <sup>5</sup>Departament de Biologia Marina i Oceanografia, Institut de Ciències del Mar—CSIC, Pg. Marítim de la Barceloneta 37–49, 08003 Barcelona, Spain and <sup>6</sup>Institució Catalana de Recerca i Estudis Avançats, 08010 Barcelona, Spain

\*Corresponding author: Department of Marine Sciences, University of Otago, PO Box 56, 310 Castle Street, Dunedin 9054, New Zealand. Tel: +64-3-479-5621; E-mail: federico.baltar@otago.ac.nz

One sentence summary: This sheds light on the potential response of marine bacteria to acidification and nutrient enrichment, comparing the responses of the most abundant/common members to the very rare ones. Editor: Patricia Sobecky

## ABSTRACT

Bacterioplankton communities are made up of a small set of abundant taxa and a large number of low-abundant organisms (i.e. 'rare biosphere'). Despite the critical role played by bacteria in marine ecosystems, it remains unknown how this large diversity of organisms are affected by human-induced perturbations, or what controls the responsiveness of rare compared to abundant bacteria. We studied the response of a Mediterranean bacterioplankton community to two anthropogenic perturbations (i.e. nutrient enrichment and/or acidification) in two mesocosm experiments (in winter and summer). Nutrient enrichment increased the relative abundance of some operational taxonomic units (OTUS), e.g. *Polaribacter, Tenacibaculum, Rhodobacteraceae* and caused a relative decrease in others (e.g. *Croceibacter*). Interestingly, a synergistic effect of acidification and nutrient enrichment was observed on specific OTUs (e.g. SAR86). We analyzed the OTUs that became abundant at the end of the experiments and whether they belonged to the rare (<0.1% of relative abundance), the common (0.1–1.0% of relative abundance) or the abundant (>1% relative abundance) fractions. Most of the abundant OTUs at the end of the experiments were abundant, or at least common, in the original community of both experiments, suggesting that ecosystem alterations do not necessarily call for rare members to grow.

Keywords: marine bacteria; rare biosphere; diversity; acidification; eutrophication

Downloaded from https://academic.oup.com/femsec/article/91/6/fiv058/2594615 by guest on 22 July 202

Received: 22 October 2014; Accepted: 20 May 2015

<sup>©</sup> FEMS 2015. All rights reserved. For permissions, please e-mail: journals.permissions@oup.com

### **INTRODUCTION**

The diversity of marine bacterial communities at a particular time and location is composed of a small set of abundant taxa and a very large collection of low-abundant organisms (Pedrós-Alio 2006; Pommier et al. 2007), the so-called rare biosphere (Sogin et al. 2006). Abundant and rare bacteria have arbitrarily been defined as populations with relative abundances of  $\geq 1\%$  and of <0.1%, respectively (Pedrós-Alió 2012). This classification of bac-</p> terial populations in terms of their relative abundance has contributed to our understanding of bacterial community structure (e.g. Pedrós-Alio 2006, 2007, 2012; Pommier et al. 2007; Galand et al. 2009). Still, the ecological significance of the large diversity of rare bacteria remains elusive-in particular, reliable estimates are lacking of the order of magnitude of the total number of bacterial taxa in the oceans or on the ecological mechanisms that allow subsistence of many species in low numbers (Pedrós-Alió 2012). Although, a priori, it would be feasible that rare community members remain permanently rare, the main current hypothesis is that they represent a largely inactive seed bank, from which some bacteria can emerge and become active in response to environmental changes (Epstein 2009; Lennon and Jones 2011). The quite predictable responses of some copiotrophic bacteria (sensu; Lauro et al. 2009) in laboratory manipulation experiments, including filtration, confinement, transplantation (Ferguson, Buckley and Palumbo 1984; Sjöstedt et al. 2012) or organic matter enrichment (Teira et al. 2007), would support this view. Since similar shifts in bacterial community structure are sometimes observed also in situ (e.g. Gilbert et al. 2011; Teeling et al. 2012), it is important to investigate how natural environmental changes and anthropogenic impacts select for or against members of the rare biosphere-i.e. what controls the responsiveness of rare compared to abundant bacteria.

Although bacteria play a paramount role in the marine carbon cycle, it is not clear to what degree bacterial diversity will be affected by anthropogenic pressures such as ocean acidification or eutrophication. Moreover, the cooccurrence of several disturbances can potentially produce synergistic/antagonistic effects on marine biota different than those caused by individual stresses. For instance, Lindh *et al.* (2013) found in a Baltic Sea experiment that although temperature increments selectively promoted the growth of specific bacterial populations, such selection was enhanced under acidified conditions. Thus, it is relevant to study the combined effect of different anthropogenic processes on microbial communities in order to better constrain the potential future response of marine ecosystem diversity and functioning to environmental perturbations.

Here we studied the response of bacterial communities to two types of relevant environmental disturbances (i.e. nutrient enrichment and acidification, and a combination of the two) in two mesocosms experiments with water from a coastal Mediterranean site conducted in winter and summer. Our objective was to determine whether bacterial diversity was affected by acidification, eutrophication and/or the combination of both. In particular, our aim was to identify, quantify and compare the proportion of rare bacteria that became abundant after disturbances. We hypothesized that eutrophication and/or acidification, alone or in combination, would differentially impact the prokaryote species distribution, allowing the identification of particular members specifically responding to each of these single or combined manipulations. Based on previous works reporting low-abundant bacteria increasing in relative abundance in response to phytoplankton blooms and/or organic matter availability (e.g. Teira et al. 2007; Gilbert et al. 2011; Teeling et al. 2012),

we expected that nutrient additions, and its associated increase in phytoplankton biomass, would favor mostly the rare bacteria. Due to differences in the *in situ* nutrient concentration and community composition between seasons (Alonso-Sáez *et al.* 2007), we anticipated that the number of rare members responding and becoming abundant would differ between experiments potentially providing insights into how the abundance of different bacterial populations is regulated. We expected to find a higher number of rare members becoming abundant in winter than in summer due to the higher level of total nutrient enrichment in winter (albeit proportional to *in situ* concentrations in the two experiments), or because the winter bacteria are more used to nutrient pulses and therefore can take advantage of nutrient enrichment in a more efficient way, or as a result of both factors.

### **MATERIALS AND METHODS**

#### **Experimental setup**

We studied the response of bacterioplankton to diverse environmental disturbances, including reduced pH (ca. 0.1–0.3 units lower than the pH in the control mesocosms) and inorganic nutrient additions (ca.  $8 \times$  nitrogen (N) and silicon (Si) concentrations found typically in situ at the time of the respective experiments, and phosphorus (P) added at Redfield ratios) (Figs S1-S3, Supporting Information). The pH reduction range was selected to mimic realistic ocean acidification scenarios by the end of this century (Stocker et al. 2013). The nitrogen concentrations used were based on the observed increase in the nutrient loading to coastal waters due to the increased production and application of nitrogen-bearing fertilizers in agriculture in the last half-century (Howarth and Marino 2006). Two mesocosm experiments were performed, one in winter (WIN [13-26 February 2010] and one in summer (SUM [5-15 July 2011]). These experiments were done using 200 L polyethylene mesocosms with water collected from the Blanes Bay Microbial Observatory (BBMO, NW Mediterranean Sea, 41°40′N, 2°48′E). The added N concentrations (as nitrate) were 16 and 4  $\mu$ M N in WIN and SUM, respectively. Si was added at 28 and 7.5  $\mu$ M in WIN and SUM, respectively. The experiments were conducted in a temperature-controlled chamber, at in situ temperature and under a 12:12 h light:dark cycle. The light conditions were set by a combination of coolwhite and gro-lux lamps, which mimic the quality of natural light. The pH treatment was performed by bubbling very small amounts of CO<sub>2</sub> (99.9% purity) directly to the mesocosms. The bubbling was regulated manually every morning to maintain the levels of pH in the acidified tanks 0.25-0.30 pH units lower than the controls, and monitored using glass electrodes (LL Ecotrode plus-Metrohm), which were calibrated on a daily basis with a Tris buffer following standard procedures (Dickson, Sabine and Christian 2007). The pH in the tanks was continuously recorded by a D130 data logger (Consort, Belgium). In order to mimic the potential physical perturbation associated with CO<sub>2</sub> bubbling, the control mesocosms were also bubbled with similar small amounts of compressed air at current atmospheric CO<sub>2</sub> concentrations. The setup included four duplicate conditions: control (KB, no nutrient addition nor pH decrease), acidified control (KA, no nutrient addition, but lowered pH), nutrients addition (NB, no pH decrease) and acidified nutrient addition (NA). We followed the daily changes in bacterial abundance and phytoplankton biomass (as chlorophyll-a). Bacterial community composition was determined at the beginning and at the end of our 8-9 days experiments (454 tag pyrosequencing of 16S rRNA gene

sequences). The total length of the experiment was determined by the duration of the bloom and was in agreement with the duration of other mesocosm experiments carried out with water from this site (e.g. Allers et al. 2007; Sandaa et al. 2009; Ray et al. 2012).

#### Chlorophyll-a concentration and bacterial abundance

Chlorophyll-a (Chl-a) was estimated fluorometrically from 50 ml samples filtered through Whatman GF/F filters. The filters were ground in 90% acetone and left in the dark at room temperature for at least 2 h. The fluorescence of the extract was measured with a Turner Designs fluorometer. Bacterial abundance was determined by flow cytometry. Samples were preserved with a mixture of 1% paraformaldehyde and 0.05% glutaraldehyde (final concentrations), and stored frozen at  $-80^{\circ}$ C. Within a few days, cell counts were obtained with a BectonDickinson FACSCalibur flow cytometer with a blue laser, after staining with a  $10\times$  final dilution of SybrGreen I (Molecular Probes, Invitrogen).

### DNA sampling collection and extraction

Around 1 L of sample from each mesocosm was filtered through a 0.2  $\mu$ m pore-size Supor-200 filter (PALL, 47 mm diameter), immediately transferred into cryovials containing TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and frozen at  $-80^{\circ}$ C until further processing. A combined treatment with enzymes (lysozyme, proteinase K) and enzyme/phenol-chloroform was used to extract the DNA as described previously, including a 30-min lysozyme digestion at 37°C and an overnight proteinase K digestion at 55°C (Boström *et al.* 2004). DNA was quantified using PicoGreen (Molecular Probes).

#### PCR and sequencing preparation

Partial bacterial 16S rRNA genes were amplified for pyrosequencing using a primer cocktail containing the degenerate primers 530F (5'-GTGCCAGCMGCNGCGGGTA-3') with TA (thymine-adenine) added at the 3-prime end to increase specificity, and 1061R (5'-CRRCACGAGCTGACGAC-3') (Dowd *et al.* 2008) labeled with specific hexamers to differentiate samples by using a different barcode for each of the samples (Sjöstedt *et al.* 2012). The PCR products were gel purified (QIAquick Gel Extraction Kit, Qiagen), concentrated (QIAquick PCR Purification Kit, Qiagen) and quantified before being mixed in equimolar amounts. Addition of adaptor and pyrosequencing on a Roche GS FLX TITA-NIUM (Roche Applied Science) were performed at LGC Genomics (Germany) according to the manufacturer's instructions.

#### Sequence analysis

In the 18 samples collected (9 samples per experiment), a total of 624 794 sequences were obtained and analyzed following previously described methods (Fierer et al. 2008; Hamady et al. 2008; Lauber et al. 2009) using the Quantitative Insights Into Microbial Ecology (QIIME v.1.2.1) pipeline (http://qiime.org). Low-quality sequences (sequences < 200 bp in length) were removed. Denoising was done via the n3phele cloud (http://www.n3phele.com) working with the QIIME toolkit. Chimera removal by Perseus is an integral part of the QIIME implementation of AmpliconNoise. Perseus was run with default settings (Egge et al. 2013). Recent studies have shown that singletons in pyrosequencing of microbial communities could to

a large extent be the result of DNA sequencing errors creating false sequence-based taxa, which suggests that they should be omitted from analyses (Kunin et al. 2010). Thus, singletons were not included in our further analyses. Although the elimination of singletons may well eliminate some real species, treating all singleton sequences as suspect and deleting singletons from analysis is considered a conservative approach (Medinger et al. 2010; Tedersoo et al. 2010). Using this approach, the final number of sequences remaining was 111 221 (average length 490 bp). It has been recently shown that 5000 denoised sequences per sample are needed for an accurate and precise estimation of trends in bacterial alpha-diversity and around 1000 for betadiversity (Lundin et al. 2012). In our study, after eliminating the singletons we still had more than enough sequences (total of 111 221 sequences; >6500 sequence per sample) to account for beta- and alpha-diversity. Similar sequences were binned into operational taxonomic units (OTUs) using UCLUST (Edgar 2010) with a minimum pairwise identity of 97%. Representative sequences for each OTU were aligned with PyNAST, the taxonomic identity of each phylotype determined using the RDP Classifier (Wang et al. 2007) and a tree built using FastTree (Price, Dehal and Arkin 2009). Subsampling to a sequencing depth determined by the minimum number of sequences in a sample (i.e. 6000 sequences) was performed in QIIME on all samples to standardize the analyses. Alpha-diversity was measured with the Shannon index using QIIME standard settings. Sequences have been deposited in GenBank under the accession numbers KM277008-KM277354

#### Statistical analyses

To compare the different sets of samples, we carried out an analysis of variance (ANOVA) followed by a post-hoc Tukey's honestly significant difference (HSD) test to compare the group means after log transformation of the data to attain normality using the JMP Statistical Software (SAS Institute Inc, Cary, NC). Normality was checked with a Shapiro–Wilk test.

## RESULTS

## Phytoplankton biomass and bacterial abundance response

The initial Chl-a concentration was significantly higher (Tukey-HSD,  $\alpha < 0.05$ ) in the winter (WIN) than in the summer (SUM) experiment (Table 1). After a lag of 1–3 days, Chl-a concentration increased in all mesocosms enriched with nutrients (NA, NB), reaching significantly higher concentrations (Tukey-HSD,  $\alpha < 0.05$ ) than in the controls (KA, KB) in both experiments. Consistent with the added nutrient concentration, the Chl-a peak was higher in WIN ( $30 \mu g l^{-1}$ ) than in SUM ( $3.6 \mu g l^{-1}$ ), while Chl-a in the controls remained below 3 and  $1 \mu g l^{-1}$  in WIN and SUM, respectively. The distribution patter of Chl-a was tightly linked to the abundance of picoeukaryotes (Sala *et al.* in preparation). Acidification resulted in slightly lower Chl-a concentrations towards the last days of the nutrient-enriched mesocosms of WIN, but in higher Chl-a concentrations during the Chl-a peak of SUM (Tukey-HSD,  $\alpha < 0.05$ ).

The initial bacterial abundance was significantly (Tukey-HSD,  $\alpha < 0.05$ ) higher in SUM (0.8 × 10<sup>6</sup> cells ml<sup>-1</sup>) than in WIN (0.5 × 10<sup>6</sup> cells ml<sup>-1</sup>) (Table 1). Cell numbers increased rapidly in the winter mesocosms (only 0–1 day after setting up the experiment), whereas there was a lag of 4–5 days before cell numbers started to increase in the summer experiment (data not shown). The highest cell abundances were found in winter (7.5  $\times$  10<sup>6</sup> cells ml<sup>-1</sup>), while maximum abundances in the summer experiment reached 1.6  $\times$  10<sup>6</sup> cells ml<sup>-1</sup>. Thus, bacterial abundance was higher in the experiments where Chl-a also showed higher concentrations. Acidification significantly affected bacterial abundance in the unamended controls only, particularly at the end of both experiments (Tukey-HSD,  $\alpha$  < 0.05; details in Sala *et al.* in preparation).

#### Responses of bacterioplankton community composition

At the class level, the summer and winter experiments began with a bacterial community structure dominated by Alphaproteobacteria (30-35%) and Cyanobacteria (10-23%), with lower contributions of Gammaproteobacteria (10-15%) and Flavobacteria (8-15%) (Fig. 1). Despite these similarities between experiments at the class level, at the level of specific OTUs we found pronounced differences between the initial communities of WIN (dominated by OTUs related to SAR11, Cyanobacteria, SAR86, other Gammaproteobacteria and Euryarchaeota) as compared to SUM (dominated by OTUs related to Cyanobacteria, SAR11, Blastopirellula, Glaciecola, Oleispira and other Rhodobacteraceae) (Fig. 2). At the end of the two experiments, all mesocosms (including the controls) went through a shift in community composition, coinciding with a decrease in the Shannon diversity index (Table 1), with Gammaproteobacteria, Alphaproteobacteria and Flavobacteria increasing in relative abundance (Fig. 1).

The response of the bacterial community to the treatments was analyzed by comparing the relative abundance of OTUs in each treatment at the end of the experiment with the corresponding relative abundance in the controls at the end of the experiment. We observed OTUs that preferentially responded to the nutrient enrichments in the two experiments by increasing in relative abundance (Table 2, S1 and S2, Supporting Information). Flavobacteria OTUs related to the genera Polaribacter and Tenacibaculum significantly increased with nutrients in WIN, along with Alphaproteobacteria OTUs related to the Rhodobacteraceae clade (Tukey-HSD,  $\alpha$  < 0.05). In contrast, Croceibacter (Flavobacteria) OTUs increased only without nutrient addition in WIN. The impact of acidification on bacterioplankton community composition only produced significant changes in the relative abundance of three OTUs (Table 2). Interestingly, these changes were found when pH reduction was combined with nutrient enrichment, causing increases of up to 10-fold in the relative abundance of OTUs related to Polaribacter (Flavobacteria), another Flavobacteriaceae and SAR86 (Gammaproteobacteria) (Table 2, S1 and S2, Supporting Information).

The above results were based on changes in relative abundance since we were interested in studying how different bacterial taxa became more or less important members of the community. As a complementary analysis, the total abundance of each bacterial OTU was also calculated taking into account the number of bacterial cells in each of the mesocosms at the time of sampling (Table S3, Supporting Information). A very similar pattern was obtained when the analysis was based on the relative or the total abundance of OTUs. The main difference was that, when looking at the total abundance, there were more bacterial taxa being positively affected in WIN by the combination of acidification plus nutrients, basically due to the higher bacterial abundance found in NA as compared to the other treatments at the end of the WIN experiment (Table 1).

## Response of the abundant, common and rare members of the bacterial community to perturbations

We defined as abundant the microbial components of the community representing  $\geq$ 1% relative abundance, and *rare* those with ≤0.1% relative abundance (Pedrós-Alió 2012). We then defined as common those microorganisms between 0.1 and 1% in relative abundance (i.e. those between abundant and rare). No significant differences were found in the number of OTUs in the different abundance fractions between the acidified and non-acidified treatments (Tukey-HSD,  $\alpha$  < 0.05). For this reason we pooled the data for the controls (KA, KB), and did the same for the nutrient treatments (NA, NB). Typical rankabundance distributions were found at the initial time of both experiments (Table 3), with few abundant OTUs (22 in WIN and 17 in SUM), followed by a long tail of remaining OTUs (205 in WIN [i.e. 94 common, 111 initially detected as rare and 133 'not initially detected members'] and 232 in SUM [i.e. 84 common, 148 initially detected as rare and 342 'not initially detected members']). At the onset of both experiments, all abundant OTUs together accounted for 66-67% of the relative abundance of the community, whereas the common and the rare OTUs represented 27-29 and 4-5%, respectively (Table 3).

At the end of the WIN experiment, originally abundant OTUs accounted for only <12% of the relative abundance in both control (11%) and nutrient-enriched conditions (6.8%) (Table 3). The initially common OTUs increased at the end of the WIN experiment from a relative abundance of 29.5% at time zero to 51.1 and 76.5% in the nutrient-enriched and control mesocosms, respectively (Table 3). OTUs initially detected as rare members of the community also increased their relative abundance until the end of WIN, particularly in response to nutrients (8.6 and 29.2% in control and nutrient-enriched mesocosms, respectively), but still represented a lower relative abundance than the initially common members (Table 3). Several OTUs that were not detected in the original community (due to their very low initial relative abundance) were found at the end of the experiments (see OTUs appearing after the gray tail ends in Figs 3 and 4) (i.e. 'not initially detected members') (Table 3). Four of these originally undetected OTUs strongly increased in relative abundance during the experiment, to the point that they became abundant at the end of WIN (a single OTU of each of the following members: Glaciecola, Polaribacter, SAR11 and other Flavobacteriales) (Table S4, Supporting Information).

At the end of the SUM experiment, in contrast to WIN, the originally abundant OTUs accounted for around half or more of the relative abundance of the bacteria (i.e. 65 and 49% in the control and nutrient conditions, respectively) (Table 3). The change in the relative abundance observed among the initially common and rare members was smaller at the end of SUM than in WIN, with increases confined to the nutrient-enriched mesocosms (from 27.5 to 38.1% and from 5.5 to 10.9% for common and rare, respectively). Only one of the not initially detected OTUs became abundant at the end of SUM (Oleispira).

After quantifying the number of OTUs that became abundant at the end of the two experiments, and identifying whether they were originally abundant, common or rare, we found that, unexpectedly, most of the OTUs that were abundant at the end of the experiments were also abundant, or at least common, in the original communities of both experiments (Figs 3C and D and 4C and D). This was mainly evident in the unamended controls, where the proportion of abundant OTUs at the end of the



Figure 1. Community composition at the Class level. Percentage of relative abundance of taxonomical classes at the initial time (TO) and at the end of the WIN (A) and SUM (B) experiments in duplicate mesocosms (1, 2). Euryarchaeota, Thaumarchaeota and Chlorophyta are not classes but they are included at the phylum level because most of their sequences could not be assigned to specific classes. Only those groups with a relative abundance >1% in any sample were included in the plot legend. KA: acidified control (no nutrient addition, but lowered pH), KB: basic control (no nutrient addition nor pH decrease), NA: acidified nutrient addition, NB: basic nutrients addition (no pH decrease).

experiments that were initially rare accounted for only 20 and 12% in WIN and SUM, respectively. In contrast, the proportion of rare OTUs becoming abundant in the nutrient-enriched meso-cosms was significantly higher (Tukey-HSD,  $\alpha < 0.05$ ) in WIN (52%) but not in SUM (17%). The proportion of abundant OTUs at the end of the experiments that were initially common was surprisingly high, accounting for around half of the responding OTUs in SUM (both in nutrients and controls) and in WIN (in the

## controls), and never less than 30% in any experiment or treatment (Figs 3C and D and 4C and D).

## DISCUSSION

We investigated the response of coastal Mediterranean Sea bacterioplankton communities to two anthropogenic perturbations, nutrient enrichment and/or acidification. Mesocosm



Figure 2. Community composition at the genus level. Percentage of relative abundance in the initial time (TO) and at the end of the WIN (A) and SUM (B) in duplicate mesocosms (1, 2). Some groups of sequences could not be characterized down to the genera level but were included in this figure as well because they were abundant (e.g. other Gammaproteobacteria, other Alphaproteobacteria, etc.). Only groups showing a relative abundance >1% in any sample were included in the plot legend. KA: acidified control (no nutrient addition, but lowered pH), KB: basic control (no nutrient addition nor pH decrease), NA: acidified nutrient addition, NB: basic nutrients addition (no pH decrease).

Table 1. Average ( $\pm$  SE) pH (total scale), chlorophyll-a concentration (Chl-a;  $\mu$ g l<sup>-1</sup>), bacterial abundance (BA;  $\times 10^6$  cells ml<sup>-1</sup>) and bacterial Shannon diversity indexes at the initial and final times of the winter (WIN) and summer (SUM) experiments in the different treatments.

itial Final
± 0.01 7.55 ± 0.0
± 0.04 0.56 ± 0.0
± 0.01 0.76 ± 0.1
$\pm 0.1$ 3.2 $\pm 0.3$
± 0.01 7.81 ± 0.0
$\pm 0.03$ 0.68 $\pm 0.04$
$\pm 0.01$ 0.55 $\pm 0.0$
$\pm 0.1$ 3.4 $\pm 0.9$
$\pm 0.01$ 7.59 $\pm 0.0$
$\pm 0.01$ 1.43 $\pm 0.1$
$\pm 0.01$ 1.28 $\pm 0.0$
$\pm 0.1$ 4.5 $\pm 0.3$
± 0.01 7.83 ± 0.0
$\pm 0.01$ 1.14 $\pm 0.1$
$\pm 0.01$ 1.60 $\pm 0.1$
;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;;

KA: acidified control (no nutrient addition, but lowered pH).

KB: basic control (no nutrient addition nor pH decrease).

NA: acidified nutrient addition.

NB: basic nutrients addition (no pH decrease).

Table 2. Affiliation of OTUs significantly (Tukey-HSD,  $\alpha < 0.05$ ) responding in relative abundance, positively (+) or negatively (–), to the nutrient addition (NUT), reduced pH (pH) and the combination of both (pH + NUT) in the winter (WIN) and summer (SUM) experiments. This was analyzed by comparing the relative abundance of OTUs in each treatment at the end of the experiment with the corresponding relative abundance in the control at the end of the experiment. We compared NB with KB to assess the effect of nutrients, KA with KB to evaluate the impact of acidification, and NA with KB to calculate the combined effect of NUT and pH.

	NUT		рН		pH + NUT	
	WIN	SUM	WIN	SUM	WIN	SUM
Polaribacter	+				+	
SAR86					+	
Tenacibaculum	+					
Croceibacter	-					
Uncharacterized Rhodobacteraceae		+				
Uncharacterized Flavobacteriaceae					+	

experiments in winter and summer were carried out to investigate the potential impact of these stressors on the diversity of bacterioplankton and the role of different components of bacterioplankton communities (i.e. abundant, common and rare members) in the community response to perturbations. The most remarkable result was that the bacteria most responsive to the treatments were the OTUs that were common already at the start of the experiment.

In the control mesocosms, similar community structures at the Class level were observed in both the WIN and the SUM experiments, with Gammaproteobacteria, Alphaproteobacteria and Flavobacteria becoming dominant at the end. However, at the OTU level, the most abundant OTUs in the controls at the end of the experiments were different between the two experiments. Incidentally, the observed increase in relative abundance of a *Glaciecola* OTU in the controls agrees with their blooming behavior observed in the natural seawater in Blanes Bay (Alonso-Sáez *et al.* 2007), where they are typically around 1% over the year but with an ability to drastically increase in summer, to reach a relative abundance of around 50%.

Also in the nutrient-enriched mesocosms, individual dominant OTUs changed over time in the two experiments, although at the Class level little differences were observed. This indicates the importance of the level of taxonomic resolution (i.e. 6000 sequences per sample allows detecting an organism that is 1/6000, thus around 0.016% of the community, or about 200 cells out of a million) necessary to uncover responses in bacterioplankton composition (Fig. 2). For instance, OTUs related to the genera Polaribacter (Flavobacteria), Tenacibaculum (Flavobacteria) and Rhodobacteraceae cluster (Alphaproteobacteria) bloomed after nutrient addition, whereas Croceibacter (Flavobacteria) preferred the unamended control. The positive responses to the nutrient-enriched conditions observed in this study are in agreement with the observed increase in relative abundance of a particular Polaribacter population (from ca. 3 to 27%) in response to a spring bloom in the German Bight of the North Sea (Teeling et al. 2012) and the increase found in the relative abundance of Rhodobacteriaceae in response to nutrient-induced phytoplankton blooms in a mesocosm experiment with water from the same location as in this study (Allers et al. 2007). Moreover, most of the members of the Tenacibaculum (in Latin meaning 'rodshaped bacterium that adheres to surfaces') genus seem to be related to high-nutrient habitats, like surfaces of marine organisms or particles (Suzuki et al. 2001). Consistent with our results, Croceibacter atlanticus was isolated using the high-throughput cultivation technique (Connon and Giovannoni 2002), designed for isolating strains adapted to highly oligotrophic ecosystems (e.g. open ocean seawater), indicating the preference of members of this genus to live under low-nutrient conditions.

In the current study, acidification provoked effects on different bacterioplankton members only when combined with

Table 3. Number of OTUs that were abundant (>1% relatively abundant), common (<1–0.1% relative abundance), rare (<0.1% relative abundance) or rare not detected at time zero, and proportion of relative abundance (%) explained by these OTUs at time zero (T = 0) and at the end of the experiment in the control (K = KA + KB) and nutrient-enriched (N = NA + NB) mesocosms.

	Groups	Initial relative abundance (%)	No. of OTUs	T = 0 (%)	K (%)	N (%)
WIN	Abundant	>1	22	66.3	11	6.8
	Common	0.1–1	94	29.5	76.5	51.1
	Rare	<0.1	111	4.2	8.6	29.2
	Rare; not initially detected	0	133	0	3.9	12.9
SUM	Abundant	>1	17	66.9	65	49.2
	Common	0.1–1	84	27.5	26.6	38.1
	Rare	<0.1	148	5.5	7.4	10.9
	Rare; not initially detected	0	342	0	3.7	6.8



Figure 3. Rank-abundance distribution of the OTUs at the initial time (in gray), and abundance of these same OTUs at the end of the experiment in the controls (blue) and in the mesocosms enriched in nutrients (red) in the WIN experiment. (A) and (B) are the same figure but with a downscaled y-axis. Inserts show that the proportion of OTUs becoming abundant at the end of the experiments that were originally rare (green,  $\leq 0.1\%$  relative abundance), common (purple, <1-0.1% relative abundance) and abundant (orange,  $\geq 1\%$  relative abundance) in the controls (C) and in the mesocosms enriched with nutrients (D) in the WIN experiment.

nutrient enrichment. In previous studies, shifts in marine bacterial community structure due to acidification have been reported (Allgaier *et al.* 2008; Thurber *et al.* 2009; Arnosti *et al.* 2011; Witt *et al.* 2011; Zhang *et al.* 2012; Maas *et al.* 2013). Other studies, however, report minimal pH effects on bacterial community composition (Allgaier *et al.* 2008; Tanaka *et al.* 2008; Newbold *et al.* 2012; Roy *et al.* 2013). Changes in the community composition of bacterioplankton in response to acidification were found in an experiments conducted in the Ross Sea (Maas *et al.* 2013), as well as in two studies conducted in a Norwegian fjord (Raunefjorden), where effects were noted on the whole bacterial community (Arnosti *et al.* 2011) or just the free-living bacteria (Allgaier *et al.* 2008). However, no specific taxa were identified as responding to acidification in those studies. An increase in the relative abundance of Bacteroidetes, Spirochaetes, Chlorobi and Cyanobacteria, and a decrease of Actinobacteria was found in a study of the metagenomic response of pH stressed coral holobionts (Thurber *et al.* 2009). The relative abundance of Bacteroidetes



Figure 4. Rank-abundance distribution of the OTUs at the initial time (in gray), and abundance of these same OTUs at the end of the experiment in the controls (blue) and in the mesocosms enriched with nutrients (red) in the SUM experiment. (A) and (B) are the same figure but with a downscaled y-axis. Inserts show the proportion of OTUs becoming abundant at the end of the experiments that were originally rare (green,  $\leq 0.1\%$  relative abundance), purple (yellow, <1-0.1% relative abundance) and abundant (orange,  $\geq 1\%$  relative abundance) in the controls (C) and in the mesocosms enriched with nutrients (D) in SUM.

(Flavobacteria) increased with reduced pH in biofilms from the Great Barrier Reef, whereas members of the Roseobacter clade decreased (Witt *et al.* 2011). Only Bacteroidetes were shown to respond to acidification (by decreasing in relative abundance) in a mesocosm experiment conducted in a fjord in Spitsbergen (Zhang *et al.* 2012). However, in another mesocosm experiment carried out in the same location, a negligible effect of ocean acidification on bacterial community structure was reported, with only minor effects on Gammaproteobacteria (Roy *et al.* 2013). These findings collectively suggest that reductions in pH do not lead to major changes in overall bacterioplankton community structure, although the abundance of particular taxa can be significantly affected.

It can be argued that observed slight effects of acidification on coastal bacterioplankton communities are in agreement with the considerably stronger natural variability in pH found in coastal ecosystems, with amplitudes of >0.3 units at scales ranging from diel to seasonal and decadal oscillations (Duarte et al. 2013). Not only extremophile bacteria but also bacteria living in environments where they primarily encounter neutral pH (e.g. pH around 7–8) have elaborate physiological mechanisms to maintain stable intracellular pH levels to enable adequate cellular functioning (Slonczewski et al. 2009). Although little studied in marine bacteria (Joint, Doney and Karl 2011; Teira et al. 2012), it is reasonable to assume that they can regulate internal pH levels to have adaptability to external pH fluctuations. The combined effect of acidification and nutrient addition on some OTUs belonging to SAR86 is noteworthy, since bacteria in this clade are among the most abundant uncultivated constituents of microbial assemblages in the surface ocean (Dupont *et al.* 2011; Molloy 2012). The observed synergistic effects of nutrient additions and acidification on these specific bacterial members highlight the importance of evaluating the combined effects of anthropogenic perturbations (e.g. acidification, eutrophication, warming) in order to better predict the impact of global change on marine bacterioplankton community composition and ecosystem functioning.

Determining the origin of the OTUs that became abundant at the end of the experiments, i.e. whether they belonged to the rare, the common or the abundant fractions of the original community, we found that the originally rare bacteria came to contribute more to the changes in bacterial community composition in the nutrient-enriched mesocosms in WIN than in any other mesocosms of the two experiments (Fig. 3). It should be noted that the enrichments in our two experiments were chosen to represent approximately an 8-fold increase in nutrients compared to averages for the months of February and July, respectively. Since the average for February is at the high end of

yearly values, the enrichment in the WIN experiment was relatively large in comparison to values naturally occurring in the Mediterranean Sea (to the point that the microbes in this sea do not encounter such values), although representative of concentrations in coastal upwelling areas. Thus, the magnitude of response of rare bacteria to enrichment in WIN may be a result of the magnitude of the nutrient enrichment in this experiment compared to SUM (i.e. four times higher). Alternatively, or rather as a complementary explanation, it could be that rare components of the bacterial community in winter are better adapted than corresponding members in the summer community at taking advantage of temporary nutrient pulses, allowing them to take more efficient advantage of the nutrient enrichment. Our results for the WIN nutrient-enriched treatment agree with the increase in abundance of rare members in response to organic carbon additions in a Baltic Sea experiment (Sjöstedt et al. 2012), and the occasional bloom of rare members observed in situ in the English Channel and the North Sea (Gilbert et al. 2011; Teeling et al. 2012). However, the proportion of rare populations becoming abundant in response to nutrient enrichments in the summer experiment was only 17% (Fig. 4). This, together with the low contribution of final abundant OTUs that were originally rare in all the unamended control experiments (12-20%), suggests that the initially abundant and common OTUs are the most prone to remain or to become even more abundant after the specific perturbations that we mimicked. Moreover, the 'common' OTUs (<0.1–1% relative abundance) constituted around  $\sim$ 50% of the abundant OTUs in most mesocosms at the end of two the experiments (except in the winter nutrient-enriched treatment). This highlights the common bacteria as important, but previously unrecognized, components for determining the responsiveness of bacterioplankton communities to perturbations in the marine environment.

Interestingly, we found that many OTUs that were abundant at the end of one experiment, but not in the in situ community of that same experiment, were actually abundant in the in situ community of the other experiment (e.g. OTUs of Polaribacter, Tenacibaculum, other Rhodobacteraceae, Glaciecola and SAR86) (Table S4, Supporting Information). This suggests that many of the responding OTUs are members that can be numerically important in the in situ assemblages during other times of the year. This finding remarks the preferential role of some specific main players in the seasonal changes of bacterioplankton communities, and suggests that these key OTUs are highly dynamic and can frequently change between being in the rare, the common or the abundant fraction of the community, depending on the season and/or the kind of perturbation. These results also bring to light the importance of repeating the same experimental design with different initial communities when studying community responses to different environmental stressors.

In summary, we show that the level of taxonomic resolution is important when analyzing the response of bacterial community structure to environmental disturbances. Interestingly, specific synergistic effects were found when acidification was combined with nutrient enrichment, selecting particular bacterial members that were not responding to acidification or nutrient enrichment alone. This pattern has implications for interpreting the impact of anthropogenic perturbations on marine ecosystem diversity and function. We also found that most of the OTUs that become abundant in response to disturbances were originally abundant or common, although the proportion of rare members becoming abundant could be relevant depending on the magnitude of the perturbation.

## SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

### ACKNOWLEDGEMENTS

We thank C. Cardelús, V. Balagué, L. Cros, J. Movilla and A. López-Sanz for efficient technical help in setting up the experiment and H. Sarmento and A. Gomes for flow cytometry. We would like to acknowledge the support and insightful comments of the reviewers, which clearly helped improving the overall merit of the manuscript.

### **FUNDING**

The experiments were funded by projects ACDC (CTM2009-08849, to EC and CP), STORM (CTM2009-09352/MAR, to CM and JMG) and ECOBAF (CTM2010-10462-E/MAR, to JMG). FB was supported by an University of Otago Research Grant (UORG). The diversity work was supported by projects from the European Science Foundation (EuroEEFG project MOCA) and the Swedish Research Council to JP.

Conflict of interest. None declared.

## REFERENCES

- Allers E, Gómez-Consarnau L, Pinhassi J, et al. Response of Alteromonadaceae and Rhodobacteriaceae to glucose and phosphorus manipulation in marine mesocosms. Environ Microbiol 2007;9:2417–29.
- Allgaier M, Riebesell U, Vogt M, et al. Coupling of heterotrophic bacteria to phytoplankton bloom development at different pCO 2 levels: a mesocosm study. Biogeosci Discuss 2008;5: 317–59.
- Alonso-Sáez L, Balague V, Sà EL, et al. Seasonality in bacterial diversity in north-west Mediterranean coastal waters: assessment through clone libraries, fingerprinting and FISH. FEMS Microbiol Ecol 2007;60:98–112.
- Arnosti C, Grossart H-P, Mühling M, et al. Dynamics of extracellular enzyme activities in seawater under changed atmospheric pCO 2: a mesocosm investigation. Aquat Microb Ecol 2011;64:285–98.
- Boström KH, Simu K, Hagström A, et al. Optimization of DNA extraction for quantitative marine bacterioplankton community analysis. *Limnol Oceanogr-Meth* 2004;2: 365–73.
- Connon SA, Giovannoni SJ. High-throughput methods for culturing microorganisms in very-low-nutrient media yield diverse new marine isolates. *Appl Environ Microb* 2002;**68**:3878–85.
- Dickson AG, Sabine CL, Christian JR (eds). Guide to Best Practices for Ocean CO<sub>2</sub> Measurements: PICES Special Publication 3. Sidney, Canada: PICES, 2007.
- Dowd SE, Sun Y, Wolcott RD, et al. Bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) for microbiome studies: bacterial diversity in the ileum of newly weaned Salmonellainfected pigs. *Foodborne Pathog Dis* 2008;5:459–72.
- Duarte CM, Hendriks IE, Moore TS, et al. Is ocean acidification an open-ocean syndrome? Understanding anthropogenic impacts on seawater pH. Estuar Coast 2013;**36**: 221–36.
- Dupont CL, Rusch DB, Yooseph S, et al. Genomic insights to SAR86, an abundant and uncultivated marine bacterial lineage. ISME J 2011;6:1186–99.

- Edgar RC. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 2010;**26**:2460–1.
- Egge E, Bittner L, Andersen T, et al. 454 pyrosequencing to describe microbial eukaryotic community composition, diversity and relative abundance: a test for marine haptophytes. PloS One 2013;8:e74371.
- Epstein SS. Microbial awakenings. Nature 2009;457:1083-3.
- Ferguson RL, Buckley EN, Palumbo AV. Response of marine bacterioplankton to differential filtration and confinement. Appl Environ Microb 1984;47:49–55.
- Fierer N, Hamady M, Lauber CL, *et al*. The influence of sex, handedness, and washing on the diversity of hand surface bacteria. P Natl Acad Sci USA 2008;**105**:17994.
- Galand PE, Casamayor EO, Kirchman DL, *et al*. Ecology of the rare microbial biosphere of the Arctic Ocean. P Natl Acad Sci USA 2009;**106**:22427–32.
- Gilbert JA, Steele JA, Caporaso JG, et al. Defining seasonal marine microbial community dynamics. ISME J 2011;6:298–308.
- Hamady M, Walker JJ, Harris JK, et al. Error-correcting barcoded primers for pyrosequencing hundreds of samples in multiplex. Nat Methods 2008;5:235–7.
- Howarth RW, Marino R. Nitrogen as the limiting nutrient for eutrophication in coastal marine ecosystems: evolving views over three decades. *Limnol Oceanogr* 2006;**51**: 364–76.
- Joint I, Doney SC, Karl DM. Will ocean acidification affect marine microbes?. ISME J 2011;5:1–7.
- Kunin V, Engelbrektson A, Ochman H, et al. Wrinkles in the rare biosphere: pyrosequencing errors can lead to artificial inflation of diversity estimates. *Environ Microbiol* 2010;**12**: 118–23.
- Lauber CL, Hamady M, Knight R, et al. Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale. Appl Environ Microb 2009;75:5111–20.
- Lauro FM, McDougald D, Thomas T, et al. The genomic basis of trophic strategy in marine bacteria. P Natl Acad Sci USA 2009;106:15527–33.
- Lennon JT, Jones SE. Microbial seed banks: the ecological and evolutionary implications of dormancy. Nat *Rev Microbiol* 2011;**9**:119–30.
- Lindh MV, Riemann L, Baltar F, et al. Consequences of increased temperature and acidification on bacterioplankton community composition during a mesocosm spring bloom in the Baltic Sea. Environ Microbiol Rep 2013;5:252–62.
- Lundin D, Severin I, Logue JB, et al. Which sequencing depth is sufficient to describe patterns in bacterial  $\alpha$ -and  $\beta$ -diversity? Environ Microbiol Rep 2012;4:367–72.
- Maas EW, Law CS, Hall JA, et al. Effect of ocean acidification on bacterial abundance, activity and diversity in the Ross Sea, Antarctica. Aquat Microb Ecol 2013;**70**:1–15.
- Medinger R, Nolte V, Pandey RV, et al. Diversity in a hidden world: potential and limitation of next-generation sequencing for surveys of molecular diversity of eukaryotic microorganisms. Mol Ecol 2010;**19**:32–40.
- Molloy S. Marine microbiology: SAR86: streamlined for success. Nat Rev Microbiol 2012;**10**:82–3.
- Newbold LK, Oliver AE, Booth T, et al. The response of marine picoplankton to ocean acidification. Environ Microbiol 2012;14:2293–307.
- Pedrós-Alio C. Marine microbial diversity: can it be determined? Trends Microbiol 2006;**14**:257–63.
- Pedrós-Alio C. Dipping into the rare biosphere. Science 2007;**315**:192–3.

- Pedrós-Alió C. The rare bacterial biosphere. Annu Rev Mar Sci 2012;4:449–66.
- Pommier T, Canbäck B, Riemann L, et al. Global patterns of diversity and community structure in marine bacterioplankton. Mol Ecol 2007;**16**:867–80.
- Price MN, Dehal PS, Arkin AP. FastTree: computing large minimum evolution trees with profiles instead of a distance matrix. Mol Biol Evol 2009;**26**:1641–50.
- Ray JL, Töpper B, An S, et al. Effect of increased pCO2 on bacterial assemblage shifts in response to glucose addition in Fram Strait seawater mesocosms. FEMS Microbiol Ecol 2012;82: 713–23.
- Roy A-S, Gibbons S, Schunck H, et al. Ocean acidification shows negligible impacts on high-latitude bacterial community structure in coastal pelagic mesocosms. *Biogeosciences* 2013;10:555–66.
- Sandaa RA, Gómez-Consarnau L, Pinhassi J, et al. Viral control of bacterial biodiversity—evidence from a nutrientenriched marine mesocosm experiment. Environ Microbiol 2009;11:2585–97.
- Sjöstedt J, Koch-Schmidt P, Pontarp M, et al. Recruitment of members from the rare biosphere of marine bacterioplankton communities after an environmental disturbance. Appl Environ Microb 2012;**78**:1361–9.
- Slonczewski JL, Fujisawa M, Dopson M, et al. Cytoplasmic pH measurement and homeostasis in bacteria and archaea. Adv Microb Physiol 2009;55:1–317.
- Sogin ML, Morrison HG, Huber JA, et al. Microbial diversity in the deep sea and the under-explored 'rare biosphere'. P Natl Acad Sci USA 2006;**103**:12115–20.
- Stocker TF, Qin D, Plattner G-K, et al. Climate change 2013: the physical science basis. In: Intergovernmental Panel on Climate Change, Working Group I Contribution to the IPCC Fifth Assessment Report (AR5). New York: Cambridge University Press, 2013.
- Suzuki M, Nakagawa Y, Harayama S, et al. Phylogenetic analysis and taxonomic study of marine Cytophaga-like bacteria: proposal for Tenacibaculum gen. nov. with Tenacibaculum maritimum comb. nov. and Tenacibaculum ovolyticum comb. nov., and description of Tenacibaculum mesophilum sp. nov. and Tenacibaculum amylolyticum sp. nov. Int J Syst Evol Mic 2001;51:1639–52.
- Tanaka T, Thingstad T, Lovdal T, *et al.* Availability of phosphate for phytoplankton and bacteria and of labile organic carbon for bacteria at different pCO2 levels in a mesocosm study. *Biogeosciences* 2008;5:669–78.
- Tedersoo L, Nilsson RH, Abarenkov K, et al. 454 Pyrosequencing and Sanger sequencing of tropical mycorrhizal fungi provide similar results but reveal substantial methodological biases. *New* Phytol 2010;**188**:291–301.
- Teeling H, Fuchs BM, Becher D, et al. Substrate-controlled succession of marine bacterioplankton populations induced by a phytoplankton bloom. *Science* 2012;**336**: 608–11.
- Teira E, Fernández A, Álvarez-Salgado XA, et al. Response of two marine bacterial isolates to high CO2 concentration. Mar Ecol-Prog Ser 2012;**453**:27–36.
- Teira E, Lekunberri I, Gasol JM, et al. Dynamics of the hydrocarbon-degrading Cycloclasticus bacteria during mesocosm-simulated oil spills. Environ Microbiol 2007;9:2551– 62.
- Thurber RV, Willner-Hall D, Rodriguez-Mueller B, et al. Metagenomic analysis of stressed coral holobionts. Environ Microbiol 2009;11:2148–63.

- Wang Q, Garrity GM, Tiedje JM, et al. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microb 2007;**73**:5261–7.
- Witt V, Wild C, Anthony K, et al. Effects of ocean acidification on microbial community composition of, and oxygen fluxes

through, biofilms from the Great Barrier Reef. Environ Microbiol 2011;**13**:2976–89.

Zhang R, Xia X, Lau S, et al. Response of bacterioplankton community structure to an artificial gradient of pCO 2 in the Arctic Ocean. *Biogeosci Discuss* 2012;**9**:10645–68.