Experimental assessment of marine bacterial respiration

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ABSTRACT: We present a systematic experimental assessment of the effect of pre-incubation filtration procedures on marine bacterial respiration (BR) measurements. The *in vivo* electron transport system (ETS) method, which enables measurements of BR using short incubation times (~1 h) and without the requirement of pre-incubation size-fractionation procedures, was employed in 20 experiments from 2 different ecosystems: the NW Iberian Peninsula shelf and shelf-break, and the North Pacific Subtropical Gyre. BR was determined in both pre-incubation size-fractionated filtered (PF) and pre-incubation unfiltered (PU) treatments. Additionally, the effect of incubation time (up to 24 h) on BR, community respiration (CR), bacterial production (BP), and picoplankton community composition was assessed in 6 of the experiments; the standard oxygen consumption method (i.e. Winkler) was also applied in PF treatments. The mean contribution of BR to total CR (%BR) obtained with the *in vivo* ETS method in PU and short-time incubated samples was $31 \pm 4\%$ (mean $\pm$ SE; $n = 20$). PF procedures increased BR by $264 \pm 46\%$ ($n = 20$). This overestimation increased with incubation time. The %BR in PF 24 h-incubated samples was $>100\%$ using either the *in vivo* ETS method (%BR = $109 \pm 31\%$, $n = 6$) or the Winkler method (%BR = $185 \pm 34\%$, $n = 6$). By contrast, incubation time did not significantly affect BR or CR rates in the PU experiments. Metabolic changes during extended incubations following PF coincided with a significant increase in the proportion of very high nucleic acid content bacteria to total heterotrophic bacteria. In this study, PF combined with extended incubation times resulted in an overestimation of BR and %BR of ~300% and an underestimation of bacterial growth efficiency of ~50% compared to PU, short-incubated samples. These results may partially reconcile bacterial carbon consumption assessments and estimates of organic carbon flow in oligotrophic waters.

KEY WORDS: Bacterial respiration · Filtration · Incubation time · Bacterial growth efficiency

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INTRODUCTION

Bacterioplankton play essential roles in energy and matter flow of planktonic food webs as a consequence of their significant biomass, efficient nutrient uptake, and large growth potential (Azam et al. 1983). Overall, $>50\%$ of the organic carbon synthesized by marine primary producers is believed to be channelled through bacterioplankton (Azam et al. 1983). Heterotrophic bacteria (HB) fulfill 2 major functions in the

oceanic carbon cycle. They utilize dissolved organic carbon (DOC) for biomass production (Azam 1998, Ducklow 2000), but, at the same time, convert a large part of this DOC into CO₂ through respiration (del Giorgio et al. 1997). Bacterial growth efficiency (BGE) is the amount of new bacterial carbon produced per unit of organic carbon substrate consumed, and is calculated using bacterial production (BP) and respiration (BR) data, i.e. $BP/(BP + BR)$ (del Giorgio & Cole 1998). Considering that BR commonly accounts for a

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major fraction of the total bacterial carbon demand (del Giorgio & Cole 1998), it represents a master variable when trying to unravel carbon budgets in marine microbial ecology (Jahnke & Craven 1995, del Giorgio & Cole 1998, Ducklow 2000).

High contributions of BR to total microbial community respiration (CR) have been reported (i.e. up to 90% or greater), especially in oligotrophic ecosystems (del Giorgio et al. 1997, Biddanda et al. 2001, Alonso-Sáez et al. 2007, Robinson 2008, Aranguren-Gassis et al. 2012), even though bacterial biomass contribution to total microbial biomass is much lower (Li & Harrison 2001, Morán et al. 2004, 2007, Marañón et al. 2007). A contemporary scientific controversy exists regarding the metabolic balance and carbon recycling in oligotrophic waters, since bacterial carbon consumption frequently surpasses estimates of primary production and organic carbon flow (del Giorgio & Cole 2000, Hoppe et al. 2006, Carlson et al. 2007, Duarte et al. 2013, Ducklow & Doney 2013, Williams et al. 2013). The scarcity of BR measurements and BGE calculations is considered to be one of the main factors limiting our understanding of carbon flow in the open ocean (Morán et al. 2007, Gasol et al. 2008, Robinson 2008).

A major difficulty when measuring BR with standard methodologies (i.e. direct oxygen consumption measurements) is the need to separate bacteria from the other components of the microbial plankton community using pre-incubation size fractionation (Robinson 2008). Potential filtration artifacts including removal of organic matter supplies (i.e. separation from autotrophic communities), separation from competitors and predators (i.e. phytoplankton and larger heterotrophs) (Fuhrman & Bell 1985, Hopkinson et al. 1989, Gasol & Morán 1999, Litchman et al. 2004, Robinson 2008), supply of organic carbon from cell breakage during filtration (Fuhrman & Bell 1985, Gasol & Morán 1999, Kiene & Linn 1999), or release of particle-attached bacteria that may undergo rapid growth during incubation, can all influence BR estimations (Pomeroy et al. 1994, Gasol & Morán 1999, Gattuso et al. 2002, Gasol et al. 2008). Microbial communities from oligotrophic environments may be more sensitive to pre-incubation filtration treatment (Gasol & Morán 1999) since disruption of plankton community linkages will be more detrimental due to the tight coupling that exists between HB and both autotrophs and predators in these environments (Azam et al. 1983, Cotner & Biddanda 2002). Gasol & Morán (1999) showed that pre-incubation filtration procedures were associated with decreasing picoplankton abundances, increasing cell-specific activ-

ity, and, in general, with changes in picoplankton community structure. Gattuso et al. (2002) also described changes in bacterial community composition and decreases of bacterial abundance, cell volume, and biomass in pre-incubation size-fractionated (PF) samples compared to pre-incubation unfiltered (PU) freshwater samples. The use of pre-incubation filtration experimental procedures is very likely to result in an overestimation of BR rates (Morán et al. 2007, Robinson 2008, Aranguren-Gassis et al. 2012).

Experimental artifacts associated with extended incubation times are also possible when BR is estimated by the direct oxygen consumption (i.e. Winkler) method. Thus, several studies have demonstrated the importance of incubation time on HB stocks and/or activity measurements in both PF (Gasol & Morán 1999, Gattuso et al. 2002, Briand et al. 2004) and PU samples (Pomeroy et al. 1994, Gasol & Morán 1999, Sherr et al. 1999, Gattuso et al. 2002, Calvo-Díaz et al. 2011).

To overcome these methodological limitations, a new approach, the *in vivo* electron transport system (ETS) activity method (Martínez-García et al. 2009), enables relatively short-term (1 to 4 h) measurements of BR without pre-incubation filtration. A recent study by Aranguren-Gassis et al. (2012) compared the bacterial contribution to CR rates (%BR) from 2 independent datasets measured using: (1) standard Winkler procedures (using PF and extended incubation times), and (2) the *in vivo* ETS method (using PU samples and short-term incubations). Their data analysis suggested that %BR might be overestimated by the Winkler oxygen consumption method, especially in oligotrophic areas. However, the role of pre-incubation filtration and/or extended incubation times as potential artifacts were not experimentally tested in that work, since data of oxygen consumption and *in vivo* ETS activity measurements were compared from 2 separate sets of samples compiled in the same areas.

To evaluate how classical methods could affect BR estimates, we experimentally tested the effect of pre-incubation filtration on BR using the *in vivo* ETS method (Martínez-García et al. 2009) in 20 experiments performed in 2 different areas: the mesotrophic NW Iberian Peninsula shelf/shelf-break and the oligotrophic North Pacific Subtropical Gyre. The effect of incubation time on BR in both PF and PU samples was also examined in 6 of those experiments. In these experiments, BR in PF samples was also measured by the Winkler method. Our results indicate an overestimation of BR rates in PF, long-incubated samples compared to PU, short-incubated samples.

MATERIALS AND METHODS

Study sites and sampling

Nine experiments were performed in on-shelf and shelf-break waters of the NW Iberian Peninsula: 1 experiment in April 2007 on board the RV 'Mytilus', and 8 experiments in July 2008 on board the RV 'Sarmiento de Gamboa' (hereinafter 'Atlantic experiments') (Fig. 1). PF and PU samples for BR measurements were taken in the Atlantic experiments. Another 11 experiments were performed in August 2010 in the North Pacific Subtropical Gyre in the vicinity of Stn ALOHA (22° 45' N, 158° 00' W) (Fig. 1) on board the RV 'Kilo Moana' (hereinafter 'Pacific experiments'). PF and PU samples for BR and BP and also for picoplankton abundance measurements were taken in the Pacific experiments. Incubation time assays and BR and CR measurements using oxygen consumption were also performed in the Pacific experiments.

Euphotic-zone vertical profiles of temperature, salinity, and *in situ* fluorescence were obtained with an SBE 911 CTD and a Seatech fluorometer attached to a rosette water sampler. Seawater samples (5 to 75 m depth) were collected in 12 and 15 l PVC bottles (in the Pacific and Atlantic experiments, respectively) attached to the CTD rosette sampling system. Location of the sampled stations as well as date, depth, temperature, and chlorophyll *a* (chl *a*) concentrations of the water samples collected for the experiments are presented in Table 1.

Pre-incubation filtration procedures

Size-fractionated filtration of seawater samples was performed immediately after sampling from CTD water bottles. All carboys, bottles, tubing, and filtration devices were washed in 10% HCl and rinsed with ultrapure water (Milli-Q) prior to each experiment. In the Atlantic experiments, vacuum (<100 mm Hg) filtration through 47 mm diameter, 0.8 µm porosity poly-

carbonate filters (Millipore) was employed. Paired, PU samples were also used in all experiments for comparison with PF samples. In 6 of the Pacific experiments (incubation time experiments, Expts Pacific 15 to 20), PF and PU seawater samples were incubated in the dark and under *in situ* temperature conditions in a shipboard surface seawater flow-through incubator and additionally sampled after 6, 12, and 24 h. Due to the large volume required for these Pacific experiments, seawater for rate determinations was collected into 20 l carboys, and pre-incubation filtration was performed using a peri-

Fig. 1. (A) NE Atlantic Ocean with locations (●) of the Atlantic experiments carried out on the coastal shelf of the NW Iberian Peninsula, and (B) North Pacific Subtropical Gyre with locations (■) of the Pacific experiments in the vicinity of Stn ALOHA (22° 45' N, 158° 00' W)

Table 1. Locations and metadata for the experiments

Expt	Date (mm/dd/yy)	Sampling location	Depth (m)	Temp. (°C)	Chl <i>a</i> (µg l ⁻¹)
Atlantic 1	07/09/08	44° 26' N, 9° 10' W	12	17.9	0.19
Atlantic 2	07/09/08	44° 26' N, 9° 10' W	40	17.3	0.18
Atlantic 3	07/10/08	44° 10' N, 8° 50' W	5	18.0	0.21
Atlantic 4	07/10/08	44° 10' N, 8° 50' W	30	17.3	0.43
Atlantic 5	07/08/08	44° 0' N, 8° 24' W	15	17.7	0.17
Atlantic 6	07/08/08	44° 0' N, 8° 24' W	50	14.9	0.38
Atlantic 7	07/13/08	43° 39' N, 8° 20' W	40	15.5	2.29
Atlantic 8	07/13/08	43° 39' N, 8° 20' W	50	15.1	2.76
Atlantic 9	04/27/07	42° 8' N, 8° 57' W	1	14.4	2.43
Pacific 10	08/22/10	22° 48' N, 158° 2' W	10	25.5	0.11
Pacific 11	08/23/10	23° 59' N, 158° 0' W	50	25.5	0.16
Pacific 12	08/25/10	24° 50' N, 160° 43' W	12	26.0	0.20
Pacific 13	08/26/10	24° 40' N, 160° 51' W	37	26.0	0.25
Pacific 14	08/27/10	25° 30' N, 160° 40' W	10	25.9	0.45
Pacific 15	08/21/10	22° 45' N, 158° 0' W	5	25.5	0.18
Pacific 16	08/22/10	22° 48' N, 158° 2' W	75	20.4	0.12
Pacific 17	08/23/10	23° 59' N, 158° 0' W	10	25.6	0.09
Pacific 18	08/25/10	24° 50' N, 160° 43' W	37	25.3	0.51
Pacific 19	08/26/10	24° 40' N, 160° 51' W	10	26.0	0.23
Pacific 20	08/27/10	25° 30' N, 160° 40' W	40	25.0	0.16

staltic pump and sequentially filtering through rinsed 47 mm diameter, 5 μm and 0.8 μm porosity polycarbonate filters (Millipore) that were changed periodically (i.e. every 2 to 3 l of filtration) in order to minimize clogging, which is more likely to occur when large volumes of water are filtered (Lee et al. 1995, Gasol & Morán 1999), and to ensure a constant flow rate of ca. 3 to 4 l h⁻¹.

BR in PF and PU samples, and BP and CR in PU samples were measured after water collection in all experiments (Expts 1 to 20) using the *in vivo* ETS method. Additionally, in the Pacific experiments, BP in PF samples, and picoplankton community composition in both PU and PF samples were measured after sampling. In the incubation time experiments (Expts Pacific 15 to 20), the effect of incubation time on CR, BR, BP, and picoplankton community composition was estimated by subsampling the same PF and PU water after 6, 12, and 24 h of confinement. CR was estimated by the Winkler oxygen consumption method in addition to the INT method (see next section, 'In vivo ETS') in all experiments except for Expt Atlantic 9, and BR was estimated by the Winkler oxygen consumption method in addition to the INT method in the incubation time experiments (Expts Pacific 15 to 20).

Since *Prochlorococcus* and *Synechococcus* cells are often recovered after pre-incubation filtration (Gasol et al. 2008), BR and BP cannot be exclusively assigned to HB, but they rather account for prokaryotic respiration and production. Also, since BR and BP are measured in the fraction smaller than 0.8 μm , they do not account for cells attached to bigger particles, but rather for the free-living fraction.

In vivo ETS

In vivo ETS activity rates were measured by the *in vivo* INT method (Martínez-García et al. 2009) and used to estimate CR and BR. The ETS method is based on the reduction of the tetrazolium salt 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT) to INT-formazan (INT-F) by ETS dehydrogenase enzymes. A previous version of the INT method measured *in vitro*, rather than *in vivo*, ETS activity by extraction of enzymes and measurements of maximum rates under saturating substrate conditions (Packard 1971). *In vitro* ETS activity was demonstrated to follow the same pattern as oxygen consumption (Packard & Williams 1981), and a significant correlation between ETS activity and oxygen has been widely reported (see review in Arístegui &

Montero 1995). However, due to variations in the availability of natural substrates, maximum potential respiration rates provided by the *in vitro* ETS assay are probably not achieved under *in situ* conditions (Robinson & Williams 2005) even after application of temperature corrections. There are a few examples in the literature of *in vivo* (i.e. using living cells) ETS activity measurements (del Giorgio et al. 1997, Posch et al. 1997, Sherr et al. 1999, Sieracki et al. 1999). *In vivo* INT reduction rates have been shown to be highly correlated with microbial oxygen consumption in soil and freshwater sediments (Trevors et al. 1982, Trevors 1984) and in marine surface waters (Martínez-García et al. 2009). *In vivo* ETS activity has also been demonstrated to be significantly correlated with CO₂ production in mixed microbial communities (Cook & Garland 1997). Martínez-García et al. (2009) demonstrated that the *in vivo* INT method is suitable to estimate respiratory rates of marine microplankton communities. An R/ETS ratio (where R is respiration) of 12.8 was empirically estimated for nonaxenic cultures of *Isochrysis galbana* with different bacterial to algal biomass ratios using the same incubation time for measuring oxygen consumption and INT-F formation (Martínez-García et al. 2009). A large set (n = 72) of field experiments using a wide range of marine microbial planktonic communities (sampled in the oligotrophic North Atlantic Subtropical Gyre and in the mesotrophic/eutrophic NW Iberian Peninsula) was also performed in order to evaluate if the oxygen consumption to INT reduction ratio (R/ETS) obtained with nonaxenic algal cultures in the lab would also hold for different natural samples (Martínez-García et al. 2009). In those field experiments, the incubation time was not the same for both techniques (Martínez-García et al. 2009). The R/ETS ratio obtained for natural waters was not significantly different from that derived from algal cultures, and a constant relationship between oxygen consumption and INT-F formation rates was found over a range spanning 2 orders of magnitude of respiration rates (0.11 to 8.3 $\mu\text{M O}_2 \text{ d}^{-1}$) (Martínez-García et al. 2009). Although the R/ETS ratio may vary between different microbial communities and this should be taken into account when predicting BR and CR from *in vivo* ETS activity data, the use of a mean ratio should not introduce large uncertainty in the estimation of oxygen consumption rates.

Samples (2 killed controls and 3 live 250 ml replicates) were inoculated with a sterile solution of INT and incubated in the dark during 2.5 to 4 h or 1 h (Atlantic and Pacific experiments, respectively) in shipboard surface seawater flow-through incubators.

Time-course experiments were performed in both environments to determine the optimal incubation time for ETS activity measurements using the *in vivo* INT reduction method (Martínez-García et al. 2009). After incubation, samples were fixed by adding formaldehyde (2% v/v final concentration) and sequentially filtered through 0.8 and 0.2 μm pore size polycarbonate filters. The reduced INT-F was extracted from the filters using propanol and its concentration determined colorimetrically on a Beckman model DU640 and an Ultra Violet-2401 PC Shimadzu spectrophotometer (Atlantic and Pacific experiments, respectively). In order to transform ETS activity into oxygen consumption, an R/ETS ratio of 12.8 was used (Martínez-García et al. 2009). In order to transform oxygen consumption into carbon utilization, a respiratory quotient (RQ) of 0.89 (Williams & del Giorgio 2005) was used.

Winkler method

Determinations of respiration were based on dark-bottle oxygen consumption measurements (Carritt & Carpenter 1966, Williams et al. 2004). Respiration was estimated as the total oxygen consumption in the dark bottle incubation relative to a time zero. Pre-incubation filtration in these experiments was performed using a peristaltic pump as described above (section 'Pre-incubation filtration procedures'). Seawater for rate determinations was collected into 20 l carboys, and 12 replicate 125 ml borosilicate flasks were subsampled from the carboys. Six replicate bottles were immediately fixed with Winkler oxygen reagents for initial oxygen concentration measurements, and 6 additional replicate bottles were filled and incubated in opaque containers in the dark for 24 h in shipboard surface seawater flow-through incubators. Rates are reported as changes in oxygen concentration per hour and converted into carbon units by applying an RQ of 0.89 (Williams & del Giorgio 2005).

Leucine incorporation rates

The [^3H]-leucine incorporation method (Kirchman et al. 1985), modified as described by Smith & Azam (1992), was used to determine leucine (Leu) incorporation rates as an estimation of BP. Samples were dark-incubated for 1 h in the same incubation chamber as the experimental bottles. To estimate BP, a theoretical Leu to carbon conversion factor of 1.5 kg

C mol $^{-1}$ Leu was used (Kirchman 2001). It is important to note here that as a theoretical carbon conversion factor was used, our estimations of BP rates should be taken with caution since they might be overestimated due to intracellular protein turnover and Leu respiration in the cell (Alonso-Sáez et al. 2007). It has been demonstrated that bacteria exhibit a significant protein turnover and also that up to 80 % of Leu taken up by cells from off-shore areas may be respired, and therefore, BP may be overestimated unless conversion factors are empirically calculated (Alonso-Sáez et al. 2007). Since the aim of the present study was to investigate BR measurement artifacts (and their effects in BGE calculations), we use a common theoretical conversion factor for all our BP estimates, although it is important to note that the absolute value of BGE may be affected by the Leu to carbon conversion factor used.

Chl *a*

Chl *a* concentrations were measured in 250 ml water samples that were filtered through 0.2 μm polycarbonate and GF/F filters in the Atlantic and Pacific experiments, respectively. In the Atlantic experiments, after extraction with 90 % acetone at 4°C overnight in the dark, chl *a* fluorescence was determined with a TD-700 Turner Designs fluorometer calibrated with pure chl *a* (Welschmeyer 1994). In the Pacific experiments, chl *a* concentrations were calculated from CTD fluorescence profiles calibrated with extracted chl *a* from natural samples. These data were obtained after extraction with 90 % acetone at 4°C during 7 to 10 d in the dark, and chl *a* fluorescence was determined with a 10-AU Turner Designs fluorometer calibrated with pure chl *a*.

Flow cytometry

Samples (2 ml) were preserved with paraformaldehyde (1.5% v/v final concentration), flash-frozen in liquid nitrogen and preserved at -80°C until analyzed. The abundances of *Synechococcus*, *Prochlorococcus*, picoeukaryotes, and HB were determined using a Cytopeia Influx Mariner flow cytometer with 488 nm and 457 nm solid-state lasers for excitation, triggering on forward scatter. Enumeration and fluorescence data were analyzed using FlowJo software (Tree Star, www.flowjo.com). Prior to analysis, cells were stained with 2.5 mM SYBR Green I DNA fluorochrome. Picoplankton groups were identified on

the basis of their fluorescence and light side scatter (SSC) signatures. *Synechococcus* and *Prochlorococcus* cyanobacteria and pigmented eukaryotic cells were identified in plots of SSC versus red fluorescence (692 nm) and orange fluorescence (580 nm) in unstained samples, whereas 3 groups of HB were distinguished by their green fluorescence (530 nm) after SYBR Green I staining: very high (vHNA), high (HNA), and low (LNA) nucleic acid content bacteria.

Data analysis

The 24 h-averaged rates and standing stocks were calculated in the incubation time experiments in PF and PU samples (PF_{24ave} and PU_{24ave} , respectively) in order to compare them with the initial values (PF_0 and PU_0). For example, in order to calculate a 24 h-averaged rate (Eq. 1) in a pre-incubation filtered sample, calculations would be made as follows:

$$PF_{24ave} = \frac{\text{average } [PF_0, PF_6, PF_{12}, \text{average } (PF_{12}, PF_{24}), PF_{24}]}{24} \quad (1)$$

where PF_0 , PF_6 , PF_{12} , and PF_{24} correspond to the PF value at 0, 6, 12, and 24 h, respectively, after confinement.

Statistical analysis

Repeated-measures ANOVA (RM-ANOVA) was conducted to assess time (within-subject factor), treatment (between-subjects factor, pre-incubation filtration), and experiment (between-subjects factor, sampling location) effects. Log transformation was applied to those datasets that did not fit a normal distribution (Kolmogorov-Smirnov test). Even after log or arcsine data transformation, the homogeneity of covariance matrices failed for some datasets. For the latter case, we applied the Huynh-Feldt adjustment to correct p-values (Scheiner & Gurevitch 1993). Analyses using *t*-test and paired *t*-test were performed in order to compare Atlantic and Pacific experiments and PF and PU samples at different sampling points, respectively, and a significance level of $p < 0.05$ was used (Sokal & Rohlf 1995). Standard errors were calculated when appropriate.

RESULTS AND DISCUSSION

Initial conditions

BR, CR, BP, and BGE in initial pre-incubation unfiltered samples (PU_0) are presented in Table 2. The corresponding contributions of BR to CR (%BR- PU_0) are represented in Fig. 2A,B. BR and %BR were significantly higher (*t*-test, $p < 0.01$, $n = 20$) in the Pacific than in the Atlantic experiments. BP was not significantly different (*t*-test, $p > 0.05$, $n = 20$) but BGE was significantly higher ($p < 0.05$, $n = 20$) in the Atlantic than in the Pacific experiments (Table 2). Initial CR measured by the *in vivo* ETS method ($CR_0 = 0.55 \pm 0.05 \mu\text{g C l}^{-1} \text{ h}^{-1}$; Table 2) were not significantly different (paired *t*-test, $p > 0.05$, $n = 19$) from those measured by the 24 h incubation Winkler method in the same samples ($0.50 \pm 0.07 \mu\text{g C l}^{-1} \text{ h}^{-1}$).

HB abundance in the Pacific experiments was 5.4×10^5 cells ml^{-1} and was dominated by HNA and LNA bacteria (2.1×10^5 and 3.3×10^5 cells ml^{-1} , respectively), while the abundance of vHNA bacteria was 2 orders of magnitude lower (7.3×10^3 cells ml^{-1}). The dominant picophytoplankton group in the Pacific experiments was *Prochlorococcus*, which accounted

Table 2. Metabolic rates (means, $\pm$ SE where applicable) in the initial pre-incubation unfiltered samples (PU_0). BR: bacterial respiration, CR: community respiration, BP: bacterial production; all in $10^{-3} \mu\text{g C l}^{-1} \text{ h}^{-1}$. BGE: bacterial growth efficiency

Expt	BR- PU_0	CR ₀	BP- PU_0	BGE- PU_0
Atlantic 1	34 $\pm$ 17	212 $\pm$ 4	13 $\pm$ 1	0.28
Atlantic 2	37 $\pm$ 14	178 $\pm$ 5	10 $\pm$ 1	0.22
Atlantic 3	29 $\pm$ 13	314 $\pm$ 14	15 $\pm$ 1	0.34
Atlantic 4	64 $\pm$ 11	540 $\pm$ 58	24 $\pm$ 2	0.28
Atlantic 5	64 $\pm$ 24	286 $\pm$ 16	21 $\pm$ 1	0.25
Atlantic 6	100 $\pm$ 19	530 $\pm$ 56	8 $\pm$ 4	0.08
Atlantic 7	80 $\pm$ 4	734 $\pm$ 84	152 $\pm$ 8	0.66
Atlantic 8	82 $\pm$ 1	516 $\pm$ 55	174 $\pm$ 8	0.68
Atlantic 9	238 $\pm$ 26	971 $\pm$ 28		
Atlantic mean	81 $\pm$ 20	476 $\pm$ 82	52 $\pm$ 22	0.35 $\pm$ 0.07
Pacific 10	432 $\pm$ 17	702 $\pm$ 18	20 $\pm$ 1	0.04
Pacific 11	171 $\pm$ 27	538 $\pm$ 125	17 $\pm$ 1	0.09
Pacific 12	73 $\pm$ 32	458 $\pm$ 89	30 $\pm$ 1	0.29
Pacific 13	222 $\pm$ 35	317 $\pm$ 107	21 $\pm$ 1	0.09
Pacific 14	295 $\pm$ 73	937 $\pm$ 46	23 $\pm$ 1	0.07
Pacific 15	129 $\pm$ 61	399 $\pm$ 67	28 $\pm$ 3	0.18
Pacific 16	167 $\pm$ 31	372 $\pm$ 67	14 $\pm$ 1	0.08
Pacific 17	403 $\pm$ 24	739 $\pm$ 58	19 $\pm$ 1	0.04
Pacific 18	277 $\pm$ 34	632 $\pm$ 35	55 $\pm$ 1	0.17
Pacific 19	301 $\pm$ 60	606 $\pm$ 42	20 $\pm$ 1	0.06
Pacific 20	398 $\pm$ 51	964 $\pm$ 39	22 $\pm$ 1	0.05
Pacific mean	261 $\pm$ 34	606 $\pm$ 62	24 $\pm$ 3	0.11 $\pm$ 0.02
Global mean	180 $\pm$ 29	547 $\pm$ 52	36 $\pm$ 10	0.21 $\pm$ 0.04

Fig. 2. Mean contribution of bacterial respiration (BR) to total community respiration (CR) in initial pre-incubation unfiltered (%BR-PU₀) and pre-incubation filtered (%BR-PF₀) samples in the (A) Atlantic and (B) Pacific experiments. (C) Percent change in BR in PF₀ compared to PU₀ samples. Horizontal solid line represents a value of 100% (no change relative to BR-PU₀). Error bars represent the standard error from triplicates

for a mean of 1.9×10^5 cells ml⁻¹. Initial *Synechococcus* and picoeukaryote abundances were on average 1.4×10^3 and 1.0×10^3 cells ml⁻¹, respectively.

Effect of pre-incubation filtration procedure on bacterial communities

BR

Previous studies have shown that BP (e.g. measured as bacterial uptake of Leu and/or thymidine) may be enhanced after filtration (Ammerman et al. 1984, Pomeroy et al. 1994, Gasol & Morán 1999, Gattuso et al. 2002, Alonso-Sáez et al. 2007, Gasol et al. 2008), but the effect on BR has never been tested before. Here we present an experimental assessment of the effect of pre-incubation filtration on BR.

The contribution of BR to total CR (%BR) significantly increased (paired *t*-test, $p < 0.05$, $n = 20$) in PF (%BR-PF₀ = $57 \pm 5\%$, $n = 20$) compared to PU samples (%BR-PU₀ = $31 \pm 4\%$, $n = 20$) (Fig. 2A,B). A statistically significant increase (paired *t*-test, $p < 0.05$, $n = 20$) in BR in the PF samples (BR-PF₀) compared to the PU samples (BR-PU₀) was observed when both datasets were considered together (% change of BR by pre-incubation filtration = $264 \pm 46\%$, $n = 20$) (Fig. 2C). This overestimation caused by pre-incubation filtration was significantly higher (*t*-test, $p < 0.01$) in the Atlantic than in the Pacific experiments. In fact, while a significant effect (paired *t*-test, $p < 0.001$) of pre-incubation filtration on BR in the Atlantic experiments (%change: $404 \pm 71\%$, $n = 9$) was found, no significant effect (paired *t*-test, $p > 0.05$, $n = 11$) was encountered in the Pacific experiments (%change: $149 \pm 29\%$, $n = 11$). These results contradict our initial prediction of a higher effect of pre-incubation filtration in

bacterial communities from oligotrophic environments (Gasol & Morán 1999, Aranguren-Gassis et al. 2012). It is important to note here that in most of our Atlantic experiments, chl *a* concentration and CR were in the range of those measured in the Pacific experiments (Tables 1 & 2). We found a significant inverse relationship ($p < 0.05$, $n = 20$) between initial CR (CR_0) and BR overestimation ($BR-PF_0/BR-PU_0$) (Fig. 3). In our experiments, mesotrophic bacterial communities from the Atlantic experiments, characterized by higher BP and BGE values and lower %BR (Table 2), appeared to be more susceptible to pre-incubation filtration than oligotrophic communities from the Pacific experiments. This suggests that more efficient bacterial communities found in the Atlantic experiments will be impacted greater by pre-incubation filtration procedures, probably because they may respond quickly following perturbations such as removal of grazing pressure or organic carbon enrichment. Sherr et al. (1992) and Gasol et al. (1995) showed that the actively growing portion of the bacterial assemblage is highly susceptible to grazing, and thus, it is reasonable to expect that this bacterial fraction would benefit from predator suppression. Also, since the amount of particles is expected to be higher in the Atlantic experiments (i.e. shelf/break environment; Turley & Mackie 1994), the concentration of particle-attached bacteria released because of the filtration procedure might be higher in these experiments, thereby increasing the effect of pre-incubation filtration on respiration. Furthermore, in 4 out of the 11 Pacific experiments, pre-incubation filtration promoted decreases in BR instead of increases (Fig. 2B). A possible explanation for this result is a decrease in the dissolved organic matter (DOM) available during the incubation period due to the removal of autotrophs (i.e. DOM exudation in the dark) and bigger heterotrophs (i.e. DOM released by predation processes). Surprisingly, these 4 experiments accounted for some of the highest %BR- PU_0 of the 20 experiments, which may suggest that the stronger the role of HB in a microbial community, the more important the effects of the disruption of community linkages. The results obtained in the present study suggest that the magnitude of the effect of pre-incubation filtration procedures on BR depends on 3 factors: (1) respiration rate, (2) production rate, and (3) growth efficiency of the bacterial populations.

BP and biomass

The effect of pre-incubation filtration on BP and picoplankton abundance was also investigated in the

Fig. 3. Percent change in bacterial respiration (BR) in pre-incubation filtered (PF_0) compared to pre-incubation unfiltered (PU_0) samples for the Atlantic and Pacific experiments versus initial community respiration rates (CR_0). Regression line including both experiments: $y = -216.8 \ln(x) + 110.6$; $r^2 = 0.25$, $p < 0.05$. Horizontal dotted line represents a value of 100% (no change relative to $BR-PU_0$)

Pacific experiments. A significant (paired t -test, $p < 0.001$), ~50% decrease (%change: $51 \pm 3\%$, $n = 11$) in mean BP in the initial PF samples ($BP-PF_0$) compared to the PU samples ($BP-PU_0$) was observed (Table 3). Accordingly, BGE calculated using $BR-PF_0$ and $BP-PF_0$ ($BGE-PF_0 = 0.04 \pm 0.01$, $n = 11$; data not shown) was significantly lower (paired t -test, $p < 0.05$) (%change: $56 \pm 12\%$, $n = 11$; Table 3) than that calculated for the PU samples ($BGE-PU_0 = 0.11 \pm 0.02$, $n = 11$; Table 2).

In the Pacific experiments, the abundance of cells in the PF_0 samples (i.e. those that passed through the $0.8 \mu m$ filter) decreased (paired t -test, $p < 0.001$, $n = 6$) to $73 \pm 2\%$, $84 \pm 1\%$, $72 \pm 5\%$, and $8 \pm 3\%$ of the initial abundances in the PU sample (PU_0) for HB, *Prochlorococcus*, *Synechococcus*, and picoeukaryotes respectively (Table 3). This is in accordance with previous studies that reveal up to 50% reduction of HB and a 20% reduction of picoalgae in the filtrate after filtration through 0.7 – $1.2 \mu m$ pore-sized filters (Gasol & Morán 1999, Kiene & Linn 1999, Gattuso et al. 2002, Reinthaler & Herndl 2005).

The observed instantaneous decrease in BP after pre-incubation filtration may be explained by 3 factors: (1) removal of large microbes that can assimilate Leu (e.g. *Synechococcus*, diatoms, dinoflagellates), (2) the observed reduction in bacterial biomass (Table 3), and (3) a reduction in the metabolic rates per cell of the PF community. In the Pacific experiments, the BP rate per cell in the PU sample ($BP-PU_0$ cell $^{-1} = 3.4 \times 10^{-2} \pm 0.4 \times 10^{-2}$ fg C h $^{-1}$ cell $^{-1}$) was significantly higher (paired t -test, $p < 0.05$, $n = 11$) than

Table 3. Bacterial production (BP), bacterial growth efficiency (BGE), and picoplankton abundances in the initial pre-incubation filtered (PF₀) samples expressed as a percentage of the initial pre-incubation unfiltered value (PU₀) in the Pacific experiments (means $\pm$ SE, where applicable). Values <100 indicate decreased values and values >100 indicate increased values of PF₀ relative to PU₀. HB: total heterotrophic bacteria; vHNA: very high nucleic acid content bacteria; HNA: high nucleic acid content bacteria; LNA: low nucleic acid content bacteria; Pro: *Prochlorococcus*; Syn: *Synechococcus*; Pico: picoeukaryotes

Expt	(PF ₀ /PU ₀) $\times$ 100									
	BP (%)	BGE (%)	HB (%)	vHNA (%)	HNA (%)	LNA (%)	Pro (%)	Syn (%)	Pico (%)	Debris (%)
Pacific 10	52	153	69	30	59	77	81	64	7	9134
Pacific 11	71	46	87	87	79	91	93	101	35	3750
Pacific 12	65	24	74	58	70	77	83	63	7	16 219
Pacific 13	51	86	73	72	66	78	82	51	8	15 953
Pacific 14	50	26	63	50	58	66	81	72	5	19 575
Pacific 15	28	14	72	174	59	78	86	63	2	4789
Pacific 16	46	23	72	87	61	79	79	57	5	8350
Pacific 17	44	65	75	60	68	78	89	76	1	2386
Pacific 18	44	41	71	60	63	78	78	59	4	14 881
Pacific 19	57	93	73	63	69	77	90	12	6	21 278
Pacific 20	49	50	73	89	64	78	84	73	4	10 682
Mean	51 $\pm$ 3	56 $\pm$ 12	73 $\pm$ 2	75 $\pm$ 10	65 $\pm$ 2	78 $\pm$ 2	84 $\pm$ 1	72 $\pm$ 5	8 $\pm$ 3	12 454 $\pm$ 2353

that in the PF sample (BP-PF₀ cell⁻¹ = $2.2 \times 10^{-2} \pm 0.3 \times 10^{-2}$ fg C h⁻¹ cell⁻¹). A possible explanation for this may be the uncoupling between the components of the microbial food web caused by the removal of the phytoplankton-mediated DOM production (Alonso-Sáez et al. 2007). It has also been suggested that this immediate decrease in BP in the filtrate may be related to the fact that the most active cells (i.e. the largest bacteria or the particle-attached bacteria) would be more easily retained on the filter (Gasol et al. 1995, 2008).

A group of events identified in the plots of SSC versus red fluorescence (Fig. 4A,B), referred to hereinafter as 'debris' (i.e. detrital particles), increased in the PF samples compared to PU samples (debris-PU₀ = 83 ± 24 events ml⁻¹, %change = $12\,454 \pm 2353$ % in PF₀ compared to PU₀ samples; Table 3). A significant relationship was found between the increase in debris and the reduction of picoeukaryotes in PF₀ compared to PU₀ samples ($n = 11$, $r^2 = 0.42$, $p < 0.05$), suggesting that the debris may have originated from picoeukaryotic cell breakage during pre-filtration (Fuhrman & Bell 1985, Gasol & Morán 1999, Kiene & Linn 1999, Sherr et al. 1999, Massana et al. 2001).

Effect of incubation time on bacterial communities

BR

Overall, no changes were observed in BR-PU (Fig. 5A) or CR rates with incubation time. Incubation time did not have a significant effect on BR-PU or on

CR ($p > 0.05$, RMANOVA test). Although BR-PU and CR average rates over a 24 h incubation (BR-PU_{24ave} and CR_{24ave}, respectively) were not significantly different (paired t -test, $p > 0.05$, $n = 6$) from the initial PU₀ values, the slight increases found (%change = 128 ± 15 % and 124 ± 20 %, $n = 6$, respectively) may be related to the extra DOM released by the degradation of autotrophic organisms during the 24 h incubation in dark conditions.

BR in PF samples (BR-PF) increased at the end of the incubations (Fig. 5A). BR-PF_{24ave} rates were significantly higher (paired t -test, $p < 0.05$, $n = 6$) than BR-PU₀ (%change of BR = 305 ± 94 %, $n = 6$). This overestimation in BR-PF_{24ave} is approximately twice that observed in the corresponding initial PF samples (%change of BR = 133 ± 27 %, $n = 6$; Fig. 2C, Expts Pacific 15 to 20). The statistical analysis showed that the incubation time (within-subject factor), as well as the experiment (i.e. initial conditions) and the pre-incubation filtration (between-subjects factors), had significant effects on BR, BP, and %BR ($p < 0.001$, RMANOVA test). Significant interactions between the factor pre-incubation filtration and the factors time and experiment were found ($p < 0.001$, RMANOVA test); i.e. the effect of pre-incubation filtration on BR, BP, and %BR differed among experiments and changed with time.

Therefore, incubation time is a crucial variable that exacerbated the effect of pre-incubation filtration on BR estimation in most of the field experiments that we performed. However, enhanced BR was not observed until at least 12 h of confinement (Fig. 5A). This is in agreement with previous studies that have shown in-

Fig. 4. Plot of side scatter (SSC) versus red fluorescence (488-RED) for (A) a pre-incubation unfiltered sample at Time = 0 h, and (B) a pre-incubation filtered sample at Time = 0 h. Plot of SSC versus green fluorescence (488-GRN) for (C) a pre-incubation unfiltered sample at Time = 0 h, and (D) a 24 h-incubated pre-incubation filtered sample. vHNA: very high nucleic acid content bacteria. Yellow and red colours represent higher abundance

creasing bacterial abundance and activity related to incubation time (Pomeroy et al. 1994, Gasol & Morán 1999, Sherr et al. 1999, Gattuso et al. 2002, Briand et al. 2004), and demonstrates that the magnitude of overestimation of BR by pre-incubation filtration increases as incubation time increases. In a review of the literature, Robinson (2008) reported that 30% of previous time-course assays with pre-incubation filtered water samples have shown that BR measured as oxygen consumption is not linear during incubation time and that increases in bacterial biomass were measured in some of the experiments in which linear oxygen consumption was registered. For example, Briand et al. (2004) reported that the respiration rate was not constant during the incubation in 18 out of 27 experiments, and registered parallel increases in bacterial abundance and thymidine incorporation. In the

present work, the effects of time and pre-incubation filtration were significant on bacterial populations' biomass, production, and respiration, although the magnitude of these effects varied between experiments (e.g. no important effects of incubation time on BR in Expts Pacific 16 and 17; Fig. 5A). It seems reasonable to conclude that incubation time and pre-incubation filtration affect, although in a variable way, bacterial abundance, production, and respiration.

The %BR increased in most experiments after 12 h of confinement in PF samples (Fig. 5B). The mean 24 h averaged %BR calculated in PU samples ($\%BR_{PU_{24ave}} = 47 \pm 3\%$, $n = 6$) and in initial PF samples ($\%BR_{PF_0} = 55 \pm 9\%$, $n = 6$) were not significantly different (paired t -test, $p > 0.05$, $n = 6$) from those resulting from initial PU samples ($\%BR_{PU_0} = 44 \pm 3\%$, $n = 6$) (Fig. 6). However, %BR calculated from PF_{24ave}

Fig. 5. Time course of mean (A) bacterial respiration (BR), (B) contribution of BR to total community respiration (%BR), and (C) bacterial production (BP) in the incubation time experiments (Expts Pacific 15 to 20). PU: pre-incubation unfiltered sample, PF: pre-incubation filtered sample. Note that different y-axis scales were used. Error bars represent the standard error from triplicates; where error bars are not visible, they are smaller than the symbol size

samples ($\%BR\text{-PF}_{24\text{ave}} = 109 \pm 31\%$) and from Winkler samples ($\%BR\text{-Winkler} = 185 \pm 34\%$, $n = 6$) were unrealistically high, yielding both significant overestimations (paired t -test, $p < 0.05$, $n = 6$) of %BR compared to the corresponding $\%BR\text{-PU}_0$ (%change of $\%BR = 275 \pm 103$ and $405 \pm 51\%$, respectively) (Fig. 6). This is in agreement with previous investigations reporting %BR of 90%, and even higher, in oligotrophic areas where pre-incubation filtration procedures and long incubation times were applied (del Giorgio et al. 1997, 2011, Sherry et al. 1999, Bidanda et al. 2001, González et al. 2003, Reinthaler et al. 2006, Alonso-Sáez et al. 2007, Robinson 2008, Aranguren-Gassis et al. 2012). By contrast, the mean %BR found in the present work when short incubation times and pre-incubation unfiltered procedures were used ($\%BR = 31 \pm 4\%$, $n = 20$; Fig. 2A,B) is in accordance with previous indirect estimates of %BR in oligotrophic areas from BGE models (Morán et al. 2004, 2007), from models of microbial carbon fluxes (Fasham et al. 1999, Nagata 2000), and from biomass budgets (Robinson & Williams 2005, Maraño et al. 2007), as well as with CR and BR datasets in which unrealistic %BR data (i.e. $\%BR > 80\%$) were discarded (Robinson 2008, Aranguren-Gassis et al. 2012). Robinson & Williams (2005) and Robinson (2008) reported a %BR of 56% and 45%, respectively, generated from a regression analysis using previously published data ($n = 56$ and $n = 119$, respectively). They specified that apart from 3 values where %BR was 23% (Obernosterer et al. 2003), the

Fig. 6. Mean contribution of bacterial respiration (BR) to community respiration (CR) calculated for initial pre-incubation unfiltered (PU) and pre-incubation filtered (PF) electron transport system (ETS) samples ($\%BR\text{-PU}_0$ and $\%BR\text{-PF}_0$, respectively), 24 h-averaged PU and PF ETS samples ($\%BR\text{-PU}_{24\text{ave}}$ and $\%BR\text{-PF}_{24\text{ave}}$, respectively), and 24 h-incubated PF Winkler samples ($\%BR\text{-Winkler}$) for the incubation time experiments (Expts Pacific 15 to 20). Error bars represent the standard error from triplicates

mean %BR for each individual study is either 40–50% or 70–80%, both of which are values beyond the average value found in the present work. Robinson & Williams (2005) and Robinson (2008) included data of $\%BR > 80\%$ in their analyses, but when these unrealistic data are taken out of the calculations (C. Robinson's public database, www.uea.ac.uk/env/

people/facstaff/robinsonc), the calculated %BR is 27 %, similar to the average value found in the present work.

To date, pre-incubation filtration and subsequent direct measurements of oxygen consumption in dark-bottle incubations lasting hours have been the most commonly used methodology to estimate total and size-fractionated microbial respiration (Robinson & Williams 2005). In the present work, %BR was overestimated by the Winkler method, which is the most commonly employed method for BR measurements performed in oligotrophic areas as well as for a majority of the BR datasets published for meso- and eutrophic environments. Fig. 7 shows the relationship between CR and BR for the datasets in C. Robinson's public database (www.uea.ac.uk/env/people/facstaff/robinsonc). Different methodologies (i.e. *in vivo* ETS and Winkler methods) and also realistic and unrealistic data (i.e. %BR < 80 % and %BR > 80 %, respectively; Aranguren-Gassis et al. 2012) from the C. Robinson database have been separated (Fig. 7). The fraction of CR attributable to bacteria can be derived from these relationships. The exponents of the power-fitted functions shown are close to 1, so factors multiplying CR represent the contribution of BR to CR (i.e. %BR). The derived %BR values are 31 % (n = 145), 27 % (n = 143), and 139 % (n = 55) for *in vivo* ETS and realistic and unrealistic Winkler data, respectively. Three different datasets obtained in the present study have also been included in this figure: BR-PU₀ (n = 20, %BR = 31 ± 4 %), BR-PF_{24ave} (n = 6, %BR = 109 ± 31 %), and BR-Winkler (n = 6, %BR = 185 ± 34 %). As expected, BR-PU₀ data fall within the range of previously published *in vivo* ETS and realistic Winkler data, while BR-PF_{24ave} and BR-Winkler data are located in the plot within the range of unrealistic Winkler data previously published. The position of these 3 respiration datasets within the plot implies that pre-incubation filtration procedures along with extended incubation times promoted an increase in the %BR measured for the same initial microbial communities, and strongly suggests that previously reported %BR > 80 % data are influenced by pre-incubation filtration and/or extended incubation time artifacts. Our view of the relevance of BR in the ocean might

have been systematically biased by unrealistically high estimates. In the context of carbon flow calculations, this is particularly relevant in oligotrophic environments, where prokaryotes play an important role in material and energy fluxes (Cotner & Biddanda 2002). It is important to note here that BR estimates that include size fractionation of the community (i.e. pre-incubation filtration and post-incubation filtration in the case of Winkler and *in vivo* ETS methods, respectively) only account for free-living cells smaller than 0.8 µm (i.e. they do not account for particle-attached bacteria), and in some environments may include other prokaryotes like *Prochlorococcus* and *Synechococcus*.

BP and biomass

Important increases in BP in PF and some of the PU samples were also found after 12 h of confinement (Fig. 5C). A significant overestimation (paired *t*-test, *p* < 0.05, *n* = 6) of BP-PF_{24ave} compared to BP-PU₀ (%change of BP = 850 ± 146 %; Table 4) was found, while the increase in BP-PU_{24ave} compared

Fig. 7. Type II linear regression between logarithms (base 10) of community respiration and bacterial respiration ($\text{mmol m}^{-3} \text{d}^{-1}$). C. Robinson database: *in vivo* electron transport system (ETS) data (n = 145), and realistic (%BR < 80 %, n = 143) and unrealistic (%BR > 80 %, n = 55) Winkler data. Present study: *in vivo* ETS data measured at Time = 0 h in pre-incubation unfiltered (PU₀) samples (n = 22), *in vivo* ETS data measured in 24 h-averaged pre-incubation filtered (PF_{24ave}) samples (n = 6), and Winkler data (n = 6). Type II regression lines are shown for data from the C. Robinson database: *in vivo* ETS ($\text{BR} = 0.31\text{CR}^{1.26}$; $r^2 = 0.81$, n = 145) (---), and realistic ($\text{BR} = 0.27\text{CR}^{1.18}$; $r^2 = 0.84$, n = 143) (---) and unrealistic Winkler data ($\text{BR} = 1.39\text{CR}^{0.92}$; $r^2 = 0.94$, n = 55) (—). The %BR = 80 % threshold is also shown (·····).

to BP-PU₀ (%change of BP = $346 \pm 85\%$) was not significant (paired *t*-test, $p > 0.05$, $n = 6$). The increase of BP-PF may be related to the same above-mentioned (see previous section) factors responsible for BR overestimations (i.e. decrease of predation and competition pressure and increase of inorganic and organic substrate availability), and is in accordance with previous results showing similar increases in BP with incubation time (Pomeroy et al. 1994, Gasol & Morán 1999, Sherr et al. 1999, Briand et al. 2004, del Giorgio et al. 2011). It is important to note here that the use of the same theoretical Leu to carbon conversion factor for PF and PU samples throughout the incubation may introduce some biases in the BP estimations, since the changes in Leu incorporation rates observed throughout the incubation could also be reflected in the conversion factor.

The abundance of total HB remained nearly constant in PU samples throughout the incubation but slightly increased with incubation time in the PF samples. These increases were due to increments in the abundance of vHNA bacteria. The relative contribution of vHNA bacteria to total HB (vHNA/HB) increased throughout the incubations (Fig. 4C,D), especially during the last 12 h, with values ranging from 9 to 47 % in PF samples at the end of the incubation period. Compared to the initial PU sample, the 24 h-averaged vHNA bacteria abundance increased in all experiments in PU (%change = $328 \pm 152\%$), and more importantly and significantly in PF (%change = $882 \pm 237\%$; $n = 6$, paired *t*-test, $p < 0.05$) samples (Table 4). The vHNA bacteria have been previously found in natural open-ocean samples (Fernández et al. 2008). In addition, Martínez-García et al. (2010) showed that HB identified as vHNA bacteria in those experiments accounted for a considerable fraction of the total bacteria standing stock

when increases in BP were observed in the first 24 h of incubation in microcosm nutrient-addition experiments in oligotrophic open-ocean waters. A significant relationship between the magnitude of increase of BP (BP-PF_{24ave}/BP-PU₀) and the magnitude of increase of vHNA bacteria (vHNA-PF_{24ave}/vHNA-PU₀) in the PF compared to the PU samples was found ($r^2 = 0.84$, $p = 0.05$, $n = 6$). This indicates that the differences in BP between PF and PU samples are likely to be associated with the activity of vHNA bacteria. These observations are consistent with the role of vHNA as rapid responders, benefiting from enhanced nutrient availability and grazing pressure release (Jacquet et al. 2002), and also with previous results derived from pre-incubation filtration experiments showing an increase of the relative abundance of HNA bacteria in the filtrate with time (Gasol & Morán 1999). The vHNA bacteria SSC signal significantly increased (paired *t*-test, $n = 6$, $p < 0.01$) at the end of incubations in both PF and PU samples, which might be related to changes in vHNA cell size (Calvo-Díaz & Morán 2006). Previous studies have also shown an effect of incubation time on cell biovolume (Gattuso et al. 2002).

The BP per cell was significantly higher (paired *t*-test, $p < 0.05$, $n = 6$) in PF_{24ave} (0.40 ± 0.12 fg C h⁻¹ cell⁻¹) than PU_{24ave} (0.13 ± 0.05 fg C h⁻¹ cell⁻¹) samples. Similarly, the BR per cell was significantly higher (paired *t*-test, $p < 0.01$, $n = 6$) in PF_{24ave} (1.09 ± 0.14 fg C h⁻¹ cell⁻¹) than in PU_{24ave} (0.41 ± 0.04 fg C h⁻¹ cell⁻¹). These results may be related to the above-mentioned enhanced nutrient availability and the grazing pressure release that allows more active bacteria to increase their biomass (i.e. vHNA bacteria) in the PF samples.

The abundance of picoeukaryotes significantly decreased (paired *t*-test, $p < 0.01$, $n = 6$) with incubation time in PU samples (%change = $75 \pm 33\%$). As shown

Table 4. Bacterial production (BP) and picoplankton abundances in the 24 h-averaged values in pre-incubation filtered samples (PF_{24ave}) in the incubation time experiments (Expts Pacific 15 to 20) expressed as a percentage of the initial pre-incubation unfiltered value (PU₀). Values <100 indicate decreased values and values >100 indicate increased values of PF_{24ave} relative to PU₀. See Table 3 for abbreviations

Expt	(PF _{24ave} /PU ₀) × 100								
	BP (%)	HB (%)	vHNA (%)	HNA (%)	LNA (%)	Pro (%)	Syn (%)	Pico (%)	Debris (%)
Pacific 15	1445	99	1960	80	71	88	65	3	3971
Pacific 16	720	71	365	64	66	75	61	7	6123
Pacific 17	538	78	651	83	68	83	63	3	1783
Pacific 18	1187	97	1261	82	81	74	65	4	11770
Pacific 19	789	79	804	76	65	81	95	5	15972
Pacific 20	421	70	249	72	64	76	68	2	7744
Mean	850 ± 146	82 ± 5	882 ± 237	76 ± 3	69 ± 2	80 ± 0.02	70 ± 5	4 ± 1	7877 ± 1939

in previous studies (Jacquet et al. 2002, Litchman et al. 2004, Morán et al. 2007, Calvo-Díaz et al. 2011), dark incubation conditions seem to affect picoeukaryotes more than any other picoplankton group.

The 24 h-averaged *Prochlorococcus*, *Synechococcus*, picoeukaryotes, HB, HNA, and LNA abundances in PF_{24ave} samples were significantly lower (paired *t*-test, $p < 0.05$, $n = 6$) than in the PU₀ samples (Table 4). By contrast, vHNA-PF_{24ave} and debris-PF_{24ave} were significantly higher (paired *t*-test, $p < 0.05$, $n = 6$) than in PU₀ samples (Table 4). Statistical analysis showed that incubation time (within-subject factor) had a significant effect on *Prochlorococcus*, picoeukaryotes, debris, HB, and vHNA ($p < 0.001$, RM-ANOVA test). Significant interactions between the factor pre-incubation filtration and time were found ($p < 0.001$, RMANOVA test) for *Prochlorococcus* and debris; i.e. the effect of pre-incubation filtration changed with time.

Debris concentration significantly decreased (paired *t*-test, $p < 0.05$, $n = 6$; data not shown) over time in PF samples compared to the initial PF value, a feature that we hypothesize may be related to breakdown and/or consumption of the debris formed in PF samples at Time = 0 h by the growing HB stock.

Potential effects of pre-incubation filtration and incubation time on BGE estimation

BGE is commonly estimated using BP from Leu uptake rates in short-term incubated PU samples and BR from oxygen consumption rates in long-term incubated PF samples (Briand et al. 2004, Reinthaler et al. 2006, del Giorgio et al. 2011). As shown in the previous section, incubation time effects on BP were not found before 12 h of incubation. Therefore, routinely measured BP rates used for BGE estimations should neither be affected by incubation time nor pre-incubation filtration since incubation times < 4 h are usually used. By contrast, BR rates are expected to be affected by both incubation time and pre-incubation filtration. Alonso-Sáez et al. (2007) examined the effect of incubation time and pre-incubation filtration on BP and the corresponding consequences for BGE estimations calculated using BR rates obtained from 24 h-incubated PF samples. As discussed in the previous section, they found important decreases of BP rates in PF compared to initial PU samples but, interestingly, they also reported higher 24 h-averaged BP-PF rates compared to the initial BP-PF₀. In order to be consistent with the long-term incubated BR-PF

rates used, Alonso-Sáez et al. (2007) considered it inappropriate to calculate BGE using BP values of PU samples measured at Time = 0 h. Instead, they decided to calculate BGE using an average of BP-PF₀ and the change in biomass throughout the incubation.

In our incubation time experiments, BGE values calculated using BP-PU₀ and BR-PU₀ rates (BGE-PU₀ = 0.097 ± 0.021 , $n = 6$; Table 2) were not significantly different (paired *t*-test, $p > 0.05$, $n = 6$) from those calculated using BR-PU_{24ave} (BGE-PU_{24ave} = 0.07 ± 0.010 , %change = $87 \pm 9\%$; Table 5). However, BGE-PU₀ values were significantly higher (paired *t*-test, $p < 0.05$, $n = 6$) than those calculated using BR-PF_{24ave} *in vivo* ETS (BGE = 0.039 ± 0.005 , %change = $53 \pm 12\%$) and BR-Winkler samples (BGE = 0.035 ± 0.006 , %change = $44 \pm 11\%$) (Table 5). Statistical analysis showed that incubation time (within-subject factor), as well as experiment (i.e. initial conditions) and pre-incubation filtration (between-subjects factors), had a significant effect on BGE ($p < 0.001$, RMANOVA test). Significant interactions between the pre-incubation filtration factor and the time and experiment factors were found ($p < 0.001$, RMANOVA test); i.e. the effect of pre-incubation filtration on BGE differed among experiments and changed with time.

In the present study, we have shown that the traditionally different time-scales used for the estimation of BR and BP as well as the use of PF procedures for BR, but not for BP estimations, lead to biased BGE estimations. Alonso-Sáez et al. (2007) and del Giorgio et al. (2011) reported average BGE

Table 5. Bacterial growth efficiencies (BGE) estimated using 24 h-averaged bacterial respiration (BR) rates in the pre-incubation unfiltered (PU_{24ave}), pre-incubation filtered (PF_{24ave}), and Winkler measurements for the incubation time experiments (Expts Pacific 15 to 20). Values are expressed as the percentage of BGE estimated from BR from initial pre-incubation unfiltered samples (PU₀). Values < 100 indicate decreased values and values > 100 indicate increased values of PU_{24ave} and PF_{24ave} relative to PU₀. Bacterial production (BP) data from PU₀ samples (BP-PU₀) were used for all calculations

Expt	BGE (%)		
	(PU _{24ave} / PU ₀) $\times 100$	(PF _{24ave} / PU ₀) $\times 100$	(Winkler/ PU ₀) $\times 100$
Pacific 15	68	15	29
Pacific 16	69	42	37
Pacific 17	119	107	31
Pacific 18	57	35	23
Pacific 19	101	42	43
Pacific 20	105	78	101
Mean	87 ± 9	53 ± 12	44 ± 11

values of 0.04 and <0.1 in North Atlantic and North Pacific offshore waters, respectively, using BR data from PF samples and long-term incubations and BP values of PU samples measured at Time = 0 h. If a mean underestimation of BGE of 53 % (found in the present work when pre-incubation filtration and long incubation times were applied; Table 5) is used to correct the above-mentioned values reported by Alonso-Sáez et al. (2007) and del Giorgio et al. (2011), the resulting average BGE values would be 0.06 and <0.15 for North Atlantic and North Pacific areas, respectively. In the experiments performed in the oligotrophic Pacific, we found an average BGE of 0.097 ± 0.021 ($n = 6$, Expts Pacific 15 to 20; Table 2). However, the BGE values encountered when pre-incubation filtration and long incubation times were applied ($BGE = 0.039 \pm 0.005$ and 0.035 ± 0.006 , $n = 6$, for *in vivo* ETS and Winkler methods, respectively) were much closer to the values found in the literature, suggesting possible biases in BGE estimates due to overestimations of BR. It is important to note here that pre-incubation filtration and incubation time have been shown to affect bacterial activity in a variable way, so the use of this underestimation correction may not be appropriate for all datasets.

CONCLUSIONS

If we aim to understand the role of bacteria in marine carbon cycles, it is important to accurately assess the links between BP and BR in different environments (Gasol et al. 2008, del Giorgio et al. 2011). We have shown here that when eliminating unrealistically high BR rates, likely related to pre-incubation filtration artifacts, from previously published large datasets (i.e. C. Robinson's database), the average contribution of BR to total CR is 27 %, which is very similar to the 31 % obtained in the present study using the *in vivo* ETS method in PU and short-time incubated samples. This finding may aid to partially reconcile total bacterial carbon consumption assessments and estimates of primary production and organic carbon flow (del Giorgio & Cole 2000, Hoppe et al. 2006, Carlson et al. 2007).

We have shown that methodological approaches avoiding pre-incubation filtration and long incubation procedures may deliver more accurate BR and BGE measurements, which may help to constrain estimates of carbon flow through bacteria and to improve our understanding of the recycling of DOC in the ocean.

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