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**Wickham SA, Steinmair U, Kamennaya N**

Ciliate distributions and forcing factors in the Amundsen and Bellingshausen Seas (Antarctic) 215–230

**Yang J, Sun J, Lee OO, Wong YH, Qian PY**

Phylogenetic diversity and community structure of sponge-associated bacteria from mangroves of the Caribbean Sea 231–240

**Kolmonen E, Haukka K, Rantala-Ylinen A, Rajaniemi-Wacklin P, Lepistö L, Sivonen K**  
Bacterioplankton community composition in Finnish lakes differs according to trophic status 241–250

**Apprill A, Rappé MS**

Response of the microbial community to coral spawning in lagoon and reef flat environments of Hawaii, USA 251–266

**Straza TRA, Kirchman DL**

Single-cell response of bacterial groups to light and other environmental factors in the Delaware Bay, USA 267–277

**Park MG, Lee H, Kim KY, Kim S**

Feeding behavior, spatial distribution and phylogenetic affinities of the heterotrophic dinoflagellate *Oxyphysis oxytoxoides* 279–287

**Pinckney JL, Long RA, Paerl HW**

Structural and functional responses of microbial mats to reductions in nutrient and salinity stressors in a Bahamian hypersaline lagoon 289–298

**Baptista I, Santos AL, Cunha Â, Gomes NCM, Almeida A**

Bacterial biomass production in an estuarine system: high variability of leucine conversion factors and changes in bacterial community structure during incubation 299–310

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# Bacterial biomass production in an estuarine system: high variability of leucine conversion factors and changes in bacterial community structure during incubation

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**ABSTRACT:** Heterotrophic bacterioplankton play a key role in the transfer of organic carbon from the dissolved pool into planktonic trophic webs. The accurate measurement of bacterial biomass production (BBP) is crucial for the establishment of carbon budgets in aquatic systems. Estimation of BBP from the incorporation of radiolabelled leucine into bacterial proteins requires the use of empirical, semitheoretical or theoretical conversion factors (CFs). In this study, our estimate of the BBP for the estuarine system of Ria de Aveiro was higher when we used empirical and semitheoretical CFs than when we used theoretical ones. Empirical CFs ranged between 9.26 and 29.81 kg C mol<sup>-1</sup> in the marine zone and between 4.25 and 16.88 kg C mol<sup>-1</sup> in the brackish zone. Semitheoretical CFs ranged from 5.06 to 9.49 kg C mol<sup>-1</sup> in the marine zone and from 5.28 to 9.34 kg C mol<sup>-1</sup> in the brackish zone. During experiments to determine empirical CFs, the structure of the bacterial community was also analyzed. Sample preparation and the addition of leucine altered the structure of the bacterial community, and this probably affected the determination of the empirical CFs. The results show that, besides the effect of the use of an inadequate CF, the estimates of BBP can be biased in a system-specific way by the alteration of the structure of the bacterial community as a result of sample manipulation (i.e. filtration/dilution, incubation, and addition of non-radiolabelled and radiolabelled leucine).

**KEY WORDS:** Heterotrophic bacterioplankton · Leucine incorporation · Isotope dilution · Conversion factors · Bacterial communities · Estuary

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## INTRODUCTION

Heterotrophic bacterioplankton play a key role in the biological transfer of carbon via the microbial loop (Ghiglione et al. 2007), represent a significant fraction of total biomass (Valencia et al. 2003) and are involved in 2 main processes of transformation of organic matter: production of new bacterial biomass and respiration of organic carbon to inorganic carbon. Bacterial biomass production (BBP) by heterotrophic bacteria reflects the overall bacterial response to the prevailing ecological conditions. Through BBP, dissolved organic

carbon (DOC) is converted into particulate organic carbon (POC) in the form of bacterial cells, and these cells become potentially available to consumers at higher trophic levels in the food web (Cole & Pace 1995). Estimations of BBP are useful because they allow the estimation of rates of metabolism, carbon budgets and the amount of organic matter metabolized by aquatic bacteria (Riemann & Søndergaard 1984). Being a key parameter for evaluating the role of heterotrophic bacterioplankton in the oceanic carbon cycle (Calvo-Díaz & Morán 2009), the development of reliable methods for accurate measurement of BBP becomes an important

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objective in microbial ecology (Bååth 1998). The importance of heterotrophic bacterioplankton is enhanced in coastal regions, mainly deltas, estuaries and bays, and river outlets (Kolm et al. 2002). This is due to the input of organic matter and nutrients and also because of anthropogenic influences such as cities, ports, factories and agriculture (Gunkel 1966, Gocke 1977, Rheinheimer 1991, Kolm et al. 2002).

Since the 1980s, several techniques have been used to estimate BBP, but the incorporation of [methyl- $^3\text{H}$ ]thymidine into bacterial DNA (Fuhrman & Azam 1980), and the incorporation of [ $^3\text{H}$ ]leucine or [ $^{14}\text{C}$ ]leucine into bacterial proteins (Kirchman et al. 1985), are the most commonly used methods. More recently, the incorporation of 5-bromo-2'-deoxyuridine (BrDU) into DNA (Nelson & Carlson 2005) has been used as an alternative to the [methyl- $^3\text{H}$ ]thymidine incorporation technique (Hamasaki et al. 2007). Although these techniques are very useful, they are not free of drawbacks. One of the main problems with the leucine method is the determination of specific conversion factors (CFs) that translate leucine incorporation rates into bacterial carbon production. These CFs are specific to each system and must be determined in order to obtain values of BBP that are the most reliable. There are 3 ways of converting leucine incorporation rates into bacterial production: (1) the theoretical approach, which is the most commonly used (Simon & Azam 1989); (2) the semitheoretical approach, in which the theoretical value of isotope dilution (ID) is replaced by an empirical value obtained in ID experiments (Pedrós-Alió et al. 1999); (3) the empirical approach, in which CFs are determined by comparing the rates of leucine incorporation with the increase in bacterial biomass over a period of time (Buesing & Marxsen 2005). According to Riemann et al. (1990), these CFs will vary between environments, over the seasons, and will depend on growth conditions and substrate composition. Several authors have determined empirical CFs for different systems; for example, Calvo-Díaz & Morán (2009) determined CFs for Atlantic coastal waters (with values between 0.49 and 1.92 kg C mol $^{-1}$ ), and Jørgensen (1992) proposed values of 2.11 kg C mol $^{-1}$  for freshwater systems. Buesing & Marxsen (2005) determined values of 1.445 kg C mol $^{-1}$  for freshwater systems, and Kirchman & Hoch (1988) reported values of 0.78 and 1.97 kg C mol $^{-1}$  for estuarine systems. Looking at this variability, it becomes clear that using CFs previously determined for other systems can result in the incorrect measurement of BBP.

Determination of empirical CFs involves long-term incubations (between 24 h and 1 wk). Changes in bacterial composition during this sort of incubation have been reported (Massana et al. 2001). Filtration and the consequent removal of bacterivores have also been

shown to result in a shift in bacterial communities after a 2 d incubation (Suzuki 1999). However, as far as we know, potential changes in the structure of the bacterial community during incubation experiments for determining empirical factors in the presence of [ $^3\text{H}$ ]leucine have never been addressed, especially in highly productive estuarine systems.

This study aimed to (1) determine specific CFs, both semitheoretical and empirical, for the estuarine system of Ria de Aveiro, Portugal; this had not been done previously. We also aimed to (2) evaluate the effect of sample treatment, incubation, and the differential use of non-radioactive and radioactive substrate on the structural diversity of the estuarine bacterial community during experiments for determining empirical CFs.

## MATERIALS AND METHODS

**Study area and water sampling.** Ria de Aveiro (Fig. 1) is a tidal estuarine system on the western coast of Portugal, connected to the Atlantic by a narrow opening (Silva 1994). The estuarine system has a maximum length of 45 km and a maximum width of 10 km, covering an area of 66 and 83 km $^2$  at low tide and high tide, respectively (Dias et al. 2001). The average depth is 1 m, although in the mouth of the estuary it can reach 30 m (Dias et al. 2000, Dias & Lopes 2006). The system has a complex topography, with 4 major channels (S. Jacinto, Ovar, Mira and Ílhavo) spreading from the mouth and branching into secondary channels. Several rivers carry fresh water into the Ria de Aveiro with an average water input of 1.8 Mm $^3$  during a tidal cycle

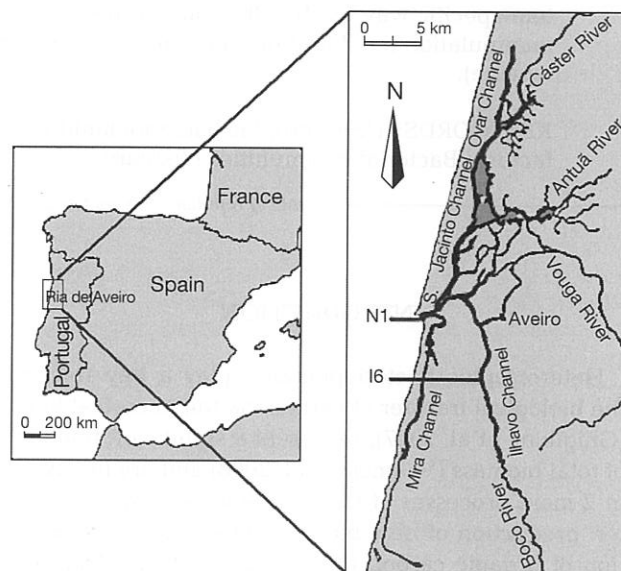


Fig. 1. The estuarine system of Ria de Aveiro and location of the sampling stations (N1 - 40° 38' 42" N, 08° 45' 25" W and I6 - 40° 35' 41" N, 08° 41' 21" W)



(Silva 1994). Water circulation is regulated by semi-diurnal tides (Lopes & Cardoso 2010) and by river discharges. The residence time varies between 2 d close to the mouth, and 2 wk in the upper areas (Dias et al. 2001). Velocities of the tidal currents near the mouth can reach  $1 \text{ m s}^{-1}$  but, in the many of the innermost small channels, the tidal currents are very weak (Martins et al. 2010). Ria de Aveiro is under high anthropogenic pressure due to naval traffic, domestic and industrial discharges. It is considered a 'moderately low' eutrophic system, with a higher trophic status in the inner sections compared with the outer ones (Lopes et al. 2007).

Two sampling sites were considered in the present study: (1) Stn N1 ( $40^{\circ}38'42'' \text{ N}$ ,  $08^{\circ}45'25'' \text{ W}$ ), located near the mouth of the estuary and representing the marine zone (maximum depth 30 m), and (2) Stn I6 ( $40^{\circ}35'41'' \text{ N}$ ,  $08^{\circ}41'21'' \text{ W}$ ), located in the Ílhavo Channel and representing the brackish water zone (maximum depth 2 m) (Fig. 1). All samples were collected at low tide at 0.5 m below the water surface with a Van Dorn Bottle (Wilco, Model No. 1930 605 0595) between October 2007 and October 2008 (Table 1). Water samples were kept cold, and in the shade, during transport to the laboratory, and were processed within 2 to 3 h of collection.

**Conversion factor (CF) experiments.** Leucine-to-carbon empirical CFs (eCFs) were estimated according to Bjørnsen & Kuparinen (1991) and Kirchman & Ducklow (1993). Water samples were collected from each sampling site. From those samples, 900 ml were filtered through polycarbonate membranes (Poretics) of  $0.2 \mu\text{m}$  pore size and then added to 100 ml of unfiltered sample (dilution 1:10). The diluted samples were then incubated in acid-cleaned bottles (Shott Duran, VWR International), covered with aluminium paper, for 36 h at room temperature with agitation (90 rpm). At regular intervals (4 h), sub-samples were taken to assess the rate of leucine incorporation (LIR) and the change in bacterial biomass. In order to calculate eCF, data were computed following the integrative method (Riemann et al. 1987):

$$\text{eCF} = \frac{\text{BM}_f - \text{BM}_0}{\int (\text{LIR} dt)} \quad (1)$$

Table 1. Sampling dates of conversion factor experiments, isotope dilution experiments and denaturing-gradient gel electrophoresis (DGGE) experiments and respective local times of low tide (in parentheses)

Conversion factor experiments	Isotope dilution experiments	DGGE experiments
31 Oct 2007 (12:36)	21 Jan 2008 (08:02)	29 Oct 2008 (08:45)
27 Feb 2008 (12:30)	22 Apr 2008 (11:47)	17 Jan 2009 (13:30)
15 Apr 2008 (07:41)	18 Oct 2008 (12:52)	

in which eCF is the empirical leucine-to-carbon conversion factor,  $\text{BM}_f$  and  $\text{BM}_0$  are the final and initial bacterial biomass, respectively, and  $\int (\text{LIR} dt)$  is the integrated leucine incorporation rate over the course of the experiment. The experiments were always performed for a period of 36 h, but we used only the values of bacterial biomass and LIR obtained before cell numbers started to decrease.

**Isotope dilution (ID) experiments.** Saturation curves were determined in order to assess ID according to the protocol described above (LIR and bacterial biomass determination) and by adding the same concentration of  $[^3\text{H}]$ leucine, but using increasing concentrations of unlabelled leucine. The concentrations used to perform the saturation curves ranged from 23.2 to 203.2  $\text{nM l}^{-1}$  for Stn N1 and from 83.2 to 323.2  $\text{nM l}^{-1}$  for Stn I6. The LIR values were plotted against the respective concentrations and the resulting incorporation velocities were fitted to the hyperbolic function of Michaelis-Menten enzyme kinetics by using nonlinear regression (SigmaPlot 11.0, Systat Software). The fitted parameters were used to determine  $V_{\text{max}}$  (maximal incorporation rate) which was used to calculate isotope dilution:

$$\text{ID} = \frac{V_{\text{max}}}{V_{\text{meas}}} \quad (2)$$

in which  $V_{\text{meas}}$  is the incorporation rate at the concentration used in the routine assays (van Looij & Riemann 1993).

Using the values of ID, semitheoretical conversion factors (sCFs) were calculated (Pedrós-Alió et al. 1999):

$$\text{sCF} = \text{PM} \cdot \left( \frac{1}{L_p} \right) \cdot C_{\text{cp}} \cdot \text{ID} \quad (3)$$

in which PM is the molecular weight of leucine ( $0.1312 \text{ kg} \cdot \text{mol}^{-1}$ ),  $L_p$  is the leucine content of cellular protein (0.073) and  $C_{\text{cp}}$  is the ratio of cellular carbon to protein (0.86).

**Determination of LIR and bacterial biomass.** LIR was determined using three 10 ml replicates and a formaldehyde-fixed control (2% final conc.) for each sample. In eCF experiments, samples were incubated with  $[^3\text{H}]$ leucine (Amersham, specific activity  $63.0 \text{ Ci mmol}^{-1}$ ; Ci = Curie) at a previously determined saturating concentration (83.2 nM). After 1 h, incubations were stopped with formaldehyde (2% final conc.). Following 15 min incubation on ice, 1 ml of ice-cold 20% trichloroacetic acid (TCA) was added. After a 10-min incubation, sub-samples and controls were filtered through polycarbonate membranes (Poretics) of pore size  $0.2 \mu\text{m}$  and washed twice with

2 ml of ice-cold TCA (5%) and 5 ml of ice-cold ethanol (90%). Membranes were then placed in 5 ml scintillation vials and 4.5 ml of scintillation cocktail UniverSol (ICN Biomedicals) was added. Radioactivity was measured after 3 d in a Beckman LS 6000 IC liquid scintillation counter. BBP was calculated from LIRs using a ratio of cellular carbon to protein of 0.86 and the fraction of leucine in protein of 0.073 (Simon & Azam 1989), using the ID of 1, previously determined by Simon & Azam (1989), and an ID determined in the ID experiments.

Bacterial biomass and total bacterial numbers were determined by the acridine orange technique (Hobbie et al. 1977). Cell counting and measurements were performed using an epifluorescence microscope (Leica DMLS), equipped with an I 2/3 filter. At least 200 cells or 10 microscope fields were counted, and 200 cells were measured, in each replicate. To determine the cell biovolume, size classes were defined according to size and shape determined using a graduated reticule giving 48 size classes ( $0.0056$  to  $5.625 \mu\text{m}^3$ ). A total of 200 cells was counted in each replicate and attributed to one of the pre-established size classes. Cell volume of individual cells was determined by considering rods as cylinders, with hemi-

spherical ends, and cocci as spheres (Fry 1988). Cell volume was converted to cell carbon using the CF determined by Norland (1993):

$$\text{pg C cell}^{-1} = 0.12 \cdot (\mu\text{m}^3 \text{ cell}^{-1})^{0.7} \quad (4)$$

In order to compare the values obtained in the estuarine system of Ria de Aveiro with those obtained in the study by Kirchman & Hoch (1988) in the Delaware Bay estuary, the calculations were performed using the same CF.

**Bacterial community fingerprint analysis.** For denaturing-gradient gel electrophoresis (DGGE), 4 different types of triplicate samples were studied (Fig. 2): (1) type O: original sample without filtration, dilution, or substrate amendment, designated as time 0 samples, and with [ $^3\text{H}$ ]leucine and cold leucine added at times 1 and 32 h (T1 and T32, respectively); (2) type A: filtered and diluted sample, such as the one used in eCF experiments, without substrate amendment; (3) type B: similar to type A samples, but amended with unlabelled leucine; (4) type C: similar to type B samples, but amended with unlabelled leucine and [ $^3\text{H}$ ]leucine. For the type O sample, subsamples were removed after 0, 1 and 32 h of incubation. For this type of sample leucine was not added at

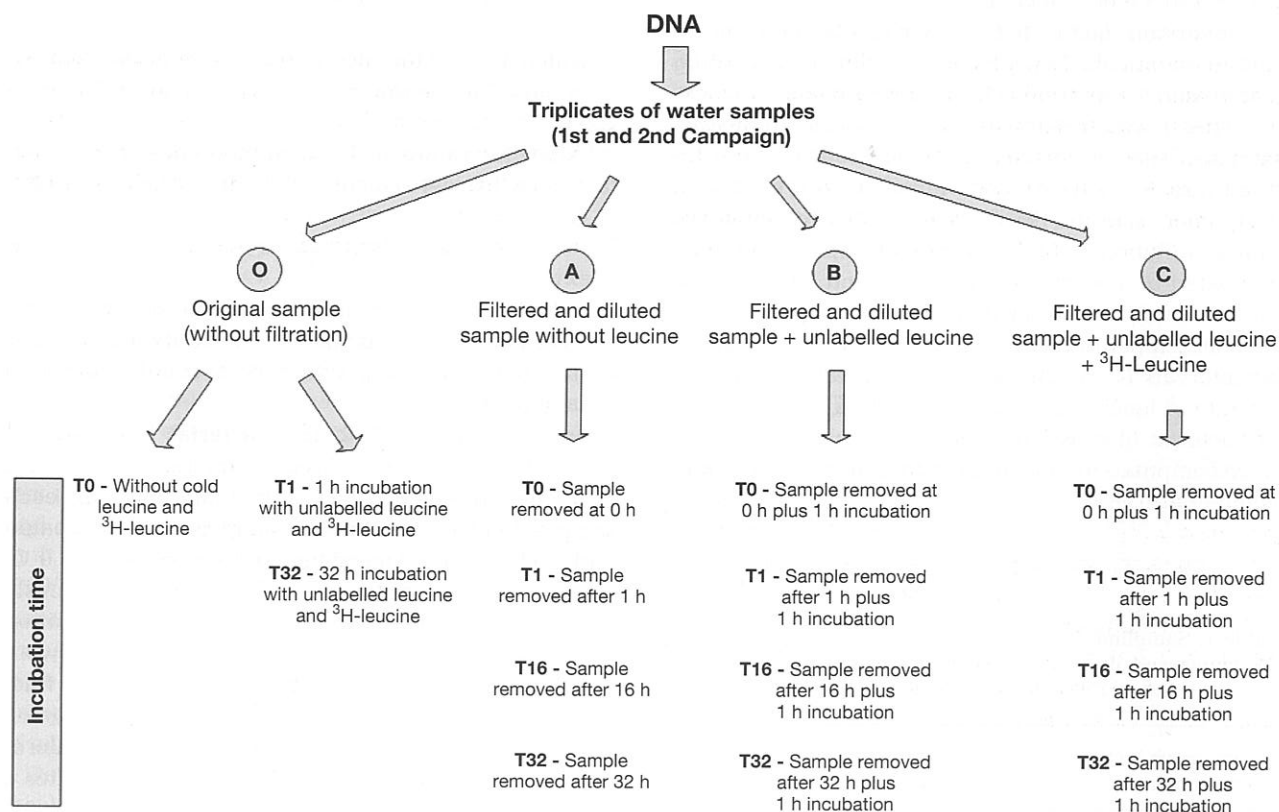


Fig. 2. Schematic drawing of the denaturing-gradient gel electrophoresis (DGGE) experiments for DNA for Stn N1 on the 2 sampling dates (29 October 2008 and 17 January 2009). O, A, B and C show the different types of treatment over the incubation times (from T0 to T32)

time T0, but for the times T1 and T32 unlabelled and [ $^3\text{H}$ ]leucine were added at the beginning of the incubation, in order to observe the effects of both types of leucine in the structure of bacterial communities. For type A, B and C treatments, aliquots were removed at times 0, 1, 16 and 32 h. Unlabelled leucine and/or [ $^3\text{H}$ ]leucine were added to the sub-samples after collection of aliquots during the different time intervals for samples B and C and incubated for 1 h. Samples without leucine (O samples at time 0 and A samples at times 0, 1, 16 and 32 h) were analyzed immediately after sampling.

DNA extraction was performed after filtering 250 ml of each sub-sample through polycarbonate filters of pore size 0.2  $\mu\text{m}$ . Collected cells were resuspended in 2 ml of Tris-EDTA (TE) buffer and centrifuged, and the pellet was preserved at  $-80^\circ\text{C}$  until processing. The pellet was treated with 1 mg ml $^{-1}$  lysozyme solution as previously described (Henriques et al. 2004) and DNA was extracted using the genomic DNA purification kit (MBI Fermentas). DNA was resuspended in TE buffer and stored at  $-20^\circ\text{C}$  until analysis. The yield of extracted DNA, and its quality, were checked by electrophoresis in a 0.8% (w/v) agarose gel.

Amplification by the polymerase chain reaction (PCR) of a ~400 bp fragment of 16S rDNA (region V6 to V8) from the extracted DNA was performed using the primer set F968GC and R1401 (Heuer et al. 1999). The reaction was conducted in a MultiGene Gradient Thermal Cycler (MIDSCI). The 25  $\mu\text{l}$  reaction mixture contained ~50 to 100 ng of extracted DNA, 1 $\times$  PCR buffer (PCR buffer without  $\text{MgCl}_2$ :PCR buffer with  $\text{KCl}_2$  in a ratio of 1:1), 2.75 mM  $\text{MgCl}_2$ , acetamide (4%), 0.2 mM of each nucleotide, 0.1  $\mu\text{M}$  of each primer, 1 U of *Taq* DNA polymerase. The PCR protocol included a 5 min initial denaturation at  $94^\circ\text{C}$ , 35 cycles at  $95^\circ\text{C}$  for 1 min,  $53^\circ\text{C}$  for 1 min and  $72^\circ\text{C}$  for 2 min, with a final extension for 10 min at  $72^\circ\text{C}$  (Heuer et al. 2001). Amplification was confirmed by agarose gel electrophoresis.

DGGE was performed with the Dcode System (C.B.S. Scientific). PCR products were loaded onto a 6 to 9% (w/v) polyacrylamide gel in 1 $\times$  Tris-acetate-EDTA (TAE) buffer. The 6 to 9% polyacrylamide gel (bisacrylamide: acrylamide ratio = 37.5:1) was made with a denaturing gradient ranging from 40 to 70%. Electrophoresis was performed at  $60^\circ\text{C}$  for 16 h at 130 V. Following electrophoresis, the gels were incubated for silver staining (Heuer et al. 2001).

GelCompar 4.0 program (Applied Maths) was used to analyze bacterial

community profiles of the images of DGGE gels (Smalla et al. 2001). The band positions and their corresponding intensities (surface) from each treatment were exported to Excel (Microsoft). The band surface was converted to relative intensity by dividing its surface by the sum of all band surfaces in a lane. Bray-Curtis similarities were calculated based on the band position and relative intensity of each sample. The matrices of similarities were then used for analysis of similarities (ANOSIM). The ANOSIM was used to test whether bacterial communities from different groups of samples are separated ( $R = 1$ ) or not ( $R = 0$ ). The  $R$  statistic in ANOSIM ranges from 0 to 1, with higher values indicating greater variation in composition among samples; values of  $R > 0.75$  correspond to well separated groups, values  $0.50 \leq R \leq 0.75$  correspond to separated but overlapping groups, values  $0.25 < R < 0.50$  correspond to separated but strongly overlapping groups and values of  $R \leq 0.25$  correspond to barely separated groups (Ramette 2007, Michelland et al. 2009).

## RESULTS

### Determination of empirical conversion factor (eCF)

Values of both the final and initial bacterial biomass ( $\text{BM}_f$  and  $\text{BM}_0$  respectively) and integrated rate of leucine incorporation  $\int(\text{LIR}dt)$  (Fig. 3) were, in general, higher at Stn I6 (Table 2). Differences between final and initial bacterial biomass were higher in April for Stn N1, while for Stn I6 these differences were higher in October. The highest  $\int(\text{LIR}dt)$  occurred, for both stations, in October.

With the exception of the October campaign, values of eCF, determined using the factor of Norland (1993) to transform biovolume into biomass, were superior in N1 samples (Table 2). For this station, the average eCF

Table 2. Calculation of empirical conversion factors for Stns N1 and I6, for the 3 sampling dates, using the integrative method of Riemann et al. (1987) and the factor of Norland (1993) to transform biovolume into biomass.  $\text{BM}_f$  = final bacterial biomass;  $\text{BM}_0$  = initial bacterial biomass;  $\int(\text{LIR}dt)$  = integrated leucine incorporation rate over the course of the experiment; eCF = empirical conversion factor

Stn	Date	$\text{BM}_f$ ( $\mu\text{g C l}^{-1}$ )	$\text{BM}_0$ ( $\mu\text{g C l}^{-1}$ )	$\int(\text{LIR}dt)$ ( $\times 10^{-8} \text{ mol h}^{-1}$ )	eCF ( $\text{kg C mol Leu}^{-1}$ )
N1	31 Oct 2007	379.06	32.39	3.70	9.36
	27 Feb 2008	240.13	19.83	1.03	21.37
	15 Apr 2008	419.96	20.21	1.34	29.81
I6	31 Oct 2007	820.21	70.12	6.47	11.59
	27 Feb 2008	185.94	22.49	3.85	4.25
	15 Apr 2008	603.86	44.19	3.32	16.88

Table 3. Biovolume-to-biomass conversion factors available in the literature and used to determine empirical conversion factors. Vol = biovolume

Conversion factor	System	Source
0.22 g C cm <sup>-3</sup>	Pure cultures	Bratbak & Dundas (1984)
0.35 pg C cell <sup>-1</sup>	Estuary native bacteria	Bjørnsen (1986)
20 fg C cell <sup>-1</sup>	Marine native bacteria	Lee & Fuhrman (1987)
0.12 · Vol <sup>0.7</sup> pg C cell <sup>-1</sup>	Freshwater and marine native bacteria	Norland (1993)
15 fg C cell <sup>-1</sup>	Marine native bacteria	Caron et al. (1995)
435 · Vol <sup>0.86</sup> fg C cell <sup>-1</sup>	Pure cultures and freshwater native bacteria	Loferer-Krößbacher et al. (1998)

was 20.18 kg C mol Leu<sup>-1</sup>, the highest value being observed in April (29.81 kg C mol Leu<sup>-1</sup>) and the lowest in October (9.36 kg C mol Leu<sup>-1</sup>). For Stn I6, the highest value of eCF was obtained in April (16.88 kg C mol Leu<sup>-1</sup>), while the lowest was obtained in February (4.25 kg C mol Leu<sup>-1</sup>), with an average eCF of 10.91 kg C mol Leu<sup>-1</sup> (Table 2). Using the the factor of Lee & Fuhrman (1987) (Table 3) to transform biovolume into biomass, the CFs obtained for the estuarine system

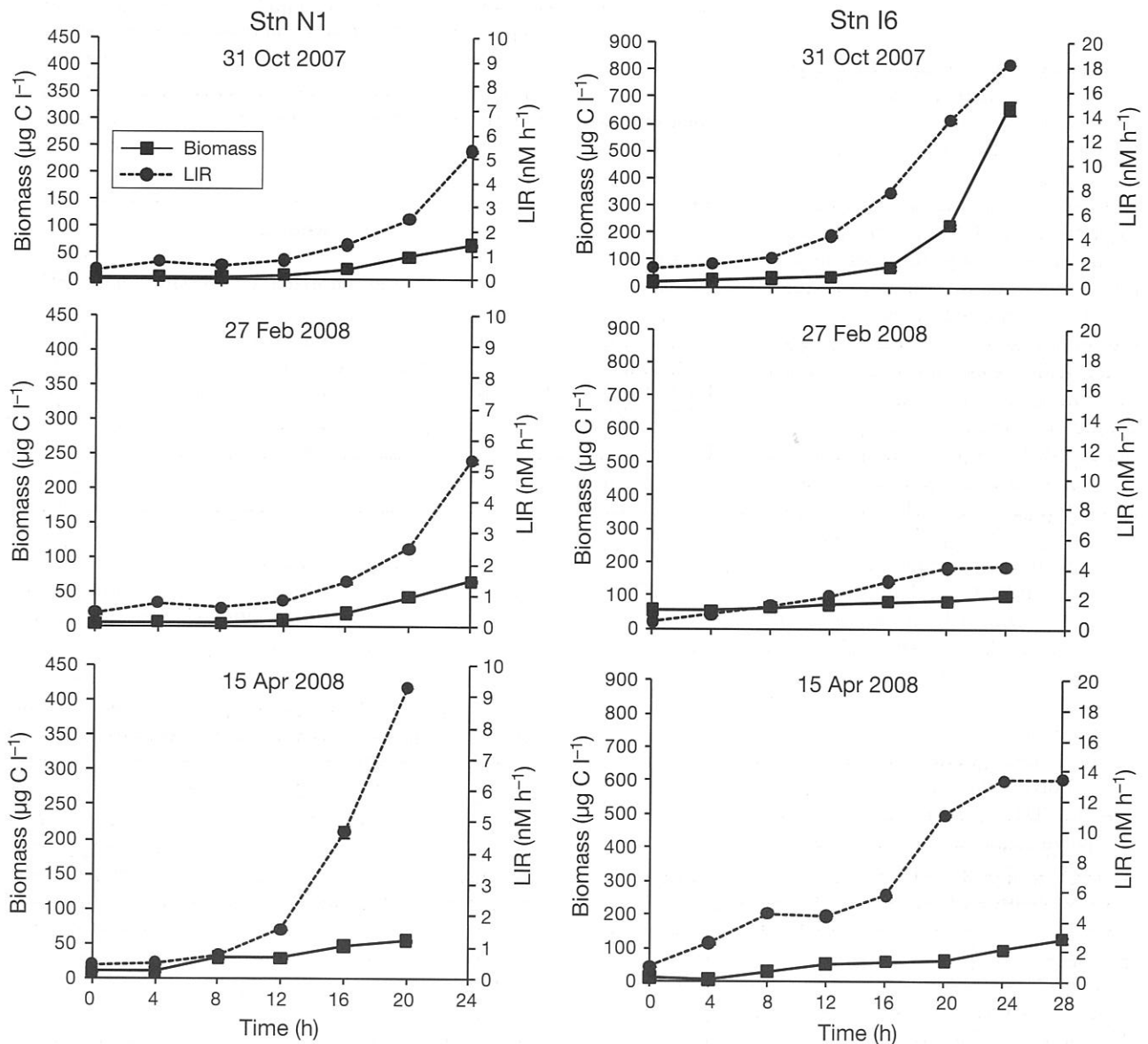


Fig. 3. Measurements of biomass ( $10^8$  pg C cell<sup>-1</sup>) and leucine incorporation rate (LIR) (nM h<sup>-1</sup>) over time (0 to 24 h for Stn N1, and 0 to 28 h for Stn I6), used to calculate empirical conversion factors (eCFs) for Stns N1 and I6 for the 3 different sampling dates



Ria de Aveiro were much lower (average 3.521 kg C mol Leu<sup>-1</sup> for Stn N1 and 1.706 kg C mol Leu<sup>-1</sup> for Stn I6).

Using the Norland (1993) factor to transform biovolume into biomass, the BBP determined by the theoretical method increased from 6.678 to 83.600 µg C l<sup>-1</sup> h<sup>-1</sup> at Stn N1 station and from 7.337 to 49.645 µg C l<sup>-1</sup> h<sup>-1</sup> for Stn I6. However, using the Lee & Fuhrman (1987) factor to transform biovolume into biomass, the BBP determined by the theoretical method increased only from 6.678 to 14.587 µg C l<sup>-1</sup> h<sup>-1</sup> at Stn N1 and from 7.337 to 7.765 µg C l<sup>-1</sup> h<sup>-1</sup> at Stn I6.

#### Determination of extracellular IDs and sCFs

Values for extracellular IDs and sCFs were generally higher for Stn N1 (Table 4). For this station the highest values were obtained in October, with an ID of 6.143 and an sCF of 9.495 kg C mol Leu<sup>-1</sup>. The average ID at this station was 5.072, and the average sCF was 7.840 kg C mol Leu<sup>-1</sup> (differences in BBP can go from 6.678 to 32.479 µg C l<sup>-1</sup> h<sup>-1</sup>). For Stn I6 the highest values were obtained in April, with an ID of 6.041 and an sCF of 9.339 kg C mol Leu<sup>-1</sup>. The average ID for this station was 5.015, and the average sCF was 7.752 kg C mol Leu<sup>-1</sup> (differences in BBP can go from 7.337 to 35.284 µg C l<sup>-1</sup> h<sup>-1</sup>).

The value of ID in the estuarine system varied over time, although a consistent pattern of variation could not be established in this study. For both study areas, different extracellular IDs were observed on the 3 sampling dates, with a 2-fold range of variation.

#### Community fingerprint analyses

The analysis of similarities (ANOSIM) of DGGE community fingerprints was used as a means to test the separation (significant differences,  $R > 0.5$ ) between the composition of the bacterial community at different incubation periods and with different types of treatment (O, A, B, C; see Fig. 2). Comparison of the bacterial community structure after incubation of the samples for BBP determination for various periods of time suggests that the bacterial communities from both the first and second campaigns (29 October 2008 and 17 January 2009, respectively) were significantly

Table 4. Calculation of isotope dilution and respective semitheoretical conversion factors (sCFs) for Stns N1 and I6 for the 3 sampling dates.  $V_{\text{meas}}$  = incorporation rate at the concentration used in the routine assays;  $V_{\text{max}}$  = maximal incorporation rate

Sampling station	Date	$V_{\text{meas}}$	$V_{\text{max}}$	Isotope dilution ( $V_{\text{max}}/V_{\text{meas}}$ )	$r^2$	sCF (kg C mol Leu <sup>-1</sup> )
N1	21 Jan 2008	0.83	4.82	5.80	0.94	8.96
	22 Apr 2008	2.19	7.17	3.27	0.93	5.06
	18 Oct 2008	1.45	8.90	6.14	0.93	9.49
I6	21 Jan 2008	7.77	26.54	3.42	0.96	5.28
	22 Apr 2008	2.12	12.81	6.04	0.89	9.34
	18 Oct 2008	2.25	12.59	5.99	0.92	8.64

Table 5. Analysis of similarities (ANOSIM), in terms of Bray-Curtis similarity measures (R), of bacterial communities at different time intervals for Stn N1 for the 2 sampling dates (see Table 1) of denaturing-gradient gel electrophoresis (DGGE) experiments performed under conditions similar to those used to determine the empirical conversion factor (eCF). Analysis involved data from 3 independent sub-samples for each treatment (see Fig. 2 for details on treatments). Degree of separation between samples (R ranges from 0 to 1): well separated,  $R > 0.75$ ; separated but overlapping,  $0.50 \leq R \leq 0.75$ ; separated but strongly overlapping,  $0.25 < R < 0.50$ ; barely separated,  $R \leq 0.25$ . nd = not determined (DGGE not performed)

Community shift	Treatment							
	O		A		B		C	
	Oct 08	Jan 09	Oct 08	Jan 09	Oct 08	Jan 09	Oct 08	Jan 09
0 h vs. 1 h	1	1	0.481	1	0.259	0.963	0.444	1
0 h vs. 16 h	nd	nd	1	1	0.741	1	1	1
0 h vs. 32 h	1	1	0.556	1	0.296	1	1	1
1 h vs. 16 h	nd	nd	1	0.926	1	1	1	1
1 h vs. 32 h	1	1	0.556	1	0.704	1	1	0.778
16 h vs. 32 h	nd	nd	0.556	0.926	0.593	1	0.593	1

affected by sampling preparation for all incubation times (0, 1 and 32 h for original samples, and 0, 1, 16 and 32 h for the treatments A, B and C) (Table 5). The only exceptions were the filtered samples (A) and the filtered samples that were incubated with unlabelled leucine (B), in which the communities tended to be more similar after 32 h of incubation in both campaigns. Furthermore, regardless of the type of treatment, comparative analyses of the bacterioplankton communities at different times of incubation also indicate significant structural shifts for nearly all sampling times (Table 6). Additionally, it is also evident that certain 16S ribotypes from the DGGE gels disappeared after manipulation of water samples, namely, the addition of [<sup>3</sup>H]leucine (C) (Fig. 4). Only R values of the comparative analyses of communities at incubation time 0 h versus 1 h, and 0 h versus 32 h, in the October campaign suggested that the communities were weakly separated. The R value of the unfiltered and undiluted samples (O) was 1 for both campaigns, showing that bacterial communities were well separated between the time intervals 0 h and 1 h, 0 h and

Table 6. Analysis of similarities (ANOSIM) as in Table 5, but for treatment effects

Treatment effects	Incubation time							
	0 h		1 h		16 h		32 h	
	Oct 08	Jan 09	Oct 08	Jan 09	Oct 08	Jan 09	Oct 08	Jan 09
A vs. B	nd	1	1	1	1	1	0.185	0.333
A vs. C	nd	1	0.704	0.889	1	1	1	1
A vs. O	nd	1	nd	1	nd	nd	nd	1
B vs. C	nd	1	0.556	0.778	1	0.778	0.481	1
B vs. O	nd	1	nd	1	nd	nd	nd	0.963
C vs. O	nd	1	nd	0.852	nd	nd	nd	1

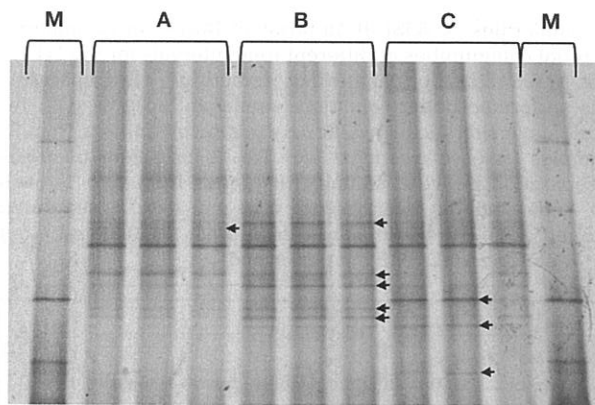


Fig. 4. Fingerprints of bacterial communities of water samples from Stn N1 during the first campaign (see Table 1) processed as for determination of empirical conversion factor (eCF) and visualized by denaturing-gradient gel electrophoresis (DGGE) of PCR-amplified partial 16S rRNA genes. A = filtered and diluted samples without [<sup>3</sup>H]leucine and without cold leucine; B = similar to type A samples but with unlabelled leucine; C = similar to type B samples but with unlabelled and [<sup>3</sup>H]leucine; M = marker. Differentiating ribotypes are marked by arrows

32 h, and 1 h and 32 h. The highest values of R occurred in the O samples between the time intervals 0 h and 1 h. For these samples, at times 1 h and 32 h, the sample was incubated with both [<sup>3</sup>H]leucine and unlabelled leucine, as opposed to the procedure at time 0 h, in which leucine was not added. Differences between treatments were also noticed when compar-

Table 7. Isotope dilution in various aquatic systems

Isotope dilution	System	Source
1.1 to 16.7	River sediments	Fischer & Pusch (1999)
1.0 to 1.4	Antarctic Península	Pedrós-Alió et al. (2002)
1.03 to 2.12	Paint Creek (inland emergent marsh wetland)	Gillies et al. (2006)
1.025 to 1.30	Freshwater ecosystem	Buesing & Marxen (2005); Miranda et al. (2007)
3.275 to 6.143	Ria de Aveiro (estuarine system)	Present study

ing the unfiltered and undiluted samples O and the other samples (A, B and C), indicating that manipulated bacterial communities are well separated from original ones during all incubation periods (Table 6).

## DISCUSSION

The current methods for measuring BBP are simple to perform and are a convenient way to follow bacterial growth over time (Bell & Kuparinen 1984), or over changing geographical or local conditions (Pace & Cole 1994). The determination of specific CFs, and changes in the structure of the bacterial community in the presence of [<sup>3</sup>H]leucine during the incubation period of CF experiments, have, to our knowledge, never been assessed in estuarine systems such as the Ria de Aveiro.

This study has shown that (1) both the eCFs and sCFs obtained for the Ria de Aveiro are high, overestimating BBP values relative to those estimated by the theoretical method; (2) both the eCF and sCF show a high variability over time and space, covering the whole range of factors referred to in the literature and resulting in a wider range of BBP values relative to those estimated by theoretical methods for the Ria de Aveiro; (3) for the estuarine system of Ria de Aveiro, the estimation of BBP can be biased by the alteration in structure of the bacterial community as a result of sample manipulation (i.e. filtration/dilution, incubation and addition of non-radio-labelled and radiolabelled leucine) during the CF experiments.

The determination of eCFs and sCFs for the Ria de Aveiro shows an underestimation of BBP in this estuarine system in works performed over the years, such as Almeida et al. (2001a, 2005, 2007), which used the theoretical approach and, consequently, an ID of 1. Because the determined values for extracellular isotope dilution were ~5, differences in BBP, without taking into account this factor, can be 5 times lower. Moreover, ID might also have been underestimated because intracellular ID was not considered in those works. However, intracellular ID is rather constant and does not exceed a factor of 2 to 3 (Simon 1991, Simon & Rosenstock 1992), usually assuming values of 1.44 to 1.55 kg C mol Leu<sup>-1</sup> (Simon & Azam 1989, van Looij & Riemann 1993, Buesing & Marxen 2005). Compared to other studies (Table 7), values for extracellular ID in the Ria de

Aveiro are high, but this is not surprising because it is an estuary that receives subsidies of organic matter, namely, during the cold season. Although, during the warm season in this system, an average of 21% of the primary production (range 0.11 to 512.9  $\mu\text{g C l}^{-1} \text{h}^{-1}$ ) is potentially sufficient to support the bacterial production, the total primary production would be either required or even insufficient to support bacterial production during the cold months (Almeida et al. 2002). In the brackish water zone, even during the warm season, allochthonous carbon accounted for 41% (on average) of the bacterial carbon demand (Almeida et al. 2002). In the marine zone, during the warm season, allochthonous carbon accounted for only 26% (on average) of the bacterial carbon demand (Almeida et al. 2002). Due to the eutrophic status of Ria de Aveiro (Lopes et al. 2007) at low tide, high values of extracellular ID can be expected because, in eutrophic conditions, it is more difficult to saturate the system, unless high concentrations of leucine (more than 200 nM) are used (van Looij & Riemann 1993).

If high values of estimated BBP emerge when ID is used, these differences become even more pronounced with the use of empirical conversion—differences that were very high, especially at Stn N1. The CFs obtained for the estuarine system Ria de Aveiro, using the factor of Norland (1993) to transform biovolume into biomass, are generally up to an order of magnitude greater than the theoretical values and higher than most of the empirical values reported in the literature. Values of eCF of the same order of magnitude (12.8 and 36.4 kg C mol Leu<sup>-1</sup>) were obtained only by Rivkin & Anderson (1997) for the Sargasso and Caribbean Seas and for the Gulf Stream. The only eCF calculated for an estuarine environment is provided by Kirchman & Hoch (1988) in the Delaware Bay estuary (0.78 and 1.97 kg C mol Leu<sup>-1</sup>). If we had used the factor of Lee & Fuhrman (1987) for transforming biovolume into biomass, the CFs obtained for the estuarine system Ria de Aveiro would have been much lower (average of 3.521 kg C mol Leu<sup>-1</sup> for Stn N1 and of 1.706 kg C mol Leu<sup>-1</sup> for Stn I6) and similar to those found in the literature. Thus, depending on the biovolume-to-biomass (Table 3) CF, the empirical CF will be different, even if determined for the same time and place.

Although it is easy to understand the higher variability of BBP estimated by the semitheoretical and empirical methods relative to the theoretical method, a systematic explanation for the variability of CFs with respect to the time and location of sampling could not be defined from the results of the present study. Nevertheless, the great variability in CFs (regardless of the different methods applied) in Ria de Aveiro is not surprising, because this lagoon is strongly influenced by

different external sources of organic matter (Almeida et al. 2002, Santos et al. 2007), by the mixing of marine, brackish and limnetic bacterial populations, by tides of different intensity, and even by drastic changes in weather conditions during the study period. Thus, it seems that the wide range of factors and of bacterial productivity measurements over the seasons is, to some extent, typical of this ecosystem. Therefore, in Ria de Aveiro and comparable environments, the CFs must be continually determined, although this may not be so urgent in more stable aquatic environments.

The results of the present study show that, for the estuarine system Ria de Aveiro, the influence of sample preparation and of leucine addition on the structure of bacterial communities may affect the rates of leucine incorporation during CF experiments and, consequently, affect the reliability of BBP estimates. The results of DGGE performed in the different types of sample treatment during empirical CF experiments demonstrate the occurrence of significant changes in the structural diversity of bacterial communities. The DGGE profiles show that the diversity of the bacterial community in the original samples (O) is significantly different from that in the samples used to determine the empirical CFs (A), which are filtered and diluted before the addition of leucine. These differences are enhanced during the incubation period. Such results are consistent with those obtained by Massana et al. (2001) and Suzuki (1999); these authors found changes in bacterial composition during incubation and filtration, respectively. The addition of unlabelled leucine (B) to the samples, used to achieve the saturation in BBP assays in an inexpensive way, resulted in changes in the structure of the bacterial community in both sampling sites within 16 h of incubation; however, after 32 h the differences were attenuated. Bottle effect can, in part, explain this pattern of variation. Furthermore, and unexpectedly, the composition of the bacterial community in samples incubated solely with unlabelled leucine (B) was different from that detected in samples incubated with both unlabelled and labelled leucine (C) corresponding to the conditions used in BBP determination assays. Moreover, the effect of adding [<sup>3</sup>H]leucine was observed within only 1 h of incubation. However, the results should be considered with caution because such an impact on the bacterial community would imply the extinction of some bacterial groups in the [<sup>3</sup>H]leucine-amended samples, and these results have been tested only in the estuarine system of Ria de Aveiro.

In contrast to DNA, short-lived RNA molecules are degraded rapidly in living bacterial cells by enzymes (RNases) which are very stable, even in harsh environments (Sela et al. 1957, Sheridan et al. 1999). So, specific RNA sequences represent an excellent target for



the detection of rapid changes in bacterial communities. In fact, in the present study, assessment of the bacterial community structure using reverse transcribed PCR products revealed significant differences between treatments after 1 h of incubation (data not shown). This suggests that radioactivity can affect the bacterial communities. The difference in bacterial community structure between incubation time 0 (without [ $^3\text{H}$ ]leucine) and incubation times 1 h or 32 h (with [ $^3\text{H}$ ]leucine) for the unfiltered/undiluted samples (samples O) could also be explained by the effect of [ $^3\text{H}$ ]leucine on bacteria. Bacterial death due to the mutagenic effect of tritium has long been known (Person & Bockrath 1964, Higo & Yamamoto 1985). However, the amount of exogenous leucine used in the present study (83.2 nM) is within the concentration range reported for dissolved free amino acids in estuarine waters (Macko & Green 1982, Coffin 1989), and thus unlikely to alter so quickly and drastically the bacterial community structure and the uptake rates.

The alterations in community composition within 1 h imply that the balance of bacterial growth and grazing/lysis changed significantly in a short period of time. Yet, the mechanisms for such a change could not be established. Such effects of [ $^3\text{H}$ ]leucine amendment on bacterial community structure would be expected as a result of massive cell lysis. However previous microautoradiography studies in this system showed that, typically 30% of the cells actively incorporate [ $^3\text{H}$ ]leucine (Almeida et al. 2001b) and cellular damage was not evident by epifluorescence microscopy observations. Similar results have been obtained in other studies (Vila et al. 2004, Sintes & Herndl 2006). Further studies on the effect of radioactivity on bacterial community structure and dynamics are necessary to clarify the underlying mechanisms of the observed effect of [ $^3\text{H}$ ]leucine on the composition of the bacterial community.

As for the CFs, the observed changes in community composition during incubation are not surprising in such an unstable environment. In this environment, higher changes can be expected during incubation than in samples where bacterial populations are stable and characterised by few clearly dominating species.

In summary, besides the effect of the use of an inadequate CF, the estimates of BBP can be biased by alteration in the structure of the bacterial community during the assessment of empirical conversion factors as a result of sample manipulation (i.e. filtration/dilution, incubation and addition of non-radiolabelled and radiolabelled leucine). The sole addition of non-radioactive leucine, and of the mixture of non-radioactive and [ $^3\text{H}$ ]leucine, caused an alteration in the structure of bacterial communities, affecting the determination of empirical CFs. The use of semitheoretical CFs allows these problems to be bypassed because manipulation

of the samples is not needed. Moreover, the determination of the semitheoretical CFs is more standardized because there is no need to transform biovolume into biomass; additionally, this does not affect the simplicity and quickness of the approach because ID experiments can be performed in parallel with BBP assays.

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