

Theoretical and empirical conversion factors for determining bacterial production in freshwater sediments via leucine incorporation

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Abstract

Bacteria play an extraordinarily important role in carbon transformations. It is therefore crucial to accurately measure bacterial production. One of the most widely used methods is the leucine method. From rates of leucine incorporation bacterial carbon production can be derived by empirical or theoretical conversion factors (CFs). However, only few empirical CFs have been established, and no estimation of the theoretical conversion factor for freshwater systems exists until today. Therefore, we determined both, the empirical and the theoretical conversion factor, of sediment bacteria from a headwater stream. The empirical conversion factor determined from exponentially growing bacteria was $1.445 \text{ kg C mol}^{-1}$. The theoretical conversion factor derived from the determination of the molar fraction of leucine in bacterial protein (0.081 ± 0.001) was $1.442 \text{ kg C mol}^{-1}$. Both conversion factors are close to each other and similar to conversion factors established for marine bacterioplankton. Therefore, results of the present study indicate that high values of bacterial production determined in freshwater sediments by the leucine method in several studies were not overestimates caused by inappropriate use of CFs from marine systems but represent true high bacterial production in these environments. For studies that apply the leucine technique in freshwaters, we recommend using the theoretical conversion factor for calculation of bacterial carbon production: $\text{BCP (kg)} = 1.44 \times \text{Leu}_{\text{inc}}$ (Leu_{inc} = leucine incorporation in mol).

Bacterial production is among the most important driving processes in the transformation of organic carbon. It is, therefore, of great importance to accurately measure bacterial production. Growth rates and production of bacteria can be estimated from the incorporation of radiolabeled nucleic or amino acids into bacterial DNA or protein, respectively (Fuhrman and Azam 1980; Kirchman et al. 1985). Despite some theoretical advantages of the thymidine technique, which follows the incorporation of thymidine into bacterial DNA (Roberts and Zohary 1993), many researchers have preferred the incorporation of radiolabeled leucine into protein as a superior technique (e.g., Riemann and Azam 1992; Thomaz and Wetzel 1995; Marxsen 1996; Fischer and Pusch 2001; Buesing and Gessner 2003). One reason for this prefer-

ence is that the leucine method is more sensitive because the production of a bacterial cell requires 10 times the incorporation of leucine into protein than thymidine into DNA (Riemann and Bell 1990). In addition, leucine appears to constitute a quite constant fraction of total amino acids in bacterial protein (Simon and Azam 1989), and protein represents a rather constant percentage of bacterial carbon (Hagström et al. 1984; Simon and Azam 1989). Thus, conversion factors (CF), which translate rates of leucine incorporation to bacterial carbon production, are more accurate than CFs based on thymidine incorporation rates into DNA (Riemann and Bell 1990; Marxsen 1996).

There are two approaches for determining CFs: theoretically or empirically. The determination of the theoretical CFs consists of measuring the molar fraction of leucine in bacterial protein. Empirical CFs are determined by comparison of leucine incorporation rates with the increase in bacterial biomass over time. Some empirical CFs have been determined for marine pelagic systems (Fuhrman and Azam 1980; Bjørnsen and Kuparinen 1991; Sherr et al. 1999) and very few for freshwater systems (Moran and Hodson 1992; Servais 1990; Tulonen 1993). The theoretical conversion factor has been derived only once from bacterial cultures (Reeck 1983) and once from the marine open water (Simon and Azam 1989). However, until now, the empirical and theoretical CFs have not been deter-

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mined simultaneously in a freshwater system and especially for particle-associated communities such as sediment bacteria.

The leucine technique has been widely applied in freshwater habitats including the pelagic and littoral water column, epiphytic, leaf litter, and sediment biofilms throughout the last years (e.g., Tulonen 1993; Thomaz and Wetzel 1995; Weyers and Suberkropp 1996; Sommaruga et al. 1997; Kirschner and Velimirov 1999; Petit et al. 1999; Theil-Nielsen and Søndergaard 1999). In most of these studies, the theoretical CF from a marine system was used to calculate bacterial carbon production from leucine incorporation into protein. Especially in freshwater sediments, surprisingly high bacterial production values were obtained (e.g., Marxsen 1996, 2001; Fischer and Pusch 2001; Buesing 2002). Thus, the question arises whether these high production values are overestimates resulting from the application of inappropriate conversion factors from marine systems. Therefore, the aim of the present study was to determine for the first time both types of CFs for freshwater sediment bacteria, in order to determine a specific CF for freshwater and especially for sediment-associated bacterial communities.

Material and procedures

Batch cultures—Experiments were conducted with aerobic sediment samples from the Breitenbach, a small Central German upland stream (Marxsen 1988; Marxsen et al. 1997). To 9 mL of sterile filtered stream water, 1 mL aerobic sediment was added. Sediment bacteria were detached from their natural substrate by briefly sonifying samples for 10 s with an ultrasonic probe (Branson Sonifier 250, standard resonator, 11.4 mm tip, 80 W output, 76 μ m amplitude). Preliminary tests showed that the activity of bacteria was only slightly affected by this short sonication. In order to exclude grazing, the suspension was filtered stepwise through filters with decreasing pore size down to 0.8 μ m. Detached sediment bacteria were grown in 0.2 μ m filtered creek water at in situ temperature (9°C) in three replicate glass flasks, which were mounted onto a shaker and gently shaken. The flasks were each amended with 20 g of sterilized (heated for 4 hours at 550°C) stream sediment as mineral substratum for bacterial growth. Subsamples of each culture flask were taken every 8 to 12 h to determine bacterial production, bacterial biomass, and amino acid composition. Subsamples were taken with sterile glass pipettes (also heated 4 h at 550°C).

To determine bacterial production, 3 mL bacterial suspension from each of the three replicate glass flasks were immediately incubated with radiolabeled leucine. Also for bacterial biomass determination, 3 mL of the suspension were taken and fixed with formaldehyde solution (final concentration 2%, buffered with 0.1% sodium pyrophosphate). For amino acid analyses, 20 mL bacterial suspension was filtered through 0.2 μ m polycarbonate filters, which were stored at -20°C until further processing.

Amino acid analysis—Bacterial samples collected on polycarbonate filters were transferred into 1.7-mL screw cap vials.

Bacterial protein was extracted in 500 μ L NaOH suprapure (0.5 mol L⁻¹) at 90°C for 1 h. Samples were cooled to room temperature and transferred to glass tubes (8 \times 40 mm), which were inserted into 25 mL Schott glass flasks containing 500 μ L 30% HCl suprapure. Samples were degassed with N₂ to avoid losses of easily oxidizable amino acids before proteins were hydrolyzed by gas phase extraction for 4 h at 140°C.

Hydrolyzates were concentrated and neutralized by evaporating the liquid in a speed vacuum centrifuge. The dried sample was dissolved in HCl (10 mmol L⁻¹) and filtered through sterile glass fiber filters (GF/F). A subsample of 37 μ L was diluted with 9 μ L borate buffer (9 mol L⁻¹) and measured in a high-performance liquid chromatography (HPLC) system. The HPLC system (Kontron, Zurich, Switzerland) consisted of two HPLC pumps (PU-422 master/slave), an autosampler (AS-465), an OPA-3 column (Grom, Rottentburg-Haiflingen, Germany) protected by a C18 guard column and a fluorescence detector (SFM 25). The excitation was set at 330 nm, and the emission measured at 450 nm. Twenty microliters of ortho-phthalaldehyde reagent (Grom, Rottentburg-Haiflingen, Germany) diluted 1:10 with borate buffer (9 mol L⁻¹) was added to the sample, and after 90 s, a subsample of 10 to 20 μ L was injected into the column.

Calibration curves for each amino acid were established by measuring individual standards at four concentrations (0.2, 0.4, 0.6, and 0.8 μ mol L⁻¹).

Time series and saturation curve for bacterial production measurement—To minimize isotope dilution a saturation curve was established to determine the leucine concentration at which incorporation is saturated. This means that the external pool of leucine is overwhelmed by adding leucine at a sufficiently high amount to maximally label the pool and, at the same time, to inhibit de novo synthesis of leucine (Kirchman et al. 1985; Simon 1991). Isotope dilution was assessed by calculating the ratio of v_{\max} to v_{measured} at the concentration applied for further measurements (van Looij and Riemann 1993). A time series was also run to determine the maximum incubation time ensuring constant leucine incorporation rates into protein.

Bacterial production measurement—Subsamples from each of the three batch cultures were incubated with ³H leucine with a final concentration of 50 μ mol L⁻¹ (Marxsen 1996; Fischer and Pusch 2001; Buesing and Gessner 2003). Incorporation of leucine was stopped after 1 h by adding trichloroacetic acid (TCA) to a final concentration of 5% w/v. Samples were then sonified for 1 min and centrifuged for 10 min at 14000g. The supernatant was removed from the sample and filtered through a 0.2- μ m polycarbonate filter supported by a backing filter. Both filter and pellet were washed twice with 5% TCA, once with 40 mM leucine, once with 80% ethanol, and once with nanopure water. The filter and pellet were combined in a centrifuge tube and protein was dissolved in an alkaline solution (0.5 N NaOH, 25 mM EDTA, 0.1% sodium dodecyl sulfate) for 60 min at 90°C (Buesing and Gessner 2003). The samples

were cooled to ambient temperature, centrifuged (10 min at 14000g), and an aliquot of 100 to 500 μL , depending on expected radioactivity, was radioassayed. Blanks (subsamples that were killed with TCA before adding ^3H leucine) were throughout the experiment less than 0.5% of the samples with active bacteria.

Bacterial counts and biomass determination—The abundance of bacteria was determined by epifluorescent counts of cells stained with Sybr Green I (Noble and Fuhrman 1998). Briefly, samples were filtered through an Anodisc filter, which was then placed on a drop of Sybr Green I (stock solution diluted 400 fold), dried carefully by placing it onto a cleansing tissue, and transferred to a slide. A 30- μL drop of antifade mounting solution (50% glycerol, 0.1% *p*-phenylenediamine, 50% PBS: 120 mM NaCl, 10 mM NaH_2PO_4 , pH 7.5) was added and a cover slip placed on top. Bacteria were counted with a Zeiss Axio-phot2 epifluorescence microscope equipped with a 100 W high-pressure bulb and a Zeiss light filter set nr 09 (excitation filter BP 450-490 nm, beamsplitter FT 510 nm, emission filter LP 520 nm). Cell numbers and biovolumes were determined in 10 to 20 microscopic fields (typically > 400 cells; Kirchman 1993) with an image analysis system. Digital images were captured with a 12-bit cooled slow scan charge coupled device (CCD) camera (Axiocam, Zeiss, Germany). The Imaging Software KS400 (Zeiss, Germany) was used for analyzing the acquired images. Cell edges were sharpened by applying a highpass filter ("Mexican Hat" function). The optimal threshold level for detecting cells from background was set manually. The resulting binary picture was then edited in an overlay mode with the original gray image.

This digital image analysis allowed us to measure the dimensions and calculate the biovolume of more than 34,000 cells over the course of the experiment.

Calculations—The empirical conversion factor was calculated by the modified derivative method as suggested by Ducklow and Hill (1985):

$$CF = \frac{B_t - B_0}{\int (Leu \, dt)} \quad (1)$$

where B_0 and B_t are the initial and final bacterial biomasses determined by nonlinear regression analysis and $\int (Leu \, dt)$ is the rate of leucine incorporation over the course of the experiment.

To convert measured biovolumes to biomass, the conversion factor of Loferer-Kröbächer et al. (1998) was used:

$$DW = 435 \cdot V^{0.86} \quad (2)$$

where DW is dry weight (fg) and V the biovolume (μm^3) of a bacterial cell. Carbon was assumed to be 55% of bacterial dry weight (Simon and Azam 1989).

The theoretical conversion factor was calculated by the equation:

$$CF = \frac{b \cdot c}{a \cdot d} \cdot ID \quad (3)$$

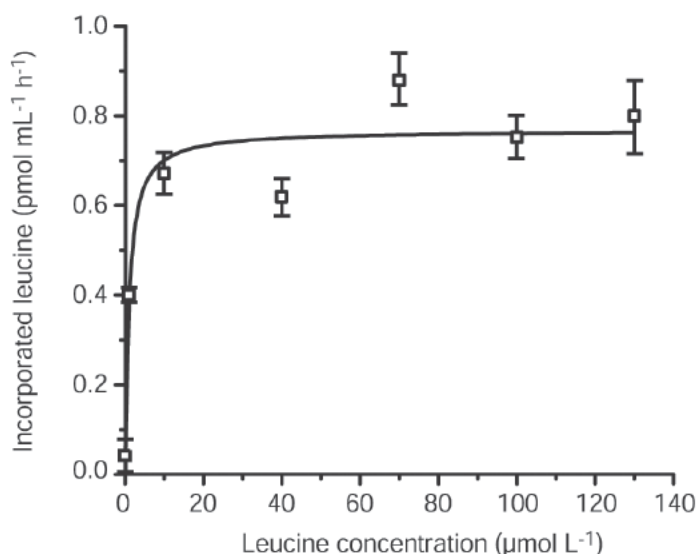


Fig. 1. Saturation curve of leucine incorporation into protein of freshwater sediment bacteria in suspension. Symbols are means of three replicates \pm SD.

where a is molar fraction of leucine in protein, b is molecular weight of leucine (131.2 g mol^{-1}), c is % dry weight of cellular carbon, d is % dry weight of proteins in bacterial cells, and ID is isotope dilution.

Two proteinogenic amino acids were not included in the HPLC measurements: proline, which cannot be detected with the ortho-phthalaldehyde reagent, and cysteine, which is destroyed during acid hydrolysis. These make up about 5% all proteinogenic amino acids (Reeck 1983).

Assessment

Preliminary experiments—The leucine concentration needed for the incubation of bacterial suspension samples was established by a saturation curve (Fig. 1). A saturating concentration of 50 $\mu\text{mol L}^{-1}$ was chosen for experiments determining the CF. This concentration is also in accordance with earlier measurements performed in the Breitenbach (Marxsen 1996). The measured leucine incorporation rate (v_{meas}) at this concentration was 0.749 $\text{pmol mL}^{-1} \text{ h}^{-1}$ and the maximum incorporation rate (v_{max}) was 0.768 $\text{pmol mL}^{-1} \text{ h}^{-1}$. Thus the ratio between v_{max} and v_{meas} at concentration 1.025, which is a measure for isotope dilution (van Looij and Riemann 1993), clearly indicates that isotope dilution was insignificant. The similarity of this saturation curve compared to saturation curves of bacteria on untreated sediment (Marxsen 1996) indicates that experimental conditions did not seem to alter greatly the metabolism of sediment bacteria.

Time series (Fig. 2) showed that an incubation time of 1 h was adequate. Incorporation of leucine declines only after more than 90 min incubation time indicating that the metabolic activity of bacteria remained constant for more than 1 h.

Theoretical conversion factor—The molar fraction of leucine expressed as percent of all amino acids was on average $8.14 \pm$

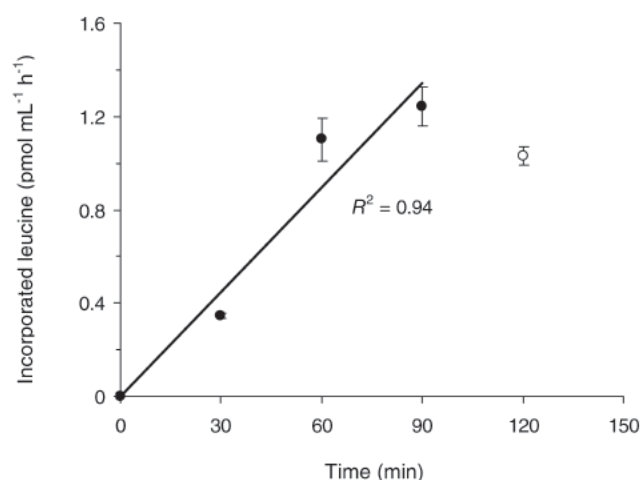


Fig. 2. Time series of leucine incorporation into protein. Symbols are means of three replicates \pm SD. For graph calculation, only the data from 0 to 90 min were used (closed circles).

0.13% (Table 1). Given a protein concentration in bacterial cells of 63% and a carbon concentration of 55% (Simon and Azam 1989) in bacteria of that size class (cell volume of 0.1 to 0.4 μm^3 , Fig. 3C), a theoretical conversion factor of 1.442 kg C mol^{-1} was calculated.

Empirical conversion factor—The parameters for calculating the empirical CF in the present study were derived by nonlinear regression analysis of the data of the exponential growth phase shown in Fig. 3 and 4. Using Eq. 1, we estimated $B_t = 1.59 \times 10^9$ and $B_0 = 2.52 \times 10^7$ and the rate of leucine incorporation integrated over time $\int \text{Leu dt} = 281559$. The estimated empirical conversion factor was 1.445 kg C mol^{-1} with an average carbon content of 260 pg per μm^3 bacterial cell. This car-

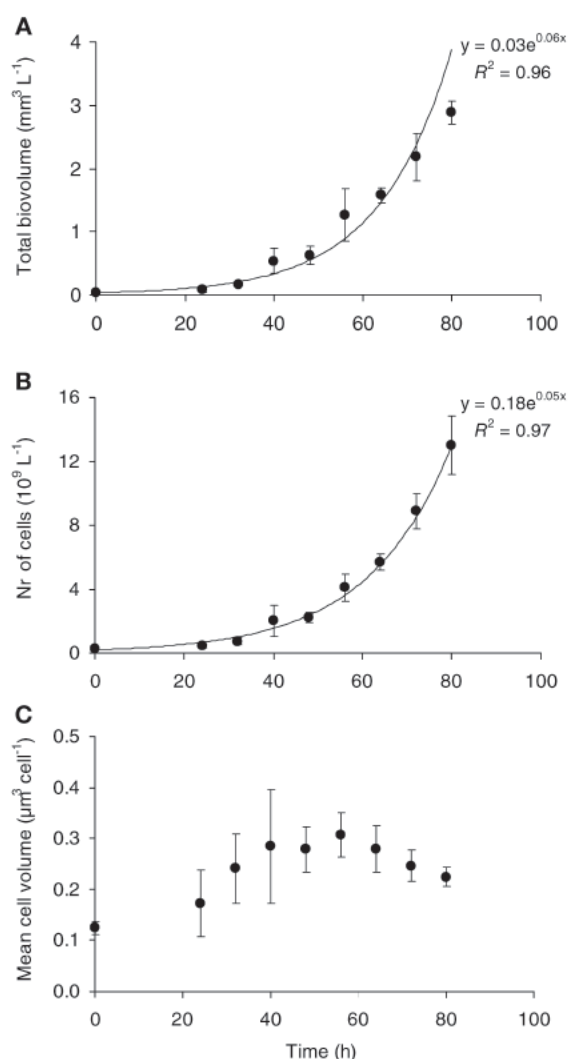


Fig. 3. Total biovolume (A), numbers (B), and average biovolumes (C) of bacteria in batch cultures throughout the experiment. Symbols are means of three replicates \pm SD.

Table 1. Molar fraction of amino acids in protein of Breitenbach freshwater sediment bacteria ($n = 27$)

Amino acid	Molar fraction	Standard error
Alanine	0.135	0.001
Arginine	0.010	0.001
Aspartic acid	0.123	0.003
Glutamic acid	0.125	0.002
Glycine	0.157	0.004
Histidine	0.010	0.001
Isoleucine	0.053	0.001
Leucine	0.081	0.001
Lysine	0.059	0.001
Methionine	0.016	0.002
Phenylalanine	0.030	0.001
Serine	0.042	0.002
Threonine	0.018	0.001
Tryptophan	0.003	0.001
Tyrosine	0.033	0.000
Valine	0.103	0.004

bon content was estimated from biovolumes of each measured cell observed in the epifluorescence microscope and subsequent image analysis. Conversion from biovolumes to carbon was performed by Eq. 2.

Discussion

The molar fraction of leucine determined in the present study for sediment freshwater bacteria (0.0814 ± 0.0013) is similar to the values determined for cultured and marine pelagic bacteria (Table 2). With Eq. 3 and using the newly determined molar fraction of leucine we estimated a theoretical CF—considering no isotope dilution—of 1.44 kg C mol^{-1} . This is close to the 1.36 kg C mol^{-1} proposed by Jørgensen (1992b), who used the molar leucine fraction of Reek (1983) established with bacterial cultures. It is somewhat lower, however, than the one calculated by Simon and

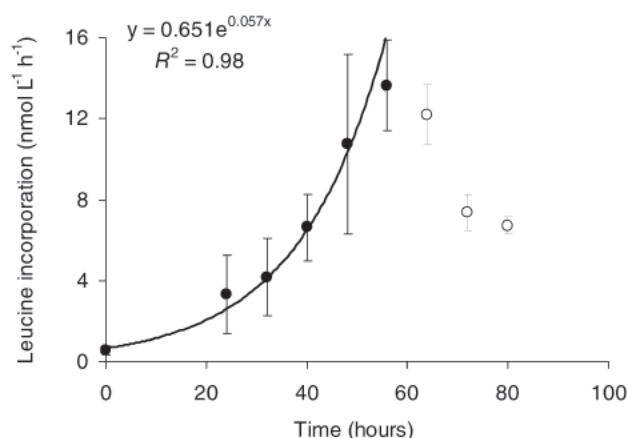


Fig. 4. Leucine incorporation rate of bacteria in batch cultures throughout the experiment. Symbols are means of three replicates ± 1 SD. Exponential growth curve was fitted through data symbolized by solid circles. Values that were not part of the exponential growth phase (given as open circles) were not included in calculation of the empirical conversion factor (Eq. 1).

Azam (1989) of $1.55 \text{ kg C mol}^{-1}$ for marine bacterioplankton (Table 2). Consequently, the application of our new CF from freshwater sediments results in bacterial production values about 93% of the values calculated with the CF from Simon and Azam (1989). Compared to the many other sources of variation associated with production measurements, this

difference can be regarded as a minor uncertainty among the published data.

Our empirical CF ($1.445 \text{ kg C mol}^{-1}$) is also within the range of CFs from investigations (0.3 to $3.95 \text{ kg C mol}^{-1}$) conducted in marine pelagic systems (Table 2), although much lower than the only CF reported from a freshwater wetland ($8.6 \text{ kg C mol}^{-1}$; Moran and Hodson 1992). However, these authors followed a different approach. As pointed out by van Looij and Riemann (1993), there are two possible approaches to correct for isotope dilution when measuring bacterial production from leucine incorporation.

Ideally, the external pool is overwhelmed by adding a high concentration of leucine, which at the same time inhibits cell internal de novo leucine synthesis (Kirchman et al. 1985; Simon 1991). This approach was taken during recent studies (this article; Marxsen 1996, 1999, 2001; Buesing and Gessner 2003; Fischer and Pusch 1999). It has the advantage that there is no measurable isotope dilution that has to be taken into account. Furthermore because one is working in the saturation range of leucine uptake, variations in uptake kinetics as well as in leucine concentrations between different sampling sites do not affect isotope dilution. Thus, only the molar fraction of leucine in protein is of relevance to convert leucine incorporation rates to bacterial protein production.

If leucine incorporation rates are measured at leucine concentrations below saturating levels, a correction for isotope

Table 2. Theoretical and empirical conversion factors in various aquatic systems

System	Theoretical CF ($\text{kg C mol}_{\text{leu}}^{-1}$)	Empirical CF ($\text{kg C mol}_{\text{leu}}^{-1}$)	Molar leucine fraction	Source
Purified bacterial proteins			0.085 ± 0.023	Reeck (1983)
Seawater culture			0.087 ± 0.004	Hagström et al. (1984)
Seawater culture	1.55		0.073 ± 0.019	Simon and Azam (1989)
Lake water	1.36*			Jørgensen (1992b)
North Sea (Belgium)		3.95		Servais (1990)
Southern Ocean		3.03		Bjørnsen and Kuparinen (1991)
Okefenokee Swamp (Georgia, USA)		8.6		Moran and Hodson (1992)
Eutrophic lakes (Northern Zealand, Denmark)		2.11†		Jørgensen (1992a)
Mesohumic lake (Southern Finland)		0.78-2.35		Tulonen (1993)
NW Mediterranean Sea				Gasol et al. (1998)
Offshore		0.3‡		
Slope		1.5‡		
Coastal		2.1‡		
Pacific Ocean (Oregon Coast, USA)				Sherr et al. (1999)
Midshelf		2.5‡		
Slope		2.3‡		
Offshore		1.6‡		
Freshwater sediment (Breitenbach, Germany)	1.44	1.45	0.081 ± 0.001	This study

*Not newly determined by Jørgensen (1992b), but calculated by this author with data on the molar leucine fraction from Reeck (1983) and on dry weight % of protein and carbon suggested by Simon and Azam (1989).

†Calculated from the CF of 6.4×10^{16} cells per mol leucine (determined by Jørgensen 1992a) using the conversion factor for bacterial biomass of Løferer-Krößbacher et al. (1998).

‡Assuming $20 \text{ fg C cell}^{-1}$ (Lee and Fuhrman 1987).

dilution is needed (Simon and Azam 1989; Riemann and Azam 1992). The size of external isotope dilution depends on the concentration of leucine added and the natural concentration. Thus differences between sampling sites need to be considered. Internal isotope dilution might also be important. Values for isotope dilution given in different studies are up to 11.5 (Simon 1991; more data summarized by van Looij and Riemann 1993). The CF determined by Moran and Hodson (1992) of 8.6 kg C mol⁻¹ may be much higher than all other CFs because isotope dilution was high. Consequently, this and other CFs determined at subsaturating leucine concentrations should not be applied to other systems.

Considering the different kind of habitats and experiments, there is a very narrow range of CFs for aquatic environments, including this study (Table 2). This is especially true if the data are compared, which were achieved when empirical CFs were determined at leucine saturation levels as well as the data for theoretical CFs. Therefore, high bacterial production determined in freshwater sediments by the leucine uptake method in recent years (e.g., Marxsen 1999, 2001; Fischer and Pusch 2001; Buesing 2002) were not overestimates caused by inappropriate use of CFs from marine systems, but represent true high bacterial production in these environments.

Even though the thymidine method theoretically represents an advantage, since transport mechanisms for assimilating thymidine and the enzyme thymidine kinase needed for nucleotide synthesis via the salvage pathway are only present in bacteria, much higher variation in CFs in the thymidine method occurs. The range of published values is much more than one order of magnitude (Riemann and Bell 1990) although most are between 1×10^{18} and 4×10^{18} cells (mol thymidine)⁻¹ (Moriarty 1988). This problem is even more pronounced because further conversion from cell number to bacterial carbon is necessary for many questions. In sediments, nearly all investigations showed significant dilution of added thymidine when very high amounts were not added (Findlay 1993).

Comments and recommendations

For studies applying the leucine technique in freshwaters, including freshwater sediments, we suggest using the theoretical conversion factor from this study to calculate bacterial production. Thus the recommended equations are

$$\text{BPP (kg)} = 1.65 \times \text{Leu}_{\text{inc}} \quad (4)$$

and

$$\text{BCP (kg)} = 1.44 \times \text{Leu}_{\text{inc}} \quad (5)$$

where BPP is bacterial protein production, Leu_{inc} is moles of leucine incorporated, and BCP is bacterial carbon production.

We consider the theoretical factor preferable because its calculation involves fewer assumptions than the empirical CF. In particular, microscopically determined bacterial biovolumes

need not be converted to bacterial biomass. This is a step that contains further assumptions and uncertainties. Many of the factors published for converting cell volume to biomass were often doubted (Watson et al. 1977; Lee and Fuhrman 1987; Nagata and Watanabe 1990).

The low variation of CFs found for the leucine uptake technique is especially valuable if studies from different environments are compared. Thus this approach for measuring bacterial production is also highly recommended for investigations that are directed to assess carbon flow and quantifying the importance of bacterial production in aquatic systems.

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