

Sample Dilution and Bacterial Community Composition Influence Empirical Leucine-to-Carbon Conversion Factors in Surface Waters of the World's Oceans

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The transformation of leucine incorporation rates to prokaryotic carbon production rates requires the use of either theoretical or empirically determined conversion factors. Empirical leucine-to-carbon conversion factors (eCFs) vary widely across environments, and little is known about their potential controlling factors. We conducted 10 surface seawater manipulation experiments across the world's oceans, where the growth of the natural prokaryotic assemblages was promoted by filtration (i.e., removal of grazers [F treatment]) or filtration combined with dilution (i.e., also relieving resource competition [FD treatment]). The impact of sunlight exposure was also evaluated in the FD treatments, and we did not find a significant effect on the eCFs. The eCFs varied from 0.09 to 1.47 kg C mol Leu⁻¹ and were significantly lower in the FD than in the F samples. Also, changes in bacterial community composition during the incubations, as assessed by automated ribosomal intergenic spacer analysis (ARISA), were more pronounced in the FD than in the F treatments, compared to unmanipulated controls. Thus, we discourage the common procedure of diluting samples (in addition to filtration) for eCF determination. The eCFs in the filtered treatment were negatively correlated with the initial chlorophyll *a* concentration, picocyanobacterial abundance (mostly *Prochlorococcus*), and the percentage of heterotrophic prokaryotes with high nucleic acid content (%HNA). The latter two variables explained 80% of the eCF variability in the F treatment, supporting the view that both *Prochlorococcus* and HNA prokaryotes incorporate leucine in substantial amounts, although this results in relatively low carbon production rates in the oligotrophic ocean.

Prokaryotic heterotrophic production (PHP), also known as bacterial production, is a key variable for evaluating the role of prokaryotes in ocean carbon fluxes. However, direct PHP measurements by means of biomass increase with time require long incubations (several days). This procedure is excessively time-consuming for routine measurements at adequate spatial and temporal scales, and therefore, PHP is typically estimated from related metabolic processes. Determining the incorporation rates of radiolabeled substrates, such as leucine and thymidine, is by far the most widespread approach due to the high sensitivity and the short incubation times required (1, 2). However, the transformation of leucine or thymidine incorporation rates into rates of prokaryotic carbon production relies on the use of conversion factors (CFs). In the case of leucine, a theoretical CF of 3.1 kg C mol Leu⁻¹ was estimated by Simon and Azam (3) based on the protein content of an average bacterial cell and the typical ratio of carbon-to-protein content, assuming a 2-fold dilution with external leucine (or 1.55 kg C mol Leu⁻¹ assuming no isotope dilution). Regardless of the systematic application of any of these two theoretical CFs in most published studies, compelling evidence indicates that the relation between leucine incorporation and carbon produced is far from constant, and thus, the variability in empirically determined CFs is large (4–8).

The determination of empirical CFs (eCFs) typically involves the facilitation of bacterial net growth by incubation of natural prokaryotic assemblages for up to several days (until they enter stationary phase) in the dark and after reduction of grazing pressure and/or increasing resource availability by dilution and/or fil-

tration, with or without added nutrients (2, 9, 10). It is not clear to what extent the experimental design may influence the obtained eCFs. For example, several studies found lower eCFs in glucose- and/or inorganic-nutrient-amended compared to unamended incubations (9–11), which suggests that a strong dilution, substantially increasing resource availability, may produce lower estimates of the leucine-to-carbon CF. The incubations under dark conditions may also affect the derived eCF, as sunlight has been shown to have a relevant impact on bacterial metabolism (12–14). However, to the best of our knowledge, the effect of light on the determination of eCFs has never been assessed.

In addition to the variability of eCFs associated with methodological factors, several studies have shown variation in relation to ecological factors, such as resource availability (5, 15), chlorophyll *a* concentration (11), prokaryotic growth efficiency (4, 6, 16), or

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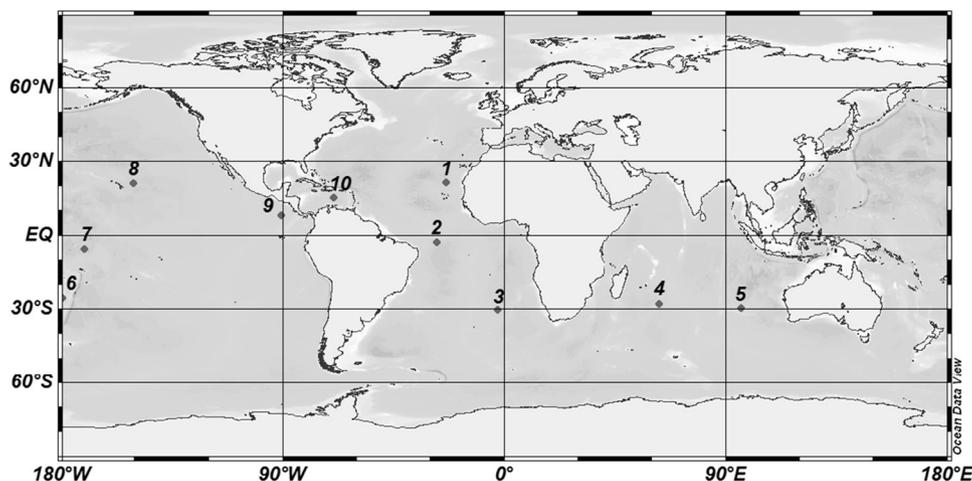


FIG 1 Map of sampling locations where leucine-to-carbon empirical conversion factor experiments were conducted. The map was generated using Ocean Data View software (R. Schlitzer, 2015 [<http://odv.awi.de>]).

bacterial community composition (11). However, only a few of these studies were conducted in open ocean surface waters (4, 6), which might hamper our ability to derive an empirical model able to predict CFs from environmental variables in vast extensions of the global ocean. Indeed, the wider the gradient of environmental conditions surveyed, the wider the range of CFs found. As an example, CFs varied by two orders of magnitude along a trophic gradient from shelf break upwelling to oligotrophic open-ocean Atlantic waters (4), and leucine-to-carbon CFs were significantly correlated only with bacterial growth efficiency, which is not a routinely measured variable. While the few published leucine-to-carbon CFs in surface oligotrophic oceanic waters are consistently low (4, 6, 17), no studies on the large-scale variability of CFs in relation to environmental factors have been conducted so far in these low-production areas.

The aim of our work was to evaluate the effect of filtration, dilution, and sunlight exposure on leucine-to-carbon CF estimates and to relate the empirically derived CFs with environmental factors in surface oceanic oligotrophic waters of the world's oceans, during the Malaspina 2010 circumnavigation expedition, designed to cover a longitudinal range of tropical and subtropical waters between 30°N and 30°S.

MATERIALS AND METHODS

Sample collection and experimental setup. Experiments were carried out at 10 stations located in the tropical and subtropical Atlantic, Indian, and Pacific oceans between 14 December 2010 and 14 July 2011 during the Malaspina 2010 circumnavigation expedition on board the R/V *Hespérides* (Fig. 1). Conductivity-temperature-depth (CTD) casts were carried out at each station with a Sea-Bird Electronics 911 Plus probe attached to a rosette equipped with Niskin bottles. Samples for phosphate and nitrate measurements were frozen, and the concentrations were determined by standard colorimetric methods with a Technicon autoanalyzer. Chlorophyll *a* concentration was fluorometrically determined after biomass concentration on 0.2- μm -pore-size polycarbonate filters and extraction in 90% acetone.

Seawater for the experiments was collected at a 3-m depth using 30-liter Niskin bottles. UV radiation-transparent 3-liter carboys were used for the incubations. Each experiment consisted of 3 treatments (in duplicate): filtration through 0.8- μm -pore-size polycarbonate filters to remove large predators while maintaining most free-living prokaryotes and expo-

sure to sunlight (referred to as light plus filtration [LF]), filtration and dilution (0.8- μm -filtered seawater diluted [1:5] with 0.2- μm -filtered seawater) to reduce both predators and resource competition and exposure to sunlight (referred to as light plus filtration plus dilution [LFD]), and filtration and dilution under dark conditions (referred to as dark plus filtration plus dilution [DFD]). A control consisting of unmanipulated seawater exposed to sunlight (light control [LC]) was also incubated in order to check for changes in bacterial community composition associated with sample manipulation. LC, LF, and LFD samples were incubated on deck under natural light conditions, and DFD samples were incubated on deck under dark conditions. The experimental carboys were kept at near *in situ* temperature by circulating surface seawater in the incubation tank. The experiments lasted 3 days, and samples were taken every 12 to 24 h for heterotrophic prokaryotic biomass (HPB) (as estimated by flow cytometry) and leucine incorporation rate measurements. The integrative method was used for the leucine-to-carbon conversion factor calculation (18) using the time intervals where an increase in heterotrophic prokaryotic biomass was observed. The conversion factor was calculated as the HPB (kg C liter^{-1}) produced over the selected time period of the experiment (essentially, the final HPB minus the initial HPB for that time period) divided by the total amount of leucine ($\text{mol leucine liter}^{-1}$) incorporated during that time period. The total amount was measured by integrating the incorporation rates over the selected time period.

Bacterial community composition was assessed at the beginning and at the end of the experiments using the automated ribosomal intergenic spacer analysis (ARISA) fingerprinting technique (see below).

Flow cytometry analyses. Samples were fixed and processed with a FACSCalibur flow cytometer (BD Biosciences) with a blue laser emitting at 488 nm. Samples of 1.2 ml of seawater were fixed with a paraformaldehyde-glutaraldehyde mix (1% and 0.05% final concentrations, respectively) and stored at -80°C until analysis in the laboratory within 7 months after the end of the cruise. Samples were stained with SYBR green I, at a final concentration of 1:10,000, for 15 min in the dark at room temperature. The average flow rate used was $12 \mu\text{l min}^{-1}$, and acquisition time ranged from 30 to 260 s depending on cell concentration in each sample. Data were inspected in a FL1-versus-light side scatter (SSC, also termed right-angle light scatter [RALS]) plot and analyzed as detailed by Gasol and del Giorgio (19), including the differentiation of the two widespread groups of low-nucleic-acid (LNA) and high-nucleic-acid (HNA) prokaryotes. Latex beads (1 μm ; Molecular Probes) were always used as internal standards. The biovolume of prokaryotic cells was estimated using the calibration obtained by Calvo-Díaz and Morán (20) relating relative light side scatter (population SSC divided by bead SSC) to cell diam-

TABLE 1 Environmental conditions at the beginning of the leucine-to-carbon conversion factor experiments^a

Expt and ocean	Temp (°C)	Chla concn (μg liter ⁻¹)	PO ₄ ⁻ concn (μmol liter ⁻¹)	NO ₃ ⁻ concn (μmol liter ⁻¹)	Leucine incorporation (pmol Leu liter ⁻¹ h ⁻¹)	HPB (μg C liter ⁻¹)	%HNA	Picocyanobacterial abundance (10 ⁴ cells ml ⁻¹)	C content (fg C cell ⁻¹)	Virus abundance (10 ⁶ ml ⁻¹)
1; N Atlantic	24.80	0.18	NA	0.509 ^b	30.2	6.39	41	9.68	13.2	13.71
2; Eq Atlantic	27.50	0.15	0.078	NA	168.6	11.14	51	13.30	13.6	5.92
3; S Atlantic	22.50	0.03	0.170	0.361 ^b	34.5	3.84	25	1.37	13.1	NA
4; S Indian	25.90	0.09	0.033	0.262 ^b	22.4	4	47	3.31	12.3	1.54
5; S Indian	21.70	0.07	0.019	0.191	6.3	2	38	5.74	9.8	2.99
6; S Pacific	24.00	0.13	0.089	0.143	5.5	4.12	53	6.23	12.9	3.10
7; Eq Pacific	28.30	0.18	0.319	2.280	2,362.4	14	51	6.87	15.9	9.91
8; N Pacific	24.02	0.09	0.083	0.028	6.2	7.67	37	1.51	12.8	4.35
9; N Pacific	28.20	0.21	0.229	0.377	68.3	7.21	38	15.00	10.2	43.44
10; N Atlantic	28.70	0.09	0.068	0.340	35.8	6	27	4.67	12.7	12.50

^a Temperature, chlorophyll *a* (Chla), phosphate concentration, nitrate concentration, and virus abundance correspond to unmanipulated seawater. Leucine incorporation rates, heterotrophic prokaryotic biomass (HPB), percent high-nucleic-acid-content prokaryotes (%HNA), picocyanobacterial abundance, and prokaryotic cell carbon content correspond to 0.8-μm-filtered seawater. Eq, equatorial; NA, not available.

^b Data from a 7- to 17-m depth within the mixed layer.

eter, assuming spherical shape. Cell biovolume was finally converted into carbon biomass with the equation of Gundersen et al. (21): cell biomass (in femtograms of C per cell) = 108.8 × V^{0.898}. Counts of *Prochlorococcus* picocyanobacteria, which tended to overlap partially with the HNA prokaryote cluster, were subtracted from total heterotrophic prokaryotic counts by independent assessment in nonstained aliquots. *Synechococcus* and *Prochlorococcus* cyanobacteria were in turn identified in plots of SSC versus red fluorescence (FL3; >650 nm) and of orange fluorescence (FL2; 585 nm) versus FL3. Picocyanobacterial biomass was calculated by using the following volume-to-carbon conversion factors: 230 fg C μm⁻³ for *Synechococcus* and 240 fg C μm⁻³ for *Prochlorococcus* (22).

Leucine incorporation rates. The [³H]leucine incorporation method (23), modified as described by Smith and Azam (24), was used to determine leucine incorporation rates. From each experimental carboy, six 1.5-ml vials (4 replicates and 2 killed controls) were filled with 1.2 ml of seawater. A total of 120 μl of cold 50% trichloroacetic acid (TCA) was added to the killed controls. After 15 min, 20 nmol liter⁻¹ of L-[4,5-³H]leucine (144.2 Ci mmol⁻¹; Amersham) was added to all samples, which were incubated for 2.5 to 6 h in the same incubation tank and under the same light conditions as the corresponding experimental carboys.

ARISA. Automated ribosomal intergenic spacer analysis (ARISA) was conducted with DNA extracted from samples taken at the beginning and the end of each experiment. Seawater samples (1 to 2 liters) were prefiltered through a 3-μm-pore-size filter and subsequently filtered through a 0.2-μm-pore-size polycarbonate filter (47-mm filter diameter; Nuclepore Whatman). Filters were then stored at -80°C until DNA extraction. Microbial community DNA was extracted using an Ultra Clean soil DNA isolation kit (MoBio Laboratories, Inc.) and quantified in a Nanodrop spectrophotometer. Bacterial ARISA was performed using the ITSf/ITSr primer set (Thermo Scientific) previously described by Cardinale et al. (25). The PCR mixture (25 μl) contained final concentrations of 1× PCR buffer (Genecraft), 2.5 mmol liter⁻¹ MgCl₂ (Genecraft), 250 μmol liter⁻¹ of each deoxynucleoside triphosphate (dNTP) (Genecraft), 250 nmol liter⁻¹ of the universal primers ITSf (5'-GTCGTAACAAGGTAGCCGTA-3') and eubacterial ITSr (5'-GCCAAGGCATCCACC-3') (8) (the former being labeled at the 5' end with fluorescein amidite dye [6-FAM]), 40 ng μl⁻¹ bovine serum albumin, 3.5 U of BioThermD *Taq* DNA polymerase (GeneCraft), and approximately 0.13 ng μl⁻¹ of template DNA. The reaction mixture was held at 94°C for 2 min, followed by 32 cycles of amplification at 94°C for 15 s, 55°C for 30 s, and 72°C for 3 min, with a final extension of 72°C for 10 min. The PCRs were conducted in duplicate for each DNA extract (this compensates for any anomalously running fragments both in the samples as well as in the standards). Amplification products were sent for capillary electrophoresis migration on a 50-cm-capillary ABI Prism 3730XL DNA analyzer (Applied Biosystems) at Genoscreen (www.genoscreen.fr/). A standardized migration cocktail contained 0.5 μl of amplification product, 0.25 μl of the internal size

standard LIZ 1200 (20 to 1,200 bp; Applied Biosystems), and 8.75 μl of deionized Hi-Di formamide (Applied Biosystems). The mixture was denatured 5 min at 95°C and kept on ice before being further processed by the sequencer. Capillary electrophoresis parameters were 10 kV (run voltage), 1.6 kV (injection voltage), 22 s (injection time), and 63°C (oven temperature). The resulting electropherograms were analyzed using the DAX software (Data Acquisition and Analysis Software; Van Mierlo Software). Internal size standards were built by using a second-order least-squares method and local Southern method. Profiles were double checked manually for perfect internal size standard fit and stable baselines. Baselines were then extracted, and subsequently, peak heights and absolute areas were determined. The same process was done for the PCR-negative sample. From the negative sample, the 95th percentile was calculated for the height measurement and used as a threshold. Samples with peak heights below the 95th percentile were discarded (the 95th percentile of each duplicated PCR negative presented relative fluorescence intensity [RFI] values of 9 and 8.7, respectively).

Profile peaks were binned and reordered by operational taxonomic units (OTUs) by using R automatic binning and interactive binning scripts (26). Binning was carried out independently of the sample (peaks from all samples together). To avoid size-calling imprecisions, a window size (WS) of 2 bp (determined by preliminary empirical tests) was used for the binned method, and only peaks in the range from 200 to 1,200 bp and with peak values above 0.09% of the total RFI were taken into account. Peaks from duplicates were manually checked using binned-OTU tables, to avoid erroneous OTU divisions due to rearrangement of all samples together.

Statistical analyses. Repeated-measures analysis of variance (RMANOVA) was performed to evaluate the significance of the differences observed in the eCFs among the different treatments (LF, LFD, and DFD). The square root-normalized OTU relative abundances assessed with ARISA were used to calculate pairwise similarities in bacterial community composition among samples based on the Bray-Curtis similarity index. Similarity patterns among samples were examined using a hierarchical cluster analysis. Dendrograms were generated using the group average method, and the SIMPROF (similarity profile) test was used to test for differences between the generated clusters at the 95% confidence level. All the ARISA statistical analyses were completed in PRIMER-E v6 (27).

RESULTS

Initial conditions. A considerable range of initial seawater conditions was observed in the experiments, both for environmental variables (Table 1) and for bacterial community composition (Fig. 2). Chlorophyll *a* concentrations in the unfiltered seawater ranged from 0.03 μg liter⁻¹ in the South Atlantic (experiment 3) to 0.21 μg liter⁻¹ in the western tropical Pacific (experiment 9) (Table 1).

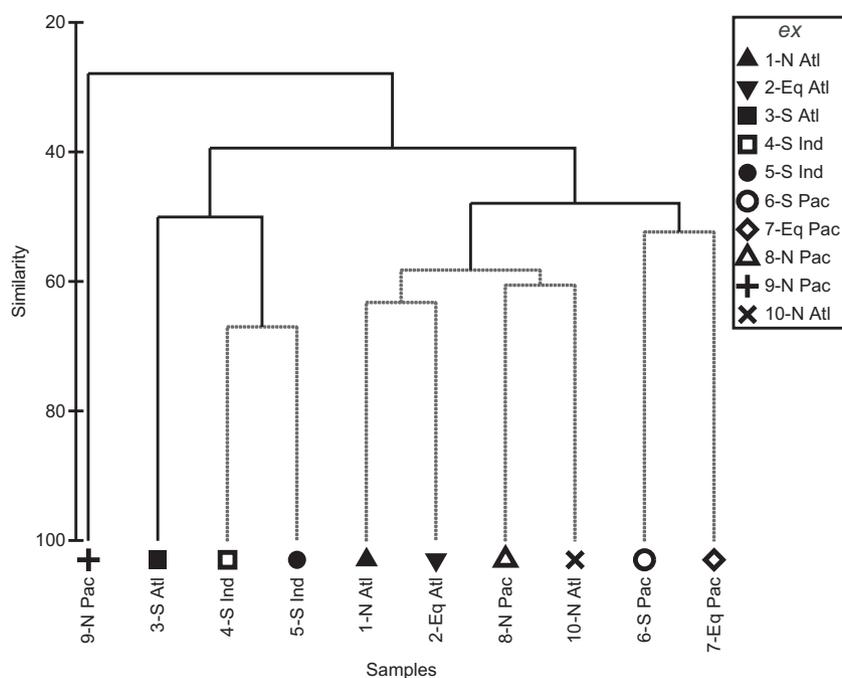


FIG 2 Dendrogram based on automated ribosomal intergenic spacer analysis (ARISA) profile similarities of unmanipulated seawater at the beginning of the experiments. Dashed lines represent statistically significant clusters ($P < 0.05$) according to the SIMPROF test. Eq, equatorial; Atl, Atlantic; Ind, Indian; Pac, Pacific.

Phosphate concentration also varied about 10-fold, from $0.02 \mu\text{M}$ in the Indian Ocean to $0.32 \mu\text{M}$ in the equatorial Pacific (Table 1). In contrast, nitrate concentration varied by two orders of magnitude, from $0.03 \mu\text{M}$ in the North Pacific to $2.28 \mu\text{M}$ in the equatorial Pacific (Table 1). Leucine incorporation rates in the $0.8\text{-}\mu\text{m}$ -filtered seawater varied by three orders of magnitude, whereas prokaryotic heterotrophic biomass (PHB) and picocyanobacterial abundance (PCA) varied by one order of magnitude (Table 1). Approximately 50% of the picocyanobacteria in the original sample were present in the $<0.8\text{-}\mu\text{m}$ fraction (data not shown). *Prochlorococcus* represented $>90\%$ of the picocyanobacteria in all the experiments except in experiment 9 (eastern tropical North Pacific), which was dominated by *Synechococcus* (details not shown). Heterotrophic prokaryotes were generally dominated by LNA cells, with the proportion of HNA cells ranging from 27 to 53%. The initial bacterial community composition as assessed by ARISA showed significant differences among sampling sites (Fig. 2), although there were no significant differences in the bacterial assemblages among experiments 1, 2, 8, and 10 (samples from Atlantic and North Pacific oceans), between experiments 6 and 7 (samples from equatorial and South Pacific oceans), and between experiments 4 and 5 (samples from the Indian Ocean). The highest bacterial community composition similarity (68%) was observed between the experiments conducted in the Indian Ocean. Bacterial community composition in the eastern tropical North Pacific Ocean (experiment 9) differed most from those in other locations ($<35\%$ similarity).

eCFs. Empirical leucine-to-carbon conversion factors (eCFs) were determined by comparison of leucine incorporation rates with the increase in bacterial biomass during the experimental incubations (Fig. 3). The resulting eCFs ranged from 0.09 ± 0.01 to $1.47 \pm 0.08 \text{ kg C mol Leu}^{-1}$, showing values close to or higher

than $1 \text{ kg C mol Leu}^{-1}$ in the filtered treatments in 4 out of 10 experiments. Overall, eCFs were higher in the filtered than the filtered and diluted treatments (Fig. 4). The eCFs in the LF, LFD, and DFD treatments followed similar variability patterns (Fig. 4), although significant differences were found among treatments (RMANOVA; $P = 0.018$). Pairwise comparisons showed that eCFs were significantly higher in the LF than in both LFD and DFD treatments (Bonferroni test; $P < 0.042$). No significant differences between LFD and DFD treatments were found (Bonferroni test; $P > 0.05$), although eCFs were lower in the light than in the dark treatments in experiments 8 and 10.

In order to relate the observed variability in eCFs with environmental factors, we conducted a correlation analysis (Table 2). The conversion factors in the FD treatment (under either light or dark conditions) did not significantly correlate with any of the considered variables. In contrast, the eCFs in the LF treatment showed significant and strong negative correlations to chlorophyll *a* concentration, picocyanobacterial abundance, and the percentage of HNA prokaryotes (r ranging from -0.67 to -0.80 ; $P < 0.05$) (Table 2). Significant semilogarithmic or linear relationships were found between the LF eCFs and picocyanobacterial abundance (Fig. 5A) or the %HNA (Fig. 5B), explaining 64% and 56% of the observed variability, respectively. A multiple linear regression model including LF eCFs as a dependent variable and both %HNA and log picocyanobacterial abundance as independent variables explained 80% of the variability in the LF eCFs ($\text{eCFs} = 4.98 [\pm 1.04] - 0.73 [\pm 0.25] \times \log \text{picocyanobacterial abundance} - 0.021 [\pm 0.09] \times \% \text{HNA}$; $r^2 = 0.80$; adjusted $r^2 = 0.75$; $P = 0.005$; $n = 10$) (standard errors are in brackets). Log picocyanobacterial abundance had a greater effect on F eCFs (beta coefficient = -0.57 ; $P = 0.022$) than %HNA (beta coefficient = -0.46 ; $P = 0.049$).

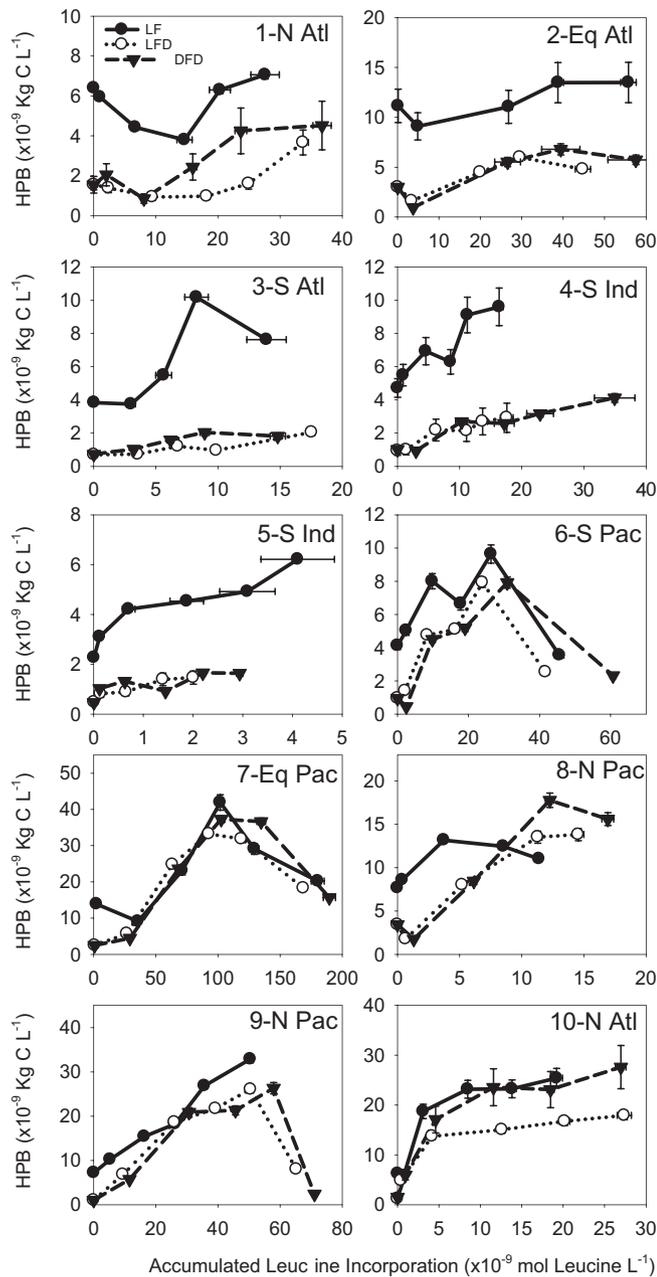


FIG 3 Heterotrophic prokaryotic biomass (HPB) versus accumulated leucine incorporation for light plus filtration (LF), light plus filtration plus dilution (LFD), and dark plus filtration plus dilution (DFD) treatments in each of the 10 experiments. Eq, equatorial; Atl, Atlantic; Ind, Indian; Pac, Pacific. Error bars represent the standard errors for duplicates.

Bacterial community composition in the unmanipulated control treatment showed, on average, 35% similarity to the initial bacterial community composition after 3 days of incubation (data not shown). The manipulation of nutrient availability (by dilution), nutrient availability and grazing pressure (filtration and dilution), and light caused changes in bacterial community composition (Fig. 6). However, the resulting community was mostly determined by the initial bacterial assemblage, since the samples clustered primarily by experiment. Some experiments showing similar eCFs also showed similar bacterial communities, such as

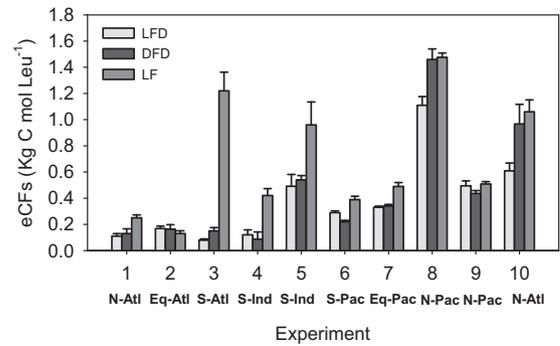


FIG 4 Mean leucine-to-carbon empirical conversion factors (eCFs) obtained for LF, LFD, and DFD treatments in each of the 10 experiments. Eq, equatorial; Atl, Atlantic; Ind, Indian; Pac, Pacific. Error bars represent the standard errors for duplicates.

experiments 1 and 2 or experiments 8 and 10 (Fig. 4 and 6). The effect of solar radiation on the bacterial community composition was negligible in most of the diluted treatments, as no significant differences were observed between LFD and DFD samples (SIMPROF test; $P < 0.05$), except in experiments 4, 8, and 10. The average similarity between LFD and DFD samples was 63%. On the other hand, when comparing the unmanipulated control samples (LC) with LF and LFD samples, we observed that filtration alone (LF) had fewer effects on bacterial community composition (average similarity of 60%) than the combination of filtration and dilution (LFD) (average similarity of 46%). Indeed, bacterial community composition in LF samples was not significantly different from that in LC samples in 4 out of 8 experiments (SIMPROF test; $P < 0.05$) (Fig. 6). In contrast, bacterial community composition in LFD samples did not significantly differ from that in LC samples in only 1 out of 8 experiments (SIMPROF test; $P < 0.05$) (Fig. 6).

DISCUSSION

Considering the widely demonstrated variability in eCFs, the use of constant theoretical CFs may produce erroneous estimates of prokaryotic heterotrophic production (PHP) by relying only on estimates of substrate incorporation rates rather than also on the fate of the incorporated compounds (i.e., fraction of substrate that is not assimilated into biomass) (6, 28). Several studies have

TABLE 2 Pearson correlation coefficients between eCFs in LFD, DFD, and LF treatments and different environmental factors^a

Factor	Coefficient for treatment		
	LFD	DFD	LF
Temp	NS	NS	NS
Chlorophyll <i>a</i>	NS	NS	-0.67*
Phosphate	NS	NS	NS
Ln nitrate	NS	NS	NS
Leucine incorporation	NS	NS	NS
HPB	NS	NS	NS
%HNA	NS	NS	-0.75*
Log picocyanobacterial abundance	NS	NS	-0.80**
C content	NS	NS	NS
Virus abundance	NS	NS	NS

^a HPB, heterotrophic prokaryotic biomass; %HNA, percentage of high-nucleic-acid-content prokaryotes. *, $P < 0.05$; **, $P < 0.01$; NS, not significant. $n = 10$, except for phosphate ($n = 9$).

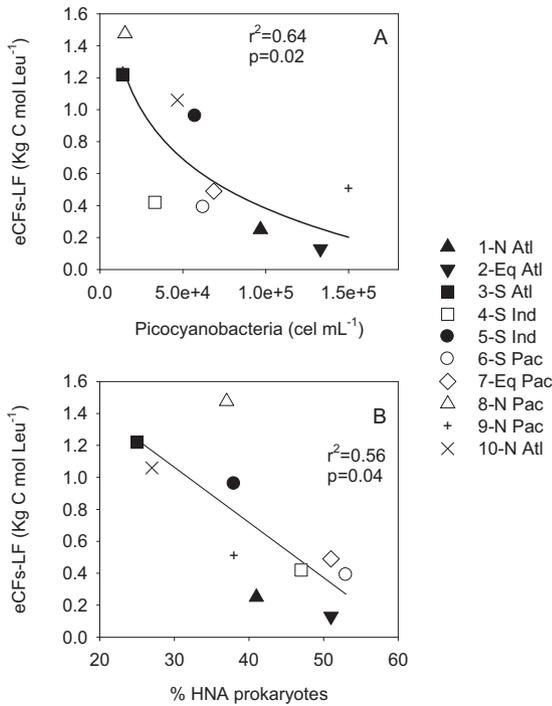


FIG 5 Relationship between LF eCFs and picocyanobacterial abundance (A) or the percentage of HNA prokaryotes (B). Eq, equatorial; Atl, Atlantic; Ind, Indian; Pac, Pacific.

shown that the use of theoretical leucine-to-carbon CFs may overestimate both temporal and spatial variability in prokaryotic biomass production (4, 5). However, as CF experiments are time-consuming and labor-intensive, unraveling which environmental factors drive changes in eCFs and deriving empirical models for predicting eCFs from basic environmental variables represent a

present challenge in aquatic microbial ecology. The broad range of environmental conditions sampled in our survey (Table 2) allowed us to find an empirical model that could be useful for deriving eCFs in surface oligotrophic oceanic waters from data obtained by flow cytometry, the usual method for estimating microbial plankton abundance.

Filtration versus filtration and dilution in eCF experiments. Although there are several extensive studies reporting leucine-to-carbon eCFs across a variety of environmental conditions, a meta-analysis is not easy to conduct due to the great variability in the prokaryotic community pretreatments. Early work by Coveney and Wetzel (9) evaluating the effects of different pretreatments (filtration, dilution, and nutrient addition) on thymidine eCFs consistently found lower eCFs associated with the addition of phosphorus. In the case of leucine, only the study by Alonso-Sáez et al. (11) systematically tested the effect of nutrient addition on the eCFs in prefiltered and 20×-diluted samples from a coastal station along an annual cycle in the Mediterranean Sea. Significantly lower eCFs were found in inorganic-nutrient-enriched than in unamended seawater samples, and substantial changes in bacterial community composition were observed in association with nutrient additions. Those authors hypothesized that addition of inorganic nutrients could lead to a situation of C limitation, where leucine would be utilized to obtain energy, leading to low net biomass production regardless of high leucine incorporation rates. Interestingly, the addition of carbon compounds such as glucose (10) or glucose and acetate (29) did not seem to affect leucine eCFs in studies performed in temperate coastal and Antarctic waters, respectively. Kirchman (30) also found that thymidine and leucine eCFs were not affected by addition of organic compounds in the subarctic Pacific.

Although we did not directly test the effect of nutrient enrichment, we did evaluate the effect of dilution of the sample with 0.2-μm-prefiltered seawater, which can also increase nutrient availability. Most of the published leucine-to-carbon eCFs derive

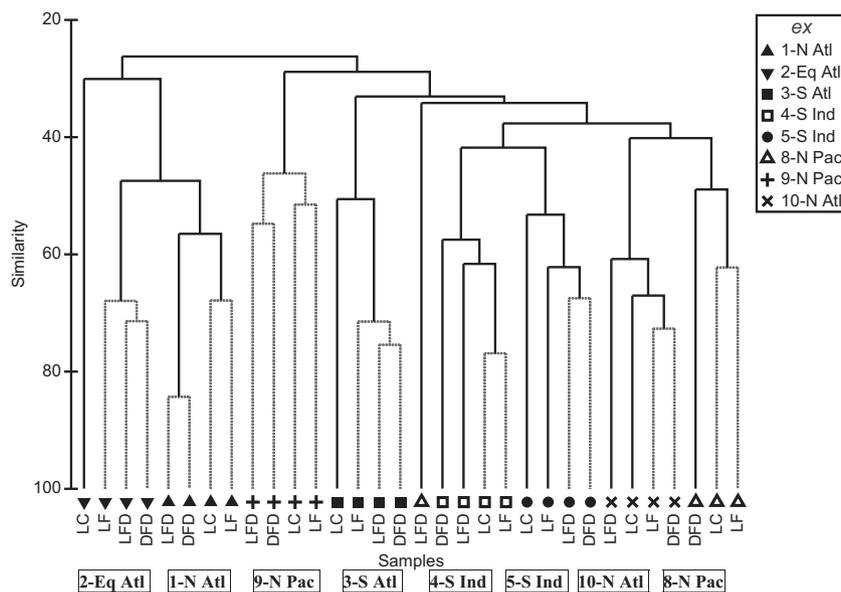


FIG 6 Dendrogram based on automated ribosomal intergenic spacer analysis (ARISA) profile similarities of seawater samples from LC, LF, LFD, and DFD treatments at the end of the experiments. Dashed lines represent statistically significant clusters ($P < 0.05$) according to the SIMPROF test. Eq, equatorial; Atl, Atlantic; Ind, Indian; Pac, Pacific.

from incubation of filtered and diluted seawater, with dilution factors ranging from $1\times$ (10) to $20\times$ (11); however, the potential influence of dilution on the leucine-to-carbon eCF estimations had not been tested so far. The significantly lower eCFs obtained in the filtered and diluted (LFD and DFD) compared to the filtered (LF) treatments are in agreement with the aforementioned negative effect of nutrient enrichment on eCF estimates. Thus, our results suggest that dilution treatments may not be appropriate in CF experiments in oligotrophic areas. The effect of dilution was variable among the experiments (Fig. 4), which may be related to the degree of nutrient limitation in each seawater incubation. According to the hypothesis of C limitation proposed by Alonso-Sáez et al. (11) and del Giorgio et al. (6), the effect of filtration and dilution on eCFs is expected to be higher when C is the primary limiting element. Under these conditions, the filtration and dilution increase mineral nutrient availability, which in turn may exacerbate C limitation, as primary production is drastically reduced after filtration, leading to an uncoupling of leucine incorporation and bacterial biomass production (i.e., low eCFs). The greatest difference between eCFs obtained with LFD and eCF obtained with LF was observed in experiment 3, where the lowest chlorophyll *a* concentration was measured (Table 1), suggesting that strong carbon limitation was the primary cause of the discrepancy.

A further argument that might discourage the dilution pretreatment in CF experiments in oligotrophic areas is the fact that the bacterial community composition developed in LFD treatments was significantly different than that in unmanipulated (LC) seawater, while LF samples were much more similar to the unmanipulated controls (Fig. 6). Although both dilution and nutrient enrichment appear to affect leucine-to-carbon eCFs, we found only one study, conducted in Antarctic waters, where eCFs were estimated in filtered but nondiluted seawater incubations (29). The eCFs derived by those authors were very similar to those obtained by Teira et al. (31) in the same sampling region using the dilution approach. Summertime Southern Ocean waters are already nutrient rich (29, 31), and an increase in nutrient availability through dilution may have little effect on eCFs, supporting the role of trophic status in the effect of the different experimental manipulations for estimating eCFs.

Only the variability in LF eCFs could be explained by any of the environmental factors measured in our study (Table 2). We are aware that other nonincluded ecological factors might also have influenced the eCFs, such as dissolved organic carbon (DOC) concentration, leucine catabolism, or bacterial growth efficiency (4, 6, 15). However, we believe that the lack of a coherent explanation for the variability in eCFs in the diluted treatments may be at least partially related to the inadequacy of the experimental design. Considering the high variability that the methodological approach may introduce in eCF estimates, and in view of the large changes in bacterial communities and the lower leucine yields associated with dilution, we strongly recommend avoiding dilution in eCF experiments, at least in oligotrophic waters.

Relationship between eCFs and environmental factors. Regardless of the potential methodological problems, some potential explanations for the leucine-to-carbon eCFs variability have been postulated to date. Overall, eCFs higher than the theoretical ($1.55 \text{ kg C mol Leu}^{-1}$, assuming no isotopic dilution) can be explained by the isotopic dilution of the radiotracer. If the radiotracer is not added at a saturating concentration, then the measured leucine

incorporation rates will be lower than the actual rates, leading to artificially high eCFs. This problem may typically occur in coastal eutrophic waters, where the ambient leucine concentration may be higher than the commonly used concentrations of added radiotracer (20 to $40 \text{ nmol liter}^{-1}$). In fact, leucine-to-carbon eCFs higher than the theoretical one have been repeatedly found at coastal sites (6, 11, 32–34).

In open ocean oligotrophic waters, the leucine-to-carbon eCFs are consistently lower than the theoretical one (4, 6, 17), which implies an unbalanced bacterial growth in which net bacterial biomass production is low regardless of relatively high leucine incorporation rates. We also measured eCFs lower than the theoretical one in all of the experiments (Fig. 4). An unbalanced bacterial growth under limiting conditions has been described, where protein synthesis is maintained in order to maximize survival rather than growth and reproduction (3, 15, 35), resulting in high turnover rates of intracellular protein (36). However, Alonso-Sáez et al. (4) measured relatively low protein turnover rates in oligotrophic waters of the subtropical Atlantic Ocean and concluded that the low eCFs found in the area were related to leucine catabolism by energy-limited bacterial cells. According to this hypothesis, leucine would be incorporated into the cell but a high portion would be respired before being used for protein synthesis; this would translate into low eCFs if the tritium signals of the incorporated and respired leucine were recovered by cold trichloroacetic acid. High percentages of leucine respiration (40 to 80%) associated to low eCFs have been found by Alonso-Sáez et al. (4) and del Giorgio et al. (6), revealing a significant negative correlation between the percentage of leucine respired and the eCFs ($r = -0.46$; $P < 0.001$; $n = 25$) (unpublished data).

Low leucine-to-carbon eCFs have been also related to a faster synthesis of transport proteins relative to cell duplication when substrate availability is low (5). Calvo-Díaz and Morán (5) obtained a significant empirical model to predict eCFs from leucine incorporation rates and cellular carbon contents. However, samples were not prefiltered in their study, and thus grazing, although reduced by dilution, could have influenced their results. In our data set, we did not find any significant correlation between eCFs and either leucine incorporation rates or bacterial cellular carbon content (Table 2). Moreover, by contrast with previous studies (11), we found a negative correlation between LF eCFs and chlorophyll *a*, indicating that higher eCFs occurred under limiting conditions. However, it is important to note that although we sampled a relatively wide range of environmental conditions, the trophic gradient was rather limited (chlorophyll *a* $< 0.21 \text{ mg m}^{-3}$), and thus, energy limitation supposedly occurred at all sampling sites.

The lower LF eCFs associated with high picocyanobacterial abundance, dominated by *Prochlorococcus*, could be related to the ability of these autotrophic bacteria to incorporate leucine (37–40). As both picoautotrophs and heterotrophs contribute to leucine assimilation, but only the biomass production of heterotrophs is taken into account in the calculation of eCFs, a high abundance of picocyanobacteria during the experiments may result in high bulk leucine incorporation rates irrespective of low heterotrophic prokaryotic biomass production. Mean picocyanobacterial biomass during the incubations ($0.86 \pm 0.18 \mu\text{g C liter}^{-1}$) was lower than heterotrophic prokaryotic biomass ($6.8 \pm 1.1 \mu\text{g C liter}^{-1}$). Even if picocyanobacteria represented only 2 to 20% of total prokaryotic abundance, their contribution to leucine

incorporation could be considerable, as *Prochlorococcus* cells may display higher cell-specific incorporation rates than heterotrophic bacteria because of their larger volume (39). The few existing estimates indicate that *Prochlorococcus* may contribute 24 to 63% of the total microbial plankton leucine incorporation into proteins (38, 39).

We also found a significantly negative relationship between LF eCFs and the percentage of HNA prokaryotes (Table 2; Fig. 5B). As HNA prokaryotes appear to have higher cell-specific leucine incorporation rates than LNA prokaryotes (39, 41) and tend to be more susceptible to viral infection than LNA prokaryotes (42–44), the low LF eCFs could also be related to high cell-specific leucine incorporation rates along with a low net biomass increase of this bacterial functional group during the incubations. As grazing was minimized by prefiltration, we hypothesize that other factors, such as viral lysis or apoptosis, could be responsible for low net biomass accumulation during our incubations. The fact that picocyanobacterial abundance and the relative abundance of HNA cells explained 80% of the variability observed in LF eCFs in these oligotrophic waters suggests that prokaryotic community composition, and particularly these two prokaryotic groups, have a strong influence on eCF estimates.

In conclusion, we have shown that whereas light exposure does not have a clear effect on the leucine-to-carbon eCFs, the dilution pretreatment tends to reduce the carbon-to-leucine yield and promotes important changes in bacterial community composition (assessed with ARISA fingerprinting) compared to unmanipulated seawater samples. Filtration alone, on the other hand, allowed bacterial biomass increase and did not imply important changes in bacterial community composition, thus appearing to be an adequate experimental approach for deriving empirical conversion factors, as the environmental characteristics would be closer to those found under *in situ* conditions compared to the filtered and diluted treatments. We also provide a new perspective to explain low eCFs in oceanic oligotrophic waters, in addition to the previously proposed hypotheses of high protein turnover and leucine catabolism. We hypothesize that eCF variability patterns could be driven, in part, by low net biomass accumulation of highly active prokaryotes during incubation. As prokaryote biomass loss cannot be totally avoided during conversion factor experiments, the prokaryotic production rates derived from the application of eCFs may not yield gross biomass production rates, which should be taken into account when microbial carbon budgets are constructed.

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