Applied and Environmental Microbiology, May 2009, p. 3216–3221 Vol. 75, No. 10
0099-2240/09/$08.00+0 doi:10.1128/AEM.01570-08

Empirical Leucine-to-Carbon Conversion Factors for Estimating Heterotrophic Bacterial Production: Seasonality and Predictability in a Temperate Coastal Ecosystem▽

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Received 10 July 2008/Accepted 16 March 2009

Leucine-to-carbon conversion factors (CFs) are needed for converting substrate incorporation into biomass production of heterotrophic bacteria. During 2006 we performed 20 dilution experiments for determining the spatiotemporal variability of empirical CFs in temperate Atlantic coastal waters. Values (0.49 to 1.92 kg C mol Leu^{-1}) showed maxima in autumn to early winter and minima in summer. Spatially averaged CFs were significantly negatively correlated with in situ leucine incorporation rates ($r = -0.91$) and positively correlated with phosphate concentrations ($r = 0.76$). These relationships, together with a strong positive covariation between cell-specific leucine incorporation rates and carbon contents ($r = 0.85$), were interpreted as a strategy to maximize survival through protein synthesis and low growth rates under nutrient limitation (low CFs) until favorable conditions stimulate cell division relative to protein synthesis (high CFs). A multiple regression with in situ leucine incorporation rates and cellular carbon contents explained 96% of CF variance in our ecosystem, suggesting their potential prediction from more easily measurable routine variables. The use of the theoretical CF of 1.55 kg C mol Leu^{-1} would have resulted in a serious overestimation (73%) of annual bacterial production rates. Our results emphasize the need for considering the temporal scale in CFs for bacterial production studies.

Bacterial production (BP) is a key parameter for evaluating the role of heterotrophic bacterioplankton in ocean carbon cycling. However, BP cannot be directly measured and is rather estimated from related metabolic processes. Incorporation of radioactively labeled substrates such as thymidine (TdR) and leucine (Leu) are by far the most widespread approaches. Both methods are based on measuring some aspect of cellular macromolecular synthesis (DNA in the case of TdR and protein in the case of Leu). Substrate incorporation rates are then converted into rates of macromolecular synthesis and eventually into rates of biomass production (i.e., cells or cellular carbon or nitrogen) (17). This final step requires some conversion factor (CF). Since CFs are not easy to measure routinely and since CF determination usually involves the incubation of natural samples for several days, literature values are still often used in spite of strong evidence of their variability (11). The values of these constant CFs are 3.1 or 1.55 kg C mol Leu^{-1} (assuming an isotope dilution of 2 or no isotope dilution, respectively) (26) and 2×10^{18} cells mol TdR^{-1} (5).

Given the reported high variability in empirically determined CFs in many ecosystems (16), it should always be preferred to estimate them rather than using a fixed theoretical value, especially in low-productivity environments (23), where empirical CFs are usually much lower than the theoretical ones (2). Sources of empirical CF variability include the design of dilution culture incubations and the choice of calculation methods (11), in addition to ecologically relevant characteristics, such as the physiological state of bacteria and the amount and quality of organic and inorganic substrates (24). Recent studies tend to include empirical CFs, but seldom has the seasonal component been taken into account. If this component is significant, there would be uncertainty in quantifications of the role of the bacterioplankton in global carbon cycling.

With the aim of determining the spatial and temporal variability of leucine-to-carbon (Leu-to-C) empirical CFs in temperate coastal waters, we conducted an annual cycle of dilution culture experiments at three stations located in the south Bay of Biscay continental shelf. On the one hand, we wanted to assess the ecological implications of this variability for quantifying carbon fluxes through the ecosystem. On the other hand, we also wanted to explore the predictability of the empirical Leu-to-C CFs in this temperate ecosystem from easily and routinely measurable environmental variables such as inorganic nutrient concentrations and bacterial activity and cellular properties.

MATERIALS AND METHODS

Sampling strategy. The study was conducted along a transect perpendicular to the north Iberian coast. Three stations (station 1 [St1], 43.58°N, 5.61°W; St2, 43.67°N, 5.58°W; St3, 43.78°N, 5.55°W) located over the continental shelf were sampled every 2 months during 2006 on board R/V *José de Rioja* as part of the Instituto Español de Oceanografía (IEO) time-series project Radiales. Maximum depths were 20 (St1), 100 (St2), and 150 m (St3). Temperature was acquired from conductivity-temperature-depth casts using a SeaBird 25. Samples for nutrients (NO_3^-, NO_2^-, PO_4^-, and SiO_2) were frozen, and their concentrations were determined with a Technicon autoanalyzer within 6 months. All experiments were performed with surface water (5-m depth) except the August

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▽ Published ahead of print on 20 March 2009.

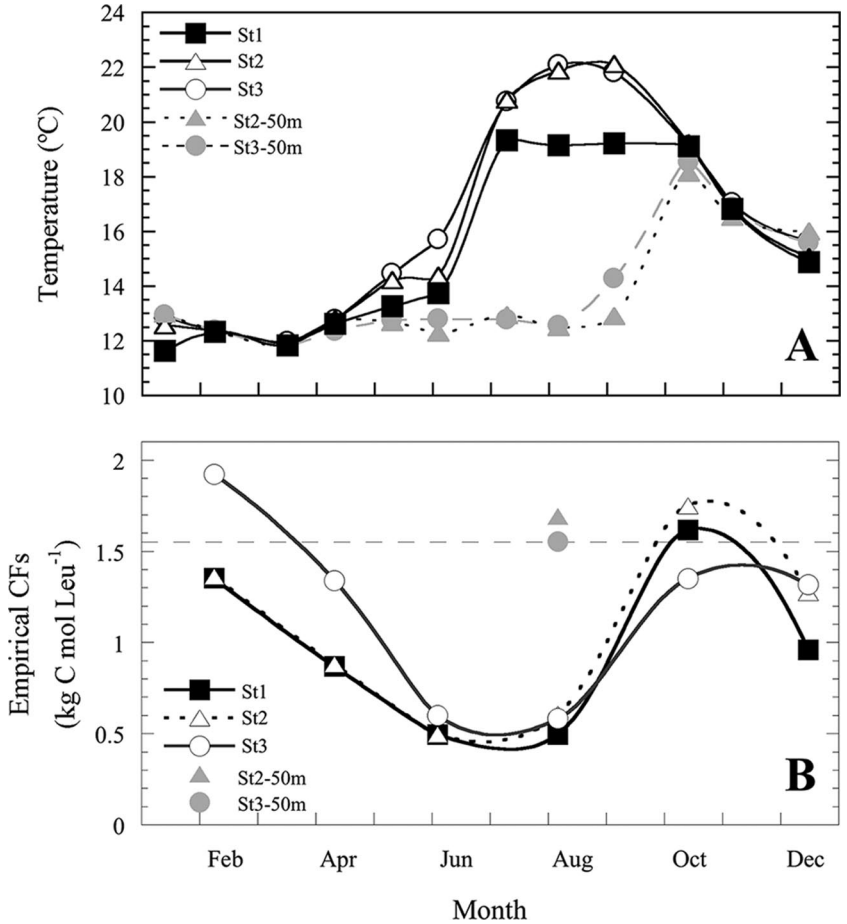

FIG. 1. (A) Seasonal variations of temperature (°C) at the surface and 50-m depth at the three stations (St1, St2, and St3) during 2006. (B) Empirical leucine-to-carbon CFs determined at the surface by the cumulative method. Additional determinations at 50-m depth in August are also shown for St2 and St3. The dashed line represents the theoretical CF of 1.55 kg C mol Leu^{-1} (25).

sampling, where due to strong thermal stratification additional sampling at 50 m was also undertaken.

Bacterial abundance and biomass. Samples for determining the abundance of heterotrophic bacteria (1.8 ml) were preserved with 1% paraformaldehyde plus 0.05% glutaraldehyde (final concentration), left for 10 min in the dark to fix, and frozen at −80°C until analysis in the laboratory with a FACSCalibur flow cytometer (Becton & Dickinson) equipped with a laser emitting at 488 nm. Prior to analysis, heterotrophic bacteria were stained with 2.5 μmol liter^{-1} Syto-13 DNA fluorochrome (Molecular Probes). Bacteria were detected by their distinct signature in a plot of side scatter versus green fluorescence (7). A solution of 1-μm fluorescent latex beads (Molecular Probes) was added as an internal standard. All cellular variables were related to values for fluorescent beads. An empirical calibration between relative side scatter and cell diameter (7) was used to estimate bacterial biovolumes (BBv), which were very similar to previous values reported for these shelf waters obtained by microscope measurements (4, 28). We used the allometric relationship of Norland (22) for converting BBv to cellular carbon content (CCC): CCC (pg cell^{-1}) = 0.12 × BBv$^{0.72}$. Although vertical and seasonal variations in cell size were observed, in agreement with previous reports as already noted (7), bacterial biomass was largely determined by cell abundances rather than changes in cell size.

Leucine-to-carbon CF experiments. Dilution experiments in order to determine the in situ Leu-to-C CFs were performed every 2 months at each station with surface water (5-m depth). Due to strong stratification (Fig. 1A), two additional experiments with water from 50 m were also performed at St2 and St3 in August. The water sample (300 ml) was diluted (1:5) with 0.2-μm-filtered (Acropack 1000; Pall) seawater and kept in 1.5-liter acid-cleaned polycarbonate bottles in the dark at in situ temperature (±1.5°C). Subsamples were taken for estimating Leu incorporation (see below) and bacterial biomass at intervals of 6 to 24 h until bacteria reached the stationary growth phase. Three different methods of estimating CFs were compared here (see Results and Discussion): modified derivative (10), cumulative (6), and integrative (25).

LIR and BP. The [^{3}H]leucine incorporation method (14), modified as described by Smith and Azam (27), was used to determine Leu incorporation rates (LIR). For each sampled depth and each time interval of the dilution experiments, four aliquots (1 ml) plus two trichloroacetic acid-killed controls were placed into Eppendorf tubes and incubated with Leu at saturating concentration (40 nmol liter^{-1}, final concentration) for 1.5 to 2 h at temperatures as close as possible to the in situ temperatures in water baths (±1.8°C). This concentration was tested for rate saturation during an earlier experiment in the sampling zone. Ambient LIR values at each station were determined in an illuminated incubator simulating the irradiance found at the sampling depths, whereas all LIR determinations in the dilution experiments were performed in the dark. A comparison of levels of production of heterotrophic bacteria was carried out; the comparison was based on empirical CFs and the theoretical Leu factor specified by Simon and Azam (26) assuming no isotope dilution, 1.55 kg C mol Leu^{-1}.

RESULTS AND DISCUSSION

Methodological considerations. There are currently four ways of calculating empirical Leu-to-C CFs (12): the derivative method (15) and the already-mentioned modified derivative, integrative, and cumulative methods. When changes in LIR equal changes in bacterial biomass, all calculation methods give the same CF (17). However, uncoupling between incor-

TABLE 1. Empirical CFs at the three sampling stations calculated by different methods for the specified time ranges, as well as bacterial biomasses and LIR

Date (2006)	Station	Depth (m)	Time range (h)	BB[a] (μg C liter^{-1})		LIR (pmol Leu liter^{-1} h^{-1})		CF by[b]:		
				Initial	Final	Initial	Final	Mod. deriv.	Integ.	Cumul.
8 February	1	5	0–41.2	7.9	13.8	11.6	260	6.58	1.28	1.35
	2	5	0–41.2	7.5	14.5	7.7	429	13.17	1.27	1.36
	3	5	0–16.1	7.7	8.3	14.3	99	4.20	1.71	1.92
11 April	1	5	0–48.2	7.5	17.7	34.2	388	2.37	0.83	0.87
	2	5	0–48.2	7.1	19.0	36.4	409	2.31	0.91	0.88
	3	5	0–48.2	5.3	20.4	20.6	364	3.64	1.37	1.34
5 June	1	5	0–48.5	6.6	24.0	76.5	881	1.24	0.56	0.50
	2	5	0–48.5	5.6	22.9	78.0	1,020	1.25	0.52	0.49
	3	5	0–48.5	3.9	20.2	34.3	750	1.92	0.63	0.60
8 August	1	5	0–22.8	11.1	19.5	137.0	896	1.30	0.65	0.50
	2	5	0–22.8	8.9	15.9	139.7	879	1.44	0.53	0.61
	2	50	0–45.9	2.9	16.9	18.5	263	3.50	1.76	1.69
	3	5	0–22.8	7.6	12.1	46.8	606	2.38	0.45	0.58
	3	50	0–45.9	4.3	21.2	41.9	442	3.19	1.57	1.56
16 October	1	5	0–43.6	5.9	25.5	18.7	385	3.90	1.74	1.62
	2	5	0–37.2	4.6	18.5	2.7	348	16.50	1.71	1.75
	3	5	0–37.2	3.9	11.1	11.3	267	5.78	1.31	1.35
19 December	1	5	0–64.0	4.9	15.3	2.1	294	15.58	0.94	0.96
	2	5	0–64.0	2.5	34.1	1.1	667	67.15	1.27	1.27
	3	5	0–64.0	3.3	23.2	4.7	448	14.32	1.39	1.32

[a] BB, bacterial biomass.
[b] Mod. deriv., modified derivative method (9); Integ., integrative method (24); Cumul., cumulative method (6).

poration and growth is very often observed, as was the case in our experiments. In this instance, the original derivative method (15) yields anomalously high values. For this reason, we restricted the model performance comparison to the remaining three approaches (Table 1).

The modified derivative method uses the change in bacterial abundance to estimate BP at the start of the experiment and puts maximum weight on cell numbers. But one of the limitations of both derivative methods is that they require that increases in incorporation accurately reflect increases in bacterial biomass. This assumption is often not met because of time lags between both processes. Hence, two alternative "model-free" approaches were proposed: the integrative method, based on the calculation of the total biomass produced and total Leu incorporation during some interval, and the cumulative method, which estimates the slope of bacterial biomass versus cumulative Leu incorporation at different time intervals. Unless growth is closely balanced, i.e., when cell- and Leu incorporation-based rates are equal, these "model-free" approaches tend to give lower values than the modified derivative method (11, 12).

The results of the cumulative and integrative methods were very similar, ranging from 0.45 to 1.76 and from 0.49 to 1.92 kg C mol Leu^{-1}, respectively (Table 1). Both methods were highly correlated ($r = 0.98$, $P < 0.001$, $n = 20$), in agreement with the findings of Li et al. (19) for the western North Atlantic Ocean and J. M. Gasol (unpublished data) for the Mediterranean Sea, and statistically indistinguishable (paired t test, $P = 0.77$, $n = 20$). In addition, CF values obtained with the aforementioned methods were much lower than those obtained with the modified derivative method. The latter method yielded sometimes unrealistically high values, especially in autumn and winter (exceeding the theoretical values by up to 67 kg C mol Leu^{-1}) (Table 1). One problem with the integrative approach is the difficulty of determining which incubation length should be used to do the integration, whereas the cumulative method takes all data into account, making thus optimal use of the data available. For this reason, we chose Leu-to-C CFs obtained by the cumulative method for subsequent analysis. These values were also used for comparison with BP estimates derived from theoretical CFs.

Seasonal changes and predictability. A clear seasonal pattern emerged from our results (Fig. 1), with high Leu-to-C CFs observed in February (1.35 to 1.92 kg C mol Leu^{-1}), close to the theoretical CF of 1.55 kg C mol Leu^{-1}, followed by a gradual decrease to minima in June and August (0.49 to 0.61 kg C mol Leu^{-1}). A subsequent rise was observed in October (1.35 to 1.75 kg C mol Leu^{-1}), with values very similar to those found in February and at 50-m depth in summer (approximately 1.6 kg C mol Leu^{-1}). Relatively higher winter CFs compared with autumn and summer values were previously found in a Mediterranean coastal site (3). The values obtained were comparable to those reported for nearby coastal waters (0.67 to 3.55 kg C mol Leu^{-1} (4, 21), and values found at the surface in June and August were similar to those reported for oligotrophic waters (e.g., 0.58 [1], 0.73 [20], and 0.02 to 1.29 [2] kg C mol Leu^{-1}), suggesting a correspondence between open ocean and summer stratified coastal waters (23). Very similar

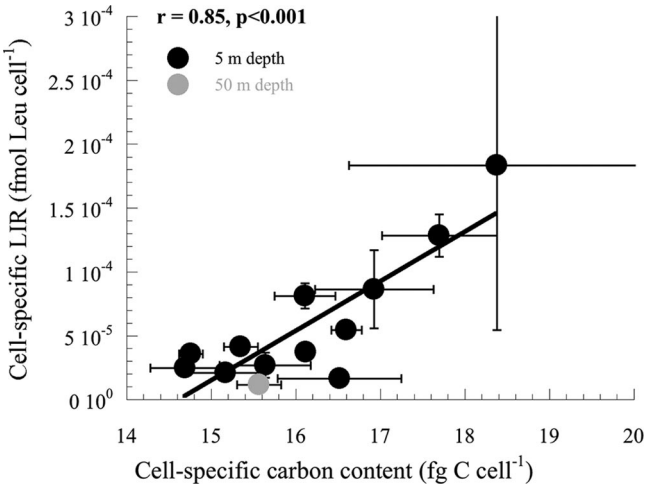

FIG. 2. Relationship between averages (±SE) for the three stations of cell-specific leucine incorporation rates and carbon contents during 2006. Surface data for every month are included together with the mean values at 50-m depth at St2 and St3 in August. The fitted line represents ordinary least-squares linear regression ($n = 13$).

temporal trends were found at the three stations during the annual cycle (Table 1 and Fig. 1). With the exception of the first two experiments at St3, spatial variability over the continental shelf was of minor importance; hence, we calculated spatially averaged CFs for each sampling time.

Leucine-to-carbon CFs integrate concurrent variations in bacterial biomass growth and substrate incorporation. Therefore, the finding of a lower biomass production than expected from the use of the theoretical CF during most of the annual cycle (Fig. 1) can be explained by a bacterial community with a slow division rate or low cell size increase. However, we must also take into account that Leu incorporation into protein could result in no net protein synthesis if there were significant protein turnover, i.e., simultaneous synthesis and degradation of cellular protein, altogether resulting in low CFs. In the same way, assimilated Leu can be used to obtain energy (leucine catabolism) rather than allocated to protein synthesis (2), which would also result in low CFs, since tritium from respired [^{3}H]Leu would still be collected by cold trichloroacetic acid (2) and its radioactivity could be measured with no real change in bacterial biomass.

Neither protein turnover nor Leu catabolism was estimated in this study. However, the significant correlation between mean cell-specific LIR and carbon content values for all data pooled (Fig. 2) could indicate that these processes were not important year-round. A higher cell-specific Leu incorporation rate would be expected in cells with a higher CCC in order to meet carbon-to-protein ratios, if we assume them constant (26). On the other hand, truly high rates of protein turnover or Leu catabolism would result in virtually no net protein synthesis, i.e., no biomass production.

A faster protein synthesis (estimated as cumulative Leu incorporation) relative to cell duplication could be a strategy for survival under unfavorable environmental conditions (9, 24). In this regard, Church (9) suggested that bacteria can regulate the number of active transporters used to acquire substrates. So, under conditions of low substrate availability, a greater fraction of energy can be devoted to substrate uptake (via synthesis of transport proteins) at the expense of cell duplication. The positive and significant correlation between phosphate concentrations and CFs that was found (Fig. 3A) is consistent with this hypothesis. Remarkably, values at 50-m depth were consistent with the general relationship found for surface values. In a Mediterranean seasonal study by Alonso-Sáez et al. (3), higher CFs were associated with increased chlorophyll *a* levels. Although we did not find a significant relationship with chlorophyll *a* (data not shown), both studies suggest that the efficiency of bacterial biomass production is higher along the gradient of trophic state.

Thus, and perhaps counterintuitively, high LIR would not necessarily imply high CFs, as shown in Fig. 3B. To our knowledge, this is the first study to report such a relationship in marine waters. Chrzanowski et al. (8) and Kirschner et al. (18)

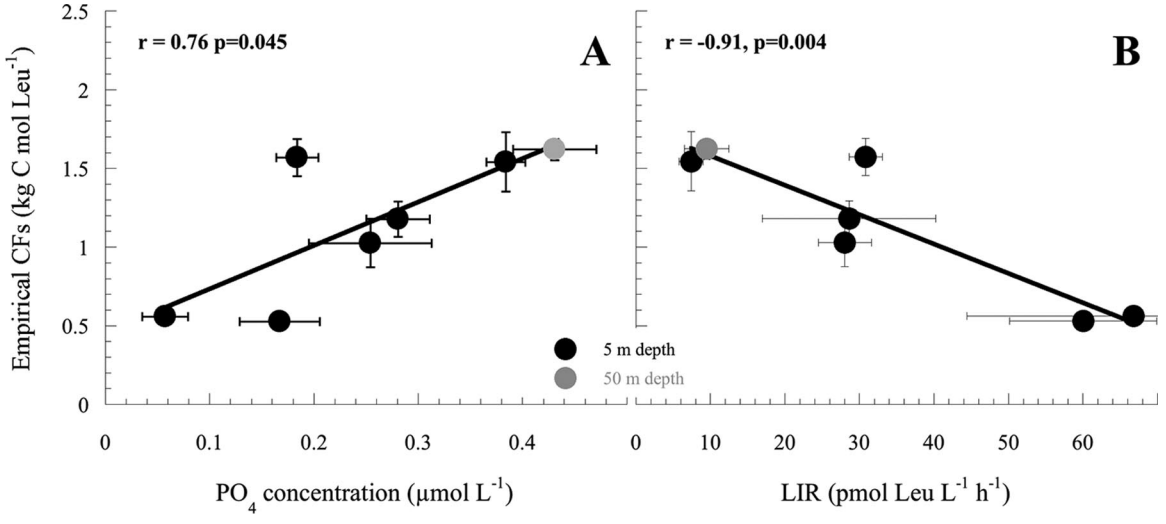

FIG. 3. Relationships between empirical leucine-to-carbon CFs averaged for the three sampled stations and mean (A) PO_4^- concentrations and (B) LIR. Fitted lines represent ordinary least-squares linear regressions ($n = 7$). Error bars represent SE.

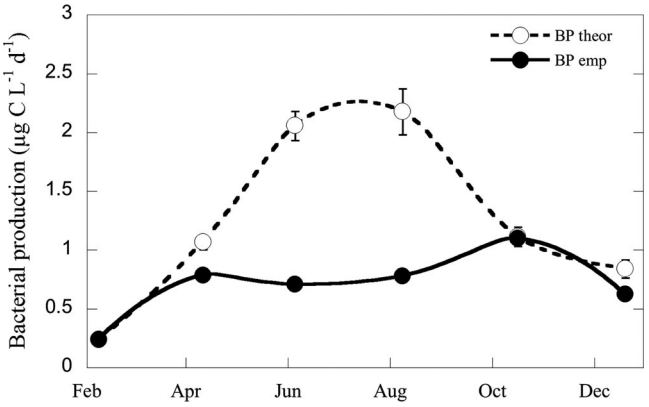

FIG. 4. Seasonal variation of mean surface BP estimated with empirical (emp) (Table 1) and theoretical (theor) (1.55 kg C mol^{-1}) (25) Leu-to-C CFs in the south Bay of Biscay continental shelf. Error bars represent SE of the weighted means for the three stations.

had reported a similar negative relationship between TdR incorporation rates and the corresponding TdR-to-cell CFs in meso- and eutrophic lakes. Relationships between bacterial size and activity are complex (13). A relatively small (~20%) increase in carbon content per cell turned into a much larger range of variation in cell-specific LIR (Fig. 2). However, these results suggest that, although under nutrient-limiting conditions larger cells may be found with higher LIR per cell, the resulting high bulk LIRs did not translate into a comparatively large effect on bacterial carbon fluxes at the ecosystem level.

Since performing dilution experiments at every BP determination can become rather tedious, we explored the possibility of predicting CFs from other routine variables by performing a stepwise multiple regression analysis with mean PO_4^-, NO_3^-, LIR, and CCC as potentially explanatory variables. The resulting equation included only LIR and CCC ($CF = 6.24\ [\pm 1.36] - 0.29\ [\pm 0.09] \times CCC - 0.01\ [\pm 0.002] \times LIR$; $r^2 = 0.96, P < 0.005$, $n = 7$) (standard errors [SE] are in brackets), which jointly explained ~95% of the variance in CFs. Generalization of this type of Leu-to-C CF empirical models based on other more easily measurable variables to other ecosystems would be of great help for marine carbon cycling models.

Ecological implications. We estimated a mean value of BP for the entire continental shelf of our sampled transect using the measured LIR and CFs at each station and compared it with the value obtained using the theoretical CF (Fig. 4). The comparison yielded radically different seasonal patterns. Thus, BP clearly peaked in late spring to early summer when the theoretical CF was used while rather homogeneous values (range, 0.24 to 1.10 μg C $liter^{-1}$ day^{-1}), characterized by a weak maximum in autumn, were found with empirical CFs. The negative relationship between LIR and CFs (Fig. 3B) was largely responsible for this result. Besides differences in seasonality, the magnitudes of BP also differed greatly depending on the choice of CF. Thus, the marked theoretical-CF-based BP peak of 2.18 μg C $liter^{-1}$ day^{-1} found in August greatly exceeded any empirically based value. These differences have a great effect in the estimated carbon fluxes through heterotrophic bacterioplankton, resulting in an annual overestimation of BP of 73% had we used the theoretical rather than the empirical CFs.

Precise measurements of BP and respiration are needed to quantify carbon fluxes in marine ecosystems given their major role in both biogenic carbon production and carbon remineralization in the ocean. In conclusion, this study illustrates the importance of estimating accurate substrate-to-carbon CFs with an appropriate temporal resolution that takes into account the specific conditions of the study system rather than assuming literature values. The use of a constant CF would have resulted in BP estimations entirely shaped by LIR values in spite of the strong negative correlation between LIR and empirical CFs found here. This work shows that temporal variations may be substantial when empirical CFs are calculated and consequently critical when quantifying the seasonal dynamics of the role of pelagic bacteria in marine carbon cycles. Finally, we demonstrate here that it was possible to predict leucine-to-carbon CFs from other basic variables with great certainty.

ACKNOWLEDGMENTS

This work was supported by the Spanish research grant Temporal Variability of the Planktonic Communities in the Central Cantabrian Sea (VARIPLACA, REN2001-0345/MAR) and the time-series project Radiales from the IEO. A.C.-D. was a recipient of a predoctoral FPI fellowship from the Spanish Ministry of Education and Science.

We are indebted to the captain and crew of R/V *José de Rioja*, to L. Á. Suárez for his help with flow cytometry analysis, and to C. Carballo from IEO-Coruña for making nutrient determinations. Comments by J. E. Hobbie, J. M. Gasol, and two anonymous reviewers are greatly appreciated.

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