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Variability in the empirical leucine-to-carbon conversion factors along an environmental gradient

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Abstract

Bacterial production is one of the key parameters to evaluate bacterial role in ocean carbon fluxes. Estimation of bacterial production requires the leucine-to-carbon conversion factors that change widely across environments. However, empirical leucine-to-carbon conversion factors (eCFs) are seldom determined *in situ* because of time consuming and little is known on regulating factors for the eCFs. During May 2015 to January 2016, fourteen dilution experiments were conducted, from the Zhujiang (Pearl River) Estuary to the coast of the northern South China Sea, to determine spatiotemporal variability in the eCFs and its potential controlling factors along an environmental gradient. The eCFs showed clear spatial variations with the highest (1.27–1.69 (kg C)/(mol Leu)) in low salinity waters (salinity<15), intermediate (1.03–1.25 (kg C)/(mol Leu)) in moderate salinity (salinity of 15–25), and the lowest (0.48–0.85 (kg C)/(mol Leu)) in high salinity waters (salinity>25). Substrate availability was responsible for spatial variability in the eCFs. In the pristine coastal waters, low eCFs was related to substrate limitation and leucine incorporated was respired to maximize the survival rather than bacterial production. Hence, the eCFs measurement was needed for estimating bacterial production accurately in various marine environments.

Key words: bacteria, leucine incorporation, conversion factors, Zhujiang (Pearl River) Estuary

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1 Introduction

Bacteria play a significant role in transferring dissolved organic carbon (DOC) to particulate organic carbon (POC) and further to higher trophic levels through microzooplankton as bacterial predator (Fuhrman, 1992; McManus and Fuhrman, 1988; Pulido-Villena and Reche, 2003). Bacterial production (BP) is used to express the transform rate of DOC to POC. It is, therefore, of great importance to accurately measure BP for understanding the ocean carbon cycling. So far, BP is mainly estimated based on the incorporation rates of radioactively labeled thymidine (TdR) or leucine (Leu) into bacterial DNA or protein, through conversion factors of thymidine-to-carbon or leucine-to-carbon (Fuhrman and Azam, 1980; Kirchman et al., 1985).

In recent years, it has been recognized that many environmental factors can cause variability of conversion factors, such as the quality and quantity of organic and inorganic substrates, and physiological state of bacteria (Ducklow et al., 1992; Pulido-Villena and Reche, 2003). However, since empirical conversion factors determinations through experimental bioassays usually last for several days and are labor-consuming, most researches prefer to use the theoretical conversion factors of 1.55 (kg C)/(mol Leu) (assuming no isotope dilution) or 3.1 (kg C)/(mol Leu) (assuming an isotope dilution of 2) (Simon and Azam, 1989) and 2×10^{18} cells/(mol TdR) (Fuhrman and Azam, 1980). Previous studies have shown that eCFs vary dramatically across environments, ranging from 0.09 (kg C)/(mol Leu) in the equatorial Atlantic (Teira et al., 2015) to 3.95 (kg C)/(mol Leu) in the Northern Ocean (Servais, 1990), even up to 29.81 (kg C)/(mol Leu) in some estuarine system of Ria de Aveiro (Baptista et al., 2011). Hence, it is necessary to determine empirical leucine-to-carbon conversion factors (eCFs) in the study area, in order to estimate BP accurately.

The Zhujiang River (Pearl River) is the second largest river in China in terms of the freshwater discharge. The annual average freshwater discharge of this river is ~3.26×10^{11} m^3 (Dai et al., 2006), 80% of which takes place in the wet season (April to September). The river water contained high nutrients (nitrate greater than 100 μmol/L) (Yin et al., 2001), and DOC concentrations (~473 μmol/L) (He et al., 2010) in the wet season, respectively. A significant amount of nutrients in the Zhujiang River water are not utilized duly, and ultimately are delivered into the adjacent shelf of the northern South China Sea (Dai et al., 2008). As a result, a clear environmental gradient occurs in the wet season from the Zhujiang Estuary to the shelf of the northern South China Sea (Xu et al., 2008). In this study, fourteen dilution experiments were conducted in areas with different environmental

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conditions during May 2015 to June 2016, in order to examine variability in the eCFs and its regulators across environments.

2 Materials and methods

2.1 *Sampling sites*

The Zhujiang Estuary and its adjacent coastal waters with gradient changes of nutrition were selected as our study waters. Total eleven stations were chosen in the Zhujiang Estuary and its adjacent coastal waters (Fig. 1), with five stations (E1–E5) in the eutrophic Zhujiang Estuary, which were visited during May and August of 2015 and during January 2016, three stations (M1, M2 and M3) in mesotrophic waters with influence of the Zhujiang River freshwater discharge, sampled during May 2016, three (P1, P2 and P3) in relatively pristine coastal waters with no influence of the Zhujiang River freshwater discharge during June 2016. Water samples were taken at the surface (1 m depth) for nutrients, chlorophyll *a* (Chl *a*), bacterial abundance and dilution experiments. The labels of the stations (starting with M and P) in this study have been renamed from the original stations for the cruise, as shown in the following pairs: M1=57, M2=60, M3=64, P1=95, P2=99 and P3=104.

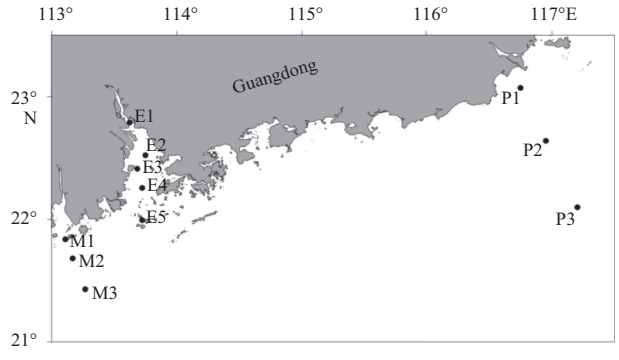

Fig. 1. Map of sampling locations.

2.2 *Salinity, nutrients and Chl a*

Water salinity was measured using YSI 556MPS (Yellow Springs) or SBE 911 plus CTD (Sea-Bird Electronics) in the Zhujiang Estuary (Stas E1, E2, E3, E4 and E5) and its adjacent coastal waters (Stas M1, M2, M3, P1, P2 and P3), respectively.

Samples for nutrients (NO_3^-, NO_2^-, NH_4^+, PO_4^{3-}, and SiO_3^{2-}) were filtered through Whatman GF/F glass fiber filters and stored at –20°C until analyzed. Nutrient samples collected from the Zhujiang Estuary were determined colorimetrically with a spectrophotometer (Metash, V-5000) following the protocols described by Grasshoff et al. (1999). Samples collected from the adjacent coastal waters in May and June 2016 were measured using an AA3 Nutrient Autoanalyzer (Bran-Luebbe, Gmbh), and the low concentrations of NO_3^- +NO_2^- and PO_4^{3-} were measured by the long-cell method (Li et al., 2008; Li and Hansell, 2008) by incorporating a 50 cm liquid waveguide cell to AA3 with detection limits of 0.02 and 0.01 μmol/L, respectively. Dissolved inorganic nitrogen (DIN) in the Zhujiang Estuary was the sum of NO_3^-, NO_2^- and NH_4^+, and DIN in the coastal water mainly refereed to NO_3^-.

Water samples (500–1 000 mL) for Chl *a* were filtered onto the Whatman GF/F glass fiber filters. Chl *a* was extracted with acetone (90%, v/v) in the dark for 24 h at 4°C and analyzed using a fluorometer (Turner Designs, Trilogy®) (Parsons et al., 1984).

2.3 *Experimental setup*

A filtration combined with dilution method was adopted to determine the eCFs. Firstly, seawater was passed through 20-μm screen mash to obtain *in situ* bacterial abundance sample. Then water sample was passed through a 1 μm filter, and the filtrate was used as the bacterial inoculum. Subsequently, about 1 L of the filtrate was filtered through a 0.2 μm polyethersulfone capsule (PALL) to obtain bacteria-free seawater. The 100 mL of the bacterial inoculum was mixed with 900 mL bacteria-free seawater in an acid-washed and Milli-Q rinsed glass bottle and incubated in the dark for 21–60 h. Running water was used to maintain the surface *in situ* temperature. Samples were taken at the interval of 2.5–22 h during the incubation to determine ^{3}H-leucine incorporation rate and bacterial abundance.

2.4 *^{3}H-leucine incorporation rate and bacterial abundance*

^{3}H-leucine incorporation rate was measured using a microcentrifugation method (Smith and Azam, 1992) and detailed sampling operations according to the JGOFS protocol (Knap et al., 1996). ^{3}H-leucine (final concentration 27 nmol/L, specific activity 54.1 Ci/mmol) was added to the control and triplicate samples. The control was prepared by adding cold trichloroacetic acid (final concentration of 5%). Samples were incubated in dark for 1 h, stopped by adding cold trichloroacetic acid and then stored at –20°C until analysis with a liquid scintillation counter (PerkinElmer).

For bacterial abundance, water samples were collected in microcentrifuge tubes, fixed with glutaraldehyde (final concentration 0.5%), kept at the room temperature for 10 min, and subsequently stored in liquid nitrogen. Bacterial abundance was determined with a flow cytometer (Becton-Dickinson Accuri™ C6) following the method of Marie et al. (1997). The data acquisition and analysis were directly performed with Becton-Dickinson Accuri™ C6 software, mainly detected on the plot of green fluorescence (FL1-A) vs. side scatter (SSC) (Xu et al., 2013).

2.5 *Calculations*

Bacterial abundance and leucine incorporation rate obtained from the exponential growth stage were used to calculate the conversion factor, according the methods described by Kirchman and Ducklow (1993) and Pulido-Villena and Reche (2003).

Changes in bacterial abundance during the exponential growth stage were fitted to the function:

$$BA_t = BA_i e^{\mu t},$$

where BA_i was initial bacterial abundance, BA_t was the bacterial abundance at time t of the exponential growth stage, and μ was the specific growth rate of bacterial abundance during this stage.

Changes in (^{3}H-leucine)-protein syntheses during the stage were fitted to the function:

$$LIR_t = LIR_i e^{bt},$$

where LIR_i and LIR_t were (^{3}H-leucine)-protein synthesis rates (i.e., ^{3}H-leucine incorporation rates) at the initial and time t of the exponential growth stage, respectively, and b was the specific growth rate of (^{3}H-leucine)-protein syntheses during exponential growth stage.

The eCFs was obtained by an integrative method:

$$eCFs = \frac{BB_f - BB_i}{\int_{t_i}^{t_f} Leu \ dt},$$

where BB_i and BB_f were the bacterial biomass at initial (t_i) and final (t_f) times during the exponential growth stage, and bacterial biomass (BB=BA×20) was calculated by multifying BA by the conversion factor of 20 (fg C)/cell (Lee and Fuhrman, 1987). $\int_{t_i}^{t_f} Leu \ dt$ was the definite integral of (^{3}H-leucine)-protein syntheses function from the initial (t_i) to final (t_f) time point. The solution of the definite integral was calculated using the following equation:

$$\int_{t_i}^{t_f} Leu \ dt = \frac{LIR_i}{b}(e^{bt_f} - e^{bt_i}).$$

The average ^{3}H-leucine incorporation rate (LIR) over the exponential growth period was obtained by the following equation:

$$LIR = (LIR_i + LIR_f)/2,$$

where LIR_i and LIR_f were ^{3}H-leucine incorporation rates at initial (t_i) and final (t_f) time point during the exponential growth stage.

The cell-specific ^{3}H-leucine incorporation rate (sLIR) over the exponential growth periodwas calculated according to the following equation:

$$sLIR = 2 \times LIR/(BA_i + BA_f),$$

where BA_i and BA_f were the initial and final abundance of bacteria during the exponential growth period, respectively.

3 Results

3.1 *Nutrients, Chl a, bacterial abundance*

Nutrients (DIN, phosphate, and silicate) concentrations decreased with an increase in salinity. DIN concentrations declined dramatically from >100 μmol/L in the upper reach of the estuary, to <12.85 μmol/L in the coastal waters (salinity>25) with little or no influence of the river discharge. Phosphate concentrations showed the same pattern as DIN, ranging from 2.59 μmol/L in freshwater (Sta. E1, May 2015) to 0.03 μmol/L in the coastal waters (Sta. P3). Silicate concentration varied by two orders of magnitude, from 137.14 μmol/L at Sta. E1 (May 2015) to 0.35 μmol/L at Sta. M3. The Chl *a* concentrations varied spatially, with high (~11 μg/L) in the estuary and low (~1 μg/L) in the coastal waters with no influence of the river discharge. Nutrients were relatively richer in the wet season (May and August) compared with the dry season (January) in the Zhujiang Estuary (Table 1).

Bacterial abundance *in situ* decreased as salinity increased, ranging from ~3×10^9 cells/L in the upper reach of the estuary to ~5×10^8 cells/L in the coastal waters with no influence of the river discharge. Leucine incorporation rates and cell-specific leucine incorporation rates varied about 30-fold and 10-fold along the salinity gradient, respectively (Table 1).

3.2 *Empirical leucine-to-carbon conversion factors*

The eCFs in the middle reach of the estuary (1.39–1.69 (kg C)/(mol Leu)) were generally the highest and showed significant difference (p<0.05), compared with the ones in the upper and lower reaches of the Zhujiang Estuary. The eCFs in the upper reach (1.27–1.38 (kg C)/(mol Leu)) and lower reach (0.80–1.44 (kg C)/(mol Leu)) of the estuary were close and not significantly different (p>0.05). The difference in the eCFs in the estuary between the wet and dry seasons was not significant (p>0.05).

The eCFs ranged from 1.27 to 1.69 (kg C)/(mol Leu) (mean= 1.45 (kg C)/(mol Leu)) in low salinity waters (salinity<15). In the intermediate salinity waters (salinity of 15–25), eCFs ranged from 1.03 to 1.25 (kg C)/(mol Leu) (mean=1.14 (kg C)/(mol Leu)). The eCFs were relatively low in the pristine coastal waters (salinity>25) with little influence of freshwater discharge, ranging from 0.48 to 0.85 (kg C)/(mol Leu) (mean=0.69 (kg C)/(mol Leu)). The eCFs were statistically significantly different among three regimes (p<0.01).

3.3 *Relationship between the eCFs and environmental factors*

The eCFs were significantly correlated with salinity, chlorophyll Chl *a*, nutrients (DIN, phosphate, and silicate) concentrations and *in situ* bacterial abundance (p<0.05 or p<0.01) (Fig. 2). Besides, the eCFs showed significant positive correlation with LIR (p<0.05, except for Sta. E5 in August 2015) and sLIR (p<0.05) in waters influenced by the Zhujiang River discharge (salinity<25),

Table 1. Ambient concentrations of Chl *a* and nutrient (DIN, PO_4^{3-} and SiO_3^{2-}), bacterial abundance (BA) and the eCFs at sampling stations, LIR and sLIR over the course of each experiment

Date	Station	Salinity	DIN/ μmol·L^{-1}	PO_4^{3-}/ μmol·L^{-1}	SiO_3^{2-}/ μmol·L^{-1}	Chl *a*/ μg·L^{-1}	BA/ 10^8 cells·L^{-1}	LIR/ 10^{-9} (mol Leu)·(L·h)$^{-1}$	sLIR/ 10^{-18} (mol Leu)·(cell·h)$^{-1}$	eCFs/ (kg C)·(mol Leu)$^{-1}$
May 2015	E1	0.19	284.00	2.59	137.14	11.06	38.00	1.97	2.99	1.38
	E2	2.78	221.79	1.15	75.71	5.66	34.25	2.32	2.53	1.69
	E5	30.28	12.85	0.13	6.07	0.68	15.88	0.45	0.42	0.85
Aug. 2015	E1	1.95	143.85	1.20	112.50	4.51	23.74	1.10	1.27	1.27
	E4	6.60	104.27	0.91	110.00	2.96	24.49	2.46	2.13	1.52
	E5	14.90	75.03	0.34	97.14	8.91	14.23	3.60	2.59	1.44
Jan. 2016	E3	4.79	138.48	1.67	80.08	11.40	12.29	0.30	1.07	1.39
	E5	32.61	8.54	0.28	12.64	1.57	10.95	0.11	1.29	0.80
May 2016	M1	21.95	107.99	0.26	38.75	4.53	14.32	0.33	0.68	1.03
	M2	21.67	na	0.13	25.61	8.04	24.48	0.43	0.70	1.25
	M3	33.51	nd	0.07	0.35	0.82	14.42	0.30	0.98	0.68
Jun. 2016	P1	33.56	1.73	0.03	14.39	1.07	9.88	0.49	1.91	0.65
	P2	28.05	1.66	0.05	0.92	0.81	6.56	0.41	2.91	0.66
	P3	28.04	3.39	0.06	0.63	1.06	5.34	0.80	4.69	0.48

Note: nd represents undetectable and na not available.

but significant negative correlation with LIR and sLIR (p<0.05) in waters with little or no influence of the Zhujiang River freshwater discharge (salinity>25) (Fig. 3).

4 Discussion

4.1 *Variability in environmental conditions*

There was a clear environmental gradient from the Zhujiang Estuary to the adjacent coastal waters, due to the influence of the Zhujiang River discharge. The river discharge delivered a huge amount of nutrients and organic carbon to the coastal waters through the estuary (Yin et al., 2001). Nitrate was disproportionately high relative to phosphate in the Zhujiang Estuary (Yin et al., 2001; Xu et al., 2008). Our observations also showed that the ratios of N:P reached up to 100:1–200:1 in the middle and upper reaches of the estuary, and even reached up to 400:1 at Sta. M1. In the Zhujiang Estuary, temporal variations in nutrients and Chl *a* were attributed to seasonal exchange between the river discharge and coastal water. In winter, oceanic water intruded into the estuary, leading to low nutrients and Chl *a* at Sta. E5. In the coastal waters during summer, the intrusion of the river plume delivered nutrients and terrestrial organic carbon, altered nutrient composition and triggered phytoplankton and bacterial growth. The input of the Zhujiang River discharge with high N:P ratios caused phosphorus limitation in the river plume-influenced coastal waters, while nitrogen limitation occurred in waters out of the river plume (Xu et al., 2008; Yin et al., 2001). Hence, considerable difference in environmental conditions from the estuary to coastal waters would potentially affect the empirical carbon-to-leucine conversion factors.

4.2 *Variability and regulators of the empirical leucine-to-carbon conversion factors*

The eCFs varied in a wide range along an environmental gradient. The eCFs in the Zhujiang Estuary with salinity being less than 15, with the average of (1.45±0.14) (kg C)/(mol Leu), agreed with theoretical CFs of 1.55 (kg C)/(mol Leu) (assuming no isotope dilution) (Simon and Azam, 1989), but were lower than those in some other coastal waters (Alonso-Sáez et al., 2010; del Giorgio et al., 2011; Franco-Vidal and Morán, 2011). The eCFs decreased as the salinity increased, which dropped to (1.14±0.15) (kg C)/(mol Leu) in the estuarine plume (salinity=15 to 25), simil-

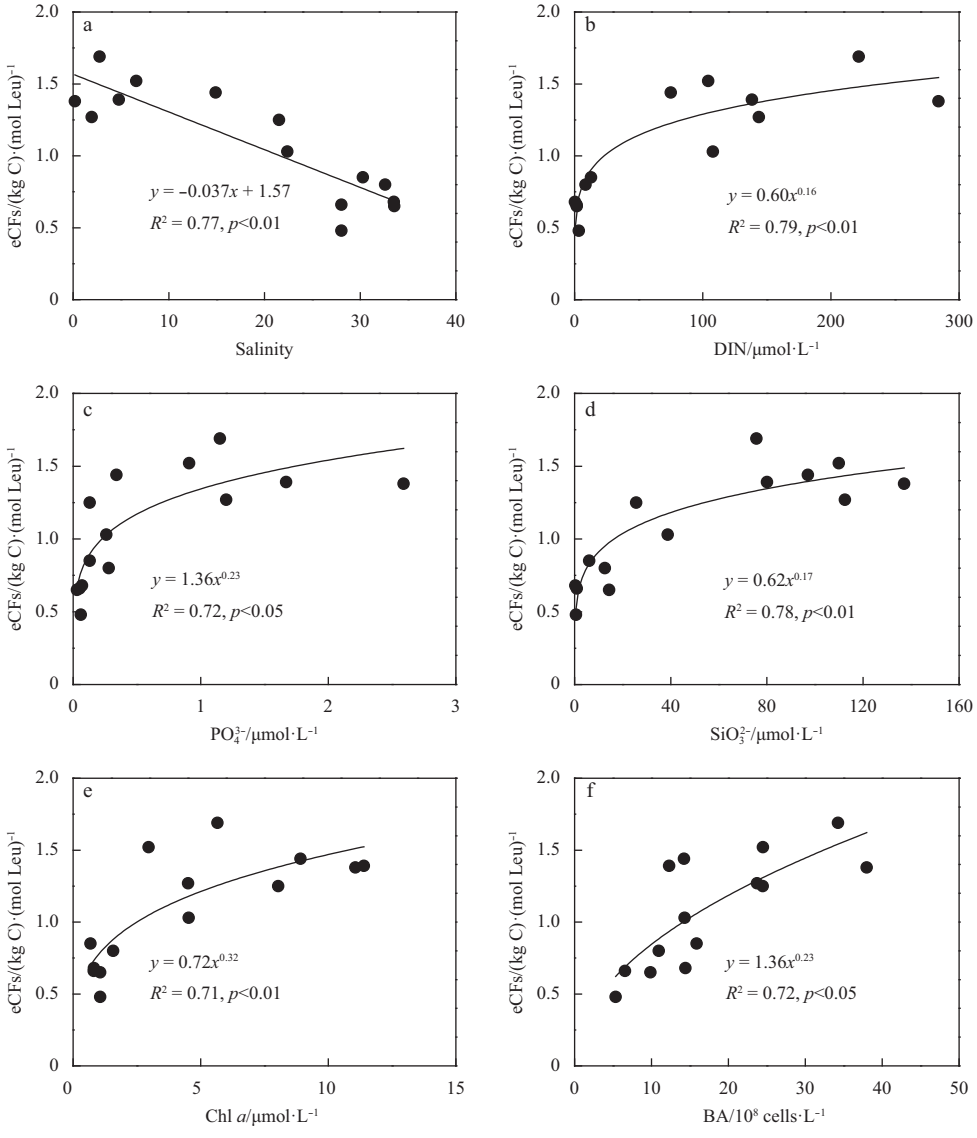

Fig. 2. Relationship between the eCFs and ambient concentrations of environmental variables.

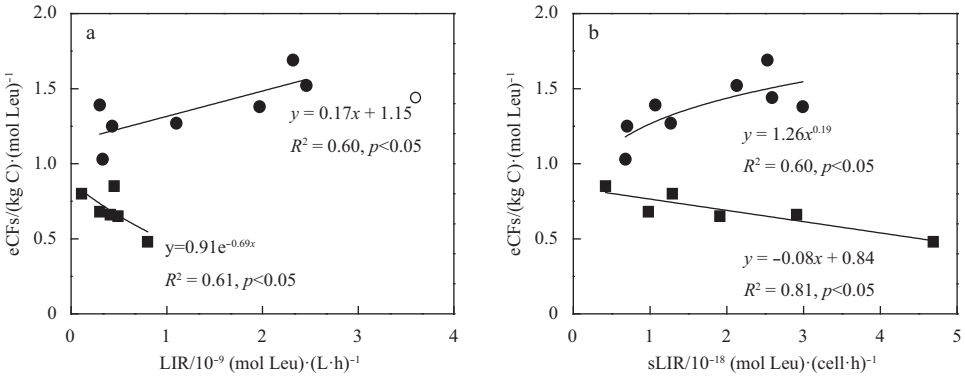

Fig. 3. Relationships of eCFs with LIR and sLIR over the course of experiments. Circles and squares denoted data from waters with low and intermediate salinity (salinity<25) and with high salinity (salinity>25), respectively. Data from Sta. E5 in August 2015 was excluded (open circle) in Fig. 3a.

ar to those in mesotrophic coastal waters (e.g. ~1.00 (kg C)/(mol Leu) in subtropical Atlantic) (Teira et al., 2015) and then rapidly descend to (0.69±0.13) (kg C)/(mol Leu) in waters out of the river plume (salinity>25), in agreement with those in oligotrophic waters (e.g., 0.58 and 0.73 (kg C)/(mol Leu) in the eastern North Atlantic) (Morán et al., 2004; Agusti et al., 2001). At Sta. E5, the eCFs demonstrated a clear seasonal variability, with the lowest (0.80 (kg C)/(mol Leu)) in winter, moderate (0.85 (kg C)/(mol Leu)) in spring and the highest (1.44 (kg C)/(mol Leu)) in summer. Seasonal variability in nutritional status was responsible for seasonality in the eCFs at Sta. E5. Spatial variability in the eCFs was attributed to changes in environmental conditions, as indicated by a significant correlation between eCFs and nutrients and Chl *a*.

The relationship between eCFs and LIR exhibited two contrasting patterns in the study areas. In the river-influenced waters (surface salinity<25, except for Sta. E5 in August 2015) with relative high substrate levels, there was a significant positive correlation of eCFs vs LIR and eCFs vs sLIR. In contrast, in waters out of the river plume (surface salinity>25) with low nutrients and Chl *a*, significant negative correlations between eCFs with LIR and sLIR were observed. These results suggested that substrate availability played a significant role in modulating bacterial metabolic activity, which ultimately affected leucine-to-carbon conversion factors.

In waters out of the plume, low eCFs was attributed to leucine catabolism in bacterial cells, indicated by a significant negative correlation between eCFs with LIR and sLIR. In hostile environments (i.e., oligotrophic waters), a larger percentage of energy is used for maintenance processes to safeguard metabolic flexibility at the cost of energetic efficiency, resulting in low net bacterial production and bacterial growth efficiency (Carlson et al., 2007). As a result, leucine incorporated was mostly respired to maximize cell survival rather than cell production (Alonso-Sáez et al., 2007; Pulido-Villena and Reche, 2003), leading to high turnover rates of intracellular protein (Mandelstam, 1958). This fraction of respired ^{3}H-leucine was recovered by TCA (del Giorgio et al., 2011; Teira et al., 2015). Hence, LIR and sLIR were high, while the eCFs was low in waters out of the river plume.

5 Conclusions

The eCFs varied in a wide range from the Zhujiang Estuary to the adjacent coastal waters. Substrate availability played a key role in regulating variability in the eCFs. In the eutrophic Zhujiang Estuary, the eCFs was close to the theoretical leucine-to-carbon conversion factors, while low eCFs in the pristine coastal waters were attributed to low substrate availability. Hence, the determination of the eCFs was needed to accurately estimate bacterial production in various marine environments.

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