Vie Et Milieu-life And Environment
2017, Volume 66 Issue 3-4 Pages 305-314
http://archimer.ifremer.fr/doc/00387/49856/

The stony road to understand isotopic enrichment and turnover rates: insight into the metabolic part

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Abstract :

Trophic enrichment factors (TEF) are essential to properly and fruitfully explore stable isotope analysis in ecology. And so is the time window of food source integration, usually estimated with the turnover rates (lambda) of isotopic incorporation. On the road to provide ecologists with a general and reliable method to obtain TEF and turnover rates for diet reconstruction, isotopists start realizing that those two parameters are ultimately linked with the physiological state of organisms and that metabolic pathways are of primary importance to understand the large ranges in TEF values. In this study, we used a diet-switching experiment for seven small marine invertebrates. Changes in isotopic compositions were fitted to an exponential decay model to estimate TEF and. values. We then partitioned the growth and the catabolism components of the turnover rate and tested how these components are correlated to the TEF among species. Results showed a significant linear negative relationship between TEF and growth values for both C and N. This ultimately means that the increase in body mass over a time window can be used to estimate the TEF values for a given species.

Key words : stable isotopes, growth rates, diet-tissue discrimination, dynamic energy budget, trophic fractionation, catabolism

1. INTRODUCTION

Ecologists have increasingly shown interest in stable isotope analysis (SIA), to provide novel insights into the trophic ecology of animals that other methods cannot. Among many examples, SIA have been used to trace pathways of organic matter into food webs, to examine intra- and inter-species trophic relationships, to track origins and migration of animals or to reconstruct organisms' diets (see review of Boecklen *et al.* 2011). Carbon and nitrogen isotope ratios in tissues of organisms closely resemble those in their diets, but with slight enrichment and after a certain lag in time when isotopic equilibrium is reached. This enrichment - classically called trophic shift, trophic fractionation, diet-tissue discrimination, or trophic enrichment factor (TEF) - and the rapidity of isotopic incorporation - classically apprehended with the turnover rate - are the two critical aspects of isotopic dynamics (Martinez del Rio *et al.* 2012).

TEF (often noted Δ) has long been recognized as a critical measurement to reconstruct diets and trophic web structure (Post 2002). TEF is explained by the fact that light isotopes (^{12}C, ^{14}N for carbon and nitrogen respectively) are preferentially used versus heavier isotopes (^{13}C, ^{15}N) in catabolism, leading to an enrichment in the tissues (Gannes *et al.* 1998, Martinez del Rio *et al.*2012). As stated by Philips & Koch (2002), "the weakest link in the application of mixing models to a dietary reconstruction relates to the estimation of appropriate Δ values". Later, Martinez del Rio *et al.* (2009) further drove the point home while writing about a "neglected complication". This enrichment has for long been considered to be consistent across species. It has been

acknowledged in numerous studies that the average values for $\Delta\delta^{13}C$ and $\Delta\delta^{15}N$ range between 0 and 1‰ and between 3 and 4 ‰, respectively (De Niro & Epstein 1978, Minagawa & Wada 1984, Peterson & Fry 1987). We know that reality is a bit more complex. Several exhaustive investigations have listed species-specific differences in $\Delta\delta^{13}C$ and $\Delta\delta^{15}N$ values from the literature, obtained both from field investigations and controlled studies (Vander Zanden & Rasmussen 2001, Vanderklift & Ponsard 2003, Mc Cutchan *et al.* 2003). They showed, for example, differences between carnivores and herbivores, or invertebrates and vertebrates (e.g. Vander Zanden & Rasmussen 2001), for $\delta^{15}N$ as well as $\delta^{13}C$ to a lesser extent. On the road to provide TEF values for all species, some authors carried out meta-analyses and found significant relationship between TEF and the isotopic composition of the diet (Caut *et al.* 2008, 2009). Even though this effort has received a great attention, it completely disregards the metabolic aspect of the isotopic incorporation (Perga & Grey 2010).

Another relevant aspect of the dynamics of isotopic incorporation is how fast an animal tissue reflects the isotopic composition of the diet, or "over what time period is resource use integrated" (Vander Zanden *et al.* 2015). The rate at which diet isotopic signature is incorporated into animal tissue can be estimated through the turnover rate (incorporation rate of an element divided by the biomass of the element). Turnover rates are assumed to be the sum of tissue growth (i.e. net anabolism, or new biomass) and catabolism (i.e. tissue replacement; Hesslein *et al*. 1993, Vander Zanden *et al.* 2015). As reviewed by Vander Zanden *et al.* (2015) exploring several taxonomic groups, there is a significant relationship between the body mass and the turnover rates for invertebrates. But the respective parts of growth and catabolism of turnover rates are yet to be addressed. It is commonly accepted that the catabolic turnover increases with body size among species (Wolf *et al.* 2009) and within species (Vander Zanden *et al.* 2015). The pattern is however more complex and the feeding level of organisms, as well as the type of tissues should be factored in (Carleton & Martinez del Rio 2010).

Diet-switching experiments, in which animals are fed with isotopically constant food source over a time period, are the best way to estimate TEF and turnover rates. But since the call for more controlled laboratory experiments 20 years ago (Gannes *et al.* 1997, Martinez del Rio *et al.* 2009), there is still a long way to provide ecologists with a general and reliable method to obtain TEF and turnover rates for diet reconstruction. The vast majority of the feeding experiments were done on terrestrial biological models, or vertebrate models (e.g. fish and mammals) for freshwater and marine species (see for example the review of Vanderklift & Ponsard 2003). Despite the need and the interest, the number of published feeding experiments is barely increasing for invertebrates (e.g. Yokoyama *et al.* 2005, Dubois *et al.* 2007, Blanchet-Aurigny *et al.* 2012, deVries *et al.* 2015), most certainly because such experiments are often expensive, logistically difficult to carried out and time-consuming. We are also convinced that many measured TEF values that deviate from commonly cited TEF from the literature are sleeping in computers because of a lack of perspective to be properly interpreted.

The aim of the present investigation is to use a feeding experiment to provide ranges of $\Delta\delta^{13}C$ and $\Delta\delta^{15}N$ values in marine invertebrates and to relate the TEF to the physiological state of the animals. Following several investigations showing that isotopic incorporation rates, maintenance metabolism and growth are strongly related (deVries *et al.* 2015) and that one can expect unexpectedly high discrimination values when organisms have a low feeding rate (Martinez del Rio & Wolf 2005, Emmery *et al.* 2011, Riemen 2015), we investigated here the growth and the catabolism components of the turnover rate and tested how these components are correlated to the TEF among species.

2. MATERIALS AND METHODS

2.1. FEEDING EXPERIMENT

We selected seven common small marine invertebrates species as experimental species: Three polychaetes, the honeycomb-worm *Sabellaria alveolata* Linnaeus 1767, the sand-mason worm *Lanice conchilega* Pallas 1766, the ragworm *Hediste diversicolor* O.F. Müller 1776, and four molluscs the slipper limpet *Crepidula fornicata* Linnaeus

1758, the Pacific oyster *Crassostrea gigas* Thunberg 1793, the blue mussel *Mytilus edulis* Linnaeus 1758 and the cockle *Cerastoderma edule* Linnaeus 1758. In many European shallow systems, these species can be found in close proximity, if not together. Individuals have been collected in spring time in soft-bottoms and hard substrata and placed in large experimental tanks (100 liters) designed to rear marine invertebrates (Sycamar®, Syndicat Mixte pour le Developpement du Littoral, Blainville sur Mer, France).

The protocol was originally described in Dubois et al. (2007) for *C. gigas* and *M. edulis*. Practically, individuals of the reef-building polychaete *S. alveolata* were kept in small reef portions, but sand mason worms *L. conchilega* were forced to reconstruct a tube in small tanks containing oven-dried sediment. Bivalves *C. gigas*, *M. edulis* and *C. edule* and gastropod *C. fornicata* were simply deposited in experimental tanks. The burrowing polychaetes *H. diversicolor* were maintained in tanks with glass tubes to mimic the burrows and to separate individuals from each other, hence preventing cannibalism.

Feeding experiments were conducted for a total of 90 days in the tanks. Animals were sampled on days 0, 4, 8, 15, 30, 45, 60 and 90, except for *H. diversicolor* which was sampled every ten days for 60 days. Tanks were maintained with filtered seawater with open-circuit under a daily light/dark regime of 16:8 h. Experimental conditions were monitored in the tanks every 2 or 3 days; temperature averaged 15.9 °C (± SD = 0.6 °C), salinity averaged 31.9 (± SD = 1.7), O_2 concentration averaged 7.9 mg L^{-1} (± SD = 0.3 mg L^{-1}), and NH_4^+ concentration averaged 8.7 µmol L^{-1} (± SD = 1.3 µmol L^{-1}). Water was continuously renewed in the tanks, and reached 12 exchanges per day to avoid particle sedimentation. In addition, tanks were completely washed every week to prevent development of bacterial populations and any accumulation of detrital material. For all species but *H. diversicolor*, living microalgae of the diatom *Skeletonema costatum*were supplied continuously from rotating batch cultures to an average concentration of 13 x 10^4 cell mL^{-1} (± 4.5 10^4 SD). Microalgae were added to the tanks at a uniform stage of the culture when the cells had reached the end of the exponential growth phase, normally 4-5 days after inoculation. To make this laboratory approach applicable to field situations, the concentration of microalgae was quite similar to what is found in natural conditions during spring or autumn phytoplankton blooms (Jouenne et al., 2007). Individuals of *H. diversicolor* were fed daily with grounded fish food flakes (Tetramin ©). Food remains were removed after 2 hours to prevent bacterial developments.

2.2. ISOTOPE ANALYSES

For each sampling date, 2 groups of 3 to 5 individuals of each species were randomly selected, carefully rinsed with distilled water, and wet weighed after removing the shells (if any). Slipper limpets *C. fornicata* were sampled as colony of 4 to 5 individuals. The entire body of the organisms were used for stable isotope analysis. A two-liter sample of the *Skeletonema costatum* culture was collected every two days, and filtered onto Whatman GF/C glass fibre filters. All animal samples and filters were freeze-dried for 48 h. Filters were kept overnight under a 1M HCl stream to remove any inorganic carbonates.

Animal samples and filters were then ground to powder using a mortar and a pestle and sealed in tin capsules. Two analytical replicates of about 1 mg sample for animals and fish food flakes and four analytical replicates of about 10 mg sample for *S. costatum* were used for CF-IRMS (Continuous Flow Isotope Ratio Mass Spectrometry) analysis using a Europa Scientific ANCA-NT 20-20 Stable Isotope Analyser with ANCA-NT Solid/Liquid Preparation Module (Europa Scientific Ltd., Crewe, UK). The analytical precision (SD, n = 5) was 0.2 ‰ for both N and C, as estimated from standards analysed along with the samples. Working standards were 1 mg leucine prepared by freeze drying 50 µl of a 20 mg ml^{-1} stock solution in tin cups, which were calibrated against 'Europa flour' and IAEA standards N1 and N2. Carbon and nitrogen stable isotopic ratios were expressed in δ notation according the following equation:

$$\delta X\ (‰) = [(R_{sample} / R_{standard}) - 1] \times 10^3$$

where X is ^{13}C or ^{15}N and R is ^{13}C / ^{12}C or ^{15}N / ^{14}N. As standards, Pee-Dee Belemnite (PDB) was used for carbon and atmospheric N_2 for nitrogen.

The isotopic composition of the food sources (δX_{diet} in further equations) were then measured (mean ± SD) for *S. costatum* ($\delta^{13}C$ = -23.0 ± 1.0 and $\delta^{15}N$ = -4.9 ± 1.1) and the Tetramin © fish food flakes ($\delta^{13}C$ = -22.1 ± 0.0 and $\delta^{15}N$ = 9.2 ± 0.1).

2.3. ESTIMATES OF TROPHIC ENRICHMENT FACTORS AND TURNOVER RATES

Variations in isotopic signatures are classically fitted against time using an exponential decay curve:

$$\delta X_{\text{whole body}}(t) = a + be^{-ct}$$

Where $\delta X_{\text{whole body}}(t)$ was the $\delta^{13}C$ or $\delta^{15}N$ values at time (t) with *a* being the asymptotic tissue isotope values (δX_∞), *b* being the difference between initial and asymptotic values ($\delta X_{\text{whole body}}(0) - \delta X_\infty$), and *c* being the turnover rate of carbon or nitrogen (λ). Following, Martinez del Rio *et al.* (2009), this equation was reformulated to provide a direct estimate of TEF by further developing δX_∞ as the sum of δX_{diet} and the TEF value ($\Delta\delta X$), so that:

$$\delta X_{\text{tissue}}(t) = \delta X_{\text{diet}} + \Delta\delta X - [\delta X_{\text{diet}} + \Delta\delta X - \delta X_{\text{tissue}}(0)]e^{-\lambda x t}$$

Curve fitting and parameter estimations ($\Delta\delta X$ and λ_X) were achieved using the downhill simplex method of the Nelder–Mead model, and standard deviations of parameters were estimated by an asymptotic method. All fittings were tested by analysis of variance ($p<0.001$), residues were tested for normality and homogeneity of variance, and parameters were tested for significance by Student's t-test ($p<0.05$). All the curve fitting processes and associated statistics were coded in MATLAB R2010b (MathWorks Natick, Massachusetts, USA).

Following Hesslein *et al.* (1993), the turnover rate λ_X can be partitioned into the fractional net growth (k_{gX}) and catabolic turnover rate (k_{cX}) for each element X, as:

$$\lambda_X = k_{gX} + k_{cX}$$

with

$$k_{gX} = \frac{\ln(W_f[X_f]) - \ln(W_i[X_i])}{t_f - t_i}$$

and $[X_f]$ the content in element X at time t and W_f the final weight.

The catabolic turnover rate (k_{cX}) can then be calculated as the difference between λ_X and k_{gX}. We also calculated the half-life of each element for each species (hereafter $t_{1/2}$) defined by $\ln(2) / \lambda_X$, and corresponding to the time required to replace 50% of the initial tissue (Hobson & Bairlein 2003).

3. RESULTS

Over the course of the experiment, species exhibited a broad range of variations in body mass. Three of them did grow significantly (*Mytilus edulis*, *Crassostrea gigas* or *Lanice conchilega*) but variances in measurements for *Crepidula fornicata* were too high to show significant level (Table 1). The three remaining species showed no obvious growth pattern. This evidenced a gradient in growth among species, spanning from species which highly benefited from the food quantity and quality to synthesize biomass, to species which allocated their energy differently. Overall, there was an increase in standard deviations in the final biomass estimates, indicating some higher heterogeneity in the food uptake between individuals during the experiment (Table 1). C:N ratios of the tissues were very similar among species and exhibited either small significant increases or decreases between

initial and final samples. Because the changes in C:N ratios were small, only small differences in k_{gC} and k_{gN} were expected.

TABLE 1. MEAN (SD) OF INITIAL (I) AND FINAL (F) BODY MASS (G, FRESH WEIGHT), CARBON AND NITROGEN CONTENT (%) AND C:N RATIO FOR THE SIX SPECIES REARED WITH *SKELETONEMA COSTATUM* AND THE RAGWORMS *HEDISTE DIVERSICOLOR* REARED WITH COMMERCIAL FISH FOOD FLAKES. P-LEVEL FOR T-TESTS BETWEEN INITIAL AND FINALVALUES: * ≤ 0.05, ** ≤ 0.01, * ≤ 0.001, NS = NON-SIGNIFICANT. DATA FOR *CRASSOSTREA GIGAS* AND *MYTILUS EDULIS* WERE RETRIEVED FROM DUBOIS *ET AL.* 2007.**

	W (g)			C (%)			N (%)			C:N		
	i	f		i	f		i	f		i	f	
	Mean (SD)	Mean (SD)		Mean (SD)	Mean (SD)		Mean (SD)	Mean (SD)		Mean (SD)	Mean (SD)	
Crassostrea gigas	0.25 (0.14)	0.65 (0.35)	*	42.0 (0.2)	41.6 (0.2)	NS	9.9 (0.1)	9.2 (0.0)	*	4.24 (0.02)	4.50 (0.01)	**
Mytilus edulis	2.17 (0.29)	2.99 (0.62)	*	41.0 (0.2)	44.3 (0.4)	**	7.7 (0.0)	8.8 (0.1)	**	5.33 (0.03)	5.04 (0.01)	**
Cerastoderma edule	1.77 (0.22)	1.52 (0.46)	NS	41.2 (0.4)	42.0 (0.5)	NS	7.7 (0.3)	9.1 (0.1)	*	5.33 (0.14)	4.59 (0.02)	*
Crepidula fornicata	1.80 (0.78)	2.70 (0.95)	NS	35.0 (0.3)	38.5 (0.4)	**	7.0 (0.1)	7.1 (0.1)	NS	4.98 (0.01)	5.40 (0.01)	***
Hediste diversicolor	0.46 (0.17)	0.67 (0.19)	NS	39.7 (0.1)	40.0 (0.8)	NS	8.7 (0.0)	8.3 (0.2)	NS	4.57 (0.02)	4.81 (0.02)	**
Lanice conchilega	1.03 (0.33)	2.14 (0.45)	***	39.0 (1.0)	43.7 (0)	*	7.1 (0.1)	8.4 (0.0)	**	5.46 (0.04)	5.20 (0.01)	*
Sabellaria alveolata	0.28 (0.07)	0.25 (0.07)	NS	39.7 (0.2)	40.1 (0.1)	NS	9.4 (0.1)	9.7 (0.0)	*	4.24 (0.02)	4.13 (0.02)	***

The isotopic signatures ($\delta^{13}C$ and $\delta^{15}N$) of suspension-feeding species changed following removal from their natural environment to a more depleted diet (*Skeletonema costatum*, $\delta^{13}C$ = -23.0 ± 1.0 and $\delta^{15}N$ = -4.9 ± 1.1). Because the nitrogen isotopic composition of the fish food flakes ($\delta^{15}N$ = 9.2 ± 0.1) was close to the initial *Hediste diversicolor*'s $\delta^{15}N$, only minor changes were observed over time, hence explaining a lower r^2 value for the model (Table 2). The rapid decrease in isotopic composition for $\delta^{13}C$ and $\delta^{15}N$ allowed a decay curve to be fitted to the data (Fig. 1). All decay curves showed a plateau after 90 days except for *L. conchilega* and *C. edule* for $\delta^{15}N$ (Table 2). The time required to replace 50% of the initial tissue ($t_{1/2}$) ranged between 4.9 and 37.8 days for the carbon and 7.3 and 38.4 days for the nitrogen. Overall, $t_{1/2}$ values were <40 days (i.e. at least 79% of the tissues have been replaced within the 90 days experiment), indicating that the isotopic compositions after 90 days reached or were very close to the asymptotic values. It is worth noticing that the ratio between turnover rates for carbon and nitrogen (λ_C and λ_N respectively) varied from 1 in *L. conchilega* to 2.3 in *C. fornicata*. Following the equations presented earlier, the trophic enrichment factor (TEF) was estimated for all the species directly, $\Delta\delta^{13}C$ spanned between 1.9 for *C. gigas* and 4.6 for *C. edule*. while $\Delta\delta^{15}N$ spanned between 3.4 for *L. conchilega* and 10.8 for *C. edule* (Table 2).

The λ values were estimated for carbon and nitrogen and partitioned into the fractional net growth (k_g) and the catabolic turnover (k_c) for each element. As expected, species with no (or negative) difference in biomass over the course of the experiment had k_g values close to zero (or slighly negative). No relationships were evidenced either between k_c and k_g values or between TEF values and λ or k_d values ($P>0.05$) . However, there was a significant linear negative relationship between TEF and k_g values for both elements ($P<0.05$; Fig. 2).

4. DISCUSSION

Contemporary research has paid increasing attention to stable isotope analysis as a key tool to understand trophic relationships and to trace pathways of organic matter among consumers (Fry 2006). Early laboratory studies showed that consumers are typically enriched in heavy isotopes (e.g. ^{13}C or ^{15}N) relative to those in their diets. This trophic enrichment factor (TEF) shows a great deal of variability in ecological studies but it is unclear whether this is a species-specific issue or if other parameters (diet, environment, nitrogenous wasting etc.) are at stakes (see review by Vander Zanden & Rasmussen 2001, McCutchan *et al.* 2003, Vanderklift & Ponsard 2003, Caut *et al.* 2008). This study calls for more controlled laboratory experiments, a call previously made by other authors (e.g. Gannes *et al.* 1997), to reveal sources of variations in isotopic incorporation and to increase literature providing quantitative data on species-specific TEF values and turnover rates. One possible explanation for the lack of available Δ values is the difficulty of conducting long-term feeding experiments, which require

extensive experimental logistics. But it is also likely that atypical results are waiting for some satisfying explanations to be held.

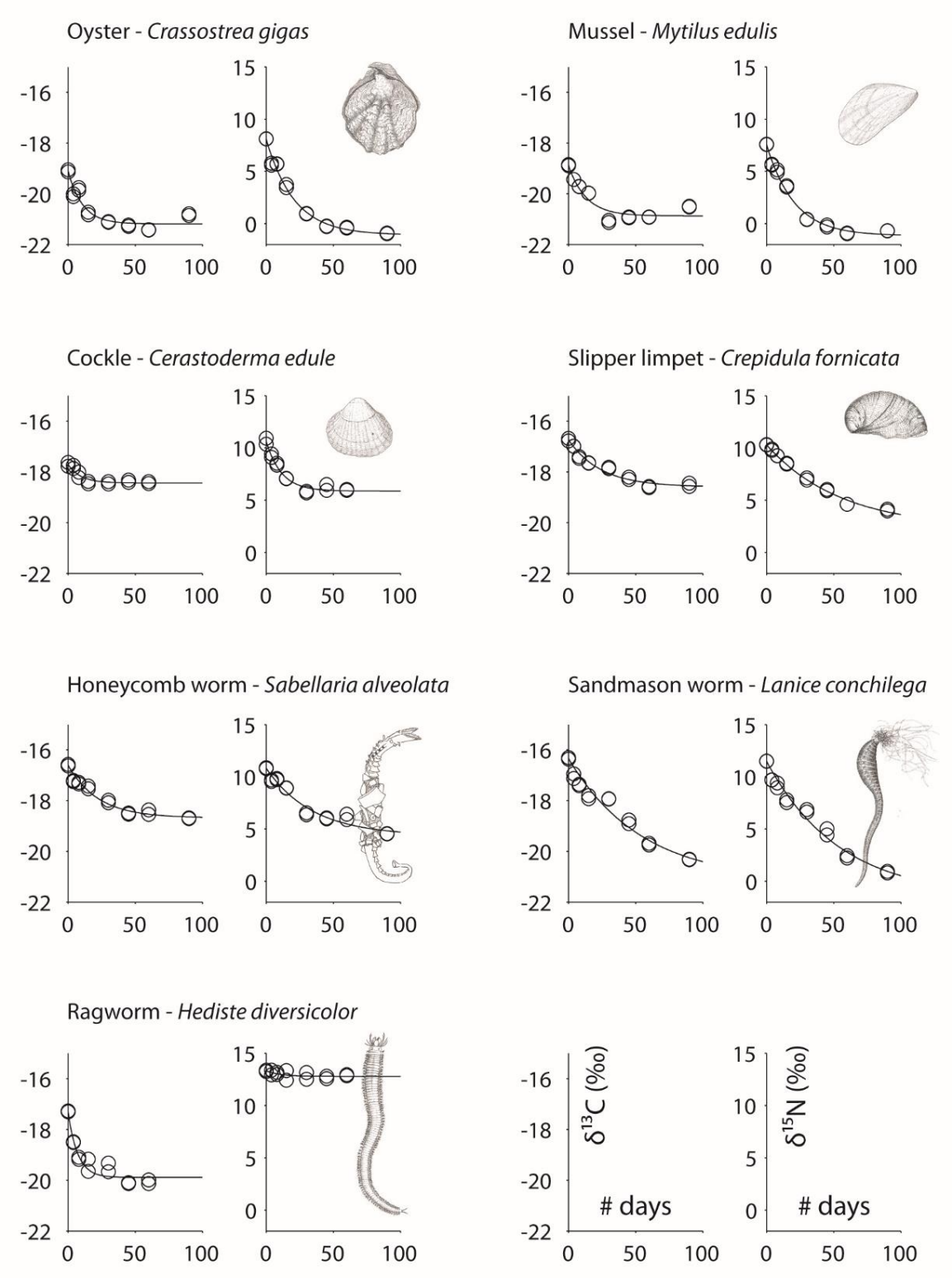

FIG. 1. EXPONENTIAL DECAY CURVES FOR $\Delta^{13}C$ (LEFT PANELS) AND $\Delta^{15}N$ (RIGHT PANELS) FOR SEVEN MARINE INVERTEBRATES REARED WITH THE MICROALGAE *SKELETONEMA COSTATUM* ($\Delta^{13}C$ = -23.0 ± 1.0 AND $\Delta^{15}N$ = -4.9 ± 1.1) FOR *CRASSOSTREA GIGAS*, *MYTILUS EDULIS*, *CERASTODERMA EDULE*, *CREPIDULA FORNICATA*, *SABELLARIA ALVEOLATA*, *LANICE CONCHILEGA* AND WITH THE COMMERCIAL FISH FOOD FLAKES ($\Delta^{13}C$ = -22.1 ± 0.0 AND $\Delta^{15}N$ = 9.2 ± 0.1) FOR *HEDISTE DIVERSICOLOR*. REPLICATE SAMPLES HAVE BEEN PLOTTED AND HALF-LIFE ESTIMATES GIVEN IN THE TEXT ARE BASED ON THESE OBSERVATIONS. LAST BOTTOM-RIGHT PANEL GIVES THE UNITS.

TABLE 2. TROPHIC ENRICHMENT FACTOR (TEF, Δ, ‰), TURNOVER RATE (Λ IN DAYS^{-1}), HALF-LIFE ($T_{1/2}$ DAYS), AND THE PARTITIONING OF Λ BETWEEN FRACTIONAL NET GROWTH (K_G) AND CATABOLIC TURNOVER RATE (K_C) AND ITS RATIO FOR CARBON (C, TOP PANEL) AND NITROGEN (N, BOTTOM PANEL) ELEMENTS FOR THE SEVEN SPECIES.

	$\Delta\delta^{13}C$ (‰)	λ_C (days^{-1})			$t_{1/2}$ (days)	k_{gC} (days^{-1})	k_{cC} (days^{-1})	k_{cC}/k_{gC}
	Mean (SD)	Mean (SD)	r^2	n	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)
Crassostrea gigas	1.9 (0.1)	0.093 (0.017)	0.90	16	7.5 (1.3)	0.011 (0.008)	0.082 (0.066)	7.8 (8.7)
Mytilus edulis	2.2 (0.1)	0.077 (0.014)	0.91	16	9.0 (1.6)	0.004 (0.001)	0.072 (0.022)	16.3 (6.4)
Cerastoderma edule	4.6 (0.1)	0.106 (0.026)	0.88	14	6.5 (1.6)	-0.001 (0.000)	0.108 (0.044)	∞ -
Crepidula fornicata	4.5 (0.1)	0.041 (0.005)	0.96	16	16.7 (2.1)	0.006 (0.003)	0.036 (0.021)	6.5 (5.2)
Hediste diversicolor	2.2 (0.1)	0.141 (0.020)	0.94	14	4.9 (0.7)	0.004 (0.002)	0.136 (0.066)	32.1 (21.6)
Lanice conchilega	1.9 (0.6)	0.018 (0.004)	0.94	16	37.8 (8.8)	0.009 (0.004)	0.009 (0.004)	1.0 (0.6)
Sabellaria alveolata	4.4 (0.1)	0.045 (0.005)	0.96	16	15.5 (1.9)	-0.001 (0.000)	0.046 (0.018)	∞ -
	$\Delta\delta^{15}N$ (‰)	λ_C (days^{-1})			$t_{1/2}$ (days)	k_{gC} (days^{-1})	k_{cC} (days^{-1})	k_{cC}/k_{gC}
	Mean (SD)	Mean (SD)	r^2	n	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)
Crassostrea gigas	3.8 (0.2)	0.048 (0.004)	0.99	16	14.4 (1.1)	0.010 (0.008)	0.038 (0.032)	3.9 (4.6)
Mytilus edulis	3.8 (0.2)	0.049 (0.003)	0.99	16	14.2 (1.0)	0.005 (0.001)	0.044 (0.010)	8.7 (2.7)
Cerastoderma edule	10.8 (0.1)	0.088 (0.009)	0.98	14	7.9 (0.8)	0.000 (0.000)	0.088 (0.009)	∞ -
Crepidula fornicata	7.2 (0.4)	0.018 (0.002)	0.99	16	38.4 (3.5)	0.005 (0.003)	0.013 (0.009)	2.9 (2.7)
Hediste diversicolor	3.6 (0.1)	0.095 (0.077)	0.37	14	7.3 (5.8)	0.004 (0.002)	0.092 (0.088)	24.8 (27.4)
Lanice conchilega	3.4 (1.3)	0.018 (0.003)	0.97	16	37.8 (6.6)	0.010 (0.004)	0.008 (0.003)	0.9 (0.5)
Sabellaria alveolata	9.3 (0.5)	0.029 (0.005)	0.96	16	23.9 (3.9)	-0.001 (0.000)	0.030 (0.016)	∞ -

According to studies addressing issue of isotopic discrimination, $\Delta\delta^{15}N$ was shown to exhibit more variation than $\Delta\delta^{13}C$ (Mc Cutchan *et al.* 2003). Some authors have even assumed that $\Delta\delta^{13}C$ was negligible among species and have considered it as 0‰ to run mixing-models (e.g. Benstead *et al.* 2006). In our study, some TEF values fall within the range of the most cited values while some others completely deviate from what is commonly expected for both $\Delta\delta^{13}C$ and $\Delta\delta^{15}N$. We showed here that the $\Delta\delta^{13}C$ values of the bivalve species, *Crassostrea gigas* and *Mytilus edulis*, and the polychaetes *Hediste diversicolor* and *Lanice conchilega* were twice what was commonly assumed, while the three other species (*Cerastoderma edule*, *Crepidula fornicata* and *Sabellaria alveolata*) are four to five times what was expected. The same trend is true for $\Delta\delta^{15}N$ values except that the lower values were close to the assumed 3.4‰ mean value. Only a dozen of laboratory studies on marine invertebrates are available for comparison with our data. To our knowledge (Table 3), this investigation broadens the ranges in $\Delta\delta^{13}C$ and $\Delta\delta^{15}N$ towards higher TEF values. To some extent, our results indicated that the discrimination is species-specific. However, we would like to emphasize that some cross-species physiological mechanisms are involved. It is now widely accepted that enrichment process are tissue-specific (e.g. Schmidt *et al.* 2004, Yokoyama *et al.* 2005, Logan *et al.* 2006), and may vary according to environmental parameters such as the diet in quantity or quality (e.g. Hesslein *et al.* 1993, Hobson & Bairlein 2003, Trueman *et al.* 2005), and the temperature (Bosley *et al.* 2002). Overall, all parameters that affect metabolism may affect discrimination processes (isotopic fractionation and routing) that are directly linked to physiological processes as predicted by Martinez del Rio & Wolf (2005). Lower TEF values could be explained by isotopic routing (Martinez del Rio et al. 2009) and/or lower fractionation during assimilation (Olive et al. 2003). As for higher TEF values, Riemen (2015) showed using a theoretical approach that metabolism shapes the dynamic of isotopic incorporation, TEF values being negatively influenced by growth, and positively influenced by nutritional stress and disease. Emmery et al. (2011) evidenced a clear effect of the feeding ration on TEF values (negative relationship) using a bioenergetical modelling approach validated with a diet-switching experiment on the Pacific oyster *Crassostrea gigas*. Besides, this authors showed that the effect of the feeding ration was much stronger than the effect of body size; an effect which is certainly reversed for large species. In this study, we showed a clear negative relationship between growth and TEF values among species and we hypothesize that physiological state is a prevalent factor in this relationship over body mass. In addition, although organisms were fed *ad libitum*, offering optimal growth conditions could be problematic when

optimal culture conditions are not fully known or controlled for each species (habitat and food quality). In other words, the quantity of food can be controlled but it is often challenging to provide qualitative food for reared animals, hence creating discrepancies in food assimilation and growth patterns among species. Our study emphasizes that growth is a key element to better predict TEF values and interpret isotopic data.

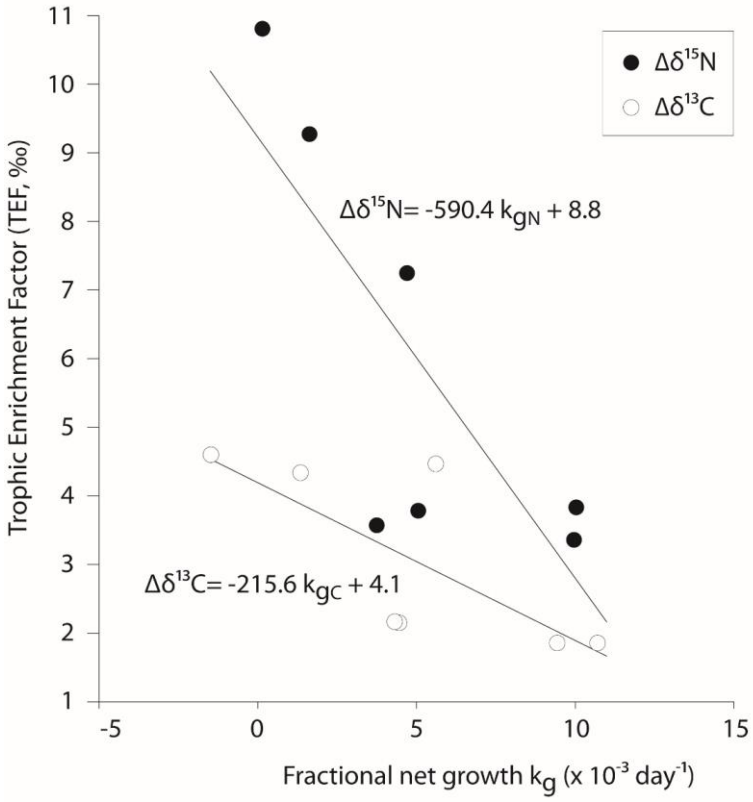

FIG. 2. RELATIONSHIP BETWEEN THE FRACTIONAL NET GROWTH K_G ($DAYS^{-1}$) AND THE TEF (‰) VALUES AMONG SPECIES, FOR CARBON (WHITE DOTS) AND NITROGEN (BLACK DOTS). LINEAR REGRESSIONS GIVE $\Delta\delta^{13}C$= -215.6 (82.9) K_{GC} + 4.1 (0.5) (N=7, R^2=0.58, F=6.8, P=0.048) AND $\Delta\delta^{15}N$= -590.4 (194.9) K_{GN} + 8.8 (1.2) (N=7, R^2=0.65, F=9.2, P=0.029).

The second pillar of isotopic incorporation is turnover rate. This parameter has renewed attention recently since it is critical to know the time window of food sources integration (Vander Zanden *et al.* 2015) and then to back-calculate the diet (Marin-Leal *et al.* 2008). The turnover rates were fine-tuned here for both C and N while it is usually not differentiated in meta-analysis (Vander Zanden *et al.* 2015). It is particularly relevant when the C/N ratio drastically changes over the course of the experiment, which was not the case here. Ln(half life) (estimated as ln(ln(2)/λ)) was shown to be linearly related to ln(body mass) with -1/4 power (Vander Zanden et al., 2015). Our results fit within this pattern (Fig. 3). Residues around the linear relationship could be related to within-species variations in metabolism, since individuals of a same species rarely exhibit the same metabolic activity. While the slope of the Vander Zanden *et al.* (2015) equation held true whatever taxonomic groups, we think the intercept accounts largely for metabolism differences depending on their physiological state. Also, we showed here that the turnover rate for carbon can be twice that for nitrogen. Similar differences between N- and C-turnover rates from feeding experiments were reported for a vertebrate, the passerine bird *Sylvia borin* (Hobson & Bairlein 2003). The authors suggested that this difference may correspond to a decoupling of the nitrogen and carbon metabolic pathways. In our study, following the switch to a microalgal diet, the more rapid uptake of carbon for some species may reflect storage of reserves (e.g. glycogen) and hence a more immediate metabolism of carbohydrates and fats.

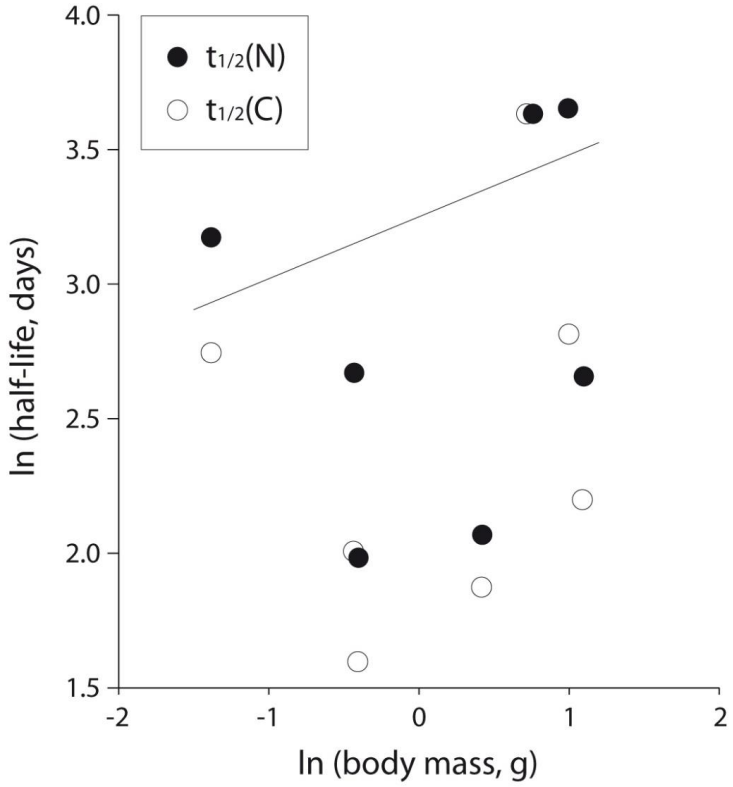

FIG. 3. RELATIONSHIPS BETWEEN LN (ANIMAL BODY MASS, IN GRAMS) AND LN (ISOTOPIC HALF-LIFE $T_{1/2}$, IN DAYS), FOR CARBON (WHITE DOTS) AND NITROGEN (BLACK DOTS). THE LINE IS THE RELATIONSHIP ESTIMATED BY VANDER ZANDEN ET AL. (2015) FOR INVERTEBRATES (Y=0.23X+3.25) WITH NO DISTINCTION BETWEEN C AND N HALF-LIVES

Assuming turnover rates are strongly dependent on metabolism ultimately implies that the partitioning between catabolic turnover and growth is more complex than expected. Catabolic turnover increases in proportion with body size between species (Wolf *et al.* 2009) and within species (Vander Zanden *et al.* 2015). Here, for marine invertebrates of similar sizes (relative to the animal kingdom), the ratio k_c/k_g is always greater than 1 which is unexpected. As evidenced by Carleton & Martinez del Rio (2010) for a fish species feeding with low food ration of a low quality diet, the ratio k_c/k_g is greater for low growth species than rapid growth species (or individuals). In our perspective, the turnover rate and the partitioning between k_c and k_g cannot be fully understood without a mechanistic bioenergetical approach. Partition between catabolic turnover and growth must depend on the energy allocation strategy of each species, along with their body mass and their physiological status. This most certainly explains why no relationship was found between k_g and k_c in our study. For example, the cockle *Cerastoderma edule* exhibited no growth pattern (k_g is near zero) but the second highest k_c and therefore a high turnover rate. Because mortalities were observed towards the end of the experiment, one can hypothesized that a large allocation of energy was diverted to immunity defence, as previously shown in for the Manila clam *Ruditapes philippinarum* suffering the ring brown disease (Flye-Sainte-Marie *et al.* 2007). We can also hypothesized that a low quality food for this species has forced individuals into using of some body tissues (e.g. muscles) to survive, hence exhibiting a high k_c.

TEF and turnover rates are separated in empirical exponential decay curves but it seems there is no reason to do so from a bioenergetical point of view. In mechanistic bioenergetical modelling - such as the DEB theory - an individual organism is described in terms of a structural body size and a reserve density (Van der Meer 2006). Assimilated food is first assimilated as a reserve pool which can fuels maintenance and growth, maintenance costs being a priority over growth. Three basic metabolic fluxes occurred, assimilation (from food to reserve), growth (from reserve to structure) and dissipation (from reserve or structure to cell activity). Each metabolic flux is regulated by catabolic and anabolic processes leading to some isotopic fractionation; light isotopes being preferentially used in catabolic reactions (Emmery *et al.* 2011). As a consequence, the reserve pool is enriched

in heavier isotopes compared to the food, and so is the structure pool over the reserve. In well-fed individuals (i.e. high turnover rate), the reserve pool is proportionally more important, while the structure pool is diluted by growth and the maintenance costs are covered by the reserve pool: the TEF for the whole body is then low. Conversely, in low-fed individuals (i.e. low turnover rate), the reserve pool is proportionally low, the structure pool is not diluted by growth and the maintenance costs can only be covered by the structure: TEF for the whole body are increasing. As a consequence, TEF values are directly linked to turnover rates. So why is it that in the present study, TEF values are significantly related to k_g but not to k_c or λ? From a DEB perspective, k_g is the growth of the reserve and the structure pools; k_g is then most likely a better proxy for the different fractionation steps occurring at the organism level, rather than the catabolic term k_c which ultimately results from three metabolic fluxes and their interactions, all highly dependent to the strategy of energy allocation. The relative growth of the species to their maximum potential growth would be a better proxy when comparing species spanning over a large range in body masses and sizes. Mechanistic bio-energetical modelling across species could definitely help further investigating the link between the physiological status and the TEF.

While the number of isotopic investigations of marine and estuarine macrobenthic species have increased exponentially over the past decade, we provide here a range of possible TEF values and turnover rates for very common marine suspension-feeding species, in order to promote better interpretation of food webs in aquatic ecosystems and to contribute to the library for marine invertebrate species. We showed evidences that TEF values are linked to growth of individuals and that it is highly relevant to estimate growths in experiments and in field studies (1) to evaluate the time window of isotopic incorporation (e.g. Marin Leal et al. 2008) and (2) to estimate TEF values. This latter aspect must need a calibration of the relationship between TEF and growth for each species that can be drawn by experiment and bioenergetic modelling in a complementary way. Also, we recommend to refine the empirical exponential decay curve parameterisation to include growth explicitly in the TEF parameter in the same manner as for λ.

ACKNOWLEDGEMENTS

This work was supported by a post-graduate fellowship (SF Dubois) funded by the regional council of Basse Normandie in the field of the POMOYSTER program financed by DIREN, Agence de l'eau and DRAM (IFOP). The authors thank Jean-Louis Blin and Bertrand Bouchaud (SMEL) for crucial involvement during the experiments. Finally, we would like to thank the two reviewers, Marie Elodie Perga and Carlos Martinez Del Rio, for their valuable comments and suggestions.

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TABLE 3. RANGES OF VARIATIONS IN $\Delta^{13}C$ AND $\Delta^{15}N$ TROPHIC ENRICHMENT FACTORS (TEF) RETRIEVED FROM THE LITERATURE FOR MARINE INVERTEBRATES: Δ $\Delta^{13}C$ AND Δ $\Delta^{15}N$ ARE PROVIDED FOR EACH SPECIES AND EACH DIET ISOTOPIC COMPOSITION. THE TISSUES SAMPLED AS WELL AS THE TOTAL DURATION (DAYS) OF THE EXPERIMENT ARE RECORDED AS WELL.

Species name	Common name	Tissue sampled	Experimental diet	$\delta^{13}C_{diet}$ (‰)	$\delta^{15}N_{diet}$ (‰)	Time (days)	$\Delta\delta^{13}C$ (‰)	$\Delta\delta^{15}N$ (‰)	Reference
Amphithoe valida	Amphipod	Whole body	*Ulva*	-14.6	8.1	42	-0.9	-0.7	Macko *et al.* 1982
-	-	-	*Gelidium*	-14.3	7.9	42	-1.5	-0.2	-
Paryhale hawaiensis	Amphipod	Whole body	*Ulva*	-14.6	8.1	42	-1.1	2.3	-
-	-	-	*Gelidium*	-14.3	7.9	42	-1.3	2.2	-
Penaeus aztecus	Brown shrimp	Blood	Shrimp	-13.7		?	-0.7		Fry & Arnold 1982
-	-	-	Squid	-15.6		?	-0.7		-
-	-	-	Brine shrimp	-19.8		?	1.0		-
Artemia sp.	Brine shrimp	Whole body	Yeast		2.8	25		4.9	Minagawa & Wada 1984
Neomysis intermedia	Mysid	Whole body	Cladocerans		-2.7	?		3.2	Toda & Wada 1990
Penaeus vannamei	Shrimp	Whole body	Artemia	-22.1	5.8	6	2.4	1.0	Dittel *et al.* 1997
-	-	-	Zooplankton	-18.2	10.2	6	0.5	2.8	-
Callinectes sapidus	Blue crab	Whole body	Meiofauna	-20.6	5.6	19	-3.4	3.2	Fantle *et al.* 1999
-	-	-	*Spartina detritus*	-15.7	5.8	19	-3.2	2.2	-
-	-	-	*Uca pugnax*	-15.7	7.4	19	0.0	0.9	-
-	-	-	*Littorina littorea*	-14.6	10.3	19	0.2	0.7	-
-	-	-	Zooplankton	-17.4	10.7	19	-0.1	0.1	-
Callinectes sapidus	Blue crab	Whole body	*Artemia*	-22.3	6.9	21	1.0	1.5	Dittel *et al.* 2000
-	-	-	*Littoraria irrorata*	-14.6	10.3	21	0.2	0.8	-
-	-	-	*Uca pugnax*	-15.7	7.4	21	-0.1	0.9	-
-	-	-	Zooplankton	-16.6	10.8	21	-0.1	0.1	-
Nereis virens (Alitta virens)	King ragworm	Whole body	dry pellets (modified)	-21.6	7.5	60	2.3	3.2	Olive *et al.* 2003
Sepia officinalis	Cuttlefish	Buccal mass	*Litopenaeus setiferus* and *Farfantepenaeus aztecus*	-18.1	11.5	210	-0.3	3.3	Hobson & Cherel 2006
Mactra veneriformis	Bivalve	Whole body	Microalgae *Chaetoceros gracilis*	-14.5	-8.9	32	0.9	3.6	Yokoyama *et al.* 2005
Ruditapes philippinarum	Manila clam	Whole body	-	-14.5	-8.9	32	0.6	3.4	-
Nihonotrypaea japonica	Ghost shrimp	Muscle	-	-21.6	-8.2	50	2.1	3.6	-
Nihonotrypaea harmandi	Ghost shrimp	Muscle	-	-21.6	-8.2	50	2.2	3.7	-
Crassostrea gigas	Pacific oyster	Whole body	Microalgae *Skeletonema costatum*	-23.0	-4.9	90	1.9	3.9	Dubois et al. 2007
Mytilus edulis	Blue mussel	Whole body	-	-23.0	-4.9	90	2.2	3.8	-
Ophiocomina nigra	Brittle star	Whole body	Fish	-17.3	13.7	92	0.5	0.1	Blanchet-Aurigny *et al.* 2012
-	-	-	Blue mussel	-18.7	7.7	92	1.4	3.2	-
-	-	-	Green macroalage	-14.0	7.5	92	-1.8	3.5	-
Ophiothrix fragilis	Brittle star	Whole body	Blue mussel	-18.7	7.7	92	1.1	3.0	-
-	-	-	Green macroalage	-14.0	7.5	92	-3.5	2.7	-
Neogonodactylus bredini	Mantis shrimp	Muscle	snail *Cerithium eburneum*	-8.7	4.6	292	3.0	0.9	deVries *et al.* 2015
-	-	Hemolymph	-	-8.7	4.6	292	1.7	0.1	-
Hediste diversicolor	Ragworm	Whole body	Commercial food flakes	-22.1	9.2	60	2.2	3.6	This study
Cerastoderma edule	Cockle	Whole body	Microalgae *Skeletonema costatum*	-23.0	-4.9	90	4.6	10.8	-
Crepidula fornicata	Slipper limpet	Whole body	-	-23.0	-4.9	90	4.5	7.3	-
Lanice conchilega	Sandmason worm	Whole body	-	-23.0	-4.9	90	1.9	3.4	-
Sabellaria alveolata	Honeycomb worm	Whole body	-	-23.0	-4.9	90	4.4	9.3	-