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Methods for assessment of body tissue composition as an indication of the energetic status in bivalve populations: A comparison of biochemical and elemental analysis

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ABSTRACT

Elemental (CHN) and proximate biochemical compositions of body tissues are useful tools among the ecological indices most commonly used in evaluation of the energetic status of animal populations. Gnaiger and Bitterlich (1984) supplied procedures for the interconversion between both these measurements based on stoichiometric relationships, that were further tested using gut contents and body tissue samples of freshwater fishes. Despite a lack of validation studies, the reported conversion factors have been broadly applied in the field of body composition analysis of marine invertebrates, especially bivalve mollusks. The aim of this study was to test the applicability of those conversion equivalents in analysis of the body tissues of two congeneric clam species (*Ruditapes decussatus* and *Ruditapes philippinarum*). To this end, proximate biochemical composition, as analyzed by fractionated extraction of tissues samples and quantification using spectrophotometric methods, was converted to elemental composition, and the resulting figures were compared with those of direct CHN analysis. The results of this comparison indicate good agreement within the ranges reported, provided that ninhydrin positive substances (NPS) are incorporated in the biochemical composition analyses. The magnitude of the nonprotein N component in bivalve tissues appears to complicate the reverse computation of biochemical components from elemental composition because no accurate estimation of proteins from N contents might be possible. Additionally, a specific correction of residual water in dried samples of bivalve tissues for CHN analysis should be applied. The absence of broad differences found between species reflects the morphological, evolutionary and functional proximity between them, whereas tissue differences display the differential role that each organ plays in the organism, although other sources of variability such as diet and sex should be addressed in future research.

1. Introduction

Many studies performed over decades in the field of ecophysiology and aquaculture of bivalves have relied on analysis of the proximate biochemical composition of body tissues (Giese, 1969). Fractionated extraction and quantification of biochemical components in bivalve tissues using conventional colorimetric methods (Bligh and Dyer, 1959; Dubois et al., 1956; Folch et al., 1957; Lowry et al., 1951; Marsh and Weinstein, 1966; Phillips and Privett, 1979) have been widely applied for different purposes (Ansell, 1974; 1972; 1967;; Ansell et al., 1980; Davis and Wilson, 1983; Giese, 1969; Taylor and Venn, 1979), including computation of the caloric (energy) content using standard equivalents (Beukema and de Bruin, 1979). Two different issues account for this interest:

1) Biochemical composition is assumed to reflect the nutritional status of individuals and can consequently be used to experimentally assess the effects of variable diets on the tissue-specific trends of growth. In this way, several works have focused on the food value and its impact on growth (Albentosa et al., 2003; 1999;; Aranda-Burgos et al., 2014; Beukema and Cadée, 1991; Uriarte and Farías, 1999) or used biochemical composition as an indicator of nutritional and/or physiological state (Baek et al., 2014; Beukema and De Bruin, 1977; Deslous-Paoli and Héral, 1988; Okumuş and Stirling, 1998; Pogoda et al., 2013; Walne and Mann, 1975).

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2) Life cycles encompass changes in biochemical composition, and the separate analysis of tissues along these cycles might aid understanding of the temporal dynamics of nutrient transference between different body systems, which is mainly associated with the specific requirements of gametogenesis. The biochemical composition of bivalves varies seasonally and depends on food availability and timing of the reproductive cycle, among other factors (Aníbal et al., 2011; Beninger and Lucas, 1984; De Zwaan and Zandee, 1972; Delgado and Pérez-Camacho, 2007; Navarro et al., 1989; Ojea et al., 2004; Robert et al., 1993; Rodríguez-Moscoso and Arnaiz, 1998; Shafee, 1981). Analysis of these changes (reviewed by Gabbott, 1976; Giese, 1969; Sastry, 1979) revealed that storage and mobilization of reserves (primarily glycogen) are closely linked to the annual reproductive cycle, suggesting a metabolic cycle of transformation of carbohydrates into lipids for the specific needs of gametogenesis in many instances (Aníbal et al., 2011; Beninger and Lucas, 1984; Dridi et al., 2006; Gabbott, 1976; 1975;; Gabbott and Bayne, 1973; Ojea et al., 2004; Zandee et al., 1980). The direct transfer of nutrients from the digestive system to the gonad has also been demonstrated (Beninger et al., 2003).

A complementary approach to these subjects relies on the use of elemental (CHN) analysis of body tissues related to the composition of potential or actual food resources. Stoichiometry principles (Sterner and Elser, 2002), which refer to the balance of nutrients (elements) within and between organisms, can be applied to account for trends in body composition during growth. This multielemental approach recognizes that the nutritional demands for maintenance and growth apply to specific proportions of the same basic components (protein, carbohydrate and lipids) that are seldom present in the required ratios within the available foods and uses elemental balance analyses (Bayne, 2009; Grant and Cranford, 1991; Hawkins and Bayne, 1985; Iglesias et al., 1996; Smaal and Vonck, 1997; Tamayo et al., 2013; Urrutia et al., 1996) to supply new insight that qualifies the already existing knowledge based on energy flow models of bivalve growth.

Biochemical composition in terms of major components (protein, carbohydrate and lipids) can be inferred on a stoichiometric basis from elemental composition (Gnaiger and Bitterlich, 1984), and quantification of biochemical components in tissues using colorimetric methods allows the elemental composition to be determined using standard equivalents (Beukema and de Bruin, 1979; Brody, 1945). The possibility of interconversion between biochemical composition measurements using both methods is interesting because each of these methods can meet different experimental demands. For instance, given that the preparation requirements for samples are minimal, CHN analysis yields immediate results while avoiding multiple-step processes for direct determination of biochemical components that are considerably time-consuming and involve large amounts of consumables and chemical disposal (mostly toxicants). Moreover, sample size requirements are much lower in CHN determinations (6–10 times), allowing the analysis of smaller samples and broad use of replicates. However, direct CHN analysis is not suited to addressing whole-body samples of bivalves (e.g., larvae or small individuals for which soft body cannot be readily dissected out of the shell) due to the distorting effect of the shell inorganic carbon, and body composition analysis in those cases forcibly relies on conventional biochemical methods.

Thus, intercalibration of these two methods using an empirical approach seems to be a desirable goal in studies aiming to integrate growth energetics data from different sample sources and life stages. Gnaiger and Bitterlich (1984) established a direct correspondence of this type between proximate and elemental composition using white muscle, liver, fat tissue and gut contents of the Chinese silver carp *Hypophtalmichthys molitrix*. The equations presented in their study have been extensively applied for the purpose of calculating biochemical composition from data on CHN composition of bivalve tissues. However, and in spite of the high demand (over 50 citations in the literature referring to bivalves), we are not aware of any studies designed to validate the main results of their study using bivalve species. Indeed, Pogoda et al. (2013) combined biochemical and elemental analysis to report the body composition of oyster species (*Ostrea edulis* and *Crassostrea gigas*), but the correspondence between both methods was not approached in a quantitative manner in that study and mainly centered on the analysis of lipid classes.

Therefore, the main aim of this study was to experimentally test the accuracy of those equivalents that relate proximate biochemical to elemental composition through a comparison of direct CHN measurements and values of elemental composition derived from biochemical measurements performed in bivalve tissues. The study was designed to compare different organs and soft-body tissues in clams sampled from populations of two con-generic species (*Ruditapes decussatus* and *R. philippinarum*), so as to evaluate the applicability of this approach in a broad range of conditions. Interorgan comparison was also performed to set the foundation for assessing nutrient transference between different body systems.

2. Materials and methods

2.1. Animal collection and maintenance

Adult specimens (Table 1) of the carpet shell clam *Ruditapes decussatus* [38.03 (1.34) mm shell length] and the Manila clam *R. philippinarum* [36.30 (0.29) mm shell length], collected by hand from the same point in the muddy flats near Santoña (Cantabria, Spain), were purchased from licensed shell-fishers and transported to our facilities by the end of July 2016. In the laboratory, the clams were kept under controlled conditions of salinity (35‰), temperature (18–19 °C) and oxygen supply (9 mg/L) and fed on a *Isochrysis galbana* (T-Iso) diet at a concentration of 20,000 cells ml^{-1} for 15 days until dissections were performed.

After cutting off the adductor muscle, the soft tissues were excised from the shell and separated into 5 different organs: gill, adductor muscle, digestive gland, gonad and remaining tissues composed primarily of the siphons, mantle and foot. Tissues were rapidly frozen by immersion in liquid nitrogen and freeze dried. Finally, samples were ground to a powder with a mortar and pestle and stored at −20 °C until analyses were performed.

2.2. Sample analysis

Tissue samples from five individuals per species were used in both proximate biochemical composition and elemental (CHN) analysis. To that end, five subsamples were taken from each sample to determine carbohydrate (2.5–3 mg), protein (2–2.5 mg), lipid (2.5–3 mg), CHN (1–1.5 mg), and ash (~10 mg.) contents. Special care was taken to keep the samples dried, particularly in those subsamples for elemental analysis, to prevent the inclusion of hydrogen from rehydration. Organic content (ash free dry weight) was estimated from the difference between

Table 1
Mean (SD) live (mg ind^{-1}) and organic weight (mg ind^{-1}) and estimated energy value of the whole animal (J g^{-1}) from both employed species.

	Ruditapes decussatus	*Ruditapes philippinarum*
Live weight	11372.5 (1697.2)	11944.5 (1161.7)
Organic weight:		
Hard tissues	101.8 (11.7)	110.8 (17.3)
Soft tissues	551.5 (33.9)	536.4 (43.8)
Gill	59.6 (10.0)	50.0 (10.4)
Gonad	126.9 (31.7)	192.4 (38.3)
Digestive gland	71.9 (12.3)	49.5 (15.6)
Adductor muscle	69.9 (11.0)	66.0 (8.0)
Remaining tissues	223.1 (37.5)	178.5 (18.7)
Estimated energy value*	25.3 (0.2)	25.8 (0.3)

*Estimations based on enthalpies of combustion of biochemical components reviewed by Gnaiger and Bitterlich (1984)

the dry weight (24 h at 100 °C) and ash weight (6 h at 450 °C) of the samples.

Gross biochemical composition was determined in triplicate using colorimetric methods. Carbohydrates were extracted in TCA (5%) and quantified according to Dubois et al. (1956) using dried glycogen from oyster as a standard. Proteins were extracted in NaOH (0.4 N) and quantified according to (Lowry et al., 1951) with a bovine sero-albumin standard. Finally, lipids were pre-extracted in acetic acid (Phillips and Privett, 1979), extracted twice in methanol:chloroform at 2:1 and 1:2 (Bligh and Dyer, 1959; Folch et al., 1957), and quantified according to Marsh and Weinstein (1966) against a tripalmitin-phosphatidylcholine 1:1 standard.

A preliminary assessment of consistent mismatches between N contents estimated from elemental analysis and those derived from protein contents in biochemical analysis drew our attention to nonprotein nitrogen. The most abundant component of this N fraction in marine invertebrates is known to be ninhydrin-positive substances (NPS), which include amino acids and taurine acting as organic osmolytes (Hochachka and Somero, 2002). Because bivalve tissues have been reported to contain at least 5% NPS over ash free dry weight (AFDW) (Navarro et al., 1989; Shumway et al., 1977), we hypothesized that these compounds could account for the observed difference between direct and protein-derived N measurements. Consequently, NPS values were determined and computed together with gross biochemical composition for a more accurate comparison of the elemental composition obtained by direct and indirect methods. Samples for NPS determinations were taken from the same 5 organs in the two species but obtained from a different pool of 5 individuals collected and processed in the same manner as for gross biochemical composition and CHN. Determinations were performed following the procedures described by Shumway et al. (1977). Approximately 10 mg of powered freeze-dried tissue from each pool was extracted in 1 ml of 80% ethanol at 95 °C, and colorimetric methods (Moore and Stein, 1954) were developed on five replicates of the extract using leucine as a standard.

Elemental analyses of carbon, nitrogen and hydrogen content were performed at the SGIker facilities (UPV/EHU) in a Euro EA Elemental Analyzer (CHNS) from EuroVector using acetanilide as standard. To compute the CHN composition of the organic fraction, three burned (450 °C) samples per tissue and species were also analyzed to correct for CHN content of ashes.

2.3. Data analysis

The biochemical elements (i.e., carbohydrates, proteins and lipids) and NPS percentage were estimated over total organic content (mean percentage of AFDW (SD): 82.56% (5.02)). The mean (SD) percent recovery of the AFDW from the added values of the four components amounted to 84.01% (14.67). Subsequently, the percentage not recovered (15.99%) was assigned to the four components according to their relative percentages. Hence, the amount of each element is expressed as the percentage of each element over the sum of carbohydrates, proteins, lipids and NPS.

Elemental components derived from biochemical composition (CHN (b)) were calculated following Gnaiger and Bitterlich (1984). CHN data from the CHN analyzer (CHN(a)) were first corrected to estimate the element percentages over AFDW (mean (SD) recovery percentage: 68.02% (3.64)) and were subsequently corrected to subtract the residual water fraction according to Gnaiger and Bitterlich (1984). Thus, the results are presented as the percentage of each element over the sum of C, H and N.

To compute an estimate of the whole soft tissues proximate composition, the dry weight of every tissue was multiplied by the fraction of each biochemical component to obtain the component weight. Each element weight was summed and subsequently divided by the total dry weight as follows:

$$Whole\ soft\ tissue\ component\ (\%) = \frac{\sum\left(DWt_i \times \frac{Component_i(\%)}{100}\right)}{\sum DWt_i} \times 100$$

Statistical analyses were performed using nonparametric tests, which were chosen due to the nature of the data (percentages). Regression analyses, paired Wilcoxon tests, two-way ANOVA and three-way ANOVA on ranks were run using R software (R Core Team, 2018). Post hoc tests (Tukey's honestly significant difference, HSD) were run after ANOVA on ranks analyses to test differences among tissues.

3. Results

Elemental composition (CHN) estimated from the proximate biochemical composition or the direct analysis of the samples yielded similar values (Fig. 1). However, slight differences were detected. For instance, although the paired Wilcoxon tests for N values revealed an absence of differences between methods ($V = 443$, p-value $= 0.06$), C values derived from biochemical components (mean: 72.09%) were significantly higher compared with direct determinations (mean: 70.24%) ($V = 1255$, p-value < 0.001), and H values (mean: 9.74%) were underestimated with respect to direct determinations (mean: 11.28%) ($V = 2$, p-value < 0.001). Indeed, the ranges of variation differed between direct (9.19–12.32%) and indirect (9.34–10.17%) estimates of H and the regression equation for the relationship between both sets of data was not significant (Fig. 1). Conversely, the corresponding regression equations for C and N were highly significant.

From these small differences, the elemental composition derived from biochemical components resulted in slight overestimations of both the C:N ratios (mean of the differences = 0.15%; paired Wilcoxon test: $V = 951$, p-value $= 0.003$) and C:H ratios (mean of the differences = 1.16%; paired Wilcoxon test: $V = 1274$, p-value < 0.001) compared with the direct CHN measurements.

The biochemical and elemental composition data of different tissues in both species are presented comparatively in Table 2. As a general outline of biochemical composition, proteins were the most abundant component (70.28%), followed by lipids (12.25%), NPS (10.83%) and carbohydrates (6.63%), irrespective of tissue and species. No consistent tendency was found for interspecific differences in the biochemical composition (Table 3).

Conversely, differences between tissues were significant for all biochemical components, including NPS (Table 3). Carbohydrates were especially abundant in the adductor muscle and secondarily in the digestive gland and remaining tissues, whereas the percentage of proteins was high in adductor muscle and gill (Fig. 2 and Table 2). Lipids showed a tissue distribution pattern approximately opposite to that of carbohydrates, with high values recorded in the gonad, gill and digestive gland and minimum contents observed in the adductor muscle. Ninhydrin-positive substances reached the highest values in the remaining tissues, followed by digestive gland and adductor muscle. Despite the lack of overall interspecific differences, certain organs such as the digestive gland and gonad exhibited noticeable differences in biochemical composition between *R. decussatus* and *R. philippinarum*. In both organs, the lipid content was higher and the protein content was lower in the former than in the latter species (Fig. 2B, C), as shown by the trends towards significant p-values of the tissue*species interaction term for proteins and lipids (Table 3). This interaction term was found to be highly significant for NPS ($p < 0.001$) as a consequence of the higher content of these substances in the digestive gland and adductor muscle of Manila clam, whereas the remainder of the tissues showed higher NPS values in the carpet shell clam.

As reported for the biochemical composition, the elemental composition and the ratios C:N and C:H varied significantly among tissues (Table 4). Both methods rendered the highest C content in the digestive gland and gonad and the lowest in the remaining tissues and the

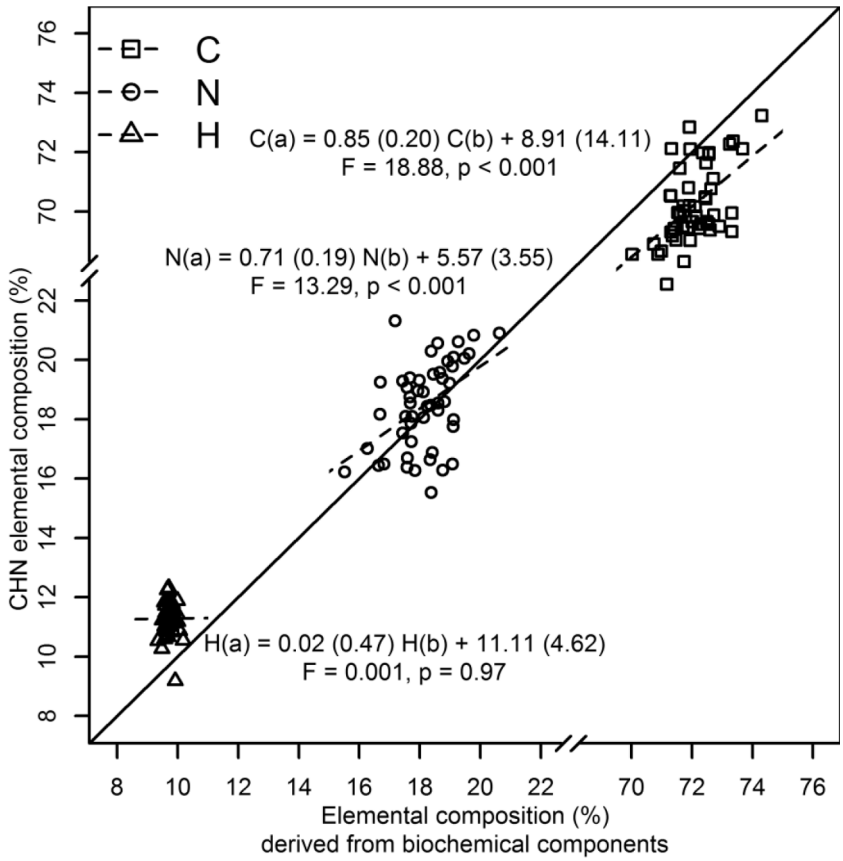

Fig. 1. Relationship between direct determinations of elemental components CHN (a) and estimations based on biochemical composition CHN (b) for carbon (□) nitrogen (○), and hydrogen (Δ). Lines represent regression fits for each element between direct determinations and estimations.

adductor muscle (Table 5), whereas the N contents followed the opposite trend. Consequently, C:N ratios ranged between a maximum (4.11–4.22) in the digestive gland to a minimum (3.38–3.49) in the adductor muscle. Hydrogen contents, although significant among tissues, varied within a narrower range of between 11.62% (mean content of gills) and 10.86% (mean content of adductor muscle).

4. Discussion

With respect to the compatibility of data on body tissue composition achieved with different analytical methods (elemental vs. biochemical analysis), this study demonstrates the suitability of the conversion factors proposed by Gnaiger and Bitterlich (1984) for application in studies with bivalves. As a consequence of the rather good level of agreement between methods, a direct correspondence was appreciated between percentages of biochemical components (Fig. 2) and elemental components (Fig. 3). Indeed, in a context of tissue- and species-specific variability, nitrogen matched the dynamics of protein plus NPS, whereas percentage carbon variation reflected a combination of lipid and carbohydrate content, with greater similarity to the lipid profile, given its higher proportion in most of the tissues. On the other hand, conversion equivalents used to estimate elemental composition (with the exception of H) could be the same, irrespective of species or tissues, as indicated by the virtual absence of significant interaction terms between the method and the other factors in the ANOVA (Table 4).

However, certain limitations of the validation procedure should be acknowledged: 1) The regression equations relating direct and biochemically estimated compositions for each element (C, H and N) (Fig. 1) differ from the Y = X relationship expected in the case of complete correspondence, even though discrepancies between both methods are not significant in the case of N and amount to <3% for C. It is highly likely that this departure from strict agreement results from the rather narrow range of values fitted with the regression equations, which sets strict limits on the possibility of applying these conversion relationships for extrapolation out of the aforementioned range. Additionally, the effect of correcting for the %AFDW not accounted for by biochemical components should be considered (see Section 2: *Data analysis*) because the efficiency of extraction might differ in the different components. 2) The data for H revealed poor agreement between methods, which resulted in a nonsignificant regression equation (Fig. 1), with direct measurements displaying a wider range of variation than estimations from biochemical components. Overestimation of %H in the direct measurement (13.7% compared with 9.74% in the indirect estimation) suggests hydration of tissue samples, not detectable in the biochemical analysis but liable to increase the H contents recorded with the elemental analyzer. It is worth noting that the above discrepancies remained after a correction for 6% residual water in dried samples was performed following recommendations by Gnaiger and Bitterlich (1984) for samples of carp tissues. This result suggests that the amount of residual water in the dried tissues of bivalves might far exceed that of fresh water species, likely due to the hygroscopic effect of higher salt content. 3) In addition to H contents, direct and estimated measurements also differed for C contents and hence the C:N and C:H indices (see ANOVA in Table 4). More specifically, the C percentages derived from biochemical composition (mean of different tissues and species: 72.09%) are higher than those from direct estimation (mean: 70.24%), and this overestimation represents 2.6% of the average %C value. It is possible that standard conversion factors for C do not accurately reflect the actual composition of biochemical components in clam tissues, but most likely the reduced %C in the direct estimate is a mere consequence of increased H content caused by residual water. 4) The N percentages estimated from protein contents (mean: 17.4%) were significantly lower than

Table 2
Mean (SD) values (%) by tissue and species (R. dec: *R. decussatus*, R. phi: *R. philippinarum*) of biochemical composition (Cho: carbohydrates, Prot: proteins, Lip: lipids and NPS) and elemental composition (C, N and H) estimated by both methods (Direct: direct analysis, Derived: derived from biochemical composition).

Tissue	Species	Cho	Prot	Lip	NPS	C		N		H		C:N		C:H	
						Direct	Derived	Direct	Derived	Direct	Derived	Direct	Derived	Direct	Derived
Gill	**R. dec**	4.77 (0.54)	69.94 (5.97)	14.22 (4.08)	11.07 (1.55)	70.32 (0.26)	72.1 (0.85)	17.96 (0.16)	18.15 (1.02)	11.71 (0.15)	9.75 (0.17)	3.91 (0.05)	3.98 (0.28)	6.00 (0.1)	7.39 (0.04)
	R. phi	4.08 (0.38)	73.16 (2.21)	14 (1.51)	8.75 (0.97)	70.1 (0.31)	71.92 (0.34)	18.38 (0.19)	18.36 (0.41)	11.52 (0.22)	9.72 (0.07)	3.81 (0.05)	3.92 (0.11)	6.08 (0.14)	7.4 (0.02)
Gonad	**R. dec**	5.55 (0.65)	71.92 (3.1)	12.44 (3.24)	10.09 (1.06)	70.08 (2)	71.91 (0.61)	18.25 (2.19)	18.38 (0.74)	11.67 (0.44)	9.71 (0.12)	3.89 (0.56)	3.92 (0.19)	6.01 (0.23)	7.41 (0.03)
	R. phi	5.37 (1.5)	68.78 (6.39)	17.85 (4.69)	8 (0.63)	71.83 (1.39)	72.89 (1.09)	17.43 (2.18)	17.2 (1.31)	10.74 (0.93)	9.91 (0.21)	4.17 (0.51)	4.26 (0.38)	6.72 (0.53)	7.36 (0.05)
Digestive gland	**R. dec**	7.63 (1.8)	70.01 (1.71)	11.69 (0.87)	10.67 (1.14)	70.94 (1.52)	72.17 (0.35)	17.37 (1.49)	18.08 (0.41)	11.68 (0.39)	9.75 (0.06)	4.11 (0.44)	3.99 (0.11)	6.08 (0.26)	7.4 (0.01)
	R. phi	7.2 (2.48)	63.82 (2.26)	16.11 (3.11)	12.87 (1.39)	71.76 (0.6)	72.92 (0.52)	17.04 (0.69)	17.18 (0.62)	11.2 (0.28)	9.9 (0.1)	4.22 (0.2)	4.25 (0.19)	6.41 (0.16)	7.37 (0.02)
Adductor muscle	**R. dec**	9.24 (4.24)	74.73 (3.94)	6.98 (1.33)	9.05 (1.04)	69.22 (0.37)	71.6 (0.59)	19.81 (0.31)	18.77 (0.68)	10.97 (0.24)	9.62 (0.09)	3.49 (0.07)	3.82 (0.17)	6.31 (0.16)	7.44 (0.01)
	R. phi	7.43 (2.99)	74.72 (5.01)	5.53 (0.97)	12.31 (1.49)	68.84 (0.28)	70.99 (0.72)	20.41 (0.46)	19.5 (0.84)	10.75 (0.38)	9.51 (0.12)	3.38 (0.09)	3.65 (0.19)	6.41 (0.23)	7.46 (0.02)
Remaining tissues	**R. dec**	7.7 (1.52)	66.17 (3.42)	11.78 (2.21)	14.35 (1.03)	69.65 (0.22)	72.2 (0.49)	19.05 (0.33)	18.04 (0.59)	11.3 (0.34)	9.75 (0.09)	3.66 (0.07)	4.01 (0.15)	6.17 (0.2)	7.4 (0.02)
	R. phi	7.34 (0.83)	69.56 (4.55)	11.93 (3.83)	11.17 (1.32)	69.65 (0.3)	72.16 (0.7)	19.13 (0.39)	18.09 (0.83)	11.22 (0.32)	9.75 (0.14)	3.64 (0.08)	4.00 (0.23)	6.21 (0.19)	7.4 (0.03)

Table 3
Results of two-way ANOVA on ranks testing the effects of tissue and species (as factors) on biochemical composition (% of Cho: carbohydrates, Prot: proteins, Lip: lipids and NPS).

	Cho	Prot	Lip	NPS
Tissue	**F = 9.543 p < 0.001**	**F = 5.486 p = 0.001**	**F = 14.387 p < 0.001**	**F = 14.670 p < 0.001**
Species	F = 1.641 p = 0.208	F = 0.330 p = 0.569	F = 3.734 p = 0.060	F = 2.365 p = 0.132
Tissue * Species	F = 0.174 p = 0.950	F = 2.315 p = 0.074	F = 2.153 p = 0.092	**F = 15.400 p < 0.001**

those from direct estimation (mean: 18.6%), but a perfect match between direct and indirect methods of N determination was achieved after the incorporation of NPS (ninhydrin positive substances) in the biochemical composition. This outcome confirms the importance of nonprotein N in assessing the body composition of bivalves, given that current estimations have accorded as much as 10% of AFDW to the fraction represented by free amino-acids and other nitrogenous compounds serving the function of osmotic compensation (Hochachka and Somero, 2002).

The incoming discussion concerning species- or tissue-specific differences in composition was decided to be based on biochemical components, considering the good correspondence between both methods and the paucity of data reporting the elemental composition of tissues in clam species of the genus *Ruditapes*. Differences among tissues are the main source of variation in every biochemical compound (Table 3). Thus, proteins and lipids were abundant in supporting tissues involved in water pumping and gas exchanges, such as the gill and mantle, including the siphons. Gills in particular were essentially composed of proteins (72%) and lipids (14%) because that organ is characterized by a high surface area, including cilia, and thus a large amount of membrane is required to satisfy their functions. The remaining tissues, that comprised the foot and mantle, also included a significant amount of carbohydrates, likely reflecting the role of the mantle in storage of glycogen (De Zwaan and Zandee, 1972) as the preferential form of energy reserves in bivalves (Laing, 1993). However, the main carbohydrate depots were found in the adductor muscle, related to the energy supply for muscular control of valve movements, although the use of this store to support gametogenesis has also been broadly documented in bivalves (Barber and Blake, 1985; 2006;; Chantler, 2016; Kang et al., 2019). Previous studies in the Manila clam (Shiraishi et al., 1995) and the carpet shell clam (Ojea et al., 2004) performed in the same period of the year, both revealed higher carbohydrate contents in the above group of tissues (gill, muscle and remaining tissues), with a consequent reduction in the lipid and protein levels with respect to this study. Although it is possible that differences in reproductive or energetic status might account for these discrepancies (Albentosa et al., 2007; Marin et al., 2003; Pérez-Camacho et al., 2003), the distorting effect of NPS (not quantified in the previous studies) on the proportions of the remainder of the components should also be considered.

No great interspecific differences in biochemical composition were found, although selected trends could be detected (Table 6). Overall, the proximate composition of each species falls within the range previously reported for *R. decussatus* (e.g., Albentosa et al., 1999; Aníbal et al., 2011; Ojea et al., 2004; Pérez-Camacho et al., 2003) and *R. philippinarum* (Baek et al., 2014; Marin et al., 2003; Robert et al., 1993), with the above-referenced exception for carbohydrates. This observation represents a minor discrepancy because this component has been reported to exhibit great seasonal and interannual fluctuations (Beninger and Lucas, 1984; Navarro et al., 1989).

Lipids reached a higher level in *R. philippinarum,* which resulted in reduced values for carbohydrates and NPS, but only differences in the lipid component were statistically significant (Table 3. Particularly, interspecies differences in lipid contents were restricted to the gonad and digestive gland, which accounts for the significance of the

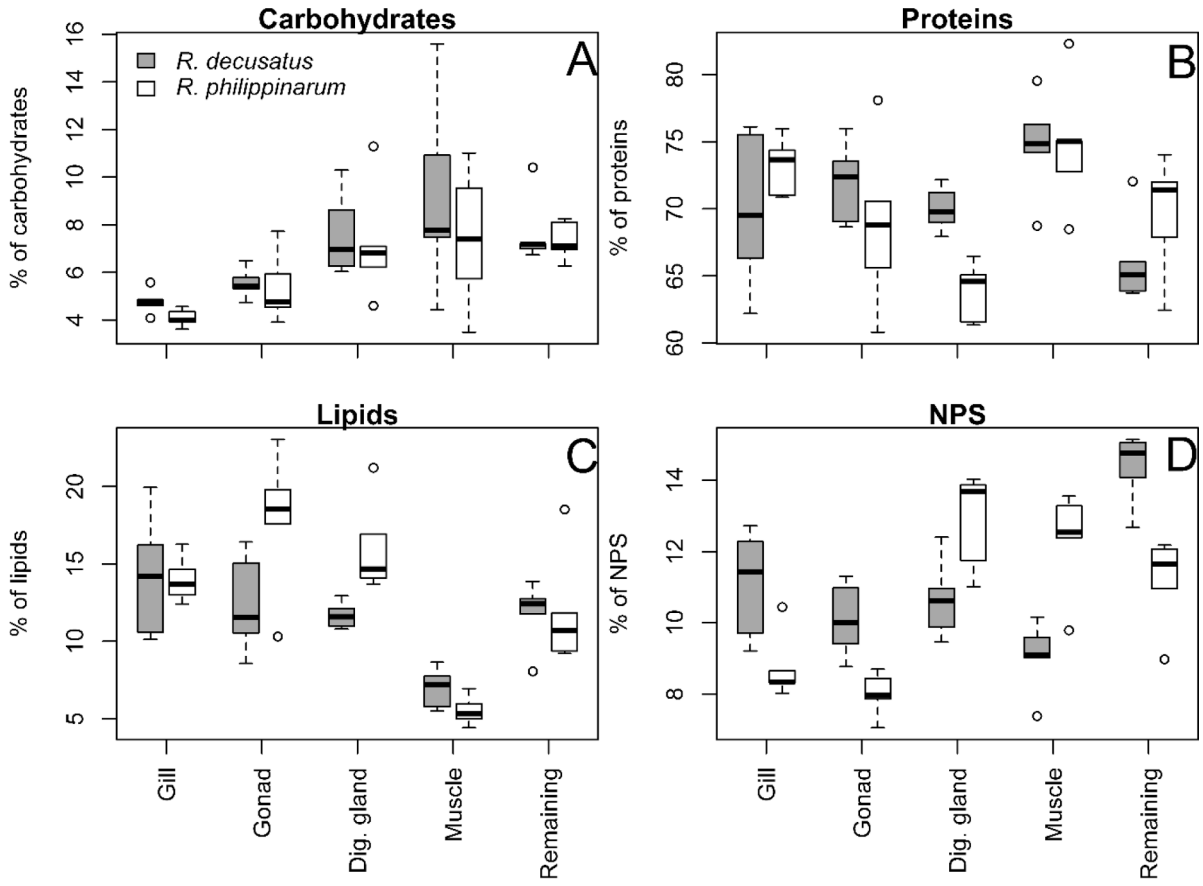

Fig. 2. A) Carbohydrate, B) protein, C) lipid and D) NPS percentage in *R. decussatus* (gray) and *R. philippinarum* (white) by tissues.

Table 4
Results of three-way ANOVA on ranks testing the effects of method, tissue and species (as factors) on the elemental composition (C, N and H) and C:N and C:H ratio.

	C	N	H	C:N	C:H
Method	**F = 109.667, p < 0.001**	F = 3.099, p = 0.08	**F = 340.285, p < 0.001**	**F = 12.082, p < 0.001**	**F = 376.323, p < 0.001**
Tissue	**F = 15.142, p < 0.001**	**F = 17.960, p < 0.001**	**F = 9.219, p < 0.001**	**F = 16.926, p < 0.001**	**F = 8.384, p < 0.001**
Species	F = 2.682, p = 0.105	F = 0.585, p = 0.447	F = 2.411, p = 0.124	F = 0.809, p = 0.371	F = 3.577, p = 0.062
M*T	F = 1.963, p = 0.108	**F = 2.786, p = 0.032**	F = 2.258, p = 0.070	**F = 2.890, p = 0.027**	**F = 4.063, p = 0.005**
M*S	F = 0.432, p = 0.513	F = 0.053, p = 0.818	**F = 8.894, p = 0.004**	F = 0.006, p = 0.940	**F = 14.066, p < 0.001**
T*S	**F = 3.824, p = 0.007**	F = 2.394, p = 0.057	F = 0.174, p = 0.951	**F = 2.501, p = 0.049**	F = 0.490, p = 0.743
M*T*S	F = 0.148, p = 0.963	F = 0.165, p = 0.955	**F = 3.517, p = 0.011**	F = 0.172, p = 0.952	**F = 4.670, p = 0.002**

tissue*species interaction term (Table 3). Lipid levels in both these organs are closely related to the reproductive cycle. The lipid contents of the gonad tend to peak during the spawning season (Bayne et al., 1982; Beninger and Lucas, 1984; Gabbott, 1983; Pieters et al., 1979), whereas digestive gland lipids are believed to be transferred to the gonad for gametogenesis purposes (Barber and Blake, 1985; 1981;; Mori, 1975; Robinson et al., 1981). Therefore, rather than constituting interspecific variability, these differences might reflect different reproductive stages

Table 5
Mean values (%) and post hoc (Tukey's HSD test) analyses for different tissues after three-way ANOVA on ranks analyses (see Table 4) calculated for elemental components (C, N and H) and C:N and C:H ratios. Tissue elements include data from both species. Different superscripts indicate significant differences among tissues at a significance level of $\alpha = 0.05$.

Tissue	Gill	Gonad	Dig. Gland	Adductor muscle	Remaining tissues
C	70.21 (0.29)^{ab}	70.95 (1.87)^{a}	71.35 (1.17)^{a}	69.03 (0.37)^{c}	69.65 (0.25)^{bc}
N	18.17 (0.27)^{bc}	17.84 (2.10)^{bc}	17.21 (1.11)^{c}	20.11 (0.48)^{a}	19.09 (0.34)^{ab}
H	11.62 (0.21)^{a}	11.20 (0.84)^{a}	11.44 (0.41)^{a}	10.86 (0.33)^{b}	11.26 (0.32)^{ab}
C:N	3.86 (0.07)^{ab}	4.03 (0.53)^{ab}	4.17 (0.33)^{a}	3.43 (0.10)^{c}	3.65 (0.07)^{bc}
C:H	6.04 (0.12)^{b}	6.37 (0.54)^{ab}	6.24 (0.27)^{ab}	6.36 (0.20)^{a}	6.19 (0.18)^{ab}

at the time of collection (end of July) because *R. decussatus* is known to spawn between June and August (Aníbal et al., 2011; Ojea et al., 2004), whereas spawning is delayed to September-October in *R. philippinarum* (Robert et al., 1993). In addition, the latter species is characterized by a longer period of spawning and more intense reproductive activity (Delgado and Pérez-Camacho, 2007). In other words, it is highly likely that the carpet-shell clams used in this study had already spawned, whereas Manila clams were on the verge of spawning. Indeed, surplus specimens of both clams maintained in our facilities for up to four months from the end of experiments exhibited differential behavior with respect to spawning, with *R. philippinarum* clams spawning massively in the first week while *R. decussatus* showed no spawning activity at all for the entire period.

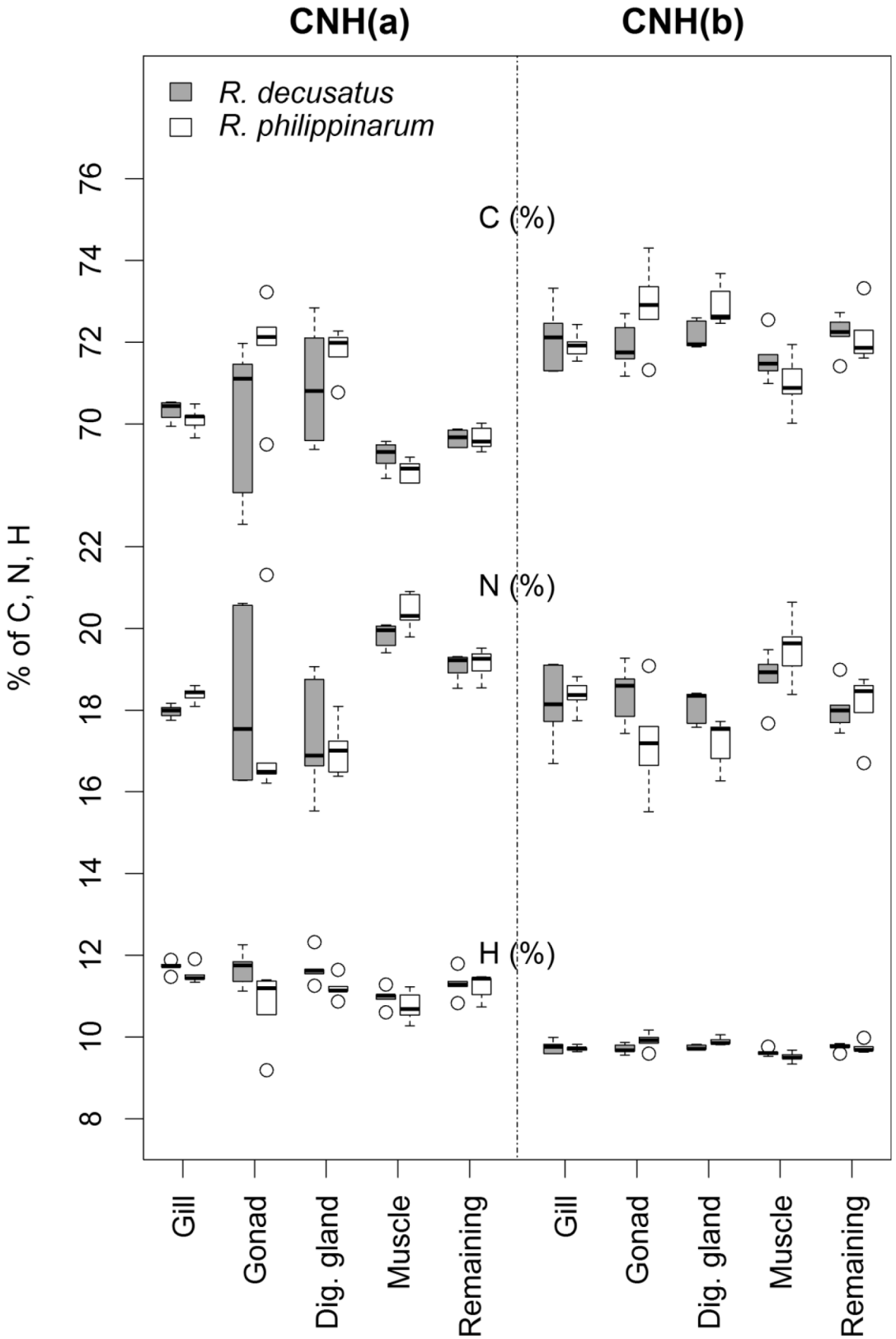

Fig. 3. Carbon, nitrogen and hydrogen percentage in *R. decussatus* (gray boxes) and *R. philippinarum* (white boxes) by tissue estimated via elemental analyzer (CHN (a), left) and biochemical methods (CHN (b), right).

In conclusion, the equivalents reported by Gnaiger and Bitterlich (1984) were suited, within the ranges we explored, to the purpose of converting proximate biochemical composition into elemental (CHN) composition in bivalve tissues, although specific changes are required to include NPS as a highly significant component of the nonprotein N fraction. This observation sets limits for the reverse computation of biochemical components from elemental composition because no accurate estimation of proteins is possible from N contents. Additionally,

Table 6
Mean (SD) values (%) of whole-flesh biochemical composition (Cho: carbohydrates, Prot: proteins, Lip: lipids, NPS: ninhydrin-positive substances) for each species.

	Cho	Prot	Lip	NPS
R. philippinarum	6.22 (0.90)	69.42 (2.87)	14.59 (2.15)	9.77 (0.532)
R. decussatus	6.90 (1.08)	69.90 (1.09)	11.52 (1.01)	11.68 (0.47)

in the case of bivalve tissues, a specific correction should be applied for residual water in dried samples for CHN analysis. The absence of broad differences found between species reflects the morphological, evolutionary and functional proximity between them, whereas the tissue differences displayed the differential role that each organ plays in the organism, even though other sources of variability such as diet and sex should be addressed in future research. Finally, the alleged interspecific differences in the biochemical and elemental compositions of specific tissues (digestive gland and gonad) might reflect a shift in timing of reproduction between both species, making plain the convenience of a seasonal approach in further studies linking physiological status to body tissue composition.

CRediT authorship contribution statement

Kristina Arranz: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Visualization. **Iñaki Urrutxurtu:** Conceptualization, Methodology, Formal analysis, Investigation, Writing - review & editing, Supervision, Project administration. **Daniel Prieto:** Writing - review & editing. **Irrintzi Ibarrola:** Writing - review & editing. **Miren Bego Urrutia:** Writing - review & editing. **Enrique Navarro:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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