An assessment of sample processing methods for stable isotope analyses of coastal foodwebs

Sirka Carabel[1], Enrique Godínez-Domínguez[2], Patricia Verísimo[1], Luís Fernández[1] and Juan Freire[1*].

[1] Departamento de Bioloxía Animal, Bioloxía Vexetal e Ecoloxía. Universidade da Coruña. Campos da Zapateira s/n. E-15071. A Coruña, Spain. Tel. +34 (981) 1670000 ext 2204 Fax. +34 (981) 167065

[2] Centro de Ecología Costera. Universidad de Guadalajara. 48980. Melaque, Mexico.

[*] Corresponding author: Ph.: 34981167000, Fax: 34981167065, e-mail: jfreire@udc.es

Abstract

The relative abundance of the light and heavy forms of stable isotopes of Carbon and Nitrogen is commonly used in the research of marine food webs. Dehydration of samples is needed for C and N isotopic analysis, and in those organisms with inorganic carbonate structures acidification with hydrochloric acid is also needed for sample preparation. There is not a consensus about the methodology appropriate for sample processing. In the present study, the effects of different sample processing methods in the relative abundance of stable isotopes of C and N in the different trophic compartments of a coastal food web are evaluated. The final purpose is to define an adequate methodology to be used in the construction of whole food webs. The results obtained after acidification show a decrease in carbon values for sedimentary organic matter, suspended particulate organic matter, plankton and invertebrates with carbonated structures, and a variable response for nitrogen values. According to our

results we recommend sampling acidification for carbon analysis in compartments in which variations of this element were observed. For nitrogen analysis acidification should not be done. Different treatments of dehydration studied provoked significant differences only in nitrogen values, thereby freeze-drying seems the most adequate method. Muscle tissue should be used in invertebrate with carbonated structures whenever body sizes make it possible.

Keywords: stable isotopes, foodweb, coastal ecosystem, sample processing

Introduction

Stable isotope analysis is commonly used to study trophic relationships in marine ecosystems (Peterson and Fry, 1987; Kwak and Zedler, 1997; Fantle et al., 1999; Peterson, 1999). However, there are large differences among the studies in the processing methods used to prepare samples for determination of isotopic ratios (Christensen et al., 2001; Kurata et al., 2001; Fry and Smith, 2002); these differences are observed both in different studies developed with similar species as in the analysis of the ecosystem trophic compartments (organisms and dead organic matter pools). This variation difficult the comparison of the isotopic ratios and the integration of the results obtained from different trophic compartments to build models of complete food webs.

Nitrogen and Carbon are two of the most commonly used elements in isotopic studies of marine ecosystems. Their use is based in the approximately constant change along the different trophic levels, with a fractionation of consumer tissue relative to their food source of approximately 3‰ in the $\delta^{15}N$ values (Owens, 1987) and minimal in $\delta^{13}C$ (0-1‰, DeNiro and Epstein, 1978; Fry and Sherr, 1984). Besides, ratios of C isotopes are closely linked to the origin of the organic matter (average values of -22‰ for planktonic

marine algae, -17 ‰ for benthic marine algae, France (1995); from -24‰ to -18‰ for suspended particulate organic matter (SPOM) of marine origin, Yokoyama and Ishihi (2003); and values from -26‰ to -1‰ for sedimentary organic matter (SOM), unpublished data).

The lack of understanding of the changes in ratio estimates, that the sample processing previous to the isotopic analytical determination may cause, could difficult the interpretation of results and induce errors in the resulting food web models. Dehydration of samples is needed for C and N isotopic analysis, and in those organisms with inorganic carbonate structures acidification with hydrochloric acid (HCl) is also needed for sample preparation (Vizzini and Mazzola, 2003). Even though there are different studies on the effects that these treatments provoke in the isotopic records of certain species (Bunn et al., 1995, Pinnegar and Poluninn, 1999), the systematic distortions that these processes could cause in the isotopic values found along the different trophic compartments has not been assessed yet. Such assessment is necessary for the data integration to develop models of complete food webs.

Dehydration is required by the analytical methods used in the determination of isotopic abundances. It can be done freeze-drying or drying at 40-70 ºC. The first method provide a total water extraction, intra and extra-cellular, whereas the drying method cannot guarantee the total elimination of water.

The presence of inorganic carbon in marine organisms can alter the carbon isotopic signatures, since it could present different isotopic values compared with those of the organic carbon, being the last one the only carbon of interest from a trophic point of view. For this reason, the elimination of inorganic carbon through a process of acidification with HCl is necessary, mainly when due to the small size of the individuals

(i.e. in many invertebrate species) it is not possible to dissect and remove the carbonated structures. The effect of acidification on the C and N isotopic values is not clear yet; thus, Bunn et al. (1995) observed some variations in the $\delta^{15}N$ after the acidification in penaeid shrimps and seagrasses, whereas Bosley and Wainright (1999) in fishes (*Pleuronectes americanus*) and penaeid shrimps (*Crangon septemspinosa*), and Chanton and Lewis (1999) in plankton, did not observe significative effects.

In the present work, the effects of different sample processing methods in the relative abundance of stable isotopes of C and N in the different trophic compartments of coastal food webs will be evaluated. To this goal, the effects of the dehydration method and of the acidification will be estimated in primary producers, consumers of different trophic levels, and in different dead organic matter pools. Also, the differences between the analysis of whole organisms and muscular tissue will be determined in invertebrates with carbonated structures. The final purpose is to define an adequate methodology to be used in the studies of the matter and energy flows in coastal food webs.

Material and Methods

Samples were obtained in different locations along the Ría de A Coruña, an oceanic bay placed in the NW of the Iberian Peninsula (43º21'N, 08º20'W), from November 2002 to January 2003. Different organic matter sources from the benthic (seaweeds and sedimented organic matter) and pelagic (suspended particulate organic matter) systems were obtained. Crustaceans, a model of an intermediate trophic level invertebrate with a high level of carbonate in their carapace, were chosen as consumers, and fishes and cephalopods as predators.

Suspended particulated organic matter (SPOM) was collected in an area nearby the beach O Castelo (Breixo), at 2 m deep using a Niskin bottle. Seaweeds were taken by divers at the same place. Sedimented organic matter (SOM) samples were obtained in the estuary of O Burgo (in the inner part of the bay) by superficial (1-2 cm) scrapings. Plankton tows of 15 minutes at 2-4 m deep using a 200 μm net were carried out along the main axis of the bay. Crustaceans, cephalopods and fishes were collected using traps in different areas of bay. Next, the experimental design and the treatments used in the preparation of samples for isotopic analysis of each trophic compartment are described (Fig. 1a).

SPOM- Two samples of water were collected and filtered through precombusted (450º during 3 h) GF/F Whatman glass fiber filters. The samples were divided into two subsamples, obtaining two filters for each initial sample.

SOM – Six sediment samples were obtained. Three of them were resuspended in 0.5 l of seawater, and they were processed in the same way as the SPOM. The other three samples were not filtered, dividing each one into two subsamples.

Plankton- Samples from three tows were obtained, dividing each one into four fractions using 750, 500, 300 and 100 μm mesh sizes. Each fraction was divided into two subsamples that were collected in 10 ml glass tubes.

Seaweeds- Four *Laminaria hyperborea* individuals were collected. After the manual elimination of their epibionts, the baldes were cut in pieces of approx. 1 cm^2 and the fragments of each individual were grouped in two subsamples and kept in aluminium boxes.

Crustaceans- two swimming crabs, *Polybius henslowii* and *Necora puber* (Decapoda, Portunidae), were collected. The muscular tissue of the cephalothorax and claws of three *P. henslowii* was dissected, whereas the whole body of three other crabs was processed. Each sample was divided into two subsamples, and was stored in aluminium boxes. In this way, the variability related to the material used in the isotopic analysis (muscular tissue vs. whole individuals) was analysed, since in many invertebrates it is not possible to isolate their muscular tissue for an independent analysis. Complementarily, crustaceans were used to test the effects of a modification of the acidification process (see below).

Cephalopods- Two individuals of *Octopus vulgaris* were collected, and a piece of 5 cm of muscular tissue was taken. Each sample was subdivided into two subsamples, that were stored in aluminium boxes.

Fishes- Four individuals of *Trisopterus luscus* were collected. From the dorsal part of each fish, a piece of muscular tissue was obtained and divided into two subsamples which were stored in aluminium boxes.

Next, all the paired samples from the different trophic compartments were dehydrated. One subsample was dried (60ºC) to constant weight, whereas the other was kept frozen (-20ºC) until its freezed-drying (using a TELSTAR CRYODOS-50 freeze-drier). After the water extraction, all the subsamples were divided into two, acidifying one of them adding HCl 1M drop-by-drop (the cessation of bubbling was used as criterion to determine the amount of the acid to add, Nieuwenhuize et al., 1994; Jacob et al., 2005). All the samples were left in acid during 3 hours. Then, they were dehydrated again, drying or freeze-drying, following the above mentioned method. Finally, the non-filtered samples were ground to a fine powder with an agate mortar and pestle.

Complementarily, a modification in the acidification method, sample washing with distilled water, developed to remove the acid from samples (Fig. 1b) was tested using both *Polybius henslowii* and *Necora puber*. Ten *Polybius henslowii* individuals were dried at 60 C in aluminium boxes to constant weight and grouped in a single sample. The cephalothoracic muscle of ten individuals of *Necora puber* was extracted, pooled in a single sample, and dried (60ºC). Both samples of crustaceans were ground to a fine powder with an agate mortar, and each sample was divided into eight parts, four of which were acidified (HCl 1M, during 3h). After acidification, two samples of each species were washed with distilled water, and centrifuged at 800 g during 5 min. Two of the four non-acidified samples of each species were also washed using this method. Next, the samples were re-dried (60º C) to constant weight.

Isotopic analyses were done at the Servicios Xerais de Apoio á Investigación (SXAIN), University of A Coruña, using an elemental analyser ThermoFinnigan FlashEA1112 connected to Finnigan MAT- DELTA plus isotope ratio mass spectrometry by a ConFlo II interface. Samples were analysed by combustion in quarz tubes at 1020ºC. Resulting gases were carried to a second tube at 650ºC in an Helio stream. The obtained products were N2, CO2 and H2O. After water vapour removal in a filter, N2 e CO2 were separated by chromatography and detected by thermal conductivity. The amounts of C and N were determined comparing peak areas with a standard (acetanilide). For isotopic analysis, gases passed to the mass spectrometer through an interface, that also carried out the injection of the reference N_2 and CO_2 gases. Once in the mass spectrometer, gas molecules were ionised by electronic impact, and separate for the action of a magnetic field, depending on the relative abundances of the molecules with different isotopic compositions.

Isotopic ratios for carbon, reported as $\delta^{13}C$, were calculated as:

$$\delta^{13}C = (^{13}C/^{12}C_{sample} / {}^{13}C/^{12}C_{standard} - 1) \cdot 1000 \ (‰)$$

Isotopic ratios for nitrogen, $\delta\ ^{15}N$, were calculated as:

$$\delta\ ^{15}N = (^{15}N/^{14}N_{sample} / {}^{15}N/^{14}N_{standard} - 1) \cdot 1000 \ (‰)$$

Standards were VPDB (Pee Dee Belemnite) for the carbon and atmospheric nitrogen for the nitrogen.

Two-way ANOVA were used for statistical analyses, using $p<0.05$ as significance criterion.

Results

The results obtained show a great variability in the effects that the treatments cause in the different trophic compartments, with variations for carbon from 0.003‰ (cephalopods) to 4.29‰ (SOM), and for nitrogen from 0.01‰ (plankton 100-300μm) to 2.40‰ (*Polybius henslowii)* among different treatments (Table 1).

SOM- Acidification caused a significative decrease (Table 2) for the filtered and non-filtered samples in the values of $\delta^{13}C$ (approx. 2‰ and 4‰, respectively) and of $\delta^{15}N$ (0.6‰ and 0.4‰). Although the method of dehydration caused significant differences in $\delta^{15}N$ values, the effects were dependent on acidification and the unbalanced design of the experiment precluded an accurate assessment.

SPOM- Acidification modified the isotopic ratios for C, with a significative decrease in the acidified samples of 5 ‰. No effects were found for N values.

Seaweeds- There was not any significative difference for the two treatments in the isotopic ratios of C and N.

Plankton- Both treatments caused modifications in the N and C isotopes, but they were dependent on the particle sizes. In the smallest fraction (100-300 μm), significant changes in N were observed only in the interaction of both treatments, though the variation between the groups was only 0.3 ‰. With C, only the acidification caused significant differences, with a diminution of approx. 0.7‰ in the acidified samples. In the fraction 300-500 μm, both the dehydration and the interaction of treatments caused significant changes only in $\delta^{15}N$ (with a maximum variation of 0.6‰). In the case of the fractions of 500-750 and >750 μm, significant differences were found only for C in the largest fraction due to the acidification process, with a diminution of 0.5‰.

Crustaceans- $\delta^{15}N$ of muscular tissue decreased in average 1.8‰ respect to samples of whole organisms ($F_{1,14}$=20.65, p=0.0004). The interaction between the type of sample (whole organisms *vs.* muscle) and dehydration was also significant ($F_{1,14}$=4.63, p=0.049). When separate analyses were performed for the two types of samples, significant differences were not found for the different treatments. In the case of C, only the acidification causes significant changes with a diminution up to 2.4‰ in the samples ($F_{1,14}$=20.35, p=0.0005).

The tests of the effect of washing with distilled water (Tables 3 and 4), done with whole individuals of *Polybius henslowii*, caused a diminution for C isotopic signatures both due to acidification and washing of samples (4‰ e 0.7‰, respectively), whereas for N isotopic ratios, this difference was only significant for acidification, with a diminution of 2‰ in $\delta^{15}N$ values. Essays done with cephalothoracic musculature of *Necora puber*

showed significant differences for $\delta^{15}N$ by washing (increase of 0.5‰ regarding the non-washed samples) and for $\delta^{13}C$ by acidification and washing (diminution of 0.4‰ for washed samples and of 0.3‰ for acidified samples).

Cephalopods- Isotopic ratios of N were significantly affected by acidification and by the interaction between acidification and dehydration, with a variation up to 1.2‰.

Fish- Different treatments did not cause significant changes in isotopic values of fish muscular tissue.

Discussion

Different authors have suggested that to avoid the effect that hydrochloric acid could cause in N isotopic values, it is advisable to divide the samples into two parts (Bunn et al., 1995), acidifying only that sample used in C analysis (Nieuwenhuize et al., 1994; Pinnegar and Polunin, 2000; Evans-White et al., 2001; Polunin et al., 2001; Bouillon et al., 2002; Cocheret de la Morinière et al., 2003). However, many authors carry out sample acidification for the two isotopes (Kang et al., 2003; Riera and Hubas, 2003; Yokohama and Ishihi, 2003), and others do not use this process (Jennings et al., 1997; Grey et al., 2001).

In this study, we observed an effect of the acidification for the isotopic ratios of C in suspended organic matter (SOM), suspended particulate organic matter (SPOM), plankton and crustaceans (both muscular tissue and whole individuals), and in N ratios in SOM, the crustacean *Polybius henslowii* and cephalopod *Octopus vulgaris*. The decrease that acidification causes in N and C isotopic ratios for SOM shows the need to acidify the samples for C analysis, leaving a non-acidified sub-sample for N analysis, as

Thornton and McManus (1994) and Bouillon et al. (2002) carried out. Both in SPOM and in the plankton, acidification only causes a decrease in the $\delta^{13}C$ values, that would allow the acidification of the whole sample. These results oppose those found in the zooplankton by Gearing et al. (1984) and Goering et al. (1990), who observed effects of the treatment with HCl in $\delta^{15}N$ values, and those obtained by Grey et al. (2001), who did not acidify as they did not find modifications in C values. These differences may be due to geographical changes in the composition of the zooplankton. The effect of acidification in the measured C isotopic ratios in SOM, SPOM and crustaceans is higher than 1‰, and in this sense the acidification of these sample types could bias the analysis of the relationships among different organisms and organic matter sources in a foodweb.

The requirement of plant washing with hydrochloric acid for the elimination of residual carbonates (Adin and Riera, 2003; Jones et al., 2003; Kang et al., 2003) is not corroborated by our results, since acidification do not modifies isotopic values. The results obtained with organisms including carbonated structures show a decrease in the N and C isotopic values in the acidified samples, what it is opposed to the results found in crustaceans by Bunn et al. (1995), who did not find variations in C and observed an increase for N, and by Bosley and Wainright (1999), who did not find modifications for none of the isotopes. The variability found for carbon could be related to the criteria used to determine the amount of HCl needed to remove calcium carbonate. In this way, it is quite common to use the stop of bubbling (provoked by the reaction of HCl with calcium carbonate) as the criterion to determine the total amount of HCl. The previous removal of C inorganic structures of the organisms, such as shells and carapaces, which allow the analysis of soft structures alone (Kline Jr., 1999; Kurata et al., 2001), is a mechanism used to try to decrease the artefacts that acidification can cause in isotopic values. However, many times, the characteristics of samples (small-sized organisms or

the impossibility to remove the carbonate particles embedded in the organic matter) makes acidification necessary (Herman et al., 2000; Frediksen, 2003). Our results show a decrease both in the samples in which whole individuals were analyzed and in which the exoskeleton was removed previously. Thus, it seems necessary to acidify even after the extraction of soft tissue, due to the remains that could be included in the samples (Christensen et al., 2001; Fry and Smith, 2002). However, the removal of the hard parts would be advisable when possible, since in this way the total elimination of carbonates is easier and the variability of the obtained results decreases.

In the case of fish muscle, our results differ from the study done by Pinnegar and Poluninn (1999), since we did not find variations caused by acidification in the isotope values, whereas they found changes in N values. These changes noticed by Pinnegar and Polunin (1999) coincide, however, with those noticed in our study for the muscle of *Octopus vulgaris*. This variable effect of the acid in N isotopic ratios in predators, probably caused by the different leaching rate of the organic nitrogen of compounds with different $\delta^{15}N$ values (Goering et al., 1990) can be avoided if the samples destined to the isotopic analysis of this element are not acidified. The washing of the samples after acidification is a common practice for the removal of acid residuals (Stoner and Zimmerman, 1988; Kang et al., 2003). However, the decrease observed in $\delta^{13}C$ values in the crustacean samples that were washed with distilled water, suggests a loss by leaching of some of the organic C components of the samples. Our results support those found by Jacob et al. (2005) who do not recommend rinsing with water after acidification.

The lack of differences between the two dehydration methods for C isotopes allows the elimination of water both by freeze-drying and drying. For nitrogen analysis, however,

differences between the two treatments were observed. Freeze-drying is a common method used for the removal of water from biologic samples (Petersen and Anger, 1997; Tuck et al., 1997) and it seems more adequate for the initial dehydration of samples, since it allows the use of these samples in other kinds of analyses (i.e. biochemical analysis), whereas drying seems a more appropriate method after the acidification process to avoid the damages that the remains of acid in the samples could cause in the freeze-drier.

The results of the present study allow to recommend that samples should not be acidified for nitrogen isotope analysis, since a decrease in $\delta^{15}N$ was observed in some groups after this treatment. Moreover, in seaweeds, fish muscle and cephalopods acidification for carbon would not be necessary, since no effect has been observed in the samples. Acidification is needed for carbon analysis in samples of SOM (filtered and non-filtered), SPOM, plankton and invertebrates with calcareous structures (both in the analysis of whole organisms and of muscle tissue, being the last one preferable when possible).

Two dehydration methods were tested and the most advisable is freeze-drying, except in acidified samples which should be dehydrated by drying. The washing treatment for elimination of HCl causes an alteration of carbon isotopic values, and for this reason we do not recommend washing and drying for sample dehydration to avoid the damages that acid could cause in the freeze-drier.

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Table 1. Mean and standard error (SE) of $\delta^{13}C$ and $\delta^{15}N$ for the different trophic compartments in relation to the acidification and dehydration treatments applied. N= number of analyzed samples.

	Treatment		$\delta^{13}C$			$\delta^{15}N$		
	Dehydration	Acidification	N	Mean	SE	N	Mean	SE
SOM (non-filtered)	Freeze-drying	No	3	-18.93	1.34	3	6.15	0.12
		Yes	2	-22.62	1.64	2	5.94	0.15
	Drying	No	3	-18.45	1.34	3	6.38	0.12
		Yes	3	-22.74	1.34	3	5.96	0.12
SOM (filtered)	Freeze-drying	No	3	-22.88	0.17	3	3.86	0.09
	Drying	No	3	-22.93	0.17	3	4.65	0.09
	Freeze-drying	No	3	-22.93	0.12	3	4.65	0.16
	Drying	Yes	3	-24.25	0.12	3	3.99	0.16
SPOM	Freeze-drying	No	2	-15.91	0.37	2	6.70	0.32
		Yes	2	-20.19	0.37	2	6.19	0.32
	Drying	No	2	-16.12	0.37	2	7.50	0.32
		Yes	2	-19.18	0.37	2	6.55	0.32
Laminaria hyperborea	Freeze-drying	No	4	-17.48	1.22	4	7.14	0.11
		Yes	4	-17.73	1.22	4	7.08	0.11
	Drying	No	4	-17.15	1.22	4	7.35	0.11
		Yes	4	-17.35	1.22	4	7.17	0.11
Plankton 100-300 µm	Freeze-drying	No	3	-17.48	0.17	3	5.64	0.07
		Yes	3	-18.04	0.17	3	5.91	0.07
	Drying	No	3	-17.58	0.17	3	5.90	0.07
		Yes	2	-18.44	0.21	2	5.66	0.09
Plankton 300-500 µm	Freeze-drying	No	3	-17.82	0.09	3	6.25	0.08
		Yes	3	-18.09	0.09	3	6.51	0.08
	Drying	No	3	-18.03	0.09	3	6.85	0.08
		Yes	1	-17.74	0.16	1	6.42	0.14
Plankton 500-750 µm	Freeze-drying	No	3	-17.87	0.12	3	6.67	0.16
		Yes	3	-18.00	0.12	3	7.35	0.16
	Drying	No	3	-17.93	0.12	3	6.90	0.16
		Yes	1	-18.42	0.20	1	7.17	0.28
Plankton >750 µm	Freeze-drying	No	3	-18.06	0.19	3	7.56	0.20
		Yes	3	-18.21	0.19	3	8.07	0.20
	Drying	No	3	-18.09	0.19	3	7.98	0.20
		Yes	3	-18.91	0.19	3	8.09	0.20
Polybius henslowii (whole organism)	Freeze-drying	No	3	-18.32	0.49	3	7.48	0.63
		Yes	3	-19.95	0.49	3	7.45	0.63
	Drying	No	3	-18.21	0.49	3	7.18	0.63
		Yes	3	-20.70	0.49	3	5.08	0.63
Polybius henslowii (muscle tissue)	Freeze-drying	No	3	-18.46	0.20	3	8.33	0.22
		Yes	3	-19.20	0.20	3	8.49	0.22
	Drying	No	3	-18.77	0.20	3	8.87	0.22
		Yes	1	-19.51	0.35	1	8.68	0.38
Trisopterus luscus	Freeze-drying	No	4	-15.71	0.17	4	12.81	0.15
		Yes	4	-15.59	0.17	4	12.78	0.15
	Drying	No	4	-15.52	0.17	4	13.01	0.15
		Yes	4	-15.67	0.17	4	12.48	0.15
Octopus vulgaris	Freeze-drying	No	2	-16.49	0.40	2	9.94	0.19
		Yes	2	-16.30	0.40	2	11.14	0.19
	Drying	No	2	-16.23	0.40	2	10.36	0.19
		Yes	2	-16.30	0.40	2	10.31	0.19

Table 2. Results of analyses of variance (F statistic and level of significance p) carried out to tests effects of the dehydration and acidification in the isotopic signatures of C and N in the different trophic compartments.

	$\delta^{15}N$						$\delta^{13}C$					
	Dehydration		Acidification		Interaction		Dehydration		Acidification		Interaction	
	F	p	F	p	F	p	F	p	F	p	F	p
SOM (non-filtered)	0.94	0.364	5.96	0.045	0.69	0.434	0.02	0.905	7.96	0.026	0.04	0.839
SOM (filtered)	40.03	0.003	8.70	0.042	------	------	0.04	0.852	63.14	0.001	------	------
SPOM	3.36	0.141	5.39	0.081	0.49	0.525	1.14	0.346	96.65	0.001	2.68	0.177
Plankton 100-300μm	0.00	0.999	0.05	0.834	11.03	0.013	1.93	0.208	15.41	0.006	0.71	0.429
Plankton 300-500μm	6.75	0.041	0.80	0.407	13.12	0.011	0.37	0.564	0.01	0.940	5.46	0.058
Plankton 500-750μm	0.02	0.898	5.88	0.052	1.03	0.350	2.80	0.145	4.67	0.074	1.58	0.256
Plankton >750μm	1.23	0.299	2.55	0.149	1.00	0.347	3.62	0.094	6.35	0.036	3.06	0.118
Laminaria hyperborea	2.13	0.170	1.26	0.285	0.31	0.585	0.08	0.778	0.03	0.856	0.00	0.984
Polybius henslowii (whole organism)	4.43	0.068	2.83	0.131	2.69	0.139	0.44	0.528	17.72	0.003	0.77	0.406
Polybius henslowii (muscle tissue)	1.84	0.257	0.01	0.024	0.42	0.996	1.57	0.257	8.93	0.024	0.00	0.996
Trisopterus luscus	0.10	0.757	3.73	0.077	2.87	0.116	0.11	0.751	0.01	0.930	0.57	0.463
Octopus vulgaris	1.20	0.334	9.55	0.037	11.35	0.028	0.10	0.772	0.02	0.554	0.10	0.767

Table 3. Mean and standard error (SE) of $\delta^{13}C$ and $\delta^{15}N$ for *Polybius henslowii* and *Necora puber* in relation to the different acidification and washing treatments. N= number of analyzed samples.

	Treatment		$\delta^{13}C$			$\delta^{15}N$		
	Acidification	Washing	N	Mean	SE	N	Mean	SE
Necora puber	No	No	4	-15.90	0.10	4	11.02	0.12
		Yes	4	-16.32	0.10	4	11.50	0.12
	Yes	No	4	-16.15	0.10	4	11.24	0.12
		Yes	4	-16.62	0.10	4	11.77	0.12
Polybius henslowii	No	No	4	-16.98	0.23	4	6.27	0.70
		Yes	4	-17.61	0.23	4	6.29	0.70
	Yes	No	6	-20.37	0.19	6	5.28	0.57
		Yes	4	-20.76	0.23	4	4.02	0.70

Table 4. Results of analyses of variance (F statistic and level of significance p) carried out to test the effects of dehydration and washing treatments for *Polybius henslowii* and *Necora puber*.

	$\delta^{15}N$						$\delta^{13}C$					
	Acification		Washing		Interaction		Acidification		Washing		Interaction	
	F	p	F	p	F	p	F	p	F	p	F	p
Polybius henslowii	5.99	0.028	0.86	0.368	0.92	0.354	214.28	0.000	5.31	0.037	0.28	0.604
Necora puber	4.47	0.056	18.85	0.001	0.04	0.849	7.09	0.021	18.31	0.001	0.04	0.844

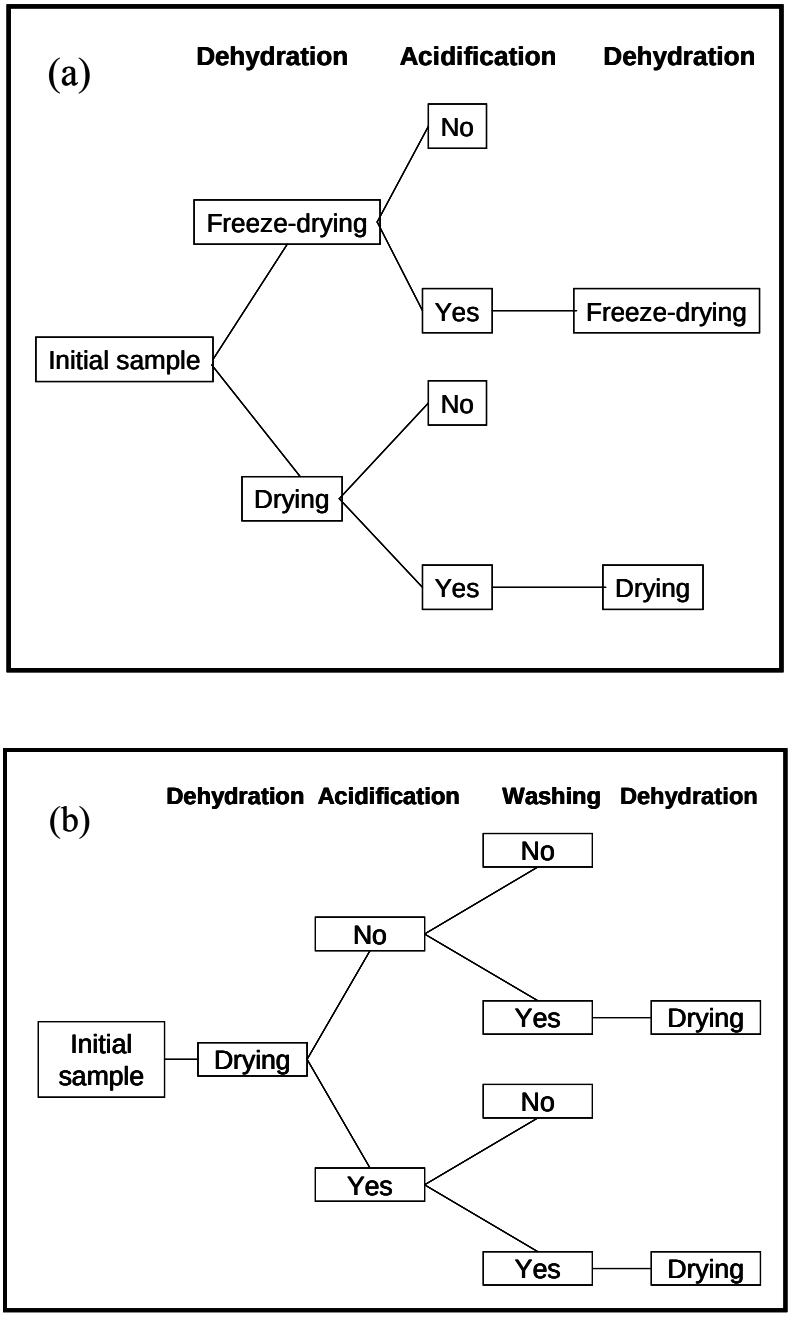

Fig. 1. (a) Experimental design and treatments used to prepare samples of the different trophic compartments for isotopic analyses. (b) Modification of the protocol to test the effect of washing using distilled water.