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# Study of the trophic web of San Simón Bay (Ría de Vigo) by using stable isotopes

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ABSTRACT

Based on the stable isotope composition in $^{15}N$ and $^{13}C$ of different potential sources of organic matter and consumers of an intertidal *Zostera marina* meadow located in San Simón Bay (Ría de Vigo, NW of Spain), a simplified food web of this community was reconstructed. For this purpose, some alternatives in different steps of the most used methodology of stable isotope dietary analysis were developed that cope with some of the limitations associated to the interpretation of isotopic signals for food web analysis, those of uncertainty on the fractionation value, mathematical model to use for the diet resolution and shortage of the isotope number for discriminating many food sources. The application of this protocol to the studied community reported similar results to those from other studies based on similar trophic webs, emphasizing the importance of local primary producers, especially microphytobenthos, which could be available for several primary consumers through resuspension forced by tidal hydrodynamic. The good agreement with previous results suggests that the proposed protocol is a feasible alternative to elucidate the most plausible trophic relationships in complex trophic webs using stable isotopes analysis.



## 1. Introduction

A trophic web describes the feeding behaviour of the organisms in a community (Cohen et al., 1990). From a functional point of view, a trophic web represents the different consumer–resource relationships in the community and the pathways of matter and energy transfer in the ecosystem. Elucidating these relationships is crucial to understanding how ecosystems work as well as predicting potential effects that different stressors such as human activity have on ecosystems (Winemiller and Polis, 1996). Usually, reconstruction of a trophic web is based on the study of anatomic structures related to feeding, direct observation of consumption and the analysis of gut contents (Polunin and Pinnegar, 2002). These methods have limited utility because they can only resolve recent feeding events, and reveal nothing about an organism's long term feeding history. They also require a large number of samples and are very difficult to apply to small consumers or to easily digestible diets. Alternative methods have recently been developed that improve and complement the previous ones (Sheppard and Harwood, 2005; De Lange and Van den Brink, 2006; Carreon-Martinez and Heath, 2010). One such method is the analysis of consumer's stable isotopes ratios. This technique finds potential sources of organic matter exploited by the consumer based on the principle "you are what you eat", which assumes that the isotopic composition of a consumer's body tissue is a direct consequence of the isotopic composition of its food sources (DeNiro and Epstein, 1978). Stable isotopes provide long-term information about a consumer's diet without requiring large sampling periods, and in addition can be applied to all organic matter sources and consumer sizes.

The assimilation of food particles by the consumer causes that the consumer isotopic composition reflects the isotopic composition of the food sources (Fry and Parker, 1979; Haines and Montague, 1979). However, differences in the physical–chemical properties and chemical reaction rates between isotopes cause an enrichment in the proportion of heavier isotopes in the consumer compared to the food sources (Ponsard and Arditi, 2000), in a process is called fractionation (Libes, 1992). By examining the isotopic ratios of various organisms in an ecosystem and accounting for fractionation, trophic ecologists can trace organic matter sources on a community (Peterson et al., 1984; Riera et al., 2000), estimate the trophic level of an organism (Vander Zanden and Rasmussen, 1999; Post et al., 2000) and reconstruct diets and trophic web of a community (Hughes et al., 2000; Pinnegar and Polunin, 2000; Ponsard and Arditi, 2000).

However, using stable isotopes to study trophic ecology presents limitations: (1) The degree to which fractionation occurs depends on different variables such as ecosystem type (France and Peters, 1997), trophic level (Vander Zanden and Rasmussen, 2001)

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and food source (Fantle et al., 1999; Adams and Sterner, 2000). (2) There is no universal methodology for reconstructing diet from stable isotopes, and the assumptions of commonly employed models are at odds with one another. For example, linear mixing models assume that the consumer isotopic composition, after correcting for fractionation, is a linear combination of the isotopic composition of all food sources weighted by their relative contributions to the diet (Fry and Sherr, 1984). Meanwhile, Euclidean distance models calculate the contribution of each food source depending on the Euclidean distance between the source and the consumer in a space defined by the isotopic composition. This model assumes that the isotopic composition of the consumer cannot be determined a priori based on the isotopic composition and relative contribution of the different sources because the implicated ecological and biological processes do not allow the prediction of the result (Ben-David and Schell, 2001). (3) Linear mixing models are additionally restricted in that they can only discriminate between a limited numbers of food sources, $n+1$ where $n$ is the number of studied isotopes.

These limitations are especially significant in trophic web studies where seagrasses are involved given the complexity of the trophic relationships associated to these ecosystems. Seagrasses meadows are considered areas of special interest given the high productivity and biodiversity as well as their role as "ecosystem engineers" (Marbà et al., 2006). The complexity of the three-dimensional structure created by *Zostera* leafs offers protection from predators and environmental stressors, increasing the biodiversity of these areas (Hemminga and Duarte, 2000). In addition, this structure is maintained by a dynamic balance between the death and formation of new leafs, which contributes significantly to carbon recycling (Törnblom and Søndergaard, 1999). The high productivity derived from this dynamic balance, increased by the microphytobenthos associated to these areas, can dominate the primary production in shallow coastal environments (Törnblom and Sondergaard, 1999). Therefore, the high biodiversity of organisms in these ecosystems results in complex trophic webs involving several food sources, including allochthonous terrestrial and marine inputs, autochthonous primary production and likely the seagrass itself by direct consumption or through channelization by detritivores (Törnblom and Sondergaard, 1999; Hemminga and Duarte, 2000). The high number of potential organic matter sources and the high diversity of trophic relationships both increase the uncertainty in the use of the limited number of isotopes in trophic web studies. In the present study the trophic web of a *Zostera marina* seagrass meadow located in an estuary area in the San Simón Bay, in the inner part of Ría de Vigo (NW Spain), is described using the stable isotopes $^{13}C$ and $^{15}N$. The trophic web of this ecosystem was reconstructed by finding the isotopic composition of many different organic matter sources and multiple consumer species with different feeding behaviours. This study suggests some alternatives in different steps of the most used methodology for studying trophic webs, revisiting well-known concepts and working in the direction of the limitations described previously, i.e. fractionation uncertainty, mathematical model and limitation on the number of isotopes. In this way, the goal of the protocol is to find the most plausible trophic relationships rather than provide a detailed and quantitative description of each consumer's diet.

## 2. Material and methods

### 2.1. Study area

San Simón Bay is located in the inner part of the Ría de Vigo (Fig. 1), and is 7 km long and 2.5 km wide, occupying a total area of 19.5 km$^2$. Tidal range is 2–4 m (mesotidal), semidiurnal periodicity and hydrodynamic conditions are low energy due to the morphology

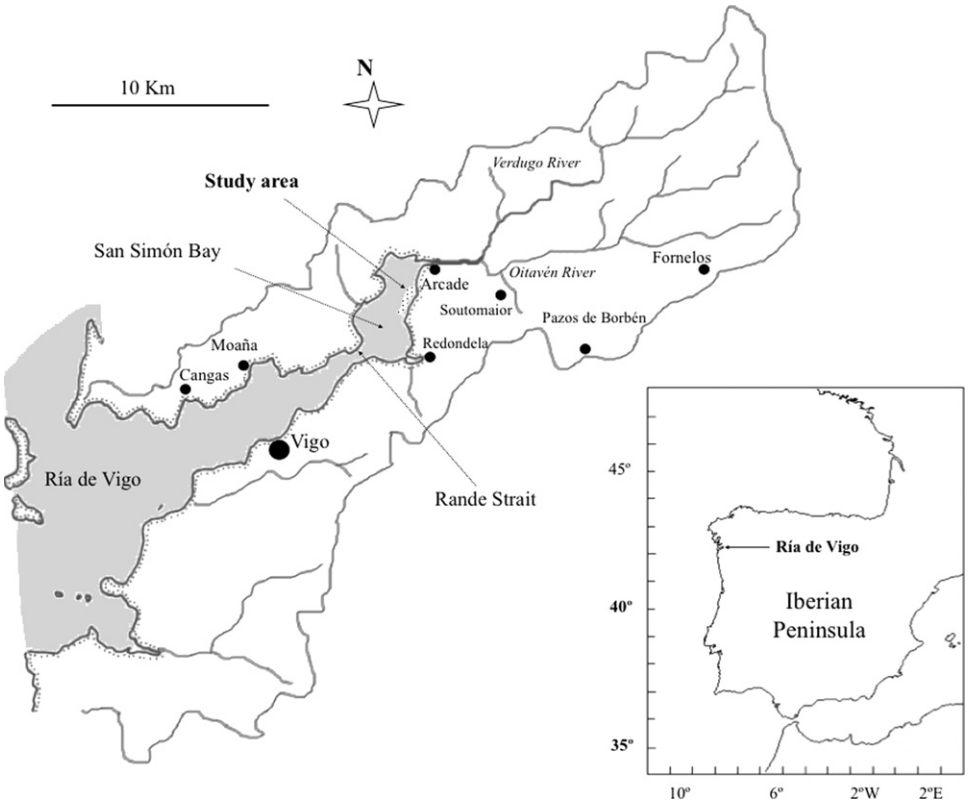


**Fig. 1.** Location of study area and main cities, villages and rivers of Ría de Vigo drainage area.

of the Rande Strait and the shallowness of the bay (Álvarez-Iglesias et al., 2006). The main freshwater input comes from the Oitavén-Verdigo River, with a mean annual discharge of 17.3 $m^{-3}\,s^{-1}$ (Álvarez-Iglesias et al., 2006). The average depth is less than 7 m, reaching 30 m in the Rande Strait. An extensive intertidal flat dominated by the seagrass *Z. marina* covers the Eastern bank of the bay, which is influenced by the Oitavén-Vergudo River. Different towns and villages are placed around the bay, reaching a population of 40,000 people, being the 85% located in the Eastern bank. Although there are three main population centers in the bay, there is a continuous mosaic of houses along the coast.

### 2.2. Collection and preparation of samples

Samples were chosen to characterize several sources of organic matter in the bay as well as consumers with different feeding behaviour and trophic level. Marine particulate organic matter (POM) was collected inside of the bay in a station close to Rande Strait (42°17′37″N 8°38′43″W). Terrestrial particulate organic matter was collected in Oitavén-Verdugo River (42°20′35″N 8°34′21″W). Sessile species and other ones with limited mobility were collected at three stations on the east bank of the bay (42°19′12″N 8°36′59″W; 42°19′26″N 8°36′59″W; 42°19′29″N 8°36′57″W). Mobile species were collected in the area delimited by those three stations. We attempted to collect all samples in triplicate with seasonal periodicity (February, May, July and October 2000 and February and May 2001), although it was not always possible to collect all of them. Each replicate of each species consisted of several individuals, with the exception of POM and *Sepia officinalis*, which were analyzed individual by individual. The results presented in this study are the average of the six sampling periods and hence represent annual means rather than seasonal periodicity.

#### 2.2.1. Sources of organic matter

*2.2.1.1. Marine particulate organic matter.* Three size fractions ($>200$ μm, 20–200 μm and $<20$ μm) were sampled. The two largest fractions were collected using a phytoplankton net towed from the bottom to the surface. The samples were transferred to a vial where 10 ml HCl 1 N were added to eliminate inorganic carbonate. The vial was shook for few seconds until the effervescence cessation. After that, the samples were transferred to a pre-burned (24 h, 550 °C) GFF filter. After that, they were dried (60 °C) until a constant weight was achieved and ground to below 100 μm in a mixer ball mill (Retsch® Mixer Mill MM 200) in order to guarantee homogenization (the drying and grounding processes are common to all samples).

The smallest fraction was collected at the peak chlorophyll depth using Niskin bottles. The samples were first filtered through a 20 μm net, then 5 l of seawater were filtered through a pre-burned GFF and rinsed with 10 ml of 33‰ saline solution (milli-Q water and NaCl—Panreac PA—ACS—ISO) to eliminate sea salts. After drying (60 °C) until a constant weight was achieved, the filters were exposed to an acid atmosphere (HCl Panreac PRS-CODEX 37%) for 4 h in order to eliminate inorganic carbonates. Thereafter, the filters were dried again and the surface was scraped to concentrate the sample before grounding.

*2.2.1.2. Terrestrial particulate organic matter.* POM samples from 12 l of freshwater were pre-filtered by 60 μm and the filtrate collected on a pre-burned GFF filter. After that, the filters were dried and exposed to acid atmosphere, following the same protocol used for the smaller fraction of marine POM.

*2.2.1.3. Sediment.* 2 $dm^3$ of sediment were collected from the top 5 mm in an area without rocks or macroalgae and sieved through 500 μm. After an exhaustive homogenization, a subsample was taken and centrifuged (3000 rpm for 3 min at 20 °C) to remove interstitial water. HCl 1 N was then added until the effervescence ceased (Nieuwenhuize et al., 1994) and the sample was rinsed several times with Milli-Q water to remove the acid leftovers (Hsieh et al., 2000). After each wash, the sample was centrifuged (3000 rpm for 3 min at 20 °C) and the supernatant was discarded. Finally, the sample was dried and ground.

*2.2.1.4. Microphytobenthos.* 2 $dm^3$ of sediment were collected from the top 2 mm in an area without rocks or macroalgae. Microphytobenthos extraction and purification was based on the positive phototrophic behaviour of the phytobenthic component of each sample. Extraction was carried out for 24 h following the method described in Couch (1989), which involves collecting the microphytobenthos in sand that is free of carbonates and organic matter. The sand was rinsed with filtered seawater (0.22 μm), and the microphytobenthos was collected in the supernatant that resulted from filtration through 60 μm and GFF filters. The GFF filters were exposed to a purification process in which they were disposed over a 60 μm filter face down in a glass container with filtered seawater (0.22 μm), which was held in such a way that its bottom could be illuminated. Given its phototrophic behaviour, the microphytobenthos migrated towards the light, being collected in the filtered seawater that was filtered again through a GFF filter. This process caused a significant enrichment in the proportion of microphytobenthos with respect to other particulates. Thereafter, the filters were dried and exposed to acid atmosphere, according to the protocol followed for the smaller fraction of marine POM.

*2.2.1.5. Ascophyllum nodosum, Enteromorpha intestinalis, Fucus vesiculosus, Ulva spp. and Zostera marina.* After the collection, the surface of each individual was cleaned of epiphytic organisms and detritic particles, then rinsed with HCl 1 N and Milli-Q water (Currin et al., 1995; Sauriau and Kang, 2000; Riera et al., 2000). Thereafter the samples were dried (60 °C) until a constant weight was achieved and ground below 100 μm in a blade mill (IKA® A11 Analytical Mill) and a mixer balls mill.

#### 2.2.2. Consumers

*2.2.2.1. Herbivores: Littorina littorea and Littorina obtusata.* In the specific case of *L. littorea*, two samples were collected from different locations; one from the *Z. marina* blades and the other from nearby 5 m high rocks without *Z. marina*. *L. obtusata* was only collected over the rocks. The individuals were kept alive for 24 h in plastic tanks with filtered seawater (20 μm) from the same area, with the aim of emptying their gut contents, which can modify the isotopic composition (Newman, 1991). Dead individuals were removed because the decomposition can also alter the isotopic values (Ponsard and Amlou in Ponsard and Arditi, 2000). Thereafter, the shells were removed and the samples were processed as in the case of macroalgae.

*2.2.2.2. Filter-feeders: Cerastoderma edule, Mytilus galloprovincialis, Scrobicularia plana and Ruditapes decussatus.* The filter-feeders were treated using the same protocol as the herbivores.

*2.2.2.3. Sub-surface feeder: Arenicola marina.* The sub-surface feeder was treated using the same protocol as the filter-feeders and

herbivores, however the individuals were kept for 48 h to guarantee their guts were empty.

*2.2.2.4. Carnivores: Nucella lapillus, Gobius niger, Symphodus bailloni and Sepia officinalis. Nucella lapillus* treatment followed the same protocol applied to the herbivore collections. In the other cases, only muscle tissue was analyzed given that muscle makes up the majority of the remaining tissue after skeletal tissue is removed, so the isotopic composition of muscle tissue is close to the whole organism composition (Peterson and Fry, 1987; Newman, 1991). After the dissection, an acidification process was carried out to remove any carbonate contamination. *G. niger* and *Symphodus bailloni* samples were ground in a blade mill, then exposed to an acidification process similar to the sediment. The *S. officinalis* tissue was rinsed first with HCl 1 N then several times with Milli-Q water. After individual-by-individual analysis, samples of *S. officinalis* were grouped into three classes depending on dorsal mantle length (DML): small (DML < 67 mm), medium (67 mm ≤ DML ≤ 120 mm) and large (DML > 120 mm) because of the size dependent variability in feeding behaviour (Castro and Guerra, 1990). All carnivores samples were ground to a size smaller than 100 μm in a blade mill and a mixer balls mill.

*2.2.2.5. Omnivores: Gammarus sp. and Palaemon adspersus.* The *P. adspersus* muscle tissue was dissected, rinsed with HCl 1 N and Milli-Q water and ground. The exoskeleton of *Gammarus* sp. contains calcium carbonate from seawater and nitrogen compounds from the diet (Bunn et al., 1995), therefore two protocols were performed after grinding the whole sample: one subsample was analyzed without acidification to determine $\delta^{15}N$ and another subsample was acidified following the protocol used to determinate $\delta^{13}C$ in the sediment.

### 2.3. Isotope analysis

Samples ground smaller than 100 μm in size were analyzed in an elemental analyzer (Carlo Erba Instruments EA 1108 CHNS/O) followed by an isotope-ratio mass spectrometer (DELTA$^{plus}$, Finningan MAT) to measure the carbon and nitrogen content as well as the relative proportion of $^{13}C$ and $^{15}N$. The isotopic relationships were expressed as

$$\delta X = (R_{sample}/R_{std} - 1) \times 1000\ (‰)$$

where $\delta X$ is $\delta^{13}C$ or $\delta^{15}N$, $R_{sample}$ is the relationship between $^{13}C$:$^{12}C$ or $^{15}N$:$^{14}N$ in the sample and $R_{std}$ is the relative abundance of the desired isotope in the standard. The standard was Vienna Pee-Dee Belemnite (VPDB) for $\delta^{13}C$ and atmospheric nitrogen for $\delta^{15}N$. The internal precision of the analyzer was $\pm 0.01‰$ and the external precision of the analysis was $\pm 0.2‰$.

### 2.4. Diet analysis

A protocol called "most plausible diet" was developed to estimate the diet of each consumer based on the isotope composition of both consumer and potential food sources. The protocol is based on the following steps:

*Step 1*: Biological characteristics and available information about the consumer.

Given the biological characteristics of the consumer: size, mobility, structures related to feeding, etc. as well as available information about its feeding behaviour and diet, different sources of food were selected as potential components of its diet.

*Step 2*: Fractionation.

The observed fractionation ranges are $-0.7‰$ through $9.2‰$ and $-2.1‰$ through $2.8‰$ for $^{15}N$ and $^{13}C$, respectively (Vander Zanden and Rasmussen, 2001). The common rule is to apply average values based on bibliography, however in this study we suggest the use of a range. In this way, different diets can be observed for the same consumer depending on the used fractionation. Three values were used for each isotope, 0.5‰, 1.0‰ and 1.5‰ for $\delta^{13}C$ and 2.2‰, 3.0‰ and 3.8‰ for $\delta^{15}N$.

*Step 3*: Mathematical modelling: Euclidean distance and linear mixing models.

The goal of this step was to select all the potential diets of a consumer and grade them according to the plausibility of being the real one of that consumer. For this purpose both linear mixing models and Euclidean distance were applied to each consumer and combination of fractionation established in Step 2. First of all, a linear mixing model (Fry and Sherr, 1984; Phillips and Gregg, 2001) was applied to each consumer, after correcting the fractionation, selecting all the combinations of food sources that can mathematically explain the isotopic composition of the consumer. Therefore, only the combinations of sources that can mathematically explain the consumer's isotopic composition are considered in the subsequent step. Each combination of food sources, thereafter called diet, was graded using a Euclidean distance model (Ben-David and Schell, 2001), discriminating the most plausible diet between all of the possible ones. The protocol employed to solve the models changed depending on the number of food sources considered: one, two or three (maximum number of sources using two isotopes).

With one food source, this was considered as a diet if consumer's average isotopic composition, after correcting for fractionation, was within one standard deviation of the food source's isotopic composition for both isotopes; a space that assuming a normal bivariate error, usually takes the form of an ellipse in the plane $\delta^{13}C$–$\delta^{15}N$ (Fig. 2A). The correlation between $\delta^{13}C$ and $\delta^{15}N$ was taken into account to determine the angle of the ellipse's axes. The Euclidean distance between the average isotopic composition of consumer and source on the plane $\delta^{13}C$–$\delta^{15}N$ was calculated for each potential diet. This distance was considered inversely proportional to the plausibility of the diet of being the real diet of the consumer.

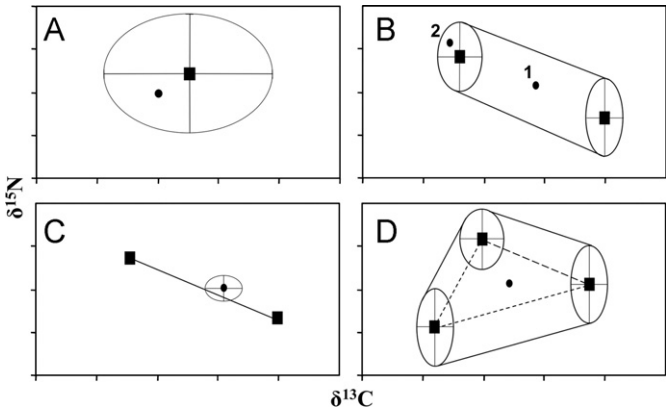


**Fig. 2.** Average isotopic composition of consumer (circle) and food sources (squares). (A) If the ellipse defined by the standard deviation of the source included the consumer after correcting the fractionation, the source was considered as a potential diet of the consumer. (B) The space delimited by both ellipses allows a mathematical explanation of the diet using linear mixing models, in the case of consumer 1 the contribution of each source is positive, however in the case of consumer 2, one of the sources has a negative contribution, which makes no biological sense. (C) The ellipse of theoretical standard deviation of the consumer calculated with the relative contribution of each source includes the straight line between the sources and therefore the sources can explain the consumer isotopic composition. (D) The space delimited by the ellipses corresponds the area where the mixing models could explain the consumer isotopic composition. The dashed line represent the solutions considered in the present study.

With two food sources, both were considered to contribute to the diet if the consumer's average isotopic composition, after correcting for fractionation, was included within the space defined by the standard deviation of both sources for both isotopes (Fig. 2B—Consumer 1). Given that the position of the consumer could provide mathematical solutions in which the contribution of one source is negative (Fig. 2B—Consumer 2), which makes no biological sense, these solutions were discarded. Using the mixing linear model, the contribution of each source to the diet was calculated for each isotope according to

$$X_{con} = p_1 \times X_1 + p_2 \times X_2$$

where $X$ is the isotope composition ($\delta^{13}C$ or $\delta^{15}N$) of the consumer ($_{con}$) and sources ($_1$ and $_2$) and $p$ the proportion of each source to the consumers' diet, taking into account that $p_1+p_2=1$. Therefore two solutions were available (one per isotope) that were more or less similar to one another depending on the distance from the consumer to the straight line between both sources, which represents the common solutions for both isotopes. This distance to the straight line is assumed to be a consequence of the standard deviation of the sources. In this way, using the calculated proportions and the standard deviation for each source, the theoretical standard deviation of the consumer (Std.Dev.$_{con}$) can be calculated:

$$\text{Std.Dev.}_{con}^2 = (\text{Std.Dev.}_1 \times p_1)^2 + (\text{Std.Dev.}_2 \times p_2)^2$$

where Std.Dev. is the standard deviation for each isotope ($\delta^{13}C$ or $\delta^{15}N$) of the consumer ($_{con}$) and sources ($_1$ and $_2$), taking into account that $p_1+p_2=1$. Using these values, the diet calculated previously was discarded if the space defined by the theoretical standard deviation of the consumer for both isotopes did not include the straight line between sources (Fig. 2C). The Euclidean distance between the average isotopic composition of consumer and each source on the plane $\delta^{13}C$–$\delta^{15}N$ was calculated for each potential diet. For each diet, the sum of the distance between the consumer and each source in the plane $\delta^{13}C$–$\delta^{15}N$ was calculated and it was considered inversely proportional to the plausibility of the diet of being the real diet of the consumer.

With three food sources, they were considered as the diet of a consumer if the average composition of this, after correcting for fractionation, can be explained by a linear mixing model in which the contribution of all food sources is positive. This solution in the plane $\delta^{13}C$–$\delta^{15}N$ is restricted to the space delimited by the averaged isotopic composition of the food sources (Fig. 2D). The Euclidean distance was calculated in the same way as that in the case with two possible food sources.

*Step 4*: "Most plausible diet".

Given that several fractionations are used for each isotope and the possibility of diets with one, two or three food sources, it is possible to obtain multiple solutions for each consumer. With the aim of simplifying these results a criterion was suggested, establishing the concept of "most plausible diet". This criterion is based on the following:

(1) For each group of diets, depending on the number of food sources considered (1, 2 or 3) and the combinations of fractionation (9 possibilities), the diet with a shorter Euclidean distance was selected. Therefore, for each consumer a table was created (Table 1) that contains in the most complex scenario 27 possible diets.
(2) The number of times that a food source appeared in a diet was counted. If two or more sources had a statistically (*t*-student) similar isotopic composition, they were considered as one and were counted together. Finally, the three highest counted sources were considered the "most plausible sources" and define the "most plausible diet" for the given consumer. The others counted sources were considered "less plausible sources".

Steps 3 and 4 were automated using Visual Basic for Application.

## 3. Results

The average isotopic composition of the organic matter sources and consumers is shown in Table 2. In some cases, the isotopic composition of different sources was statistically similar: *A. marina* and *N. lapillus* ($\delta^{13}C$: $t=1.108$, d.f.$=22$, $p=0.280$; $\delta^{15}N$: $t=1.758$, d.f.$=22$, $p=0.093$), *A. nodosum* and *C. edule* ($\delta^{13}C$: $t=0.356$, d.f.$=26.219$, $p=0.725$; $\delta^{15}N$: $t=0.787$, d.f.$=31$, $p=0.437$), *A. nodosum* and *S. plana* ($\delta^{13}C$: $t=1.686$, d.f.$=28$, $p=0.103$; $\delta^{15}N$: $t=0.349$, d.f.$=28$, $p=0.729$), *C. edule* and *R. decussatus* ($\delta^{13}C$: $t=1.047$, d.f.$=34$, $p=0.302$; $\delta^{15}N$: $t=0.464$, d.f.$=34$, $p=0.645$), *E. intestinalis* and *F. vesiculosus* ($\delta^{13}C$: $t=0.682$, d.f.$=15.780$, $p=0.505$; $\delta^{15}N$: $t=1.125$, d.f.$=28$, $p=0.270$) and *L. littorea* that grows over rocks and *L. obtusata* ($\delta^{13}C$: $t=0.465$, d.f.$=19.414$, $p=0.647$; $\delta^{15}N$: $t=1.575$, d.f.$=34$, $p=0.125$). These couples cannot be distinguished from a statistical point of view, therefore, when composition in both isotopes is the same, it is not possible to discriminate between them in diet analysis process.

**Table 1**
Structure of the table in which all the possible diets for a consumer are summarized according to the 9 combinations of fractionation and the possibility of including one, two or three food sources in the solution.

| Fractionation combination | Diets with 3 food sources | | | Diets with 2 food sources | | Diets with 1 food source |
|---|---|---|---|---|---|---|
| 0.5‰C, 2.2‰N | | | | | | |
| 0.5‰C, 3.0‰N | | | | | | |
| 0.5‰C, 3.8‰N | | | | | | |
| 1.0‰C, 2.2‰N | | | | | | |
| 1.0‰C, 3.0‰N | | | | | | |
| 1.0‰C, 3.8‰N | | | | | | |
| 1.5‰C, 2.2‰N | | | | | | |
| 1.5‰C, 3.0‰N | | | | | | |
| 1.5‰C, 3.8‰N | | | | | | |

**Table 2**
Studied samples, code used in consecutive figures, average values of $\delta^{13}C$ and $\delta^{15}N$ as well as their standard deviations, Std. D. $\delta^{13}C$ and Std. D. $\delta^{15}N$, and number of cases ($n$).

| Sample | Cod | $\delta^{13}C$ | Std. D. $\delta^{13}C$ | $\delta^{15}N$ | Std. D. $\delta^{15}N$ | $n$ |
|---|---|---|---|---|---|---|
| Marine POM > 200 μm | LAR | −19.31 | 1.391 | 7.44 | 0.913 | 18 |
| Marine POM 20–200 m | MID | −17.68 | 1.715 | 6.90 | 0.488 | 18 |
| Marine POM < 20 μm | SMA | −21.29 | 1.377 | 6.13 | 0.736 | 20 |
| Terrestrial POM < 60 μm | TER | −24.92 | 1.799 | 5.32 | 0.786 | 16 |
| Sediment | SED | −24.84 | 4.740 | 6.06 | 0.593 | 18 |
| Microphytobenthos | PB | −17.80 | 2.170 | 5.56 | 1.045 | 13 |
| *Ascophyllum nodosum* | AN | −16.11 | 0.943 | 9.39 | 0.499 | 18 |
| *Enteromorpha intestinalis* | EI | −16.28 | 2.205 | 8.43 | 0.742 | 12 |
| *Fucus vesiculosus* | FV | −15.80 | 1.254 | 8.71 | 0.599 | 18 |
| *Ulva* spp. | UL | −10.46 | 2.886 | 8.48 | 0.655 | 18 |
| *Zostera marina* | ZM | −8.34 | 0.691 | 7.22 | 0.607 | 18 |
| *Littorina littorea* from rocks | LLRO | −13.80 | 1.047 | 10.93 | 0.264 | 18 |
| *Littorina littorea* from *Z. Marina* | LLZO | −10.57 | 1.030 | 11.05 | 0.323 | 14 |
| *Littorina obtusata* | LO | −13.92 | 0.280 | 10.77 | 0.343 | 18 |
| *Cerastoderma edule* | CE | −16.20 | 0.483 | 9.53 | 0.509 | 15 |
| *Mytilus galloprovincialis* | MG | −17.14 | 0.455 | 8.98 | 0.700 | 18 |
| *Scrobicularia plana* | SP | −15.54 | 0.829 | 9.48 | 0.786 | 12 |
| *Ruditapes decussatus* | RD | −16.41 | 0.634 | 9.60 | 0.408 | 21 |
| *Arenicola marina* | AM | −15.02 | 0.604 | 11.21 | 0.864 | 18 |
| *Gobius niger* | GN | −13.92 | 0.784 | 13.39 | 0.389 | 15 |
| *Nucella lapillus* | NL | −15.34 | 0.614 | 11.85 | 0.393 | 6 |
| *Sepia officinalis* < 67 mm | SOSMA | −12.49 | 0.489 | 13.57 | 0.344 | 10 |
| *Sepia officinalis* 67–120 mm | SOMID | −15.43 | 0.653 | 12.72 | 0.483 | 10 |
| *Sepia officinalis* > 120 mm | SOLAR | −15.67 | 0.985 | 12.69 | 0.571 | 10 |
| *Symphodus bailloni* | SB | −13.61 | 0.626 | 13.83 | 0.472 | 8 |
| *Gammarus* sp. | GM | −17.48 | 0.783 | 10.34 | 0.450 | 18 |
| *Palaemon adspersus* | PA | −12.66 | 0.508 | 12.92 | 0.368 | 18 |

**Table 3**
Consumer (column)–Resource (row) trophic relationships. For each consumer are shown the food sources considered a priori as potential food sources (grey cells), those included on the most plausible diet (●) and less plausible diets (○). See Table 2 for consumer's code.

| Sample | CE | RD | MG | SP | LLRO | LLZO | LO | GM | PA | AM | NL | GN | SB | SOSMA | SOMID | SOLAR |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Marine POM >200 μm | ● | ● | ● | | | | | | | | | ○ | | ● | | |
| Marine POM 20-200 μm | ● | ● | ● | ● | | | | | | | | | | | | |
| Marine POM <20 μm | | | ○ | | | | | | | | | | | | | |
| Terrestrial POM <60 μm | | | | | | | | | | | | | | | | |
| Sediment | | | | | | | | ● | | ○ | | | | | | |
| Microphytobenthos | ● | ● | ● | ● | ● | ○ | ● | ● | | ● | | | | | | |
| *Ascophyllum nodosum* | | | | | ○ | ○ | | ○ | | ○ | | | | | | |
| *Enteromorpha intestinalis* | | | | | ○ | ○ | ○ | ● | | ● | | | | | | |
| *Fucus vesiculosus* | | | | | ● | ● | ● | | ○ | ● | | | | | | |
| *Ulva* spp. | | | | | ● | ● | ● | ○ | ● | ○ | | | | | | |
| *Zostera marina* | | | | | ○ | ● | ○ | | | ○ | | | | | | |
| *Littorina littorea* from rocks | | | | | | | | | ○ | | | ○ | ○ | | | |
| *Littorina littorea* from *Z. Marina* | | | | | | | | | ○ | | | ○ | ○ | | | |
| *Littorina obtusata* | | | | | | | | | ● | | ● | ● | ● | | | |
| *Cerastoderma edule* | | | | | | | | | | | | ○ | | | | |
| *Mytilus galloprovincialis* | | | | | | | | | | | ● | | | | | |
| *Scrobicularia plana* | | | | | | | | | ● | | | ● | ● | | | |
| *Ruditapes decussatus* | | | | | | | | | | | | | | | | |
| *Arenicola marina* | | | | | | | | | ○ | | | ● | ● | | | |
| *Gobius niger* | | | | | | | | | | | | | | | ● | ● |
| *Nucella lapillus* | | | | | | | | | | | | ○ | ○ | | | |
| *Sepia officinalis* <67 mm | | | | | | | | | | | | | | | | |
| *Sepia officinalis* 67-120 mm | | | | | | | | | | | | | | | | |
| *Sepia officinalis* >120 mm | | | | | | | | | | | | | | | | |
| *Symphodus bailloni* | | | | | | | | | | | | | | | | |
| *Gammarus* sp. | | | | | | | | | | | | | | | ● | ● |
| *Palaemon adspersus* | | | | | | | | | | | | | ○ | ● | ● | |

Potential food sources as well as most and less plausible sources for each consumer are summarized in Table 3. In the same way, Fig. 3 highlights the most plausible trophic interactions in San Simón Bay.

### 3.1. Herbivores: Littorina littorea and Littorina obtusata

There was a significant difference between the $\delta^{13}C$ of *L. littorea* growing on a rocky substrate and the $\delta^{13}C$ of the same species

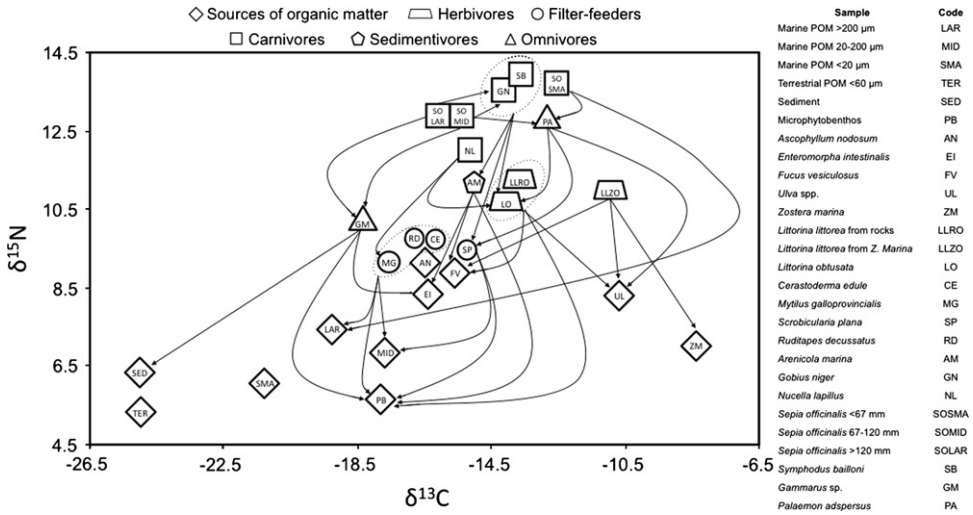


**Fig. 3.** Trophic web of San Simón. The arrows represent the most plausible consumer–resource relationships. Dot lines group species with similar diet.

collected on *Z. marina* blades ($t=8.730$, d.f.$=30$, $p<0.001$), but there was no effect of substrate on $\delta^{15}N$ ($t=1.210$, d.f.$=30$, $p=0.239$). All the macroalgae and microphytobenthos were used as potential food sources. The most plausible diet varied depending on the substrate. In case of *L. littorea* from rocky substrate the diet included *Ulva* spp., *F. vesiculosus* and microphytobenthos, while *Ulva* spp., *F. vesiculosus* and *Z. marina* was the most plausible diet for *L. littorea* from *Z. marina*.

The same potential food sources were considered in case of *L. obtusata* and the most plausible diet was similar to that of *L. littorea* from rocks.

### 3.2. Filter-feeders: Cerastoderma edule, Mytilus galloprovincialis, Scrobicularia plana and Ruditapes decussatus

All the sources of organic matter with the exception of macroalgae were considered as potential sources of food for filter-feeders. The most plausible diet for *C. edule* and *R. decussatus* included the two largest size classes of marine POM and microphytobenthos. *M. galloprovincialis* had the same diet as the former two species. However, the smallest marine POM fraction was also occasionally selected as a less plausible source for this species. The diet analysis for *S. plana* only yielded results when one food source, either microphytobenthos or marine POM between 20 and 200 µm, was considered as a potential diet.

### 3.3. Sub-surface feeder: Arenicola marina

Sediment, microphytobenthos and macroalgae were selected as potential food sources for *A. marina*, and the most plausible diet included microphytobenthos, *E. intestinalis* and *F. vesiculosus*.

### 3.4. Carnivores: Nucella lapillus, Gobius niger, Symphodus bailloni and Sepia officinalis

*L. littorea* that grows over rocks, *L. obtusata* and *M. galloprovincialis* were selected as potential food sources for *N. lapillus*, and the most plausible diet included *M. galloprovincialis* and *L. obtusata*. The isotopic composition of *L. obtusata* is similar to *L. littorea*, therefore the latter could be included as a plausible food source for *N. lapillus*.

Marine POM larger than 200 µm, herbivores, filter-feeders, *A. marina*, *N. lapillus*, *P. adspersus* and *Gammarus* sp. were considered potential food sources for *G. niger* and *S. bailloni*, and the most plausible diet was composed by the same sources for both species, that is, *S. plana*, *A. marina* and *L. obtusata*. However, *N. lapillus* and *L. littorea* could be also considered in the most plausible diet because their isotopic compositions are statistically similar to *A. marina* and *L. obtusata*, respectively.

The potential food sources and most plausible diets for *S. officinalis* depended on the dorsal mantle length (DML). For *S. officinalis* with DML $<67$ mm, marine POM larger than 200 µm, *G. niger*, *S. bailloni*, *Gammarus* sp. and *P. adspersus* were considered as potential food sources. The most plausible diet included *P. adspersus* and marine POM larger than 200 µm. For other DML sizes the same potential food sources as the smaller size class were considered, although marine POM was changed by *S. officinalis* smaller than 67 mm. The most plausible diet was *G. niger*, *Gammarus* sp. and *P. adspersus* for *S. officinalis* with 67 mm $\leq$ DML $\leq$ 120 mm, while the diet included *G. niger* and *Gammarus* sp. for *S. officinalis* with DML $>120$ mm.

### 3.5. Omnivores: Gammarus sp. and Palaemon adspersus

Sediment, microphytobenthos and macroalgae were considered potential food sources of *Gammarus* sp., and the most plausible diet was composed of *E. intestinalis*, sediment and microphytobenthos.

Marine POM larger than 200 µm, microphytobenthos, macroalgae, herbivores, filter-feeders, *A. marina*, *N. lapillus*, *Gammarus* sp. and *P. adspersus* were the potential food sources for *P. adspersus*. The most plausible diet included *S. plana*, *Ulva* spp. and *L. obtusata*. *A. nodosum* and *L. littorea* are isotopically similar to *S. plana* and *L. obtusata*, respectively, therefore they can be considered plausible sources too.

## 4. Discussion

### 4.1. Diet analysis methodology

The use of isotope analysis to reconstruct diets presents three methodological problems: (1) choosing the fractionation given the sources of variability that may exert an effect on it, (2) finding the best mathematical model that describes the most plausible diet, and (3) figuring out how to analyze complex trophic webs when the number of potential food sources is higher than $n+1$, where $n$ is the number of studied isotopes.

The fractionation ranges have been established in the literature to be $-0.7$‰ through 9.2‰ and $-2.1$‰ through 2.8‰ for $^{15}N$ and $^{13}C$, respectively (Vander Zanden and Rasmussen, 2001), however,

the degree of fractionation depends on consumer nutritional status, feeding behaviour (herbivorous vs carnivorous), diet quality, size, age and analyzed tissue (DeNiro and Epstein, 1981; Gannes et al., 1998; Fantle et al., 1999; Pinnegar and Polunin, 2000; Adams and Sterner, 2000; Vander Zanden and Rasmussen, 2001; Phillips and Koch, 2002; Post, 2002; Caut et al., 2009), making it difficult to establish the correct value. It is therefore normal to use average values and apply them to different trophic levels, ignoring the well-established uncertainty (e.g. Michener and Schell, 1994; Caut et al., 2009). In order to take this variability into account, a range of values was used instead of a single average value: 0.5‰, 1.0‰ and 1.5‰ for $\delta^{13}C$ and 2.2‰, 3.0‰ and 3.8‰ for $\delta^{15}N$. Both the most common values and a confidence interval were considered, which allowed us to establish the different diets for each consumer while taking the variability in fractionation into account. Although these common values of fractionation offer a valuable solution, the ranges of variation for different species can be reduced based on specific experiments, as was suggested by Gannes et al. (1997). In this way, recent studies carried out controlled feeding experiments to determine the isotopic fractionation of different species (e.g. Yokoyama et al., 2005 in *Mactra veneriformis*, *Ruditapes phillippinarum*, *Nihonotrypaea japonica* and *N. harmandi*; Dubois et al., 2007 in *Mytilus edulis* and *Crassostrea gigas*; Dang et al., 2009 in *Ruditapes phillippinarum*). These controlled feeding experiments can reduce the fractionation uncertainty increasing the reliability of the diet resolution, however these studies highlight that the fractionation is species- and tissue-specific, and that the accepted fractionation values may not be universally applicable (Yokoyama et al., 2005). These results support the approach of using a range of values for fractionation instead of a fixed one.

The mathematical models used in diet resolution are linear mixing models (Fry and Sherr, 1984) and Euclidean distance models (Ben-David and Schell, 2001). In the former, the consumer isotopic composition reflects that of its diet after correcting the fractionation. Therefore, a diet has to mathematically explain consumer isotopic composition according to a linear mixing model that provides the proportion of each food source. Euclidean distance models assume that the isotopic composition of the consumer is influenced by ecological, physiological and biochemical processes (Gannes et al., 1997), therefore a mathematical linear mixing model cannot explain the consumer isotopic composition. These models assume that the contribution of a source to the diet of a consumer is inversely proportional to the distance between the source and the consumer in the $\delta^{13}C$–$\delta^{15}N$ plane. In addition, the inclusion of a source in the diet is a process previous to the resolution of the model, not a result of the model itself. The protocol suggested in the present paper combines the information reported by both kinds of models, resulting in the definition of the "most plausible diet" (see Section 2). The linear mixing model was used to elucidate whether a combination of food sources can be considered as a diet of the consumer and the Euclidean distance model was used to assign different grade of plausibility to each combination of sources. In this process a bivariate normal error distribution was considered instead of the usual rectangular equiprobable region defined by the standard deviation of $\delta^{13}C$ and $\delta^{15}N$. Both approaches provide similar results, however the one used in the present study is more suitable to the statistical theory according to the normal error distribution. In addition, an a priori criterion was used to choose the potential food sources depending on biological characteristics and previous knowledge about the consumer.

The third limitation only affects mixing models given that according to the mathematical definition, the maximum number of food sources that can be considered is $n+1$, where $n$ is the number of studied isotopes. The methodology presented in this study addresses the diet resolution in a similar way as a method in the package Isosource, a program developed by Phillips and Greggs (2003), which allows the estimation of the contribution of $n$ different sources to a consumer's diet using linear mixing models. Their method provides the percent contribution of different food sources that were selected a priori as the diet of a consumer. Isosource predicts the isotopic composition for a consumer fed with all possible combinations of sources. The program changes the contribution of each source in small steps, conserving a total contribution of all the sources in 100%. The calculated isotopic composition for the consumer is compared with the real one, accepting the diet as a possible one if both values are included in a predefined tolerance range. Finally, Isosource shows the results of all the possible solutions, which is a range of contributions of each food source to the diet of the consumer. The methodology presented in this study addresses the diet resolution in a similar way as Isosource, considering the combinations of sources that provide a solution using linear mixing models as a diet. However, there are some differences between both methods: (1) Isosource does not take into account standard deviations, therefore it is not possible to find a solution when the consumer isotopic composition is outside the space defined by the average isotopic values of the sources. (2) Isosource allows the fractionation to change, which is one of the most uncertain variables in diet analysis. However it does not allow different combinations simultaneously. (3) Isosource provides a range of contributions for each source using linear mixing models. The method described in this study does not provide a range given that is focused on highlighting the most plausible food sources by employing Euclidean distance models, considering an inverse relationship between plausibility and Euclidean distance between food source and consumer in the space defined by the isotopic composition.

The suggested methodology, which considers a priori selection of possible food sources, fractionation uncertainty and the simultaneous use of linear mixing models and Euclidean distance models, allows an objective approach to determine the diet of a consumer avoiding the most important limitations of the stable isotopes technique. However, and like all stable isotopes studies that are used to discriminate objectively between two food sources, this protocol potentially underestimates the contribution of sources with extreme isotopic values. In other words, the closer the food source is to the consumer, the greater the assumed contribution of that source to the consumer's diet. The application of this methodology results in a frequentist approach to highlight the most plausible diet of a consumer.

### 4.2. Diet analysis (Table 3, Fig. 3)

It is well known that stable isotope values of both food sources (e.g. Cloern et al., 2002) and consumers (e.g. Malet et al., 2008) change through time, therefore a seasonal approach to elucidate trophic relationships would potentially provide more information about the ecosystem functioning. However, some limitations in "isotopic routing" have to be taken into account to analyze seasonally a trophic web using stable isotopes: (1) animals assimilate dietary components with varying efficiencies (Gannes et al., 1997); (2) isotopic fractionation and isotopes turnover rates are not uniform among tissues within an individual (Tieszen et al., 1983; Suring and Wing, 2009) or (3) among species with different physiologies (Suring and Wing, 2009). Therefore, although stable isotope analysis provides long-term information about consumer's diet, this integrative time varies from days to years according to the ecosystem, the species, their growth rate and the tissue considered (Pasquaud et al., 2010), which may obscure the relative importance of dietary components if an animal's diet varies through time (Tieszen et al., 1983). This study is focused in a complex trophic web that contents individuals from different trophic levels, feeding

habits, life cycle and life expectancy, which is reflected in their stable isotope values. Therefore a time-averaged approach accumulates this variability through time, minimizing the effect of "isotopic routing" on the diet resolution. This approach is not focused on seasonal dynamics, however it increases the robustness and accuracy in the understanding of the average trophic relationships, providing a general view of the bay's trophic web.

The analysis of diets using stable isotopes depends on the signatures of consumer and sources, therefore a reliable interpretation can only be made if all dietary sources are included in the study (Pitt et al., 2009). This is a limitation of the method and it becomes more important in high trophic levels given that these consumers usually present a wide spectrum of food sources. However, the analysis of all potential food sources is usually impossible to carry out for obvious logistic reasons in complex trophic webs like San Simón Bay. Therefore, the most plausible diets summarized in this study present an inherent uncertainty caused by the number of analyzed food sources. In addition, high trophic levels are vulnerable to violate an implicit assumption of stable isotopes technique given that is assumed that the C:N ratio of food sources are similar (Phillips and Koch, 2002). However, this can be violated in the case of omnivores. This potential flaw can be analyzed by means of concentration-weighted models (Phillips and Koch, 2002), which are particularly useful for quantifying the contribution of food sources (e.g. Kasai et al., 2004). However, given that the suggested protocol is directed to find the most plausible diet rather than a quantitative description of the diet, this modelling approach was not considered.

#### 4.2.1. Herbivores: Littorina littorea and Littorina obtusata

The differences in $\delta^{13}C$ between *L. littorea* in different intertidal positions could be explained by variability in diet according to food availability. Whereas *Ulva* spp. and *F. vesiculosus* are common food sources, individuals that grow over rocks consume microphytobenthos, while individuals from *Z. marina* consume this plant. Although the presence of *Z. marina* in the diet of different grazers has been recently reported (Schaal et al., 2008), this is not common in the literature and at most it has been identified as a minor important food source (Jaschinski et al., 2008) given its low digestibility (Nienhuis and Groenendisk, 1986). However, the inclusion of the *Z. marina* in the most plausible diet of this gastropod could be indirectly caused by consumption of epiphytic organisms of its leaves, which may have an isotopic composition similar to *Z. marina* (Winning et al., 1999; Jaschinski et al., 2008). The channelization of carbon, nitrogen and phosphorus, and consequently the isotopic marker, from *Z. marina* to the epiphytes (Hemminga and Duarte, 2000) could explain the *L. littorina* diet in terms of *Ulva* spp., *F. vesiculosus* and a microalgae. This microalgae would depend on the position of the gastropod on the intertidal, either microphytobenthos or epiphytes for individuals that grow over rocks or *Z. marina*, respectively. In this way, seagrasses are particularly prone to colonization by epiphytic microalgae (Mann, 2000). This most plausible diet is in good agreement with observations in the literature (Lubchenco, 1983; Petraitis, 1983; Barker and Chapman, 1990; Sommer, 2000). In addition, this would represent a non-detritivorous way of introducing *Z. marina* in the trophic web.

The most plausible diet of *L. obtusata* is the same as that observed for *L. littorea* from a rocky substratum, and hence similar to the diets reported in the literature (Hunter, 1981; Hawkins and Hartnoll, 1983; Watson and Norton, 1987).

#### 4.2.2. Filter-feeders: Cerastoderma edule, Mytilus galloprovincialis, Scrobicularia plana and Ruditapes decussatus

The diet analysis for the four bivalves suggested that terrestrial POM is not an important component of the filter-feeders' diet and emphasized the contribution of the microphytobenthos as well as marine POM. This conclusion is independent on feeding behaviour, suspensivore (*C. edule*, *M. galloprovincialis* and *R. decussatus*) or facultative depositivore (*S. plana*), or position on the substrate, epibenthic (*M. galloprovincialis*), superficial infaunal (*C. edule*) or subsuperficial infaunal (*S. plana* and *R. decussatus*). The differences in isotopic composition as well as between diets observed in the filter-feeders could be caused by differences in their ability to retain and select particles based on anatomic differences between species (Ward et al., 1998). In general, similar results have been observed using stable isotopes techniques: *Crassotrea gigas* in Marennes—Oléron Bay (Riera and Richard, 1997), *C. edule* in Marennes—Oléron Bay (Kang et al., 1999), *Macoma balthica* in Aiguillon Bay (Riera et al., 1999) and *C. edule*, *R. decussatus* and *M. galloprovincialis* in a similar ecosystem to this study (Page and Lastra, 2003).

The high contribution of microphytobenthos to the diet of filter-feeders has been emphasized using stable isotopes (Sauriau and Kang, 2000) and traditional analysis of gut content (Kamermans, 1994). The presence of microphytobenthos in the diet of epibenthic organisms suggests that resuspension processes caused by bottom currents could be important (Lucas et al., 2001) forced by tides or by the effect of the wind (de Jonge and Van Beusekom, 1995). Although this process can also resuspend detrital material derived from *Z. marina*, the contribution of this vascular plant to filter-feeders' diets is negligible, probably because of its poor nutritional quality (Williams, 1981). Resuspension could also make available detritus of other macroalgae that is part of the sediment, and although the sediment is not the most plausible diet, its presence as a minor part of the diet cannot be discarded (Duggins and Eckman, 1997; Cardona et al., 2007; Riera et al., 2009).

#### 4.2.3. Sub-surface feeder: Arenicola marina

The most plausible diet of *A. marina* included microphytobenthos and the macroalgae *E. intestinalis* and *F. vesiculosus*. One of the most important food sources for this species is the bacterial community in sediment (Grossman and Reichardt, 1991), which was not directly considered in this study, but was likely represented in the sediment sample which contains potential food sources such as meiofauna, microphytobenthos and detritus (Retraubun et al., 1996). The sediment was included as component of the *A. marina*'s diet as a low plausibility food source (Table 3). This result can be caused by (1) the potential sources that were analyzed together (bacteria, meiofauna and detritus) and potential sources that were analyzed separately (microphytobenthos) having similar isotopic compositions; (2) a high percentage of refractory matter, not assimilable by *A. marina*, occurring in the sediment; for example terrestrial organic matter, that could also explain the low $\delta^{13}C$ of the sediment (Table 2); and (3) the layer or sediment considered in this study, top 5 mm, is not deep enough to evaluate the potential depth that *A. marina* can use. However, the strong contribution of microphytobenthos to the diet of this lugworm (Herman et al., 2000) and the ability to subduct surface sediment very rapidly in its burrow (Rijken, 1979) suggest that the surface sediment plays a more important role than the subsurface one as food source. The presence of macroalgae in the diet of *A. marina* is probably not related to direct assimilation of macroalgae fragments but it might be indirectly caused by the assimilation of epiphytic bacterial communities involved in the degradation of macroalgae detritus and not the detritus itself. In fact, macrophytes support bacterial production in *Zostera* spp. meadows (Boschker et al., 2000) and those bacterial communities have an isotopic signature similar to that of the assimilated substrate (see review in Jones et al., 2003). Therefore, a consequent transfer of the macroalgae's isotopic signature to the bacterial community's consumer is expected, i.e., *A. marina*. This hypothesis is in good agreement with the expected

contribution of the bacterial community in sediment to the diet of *A. marina* (Grossman and Reichardt, 1991).

#### 4.2.4. Carnivores: Nucella lapillus, Gobius niger, Symphodus bailloni and Sepia officinalis

The most plausible diet of *N. lapillus* included *M. galloprovincialis* and *L. obtusata*, although *M. galloprovincialis* could constitute the diet by itself according to a fractionation of 1.5 for $\delta^{13}C$ and a range between 2.2 and 3.0 for $\delta^{15}N$. This diet is consistent with values observed in the literature, where barnacles and mussels were identified as the diet for this gastropod (Davenport et al., 1998). However, given the importance of barnacles in its diet (Dunkin and Hughes, 1984) and the absence of that crustacea in this study for methodological reasons – small size of available barnacles in the area – the most plausible diet might reflect only partially the trophic relationships of the dogwhelk.

The most plausible diet for *G. niger* included *S. plana*, *A. marina*, *Littorina* spp. and *N. lapillus*, which is in good agreement with the literature, where the species is described as a feeding generalist (Whitehead et al., 1986; Fjosne and Gjosaeter, 1996). According to a recent review, Mollusca, Crustacea and Polychaeta constitute of 98.73% of its diet (Filiz and Togulga, 2009), however in this study the crustaceans *Gammarus* sp. and *P. adspersus* are not in the most plausible diet. The absence of crustaceans could be a limitation in the number of species considered in this study, and the analysis of other crustaceans could include them in the most plausible diet. *G. niger* is an example where the ability of stable isotopes to discriminate between food sources is limited because only two isotopes are available.

The same food sources suggested for *G. niger* were identified as the most plausible diet for *S. bailloni*, which is consistent with the diet observed for *S. melops* in the same ecosystem (Fernández, 1995) and *S. tinca* in the southern coast of Tunisia (Ouannes-Ghorbel and Bouain, 2006), showing the highly diversified feeding behaviour of this fish.

The most plausible diet of *S. officinalis* is *P. adspersus* and POM larger than 200 μm for individuals smaller than 67 mm (DML); *P. adspersus*, *G. niger* and *Gammarus* sp. for individuals between 67 and 120 mm; and *G. niger* and *Gammarus* sp. for individuals larger than 120 mm. These diets are in good agreement with the patterns observed by Castro and Guerra (1990) for the same size classes. Both studies show that *Palaemon* sp. is an important food source for the two smaller groups and its contribution decreases in the larger group. On the contrary, the contribution of *G. niger* increases with the *S. officinalis* size. One of the most important food sources, especially for larger individuals, are portunids, which have not been analyzed in the present study. Ignoring this important food source may have falsely increased the plausibility of detecting in the model the crustacean *Gammarus* sp., which a priori should be a less plausible source. Stable isotope analysis improves on gut content studies, which would not have been able to detect the presence of marine POM in the most plausible diet of the smaller group (Blanc et al., 1998; Blanc and Daguzan, 2000), as a consequence of the difficulty in identifying small particles.

#### 4.2.5. Omnivores: Gammarus sp. and Palaemon adspersus

The most plausible diet of *Gammarus* sp. included sediment, microphytobenthos and *E. intestinalis*. *Gammarus* sp. is a generalist species in terms of food behaviour (Delong et al., 1993), with filamentous algae, fine detritus and diatoms as preferential food sources (Delong et al., 1993; Lotze and Worm, 2000), which is in good agreement with the results observed in the present study.

In the case of *P. adspersus*, the most plausible diet included molluscs (*S. plana*, *L. littorea* and *L. obtusata*) and macroalgae (*Ulva* spp. and *A. nodosum*), which differs from results obtained from gut content analysis of this species in the same geographical area (Figueras, 1986). In the latter study, marine POM and small crustaceans like *Gammarus* sp. were identified as the preferential food sources, although molluscs and macroalgae are also identified as possible food sources. These differences can be caused by the limitation of the isotopes in discriminating between a wide set of possible food sources as in the cases of *G. niger* and *S. bailloni*.

### 4.3. Analysis of organic matter sources

The structure of the studied trophic web can be described in a stable isotope space as a triangle in the plane $\delta^{13}C$–$\delta^{15}N$, abscissa–ordinate, respectively (Fig. 3). The altitude of the triangle represents the difference in trophic level between the organic source and the apex consumers and the wide $\delta^{13}C$ base illustrates the high variability in isotope composition between the different sources of organic matter. This trophic structure describes a community with a high diversity of organic matter sources, although the contribution of each source varies depending on the consumer, as is suggested by the most plausible diets (Table 3, Fig. 3). The results emphasized the important contribution of microphytobenthos as a food source for several consumers (Table 3, Fig. 3), because microphytobenthos were one of the most plausible sources for herbivores (*L. littorea* and *L. obtusata*), omnivores (*Gammarus* sp. and *A. marina*) and all of the filter-feeders (*C. edule*, *R. decussatus*, *S. plana* and *M. galloprovincialis*). This is in good agreement with other studies that demonstrated the importance of microphytobenthos to estuarine benthic organisms (Herman et al., 2000). On the contrary, although *Z. marina* is the dominant species in the study area in terms of biomass, its contribution seems to be limited to *L. littorea*, and probably channelized indirectly through epiphytic organisms, which have an isotopic composition similar to *Z. marina* (Winning et al., 1999). This small contribution could be caused by its low digestibility and nutritive quality (Hemminga and Duarte, 2000), although its contribution through detritivore trophic chains cannot be discarded (Alongi, 1998).

Similar to *Z. marina* and contrary to marine POM larger than 20 μm, marine POM below 20 μm contributed less to the consumers' most plausible diets, especially in the case of filter-feeders, which are known to consume particulates in this size range (Møhlenberg and Riisgård, 1978). A detailed study is necessary to investigate possible reasons for the unexpectedly low contribution, but it could be caused by (1) the described methodology or (2) selective retention of particles below 20 μm. In the first case, using higher fractionation for $^{13}C$ would allow this food source to be included in the most plausible diet of filter-feeders. The diet analysis protocol described in this study suggests as the most plausible source the closest one to the consumer in the plane $\delta^{13}C$–$\delta^{15}N$, which could be the reason for discarding smaller POM in place of larger fractions. In the second case, selective retention of particles from the whole fraction below 20 μm (Ward et al., 1998; Martineau et al., 2004) could result in a differential isotopic composition of the retained particles compared to the whole fraction, and therefore the isotopic composition of the marine POM below 20 μm would not be representative of the ingested fraction.

Another potential food source, terrestrial POM below 60 μm, is not relevant in the most plausible diets (TER in Table 3 and Fig. 3). The isotopic composition of this source is similar to the sediment (Table 2), therefore it could be assumed that the sediment is derived, at least partially, from terrestrial detritus. However, the indirect contribution of terrestrial POM to the most plausible diets is still not significant given the low plausibility of the sediment as a food source. In spite of this result, the contribution of the terrestrial matter through dissolved matter cannot be discarded. Marine POM and macroalgae $\delta^{15}N$ values (Table 2) correspond with those found

in other geographical areas with intermediate values of anthropogenic wastewater input (Cole et al., 2004). Wastewater discharge has also been considered as the cause of high $\delta^{15}N$ values on other areas of the Northwest Iberian Peninsula (Bode et al., 2006). The studied *Z. marina* meadow is on the Eastern bank of the San Simón Bay, where the 85% (35,000 people) of the total population lives in the bay, widely spread along the coastline, as well as where the Oitavén-Verdugo River discharges wastewater from the other minor populations located in its fluvial basin (Fig. 1), which could cause the enrichment in $\delta^{15}N$ compared to areas without anthropogenic influence. However, the use of recycled nitrogen within the bay could be an alternative hypothesis to wastewater contribution for explaining the $\delta^{15}N$ enrichment (Riera et al., 2000). This was observed in the Schelde Estuary, where suspended organic matter composition could not be explained in terms of conservative mixing of riverine and terrestrial sources on the one hand and marine sources on the other one, suggesting a significant contribution of autochthonous production (Middelburg and Nieuwenhuize, 1998). Although the characterization of anthropogenic sources in this area is challenging given human population distribution and the consequent diffusive inputs, further research is necessary to determine the contribution of urbanization and local production as well as nutrient recycling.

### 4.4. Conclusions

The similarities between the results presented here and those from other studies based on similar trophic webs (Middelburg et al., 2000; Sauriau and Kang, 2000; Page and Lastra, 2003; Jaschinski et al., 2008) suggest that the described protocol, which takes into account the fractionation uncertainty and is based on both linear mixing and Euclidean distance models, is a feasible alternative to elucidate the most plausible trophic relationships in complex trophic webs. The application of this protocol to an intertidal flat dominated by *Z. marina* emphasized the importance of local primary production, especially microphytobenthos production, which could be available for several primary consumers through resuspension forced by tidal hydrodynamic.

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