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The role of aquatic and terrestrial material flows to an estuarine food web using stable isotopes

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“Não faças do pensamento, blocos de duro cimento.”

(anónimo)

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Abstract

Estuarine food webs function within a mosaic of habitats and are supported by a mix of primary producers from both local and distant sources. Understanding the factors that govern the exchange and consumption of different organic matter (OM) sources is of paramount importance for the management and conservation of estuaries, because secondary production and food web diversity are thought to depend on the quality of the basal sources fuelling it. Therefore the studies presented in this PhD thesis aimed to improve the knowledge on the role of material flows in estuarine food webs, using the River Minho estuary as a model ecosystem, and carbon and nitrogen stable isotope analyses for estuarine biogeochemical characterization.

In the first chapter, the most common analytical approaches used in the study of estuarine food webs were reviewed around three main subjects: 1) methods used to identify the pools that support consumers; 2) characterization of end-member (i.e. sources); 3) dominant assumptions in the analysis of food webs. The most commonly used approaches are qualitative approaches, combined with basic statistical analyses (e.g. ANOVA), although there has been an increase in the use of quantitative approaches such as linear mixing models (e.g. IsoError, IsoSource, SIAR). The quantitative approaches more commonly used in the study of estuarine food webs are the IsoError (determined model) and IsoSource (undetermined model) mixing models. Also, the potential of all the existing qualitative and quantitative approaches depends on the quality of the input data and on the proper acknowledgment of the assumptions/limitations of each approach. Thus, the proper characterization of the end-members and baselines is crucial for the characterization of food web dynamics.

In the second chapter, a high spatial resolution study was conducted in the Minho River estuary to characterize the contribution of different OM sources to the lower food web, under naturally varying river discharge scenarios. It was possible to conclude that Minho River estuarine habitats are connected with each other and with its end-members. The degree of connectivity and the importance of allochthony varied with the river discharge and with the occurrence of a major winter flood. The contribution of terrestrial-derived OM to the POM pool was higher during high river discharge conditions, and during a summer following an unusual winter flood.

In the third chapter, it was verified that there was a temporal and spatial variability in the Minho river estuarine benthic food web dynamics. Spatial variability was essentially related with the characteristics of the organic matter sources available in each estuarine area, which was also influenced by the proximity to other ecosystems (i.e. higher terrestrial influence in the tidal freshwater portion and higher marine influence in the

brackish portion of the estuary). Temporal changes in the stable isotope ratios of consumers were driven essentially from changes occurring at the base of the food web, although there were also some evidences for a combination with changes in the resources used by consumers. River discharge was also an important factor influencing the changes at the base of the estuarine food web, both spatially and temporally.

In the fourth chapter were presented evidences of how man induced activities, in particular the introduction of invasive species, can potentially change estuarine food webs. The *Corbicula fluminea*'s functional response was analyzed in terms of feeding behavior and food selectivity, using the natural variation in OM sources that occur in estuarine environments. It was possible to conclude that *C*. *fluminea* has the ability to adapt to environments with low food quality because it can consume terrestrial-derived OM. This can be a competitive adaptation in systems with perennial low food quality such as the Minho River estuary. Moreover, its ability to couple benthic and pelagic environments and terrestrial ecosystems demonstrates a strong potential to alter food web flows in aquatic ecosystems.

In conclusion, Minho estuary food web relies on a strong connectivity between the different components, namely between the pelagic and benthic compartments, and also between the estuarine, terrestrial and marine ecosystems. Yet, further studies will be needed to disclose the impacts of disrupting ecosystems connectivity on estuarine productivity.

Resumo

As cadeias tróficas estuarinas estão inseridas em diferentes tipos de habitats, sendo suportadas por um conjunto de produtores primários com origem local ou provenientes de outras áreas. Para uma adequada gestão e conservação dos estuários, é essencial compreender quais os fatores que condicionam os movimentos da matéria orgânica nestes ecossistemas, bem como a sua utilização pelos consumidores, pois a produção secundária e a diversidade nas cadeias tróficas, depende da qualidade das fontes de matéria orgânica que as suportam. Assim, com os trabalhos científicos presentes nesta tese de Doutoramento, espera-se contribuir para uma melhor compreensão do papel que a dinâmica da matéria orgânica tem no funcionamento das cadeias tróficas estuarinas. Para realizar este trabalho, utilizou-se o estuário do rio Minho como sistema modelo e para a sua caracterização biogeoquímica, efetuaram-se análises de isótopos estáveis de carbono e de azoto.

No primeiro capítulo foi feita uma revisão aos métodos mais utilizados na análise e interpretação de dados no estudo das cadeias tróficas estuarinas, tendo esta sido estruturada da seguinte forma: 1) métodos mais utilizados na identificação das fontes que suportam a produção dos consumidores; 2) caracterização das fontes de energia; 3) principais pressupostos na análise de cadeias tróficas. As abordagens mais utilizadas são as qualitativas, combinadas com análises estatíticas (e.g. ANOVA), embora a utilização de métodos quantitativos (e.g. IsoError, IsoSource e SIAR), tenha aumentado nos últimos anos. As abordagens quantitativas mais utilizadas no estudo das cadeias tróficas estuarinas são os modelos IsoError (modelo determinado) e o IsoSource (modelo indeterminado). Não obstante, o potencial de qualquer uma das abordagens existentes atualmente, depende da qualidade dos dados que são utilizados e do reconhecimento dos pressupostos e das limitações inerentes a cada uma delas. Assim, uma correta caracterização de todas as fontes possíveis é crucial, para a correta avaliação da dinâmica das cadeias tróficas estuarinas.

No segundo capítulo, foi realizado um estudo com elevada resolução espacial no estuário do rio Minho, por forma a caracterizar o efeito que diferentes níveis de caudal têm na contribuição das fontes de matéria orgânica para os níveis tróficos inferiores. Concluiu-se que os habitats no estuário do rio Minho estão interligados entre si e também com os ecossistemas adjacentes. O grau de conetividade e a importância dos subsídios alóctones variaram com o caudal. A contribuição de origem terrestre para a matéria orgânica particulada na água, foi mais elevada durante períodos em que o caudal foi também mais elevado, e durante um verão de um ano em que ocorreram cheias no inverno.

No terceiro capítulo verificou-se que a cadeia bentónica do estuário do rio Minho apresenta uma dinâmica espacial e temporal variável. A variação espacial esteve essencialmente relacionada com as características das fontes de matéria orgânica disponíveis em cada uma das áreas analisadas, e que por sua vez, foram influenciadas pela proximidade a outros ecossistemas (i.e. a influência terrestre foi maior a montante e a influência marinha foi mais relevante nas zonas próximas da foz). As variações temporais nos ratios dos isótopos estáveis dos consumidores deveram-se essencialmente a variações temporais que ocorreram na base da cadeia trófica e/ou alterações no tipo de recursos utilizados pelos consumidores. A hidrologia foi também um factor determinante nas alterações espaciais e temporais que ocorreram na base da cadeia trófica estuarina.

No quarto capítulo são apresentadas evidências do impacto que as atividades humanas, através da introdução de espécies invasoras, podem ter na alteração da dinâmica das cadeias tróficas. A resposta funcional da *Corbicula fluminea* à variação das fontes de matéria orgânica que ocorrem naturalmente nos estuários, foi avaliada através da análise do seu comportamento alimentar e selectividade alimentar. Foi possível concluir que a *C. fluminea* tem a capacidade de se adaptar a ambientes com pouca qualidade de alimento, uma vez que conseguem consumir matéria orgânica com origem terrestre. Tal, poderá constituir uma adaptação competitiva a sistemas com períodos prolongados de alimento de baixa qualidade, tais como o estuário do rio Minho. A capacidade para ligar os ambientes pelágicos, bentónicos e terrestres, demonstra o seu forte potencial para alterar a dinâmica das cadeias tróficas aquáticas.

Assim, com esta tese, foi possível constatar que existe uma forte conetividade entre os diferentes componentes das cadeias tróficas estuarinas, nomeadamente entre os ambientes estuarinos pelágicos e bentónicos, e entre o estuário e os ecossistemas terrestre e marinho. No entanto, estudos mais aprofundados sobre este tema são necessários, por forma a compreender de que forma alterações na conetividade das cadeias tróficas estuarinas poderão influenciar a produtividade destes ecossistemas.

Objectives

Characterizing food webs is useful to describe relationships between species and their environment, however the dynamic of the landscape in which they occur, can cause them to have a high degree of spatial and temporal complexity (Huxel et al. 2004). Estuaries are among the most productive ecosystems on the planet and produce highly variable food webs associated with diverse habitats, with different organic matter (OM) sources, which in turn are influenced by the variability in hydrodynamics and physicochemical conditions at various spatial and temporal scales (Antonio et al. 2012, Ubertini et al. 2012). The current consensus for river-estuary complexes is that phytoplankton is the predominant source supporting upper trophic levels, even though phytoplankton generally comprised less than 10% of the particulate OM (Deegan and Garritt 1997, Hughes et al. 2000, Chanton and Lewis 2002). Nonetheless, there is a growing evidence that primary consumers rely on terrestrial-derived OM, when phytoplankton is less available (Howarth et al. 1996, Hoffman et al. 2008, Cole and Solomon 2012). Thus, understanding the dependence of estuarine food webs from adjacent ecosystems is of paramount importance for management and conservation of estuaries, because allochthonous subsidies (i.e. riparian and upland vegetation, marine OM) may have the potential to alter the carrying capacity of these ecosystems.

The description of any food web requires the establishment of trophic relationships, the identification of OM sources, and to quantify the energy flow between ecosystems' components. Traditional approaches, such as field and laboratory observations and stomach content analyses, cannot provide information about assimilation or identify the main energy sources supporting consumers' biomass. Alternatively, stable isotope techniques enabled scientists to obtain time-integrated information about feeding relationships and energy flow through food webs (Pasquaud et al. 2007). This is based on the relationship between the stable isotope composition of OM in the ecosystem and in the tissues of consumers that incorporate it into structural components and energy reserves. The naturally occurring stable isotope ratios of nitrogen ($\delta^{15}N$) and carbon ($\delta^{13}C$) in animal tissue reflect the composition of those food sources that were assimilated over time. The $\delta^{15}N$ of an organism is typically enriched by 2.2-3.4‰ relative to its diet, being used to determine the trophic position of an organism. The $\delta^{13}C$ changes little (1-2‰) as carbon moves through the food web, being used to evaluate the sources of energy for an organism (Peterson and Fry 1987).

In this context, the studies presented in this PhD thesis aimed understanding the processes that govern the exchange and consumption of different OM sources in estuarine benthic food webs. The Minho River estuary was used as a model ecosystem

because presents low phytoplankton availability (Brito et al. 2012), and maintains high benthic and epibenthic productions (Sousa et al. 2005, Souza et al. in press). This thesis is structured around five main objectives within four chapters:

1) report the main strategies currently used to analyze and interpret stable isotope data in food web studies;

2) identify the factors governing the exchange and consumption of OM sources, with different origins, in the Minho River estuarine habitats;

3) determine the degree of connectivity within estuarine habitats and between the estuary and its end-members (i.e terrestrial, riverine and marine ecosystems), and how ecosystem connectivity may influence estuarine food web dynamics;

4) infer the patterns and processes that structure benthic food web's spatial and temporal variability in Minho River estuary;

5) investigate how anthropogenic induced changes, as the introduction of non-indigenous species, may affect the flow of OM in estuarine food webs.

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Chapter 1

Using stable isotopes to untangle trophic relationships in estuarine food webs

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Abstract

Stable isotopes have emerged as a useful tool to understand the structure and dynamics of estuarine food webs, because they provide time-integrated information about the trophic relationships and energy flow through food webs. Two main approaches have been used to analyze and interpret stable isotope data in the context of estuarine food webs: qualitative and quantitative approaches. In the qualitative approaches, the analysis is based on the inspection of the position occupied by the stable isotope ratios of a certain consumer in relation to the stable isotope ratios of its most likely sources. In the quantitative approaches, it is calculated the proportional contribution of each source to the consumer's biomass. Based on the analysis of studies addressing the trophic interactions in estuarine food webs between 2000 and 2011, it was possible to verify that the most commonly used approach is still the qualitative, combined with statistical analysis (e.g. ANOVA). Nonetheless there has been an increase in the use of quantitative approaches such as linear mixing models (e.g. IsoError, IsoSource, SIAR). Also, when analyzing multiple trophic levels, researchers tend to estimate the stable isotope ratios of phytoplankton, by establishing the isotopic baseline from some other biological material near the base of the food web or by using estimates from other ecosystems. Further, the interpretation of stable isotope data generally relies on several assumptions, such as that the isotopic composition of consumer's tissues equals the weighted average of the isotopic composition of its sources, and trophic fractionation moves constantly through the food web. Since several studies contradict those assumptions, the assumptions should be acknowledge when interpreting trophic interactions based on stable isotope data in the absence of species-specific estimates.

Keywords: isotope mixing model, baseline, trophic fractionation, assumptions.

1.1 Introduction

Estuaries are among the most productive ecosystems in the world, and are characterized by complex trophic dynamics associated with their diverse habitats and organic matter (OM) sources.

Earlier studies of trophic dynamics in estuaries were usually based on consumers' stomach content analysis (Whitfield 1988). Although this can reveal consumers' diets and trophic relationships with higher taxonomic resolution, they do not provide information about assimilation. Stable isotopes emerged as a useful tool to understand the structure and dynamics of estuarine food webs, because they provide time-integrated information about the trophic relationships and energy flow through food webs (Pasquaud et al. 2007). The first applications of stable isotopes in food web studies were qualitative, providing inferences about the relationships between consumers and their resources based on inspections of species' position in bi-plots (e.g. Haines 1976, Peterson et al. 1985). During the last 20 years, quantitative approaches have emerged to quantify the differences in the stable isotopes ratios of individuals, species or others (e.g. Hsieh et al. 2002, Richoux and Froneman 2007, Vinagre et al. 2011), and more complex mixing models to determine dietary contributions of different sources to a certain consumer (e.g. Phillips 2001, Lubetkin and Simenstad 2004, Parnell et al. 2010). These approaches have dramatically improved our understanding on the complexity of estuarine food webs, providing new insights on its spatial and temporal variability (e.g. Chanton and Lewis 2002, Baeta et al. 2009, Atwood et al. 2011, Antonio et al. 2012), food chain length (e.g. Akin and Winemiller 2008), and changes in the food web subsidies and structure induced by human activities (e.g. Cole et al. 2004, Wu et al. 2009). Nonetheless the use of more quantitative approaches has raised a prolific debate due to the common underlying assumptions of each approach, especially how they incorporate sources' and consumers' variability or uncertainty (Phillips and Gregg 2001, 2003, Parnell et al. 2010, Semmens et al. 2013).

In this chapter we provide an overview on the most common analytical approaches used to interpret stable isotope data on food webs, structured around three main subjects: 1) methods used to identify the pools that support consumers' biomass; 2) characterization of end-members (i.e. sources); 3) dominant assumptions in food web analysis. To identify the most common analytical approaches used to interpret stable isotope data in estuarine food web studies we analyzed the papers published between 2000 and 2011 and cataloged in *Scopus*. The papers were identified using the keywords "food web", "estuary" and "stable isotopes".

1.2 Stable isotope ratios and estuarine food webs

Stable isotopes are atoms of the same element with different masses, because of differences in the number of neutrons. Atomic mass is essentially the sum of the masses of the protons and neutrons (each of a weight equal to about 1 atomic mass unit), because electrons have a relatively insignificant mass. For instance, the common isotope of carbon has six protons and six neutrons. It thus has an atomic mass of about 12, designated as ^{12}C. It has a rare isotope with seven neutrons, designated as ^{13}C. Stable isotopes, unlike radioisotopes, do not decay (Criss 1999).

The most commonly used elements in food web studies are nitrogen (N), carbon (C) and sulphur (S). The C isotope ratios ($\delta^{13}C$: $^{13}C/^{12}C$) are useful to discriminate between primary producers with different photosynthetic pathways (e.g. C3 and C4 plants), and since they exhibit little or no differences between trophic levels, they are useful to determine the original sources of dietary C (Peterson and Fry 1987). Similarly, the S isotope ratios ($\delta^{34}S$: $^{34}S/^{33}S$) vary substantially among primary producers, but it changes little between trophic levels and therefore are also useful tracers to identify organic matter sources (Peterson et al. 1985, Peterson and Howarth 1987). These stable isotope ratios are particularly useful in the identification of organic matter (OM) sources in estuaries, because estuaries receive inputs from multiple sources, including riparian vegetation, marsh vegetation, submerged and emergent aquatic vegetation and phytoplankton (Cloern et al. 2002, Hoffman and Bronk 2006). Riparian plants that utilize the C3 pathway have a $\delta^{13}C$ of about -28‰ because there is an uptake fractionation of about -21‰ over atmospheric CO_2 ($\delta^{13}C$: -7‰) (Smith and Epstein 1971). In contrast, plants associated with the C4 pathway (e.g. *Spartina* spp.) are more ^{13}C- enriched ($\delta^{13}C$: -13‰) owing to reduced fractionation (Smith and Epstein 1970, Fry and Sherr 1984). Freshwater and estuarine phytoplankton will also differ, because they utilize isotopically distinct pools of dissolved inorganic carbon (DIC). In general, microphytobenthos (MPB) are more ^{13}C-enriched than phytoplankton, due to the existence of a diffusive boundary layer at the sediment-water interface that reduces isotopic fractionation (France 1995). Upland plants, phytoplankton and marsh plants also derive their sulfate from different origins, thus producing distinguishable $\delta^{34}S$ values. Upland plants use the sulfate available from the rain water ($\delta^{34}S$: 2‰ to 8‰), while estuarine phytoplankton derived their sulfate from seawater ($\delta^{34}S$ ~ +18‰). Marsh plants derive sulfite from a more variable and usually light $\delta^{34}S$ inorganic sulfur pool in the sediments ($\delta^{34}S$ typically -10‰ to +5‰). For MPB the values are intermediate ($\delta^{34}S$: 4‰ to 14‰), depending upon the proportions of reduced sulfur and seawater at the sediment/water interface (Peterson and Howarth 1987, Sullivan and Moncreiff 1990, Currin et al. 1995, Newell et al. 1995). Thus, stable C and S isotopes

can help to differentiate between benthic and pelagic environments and between vascular plant and phytoplankton sources.

The N isotope ratios ($\delta^{15}N$: $^{15}N/^{14}N$) become enriched in 3-4‰ between two consecutive trophic levels and therefore are a powerful tool to estimate consumers' trophic position (Minagawa and Wada 1984, Peterson and Fry 1987, Vander Zanden et al. 1999). They can also help to separate ^{15}N-depleted terrestrial OM ($\delta^{15}N$: -4‰ to 4‰) from ^{15}N-enriched aquatic OM sources ($\delta^{15}N$: 6‰ to 10‰) (Peterson and Fry 1987, Cloern et al. 2002). However, $\delta^{15}N$ can be altered by changes in the isotope value of N substrates due to preferential uptake of isotopically light N (Cifuentes et al. 1988), nitrification, denitrification (Mariotti et al. 1981) or by microbially mediated degradation (Miyake and Wada 1971; Altabet 1988). Also the form and amount of anthropogenic nitrogen addition can influence the baseline $\delta^{15}N$. Nitrate from atmospheric sources typically have $\delta^{15}N$ values varying between 0‰ and 8‰ (Kreitler and Jones 1975, Kellman and Hillaire-Marcel 2003). The $\delta^{15}N$ values of nitrate from synthetic fertilizers range from -3‰ to 3‰ (Macko and Ostrom 1994, Kellman and Hillaire-Marcel 2003) and nitrate from human and animal waste typically have $\delta^{15}N$ values varying from 9‰ to 25‰ (Kreitler and Browning 1983, Rolston et al. 1996).

We conducted a literature survey, on *Scopus*, to assess the number of studies published that investigated the estuarine food webs dynamics using stable isotopes, from 2000 to 2011. In this period, a total of 113 studies were published. The number of publications increased through time, with the most numbers of papers published in 2011 (n= 17) (Fig. 1.1). Overall, North America (United States and Canada), Europe, and Australia produced the majority of the publications with 33%, 13% and 12% of the total, respectively. The main topics studied were the identification of OM sources supporting the estuarine food web and their structure (89% of total), although other subjects were evaluated, such as the effects of anthropogenic inputs to the food web and consumers' movements. The stable isotope ratios most commonly used were $\delta^{13}C$ and $\delta^{15}N$ combined (52% of total), though in some studies one of those, or both, were used in combination with other elemental tracers, such as $\delta^{34}S$ or fatty-acids. Details on the main approaches used to characterize estuarine food webs are discussed below.

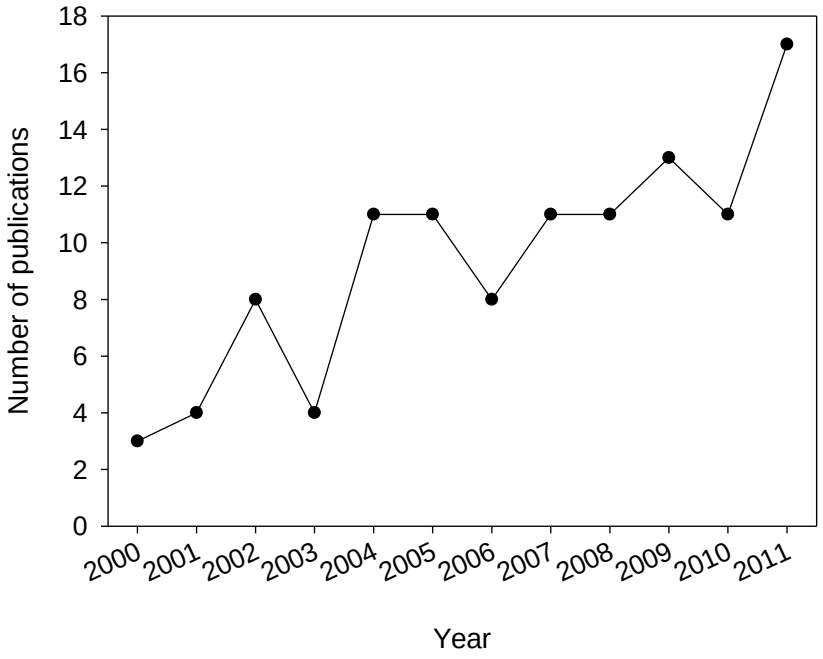

Fig 1.1 Number of studies published between 2000 and 2011 and indexed at *Scopus*, using the keywords “food web”, “estuary” and “stable isotopes”.

1.3 Methods to identify which sources support consumers in food web studies

There were two main approaches used to identify the resources supporting the production of consumers: qualitative and quantitative approaches. In a qualitative approach, the analysis is based on the inspection of the position occupied by the stable isotope ratios of a certain consumer, usually in a bi-plot (2 tracers), in relation to the stable isotope ratios of its most likely sources. In a quantitative approach, the proportional contribution of each source is estimated using a simple calculation or a mixture of more complex equations.

1.3.1 Qualitative methods

The most used method to identify trophic relationships in estuarine food webs, was the qualitative approach combined with statistical analyses (i.e. t-test, ANOVA, ANOSIM), which accounted for 61% of the papers published. Though we recognize that a visual analysis should always be done to constrain the possible sources to a consumer, this methodology is particularly limiting when studying complex food webs (i.e. multiple sources, several trophic levels and feeding strategies). Also, statistical analyses can be very informative in quantifying the differences in the stable isotope ratios of producers and/or consumers, or in assessing spatial and temporal variability in the stable isotope

ratios of a certain consumer or group of consumers. Yet, the strength of statistical analyses depends on the sample size and on within-group variability. As the number of potential food sources increases, the ability to identify dietary contributions becomes more difficult. So, in the last decades, several models have been developed to identify and quantify the importance of various food sources to the consumers' biomass.

1.3.2 Quantitative methods

Several approaches have been proposed to quantify the contribution of multiple sources to consumers' biomass: geometric (Ben-David et al. 1997), linear mixing models (Phillips 2001, Lubtekin and Simenstad 2004, Hall-Aspland et al. 2005, Parnell et al. 2010), and spatially-based approaches (Rasmussen 2010).

The geometric approaches use δ values to determine Euclidean distances between consumers and sources, and an inverse relationship is assumed between those distances and the contribution of each source to consumers' diet, i.e. the shorter the distance, the greater the contribution (Ben-David et al. 1997). However, this method overestimate rare food sources (Ben-David et al. 1997), and the equations provided are inaccurate to identify dietary contributions (Phillips 2001). For this reason, geometric approaches have been replaced by more recent quantitative approaches, such as the linear mixing models. In fact, the most commonly used quantitative approaches, in the surveyed studies of estuarine food webs, were the linear mixing models- 74% of those that used quantitative approaches. Since linear mixing models are the most widely used models, they will be discussed in more detail.

The linear mixing models have become a fundamental method to quantify the contribution of various data sources to a consumer based on their respective stable isotope ratios. These models use a set of mass-balance equations to estimate the proportional contribution of each food source to a consumer, assuming that all sources sum to 1 (Phillips 2001). For example, in a system with *n* tracers and *n+1* sources, solving the mass-balance equations will give the exact proportional contribution of each source (Phillips 2001). Assuming that we have two stable isotope ratios ($\delta^{15}N$, $\delta^{13}C$) and three sources (A, B, C), the equations would be represented as follows:

$$\delta^{13}C_M = f_A.\delta^{13}C_A + f_B.\delta^{13}C_B + f_c.\delta^{13}C_C \quad (1)$$

$$\delta^{15}N_M = f_A.\delta^{15}N_A + f_B.\delta^{15}N_B + f_c.\delta^{15}N \quad (2)$$

$$f_A + f_B + f_C = 1 \quad (3)$$

where δ_M is the isotopic composition of a consumer's tissue and f_A, f_B and f_C are the proportional contributions of sources A, B and C. These are the main principles underlying the linear mixing model IsoError (Phillips and Gregg 2001), which takes into account the source's and consumer's stable isotope ratios variability. An alternative formulation, the IsoConc linear mixing model (Phillips and Koch 2002) also considers the elemental concentration (e.g. %C,% N) of each source to estimate its proportional contribution to the consumer.

However, most food webs are very complex, with the number of sources frequently exceeding the number of tracers plus one. In this situation, researchers can constrain the possible sources using prior information to allow a determinate model (e.g. Schwamborn et al. 2002), or they will have to use an indeterminate model. The latter implies that there is not a unique solution to the model. The most commonly used mixing model is IsoSource- 40% (Phillips and Gregg 2003). This model estimates all multivariate solutions that fit the model bounding conditions (all contributors sum to 1) within a specific tolerance, and then outputs the probability distribution of the all univariate estimates under the domain of acceptable multivariate solutions. However, IsoSource does not explicitly incorporate uncertainty in the variables. The formula only requires the mean values of consumers, diet sources and trophic fractionation; the tolerance term implicitly recognizes variability in the diet sources.

Alternative modelling approaches were developed to estimate proportional source contributions including SOURCE and STEP (Lubtekin and Simenstad 2004) or the Moore Penrose pseudoinverse model (Hall-Aspland et al 2005). SOURCE is used to estimate consumers´ direct and indirect uptake of autotrophic sources and consumer trophic levels (Lubtekin and Simenstad 2004). STEP estimates a consumer's diet, which may include autotrophs or heterotrophs, or both (Lubtekin and Simenstad 2004). Both models require that the diet source isotope ratios included do not overlap, so they use a nearest neighbor distance measurement (NND^2) to determine whether two sources are sufficiently distinct to be considered individually in model calculations (Lubtekin and Simenstad 2004). These models identify the outer bounds of possible mixtures instead of examining every possible biological solution as with IsoSource, making it computationally less demanding (Lubtekin and Simenstad 2004).

The Moore Penrose pseudo-inverse model attempts to provide a unique solution of source contribution to a consumer using a single isotopic tracer and matrix algebra (Hall-Aspland et al 2005). However, the model yields a similar solution to the mean generated by IsoSource (Hall-Aspland et al 2005), but fails to acknowledge other feasible solutions as with IsoSource.

None of these indeterminate models allows for uncertainty in the variables because they all require mean values without distribution information for the stable isotope ratios of dietary sources and consumers, as well as for the estimates of trophic fractionation and trophic level. Alternative approaches, such as the Bayesian mixing models MixSIR and SIAR, have emerged to cope with these limitations. Bayesian inference incorporates variability in all the inputs, while allowing for multiple dietary sources and generates potential dietary solutions as assigned probability distributions (Parnell et al. 2010). SIAR (Parnell et al. 2010) and MixSIR (Moore and Semmens 2008) basically use the same approach, aside from minor differences in the fitting algorithms implemented (SIAR uses Markov Chain Monte Carlo sampling methods and MixSIR uses Sample Importance Resampling) (Moore and Semmens 2008, Parnell et al. 2010). The main difference between these methods is that SIAR includes a residual error term (Parnell et al. 2010). Possible unknown sources of error can be related to the physiology or ecology of the consumers studied. For instance, if the consumer under study goes through ontogenic shifts, the residual error likely will be high and thus, further analysis (e.g. literature review and/or basic statistics such as segmented linear regressions analysis) and modelling are required. An extension of the Bayesian approach has been developed to incorporate intra-population variability, where the variance in the diet of individual consumers across multiple levels of population structure can be acknowledged (Semmens et al. 2009).

There are some limitations that should be considered before applying Bayesian mixing models, namely: 1) dietary source variability and trophic fractionation may not be normally distributed, 2) Bayesian mixing models do not assume isotope routing (i.e. isotopes can be assimilated differently within the body of the consumer (which is a caveat in all the food web approaches), 3) the model will always attempt to fit a model, even if the sources lie outside the isotopic mixing polygon (Parnel et al. 2010). Another problem, raised by Fry (2013), is that likelihood densities can be multimodal in underdetermined mixing models, so the credibility intervals estimated by the Bayesian mixing models (usually 95%) can be misleading. Several strategies have been proposed to reduce multi-modality: incorporate more consumer data or prior information, change source geometry by re-grouping sources and include residual error (Semmens et al. 2013 and references therein).

One common problem that researchers have to deal with is the interpretation of the output of indeterminate mixing models. The most intuitive approach is to interpret measures of central tendency such as means, median or modes (most likely values), but this is not justified by the structure of the most common mixing models and when used alone they can be misleading. Although some unique solutions may be more likely than

others, it is advocated that the outputs should be characterized by the distribution of possible solutions (Phillips and Gregg 2003, Semmens et al. 2013).

Several approaches have been suggested to solve indeterminate mixing models: a) statistically subsample the range of feasible solutions and then calculate averages from the subsample (approach used in IsoSource); b) geometric sector approach, where the contribution of each source is inversely proportional to sector areas in a two dimensional space; c) extension of the previous approach, where a secondary polygon (danger zone) is included and defined based on halfway points between the exterior polygon and a centroid. The mixture data points falling within this danger zone are dominated by the assumed centroid contributions and little constrained by the measured data. All these approaches are detailed by Fry (2013). However the graphical analysis proposed by Fry (2013) has no statistical inferences, ignores uncertainty in both sources and mixture data, and do not provide probabilistic estimates of source contributions (Semmens et al. 2013). Nonetheless, other approaches can be used to try constraining mixing models, such as adding more tracers or conduct a prior source selection for aggregating and reducing the number of sources (Philips and Gregg 2003) based, for instance, on consumer's feeding behavior.

Although some models like SOURCE and STEP may help to decide which sources can be aggregated, there are some examples for which the existing models do not seem to have a satisfactory solution. For example, in a study aiming to identify the main dietary sources of the invasive bivalve *Corbicula fluminea* (Dias et al. in press), the authors identified five possible dietary sources based on the bivalves' feeding modes: estuarine phytoplankton (EP), marine phytoplankton (MP), particulate organic matter (POM; mixture of marine and freshwater POM), sediment organic matter (SOM) and microphytobenthos (MPB) (Fig. 1.2).

The MP and MPB have similar isotope ratios, similar C/N values (~7) and both are possible sources, based on a first inspection of the bi-plot (i.e. some *C*. *fluminea* present high $\delta^{15}N$ values). All the existing mixing models would have difficulties to solve these two sources, because they are highly correlated, i.e. the increasing proportional contribution of MP would implicate the decrease in the proportional contribution of MPB, and vice-versa. The aggregation of these sources would not be realistic because they represent completely different compartments of the system (pelagic vs. benthic), and the use of one or the other would imply differences in feeding strategies (filter feeding vs. pedal feeding). In this case, the existing knowledge on the species' physiology was crucial to discard MP as a likely source (Dias et al.in press).

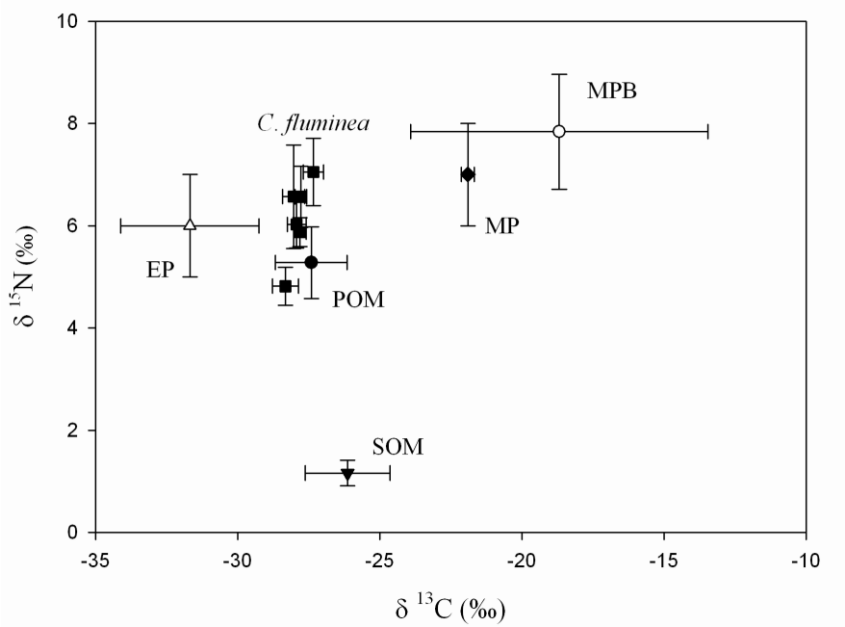

Fig. 1.2 Average $\delta^{13}C$ and $\delta^{15}N$ values of *Corbicula fluminea* adjusted for one trophic level fractionation (+0.4‰ $\delta^{13}C$, +3.4‰ for $\delta^{15}N$) and potential organic matter sources in the brackish estuary. Error bars represent one standard deviation. The sources considered relevant for *Corbicula fluminea* (closed square; each represents a station-specific average), in this portion of the estuary, were marine phytoplankton (MP; closed diamond), estuarine phytoplankton (EP; open triangle), microphytobenthos (MPB; open circle), sediment organic matter (SOM; closed triangle) and particulate organic matter (POM; closed circle). Figure *in* Dias et al. (in press).

Other models use spatial variability to estimate sources' proportional contribution when their isotope ratios are not necessarily distinct (Rasmussen 2010). This approach may be useful in systems where sources' variability is predictable across some spatial axis (e.g. altitude, river distance), which use the slopes of change along the spatial axis to estimate sources' contribution to the consumer by assuming that the consumer's stable isotope ratios is a weighted mixture of the sources along the linear gradients (Rasmussen 2010, Gray et al. 2011). The main disadvantages of this approach are that the proportions of the sources in a consumer's diet must be constant along the gradient, and the knowledge about the isotope gradients may be difficult to acquire (Layman et al. 2012).

1.4 Characterization of end-members

It is critical to properly characterize the end-members for stable isotope-based food web analyses. End-members are the source materials used in the mixing models to determine the proportional contribution of the two sources, or more, comprising the mixture being evaluated (Peterson 1999). For example, when studying the diet of a suspension feeding organism, all the sources available in the water column must be sampled. The number of end-members may increase with the complexity of the

ecosystem. For instance, estuaries receive inputs from several origins, such as rivers, sea and terrestrial ecosystems, while the main sources supporting food webs in lakes will be essentially a mixture of locally produced OM and terrestrial-derived OM.

The best approach to characterize the sources fuelling the food web is to sample all the likely sources and the consumers, on a spatial and temporal scale that reflects the relative incorporation rates of the elements and the turnover rates of tissues (O'Reilly et al. 2003). However, that might not be possible in every occasions. Phytoplankton is an important source at the base of any aquatic food web, but only in 9% of the studies published between 2000 and 2011, analyzed phytoplankton stable isotopes. Accurately measuring the stable isotope composition of phytoplankton is challenging, because it requires difficult physical separation techniques and taxonomic knowledge (i.e. different taxonomic groups of algae use different energy and nutrient sources). Thus, researchers commonly establish an isotopic baseline from some other biological material near the base of the food web (e.g. DIC, POM, plants, zooplankton; Zeng et al. 2007, Akin and Winemiller 2008, Wilson et al. 2010, Pasquaud et al. 2010, França et al. 2011, Hoeinghaus et al. 2011), or use values previously published for the studied ecosystem or from other similar ecosystems (e.g. Eddins 2001, Mulkins et al. 2002, Ruensik et al. 2003, Dierking et al. 2011).

The most commonly used approach, in estuarine food web studies published between 2000 and 2011, was to use POM stable isotope ratios as a proxy for phytoplankton. Bulk POM is a mixture of live and detrital OM with aquatic and possibly terrestrial origins. The use of POM stable isotope as a proxy to phytoplankton isotopic ratios in ecosystems dominated by phytoplankton production is likely a valid estimate, but not so valid in oligotrophic ecosystems where terrestrial-derived OM may represent a significant proportion of the bulk POM. In these situations, the alternative approach is to estimate the phytoplankton $\delta^{13}C$, by using a mixing model that includes the ratio between phytopankton C and total particulate organic carbon (POC), which yielded similar $\delta^{13}C$ estimates to those obtained from isolated phytoplankton samples (Marty and Planas 2009).

Phytoplankton $\delta^{13}C$ can also be determined based on the dissolved inorganic carbon (DIC) $\delta^{13}C$, assuming the uptake fractionation that occurs during the photosynthesis (usually -21‰; Mook and Tan 1991). However, the DIC $\delta^{13}C$ and concentration values may be influenced by biotic (photosynthesis, respiration) and abiotic (inflowing waters, methane oxidation, phytolysis) processes which will be reflected in the C stable isotope ratios of autotrophs (Marty and Planas 2009). Phytoplankton uptake fractionation can also be influenced by the supply and demand of DIC, and by

phytoplankton physiological characteristics (e.g. growth rate, cell geometry) (Laws et al. 1997, Popp et al. 1998, Burkhardt et al. 1999).

Similarly, estimates have been proposed to determine phytoplankton $\delta^{15}N$ values. For example, marine phytoplankton $\delta^{15}N$ can be estimated from chlorophyll *a* (Chl *a*), after removing seston and analyzed for $\delta^{15}N$ (Sachs et al. 1999). Chl *a* is a ubiquitous pigment in phytoplankton and degrades rapidly, thus representing live organisms, so Chl *a* $\delta^{15}N$ is considered an ideal indicator of $\delta^{15}N$ phytoplankton (York et al. 2007). There is a near constant nitrogen (N) depletion of 5.1‰ between Chl *a* relative to total N in laboratory reared phytoplankton species, which suggests that by adding this value to the Chl *a* $\delta^{15}N$ values, it is possible to obtain a good proxy for phytoplankton $\delta^{15}N$ values in the water column or sediments (Sachs et al. 1999). Although there is interspecific variability in the differences between Chl *a* and total cellular N, and because growth rate had a small effect on it, it is argued that this may be minimized when analyzing nature samples where multiple species and growth rates occur (Sachs et al. 1999). This method has been applied to estimate the $\delta^{15}N$ values of estuarine phytoplankton (York et al. 2007).

Phytoplankton $\delta^{15}N$ can also be estimated from ammonium $\delta^{15}N$ uptake fractionation. While nitrate can be assimilated by phytoplankton, ammonium is generally the preferred N source because this is the reduced form, whereas the oxidized forms (nitrate, nitrite) have to be reduced before assimilation (McCarthy 1980). Yet, isotopic fractionation range from 0‰ (Waser et al. 1999) to 16‰ (estimated by Liu et al (2013) based on De Brabandere et al. (2007)) in field measurements, and from 5‰ to 29‰ in laboratory measurements (Pennock et al. 1996). As there is evidence for a relationship between the fractionation values and the ammonium concentrations, Liu et al. (2013) developed a model, based on field observed fractionation values, that estimates the fractionation resulting from ammonium uptake by phytoplankton, based on the ammonium concentration values (µM) in the environment (Eq. 17; Liu et al. 2013). Yet, the magnitude of fractionation is dependent on species, light and growth rate (Needoba et al. 2003).

Although there are several alternatives available to estimate the isotopic baseline in estuarine food webs (models, primary consumers or literature values), unless the stable isotopes of phytoplankton (or other producers) are actually measured, the researcher will have to acknowledge several assumptions in data interpretation regarding the baseline characterization.

1.5 Dominant assumptions in food web analysis

The measurement of stable isotope ratios has contributed greatly to the understanding of trophic relationships; however, the interpretation of these ratios rely on several assumptions that, if not well recognized, may compromise their reliability.

An important assumption, when estimating the proportional contribution of different sources to a certain consumer, is that the isotopic composition of consumer's tissues equals the weighted average of the isotopic composition of its sources. This assumption is rarely valid, because the efficiency of assimilation by consumers depends on dietary components, nutrients are allocated differently to specific tissues, and consumers' tissue fractionate the diet components (Gannes et al. 1997).

Fibers (structural carbohydrates) are utilized by animals that have hemicellulase and cellulase enzymes, or by species capable of gastrointestinal fermentation of structural carbohydrates (Boyd and Goodyear 1971). Even for such animals, food with high structural carbohydrate content are less efficiently utilized than materials of lower fiber content (van Soest and Wine 1967, Varo and Amat 2008).

The interpretation of isotopic composition can be further complicated by isotopic routing, i.e. isotopes contained in different dietary components are routed differentially to specific tissues and body compartments (Tieszen and Fagre 1993 *in* Gannes et al. 1997). Thus, tissues often do not reflect the isotopic composition of the bulk diet, but the isotopic composition of the nutrient of the diet from which the tissue was synthesize. One example of isotopic routing is the incorporation of amino acid directly from dietary protein (Schwarckz and Scoeninger 1991, Ambrose and Norr 1993, Kelly and Martinez del Rio 2010, Newsome et al. 2011). An implication of these observations is that contrary to the predictions of mixing models, the $\delta^{13}C$ isotope values of animal protein in tissues (e.g muscle, blood) will resemble not that of bulk diet, but should be closer to that of dietary protein (Newsome et al. 2011). Also, different biochemical synthetic pathways for amino acids fractionate to varying extent, which will be reflected in animal tissues. For example, bone collagen contains ca. 33% glycine, which is a ^{13}C-enriched amino acid (Jim et al. 2006). That justifies the usually ca. 4‰ more positive $\delta^{13}C$ values of bone collagen when compared to other tissues commonly analyzed in ecology (Koch 2007).

Another common assumption in food web studies is that stable isotopes fractionation (Δ) is constant through the food web. It has been widely accepted that the averages for $\Delta\delta^{13}C$ and $\Delta\delta^{34}S$ are 0-1‰ (DeNiro and Epstein 1981, Peterson and Fry 1987) and that the average value of $\Delta\delta^{15}N$ are 3-4‰ (DeNiro and Epstein 1981, Minagawa and Wada 1984, Peterson and Fry 1987).

Several estimates have emerged from comprehensive studies combining field and laboratory data from the literature, and usually, the variability of $\Delta\delta^{15}N$ and $\Delta\delta^{13}C$ values seem to be mainly dependent on tissue type and diet (Vander Zanden and Rasmussen 2001, McCutchan et al 2003, Caut et al. 2009). Still, the estimates differ between reviews. For example, Vander Zanden and Rasmussen (2001) estimated that the mean (± SD) $\Delta\delta^{15}N$ and $\Delta\delta^{13}C$ values were 2.9 ± 0.3 ‰ and 0.5 ± 0.2 ‰, respectively. The main differences were found between carnivores and herbivores, where carnivores ($\Delta\delta^{15}N$: 3.4 ± 0.4 ‰; $\Delta\delta^{13}C$: 0.9 ± 1.0 ‰) had higher $\Delta\delta^{15}N$ and $\Delta\delta^{13}C$ values than herbivores ($\Delta\delta^{15}N$: 2.5 ± 2.5 ‰; $\Delta\delta^{13}C$: -0.41 ± 1.1 ‰). They argue that since the differences in the trophic fractionation values of organisms throughout the food web, the trophic position estimates should not be based on a fractionation value from a single prey-predator linkage, but should incorporate the number of trophic links leading to a given consumer (Vander Zanden and Rasmussen 2001).

McCutchan et al (2003) found differences in the trophic fractionation values of consumers regarding diet type (vascular or others), protein content, environment (aquatic or terrestrial), and tissue analyzed (muscle or whole). The main differences were found in the $\Delta\delta^{34}S$ values (for all the above parameters), although $\Delta\delta^{13}C$ of muscle was 1‰ higher than when analyzing the whole organism. The average $\Delta\delta^{13}C$ and $\Delta\delta^{34}S$ for all the animals analyzed (excluding fluid-feeding consumers) were +0.5 ± 0.1 ‰ and +0.5 ± 0.6 ‰, respectively, and the average $\Delta\delta^{15}N$ was +2.3 ± 0.2 ‰ (McCutchan et al. 2003).

One of the most commonly used estimates for trophic fractionation is the one proposed by Post (2002), where $\Delta\delta^{15}N$= +3.4 ± 1.0 ‰ and $\Delta\delta^{13}C$= +0.4 ± 1.3 ‰. Contrary to the reviews above, Post (2002) did not find any differences between aquatic and terrestrial organisms, between laboratory and field observations, or between carnivores and herbivores/detritivores.

More recently, Caut et al. (2009) proposed an alternative method called “Diet-Dependent Discrimination Factor”, which enables the calculation of trophic fractionation from data on diet isotope ratios, when laboratory estimates are not available.

To date, the lack of species-specific trophic fractionation estimates, for different diets and tissues, has led ecologists to rely their estimates on source proportional contribution to consumers’ biomass, on trophic position and on fixed trophic fractionation values often obtained from literature reviews. In the studies published between 2000 and 2011, the most used isotopic fractionation estimates were those from Post (2002) and McCutchan et al. (2003), and generally consisting in a single average value for each isotope used along multiple trophic levels.

Thus, uncertainty in $\Delta\delta^{13}C$ and $\Delta\delta^{34}S$ can cause errors in estimates of partitioning of food sources, and uncertainty in $\Delta\delta^{15}N$ can lead to biases in the estimation of sources’

and trophic position's data. An equation with three unknowns has to be solved to estimate a consumer's trophic position (Eq. 4):

$$\text{Trophic position} = \lambda + (\delta^{15}N_{consumer} - \delta^{15}N_{baseline})/\Delta \quad (4)$$

where λ is the trophic position of the organism/organisms used to estimate $\delta^{15}N_{baseline}$ (e.g. λ=1 for primary producers), $\delta^{15}N_{consumer}$ is measured directly and Δ is the $\delta^{15}N$ fractionation value per trophic level. When using this equation, three important assumptions are commonly made: the trophic fractionation of $\delta^{15}N$ is a fixed value (usually from the literature, as discussed above), N moves through the food web with a similar stoichiometry, and the chosen isotopic baseline ($\delta^{15}N_{baseline}$) integrates the spatial and temporal variability appropriate for the question being studied. In aquatic systems, most primary producers have high temporal variability in the $\delta^{15}N$, complicating their direct use as indicators of $\delta^{15}N_{baseline}$. In contrast, secondary consumers integrate $\delta^{15}N$ over longer time periods (Cabana and Rasmussen 1996). Several authors proposed the use of long-lived primary consumers (e.g. mussels) to quantify the $\delta^{15}N_{baseline}$ in aquatic food webs, because they are well-aligned with temporal and spatial environmental variability (Fry and Allen 2003).

The temporal scale on which the study will elapse must influence the choice of the tissue to be analyzed, because different tissues have different turnover rates. For instance, hairs, feathers and the dentine of teeth, are metabolically inert once they are deposited, and therefore represent consumers' diet isotopic ratio at the time of deposition. If the rate of tissue deposition is known, these tissues will represent a timeline of consumers' dietary history (Hobson and Sease 1998, Newsome et al. 2009). In contrast, blood or muscle tissue integrate diet over a time scale of days to months (Hobson and Clark 1992). Moreover, tissue turnover of the organism will also vary with growth rate (i.e. biomass gain) and the degree of metabolic turnover (Fry and Arnold 1982). Thus, this information should always be acknowledge, especially when conducting food web studies on a temporal scale.

1.6 Conclusions

Stable isotopes ratios are a useful tool to understand trophic relationships and energy flows in estuarine ecosystems. Although qualitative approaches provide useful information about the trophic relationships, quantitative approaches allow to quantify the contribution of each source to consumers' biomass. The use of quantitative approaches

have increased during the last decade however, its potential depends on a proper characterization of sources and consumers, i.e. all the possible sources have to be sampled, and sources and consumers have to be spatially and temporally aligned. Also, stable isotope analyses do not substitute the basic understanding of species' natural history. Thus, whenever possible, stable isotope studies should be enhanced with additional data on the feeding behavior of the studied species, and in conjunction with other dietary tracers, as fatty acids. Most of the quantitative approaches, are more powerful if combined with *a priori* information (e.g. reduce the number of inputs to the isotope mixing models).

The analyses of stable isotope ratios usually rely on several assumptions, such as isotopic composition of consumer's tissues equals the weighted average of the isotopic composition of its sources, and that fractionation is constant throughout the food web. Some studies showed that these assumptions are not valid. Thus, regardless the method used to interpret stable isotope ratios (qualitative and/or quantitative), assumptions have to be acknowledged when analyzing trophic interactions.

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Chapter 2

The importance of cross-ecosystem subsidies to the lower food web of an oligotrophic estuary

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Abstract

Understanding the factors that govern the exchange and consumption of different organic matter (OM) sources is of paramount importance for management and conservation of estuaries. Our objective was to determine the importance of autochthonous and allochthonous OM in supporting the production of the Minho River estuary lower food web, under different river discharge conditions. Using stable isotopes of carbon (C) and nitrogen (N) we observed that pelagic (zooplankton) and benthic (*Corbicula fluminea*) primary consumers rely on a mixture of autochthonous and allochthonous OM, which included terrestrial-derived OM. Owing to an increase in the phytoplankton fraction in the POM pool during typical low river discharge conditions, autochthonous OM was the most important contributor to consumers' biomass, declining with increasing river discharge. Based on SIAR mixing model, reliance on phytoplankton ranged from 11% to 60% during low flow discharge and from 2% to 30% (mode values) during high flow discharge. Allochthony increased with increasing river discharge, especially for consumers associated with benthic pathways. However, the highest contribution of terrestrial-derived OM (up to 70%) was observed during low river discharge conditions of an atypical hydrological year, preceded by winter high floods. We hypothesized that during high flow pulses, C loads into the estuary increased and phytoplankton biomass decreased, thereby decreasing phytoplankton availability to the food web. Although the majority of food sources were being filtered from the water column, primary consumers' stable isotope data reveal that both pelagic and benthic consumers rely on sediment OM and microphytobenthos, providing evidence for reliance on benthic C. Its ability to consume terrestrial-derived OM and to access both pelagic and benthic food sources couple the terrestrial ecosystems with the estuarine pelagic and benthic environments.

Keywords: allochthony, connectivity, river discharge, stable isotopes, pelagic, benthic.

2.1 Introduction

Estuarine food webs function within a mosaic of habitats, which include intertidal sand or mud flats, tidal creeks and channels, saltmarshes, mangroves, and habitats defined by the salinity and depth gradient (Hagy III and Kemp, 2013). Connectivity between habitats is facilitated by physical exchanges of water (with associated nutrients, plankton and other particulate organic matter [OM] sources) and movements or migrations of animals for feeding, spawning or other ontogenetic changes (Sheaves, 2009; Hagy III and Kemp, 2013).

Understanding the factors that govern the exchange and consumption of different OM sources is of paramount importance for management and conservation of estuaries, because secondary production and food web diversity depend on the quality of the basal OM sources (Rooney and McCann 2011). Three influential conceptual models have tried to explain the processes that govern the exchange and consumption of different OM sources in riverine food webs: the River Continuum Concept (RCC), the Flood Pulse Concept (FPC), and the Riverine Productivity Model (RPM).

The RCC (Vannote et al. 1980) was the first conceptual model to link the physical characteristics of stream reaches with changes in consumers' composition. According to this model, large rivers receive the majority of their OM from upstream processing of dead leaves and woody debris. Downstream transport of recalcitrant material influences trophic dynamics of large rivers because autochthonous primary production is limited by depth and turbidity. The RCC generally relied on inferences based on data collected in headwater streams; later, it was recognized that the potential to extrapolate to downstream areas was limited (Sedell et al. 1989). The FPC (Junk et al. 1989) was based on large river-floodplain systems, such as the Amazon and Mississippi Rivers, and states that the pulsing of river discharge into the floodplain influences primary and secondary production in large, lowland rivers. Thus, most animal biomass would be derived from production within the floodplain (e.g. live plant tissue and detritus) and not from downstream transport. In contrast, the RPM (Thorp and Delong 1994) hypothesized that metazoan production in large rivers is primarily fuelled by autochthonous production (phytoplankton, benthic phytoplankton, and aquatic vascular plants and mosses), and also by direct inputs from the riparian zone. The RPM states that labile autochthonous OM and moderately labile OM subsidies from the riparian zone will compensate for the typically greater abundance of recalcitrant OM from upstream leakage or floodplain inputs.

The current consensus for river-estuary complexes is that phytoplankton predominantly supports upper trophic levels, although consumers may assimilate plant (C_3) material in rivers with high sediment loads and low transparency during high-flow

pulses (Roach 2013). Nevertheless, there is growing evidence that primary consumers rely on terrestrial-derived OM when phytoplankton is not available (Howarth et al. 1996, Hoffman et al. 2008, Cole and Solomon 2012, Dias et al. in press). Hydrology is a major factor influencing OM inputs, concentration, and use by primary consumers. In rivers with strong seasonal hydrology, the inundation of the floodplain is associated with high dissolved nutrient concentrations, which promotes rapid growth of phytoplankton (Lewis et al. 2000). In contrast, consumers in rivers with stochastic flow regimes may derive more energy from terrestrial-derived OM because unpredictable and rapid flood pulses inhibit algal production and export terrestrial invertebrates into the river channel where they can be consumed by aquatic consumers (Zeug and Winemiller 2008).

Characterizing estuarine food webs is difficult because estuarine habitats incorporate OM inputs from many sources, including riparian vegetation, submerged and emergent aquatic vegetation (and associated epiphytic algae), phytoplankton, and microphytobenthos (Cloern et al. 2002, Hoffman and Bronk 2006). Carbon (C) and nitrogen (N) stable isotope analyses are powerful tools to characterize the energy flow through estuarine food webs (Pasquaud et al. 2007, Hoffman et al. 2008). For consumers, the stable isotope composition ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) of tissues is a time-integrated signal of the food sources in the ecosystem that were incorporated into an organism's structural components and energy reserves (Peterson and Fry 1987). Thus, the stable isotope ratio ($\delta^{13}C$, $\delta^{15}N$) of a consumer reflects its diet, demonstrating an average trophic fractionation (i.e. the difference between the consumer and its diet) of +0.4‰ $\delta^{13}C$ and +3.4‰ $\delta^{15}N$ per trophic level (Post 2002).

The $\delta^{13}C$ and $\delta^{15}N$ values among upland plants, marsh vegetation, and freshwater and estuarine algae differ with respect to C and N source and method of C fixation (Table 2.1). The riparian plants that utilize the C_3 pathway have a $\delta^{13}C$ value of about -28‰ whereas plants associated with the C_4 pathway (e.g. *Spartina* spp.) are more ^{13}C-enriched ($\delta^{13}C$ -13‰) owing to reduced fractionation (Smith and Epstein 1970, Fry and Sherr 1984). Freshwater and estuarine phytoplankton may differ, as well; however, as with C3 vascular plants, their ^{13}C fractionation can vary with DIC concentration, phytoplankton growth rate, and nutrient availability (Goericke et al. 1994). In freshwater systems where the $\delta^{13}C$ value of DIC is less than atmospheric CO_2 (< -10‰), phytoplankton may also be distinguished from riparian vegetation (Hoffman and Bronk 2006). In general, microphytobenthos (MPB) are more ^{13}C-enriched than phytoplankton due to the existence of a diffusive boundary layer at the sediment-water interface that reduces isotopic fractionation (France 1995). The N stable isotope composition can also help to separate ^{15}N-depleted terrestrial OM ($\delta^{15}N$ -4‰ to 4‰) from ^{15}N-enriched aquatic OM sources ($\delta^{15}N$ 6‰ to 10‰) (Peterson and Fry 1987, Cloern et al. 2002). However, OM $\delta^{15}N$ values can

be altered by changes in the isotope value of N substrates due to preferential uptake of isotopically light N (Cifuentes et al. 1988), nitrification, denitrification (Mariotti et al. 1981) or by microbially mediated degradation (Miyake and Wada 1971; Altabet 1988). Moreover, the form and amount of anthropogenic nitrogen addition can affect the $\delta^{15}N$ baseline value (Kendall et al., 2007), so $\delta^{15}N$ source values should be interpreted cautiously.

Table 2.1 Stable isotope ratios of organic matter sources commonly found in estuaries.

Organic matter sources	**$\delta^{13}C$ (‰)**	**$\delta^{15}N$ (‰)**	**References**
Woody vegetation (C_3)	-30 to -20	-4 to 4	Peterson and Fry (1987), Fry (1991), Cloern et al. (2002)
Emergent vascular plants (C_3)	-30 to -28	6 to 10	Cloern et al. (2002), Hoffman et al. (2006)
Submerged aquatic vegetation (C_3)	-30 to -16	8 to 16	Cloern et al. (2002)
Humic-rich soils	-26	0 to 4	Fry (1991), Hoffman et al. (2006)
Saltmarsh vegetation (C_4)	-16 to -10	2 to 14	Fry and Sherr (1984), Cloern et al. (2002)
Freshwater phytoplankton	-32 to -26	0 to 10	Fry (1991), Cloern et al. (2002), Hoffman et al. (2006)
Brackish phytoplankton	-26 to -18	5 to 10	Fry and Sherr (1984), Cloern et al. (2002), Hoffman et al. (2006)
Benthic diatoms	-28 to -20	2 to 10	Cloern et al. (2002)

The objective of this research was to characterize the contribution of the different OM sources fuelling the lower food web of an oligotrophic estuary under naturally varying river discharge. Specifically, we aimed to test if the contribution of autochthonous (local photosynthetic autotrophic production) and allochthonous (riparian and upland vegetation) OM sources to primary consumers varies with respect to river discharge. We hypothesized that allochthonous OM would contribute more to consumers collected at the end of the high river discharge period (late winter) than those collected at the end the low river discharge period (late summer). Also, we wanted to determine if there were persistent differences along the salinity mixing gradient. We hypothesized that allochthonous OM would contribute more to consumers in the freshwater portion of the estuary than those in the brackish portion. To test our hypothesis, we identified and quantified the contribution of different OM sources supporting the production of primary consumers, zooplankton and Asian clam *Corbicula fluminea* in the Minho River estuary

(NW-Iberian Peninsula, Europe), using C and N stable isotope analyses. These consumers were chosen because they represent, respectively, pelagic (zooplankton) and benthic (*C*. *fluminea*) food web pathways, and because they are sessile (*C*. *fluminea*) or have limited longitudinal movements (zooplankton), so they should reflect the conditions of the habitat in which they were collected. To accomplish these objectives, the study was conducted over the entire salinity gradient, during two consecutive summer low discharge periods and a contrasting winter high discharge period.

2.2 Methods

2.2.1 Study area

The Minho River is located in the NW-Iberian Peninsula (SW Europe; Fig. 2.1). The annual average discharge is 300 m^3 s^{-1} (Ferreira et al. 2003). Its watershed is 17,080 km^2, of which 95% is located in Spain and 5% in Portugal. The river is 343 km long; 76 km serves as the northwestern Portuguese-Spanish border (Antunes et al. 2011). The limit of tidal influence is about 40 km inland, and the uppermost 30 km are a tidal freshwater wetland (TFW; Sousa et al. 2008). Its estuary has an area of 23 km^2, of which only 9% is intertidal. The estuary is mesotidal with tides ranging between 0.7 m and 3.7 m (Alves 1996). The mean depth of the estuary is 2.6 m and the maximum depth is ca. 26 m (Antunes et al. 2011). Due to its ecological importance, the Minho River estuary and the international section of the River Minho were designated as a Natura 2000 site (EIONET 2012) and as an Important Bird Area (BirdLife International 2012).

Fig. 2.1 Location of the sampling stations along the salinity gradient of the Minho River estuary.

2.2.2 Field sampling

During each cruise, we sampled 21 stations located every kilometer along the main river channel during full-moon spring tide (Fig. 2.1). Sampling was conducted at relatively small spatial scale (1 km intervals) to guarantee a proper characterization of the estuarine mixing zone. To characterize conditions during the low discharge period, we sampled in September (2010) and August (2011) near the end of the annual base flow period (typically, from July through October; SNIRH 2012; Fig. 2.2), for a number of reasons. First, salt water intrusion is maximized, which favors connectivity between the estuarine and marine environments. Second, residence time is elongated during base flow, which potentially allows phytoplankton biomass to accumulate (Hoffman and Bronk 2006). To characterize conditions during the high discharge period, we sampled in March (2011), which typically represents the end of the wet season (Fig. 2.2). During high discharge, residence time decreases, flushing algae downriver and suppressesing phytoplankton production (Sin et al. 1999). Also, the high river flow delivers riverine and terrestrial OM to the estuary (Hoffman and Bronk 2006).

The OM sources sampled were selected based on the feeding modes of the primary consumers selected, zooplankton and *C. fluminea* (e.g. Boltovskoy et al. 1995, Cole et al. 2011): phytoplankton (freshwater, estuarine and marine), macroalgae, microphytobenthos (MPB), sediment organic matter (SOM), particulate organic matter (POM), and vascular plants (terrestrial and aquatic).

At each station, surface (50-100 cm below the surface) and bottom water samples (0.5 m off the bottom) were collected using a 2-L Ruttner bottle. From these samples, we measured the concentration of chlorophyll *a* (Chl *a*: μg L^{-1}), concentration and isotopic composition of POM ([POM]: mg L^{-1}, particulate organic carbon (POC) $\delta^{13}C$, particulate nitrogen (PN) $\delta^{15}N$, molar C/N), and isotopic composition of total dissolved inorganic carbon (ΣCO_2: $\delta^{13}C_{DIC}$). Salinity was measured with an YSI model 6820 QS probe and reported using the Practical Salinity Scale.

The POM and Chl *a* water samples (POM: 1L, Chl *a*: 0.5L) were pre-filtered with a 150 μm sieve and filtered onto a pre-combusted (500ºC for 2 h) Whatman GF/F and Whatman GF/C filters, respectively , and kept frozen (-20ºC) until analysis.

The DIC samples (taken from replicate water samples) were injected (6 mL) into a 10 mL exetainer (Labco Limited), containing 0.5 ml of phosphoric acid 85% (v/v), with a sterile syringe filter coupled with a 0.2 μm cellulose acetate membrane acrodisc. Samples were kept cool during field sampling and then stored at 4 ºC until $\delta^{13}C_{DIC}$ analysis.

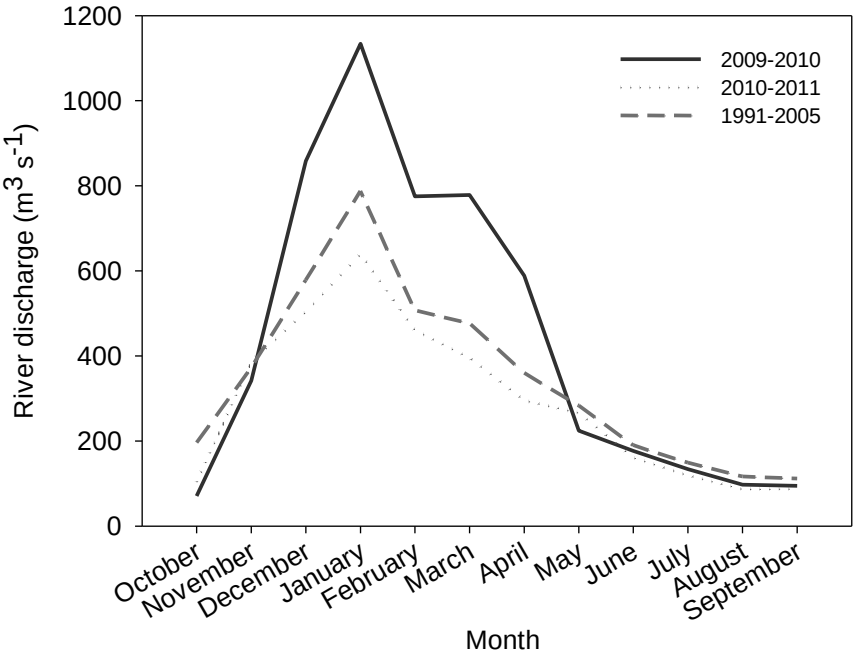


Fig. 2.2 Average monthly river discharge of Minho estuary measured between 1991 and 2005 (dashed line; SNIRH, 2012) and October 2009 and September 2011 (solide line: 2009-2010, dotted line: 2010-2011; Confederación Hidrográfica del Miño-Sil, 2012).

The MPB samples were collected at four stations situated across the salinity gradient (stations 1, 9, 16, 21). At each station, one PVC pipe was layed down and fixed in the sediment and left in the estuary for two weeks. At the end of the period, colonizing algae were scraped from each pipe into a clean vial, stored on ice, and returned to the laboratory where we applied the same procedure used for POM samples. Macroalgae, were collected in stations 1 and 9 (higher salinity influence) and vascular plants in stations, 9, 16 and 21 (areas with vegetation). The SOM samples were collected using a van Veen grab and were kept frozen (-20 ºC) until analysis.

For consumer data, at each station, zooplankton were collected using a plankton net (200 μm mesh size) towed near the surface at a constant speed for 3 minutes. Samples were immediately preserved in 70% ethanol. Ten specimens of *C*. *fluminea* were collected with a van Veen grab, and randomly selected, at each sampling station and sampling period (420 specimens in total), and kept frozen (-20 ºC) until analyses.

2.2.3 Laboratory analyses

Filters collected for Chl *a* analysis were extracted in 90% acetone and analyzed on a Spectronic 20 Genesys spectrophotometer. Chl *a* concentration was calculated following the equations proposed by Lorenzen (1967).

Filters for POM and MPB analysis were fumigated with concentrated HCl to remove inorganic carbonates, rinsed with deionized water, placed in a sterile Petri dish, and dried at 60 ºC for 24 h (Lorrain et al. 2003). Sediment samples were rinsed with 10% HCl (also to remove carbonates), rinsed with deionized water, and dried at 60 ºC for 48h. Both acidification methods are expected to produce only slight changes in sample $\delta^{15}N$ values (ca. 0.4‰; Lorrain et al. 2003, Carabel et al. 2006). Macroalgae and vascular plants were cleaned with deionized water to remove epiphytes, dried (60 ºC), and ground to a fine powder with a mixer mill for stable isotope analysis.

Zooplankton individuals were sorted, identified, grouped by the lowest taxonomic level feasible, and dried (60 ºC). For *C. fluminea,* the shell length of each specimen was measured (± 0.01 mm). Then, the foot was excised, dried (60 ºC), and ground to a fine powder with a mortar and pestle for stable isotope analysis.

Stable isotope ratios were measured using a Costec 4010 EA and Thermo Delta Plus XP isotope ratio mass spectrometer (IRMS) (United States Environmental Protection Agency, Mid-Continent Ecology Divison, Duluth, Minnesota) and Thermo Scientific Delta V Advantage IRMS via a Conflo IV interface (Centro Interdisciplinar de Investigação Marinha e Ambiental, Universidade do Porto). Stable isotope ratios are reported in δ notation:

$$\delta X{:}\delta X = (R_{sample}/R_{standard} - 1) \times 10^3 \qquad (1)$$

where X is the C or N stable isotope, *R* is the ratio of heavy: light stable isotopes, and Pee Dee Belemnite and air are standards for $\delta^{13}C$ and $\delta^{15}N$, respectively. The analytical error, the mean standard deviation of replicate reference material, was ±0.1‰ for $\delta^{13}C$ and $\delta^{15}N$. DIC $\delta^{13}C$ was measured using a GasBench II system interfaced to a Delta V Plus IRMS (Davis Stable Isotope Facility, University of California). The analytical error was ±0.2‰ $\delta^{13}C$.

2.2.4 Data analyses

In order to assess the influence of environmental variables on the $\delta^{13}C$ and $\delta^{15}N$ values of the primary consumers collected, a distance-based linear modeling (DistLM) was performed (Anderson et al. 2008). The selection criterion adopted was the AIC (Akaike Information Criterion), and a step-wise selection procedure was used, which optimizes selection of variables explaining most variation in the biotic data.

Multivariate analyses were performed to reveal natural groupings in the data according to sampling period and portion of the estuary. A cluster analyses was performed using Euclidean similarity distance based on $\delta^{13}C$ and $\delta^{15}N$ stable isotope values of all the consumers collected. A similarity profile routine (SIMPROF) was used to test for the presence of sample groups (Clarke et al. 2008). DistLM and cluster analyses were performed using PRIMER v.6.1.11 (Clarke and Gorley 2006) with PERMANOVA+1.0.1 add-on package (Anderson et al. 2008).

One-way ANOVA (factors “sampling period” or “portion of the estuary”) were performed to determine bulk zooplankton and *C*. *fluminea* changes in stable isotope values through time and space. When data did not meet the assumptions, the non-parametric Kruskal-Wallis was used. These analyses were performed using STATISTICA 7, Statsoft.

To quantify OM source contributions to the most frequently sampled zooplankton groups (Calanoids and Caridea larvae) and *C. fluminea* tissue, we used a dual-stable isotope mixing model. The mixing model estimates a proportional contribution for each food source, assuming all sources sum to 1. However, the model equations are indeterminate when there are more than *n+1* sources relative to stable isotopes (i.e. more than three sources for two stable isotopes). Because there were more than three potential food sources in the estuary, we used a stable isotope mixing model that uses Bayesian inference to solve the indeterminate equations and produce a probability distribution that represents the likelihood a given source contributes to the consumer (Parnell et al. 2010). The model, Stable Isotope Analysis in R (SIAR), allows each of the sources and the trophic enrichment factor (TEF; or trophic fractionation) to be assigned as a normal distribution, rather than a single datum (Parnell et al. 2010). We used the SIAR package (Stable Isotope Analysis in R; Parnell et al. 2010), which is part of the open source statistical language R (R Development Core Team 2007). SIAR will produce a range of feasible mixing problem solutions to which are assigned credibility intervals (CI), analogous to the confidence intervals used in frequentist statistics (in this study, 95% CI; Parnell et al. 2010). SIAR also includes a residual error term. For modelling purposes, the estuary was divided into three portions based on salinity, as a result fro the DistLM routine: tidal freshwater (TFW), brackish and marine portions.

For the mixing model, we estimated the average (± SD) $\delta^{13}C$ and $\delta^{15}N$ values for the various OM sources. At each station, the phytoplankton $\delta^{13}C$ ($\delta^{13}C_{phytoplankton}$) value was estimated from the DIC $\delta^{13}C$ ($\delta^{13}C_{DIC}$) value, assuming an uptake fractionation of -21‰ (i.e. $\delta^{13}C_{phytoplankton} = \delta^{13}C_{DIC}$ - 21‰; Peterson and Fry 1987). For each portion of the estuary, the average $\delta^{13}C_{phytoplankton}$ value used in the SIAR mixing model was the mean of station-specific average surface and bottom $\delta^{13}C_{phytoplankton}$ values (based on station

replicates) for those stations within each portion. We obtained $\delta^{15}N_{phytoplankton}$ values for the SIAR mixing model from POM sampled during August 2011 at all stations where the POM samples were comprised of nearly 100% phytoplankton based on phytoplankton C:Chl *a* and Chl *a* and POC concentrations (Canuel et al 1995, Marty and Planas 2008). For the model, the value used in the TFW and brackish portions of the estuary was the mean station-specific average $\delta^{15}N_{phytoplankton}$ values for those stations within each portion. For the marine stations, we used the values described by Bode et al. (2007) for the Iberian Peninsula Coast (6 ± 1.5 ‰).

The average MPB, POM, and SOM $\delta^{13}C$ and $\delta^{15}N$ values were calculated from all the values for those stations within each portion.

The zooplankton and *C. fluminea* $\delta^{13}C$ values were corrected for lipid content because lipids are depleted in ^{13}C compared to protein and carbohydrates (DeNiro and Epstein 1977). Variability in lipid content can bias bulk tissue $\delta^{13}C$ values and thereby cause dietary or habitat shifts to be incorrectly interpreted. We corrected zooplankton tissue data for lipid content using the mass balance correction model proposed for zooplankton by Smyntek et al. (2007; Eq. 5), and for *C. fluminea* muscle tissue data the mass balance correction for fish muscle tissue proposed by Hoffman and Sutton (2010; Eq. 6), which uses estimates of $C:N_{protein}$ and $\Delta\delta^{13}C_{lipid}$ that are similar to those from the muscle tissue found for other fish (e.g. Sweeting et al. 2006) and taxonomic groups (e.g. shrimp and zooplankton; Fry et al. 2003, Smyntek et al. 2007). Zooplankton were also corrected for ethanol preservation (+0.4 ‰ $\delta^{13}C$, +0.6 ‰ $\delta^{15}N$; Feuchtmayer and Grey 2003). For the SIAR mixing model, we adjusted the $\delta^{13}C$ and $\delta^{15}N$ values for one trophic level using the TEF estimates from Post (2002; +0.4 ± 1.3 ‰ $\delta^{13}C$, +3.4 ± 1.0 ‰ $\delta^{15}N$).

2.3 Results

2.3.1 Environmental data

The average monthly river discharge during the sampling period was similar to the average monthly estimates for the historical data set (1991-2005) (Fig. 2.2). Thus, September 2010 and August 2011 average river discharge values were close to the typical low river discharge conditions of Minho River estuary, and March 2011 average river discharge values were typical for the end of the wet season (Fig. 2.2).

In September 2010, saltwater intrusion was detected up to 12 km from the river mouth (stations 1-13 were brackish, stations 14-21 were within the tidal freshwater (TFW) portion of the estuary; Fig. 2.3 A). In March 2011, owing to high river discharge, stations

1-6 were brackish and 7-21 were freshwater (Fig. 2.3B). In August 2011, saltwater intrusion resulted in measurable salinity up to 16 km from the mouth. Stations 1-6 were marine, 7-16 were brackish and 17-21 were within the TFW (Fig. 2.3C).

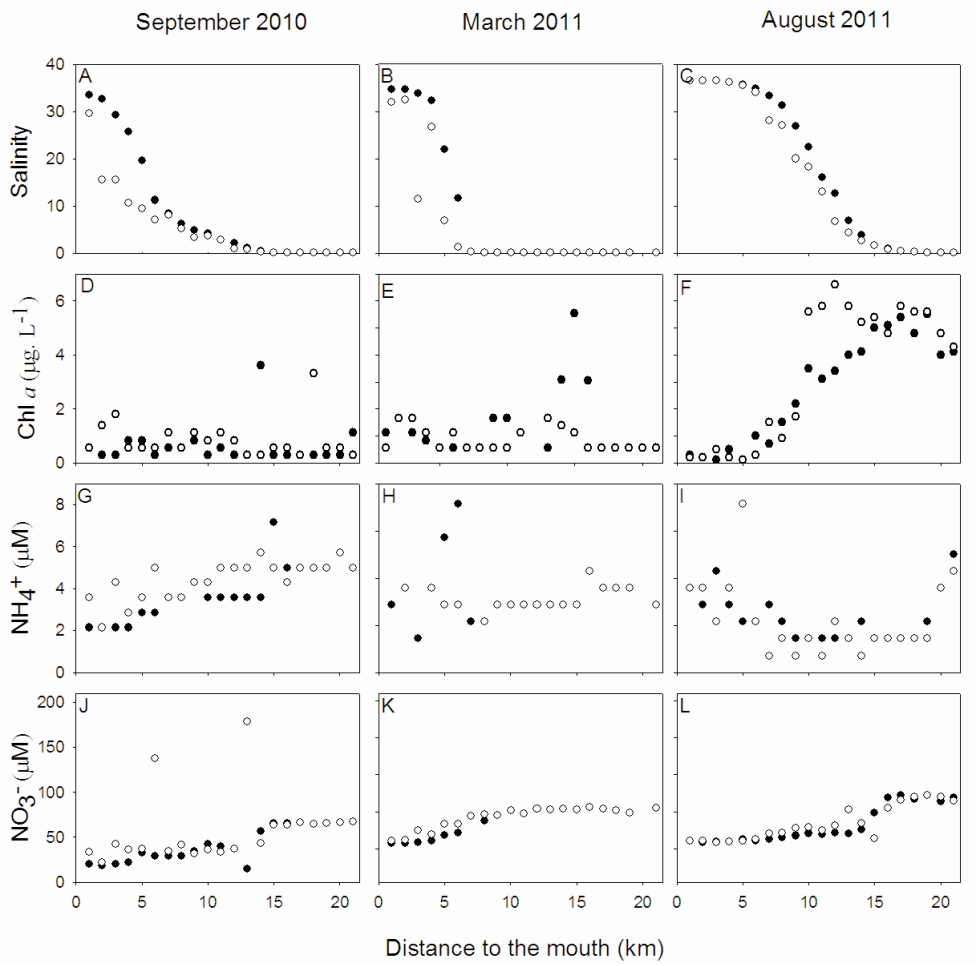


Fig. 2.3 Bottom (closed circles) and surface (open circles) salinity (A-C), chlorophyll *a* (Chl *a*) concentration (µg L^{-1}; D-F), ammonia (NH_4^+; G-I) and nitrates (NO_3^-; J-L) concentrations (µM) determined for the estuarine salinity mixing for the river Minho estuary in September 2010 (low discharge), March 2011 (high discharge), and August 2011 (low discharge).

The quality and quantity of POM varied between sampling periods and among portions of the estuary. The C/N_{POM} above 10 in September 2010 indicates a substantial contribution of terrestrial-derived OM to the POM pool in the TFW (Hedges et al. 1986, 1997; Table 2.2). In the brackish estuarine area, C/N_{POM} (7-9) varied between the C:N of terrestrial-derived OM (> 10) and of marine phytoplankton (~7; Hedges et al. 1986, 1997), indicating that brackish POM was a mixture of riverine and marine POM (Table 2.2). In March 2011, the $C:N_{POM}$ varied between 6 and 12 indicating that the POM pool was a mixture of phytoplankton and terrestrial-derived OM (Table 2.1). In August 2011, the $C:N_{POM}$ averaged above 7, the estimates of the phytoplankton proportion in the POM pool (> 50%) and high chlorophyll *a* (Chl *a*) concentrations indicate that phytoplankton was a major contributor to the POM pool (Table 2.2, Fig. 2.3 D-F).

Table 2.2 Characterization of the POM pool in the Minho River estuary, along the salinity mixing gradient in September 2010, March and August 2011.

Month	Location	$C:N_{POM}$ average (SD)	POC (µM) average (SD)	Phytoplankton's proportion % POC (SD)
September 2010	TFW	10.2 (0.9)	29.0 (11.9)	17.2 (18.8)
	Brackish	8.3 (1.1)	25.8 (13.1)	18.7 (14.0)
March 2011	TFW	8.4(1.6)	17.9 (6.3)	30.0 (21.4)
	Brackish	9.2 (1.9)	11.9 (4.9)	36.8 (26.2)
August 2011	TFW	7.0 (0.5)	27.0 (4.8)	74.4 (19.0)
	Brackish	7.0 (1.1)	27.5 (7.8)	56.6 (25.6)
	Marine	8.7 (2.6)	16.3 (4.6)	7.7 (5.1)

The POC concentrations in September 2010 and August 2011 were almost the double that measured in March 2011, and concentration values were similar along the estuarine salinity gradient (Table 2.2).

The average NH_4^+ concentration (µM; bottom and surface samples combined) was the highest in March 2011 (5.7 ± 6.6 µM) and decreased during in the low flow months (September 2010: 4.0 ± 1.2 µM; August 2011: 2.3 ± 1.3 µM; Figs. 2.3 G-I). The average NO_3^- concentration (µM; bottom and surface samples combined) were higher than NH_4^+ for all the sampling periods with values increasing towards TFW (Figs. 2.3, J-L).

2.3.2 Food web characterization

The results from the DistLM indicate that the environmental variables that accounted for the most variability in the $\delta^{13}C$ and $\delta^{15}N$ values of both zooplankton and *C. fluminea* were salinity and Chl *a* concentration (50% of the total variance; Table 2.3). The $\delta^{13}C$ and $\delta^{15}N$ values of zooplankton and *C. fluminea* did not demonstrate a significant association with river discharge (Table 2.3).

However, significant differences were observed between sampling periods for both zooplankton and *C. fluminea*. Bulk zooplankton samples collected in the TFW were significantly depleted in ^{13}C in March 2011 ($H_{(2,25)}$= 9.70, p= 0.008) and ^{13}C-enriched in the brackish stations ($H_{(2,23)}$= 6.79, p= 0.03) compared to the other sampling periods. The $\delta^{15}N$ values were significantly higher during August 2011 (TFW: $H_{(2,25)}$= 18.45, p= 0.0001; brackish: $H_{(2,23)}$= 8.87, p= 0.012). The *C. fluminea* $\delta^{13}C$ values were not significantly different (p> 0.05). However, those collected in August had the highest $\delta^{15}N$ values (TFW: $H_{(2,25)}$= 14.15, p= 0.0008; brackish: t= -2.54, df= 13, p= 0.025).

Table 2.3 DistLM model based on the $\delta^{13}C$ and $\delta^{15}N$ values of zooplankton and *Corbicula fluminea* and fitted abiotic data.

Marginal tests				
Variable	**SS (trace)**	**pseudo-F**	**p**	**proportion (%)**
Salinity	58.26	41.76	< 0.05	31.0
River discharge	5.17	2.63	0.075	2.75
Chl *a*	39.41	24.66	< 0.05	21.0

Sequential tests					
Variable	**AIC**	**SS (trace)**	**pseudo-F**	**p**	**proportion (%)**
Salinity	33.61	58.26	41.76	< 0.05	31.0
Chl *a*	5.61	35.12	34.15	< 0.05	19.0

The cluster analysis results corroborate the finding that salinity was the main factor structuring consumers' $\delta^{13}C$ and $\delta^{15}N$ values (Fig. 2.4), owing to either differences in the OM sources available or differences in the stable isotope values of the OM sources, or both. The first branch separates consumers enriched in ^{13}C and depleted in ^{15}N (A), from those showing the opposite trend (B) (Figs. 2.4, 2.5). Group A was essentially comprised of zooplankton collected in marine and brackish stations during August 2011 and of zooplankton collected in the brackish stations during March 2011 (Fig. 2.4). Group B included all the organism collected in the TFW (benthic and pelagic), and some ^{15}N-enriched consumers, such as zooplankton predators (e.g. fish larvae collected in the marine and brackish stations– FI; Fig. 2.4).

Group A clusters into group AA, which includes Caridea larvae collected in September 2010, and group AB, which includes pelagic zooplankton. Caridea larvae from group AA had the lowest $\delta^{15}N$ values, suggesting a great contribution of the ^{15}N depleted SOM to their biomass (Fig. 2.5). Group AB cluster into group ABA and ABB, essentially separating the zooplankton collected in August from those collected in March, which were more ^{13}C-depleted and ^{15}N-enriched, owing to the existence of differences in the diet (Figs. 2.6, 2.7). For instance, assuming typical trophic fractionation (+0.4 $\delta^{13}C$, +3.4 $\delta^{15}N$), it is likely that zooplankton collected in the marine stations in August 2011 were using a ^{15}N- depleted and ^{13}C- enriched source such as MPOM or MPB; in the brackish stations in March 2011 they were using a more ^{15}N- enriched source such as macroalgae and/or a more ^{13}C- depleted source such as brackish phytoplankton (Fig. 2.6).

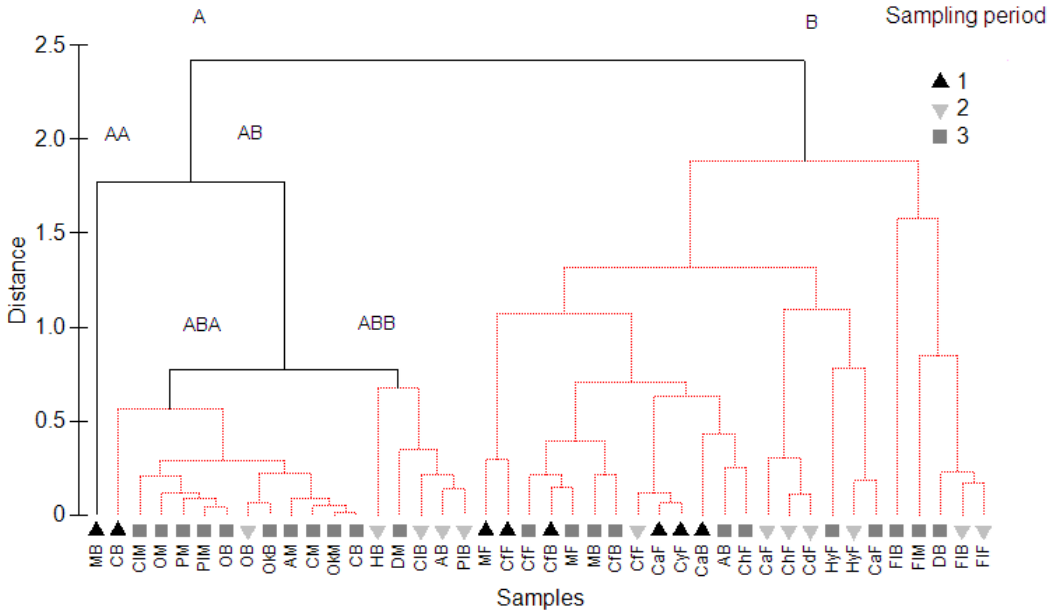


Fig. 2.4 Dendograms from clustering analyses of primary consumers $\delta^{13}C$ and $\delta^{15}N$ values collected in September 2010 (black triangle), March 2011 (inverted grey triangle) and August 2011 (grey square) in the marine (M), brackish (B) and TFW (F) portions of the Minho River estuary. The first letters (one or two) represent the species codes (listed in Appendix A) and the last letter represents the portion of the estuary where they were collected (M, B or F). Grey lines indicate groups of samples not separated (at $\alpha < 0.05$) by SIMPROF.

The SIAR mixing model indicates that OM source nutritional contribution to the primary consumers varied by sampling period, portion of the estuary, and taxonomic group (Figs. 2.8, 2.9 and 2.10). The main difference among sampling periods was the substantial phytoplankton contribution to all the consumers modelled during August 2011 (Figs. 2.8, 2.9 and 2.10).

In September 2010 and March 2011, calanoid copepods (calanoid copepods not identified in TFW and *Acartia* spp. in the brackish and marine portions of the estuary) and Caridea larvae were generally supported by terrestrial-derived OM (September 2010), MPB (September 2010) and SOM (September 2010 and March 2011). The proportion of phytoplankton in the tissues was always lower than 40% (Figs. 2.8 and 2.9). Calanoid copepods collected in the TFW portion of the estuary in March 2011 were not modelled because they had $\delta^{13}C$ values outside the range of $\delta^{13}C$ values from the OM sources sampled (Fig. 2.6). Also, cladocerans and Chironomidae larvae had low $\delta^{13}C$ values, suggesting that they were feeding on a ^{13}C-depleted source that may have not been sampled (Fig. 2.6). Differences were observed between portions of the estuary, with terrestrial-derived OM contribution increasing in the TFW stations (Figs. 2.8, 2.9).

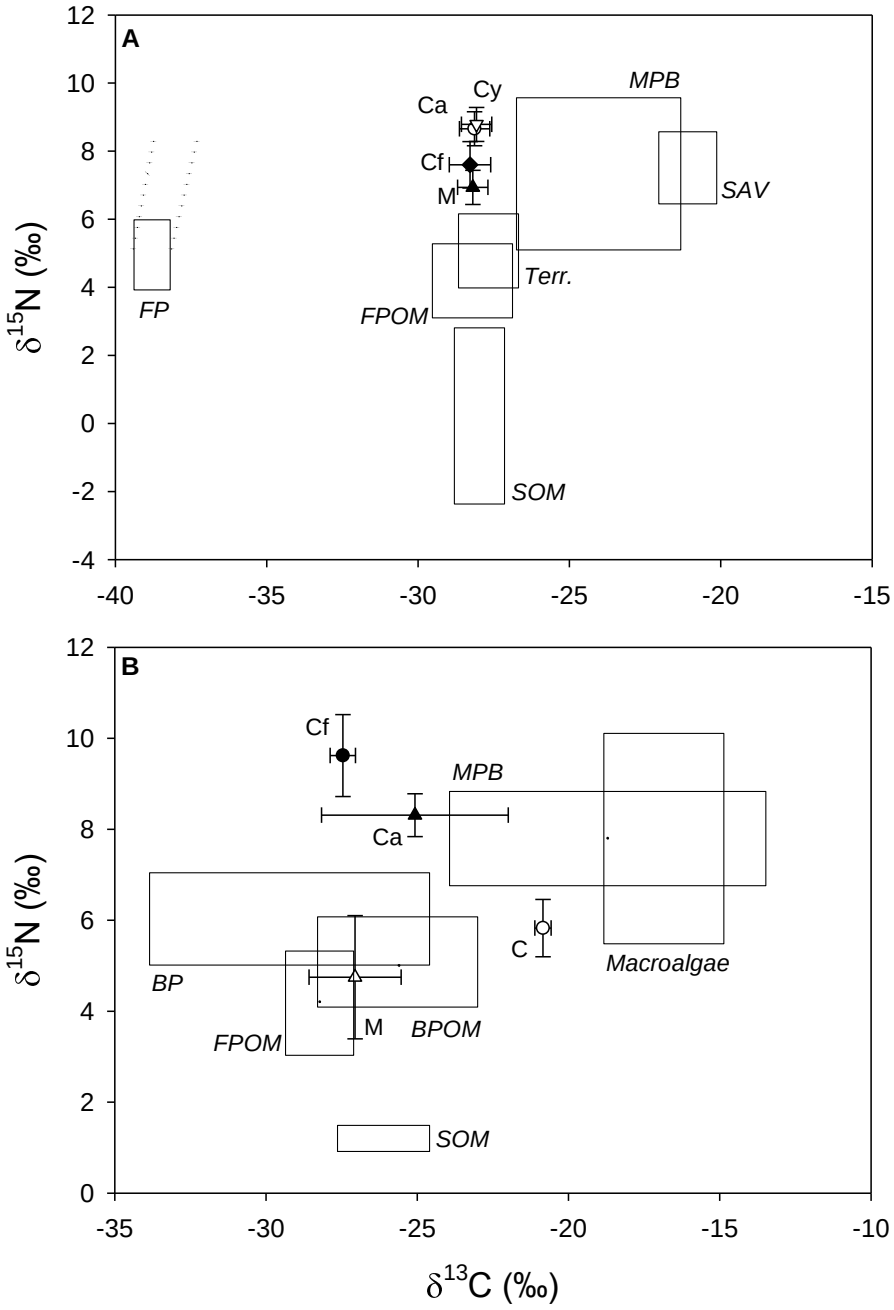


Fig. 2.5 Average stable isotope ratios (±SD) of common Minho River estuary zooplankton species (codes in Appendix A) and *Corbicula fluminea* collected in the tidal freshwater (A), brackish (B)portions of the estuary in September 2010. Organic matter (OM) sources include freshwater (FP) and brackish (BP) phytoplankton, freshwater (FPOM) and brackish (BPOM) particulate OM, microphytobenthos (MPB), submerged aquatic vegetation (SAV), terrestrial plants (Terr), macroalgae, and sediment OM (SOM). The dotted lines indicate the expected stable isotope ratio of consumers utilizing phytoplankton, assuming typical trophic fractionation (+0.4‰ $\delta^{13}C$ and +3.4‰ $\delta^{15}N$ per trophic level).

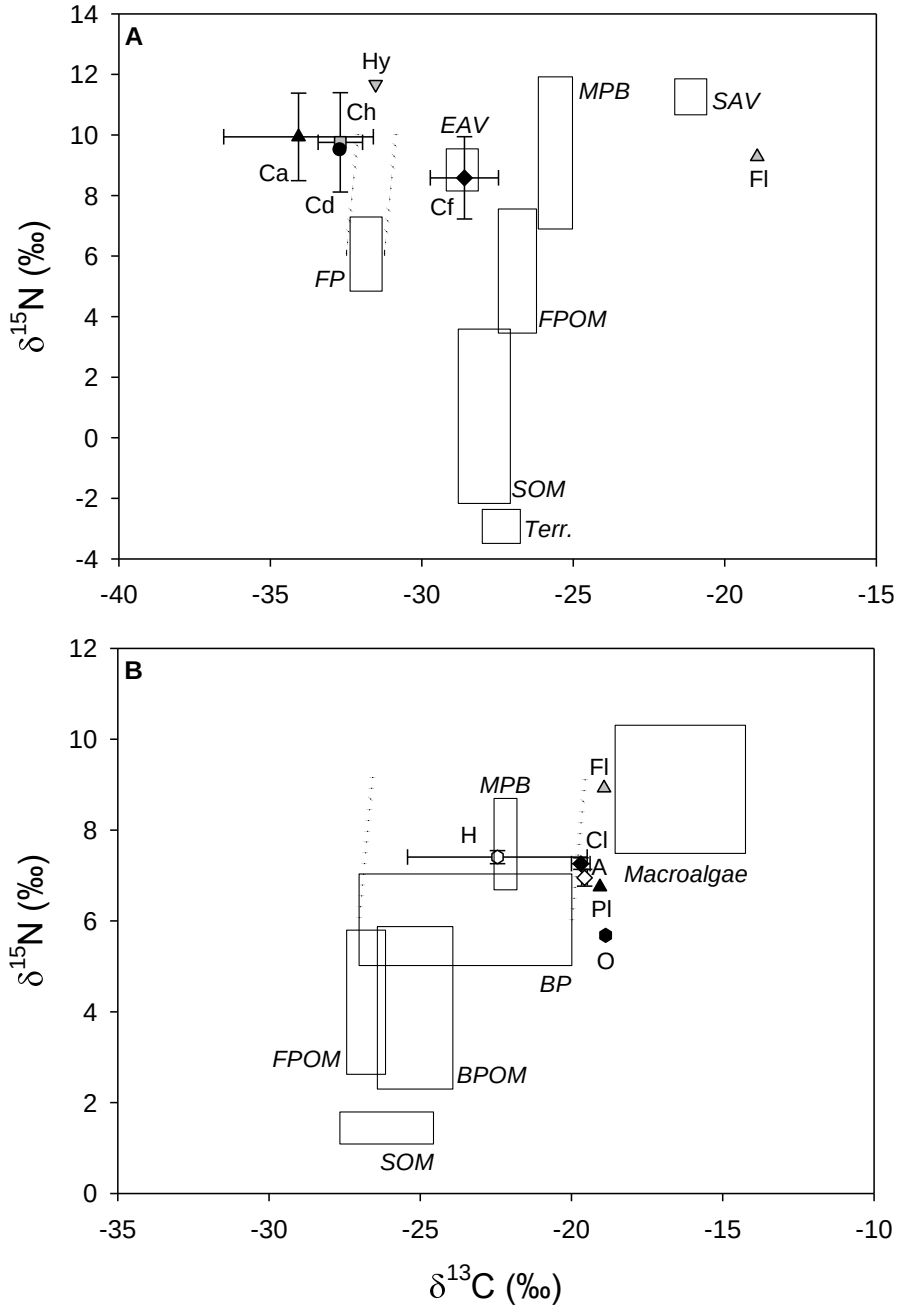


Fig. 2.6 Average stable isotope ratios (±SD) of common Minho River estuary zooplankton species (codes in Appendix A) and *Corbicula fluminea* collected in the tidal freshwater (A), brackish (B) portions of the estuary in March 2011. Organic matter (OM) sources include freshwater (FP) and brackish (BP) phytoplankton, freshwater (FPOM) and brackish (BPOM) particulate OM, microphytobenthos (MPB), submerged (SAV) and emergent (EAV) aquatic vegetation (SAV), terrestrial plants (Terr), macroalgae, and sediment OM (SOM). The dotted lines indicate the expected stable isotope ratio of consumers utilizing phytoplankton, assuming typical trophic fractionation (+0.4‰ $\delta^{13}C$ and +3.4‰ $\delta^{15}N$ per trophic level).

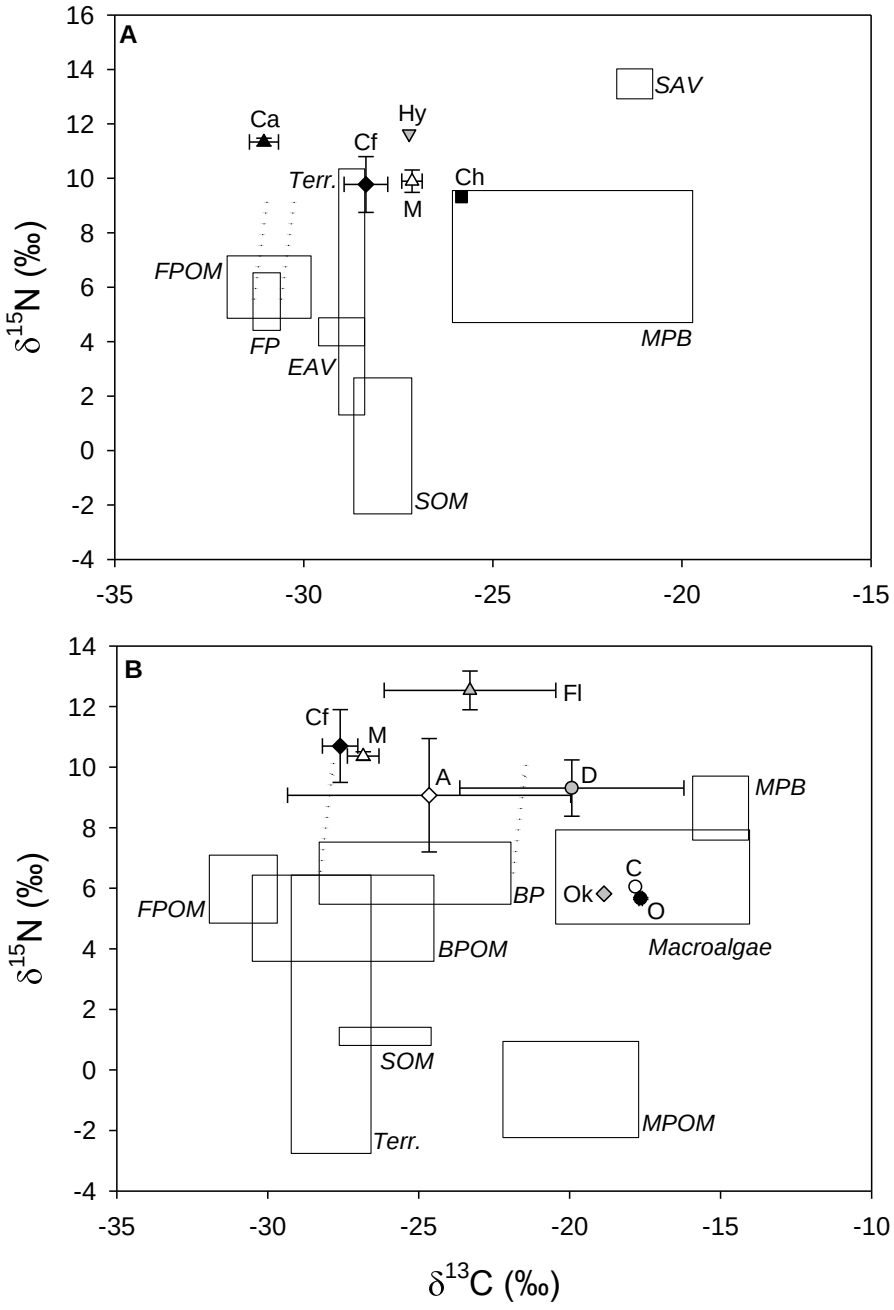


Fig. 2.7 Average stable isotope ratios (±SD) of common Minho River estuary zooplankton species (codes in Appendix A) and *Corbicula fluminea* collected in the tidal freshwater (A), brackish (B) and marine (C) portions of the estuary in August 2011. Organic matter (OM) sources include freshwater (FP), brackish (BP) and marine (MP) phytoplankton, freshwater (FPOM), brackish (BPOM) and marine (MPOM) particulate OM, microphytobenthos (MPB), submerged (SAV) and emergent (EAV) aquatic vegetation (SAV), terrestrial plants (Terr), macroalgae, and sediment OM (SOM). The dotted lines indicate the expected stable isotope ratio of consumers utilizing phytoplankton, assuming typical trophic fractionation (+0.4‰ $\delta^{13}C$ and +3.4‰ $\delta^{15}N$ per trophic level).

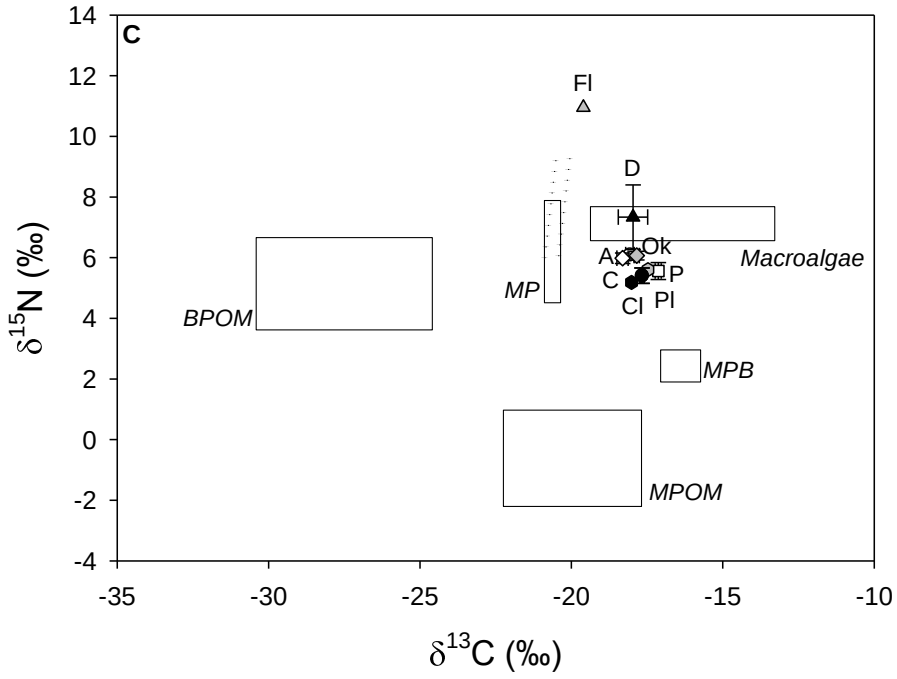


Fig. 2.7 (continuation) Average stable isotope ratios (±SD) of common Minho River estuary zooplankton species (codes in Appendix A) and *Corbicula fluminea* collected in the tidal freshwater (A), brackish (B) and marine (C) portions of the estuary in August 2011. Organic matter (OM) sources include freshwater (FP), brackish (BP) and marine (MP) phytoplankton, freshwater (FPOM), brackish (BPOM) and marine (MPOM) particulate OM, microphytobenthos (MPB), submerged (SAV) and emergent (EAV) aquatic vegetation (SAV), terrestrial plants (Terr), macroalgae, and sediment OM (SOM). The dotted lines indicate the expected stable isotope ratio of consumers utilizing phytoplankton, assuming typical trophic fractionation (+0.4‰ $\delta^{13}C$ and +3.4‰ $\delta^{15}N$ per trophic level).

The contribution of terrestrial-derived OM and OM in the sediment (SOM and MPB) to *C*. *fluminea* production was higher in September 2010 and March 2011 than August 2011 ($F_{phytoplankton} \leq 50\%$; Fig. 2.10). As with zooplankton, the proportion of phytoplankton in the tissue muscle was higher in August 2011 ($F_{phytoplankton}$: 50-100%; Fig. 2.10). Differences were also observed between portions of the estuary. In the TFW stations, the terrestrial-derived OM (FPOM) was a relevant alternative to phytoplankton (especially in September 2010 and March 2011), whereas in the brackish stations, (brackish and freshwater), phytoplankton contributed the most to *C*. *fluminea* biomass (September 2010: 30-55%; August 2011: 67-100%; Fig. 2.10).

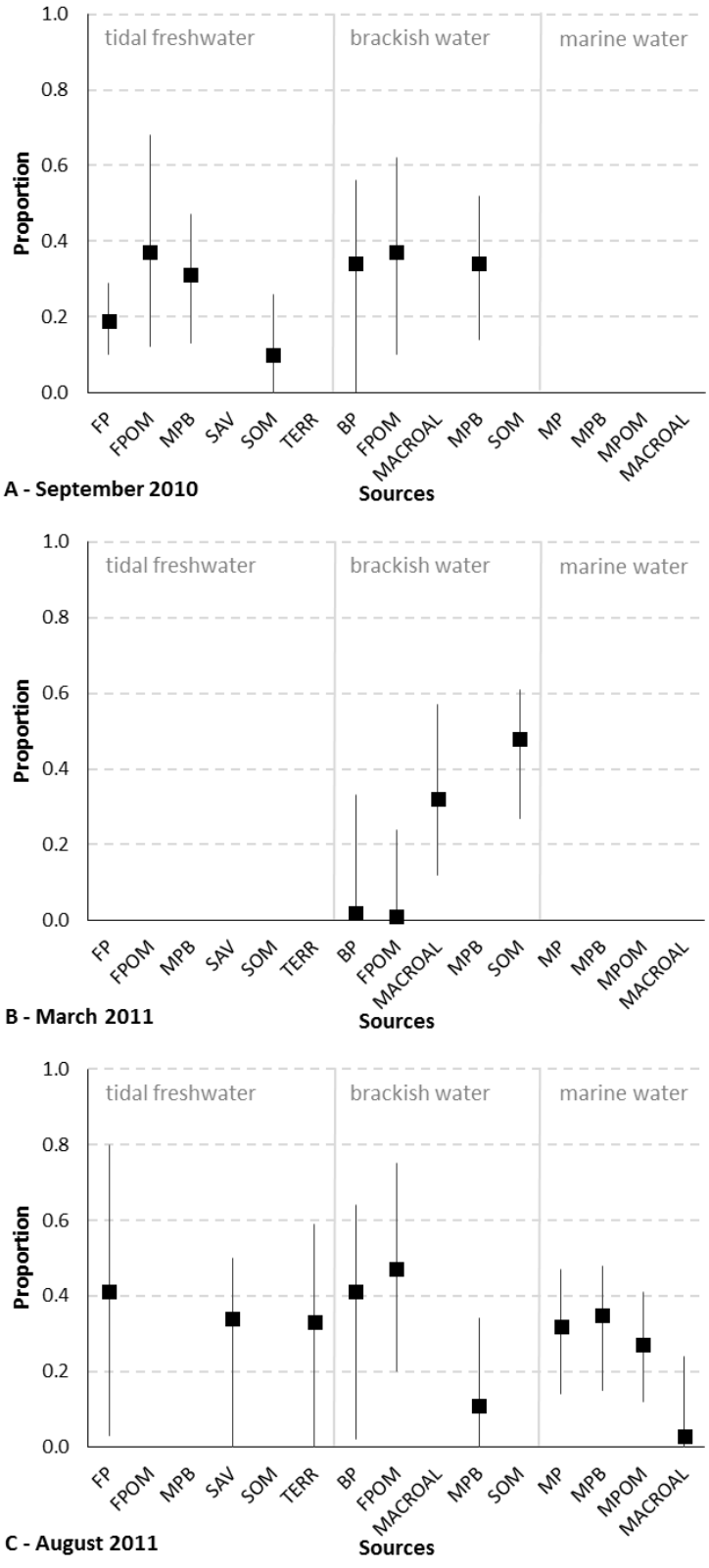
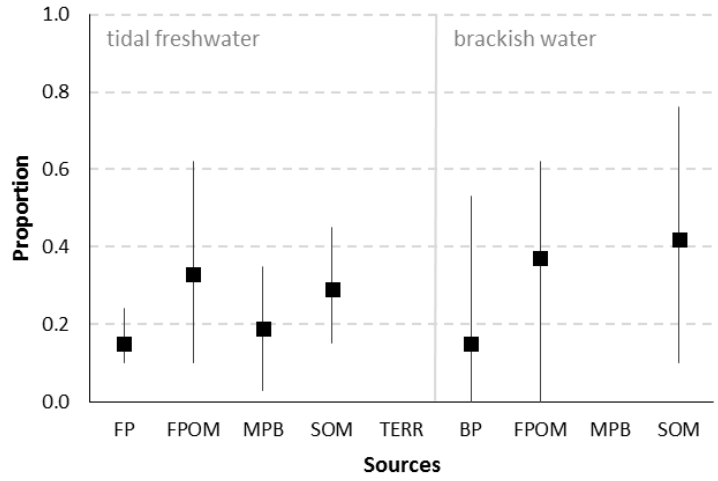
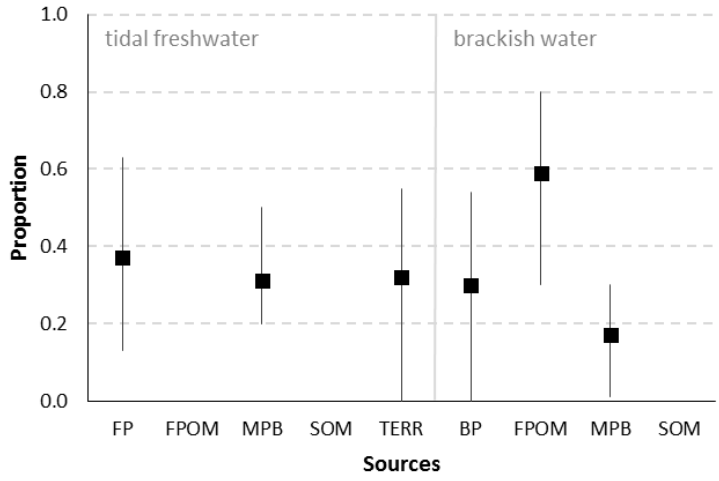


Fig 2.8 Proportion of each food source to calanoid copepod biomass collected in the tidal freshwater, brackish and marine portions of the estuary in September 2010 (A), March 2011 (B) and August 2011 (C). The food sources included in the model were freshwater, brackish and marine phytoplankton (FP, BP and MP), freshwater and marine particulate organic matter (FPOM and MPOM), microphytobenthos (MPB), submerged aquatic vegetation (SAV), sediment organic matter (SOM), terrestrial plants (TERR) and macroalgae (MACROAL). Closed squares indicate the most likely value (mode) and lines indicate the 95% Bayesian credibility intervals.

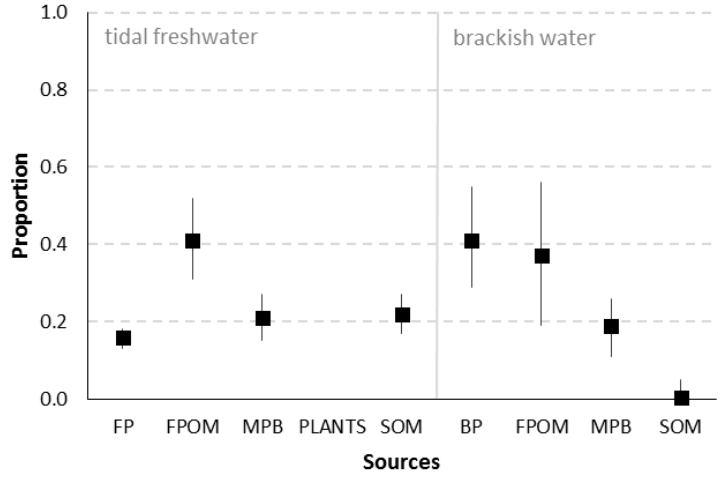


A - September 2010

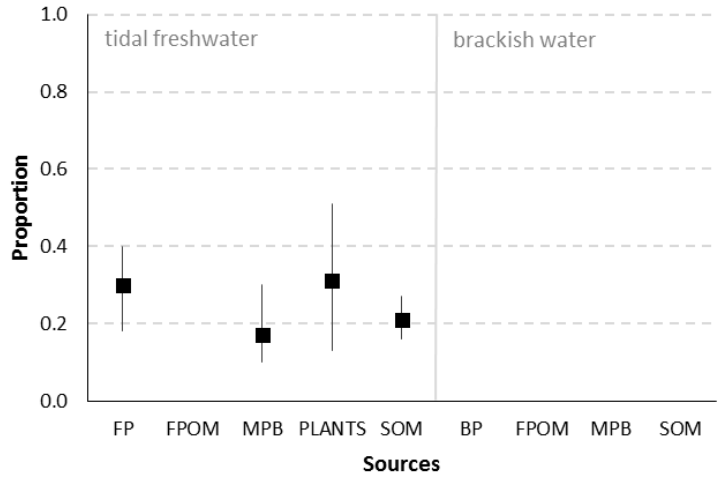


B - August 2011

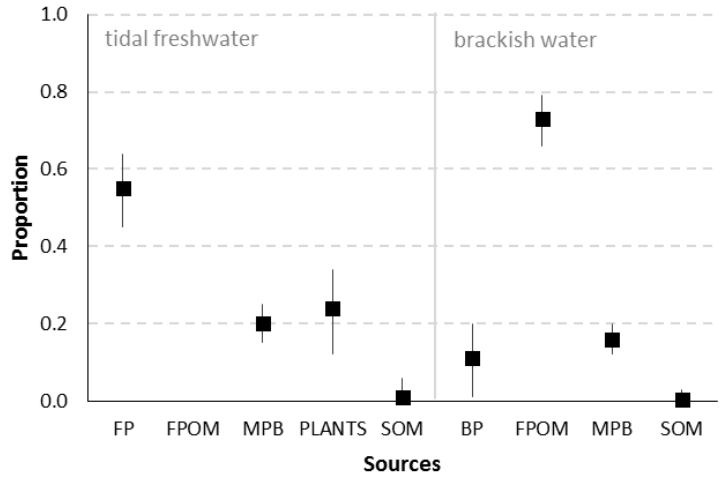
Fig 2.9 Proportion of each food source to Caridea larvae biomass collected in the tidal freshwater and brackish portions of the estuary in September 2010 (A) and August 2011 (B). The food sources included in the model were freshwater and brackish phytoplankton (FP and BP), freshwater particulate organic matter (FPOM), microphytobenthos (MPB), sediment organic matter (SOM) and terrestrial plants (TERR). Closed squares indicate the most likely value (mode) and lines indicate the 95% Bayesian credibility intervals.



A - September 2010



B - March 2011



C - August 2011

Fig 2.10 Proportion of each food source to *Corbicula fluminea* biomass collected in the tidal freshwater and brackish portions of the estuary in September 2010 (A), March (B) and August 2011 (C). The food sources included in the model were freshwater and brackish phytoplankton (FP and BP), freshwater particulate organic matter (FPOM), microphytobenthos (MPB), terrestrial or emergent aquatic vegetation (PLANTS) and sediment organic matter (SOM). Closed squares indicate the most likely value (mode) and lines indicate the 95% Bayesian credibility intervals.

2.4 Discussion

There was a pronounced variability in the quality of OM available for primary consumers in the Minho River estuary between sampling periods. The stable isotope analysis in concert with POM C/N ratios and estimates of phytoplankton fraction in the POC indicate that terrestrial-derived OM, a refractory source, was the main component of the POM pool in September 2010, and that phytoplankton, a highly palatable food source, dominated the POM pool in August 2011. Both were summer low discharge months. A corresponding shift was observed in the isotopic composition of pelagic and benthic consumers, indicating a shift in the food sources used in response to the change in food quality. The SIAR mixing model attributed most pelagic (calanoid copepods and Caridea larvae) and benthic (*C*. *fluminea*) biomass to terrestrial-derived OM in September 2010 and to phytoplankton in August 2011. The POM data also indicated an increasing contribution of phytoplankton to the POM pool towards the river mouth in September 2010 and March 2011. An opposite pattern was observed in August 2011. Accordingly SIAR mixing model results overall reflected the same pattern in phytoplankton consumption, except in August 2011 where freshwater phytoplankton was a major source contributing to the biomass of organisms collected in the brackish portion of the estuary. Notably, the stable isotopes of the primary consumers indicate that they have the ability to consume terrestrial-derived OM and they use both pelagic and benthic pathways to acquire energy, thus linking terrestrial with estuarine ecosystems and pelagic with benthic environments. The shifts in the OM sources used by primary consumers between sampling periods and along the estuarine mixing gradient will be discussed below.

2.4.1 Influence of river discharge

The contribution of allochthonous and autochthonous OM to consumer biomass varied between sampling periods. Autochthonous OM (phytoplankton, MPB and aquatic plants) supported more than 50% of primary consumer biomass in August 2011. In March 2011 and September 2010, allochthonous OM was a major contributor to the biomass of Caridea larvae and *C*. *fluminea*. In September 2010, autochthonous OM sources were still major contributors to calanoid copepod biomass (≥ 50%).

In August 2011, during low river discharge, the proportion of phytoplankton in the POM pool was higher than in the other sampling periods (average values above 50%, except in the marine stations), supporting more than 40% of the pelagic and benthic production. In contrast, in March 2011, during high discharge, the average phytoplankton proportion in the POM pool was lower (up to 40%). In concert, the proportion of

phytoplankton supporting pelagic and benthic production also decreased (< 40%). Consumers were using macroalgae and plants detritus, and SOM.

High river discharge can limit phytoplankton availability, production and accumulation due to an increase in allochthonous C loadings, low light penetration and decrease in residence time (Sin et al. 1999, Roach 2013). During high river discharge, the C loads to the Minho River estuary increased and Chl *a* concentration values were ca. 1 µg L^{-1} (33% of concentration values determined in August 2011). Phytoplankton production may have been limited due to low light penetration owing to the increase in C loads to the system, or else phytoplankton was growing but rapidly exported from the estuary.

September 2010 and August 2011 had similar discharge; however, the quality of OM available was quite different. Contrary to August 2011, in September 2010, the environmental data indicate that phytoplankton standing stock was low: phytoplankton proportion in the POM pool was lower than 20% and Chl *a* concentration values were lower than 1 µg L^{-1}. The reason for that might be in the existing conditions during the months prior to sampling. During the winter 2009-2010, a major flood occurred in the Minho River estuary, River discharge values almost doubled the historical values for this ecosystem (Fig. 2.2). A flood pulse event such as this can increase the amount of terrestrial-derived OM delivered to the aquatic ecosystem (Huryn et al. 2001, Kendall et al. 2001, Hoffman et al. 2008). Although we did not measure allochthonous C loads to the estuary during the flood of 2009-2010, there was evidence that terrestrial-derived OM increased in the estuary, during this period. First, the low $\delta^{13}C_{DIC}$ values during September 2010 (between -2‰ and -18‰; Dias et al. in press) suggest that the system was net heterotrophic. Second, the C/N_{POM} was >10 (TFW), indicating a substantial contribution of terrestrial-derived OM to the POM pool (Hedges et al. 1986, Hedges et al. 1997). The terrestrial signal was reflected in the primary consumers analyzed, especially in Caridea larvae and *C*. *fluminea*, which had $\delta^{13}C$ values similar to that of freshwater POM and were more ^{13}C-enriched than would be expected if they were consuming phytoplankton. Accordingly, we estimated that terrestrial-derived OM, as FPOM, contributed up to 70% of zooplankton biomass and up to 60% of *C*. *fluminea* biomass during this period. Thus, while terrestrial-derived OM contributed substantially to some consumers (Caridea larvae and *C*. *fluminea*), autochthonous sources largely contributed to others (calanoid copepods). A critical assumption for this food web analysis is that the OM source values measured were similar over the prior 2-3 months because the data collected during this study were obtained during single sampling events. For individuals, the isotopic turnover period depends on both somatic growth and metabolic turnover rates; in organisms that grow rapidly, somatic growth rates essentially determine the isotopic turnover period (del

Rio et al. 2009). Zooplankton isotopically turnover within a month (Hoffman et al. 2007), thus reflecting temporal environmental variability. However, it is possible that zooplankton collected in the brackish portion of the estuary were displaced downriver, especially during high discharge conditions, thus compromising spatial analysis. Our stable isotope data do not support this hypothesis because calanoid copepods and Caridea larvae collected in the brackish portion of the estuary resembled the stable isotope ratios of other taxa such as polychaeta larvae or *Calanus* spp. (March 2011) that are characteristic of brackish and marine water or were close to the stable isotope values of *C*. *fluminea*, which is a sessile benthic species (September 2010 and August 2011). Moreover, calanoid copepods and Caridea larvae collected in the brackish portion of the estuary were more ^{13}C-enriched than those collected in the TFW, reflecting the influence of the marine environment.

Although isotopic turnover rate studies of *C. fluminea* have not been conducted, it is likely that the isotopic turnover period of the *C*. *fluminea* population is about three months. *Corbicula fluminea* has a rapid growth throughout their life and, owing to their rapid sexual maturity, have a population-level turnover time (in biomass) of 73-91 days (McMahon 2002). Moreover, previous studies have demonstrated that stable isotope values of other bivalves *Dreissena polymorpha* (zebra mussel) and *Corbula amurensis* (Asian clam; formerly known as *Potamocorbula*) are spatially and temporally well aligned with environmental processes (e.g. watershed inputs, chemistry of river water; Fry 2002, Fry and Allen 2003). The same is likely true for *C*. *fluminea* owing to its rapid growth rate and similarly short life span (McMahon 2002, Thompson and Parchaso 2010).

2.4.2 Cross-ecosystem subsidies

We estimate that terrestrial-derived OM contributed up to 70% of consumers' biomass in the TFW, but that its importance declined seaward. Terrestrial inputs are often equal or larger than the autochthonous primary production in aquatic ecosystems (Meili et al. 1996), nonetheless numerous studies have demonstrated that estuarine secondary production is primarily supported by autochthonous production, especially phytoplankton (Deegan and Garritt 1997, Chanton and Lewis 2002). There is growing evidence that terrestrial inputs can subsidize estuarine food webs, supporting up to 80% of invertebrates and fish biomass (Kasai and Nakata 2005, Hoffman et al. 2008, Cole and Solomon 2012). Although the contribution of terrestrial-derived OM was higher in the TFW, some consumers collected in the brackish portion of the estuary presented low δ^{13}C values (calanoid copepods, Caridea larvae and *C*. *fluminea*), suggesting the consumption of a ^{13}C-depleted source. Based on our source data those could be brackish phytoplankton, SOM or terrestrial-derived OM (i.e. FPOM). Accordingly, the SIAR mixing model attributed

up to 60% of terrestrial-derived OM to primary consumers' biomass in the brackish estuary, thus suggesting that OM from the upper portion of the estuary was being exported and consumed in the lower portion of the estuary.

In August 2011, the contribution of phytoplankton to consumer biomass in the TFW was ≤80%, and ≤100% in the brackish portion of the estuary. The $\delta^{13}C$ and $\delta^{15}N$ values from calanoid copepods, Caridea larvae and *C. fluminea*, suggest that they were using a more ^{13}C-depleted source than brackish phytoplankton, which could be terrestrial plants or freshwater POM. Their high $\delta^{15}N$ values, however, indicate that the most likely source was freshwater POM, which was largely comprised of phytoplankton. Thus, as in September 2010, the freshwater OM was exported from the TFW portion of the estuary, and functioned as an important subsidy to organisms in the lower portion of the estuary. Accordingly we estimate that up to 80% of consumer biomass was derived from FPOM. Also in the brackish portion of the estuary, there was another group of consumers that consumed a more ^{13}C-enriched mixture of OM sources (Centropages, *Oikopleura* sp. and Ostracoda), indicating consumption of macroalgae and marine POM. The importance of marine material (phytoplankton and POM), increased with the proximity to the mouth of the river, where it comprised 26-88% of calanoid copepod biomass. Therefore, marine ecosystem can also subsidize the estuarine food web during low flow conditions.

In March 2011, the consumers collected in the brackish area of the estuary relied on ^{13}C-enriched sources, such as macroalgae and brackish phytoplankton, and ^{15}N-depleted sources (e.g. SOM). We estimated that SOM was the most important source contributing to calanoid copepod biomass (27-61%). Because the $\delta^{13}C$ and $\delta^{15}N$ values of calanoid copepods were similar to those from polychaeta larvae and ostracods, they could be also using the same OM sources. Because marine intrusion was confined to the first 6 km of the estuary, it is possible that during high discharge, the marine subsidies to the estuary are minor. If consumers were using marine phytoplankton, which has a typical $\delta^{15}N$ of 6 ± 1.5‰ (Bode et al. 2007) and $\delta^{13}C$ of -20.5 ± 1.3‰ (McMahon et al. 2013), and assuming a trophic fractionation of +3.4 ‰ for N and +0.4‰ for C, their $\delta^{13}C$ and $\delta^{15}N$ values would have to be close to -21‰ for $\delta^{13}C$ and higher than 8‰ for $\delta^{15}N$, which was not the case.

2.4.3 Possible consequences of allochthony to the estuarine food web

Calanoid copepods, Caridea larvae and *C. fluminea* obtained the majority of their food sources from the water column. The quality of these food sources varied with river discharge and along the estuarine mixing zone, revealing that primary consumers have the ability to use alternative and less labile OM sources (terrestrial-derived OM and SOM) when phytoplankton availability is low.

Although the mechanisms underlying the use of refractory material are poorly understood, previous studies proposed that terrestrial OM could become available to pelagic consumers via several mechanisms: microbial uptake of terrestrial dissolved organic carbon followed by direct or indirect consumption of the microbes (Berggren et al. 2010), direct consumption of dissolved terrestrial organic carbon (Speas and Duffy 1998) or direct consumption of terrestrial-derived particles (Cole et al. 2006). *Corbicula fluminea* has a preference for small-sized living and suspended POM (Atkinson et al. 2011), and detritus have been identified in gut content analysis (Hill 1985 cited in Foe and Knight 1985). Therefore, it is likely that they are able to consume directly terrestrial-derived particles. If so, it is possible that *C*. *fluminea* may also act as initiators to break down plant cell wall structural polysaccharides, thus providing more labile OM for benthic organisms through the production of faeces and pseudofaeces. However, further studies are needed to clarify the presence of an enzymatic mechanism that enables *C*. *fluminea* to perform this function (Dias et al in press).

Thus, primary consumers in the Minho River estuary have the ability to adapt to low food quality conditions, especially during high-flow pulses, by using less labile OM sources such as terrestrial-derived OM or SOM. Its ability to consume terrestrial-derived OM and to access both pelagic and benthic food sources couple the terrestrial ecosystems with the estuarine pelagic and benthic environments. Nonetheless, further studies are needed to understand how the use of less labile OM sources affects the productivity of primary consumers and upper trophic levels.

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Appendix A. $\delta^{15}N$ and $\delta^{13}C$ values of zooplankton taxa collected in September 2010, March 2011 and August 2011 in the tidal freshwater (TFW), brackish and marine portions of the Minho River estuary.

Zooplankton taxa	Sampling month	Location	$\delta^{15}N$ (±SD) ‰	$\delta^{13}C$ (±SD) ‰	Code
Copepoda					
Calanoida					
Calanus sp.	March 2011	Brackish	8.5 (0.1)	-18.9 (0.3)	Cl
Acartia sp.	March 2011	Brackish	8.1 (0.2)	-18.8 (0.04)	A
	August 2011	Brackish	10.7 (1.8)	-24.9 (4.5)	
	August 2011	Marine	7.2 (0.2)	-17.5 (0.2)	
Centropages sp.	September 2010	Brackish	7.0 (0.6)	-20.1 (0.3)	C
	March 2011	Brackish	7.9	-19.1	
	August 2011	Brackish	7.3	-17.0	
	August 2011	Marine	7.3 (0.1)	-17.3 (0.1)	
Calanoida n.i.	September 2010	TFW	9.9	-27.3	Ca
	September 2010	Brackish	9.5 (0.5)	-24.2 (3.1)	
	March 2011	TFW	11.2 (1.0)	-33.9 (1.7)	
	August 2011	TFW	12.5 (0.1)	-30.3 (0.4)	
	August 2011	Brackish	12.3 (0.2)	-30.5 (0.1)	
Cyclopoida n.i	September 2010	TFW	10.0	-27.3	Cy
	August 2011	Brackish	17.7 (0.1)	-21.0 (0.4)	
Harpaticoida n.i.	March 2011	Brackish	8.6 (0.2)	-21.2 (3.9)	H
Branchiopoda					
Cladocera n.i.	March 2011	TFW	10.7	-32.0	Cd
Ostracoda n.i.	August 2011	Brackish	6.9 (0.03)	-16.9 (0.1)	O
	August 2011	Marine	6.5 (0.2)	-16.9 (0.1)	
Malacostraca					
Caridea n.i.	September 2010	TFW	8.1	-27.4	M
	September 2010	Brackish	5.9 (1.4)	-26.3 (1.5)	
	August 2011	TFW	11.1 (0.4)	-26.4 (0.3)	
	August 2011	Brackish	11.6 (0.1)	-26.1 (0.5)	

Appendix A (continuation)

Zooplankton taxa	Sampling month	Location	$\delta^{15}N$ (±SD) ‰	$\delta^{13}C$ (±SD) ‰	Code
Podon sp.	August 2011	Marine	6.8 (0.3)	-16.3 (0.1)	P
Hydracaridae n.i.	August 2011	TFW	12.8	-26.4	Hy
Polychaeta					
Polychaeta larvae n.i.	March 2011	Brackish	8.0	-18.3	Pl
	August 2011	Marine	6.8	-16.7	
Ascidiacea					
Oikopleura n.i.	August 2011	Brackish	7.0	-18.1	Ok
	August 2011	Marine	7.3 (0.1)	-17.1 (0.1)	
Osteichthyes					
Fish larvae n.i.	March 2011	Brackish	10.3 (0.2)	-18.1 (0.0)	Fl
	August 2011	Brackish	13.4 (0.6)	-23.0 (2.8)	
	August 2011	Marine	12.2	-18.8	

Chapter 3

Spatial and temporal variability of an estuarine benthic food web: patterns and processes in an oligotrophic ecosystem

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Abstract

Benthic organisms are an important link between organic matter (OM) sources and predators in estuarine food webs. Thus, determining the variability of energy pathways in the benthic communities will improve our knowledge on the factors that may affect the ecosystems' functioning and productivity. We investigated the spatial and temporal variability of carbon and nitrogen stable isotope ratios in the Minho River estuary food web. For this purpose, we analyzed the stable isotope ratios of organisms that occupy different habitats (pelagic, benthic and epibenthic), and also of their potential food sources to identify the main pathways of energy flow in the benthic estuarine food web. This study was conducted along the estuarine salinity gradient, and samples were collected between January and September 2011, for seasonal comparisons, and in August 2010, for inter-annual comparisons. Overall, consumers were ^{13}C-enriched towards the mouth of the estuary in all seasons, with average $\delta^{13}C$ values varying between -21‰ and -14‰ in the stations closer to the river mouth, and between -28‰ and -22‰ in the tidal freshwater area (TFW). On the contrary, the $\delta^{15}N$ values of consumers were lower towards the mouth of the river, increasing during the summer for pelagic and benthic consumers. The seasonal variability in the $\delta^{15}N$ values of epibenthic consumers was species-specific. Based on the analysis of the stable isotope ratios of pelagic and benthic consumers and their likely OM sources, it was possible to verify that consumers relied on a mixture of OM sources with different origins. Consumers collected in the brackish portion of the estuary were influenced by the proximity to the sea, and were supported by marine phytoplankton, among other OM sources. Consumers collected in the TFW were supported by terrestrial-derived OM owing to the proximity of riverine ecosystems. Hydrology played a crucial role in food web dynamics. During high river discharge periods, the $\delta^{13}C_{POC}$ and $C:N_{POM}$ values suggested an increase of terrestrial-derived OM to the particulate OM pool, which was then used by suspension feeders. On the other hand, during low river discharge periods, marine intrusion increased upriver, which was reflected in the ^{13}C-enriched stable isotope values of pelagic and benthic consumers. Although epibenthic consumers stable isotope ratios varied spatially and temporally, the main pathways supporting their biomass did not change. Thus, this study highlighted the role of benthic consumers in linking pelagic and benthic environments in estuarine ecosystems, and the role of marine and terrestrial ecosystems in subsidizing the benthic estuarine food web.

Keywords: primary consumers, terrestrial-derived OM, stable isotopes, river discharge.

3.1 Introduction

Estuaries are among the most productive ecosystems on the planet and produce highly variable food webs associated with diverse habitats with different organic matter (OM) sources, which in turn are influenced by the variability in hydrodynamics and physicochemical conditions at various spatial and temporal scales (Livingston 1997, Antonio et al. 2012, Ubertini et al. 2012).

Understanding the factors that govern the exchange and consumption of different OM sources is of paramount importance for estuarine management and conservation, because secondary production and food web's diversity are thought to depend on the quality of the basal sources. Pelagic pathways, associated with phytoplankton consumption, are thought to be more productive than the benthic pathways associated to the consumption of detritus, which are considered less labile OM sources (Rooney and McCann 2011).

In estuaries, benthic organisms are an important link between OM and predators. Most benthic invertebrates are detritivores, such as suspension and deposit feeders (Heip et al. 1995) feeding, in general, on particulate and sedimentary OM. Several OM sources are known to support benthic communities, and include terrestrial vegetation (e.g. Kasai and Nakata 2005), marine phytoplankton (e.g. Yokoyama et al. 2005), benthic microalgae (e.g. Kang et al. 2003) and coastal algae (e.g. Currin et al. 1995). Although, little is known about the role of benthic communities in linking adjacent aquatic ecosystems (land-river-sea), it is known that benthic organisms have the potential to influence spatial patterns of trophic relationships in aquatic ecosystems, due to its feeding behavior. Since benthic organisms often feed opportunistically, they can change diet in accordance with changes of habitat (e.g. Deegan and Garritt 1997). However, few studies attempted to describe the spatial (e.g. Deegan and Garritt, 1997, Yokoyama and Ishihi 2007, Antonio et al. 2010) and temporal (Fisher et al. 2001, Molina et al. 2011) variability in the use of various food sources by benthic organisms. Determining the variability of energy pathways in the benthic communities along the river-sea transition zone will contribute to understand the factors that may affect the functioning and productivity of these ecosystems.

Carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) stable isotopes have emerged as useful tools to disclose the structure and dynamics of estuarine food webs, because they provide time-integrated information about the trophic relationships and energy flow through food webs (Pasquaud et al. 2007). The $\delta^{15}N$ of an organism is typically enriched by 2.2-3.4‰ relative to its diet, and is usually used to determine the trophic position of an organism. The $\delta^{13}C$ changes little (1-2‰) as carbon moves through the food web, being used to evaluate the sources of energy used by an organism (Peterson and Fry 1987).

In this context, we aimed to characterize the spatial and temporal variability of C and N isotope ratios of benthic and epibenthic organisms and identify the main pathways of energy flow in the benthic estuarine food web, using the Minho River estuary as a model ecosystem. Pelagic, benthic and epibenthic consumers were collected in five sampling stations along the estuarine salinity gradient to analyze the spatial variability. For the temporal analyses, the above groups of consumers were collected monthly, between January and September 2011 for inter-seasonal comparisons, and also in August 2010 for inter-annual comparisons. We hypothesize that consumers would rely mainly on the autochthonous production of local estuarine food webs, and that seasonal variation would be more prevalent in the isotopic composition of OM sources and consumers than monthly variations.

3.2 Methods

3.2.1 Study area

The Minho River is located in the NW-Iberian Peninsula (Europe; Fig. 3.1). Its watershed is 17,080 km^2, of which 95% is located in Spain and 5% in Portugal. The river is 343 km long; 76 km serves as the northwestern Portuguese-Spanish border (Antunes et al. 2011). The limit of tidal influence is about 40 km inland, and the uppermost 30 km are a tidal freshwater wetland (TFW) (Sousa et al. 2008a). The estuary has an area of 23 km^2, 9% of which are intertidal areas. The estuary is mesotidal, with tides ranging between 0.7 m and 3.7 m (Alves 1996).

The annual average river inflow is 300 $m^3\ s^{-1}$ (Ferreira et al. 2003); it can vary between 100 $m^3\ s^{-1}$ during summer, and over 600 $m^3\ s^{-1}$ during winter (Confederación Hidrográfica del Miño-Sil, 2012). Typically low chlorophyll *a* (Chl *a*) concentration indicate that this is an oligotrophic ecosystem: from 1.3 $\mu g\ L^{-1}$ in low salinity areas to 2.1 $\mu g\ L^{-1}$ in brackish areas (average values from 2000-2010; Brito et al. 2012).

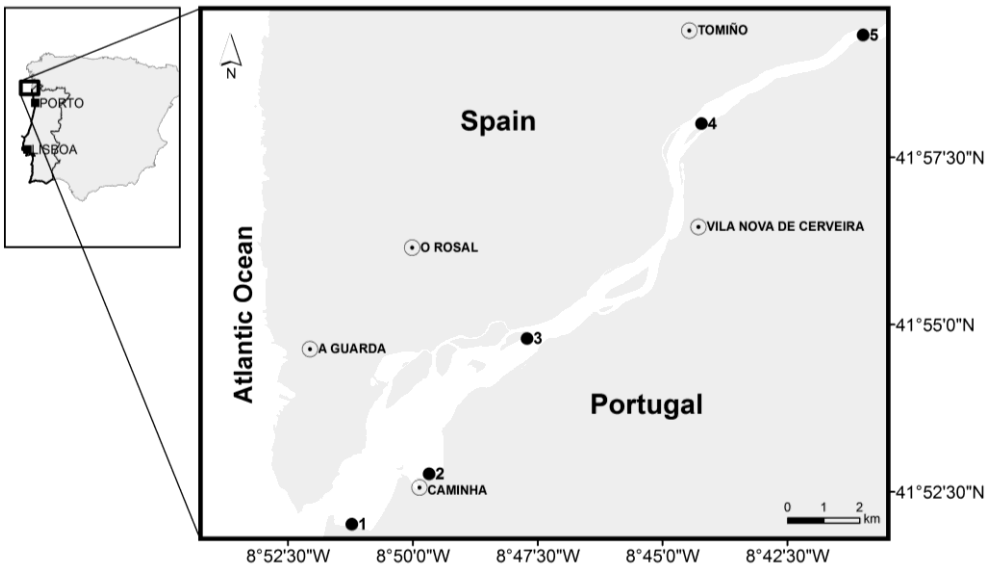

Fig. 3.1 Location of the sampling stations along the Minho River estuary.

Previous studies indicate the existence of low subtidal macrozoobenthos and epibenthos diversity (Sousa et al. 2008b, Costa-Dias et al. 2010). According to those studies, salinity is the abiotic factor that contribute the most to explain abundance and distribution patterns of subtidal macrozoobenthos and epibenthos assemblages along the Minho River estuary. Sediment characteristics (granulometry and organic matter content) also influence the distribution of macrozoobenthos species (Sousa et al. 2008b). At the estuary mouth, salinity varies between 25 in winter to 32 in summer, during high tide periods, and here subtidal habitats are sandy, with low organic matter content, though this area might be often covered by upriver debris. The dominant macrozoobenthos species is the polychaete *Hediste diversicolor* and the amphipod *Hautorius arenarius* (Sousa et al. 2008b). At the adjacent saltmarsh, the average grain size is smaller and the organic content is higher than in the estuary mouth, and the dominant species are *H. diversicolor*, the isopod *Cyathura carinata* and the bivalve *Scrobicularia plana* (Sousa et al. 2008b). In the middle estuary, salinity fluctuates between 0 in winter up to 20 during summer, and the dominant macrozoobenthos species are the amphipods *Corophium multisetosum* and *Gammarus chevreuxi*, and the invasive bivalve *Corbicula fluminea* (Sousa et al. 2008b). The dominant epibenthic species along the lower portion of the estuary, from the mouth to the middle estuary,are the crustaceans *Crangon crangon* and *Carcinus maenas*, and the fish *Pomatoschistus microps* (Costa-Dias et al. 2010, Souza et al. 2013a). In the tidal freshwater portion (TFW), the substrate is sandy and often covered by aquatic vegetation (e.g. *Elodea canadensis*). These areas are dominated by *C.fluminea*, where it represents more than 90% of the total benthic macrofauna biomass (Sousa et al. 2005, 2008b). The epibenthic community in the TFW is dominated by the freshwater shrimp *Atyaephyra*

desmaresti and by the epibenthic fishes *Cobitis paludica* and *Platichthys flesus* (Costa-Dias et al. 2010).

3.2.2 Field sampling

Sampling was conducted in August 2010 and from January to September 2011, at five stations (S) along the salinity gradient (Fig. 3.1): S1- near to the Minho river mouth, S2- at the mouth of Coura river, an estuarine tributary of Minho river; ca. 4 km away from the river mouth, S3- located at the salinity transition zone, and at 8 km upstream from the river mouth, S4 and S5- located at 15 km and 21 km, respectively, upstream the river mouth (tidal freshwater area; TFW). All the stations were sampled during full-moon spring tides.

At each station, surface (50-100 cm below the surface) and bottom water samples (0.5 m off the bottom) were collected using a 2 L Ruttner bottle. From these samples, we determined the concentration of chlorophyll *a* (Chl *a*: μg L^{-1}), concentration and isotopic composition of POM ([POM]: mg L^{-1}, particulate organic carbon (POC) $\delta^{13}C$, particulate nitrogen (PN) $\delta^{15}N$, molar C:N). Salinity was measured with an YSI model 6820 QS probe and reported using the Practical Salinity Scale. The POM and Chl *a* water samples (POM: 1L, Chl *a*: 0.5L) were pre-filtered with a 150 μm sieve and filtered onto a pre-combusted (500 ºC for 2 h) Whatman GF/F and Whatman GF/C filters, respectively, and kept frozen (-20 ºC) until analysis.

The microphytobenthos (MPB) samples were collected in each station, and at every sampling period, by scraping artificial substrates that were fixed in the sediment and left in the estuary during the period of this study. Macroalgae were collected in S1 and S2, and vascular plants in S3, S4 and S5.

Zooplankton samples were collected using a plankton net (200 μm mesh) towed near the surface. Samples were immediately preserved in 70% ethanol. Macrozoobenthos were sampled using a van Veen grab. Epibenthic organisms, such as *C*. *crangon*, *C*. *maenas*, *A*. *desmaresti* and fish species, were sampled in August 2010 and January, March, April, July-September 2011 using a 1 m beam trawl (5 mm mesh size) towed at 2 km h^{-1}. All the consumers, except zooplankton, were kept frozen (-20 ºC) until analyses.

3.2.3 Laboratory analyses

Filters collected for Chl *a* analysis were extracted in 90% acetone and analyzed on a Spectronic 20 Genesys spectrophotometer. Chl *a* concentration was calculated following the equations proposed by Lorenzen (1967).

Filters for POM and MPB analysis were fumigated with concentrated HCl to remove inorganic carbonates, rinsed with deionized water, placed in a sterile Petri dish, and dried at 60 ºC for 24 h (Lorrain et al. 2003). Sediment samples were rinsed with 10% HCl to remove carbonates, rinsed with deionized water, and dried at 60 ºC for 48h. Both acidification procedures are expected to produce only slight changes, ca. 0.4‰, in sample $\delta^{15}N$ values (Lorrain et al. 2003, Carabel et al. 2006). Macroalgae and vascular plants were cleaned with deionized water to remove epiphytes, dried at 60 ºC, and ground to a fine powder with a mixer mill for posterior stable isotopic analyses.

All the consumers were sorted, identified, measured when applicable, dried in an oven at 60 ºC and ground to a fine homogeneous powder using a mortar and pestle. The macrozoobenthos samples consisted of whole individuals, except for bivalves where we used the foot muscle for stable isotopes analyses, while for epibentic crustaceans and fish we used muscle tissue.

Stable isotope ratios were measured using a Thermo Scientific Delta V Advantage IRMS via a Conflo IV interface (Centro Interdisciplinar de Investigação Marinha e Ambiental, Universidade do Porto). Stable isotope ratios are reported in δ notation, δX: $\delta X = (R_{sample}/R_{standard} - 1) \times 10^3$, where X is the C or N stable isotope, R is the ratio of heavy:light stable isotopes. Pee Dee Belemnite and air are standards for $\delta^{13}C$ and $\delta^{15}N$, respectively. The analytical error, the mean standard deviation of replicate reference material, was ±0.1‰ for $\delta^{13}C$ and $\delta^{15}N$.

3.2.4 Data analyses

Multivariate analyes were performed to reveal natural groupings in the data according to time and space. Multiple cluster analyses were performed using Euclidean similarity distance based on $\delta^{13}C$ and $\delta^{15}N$ values of consumers. Organisms were grouped according to their functional feeding group, which included pelagic feeders (e.g. zooplankton; Kleppel 1993), suspension feeders (e.g. clams and poliquetes; Verdelhos et al. 2005, Nordström et al. 2009, Atkinson et al. 2011), benthic grazers (e.g. gastropods; Bode et al. 2006), collectors (e.g. Diptera larvae, amphipods, isopods; Gerdollf and Hughes 1994, Henriques-Oliveira et al. 2003, Cardoso et al. 2004), shredders (e.g. *A. desmaresti*; Pestana et al. 2007) and predators (e.g. fish, *Carcinus maenas*, *Crangon crangon*; Phil 1985). The polychaete *H. diversicolor* and the isopod *C. carinata* can also be carnivorous, so they were considered to belong to two distinct functional groups (Wägele et al 1981, Nordström et al. 2009). Cluster analyses were then performed pooling functional groups by station or by month. For these analyses, the months were only used when all the benthic and epibenthic functional groups where available. A similarity profile

routine (SIMPROF) was used to test for the presence of sample groups (Clarke et al. 2008). These analyses were performed using PRIMER v.6.1.11® (Clarke and Gorley 2006).

One-way ANOVA analyses (factor “station” or “season”) were performed to determine species-specific (epibenthic organisms) or functional group (zooplankton and benthic organisms) changes in stable isotope ratios through time and space. When data did not meet the assumptions (i.e. normality and homogeneity of variances), the non-parametric Kruskal-Wallis test was used. These analyses were performed using STATISTICA 7, Statsoft.

Carbon (C) and nitrogen (N) stable isotope ratios bi-plots were used to examine the most likely OM sources for zooplankton and benthic macroinvertebrates. The isotopic ranges used for POM, MPB, EAV, SAV and terrestrial plants were determined in this study. The isotopic ranges for phytoplankton, sediment organic matter (SOM) and C4 saltmarsh plants were compiled from previous studies conducted in this ecosystem (freshwater and estuarine phytoplankton and SOM; Dias et al. in press, Dias et al. unpublished data), and compiled from the literature (marine phytoplankton and C4 saltmarsh plants: Fry and Sherr 1984, Deegan and Garritt 1997, Bode et al. 2007, Vinagre et al. 2008, MacMahon et al 2013). These bi-plots were interpreted in combination with ANOVA, and considering the foraging behavior of each species or functional feeding groups, to infer food web relationships.

We used a dual-stable isotope mixing model, that uses Bayesian inference to solve indeterminate linear mixing equations (i.e for two stable isotopes and more than three diet sources), to quantify the importance of pelagic and benthic pathways supporting epibenthic production. Indeterminate linear mixing equations produce a probability distribution that represents the likelihood of a given source (e.g. primary consumer) to contribute to the secondary consumer (Parnell et al. 2010). We used the model Stable Isotope Analysis in R (SIAR), which allows each of the sources and the trophic enrichment factor (TEF; or trophic fractionation) to be assigned a normal distribution (Parnell et al. 2010). The SIAR package (Parnell et al. 2010) is part of the open source statistical language R (R Development Core Team 2007). SIAR produces the distribution of feasible solutions to the mixing problem and estimates credibility intervals (95% CI in this study), which is analogous to the confidence intervals used in frequentist statistics. SIAR also includes a residual error term. In the SIAR mixing model, we adjusted the $\delta^{13}C$ and $\delta^{15}N$ values for one trophic level using the TEF estimates from Post (2002; +0.4 ± 1.3‰ $\delta^{13}C$, +3.4 ± 1.0‰ $\delta^{15}N$).

The consumers $\delta^{13}C$ values were corrected for lipid content, because lipids are depleted in ^{13}C compared to protein and carbohydrates (DeNiro and Epstein 1977).

Variability in lipid content can bias bulk tissue $\delta^{13}C$ values and thereby cause dietary or habitat shifts to be incorrectly interpreted. We corrected zooplankton tissue data for lipid content using the mass balance correction model proposed for zooplankton by Smyntek et al. (2007; Eq. 5). In the case of benthic and epibenthic consumers, we used the mass balance correction for fish muscle tissue, as proposed by Hoffman and Sutton (2010; Eq. 6), which uses estimates of $C:N_{protein}$ and $\Delta\delta^{13}C_{lipid}$ that are similar to those from the muscle tissue found for other fish (e.g. Sweeting et al. 2006) and taxonomic groups (e.g. shrimp and zooplankton; Fry et al. 2003, Smyntek et al. 2007). Zooplankton was also corrected for ethanol preservation (+0.4‰ $\delta^{13}C$, +0.6‰ $\delta^{15}N$) (Feuchtmayer and Grey 2003).

3.3. Results

3.3.1 Spatial heterogeneity of estuarine food web components

The stations sampled presented distinct salinity patterns (Fig. 3.2). S1 and S2 were overall marine to brackish, with average (±SD) salinity varying between of 23.1 ± 11.3 (S1) and 20.5 ± 12.4 (S2). Salinity in S4 and S5 was always lower than 0.5 (Fig. 3.2), and so they are considered to be freshwater stations. In S3, the salinity values were intermediate, 5.4 ± 8.2 (Fig. 3.2), owing to daily marine water intrusion and its central position in the estuary.

The isotopic composition of primary producers varied markedly along the salinity gradient in the Minho River estuary, with $\delta^{15}N$ values ranging from -3.0‰ to 14.0‰, and $\delta^{13}C$ values ranging from -31.5‰ to -12.8‰ (Fig. 3.3A). These ranges encompass the spatial and temporal variability of all the primary producers sampled. The highest $\delta^{15}N$ values were observed for SAV collected in S5, which varied between 7.6‰ and 14.0‰, and the lowest $\delta^{15}N$ values were observed for terrestrial plants in the TFW, which varied between -3.1‰ and 9.0‰ (Fig. 3.3A).

The highest $\delta^{13}C$ values were observed for macroalgae (-19.0‰ to -12.8‰ in S1) and MPB (-20.3‰ to -16.4‰ in S1), and the lowest were observed for freshwater phytoplankton (-32.2‰ to -29.2‰ in the TFW) (Fig. 3.3A). The $\delta^{13}C$ values of primary producers (phytoplankton, MPB) collected along the entire gradient increased towards the river mouth (Fig. 3.3A).

The POM pool, which is a mixture of different sources, including phytoplankton and vascular material, also varied along the salinity gradient. The $\delta^{15}N$ values of POM inceased ca. 2‰ towards the TFW (Fig. 3.4A), while the $\delta^{13}C$ values followed an inverse

pattern. On average (± SD), POM $\delta^{13}C$ values in stations closer to the river mouth (S1 and S2) were higher than those in the TFW: -24.0 ± 2.1 ‰ and -28.0 ± 1.0 ‰, respectively (Fig. 3.4B). The average (± SD) POM $\delta^{13}C$ values in S3 were intermediate between S1-S2 and S4-S5: -26.0 ± 1.9 ‰ (Fig. 3.4B). The quality of the POM pool, as indicated by $C:N_{POM}$, was similar between stations and varied between 8.6 ± 2.3 (S3) and 10 ± 1.7 (S4) (Fig. 3.4C). The $C:N_{POM}$ close to 10 in TFW indicates that terrestrial-derived OM contributed substantially to the POM pool in this portion of the estuary (Hedges et al. 1986, 1997). In the brackish portion of the estuary (S1-S3), $C:N_{POM}$ varied between 8 and 9 (Fig. 3.4C), which is within the range of terrestrial-derived OM (>10) and marine phytoplankton (~7) (Hedges et al. 1986, 1997), thus suggesting that brackish POM was a mixture of riverine and marine POM.

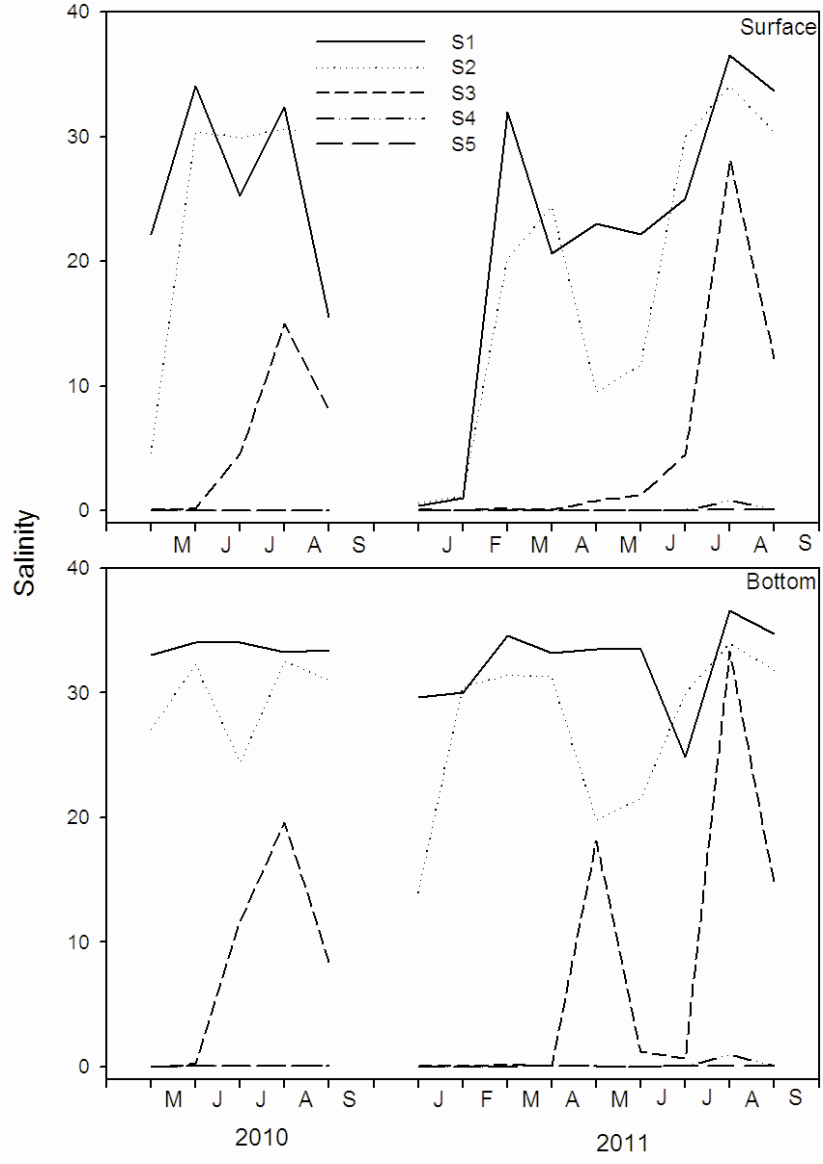

Fig. 3.2 Salinity variation along the Minho River estuary, in the 5 sampling stations (S) sampled along the estuarine mixing gradient during the studied period (2010-2011).

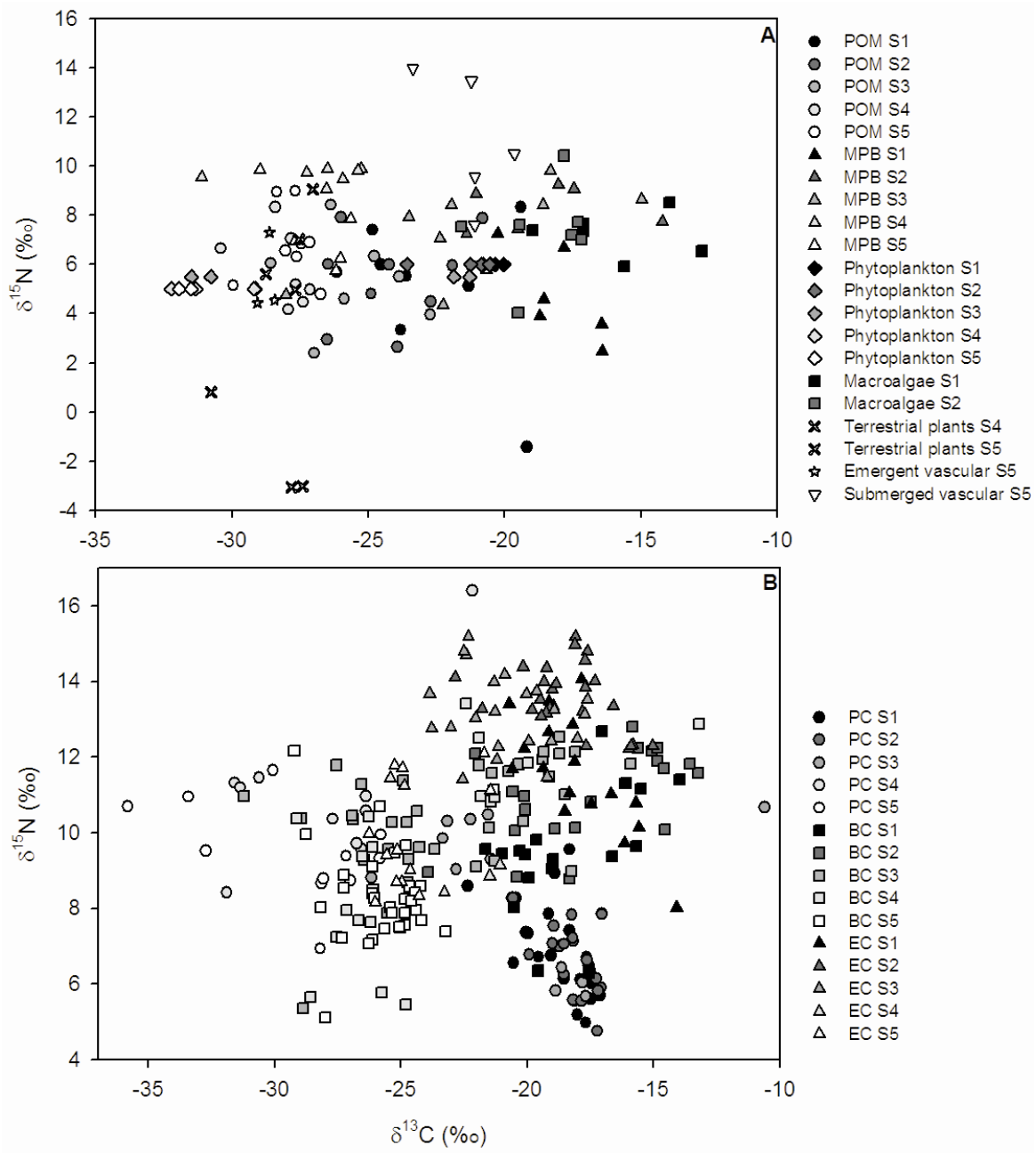

Fig. 3.3 $\delta^{15}N$ and $\delta^{13}C$ values of primary producers (A) and primary and secondary consumers (B) collected in five stations (S1-S5) along the salinity gradient of the Minho River estuary, between 2010 and 2011. The primary producers include particulate organic matter (POM), microphytobenthos (MPB), phytoplankton, macroalgae, Terrestrial plants, emergent (non identified) and submerged (*Elodea canadensis*) aquatic vegetation. Consumers include the pelagic primary consumers (PC), benthic consumers (BC) and epibenthic consumers (EC). The y-axis scale varies between graphs.

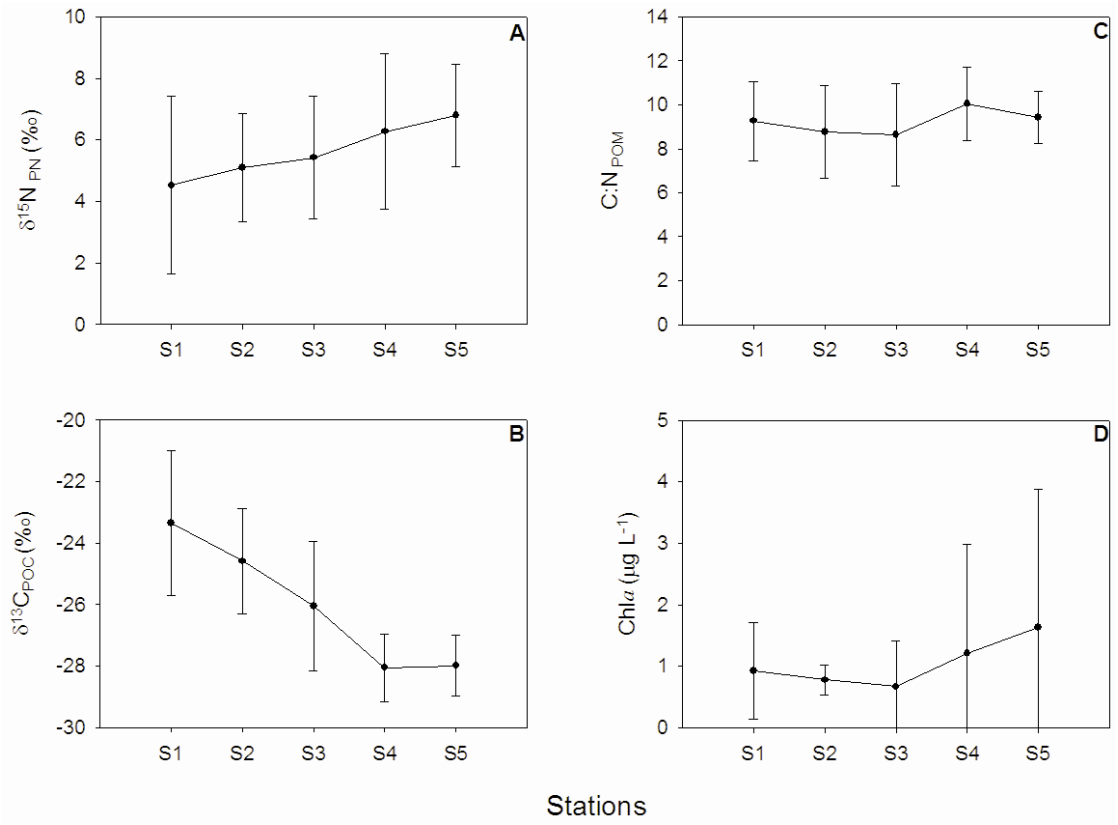

Fig. 3.4 Monthly average values (±SD) of particulate nitrogen ($\delta^{15}N_{PN}$; A), particulate organic carbon ($\delta^{13}C_{POC}$; B), particulate organic matter C:N ratios (C:N_{POM}; C) and chlorophyll *a* (Chl *a*) concentration (µg L^{-1}; D) in each sampling station, along the Minho River estuary, during the sampling period.

Overall, consumers were ^{15}N-depleted and ^{13}C-enriched towards the river mouth. Pelagic consumers were ^{15}N- depleted towards the river mouth ($H_{(4,77)}$= 40.46, p< 0.001), with average (±SD) values varying between 8.9 ± 0.9 ‰ (S1) and 11.5 ± 0.5 ‰ (TFW) (Fig. 3.5A). Although some benthic consumers (*C*. *carinata* and *H*. *diversicolor*) were ^{15}N-depleted towards the river mouth (Fig. 3.5B), the overall differences were not statistical significant ($F_{(4,115)}$= 1.34, p= 0.25). In the brackish portion of the estuary, *C*. *carinata* was the benthic consumer with the highest $\delta^{15}N$ values (close to 12‰), and *Scrobicularia plana* was the benthic consumer with the lowest $\delta^{15}N$ values (7.3 ± 1.8 ‰) (Fig. 3.5B). In the TFW, insect larvae had the highest $\delta^{15}N$ values (11.1 ± 1.5 ‰), while *C. fluminea* (9.0 ± 0.7 ‰), Gastropoda (9.0 ± 1.9 ‰) and Oligochaeta (9.1 ± 1.1 ‰), had the lowest (Fig. 3.5B). Overall, epibenthic consumers were ^{15}N-depleted towards the river mouth ($F_{(3,26)}$= 5.99, p= 0.003), with the exception of *P. flesus*. Nonetheless, the highest $\delta^{15}N$ values were observed in S2, varying between 12.6 ± 0.5 ‰ (*C. crangon*) and 14.6 ± 0.4 ‰ (*P. microps*) (Fig. 3.5C). In stations S1, S2 and S3, *P*. *microps* had the highest $\delta^{15}N$ values and *C*. *crangon* had the lowest $\delta^{15}N$ values (Fig. 3.5C). In the TFW, *A. desmaresti* had the lowest $\delta^{15}N$ values (8.0 ± 0.4 ‰) and *Gasterosteus aculeatus* had the highest $\delta^{15}N$ values (14.8 ± 0.5 ‰) (Fig. 3.5C).

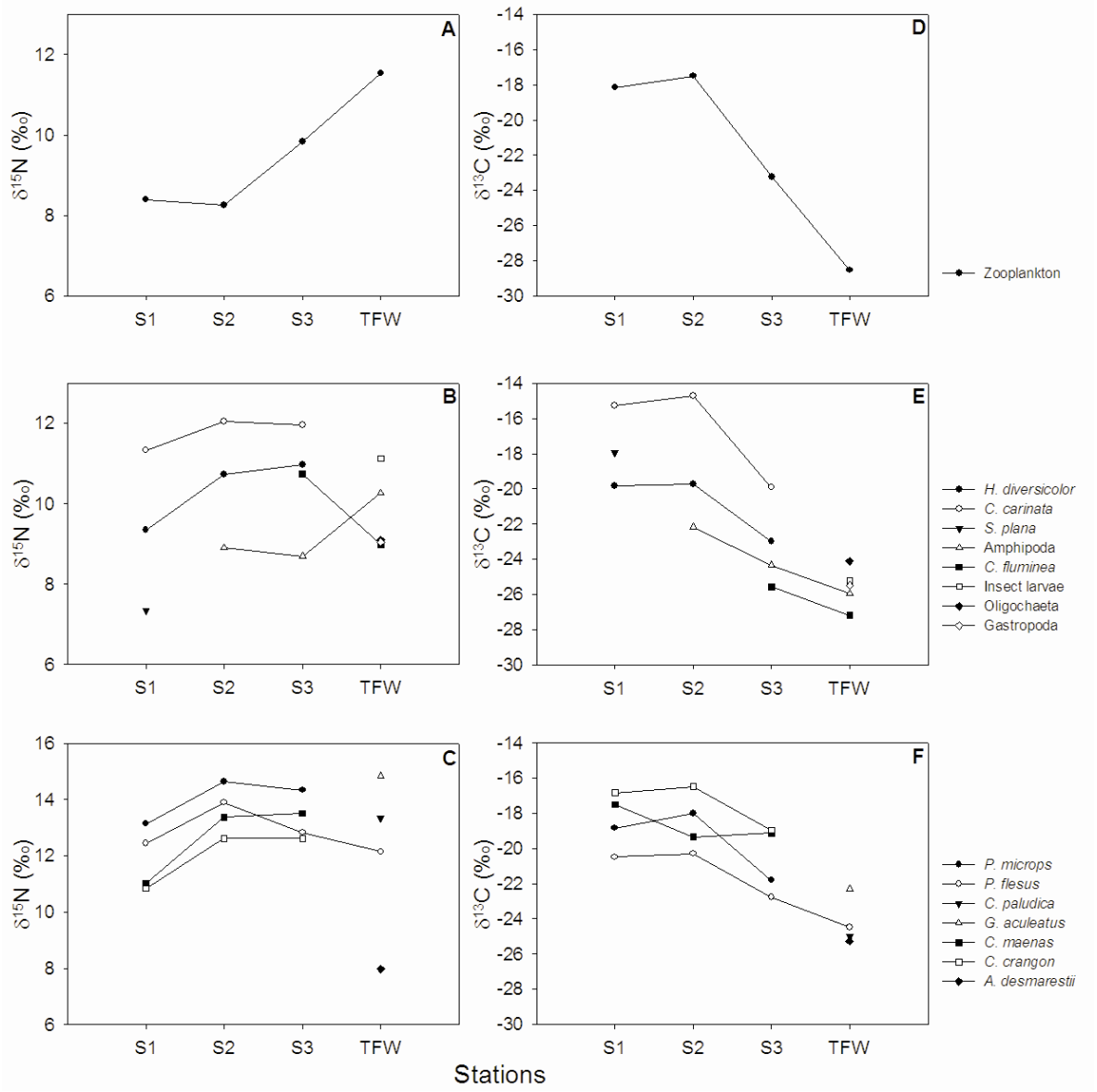

Fig. 3.5 Monthly $\delta^{15}N$ (A-C) and $\delta^{13}C$ (D-F) average values (‰) of pelagic (A, D), benthic (B, E) and epibenthic consumers (C, F), collected along the Minho River estuary during 2011.

Pelagic consumers were ^{13}C-enriched towards the river mouth ($H_{(4,77)}$= 44.0, p< 0.001) with values varying between -17.5 ± 1.1 ‰ (S2) and -28.5 ± 3.4 ‰ (TFW) (Fig. 3.5D). The $\delta^{13}C$ values of benthic consumers were significantly enriched towards the river mouth ($H_{(4,\ N=120)}$= 86.33, p< 0.001), ranging from -27.2 ± 1.0‰ (*C.fluminea*) to -24.1 ± 1.6‰ (insect larvae) at the TFW, and from -22.2 ± 2.5 ‰ (*Corophium* sp.) to -14.7 ± 0.9 ‰ (*C. carinata*) at S1 and S2 (Fig. 3.5E). Overall, the $\delta^{13}C$ values of *C. carinata* were the highest among benthic consumers in the stations S1-S3 (-21.9‰ to -13.2‰), and the $\delta^{13}C$ values of *H. diversicolor* (-31.2‰ to -17.5‰) in S1-S2, and *C. fluminea* (-27.5‰ to -24.4‰) in S3, were the lowest (Fig. 3.5E).

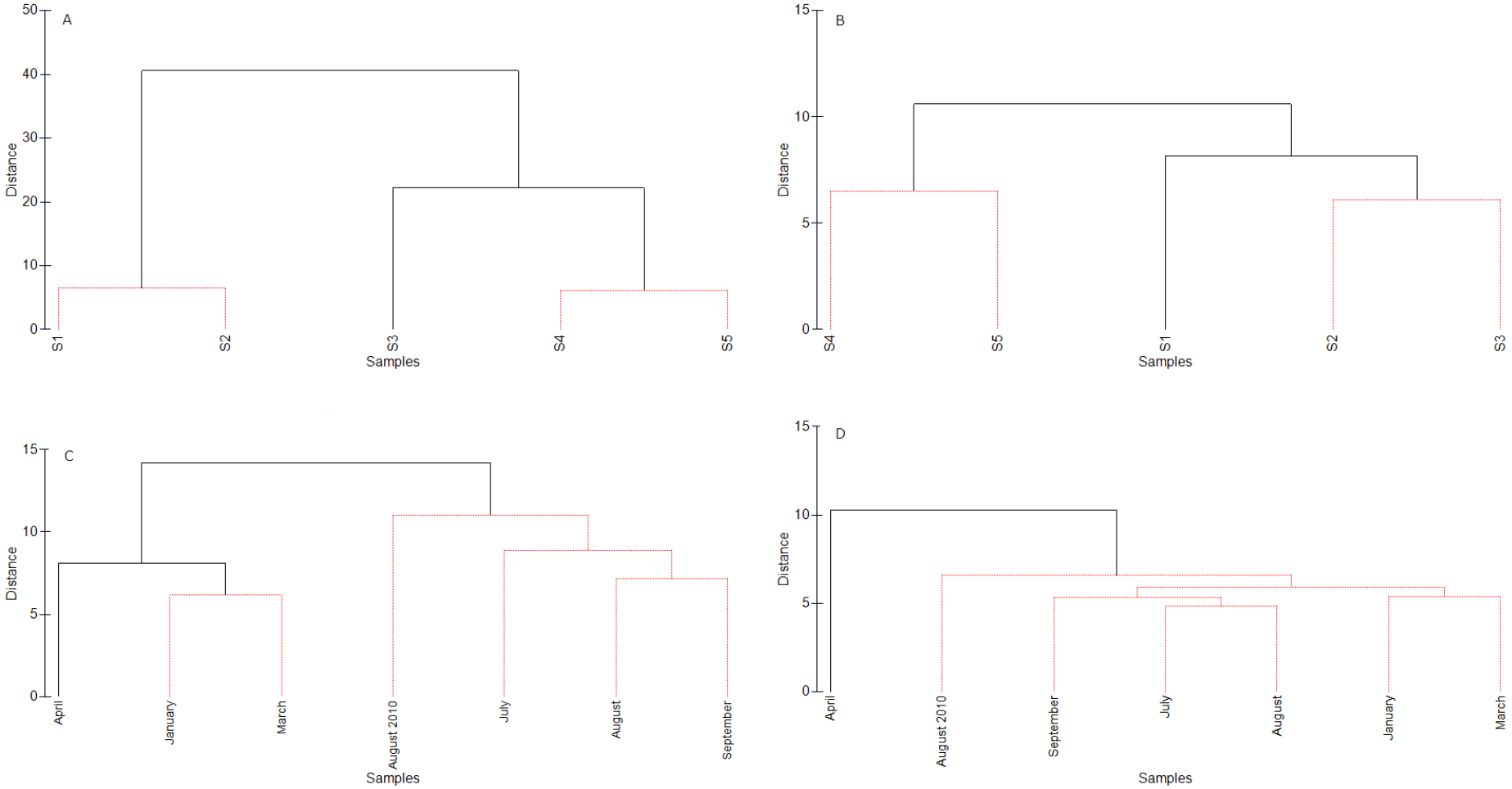

Fig. 3.6 Clustering analyses performed by station (A, B) and month (C, D) for $\delta^{13}C$ (A, C) and $\delta^{15}N$ (B, D) values for assemblages consisting of the main functional groups of consumers. Grey lines indicate groups of samples not separated (at $\alpha < 0.05$) by SIMPROF.

In the TFW the benthic consumers with higher $\delta^{13}C$ values were the insect larvae ($\delta^{13}C$: -25.2 ± 2.1 ‰) and the oligochaeta ($\delta^{13}C$: -24.1 ± 1.6 ‰) (Fig. 3.5E). The benthic consumer with the lowest $\delta^{13}C$ values was *C. fluminea* ($\delta^{13}C$: -27.2 ± 0.9 ‰) (Fig. 3.5E). The $\delta^{13}C$ values of epibenthic consumers were enriched towards the river mouth ($F_{(3,26)}$= 53.05, p< 0.001), and ranged from -25.0 ± 0.2 ‰ (*Cobitis paludica*) in the TFW to -16.5 ± 1.1 ‰ (*C. crangon*) in S2 (Fig. 3.5F). Overall, *P. flesus* was the epibenthic predator with the lowest $\delta^{13}C$ values (S1, S2, S3), contrarily to *C. crangon* (S1, S2, S3) and *G. aculeatus* (TFW) which presented the highest $\delta^{13}C$ values (Fig. 3.5F).

The cluster analyses grouped consumers collected in S4 and S5 (Fig. 3.6). Consumers collected in S1 and S2 were also clustered, but consumers collected in S3 were grouped differently according to the stable isotope ratio analyzed.

3.3.2 Temporal variability of estuarine food web components

The $\delta^{15}N$ and $\delta^{13}C$ values of the majority of the estuarine food web components varied seasonally. For POM samples (Fig. 3.7), the $\delta^{15}N_{PN}$ values increased in the summer, although the lowest values were observed in all stations in August 2011, except in S4 (Fig. 3.7A). The $\delta^{13}C_{POC}$ values increased in the summer in brackish stations, but decreased in the summer in the TFW area (Fig. 3.7B). The period when the largest differences between stations S1 and S2 versus the TFW stations in $\delta^{15}N_{PN}$ and $\delta^{13}C_{POC}$ values were observed corresponded to the occurrence of a Chl *a* maximum in the estuary (August 2011: 2.3 ± 2.2 µg L^{-1}) (Fig. 3.7D).

Inter-annual differences were observed in the POM values in all stations (Fig. 3.7). In August 2010, the POM pool was ^{13}C- enriched in relation to August 2011 in S2 and in TFW stations, and ^{13}C- depleted in S3. The POM $\delta^{15}N$ values were similar in all stations in August 2010 (between 4.8‰ and 4.9‰), but were ^{15}N-enriched in relation to samples collected in S1 in August 2011 (Fig. 3.7).

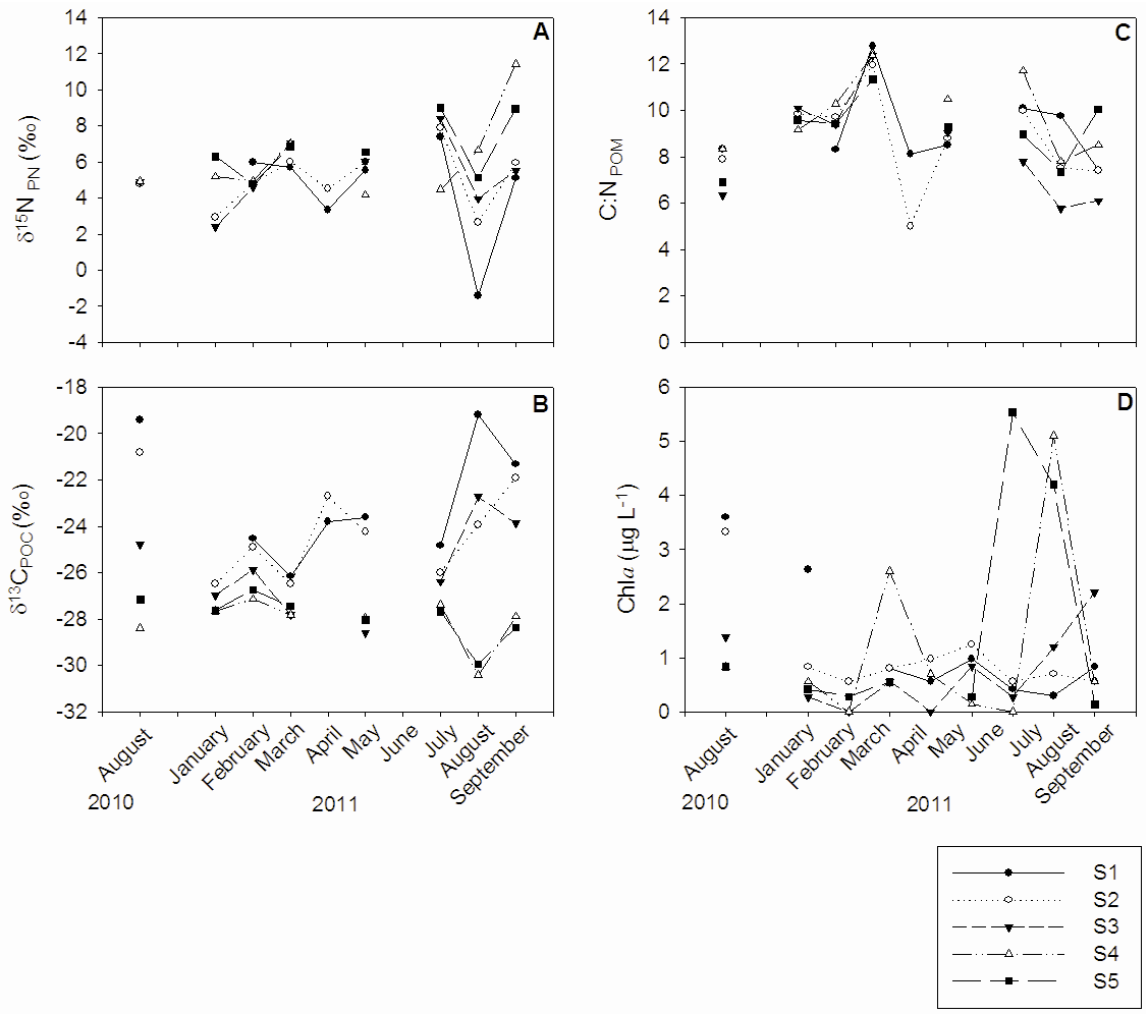

Fig. 3.7 Average values of particulate nitrogen ($\delta^{15}N_{PN}$) (A), particulate organic carbon ($\delta^{13}C_{POC}$) (B), particulate organic matter C:N ratios (C:N_{POM}) (C) and chlorophyll *a* (Chl *a*) concentration (μg L^{-1}) (D) in each station located along the Minho river estuary, in August 2010 and during 2011.

Across the estuary, the average C:N_{POM} were highest during winter, with ratios varying between 8.3 (February 2011) and 12.8 (March 2011; Fig. 3.7C). The C:N_{POM} ratios indicate that the POM pool throughout the estuary was substantially comprised of terrestrial-derived OM (> 10) during winter (Hedges et al. 1986, Hedges et al. 1997). Low $\delta^{13}C_{POC}$ values observed in S1 ($\delta^{13}C$: -25‰), particularly when compared to marine phytoplankton ($\delta^{13}C$: -20‰; McMahon et al. 2013), corroborates the interpretation. During summer, especially in August 2011, there was an increased contribution of phytoplankton to the POM pool: 1) C:N_{POM} values were close to those expected for phytoplankton (C:N ~7; Hedges et al. 1986, Hedges et al. 1997); 2) $\delta^{13}C_{POC}$ values in S1 were similar to those expected for marine phytoplankton ($\delta^{13}C$: -20‰; McMahon et al. 2013), 3) $\delta^{13}C_{POC}$ values were similar to those estimated for freshwater phytoplankton in this estuary ($\delta^{13}C$: -31‰; unpublished) in TFW stations; and 4) Chl *a* concentration increased during summer (except for S1 and S2; Fig. 3.7D).

Microphytobenthos $\delta^{15}N$ and $\delta^{13}C$ values were higher during spring and summer than during winter (Table 3.1). Vascular plants were ^{13}C-depleted and ^{15}N-enriched towards the summer, except for EAV, which presented lower $\delta^{15}N$ values during summer (Table 3.1). However, because vascular plants were pooled by group (EAV, SAV or terrestrial), it is possible that these differences are biased by different species composition between months.

In the cluster analyses conducted using $\delta^{13}C$ (Fig. 3.6C) values, winter (January and March 2011) and summer months (August 2010, July, August and September 2011) were grouped separately. Although April 2011 was grouped with the winter months, the SIMPROF analysis separated this month from the others. The same analysis conducted using the $\delta^{15}N$ values (Fig. 3.6D), did not reveal the existence of different groups; however, April 2011 appears to be different from the other months, suggesting the existence of some differences in the stable isotope ratios of consumers during this month. Thus, for further analysis, months were grouped into seasons (when applicable): winter (January-March), spring (April-May) and summer (July-September).

Overall, the highest $\delta^{15}N$ values for zooplankton were observed during summer, except in August 2011, where a minimum occurred in S1, S2 and S3 (Fig. 3.8). There was no clear temporal pattern in the $\delta^{15}N$ values of benthic consumers, although some species presented seasonal differences. For example, the $\delta^{15}N$ values of *H*. *diversicolor* were higher during spring (S2) and summer (S1 and S3) (Fig. 3.8). Amphipods were significantly ^{15}N- depleted in S3 only during winter ($F_{(2,40)}$= 3.59, p=0.04; Fig. 3.8).

Each epibenthic consumer species $\delta^{15}N$ values followed a distinct temporal pattern. In general, *C*. *crangon* and *P*. *flesus* $\delta^{15}N$ were higher during winter-spring and decreased during summer in S1, S2 and S3 (Fig. 3.9; p> 0.05). *P*. *microps* $\delta^{15}N$ values increased towards summer (S2: $H_{(2,46)}$= 16.90, p= 0.002; S3: $H_{(2,39)}$= 6.81, p= 0,03), although a minimum occurred in August 2011 in S1 (Fig. 3.9). *C*. *maenas* were more ^{15}N- enriched during the winter-spring in S1, and in the summer in S2 and S3 (Fig. 3.9; p> 0.05). In the TFW area, *P. flesus* ($H_{(2,72)}$= 11.72, p= 0.003) and *G*. *aculeatus* were more ^{15}N-enriched during the summer (Fig. 3.9).

Table 3.1 Mean (±SD) $\delta^{13}C$ and $\delta^{15}N$ values (‰) for particulate organic matter (POM), microphytobenthos (MPB), macroalgae (mixture of Chlorophyceae and Phaeophyceae), emergent aquatic vegetation (EAV; mixture of plants from the margin), submerged aquatic vegetation (SAV; *Elodea canadensis*) and terrestrial plants collected in the Minho River estuary during winter, spring and summer of 2011 in stations (S) S1, S2, S3 and in the tidal freshwater (TFW) area.

		Source											
		POM		**MPB**		**Macroalgae**		**EAV**		**SAV**		**Terrestrial plants**	
	Station	**$\delta^{13}C$ (‰)**	**$\delta^{15}N$ (‰)**	**$\delta^{13}C$ (‰)**	**$\delta^{15}N$ (‰)**	**$\delta^{13}C$ (‰)**	**$\delta^{15}N$ (‰)**	**$\delta^{13}C$ (‰)**	**$\delta^{15}N$ (‰)**	**$\delta^{13}C$ (‰)**	**$\delta^{15}N$ (‰)**	**$\delta^{13}C$ (‰)**	**$\delta^{15}N$ (‰)**
Winter	S1	-25.4 (±1.1)	5.8 (±0.2)	-18.6 (±0.1)	4.2 (±0.5)	-13.9 (±1.0)	8.5 (±1.0)						
	S2	-26.0 (±0.9)	4.6 (±1.5)	-14.2	7.7	-26.9	5.3	-26.9	5.3				
	S3	-26.9 (±1.0)	4.7 (±2.3)	-22.2 (±0.3)	7.7 (±1.0)								
	TFW	-27.4 (±0.4)	5.9 (±1.0)	-25.6 (±0.4)	8.6 (±2.1)			-28.6	7.3			-27.4	-3.0
Spring	S1	-23.7 (±0.1)	4.4 (±1.5)			-13.9 (±0.0)	8.6 (±0.1)						
	S2	-23.5 (±1.1)	5.2 (±1.1)	-18.3	9.2	-17.6 (±0.4)	9.1 (±1.9)						
	S3	-28.6	6.1	-25.8 (±3.2)	6.3 (±2.2)								
	TFW	-28.0 (±0.1)	5.4 (±1.7)	-27.8 (±1.7)	9.5 (±0.5)					-20.4 (±1.0)	10.0 (±0.7)		
Summer	S1	-21.8 (±2.9)	3.4 (±4.6)	-17.7 (±1.8)	5.0 (±2.3)	-16.9 (±2.9)	6.8 (±0.8)						
	S2	-24.0 (±2.0)	5.5 (±2.7)	-19.9 (±1.8)	8.2 (±1.0)	-18.4 (±1.6)	5.5 (±2.1)	-26.7 (±0.4)	4.4 (±0.3)				
	S3	-24.3 (±1.9)	6.0 (±2.3)	-20.0 (±1.9)	7.1 (±2.5)							-28.2 (±0.4)	3.1 (±1.8)
	TFW	-28.8 (±1.4)	6.8 (±2.1)	-26.3 (±0.7)	9.2 (±0.9)			-28.8 (±0.5)	4.5 (±0.1)	-22.3 (±1.5)	13.7 (±0.4)	-29.1 (±1.5)	1.1 (±2.0)

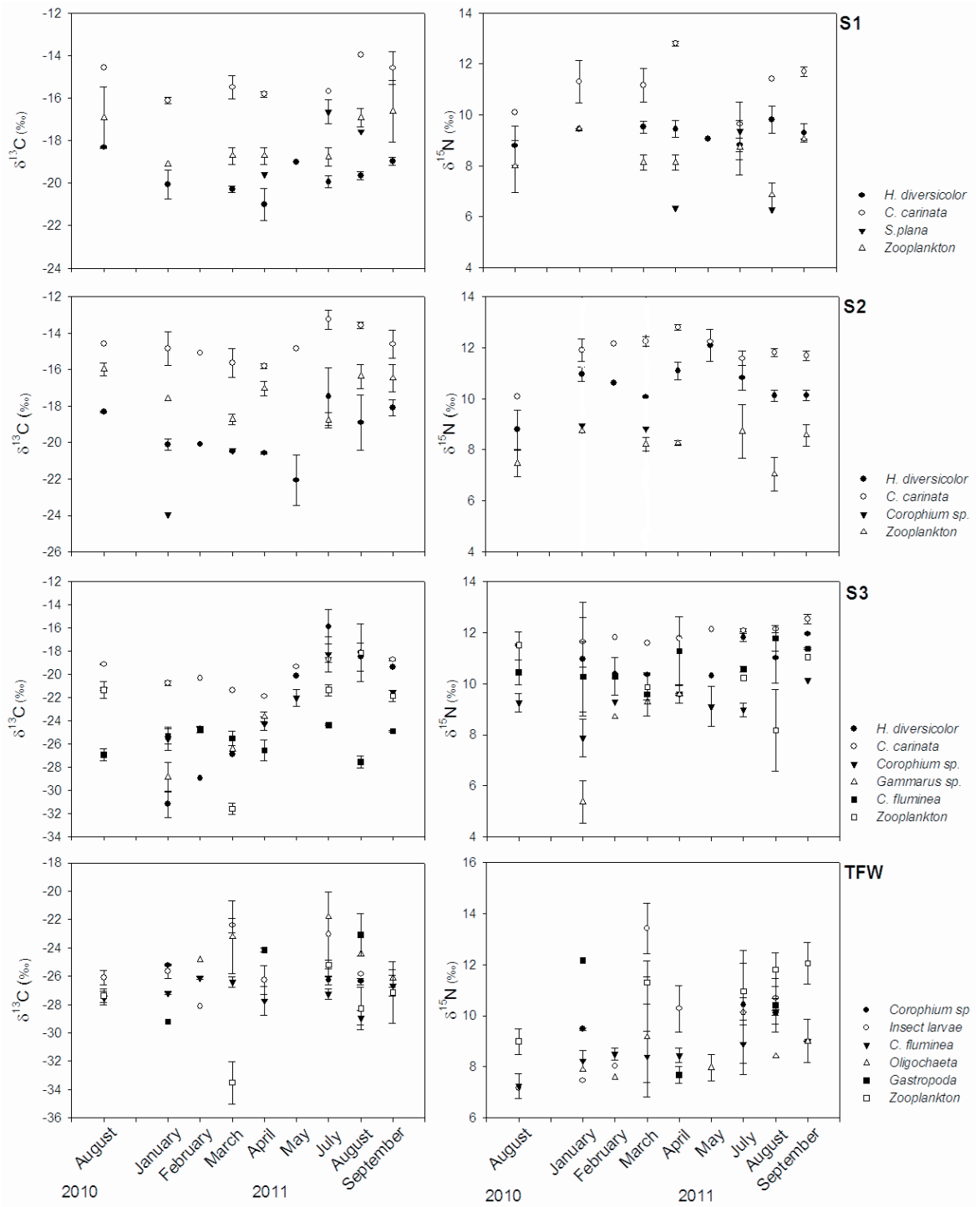

Fig. 3.8 Within-seasonal and inter-annual variation of $\delta^{13}C$ and $\delta^{15}N$ (‰, mean ± SD) for benthic and pelagic consumers, collected in the Minho River estuary in stations (S) S1, S2, S3 and in the TFW area. Note that the y-axis scale differs between plots.

For pelagic and benthic consumers, the highest $\delta^{13}C$ values in S1 and S2 were observed during summer (Fig. 3.8); the difference was statistically significant only for *H. diversicolor* in S2 ($H_{(2,12)}$= 8.13, p= 0.02). The pattern was similar for benthic consumers from S3; $\delta^{13}C$ values of the main functional feeding groups were all significantly different between winter and summer (p< 0.05). However, *C. fluminea* presented a minimum in

August 2011 (-28.9 ± 0.3 ‰) (Fig. 3.8). In the TFW area, $\delta^{13}C$ values varied throughout the sampling period, but were generally more ^{13}C-enriched during summer, with the exception of *C. fluminea*, which was ^{13}C-depleted during this season ($H_{(2,78)}$= 7.84, p=0.02) (Fig. 3.8).

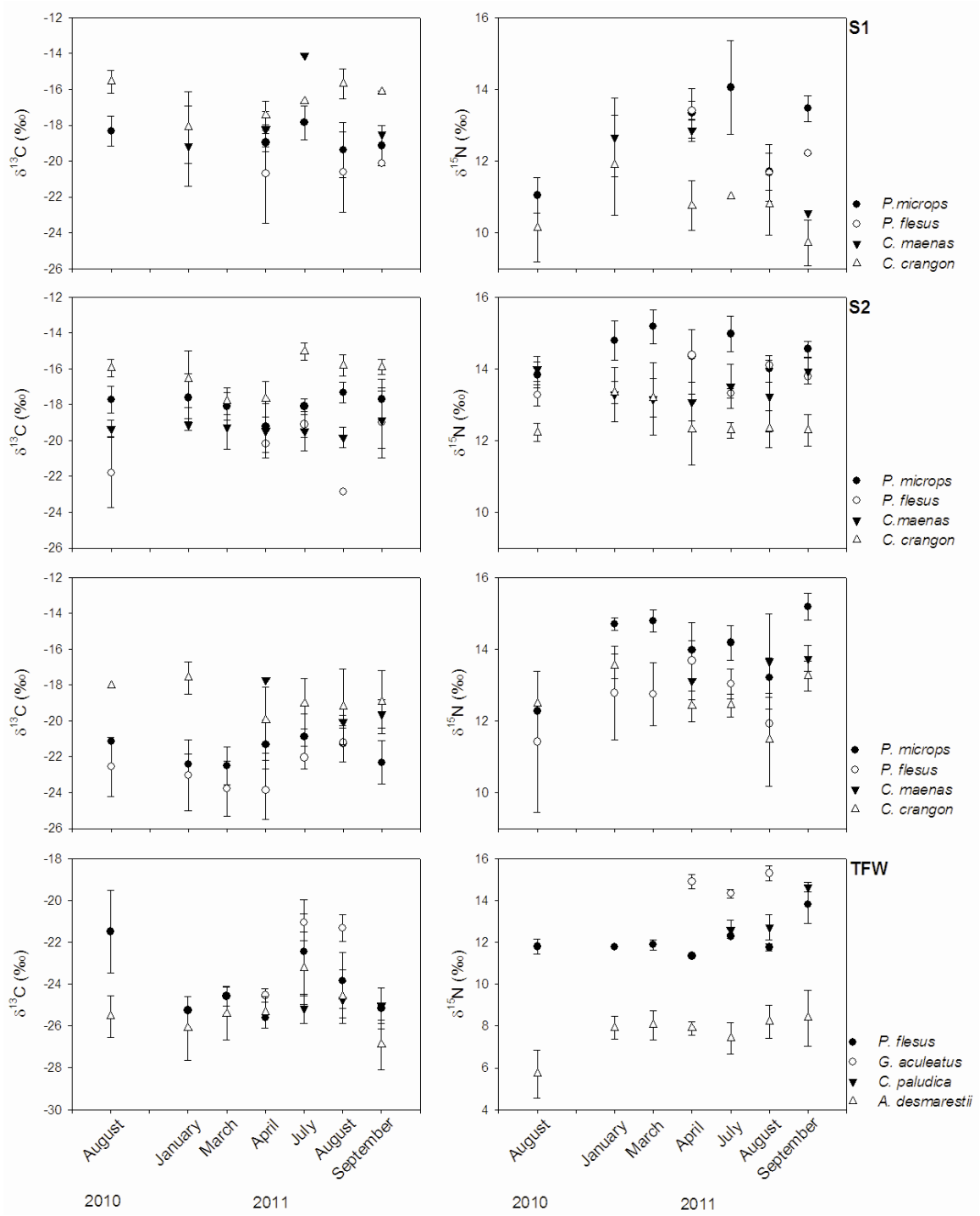

Fig. 3.9 Within-seasonal and inter-annual variation of $\delta^{13}C$ and $\delta^{15}N$ values (‰, mean ± SD) for epibenthic consumers, collected in the Minho River estuary in stations (S) S1, S2, S3 and in the TFW area. Note that the y-axis scale differs between plots.

Generally, epibenthic consumer $\delta^{13}C$ values increased towards the summer, although some exceptions to this overall pattern were observed. For example, *P. microps* (S1) and *P. flesus* (S1, S2) $\delta^{13}C$ values decreased from July to August 2011 (Fig. 3.9).

Although some inter-annual variability was observed for all the functional feeding groups, there were no significant between-year differences for either $\delta^{15}N$ or $\delta^{13}C$ values.

3.3.3 Food web characterization

In S1 and S2, pelagic and benthic consumers relied on a mixture of marine and brackish phytoplankton, macroalgae detritus and benthic OM (MPB and SOM) (Fig. 3.10). Zooplankton and *H*. *diversicolor* apparently consumed phytoplankton, whereas *C*. *carinata* likely consumed a mix of MPB and saltmarsh plants detritus (Fig. 3.10). However, the high $\delta^{15}N$ and $\delta^{13}C$ values of *H*. *diversicolor* (S2) and *C*. *carinata* (S1) during the summer suggest the possibility of a predatory behavior during this period (Fig. 3.10).

In S3, zooplankton low $\delta^{13}C$ values during winter-spring suggest consumption of freshwater phytoplankton flushed from upstream areas. Also, the high $\delta^{13}C$ values of some consumers (e.g. zooplankton, *Corophium* sp.) during summer suggests the contribution of marine POM to the intermediate areas of the estuary (Fig. 3.10). Suspension feeders, such as *C*. *fluminea*, and collectors consumed POM; the $\delta^{13}C$ values of these consumers were similar to $\delta^{13}C_{POC}$ values (ca. -26‰) and also showed the same trend as POM during winter (Figs 3.7B, 3.8). During summer, there was as an apparent shift towards phytoplankton consumption by these suspension feeders and collectors (Fig. 3.10).

In the TFW area, benthic consumers presented similar $\delta^{13}C$ and $\delta^{15}N$ values during winter-spring, suggesting that they were feeding on a similar mixture of sources, including POM and MPB. Phytoplankton was not likely a major contributor to their biomass during this period, because benthic consumers were too ^{13}C-enriched to rely on freshwater phytoplankton (> 5‰) and too ^{13}C- depleted to rely on estuarine phytoplankton (ca. 5‰) (Fig. 3.10). We were not able to resolve which OM sources were being used by pelagic consumers, because their $\delta^{13}C$ values fell outside the range of all the sources sampled during this study. During summer, the $\delta^{13}C$ and $\delta^{15}N$ values of consumers, such as zooplankton, insect larvae and *C*. *fluminea*, indicate that they were feeding on POM

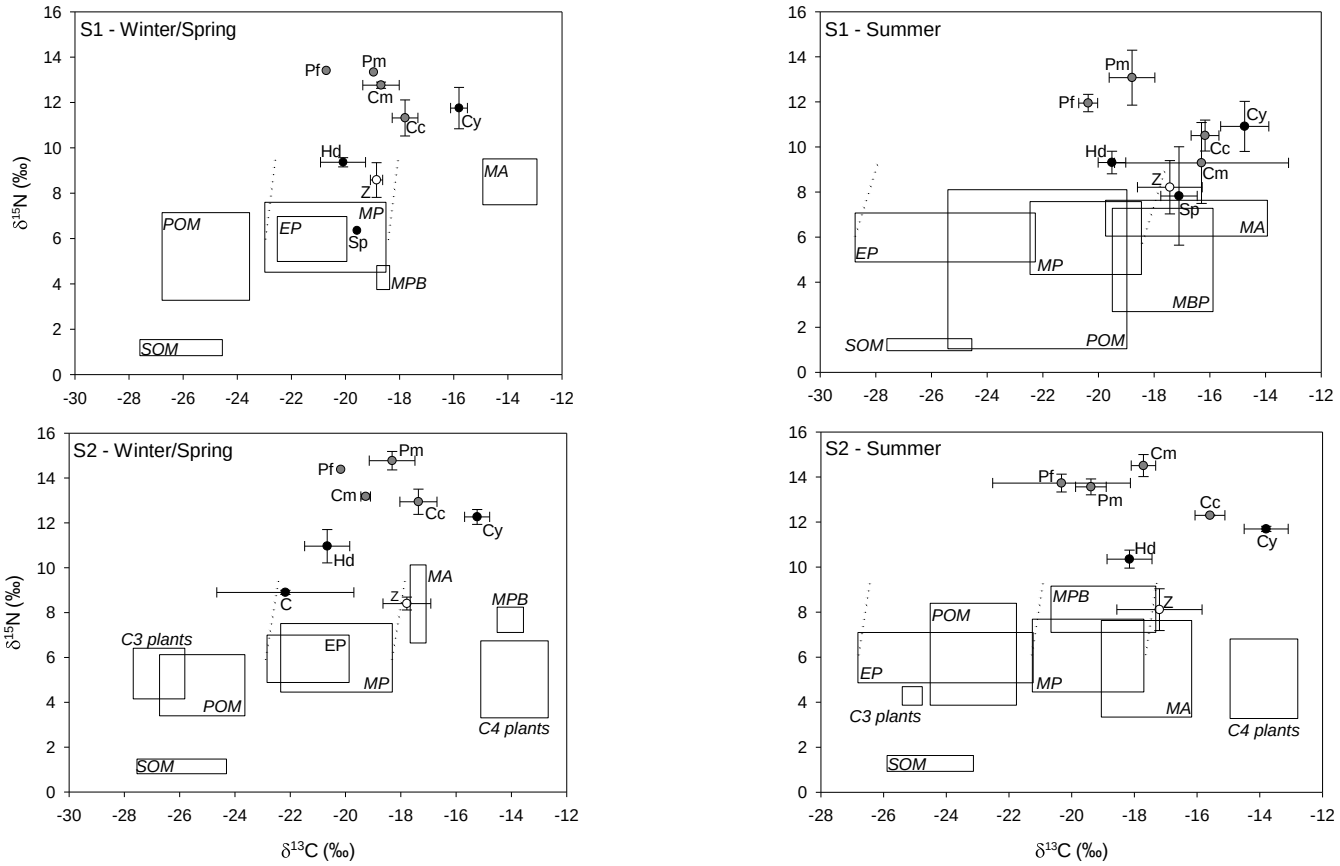

Fig. 3.10 Average (±SD) $\delta^{15}N$ and $\delta^{13}C$ values (‰) of pelagic (white symbols), benthic (black symbols) and epibenthic consumers (grey symbols) in the brackish (S1-S3) and freshwater (TFW) areas of the Minho River estuary during winter-spring and summer 2011. The consumers shown are: zooplankton (Z), *Scrobicularia plana* (Sp), *Hediste diversicolor* (Hd), *Cyathura carinata* (Cy), *Corophium* sp. (C), *Corbicula fluminea* (Cf), Insect larvae (In), Oligochaeta (O), Gastropoda (G), *Crangon crangon* (Cc), *Carcinus maenas* (Cm), *Atyaephyra desmaresti* (Ad), *Pomatoschistus microps* (Pm), *Platichthys flesus* (Pf), *Gasterosteus aculeatus* (Ga) and *Cobitis paludica* (Cp). Boxes represent the ranges for the sources collected during this study, and also the estimates values for C4 saltmarsh plants and phytoplankton (see text). The dotted lines indicate the expected stable isotope ratio of consumers utilizing phytoplankton, assuming typical trophic fractionation (+0.4‰ $\delta^{13}C$ and +3.4‰ $\delta^{15}N$ per trophic level).

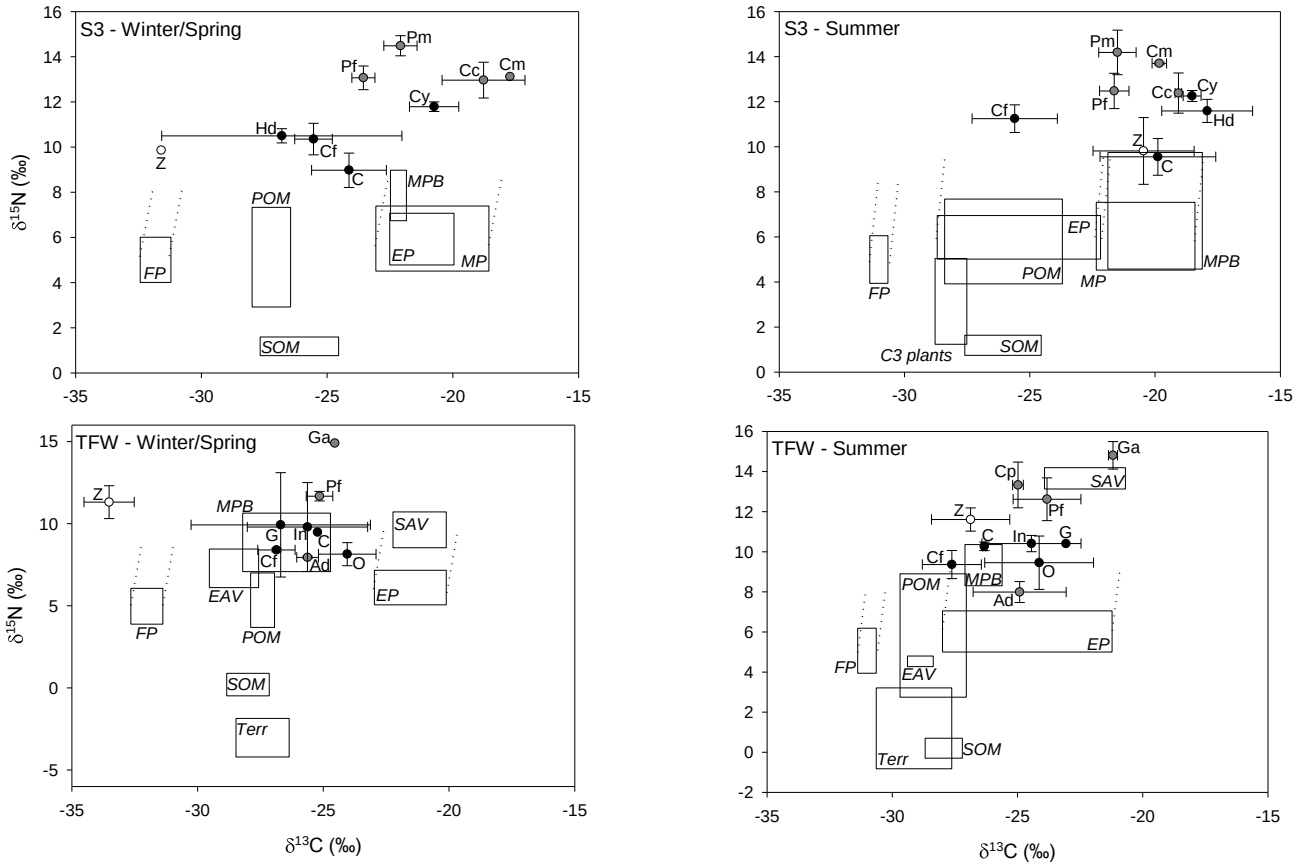

Fig. 3.10 (continuation) Average (±SD) $\delta^{15}N$ and $\delta^{13}C$ values (‰) of pelagic (white symbols), benthic (black symbols) and epibenthic consumers (grey symbols) in the brackish (S1-S3) and freshwater (TFW) areas of the Minho River estuary during winter-spring and summer 2011. The consumers shown are: zooplankton (Z), *Scrobicularia plana* (Sp), *Hediste diversicolor* (Hd), *Cyathura carinata* (Cy), *Corophium* sp. (C), *Corbicula fluminea* (Cf), Insect larvae (In), Oligochaeta (O), Gastropoda (G), *Crangon crangon* (Cc), *Carcinus maenas* (Cm), *Atyaephyra desmaresti* (Ad), *Pomatoschistus microps* (Pm), *Platichthys flesus* (Pf), *Gasterosteus aculeatus* (Ga) and *Cobitis paludica* (Cp). Boxes represent the ranges for the sources collected during this study, and also the estimates values for C4 saltmarsh plants and phytoplankton (see text). The dotted lines indicate the expected stable isotope ratio of consumers utilizing phytoplankton, assuming typical trophic fractionation (+0.4‰ $\delta^{13}C$ and +3.4‰ $\delta^{15}N$ per trophic level).

largely comprised of phytoplankton ($C:N_{POM}$ ~7). The $\delta^{13}C$ values of these consumers were similar to $\delta^{13}C_{POC}$ values and also followed the same trend as POC (Figs. 3.7B, 3.8).

The benthic primary consumers were the most relevant prey for the epibenthic secondary consumers analyzed, except for *C*. *crangon*, according to the SIAR mixing model results Overall, pelagic consumers (PC) were the most important prey for *C*. *crangon* in S1 and S2, with the highest values (95% CI) in April (S2: 39.5-87.2%) and August 2011 (S1: 54.3-93.4%; S2: 43.3-61.2%) (Fig. 3.11). Inter-annual variability was observed in S2. In August 2010, PC were the source with the lowest contribution (0.0-27.3%) to *C*. *crangon* biomass, whereas the contribution of the suspension feeder (SF/C) *H*. *diversicolor* (12.3-60.5%) and of the deposit feeder (Cl/C) *C*. *carinata* (34.8-64.6%) increased, when compared to August 2011 estimates (Fig. 3.11). In S3, collectors (Cl) were the most important prey for *C*. *crangon*, followed by PC, and their importance decreased throughout summer (Fig. 3.11), in opposition to SF/C and Cl/C.

The most important prey for *C*. *maenas* and *P*. *microps* in S1 and S2 were the SF/C, but the proportion of this source varied monthly (Figs. 3.12, 3.13). In S3, the most important prey identified for *C*. *maenas* were the Cl (20.3-65.5%) and SF/C (1.3-58.2%) (Fig. 3.12). For *P*. *microps*, the Cl/C were the most important prey during winter, decreasing towards summer (Fig. 3.13).

We ran two models for flounder, one for the brackish estuary (S1-S3) and another for the TFW area (S4-S5), because we noticed that some flounders $\delta^{13}C$ and $\delta^{15}N$ values did not represent the expected stable isotope ratios for the station where they were collected. For instance, some flounders collected in S5, which is the uppermost station, had $\delta^{13}C$ values between -24‰ and -22‰, which are similar to those shown by *P*. *microps* collected in S3 (13 km downstream). Therefore, we selected the flounders that had stable isotope ratios (especially $\delta^{13}C$) similar to those from other less mobile species, such as *P*. *microps* for the brackish water stations and *C*. *paludica* (*A*. *desmarestii* in the absence of *C*. *paludica*) for the TFW area. Thus, in the brackish estuary, the main prey of *P*. *flesus* were the benthic invertebrates, especially collectors, with its importance decreasing during summer. The opposite trend occurred for PC and SF/C (Fig. 3.14). Overall, the most important prey for *P*. *flesus* were the shredders (S) in the TFW area, with the highest contributions in March (68.1-90.9%) and August 2011 (46.7-89.6%), followed by the Cl and benthic grazers (BG). The PC were only relevant in July 2011 (1.7-49.8%) (Fig. 3.14).

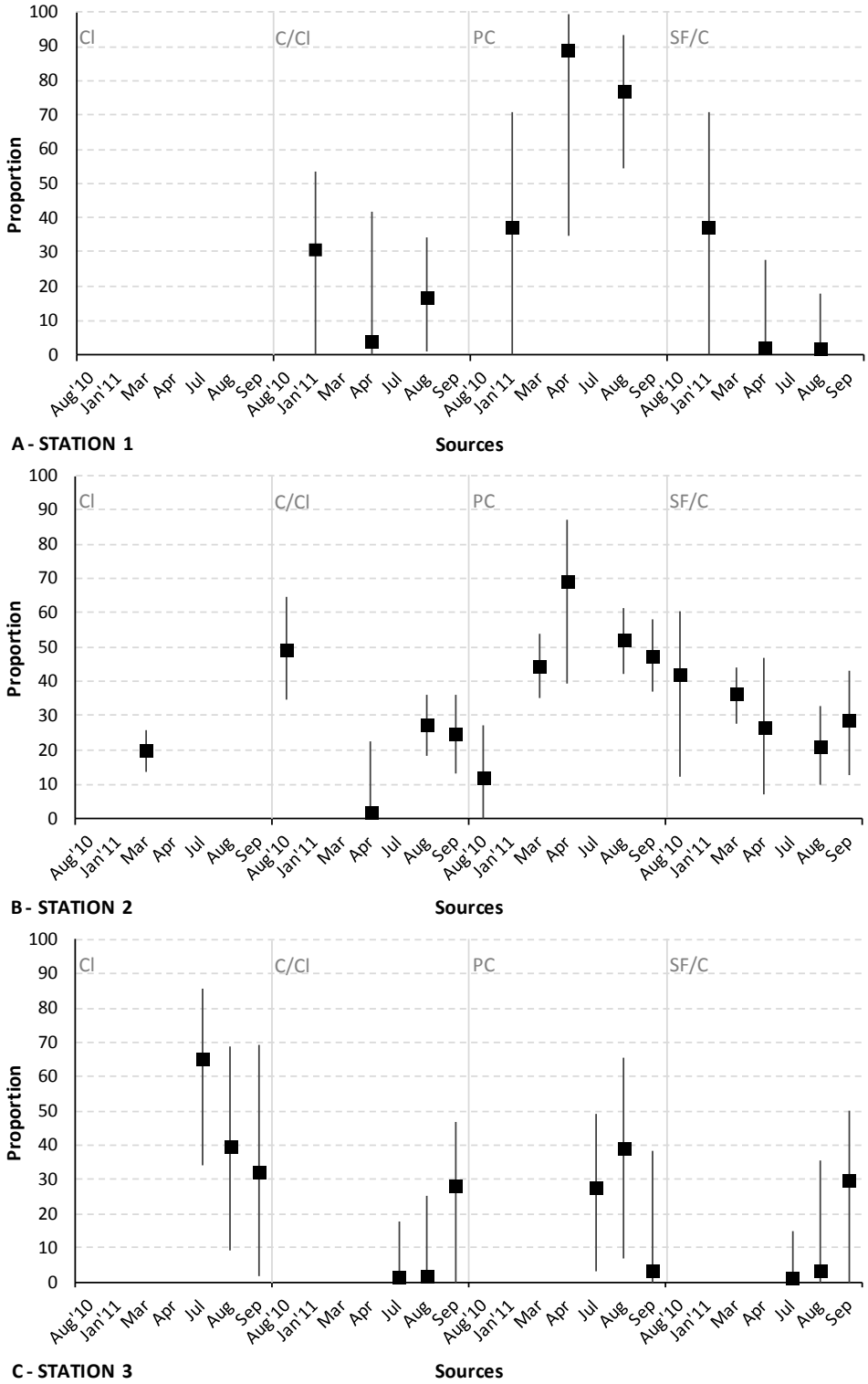

Fig. 3.11 Proportion of each food source to *Crangon crangon* biomass in stations 1, 2 and 3 in August 2010 (August'), and January-April 2011, July-September 2011, based on the stable isotope mixing model results. The food sources included in the model were the pelagic consumers (PC), suspension feeders and carnivorous (SF/C), collectors and carnivorous (Cl/C), and collectors (Cl). Data presented include the most likely solution (mode) and the 95% Bayesian credibility intervals.

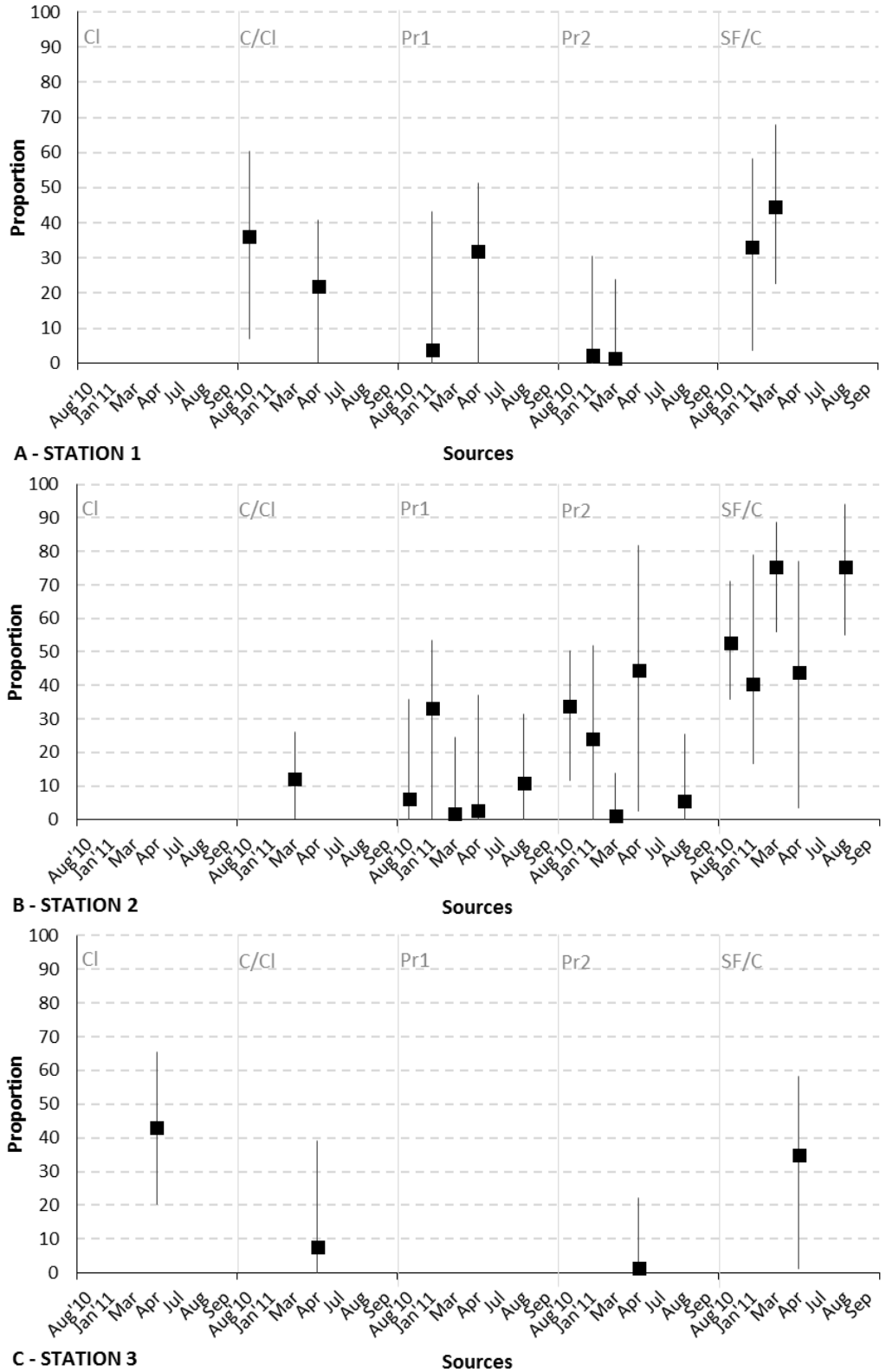

Fig. 3.12 Proportion of each food source to *Carcinus maenas* biomass in stations 1, 2 and 3 in August 2010 (August'), and January-April 2011, July-September 2011, based on the stable isotope mixing model results. The food sources included in the model were the suspension feeders and carnivorous (SF/C), collectors and carnivorous (Cl/C), collectors (Cl), crustacean predators (Pr1), and fish predators (Pr2). Data presented include the most likely solution (mode) and the 95% Bayesian credibility intervals.

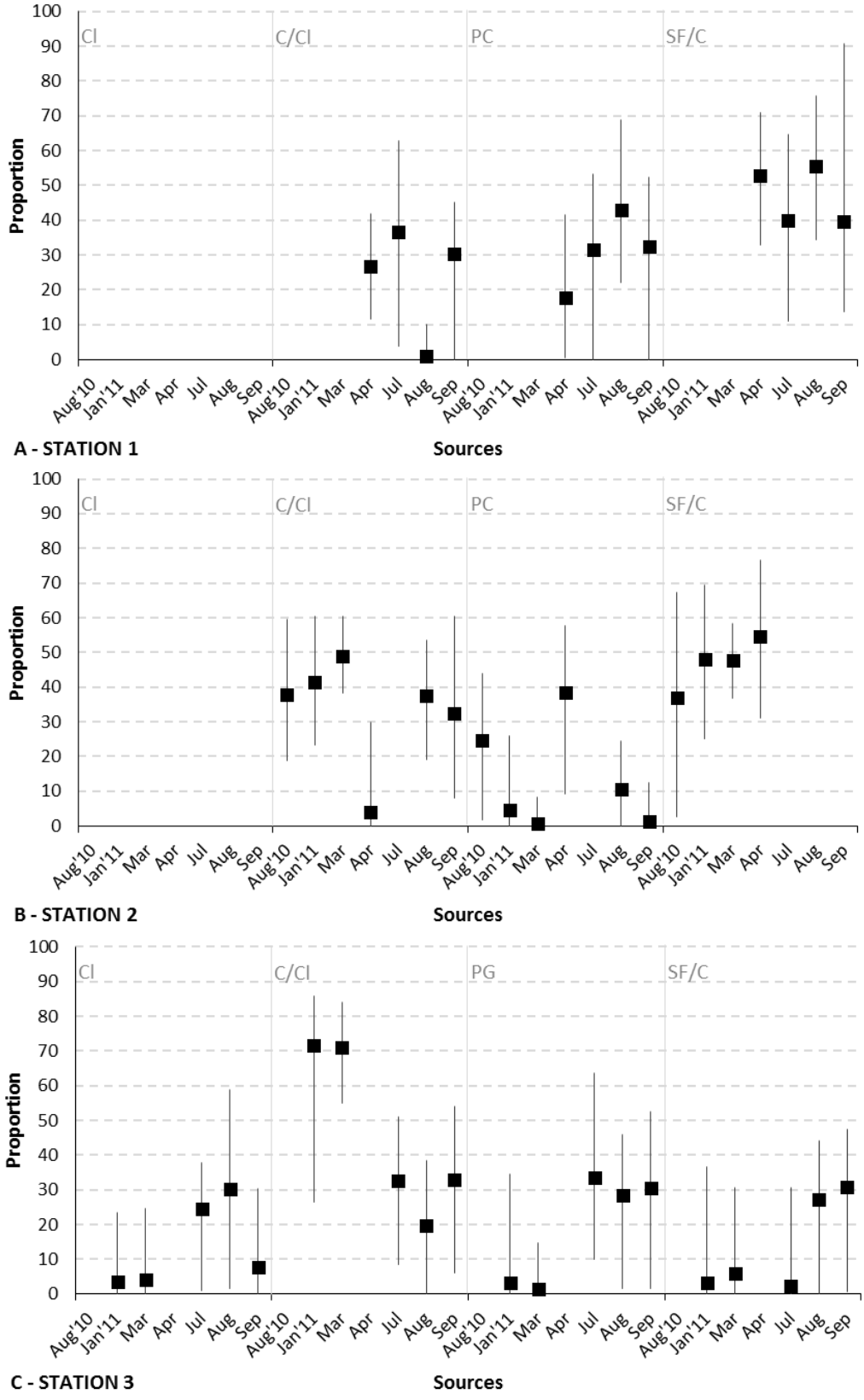

Fig. 3.13 Proportion of each food source to *Pomatoschistus microps* biomass in stations 1, 2 and 3 in August 2010 (August'), and January-April 2011, July-September 2011, based on the stable isotope mixing model results. The food sources included in the model were the pelagic consumers (PC), suspension feeders and carnivorous (SF/C), collectors and carnivorous (Cl/C), and collectors (Cl). Data presented include the most likely solution (mode) and the 95% Bayesian credibility intervals.

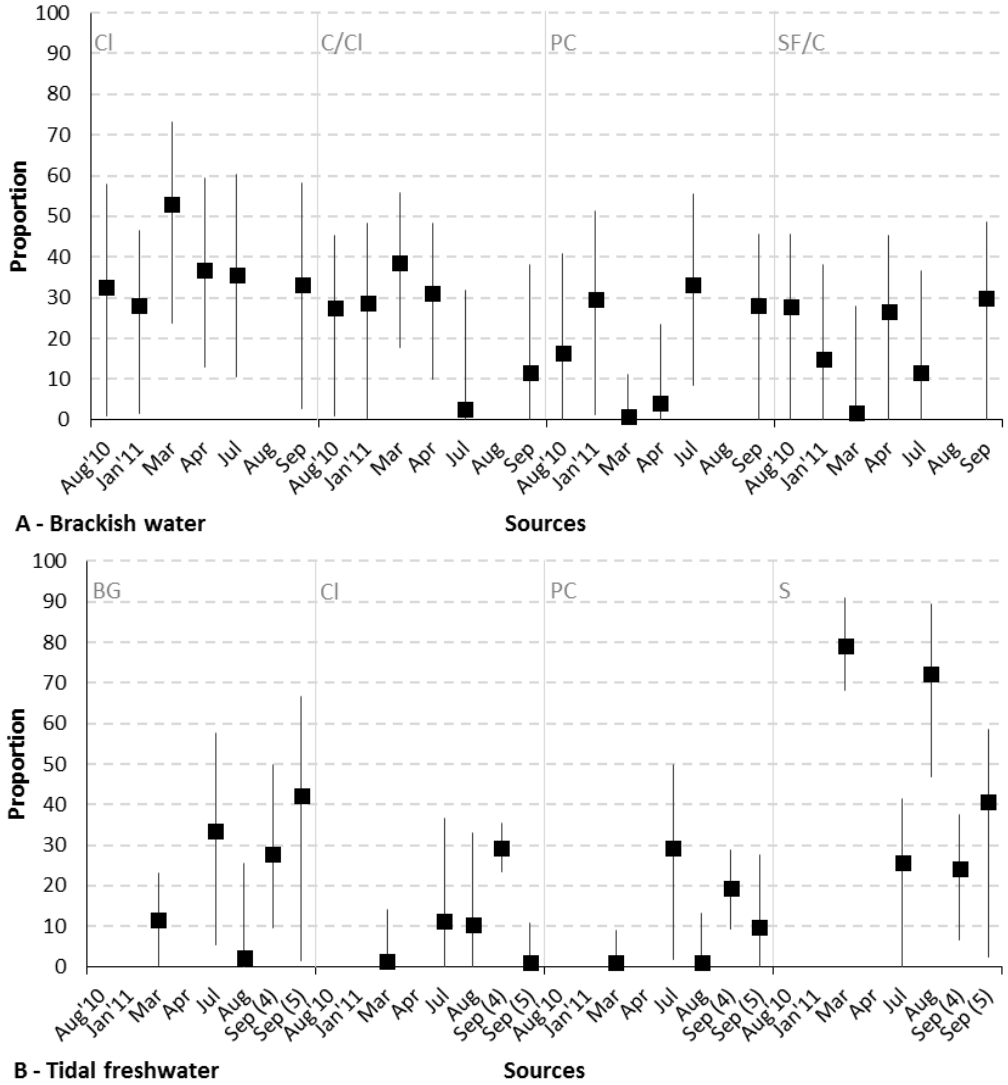

Fig. 3.14 Proportion of each food source to *Platichthys flesus* biomass in the brackish and TFW portions of the Minho River estuary in August 2010 (August'), and January-April 2011, July-September 2011, based on the stable isotope mixing model results. The food sources included in the model were the collectors (Cl), collectors and carnivorous (Cl/C), pelagic consumers (PC), suspension feeders and carnivorous (SF/C), benthic grazers (BG), and shredders (S). Data presented include the most likely solution (mode) and the 95% Bayesian credibility intervals. [4] and [5] refer to the results from the isotope mixing model for stations 4 and 5, respectively, due to the existence of significant differences between sessile (*Corbicula fluminea*) or less mobile (*Atyaephyra desmaresti*) stable isotope values in August 2011.

3.4 Discussion

This study provides evidence for spatial and temporal variability in the River Minho estuarine food web. Spatial variability was essentially related with the characteristics of the organic matter sources available in each area, which was also influenced by the proximity to other ecosystems, namely higher terrestrial influence in the tidal freshwater portion and higher marine influence in the brackish portion of the estuary. Temporal changes in the stable isotope ratios of consumers were driven essentially from changes

occurring at the base of the food web, although there were also some evidences for a combination with changes in the use of resources by the consumers. The possible mechanisms behind the spatial and temporal variability are discussed below.

3.4.1 Spatial heterogeneity of estuarine food web components

During this study, a large variability in the isotopic composition of primary producers was observed along the salinity gradient in the Minho River estuary, with $\delta^{15}N$ values ranging from -3.0‰, to 14.0‰, and $\delta^{13}C$ values ranging from -31.5‰ to -12.8‰. The variability in the $\delta^{13}C$ values of primary producers were essentially related with differences in the source of carbon dioxide used. Commonly, C uptake by C3 plants, on land, involves a net fractionation of about 21‰ between the atmosphere (-7‰) and plant biomass resulting in $\delta^{13}C$ values of ca. -28‰ (Peterson and Fry 1987). Primary producers uptaking C from solution will display more variable $\delta^{13}C$ values owing to the higher variability in sources of dissolved CO_2, which include carbonate rock weathering, atmosphere or respired OM (Peterson and Fry 1987). In consequence of that variability, several studies showed a direct relationship between dissolved inorganic carbon (DIC) $\delta^{13}C$ values and salinity, where the mixing between riverine DIC ^{13}C-depleted with the marine DIC ^{13}C-enriched, creates a gradient in the $\delta^{13}C_{DIC}$ values along the estuarine mixing gradient (Chanton and Lewis 2002, Fry 2002). Thus, the primary producers, that obtain inorganic carbon from solution, will be influenced by the portion of the estuary where they were collected.

However, the composition of the OM pool available in each portion of the estuary was also different, and contributed to the observed variability. Marine phytoplankton and macroalgae (^{13}C-enriched) were only available in the brackish portion of the estuary, while in the TFW there was an increasing contribution of vascular material, which included aquatic and terrestrial vegetation (in general, ^{13}C-depleted). River discharge also influenced the quality of the POM available in each portion of the estuary. During high river discharge periods, the contribution of terrestrial-derived OM increased in the TFW, and during low river discharge, marine intrusion was detected up to station S3, which likely explains some of the highest $\delta^{13}C_{POC}$ values observed ($\delta^{13}C$: -24‰ to -22‰) as ^{13}C-enriched marine POM ($\delta^{13}C$ ca. -20‰, typical of marine phytoplankton; MacMahon 2013) mixed with ^{13}C-depleted estuarine POM.

Primary consumers were ^{13}C-enriched towards the mouth of the estuary, reflecting the variability in the OM sources available in each station. In the TFW, terrestrial-derived OM was likely an important contributor to pelagic and benthic consumers, especially

during winter. In the brackish estuary, marine phytoplankton (or MPOM) was an important energy source, especially during summer owing to high marine intrusion.

Another factor influencing both $\delta^{15}N$ and $\delta^{13}C$ values was the habitat (pelagic vs. benthic) from where primary consumers obtained their energy. Primary consumers relying on benthic or detrital C, such as *C*. *carinata* or insect larvae, were generally more ^{15}N- and ^{13}C- enriched than those that relied mainly on OM sources in the pelagic pathway, such as zooplankton or *H*. *diversicolor*. One plausible explanation is related with the existence of a diffusive boundary layer at the sediment-water interface that reduces isotopic fractionation (France 1995).

The between-habitat differences in the stable isotope ratios of primary consumers appear to influence the isotopic composition of both crustacean and fish epibenthic consumers, which were also ^{13}C-enriched towards the river mouth. Despite these differences, SIAR mixing results indicate that *P. microps* and *C*. *maenas* relied essentially on benthic filter feeding organisms (i.e. polychaetes), regardless of the station where they were collected. However, *C. crangon* and *P. flesus* presented some differences according to the station where they were collected. Based on stable isotope ratios, *C*. *crangon* was feeding on ^{13}C-enriched and ^{15}N-depleted sources in S1 and S2 (zooplankton and/or *H.diversicolor*), and on ^{13}C- and ^{15}N-enriched sources in S3 (collectors and/or carnivorous benthic consumers). SIAR mixing model results suggest that *C*. *crangon* was supported essentially by pelagic primary consumers in S1 and S2, and by benthic primary consumers (collectors) in S3, shifting from a general pelagic pathway to an increase reliance on benthic pathway upriver. Prey abundance may explain the differences observed between stations, because gut content analyses indicated that epibenthic predators, like *P*. *microps*, *C*. *crangon* or *C. maenas,* are opportunistic carnivore that select prey based on relative availability (Phil 1985). However, to the best of our knowledge, thorough studies are missing on the relative abundance of zooplankton and benthic macroinvertebrates along the salinity mixing gradient in the Minho River estuary.

Platichthys flesus relied mostly on the detrital pathway, by feeding on collectors in the brackish portion of the estuary, and on shredders in the TFW portion of the estuary. Collectors essentially feed on fine POM (0.5µm-1 mm) from a great variety of resources including coarse POM (> 1 mm) fragmentation, periphyton, phytoplankton and microorganisms (Henriques-Oliveira et al. 2003). Shredders feed on coarse POM, which includes leaves from the riverine vegetation and macrophytes (Henriques-Oliveira et al. 2003).

3.4.2 Temporal variability in the estuarine food web

Seasonal changes were observed in the stable isotope ratios of POM, MPB and SAV. Although cluster analysis did not reveal any clear seasonal pattern in the stable isotope ratios of consumers, there was an overall trend for ^{13}C-enrichment during summer.

Temporal variation in C and N isotope ratios of OM sources were expected to occur due to hydrologic variability and to annual senescence of vascular plants. Cloern et al. (2002) found high variability (approximately 5‰ to 10‰) in monthly $\delta^{13}C$ and $\delta^{15}N$ values within some plant groups, associated with annual cycles of growth and senescence in the San Francisco Bay estuary. We cannot describe well the temporal variability in isotopic composition of aquatic plants because they were sampled according to habitat (i.e. aquatic, emergent or submerged), and thus a species-specific analysis it is not possible. However, seasonal variability was observed in the stable isotope ratios of MPB and POM. MPB were ^{13}C-enriched during summer (except in S2), suggesting that benthic phytoplankton was using a ^{13}C-enriched marine dissolved inorganic pool, which can be associated with a higher marine intrusion occurring under low river discharge periods.

The $\delta^{13}C_{POC}$ values increased towards summer in brackish portion of the estuary, while the opposite pattern was observed in stations located at the TFW portion of the estuary. During summer, river discharge declined, which decreases terrestrial inputs and increases residence time, favoring the accumulation of living and detrital phytoplankton (Hoffman and Bronk 2006). Our results suggest that during summer, and especially in August 2011, there was an increase in the contribution of phytoplankton to the POM pool. The concentration of Chl *a* increased during summer, peaking in August 2011 in the TFW stations (up to 8 times) and in the middle of the estuary (up to 6 times), suggesting the occurrence of a phytoplankton bloom during this month. Moreover, the $\delta^{13}C_{POC}$ values in the TFW ($\delta^{13}C_{POC}$: -30‰) were similar to those estimated for freshwater phytoplankton in this estuary ($\delta^{13}C$: -31‰; unpublished) and the $C:N_{POM}$ lower than 10, which suggests a decrease in the contribution of terrestrial-derived OM to the POM pool. The opposite occurred during winter, when the river discharge increased. The $\delta^{13}C_{POC}$ values ($\delta^{13}C$: -28‰ to -24‰) and $C:N_{POM}$ ratio (> 10) suggest a substantial contribution of terrestrial-derived OM to the POM pool, advected from upland or riparian habitats or both (Hedges et al. 1997, Hoffman et al. 2008). The low Chl *a* concentrations (0.8 ± 0.8 µg L^{-1}) during this period also suggests that the contribution of phytoplankton to the POM pool decreased owing to the suppression of primary production as a consequence of increased turbidity and rapid flushing rates (Sin et al. 1999, Hoffman and Bronk 2006).

The temporal variability of the POM pool was reflected in the stable isotope ratios of suspension feeders and collectors, suggesting that estuarine benthic food webs assimilate terrestrial-derived OM. This finding is in opposition to results obtained in other estuarine ecosystems that have shown little or no dependence on terrestrial OM (e.g. Deegan and Garritt 1997). However, our results are parallel to those from benthic food webs in the Yura estuary (Japan), where the contribution of terrestrial OM supporting benthic organisms was up to 80% in the upper estuary (Antonio et al. 2012). During low phytoplankton availability, *C*. *fluminea* can consume terrestrial-derived OM, thereby functioning as an important link between terrestrial and estuarine ecosystems (Dias et al. in press). Moreover, benthos feeding of microbially-mediated terrestrial OM may have a significant impact on the use of decomposed terrestrial organic materials because bacterial colonization and growth can improve the quality of POM, even for terrestrial-derived detritus (Edwards,1987), and has the potential to enhance terrestrial-derived material transfer in aquatic food webs (Zeug and Winemiller, 2008).

The hydrological changes were mirrored along the food web, with pelagic, benthic and epibenthic consumers presenting higher $\delta^{13}C$ values during summer. This fact suggests that the variability of the consumers stable isotope ratios were influenced by changes occurring at the base of the food web. The $\delta^{15}N$ values of consumers did not follow any clear pattern, however some consumers (*H*. *diversicolor*, *C*. *carinata*, *P*. *microps*) had higher $\delta^{15}N$ values during summer.

The temporal variability in the stable isotope ratios of some primary consumers could also have been influenced by changes in the feeding strategies. *Cyathura carinata* had higher $\delta^{15}N$ values (ca. 6‰) than average MPB $\delta^{15}N$ values, which was the most ^{15}N-enriched source sampled, during summer in S3. The difference in the $\delta^{15}N$ values of *C*. *carinata* and MPB were almost 2 trophic levels (assuming typical fractionation of +3.4‰ for $\delta^{15}N$ and +0.4‰ for $\delta^{13}C$ (Post 2002)), we suggest two possible explanations: 1) all the available OM sources were not sampled, or 2) *C*. *carinata* is preying on other consumers (Wägele et al. 1981). A similar reasoning can be applied to *H. diversicolor*. Stable isotope ratios of *H*. *diversicolor* were high during summer in S3. As filter feeding organisms, their $\delta^{15}N$ values should be similar to those of *C*. *fluminea* or zooplankton, but a difference of 2‰ and 4‰ was detected, respectively. Thus, our results suggest that *H. diversicolor* could also be a predator, as an alternative feeding strategy.

Overall, the temporal variability in the stable isotope ratios of epibenthic consumers was not followed by changes in the relative contribution of the different functional feeding groups to their biomass, suggesting temporal trophic stability of the estuarine epibenthic food web. Nonetheless, an increase in the proportion of pelagic consumers to the *P. microps*' biomass was observed during summer in S1 and S3.

This study provides evidence for spatial and temporal variability in the River Minho estuarine food web, which was essentially influenced by the characteristics of the OM sources available in each area: marine POM and macroalgae were only available in the brackish portion of the estuary, and vascular-derived OM was more relevant for the consumers in the TFW. There are also clear evidences that there is a strong connectivity between different components of the estuarine food web: between pelagic and benthic food webs, and also between estuarine, terrestrial and marine ecosystems. Primary consumers had a crucial role in connecting the different food web components. Our stable isotope ratios data suggest that primary consumers have the ability to use both pelagic (phytoplankton) and detrital pathways. Moreover, suspension feeders from the brackish portion of the estuary, such as zooplankton, polychaetes and bivalves, were supported by marine POM. On the other hand, suspension feeders and collectors in the TFW, relied on a POM pool that was increasingly comprised in terrestrial-derived OM during the winter, thus linking the terrestrial and the estuarine ecosystems.

Hydrology played an important role in food web dynamics. During high river discharge periods, the $\delta^{13}C_{POC}$ and $C:N_{POM}$ values suggest an increase of terrestrial-derived OM to the POM pool, which was then used by suspension feeders. On the other hand, during low river discharge periods, marine intrusion increased upriver, which was detected in the stable isotope values of pelagic and benthic consumers. Although epibenthic consumers stable isotope ratios varied spatially and temporally, the main pathways from which they acquire energy did not. Benthic consumers were the main sources supporting the epibenthic predators analyzed, with the exception of *C*. *crangon*, which was essentially feeding in the pelagic food web. Although we cannot conclude about the consequences of a mixture between pelagic and benthic pathways to the estuarine secondary production, the epibenthic predators analyzed showed some of the highest densities found for these species in European estuaries (Souza et al. 2013a, Souza et al. 2013b).

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Chapter 4

Linking terrestrial and benthic estuarine ecosystems: organic matter sources supporting the high secondary production of a non-indigenous bivalve

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Abstract

The Asian clam, *Corbicula fluminea*, is among the most pervasive invasive species in freshwater ecosystems worldwide. Our objective was to study *C*. *fluminea*'s functional response in terms of feeding behavior and food selectivity, using the natural variation in organic matter (OM) sources that occur in estuarine environments. Using C and N stable isotopes, we identified and quantified the contribution of different OM sources supporting the production of *C*. *fluminea* along the salinity gradient occupied in the Minho River estuary (NW-Iberian Peninsula, Europe), where this species presently dominates the benthic macrofauna biomass. We observed a pronounced shift in the quality of OM available for *C. fluminea* along the estuarine mixing zone. Stable isotope analysis, POM C/N, and phytoplankton contribution estimates based on C: Chl *a* revealed that POM was largely comprised of terrestrial-derived OM in freshwater stations (TFW) and was increasingly comprised of phytoplankton, a more palatable food source, towards the polyhaline estuary. A similar shift in the isotopic composition along the estuarine mixing zone was observed in *C*. *fluminea*, suggesting a shift in food resources. Accordingly, based on a Bayesian stable isotope mixing model, there was an upstream-downstream counter gradient in the contribution to *C. fluminea* biomass from terrestrial-derived OM (41-64% in TFW) and phytoplankton (29-55% in the brackish estuary). Although the majority of the food sources identified were filtered from the water column (70-80%), reliance on sediment OM and microphytobenthos provided evidence for deposit feeding by *C*. *fluminea*. We conclude that *C*. *fluminea* has the ability to adapt to environments with low food quality because it can consume terrestrial-derived OM. This can be a competitive adaptation in systems with perennial low food quality such as the Minho River estuary. Moreover, its ability to couple benthic and pelagic environments and terrestrial ecosystems demonstrates a strong potential to alter food web flows in aquatic ecosystems.

Keywords: *Corbicula fluminea*, stable isotopes, benthic food web, Minho estuary.

4.1 Introduction

The Asian clam, *Corbicula fluminea,* is among the most widespread invasive species in freshwater ecosystems (Sousa et al. 2008a). Native to Southeast Asia, this species has dispersed worldwide. It was reported for the first time in Europe in the early 1980s (Mouthon 1981) and presently is spread throughout the continent (e.g. Araujo et al. 1993, Morais et al. 2009, Munjiu and Shubernetski 2010, Caffrey et al. 2011).

Corbicula fluminea can cause several ecological and economic impacts: it can compete with and displace native mollusc populations (Hakenkamp and Palmer 1999, Vaugh and Hakenkamp 2001), influence biogeochemical cycles (Phelps 1994, Cherry et al. 2005, Cooper et al. 2005) and biofoul human water systems (Darrigran 2002). For these reasons, it has been listed as one of the 100 worst invasive species in Europe (DAISIE 2008). However, *C. fluminea* can also benefit benthic species; shell production can increase substrate availability for attachment and refugia (Gutiérrez et al. 2003), and *C. fluminea* faeces and pseudo-faeces may constitute a food resource for other organisms (Howard and Cuffey 2006, Gergs et al. 2011).

Its high salinity tolerance (up to salinities of 10-14) enables the clam to colonize both freshwater and brackish habitats (Morton and Tong 1985) within estuaries. High densities have been documented in numerous estuaries: 4,185 clams m^{-2} in the Minho River estuary (Sousa et al. 2005), >5,000 clams m^{-2} in the Paraná River delta (Boltovskoy et al. 1995), and 11,142 clams m^{-2} in the Mondego River estuary (Franco et al. 2012). The invasion success of this species has been attributed to their life history characteristics, including rapid individual growth, early sexual maturity, high fecundity, broad environmental tolerance, wide genetic variability and phenotypic plasticity (Morton 1997).

Corbicula fluminea has numerous feeding strategies and this allows it to compete with native species through multiple trophic pathways. This species has high filtering capacity (0.3-10 $m^3.m^{-2}.d^{-1}$; Strayer et al. 1999), can non-selectively suspension feed (Way et al. 1990, Boltovskoy et al. 1995), and has the ability to deposit feed when little planktonic food is available (Hakenkamp and Palmer 1999; Vaugh and Hakenkamp 2001). Previous studies revealed that *C. fluminea* can select components present within seston but has a low trophic fidelity (Atkinson et al. 2010), which probably favours its successful invasion.

In estuaries, characterizing the food sources of *C. fluminea* is complicated by the spatial and temporal complexity of estuarine food webs (Canuel et al. 1995, Deegan and Garritt 1997). Estuarine food webs can incorporate organic matter (OM) inputs from many sources, including riparian vegetation, submerged and emergent aquatic vegetation (and associated epiphytic algae), as well as phytoplankton and microphytobenthos produced in

situ (Cloern et al. 2002, Hoffman and Bronk 2006). Carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) stable isotope analysis is a powerful tool to characterize energy flow through estuarine food webs (Pasquaud et al. 2007, Hoffman et al. 2008). For consumers, the stable isotope composition ($^{13}C/^{12}C$ and $^{15}N/^{14}N$) of tissues is a time-integrated signal of the food sources in the ecosystem that were incorporated into an organism's structural components and energy reserves (Peterson and Fry 1987). Thus, the stable isotope ratio of a consumer reflects its diet, demonstrating an average trophic fractionation (i.e., the difference between the consumer and its diet) of +0.4‰ $\delta^{13}C$ and +3.4‰ $\delta^{15}N$ per trophic level (Post 2002). The C and N stable isotope ratios of upland plants, marsh vegetation, and freshwater and estuarine algae differ with respect to C and N source and method of C fixation (Goerike et al. 1994, Lajtha and Marshall 1994). The riparian plants that use the C3 pathway have a $\delta^{13}C$ of about -28‰ because there is an uptake fractionation of about -21‰ over atmospheric CO_2 ($\delta^{13}C$ -7‰) (Smith and Epstein 1971). In contrast, C4 plants (e.g. *Spartina* spp.) are more enriched in the heavy isotope ($\delta^{13}C$ -13‰) owing to reduced fractionation (Smith and Epstein 1970, Fry and Sherr 1984). Freshwater and estuarine phytoplankton will also differ where they utilize isotopically distinct pools of dissolved inorganic carbon (DIC). As with C3 vascular plants, their ^{13}C fractionation is about -21‰, but can shift with DIC concentration, phytoplankton growth rate, and nutrient availability (Goericke et al. 1994). In freshwater ecosystems, where the DIC $\delta^{13}C$ is highly depleted (lower than -10‰), phytoplankton may also be distinguished from riparian vegetation (Hoffman and Bronk 2006). In general, microphytobenthos (MPB) are more ^{13}C-enriched than phytoplankton due to the existence of a diffusive boundary layer at the sediment-water interface that reduces isotopic fractionation (France 1995). The N stable isotope composition can also help to separate ^{15}N-depleted terrestrial OM ($\delta^{15}N$ -4‰ to 4‰) from ^{15}N-enriched aquatic OM sources ($\delta^{15}N$ 6‰ to 10‰) (Peterson and Fry 1987, Cloern et al. 2002). The C/N ratios can aid source discrimination. For example, higher plant tissues are characteristically carbon-rich (C/N: 20-500) compared to algae (C/N ~ 7) owing to the predominance of N-free biomacromolecules (e.g. lignin, tannin, hemicellulose, cellulose, cutin, and suberin) over proteins (C/N: 3-4) in higher plants (Hedges et al. 1986). However, this distinction can be blurred as vascular plant tissues preferentially gain N during microbial decay, and as plankton preferentially lose N versus C during decay (Hedges et al.1997 and references therein).

The objective of this research was to study *C. fluminea*'s functional response in terms of both feeding behavior and food selectivity by using the natural variation in available OM sources that occurs in an estuarine environment over the salinity gradient occupied by *C. fluminea*. We studied this species in the Minho River estuary (NW-Iberian Peninsula, Europe), where it represents more than 90% of the total benthic macrofauna

biomass and is present at high densities (up to 4,185 clams m^{-2}). In 2005, its production was estimated to be about 465 g ash free dry weight (AFDW) m^{-2} $year^{-1}$ (Sousa et al. 2005, 2008b). Because *C*. *fluminea* has high filtering capacity (Strayer et al. 1999), we hypothesized that where phytoplankton contribution to the suspended OM pool was high, *C*. *fluminea* would respond by preferentially deriving energy from this high-quality food source; however, where the available suspended OM quality was low (e.g. due to high contributions of detritus derived from terrestrial OM) or quantity limited, *C*. *fluminea* would increase the relative proportion of deposit feeding. To test our hypothesis, we identified potential OM sources to the food web and quantified their respective contributions to the production of *C*. *fluminea* along the estuarine mixing zone in the Minho River estuary using C and N stable isotope analysis. The impact that an invasive species may have in an ecosystem is related to its abundance or biomass (or both; Parker et al. 1999, Ricciardi 2003). Thus, knowledge about the feeding strategies that are utilized by this highly abundant population can further our understanding of the underlying mechanisms by which *C*. *fluminea* can alter energy flows in an invaded ecosystem.

4.2 Methods

4.2.1 Study area

The Minho River is located in the NW-Iberian Peninsula (SW Europe; Fig. 4.1). The annual average discharge is 300 m^3 s^{-1} (Ferreira et al. 2003). Its watershed is 17,080 km^2, of which 95% is located in Spain and 5% in Portugal. The river is 343 km long; 76 km serves as the northwestern Portuguese-Spanish border (Antunes et al. 2011). The limit of tidal influence is about 40 km inland, and the uppermost 30 km are a tidal freshwater wetland (TFW; Sousa et al. 2008b). Its estuary has an area of 23 km^2, of which only 9% is intertidal. The estuary is mesotidal with tides ranging between 0.7 m and 3.7 m (Alves, 1996). The mean depth of the estuary is 2.6 m and the maximum depth is *ca*. 26 m (Antunes et al. 2011). Due to its ecological importance, the Minho River estuary and the international section of the River Minho were designated as a Natura 2000 site (EIONET 2012) and as an Important Bird Area (BirdLife International 2012).

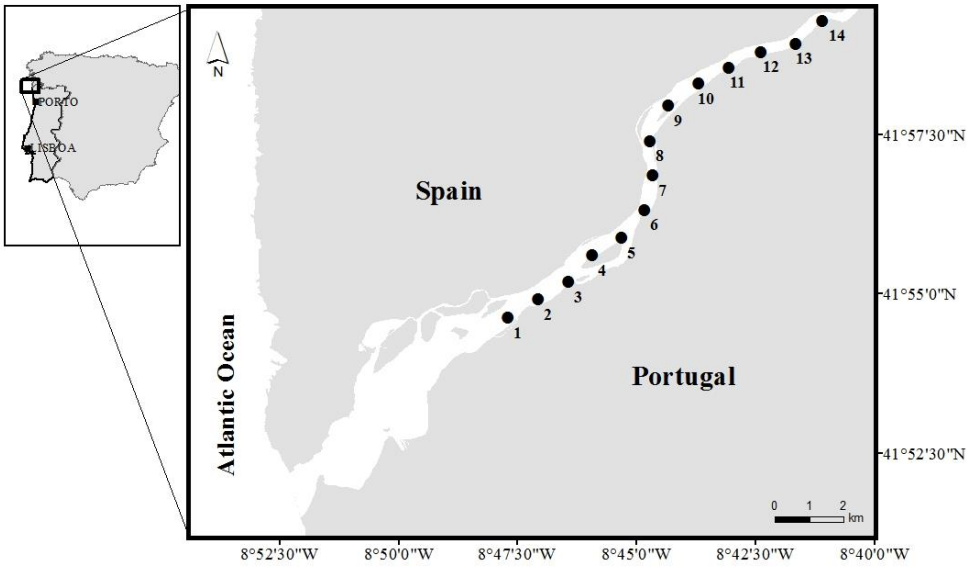

Fig. 4.1 Location of the sampling stations along the Minho River estuary.

4.2.2 Field sampling

In September 2010, 14 stations located at 1 kilometer intervals along the main river channel were sampled during full-moon spring tide. The first and the last sampling stations were located at 8 km and 21 km away from the river mouth, respectively (Fig. 4.1). These stations encompass the distribution range of *C. fluminea* along the brackish and tidal freshwater areas. Sampling was conducted at a small spatial scale to guarantee a proper characterization of the estuarine mixing zone. We chose to sample in September, near the end of the annual low flow period (typically, from July through October; SNIRH 2012), for a number of reasons. First, salt water intrusion is maximized, which favors the connectivity between the estuarine and marine environments. Second, residence time is elongated during low flow, which potentially allows phytoplankton biomass to accumulate. Third, without pronounced and varying riverine inputs, biogeochemical variability is reduced, which results in reduced variability in the OM sources and isotopic character of suspended particles (Hoffman and Bronk 2006).

Based on the feeding modes of *C. fluminea* (e.g. Boltovskoy et al. 1995, Hakenkamp and Palmer 1999), the OM sources of interest were phytoplankton (freshwater, estuarine and marine), microphytobenthos (MPB), sediment organic matter (SOM), and particulate organic matter (POM; a mixture of OM inputs to the estuary). We directly sampled MPB, SOM and POM, but used a proxy for phytoplankton (using DIC $\delta^{13}C$; $\delta^{15}N$ values were obtained from POM in a similar cruise done in August 2011) since it is difficult to isolate algae from the complex bulk POM present in estuarine environments.

At each station, surface (50-100 cm below the surface) and bottom water samples (0.5 m off the bottom) were collected using a 2-L Niskin bottle. From these samples, we measured the concentration of chlorophyll *a* (Chl *a*: μg L^{-1}), concentration and isotopic composition of POM (mg L^{-1}, $\delta^{13}C_{POC}$, $\delta^{15}N_{PN}$, molar C/N), and isotopic composition of total dissolved inorganic carbon (ΣCO_2: $\delta^{13}C_{DIC}$). Salinity was measured with an YSI model 6820 QS probe and reported using the Practical Salinity Scale.

Replicate POM and Chl *a* water samples (POM: 1L, Chl *a*: 0.5L) were pre-filtered with a 150-μm sieve and filtered onto a pre-combusted (500 ºC for 2 h) Whatman GF/F and Whatman GF/C filters, respectively , and kept frozen (-20 ºC) until analysis.

To measure $\delta^{13}C_{DIC}$ values, replicate water samples were injected (6 mL) into a 10 mL exetainer (Labco Limited), containing 0.5 ml of phosphoric acid 85% (v/v), with a sterile syringe filter coupled with a 0.2 μm cellulose acetate membrane acrodisc. Samples were kept cooled during field sampling and then stored at 4 ºC until analysis.

MPB samples were collected at three stations situated across the salinity gradient (stations 1, 8 and 14). At each station, one PVC pipe (5 cm diameter; 50 cm length) was fixed in the sediment and left in the estuary for two weeks. At the end of the period, algae colonizing at the sediment interface were scraped from each pipe into a separate vial, stored on ice, and returned to the laboratory where we applied the same procedure used for POM samples.

At each station, we used a van Veen grab to collect *C. fluminea* specimens and sediment. Ten clams per station were randomly selected for analysis (140 specimens total). Both *C. fluminea* specimens and SOM samples were kept frozen (-20 ºC) until analysis of sediment organic carbon (SOC) $\delta^{13}C$ values and nitrogen (SN) $\delta^{15}N$ values.

4.2.3 Laboratory analyses

Filters for POM and MPB analysis were fumigated with concentrated HCl to remove inorganic carbonates, rinsed with deionized water, placed in a sterile Petri dish, and dried at 60 ºC for 24 h (Lorrain et al. 2003). Sediment samples were rinsed with 10% HCl (also to remove carbonates), rinsed with deionized water, and dried at 60 ºC for 48h. Both acidification methods are expected to produce only slight changes in sample $\delta^{15}N$ values (*ca*. 0.4‰; Lorrain et al. 2003, Carabel et al. 2006).

The shell length of each *C. fluminea* specimen was measured (± 0.01 mm). Then, the foot was excised, dried in an oven at 60 ºC and ground to a fine powder with a mortar and pestle for stable isotope analysis.

Stable isotope ratios were measured using a Costec 4010 EA and Thermo Delta Plus XP isotope ratio mass spectrometer (IRMS) (United States Environmental Protection Agency, Mid-Continent Ecology Divison, Duluth, Minnesota). Stable isotope ratios are reported in δ notation, δX: $\delta X = (R_{sample}/R_{standard} - 1) \times 10^3$, where X is the C or N stable isotope, *R* is the ratio of heavy: light stable isotopes, and Pee Dee Belemnite and air are standards for $\delta^{13}C$ and $\delta^{15}N$, respectively. The analytical error, the mean SD of replicate reference material, was ±0.1‰ for $\delta^{13}C$ and $\delta^{15}N$. To control for sample processing quality, we did not include those *C. fluminea* samples with a SD between replicates (i.e. two sub-samples of the same sample) >0.2‰ $\delta^{13}C$ or $\delta^{15}N$ in subsequent data analyses. DIC $\delta^{13}C$ was measured using a GasBench II system interfaced to a Delta V Plus IRMS (Davis Stable Isotope Facility, University of California). The analytical error was ±0.2‰ $\delta^{13}C$.

Filters processed for Chl *a* analysis were extracted in 90% acetone and analyzed on a Spectronic 20 Genesys spectrophotometer. Chl *a* concentration was calculated following Lorenzen (1967).

4.2.4 Data analyses

We used a segmented linear regression to investigate if there was a continuum of *C. fluminea* $\delta^{13}C$ and $\delta^{15}N$ values with respect to salinity or clam size. Segmented linear regression analysis performs distinct linear regressions to determine values in the x-axis that are smaller or greater than a given breakpoint. The selection of the breakpoint and function type (among 6 options) is based on maximizing the statistical coefficient of explanation, and performing tests of significance (Oosterbaan 1994). A range of breakpoint values are analyzed. The breakpoint with the highest coefficient of explanation is selected. We conducted the segmented regression analyses using SegReg software (Waterlog 2012). If a breakpoint was identified between either stable isotope ratio ($\delta^{13}C$ and $\delta^{15}N$ values were analyzed separately) and salinity or shell length, two (or more) groups were defined based on the independent variable (i.e., salinity, shell length).

The segmented linear regression analysis did not identify a breakpoint with respect to clam size; however, a positive relationship was found between clam size and the stable isotope values. We then tested the differences between *C. fluminea*'s stable isotope values collected in areas subjected to different salinity regimes (the lower and upper estuary, as identified by the SegReg analysis) using an analysis of covariance (ANCOVA). The test was performed using salinity as the grouping variable (upper and lower estuary groups) and shell length as a covariate. Statistical analyses were performed using STATISTICA 7 software.

To quantify OM source contributions to *C. fluminea* tissue, we used a dual-stable isotope mixing model. The mixing model estimates a proportional contribution for each food source, assuming all sources sum to 1. However, the model equations are indeterminate when there are more than *n + 1* sources relative to stable isotopes (i.e., more than three sources for two stable isotopes). Because there were more than three potential food sources in the estuary, we used a stable isotope mixing model that uses Bayesian inference to solve the indeterminate equations and produce a probability distribution that represents the likelihood a given source contributes to the consumer (Parnell et al. 2010). The model, Stable Isotope Analysis in R (SIAR) allows each of the sources and the trophic enrichment factor (TEF; or trophic fractionation) to be assigned as a normal distribution, rather than a single datum (Parnell et al. 2010). We used the SIAR package (Stable Isotope Analysis in R; Parnell et al. 2010) which is part of the open source statistical language R (R Development Core Team 2007). SIAR will produce a range of feasible solutions to the mixing problem to which are assigned credibility intervals (CI), analogous to the confidence intervals used in frequentist statistics (in this study, 95% CI; Parnell et al. 2010). SIAR also includes a residual error term. For modelling purposes, the estuary was divided into two portions based on evidence from the segmented regression analysis: upper and lower estuary.

We estimated the average (± SD) $\delta^{13}C$ and $\delta^{15}N$ values for the various OM sources differently for each OM source. At each station, the phytoplankton $\delta^{13}C$ ($\delta^{13}C_{phytoplankton}$) value was estimated from the DIC $\delta^{13}C$ ($\delta^{13}C_{DIC}$) value, assuming an uptake fractionation of -21‰ (i.e., $\delta^{13}C_{phytoplankton} = \delta^{13}C_{DIC}$-21‰; Peterson and Fry 1987 and references there in). However, isotopic fractionation can vary with DIC concentration, phytoplankton growth rate, and nutrient availability (Mook and Tan 1991). To corroborate the DIC-based estimates, we used an independent method to estimate $\delta^{13}C_{phytoplankton}$ values; this alternative method was shown to yield values similar to isolated algae samples (Marty and Planas 2008). A two source, single stable isotope mixing model was used to estimate $\delta^{13}C_{phytoplankton}$ by correcting particulate organic carbon $\delta^{13}C$ ($\delta^{13}C_{POC}$) for algal biomass based on Chl *a* concentration (C:Chl *a*= 80; Marty and Planas 2008):

$$\delta^{13}C_{POM} = x \cdot (\delta^{13}C_{phytoplankton}) + (1 - x) \cdot (\delta^{13}C_a)$$

where *x* represents the proportion of algal carbon in the POM pool and $\delta^{13}C_a$ = -27.0‰, which is the typical value for C3 plants in the TFW portion of the estuary, for the TFW portion and $\delta^{13}C_a$ = -20‰ for marine phytoplankton in the polyhaline estuary (McMahon et al 2013). The proportion of algal carbon in the POC pool was calculated according to the modified relationship from Wienke and Cloern (1987) in Canuel et al. (1995). For this

second method, we estimated the $\delta^{13}C_{phytoplankton}$ value for only the most upriver TFW and most seaward polyhaline stations to best meet the two source mixing model assumption that only two OM sources contribute to the POM pool (TFW: phytoplankton and terrestrial OM; polyhaline: marine and estuarine phytoplankton).

For both portions of the estuary, the average $\delta^{13}C_{phytoplankton}$ value used in the SIAR mixing model was the mean of station-specific average bottom $\delta^{13}C_{phytoplankton}$ values (based on station replicates) for those stations within each portion. We obtained $\delta^{15}N_{phytoplankton}$ values for the SIAR mixing model from POM sampled during a replicate sampling effort conducted in August 2011 at all stations where the POM samples were comprised of nearly 100% phytoplankton (unpublished data) based on phytoplankton C:Chl *a* and Chl *a* and POC concentrations (Canuel et al 1995, Marty and Planas 2008). For the model, the value used in either portion of the estuary was the mean station-specific average $\delta^{15}N_{phytoplankton}$ values for those stations within each portion.

The average MPB, bottom POM, and SOM $\delta^{13}C$ and $\delta^{15}N$ values were calculated from all the values for those stations within each portion (upper and lower estuary). For MPB, the upper estuary portion included stations 8 and 14; the lower estuary portion included station 1.

For each station, stable isotope data from 5-10 clams (total, 111 clams) were included in the analyses. The *C. fluminea* $\delta^{13}C$ values were corrected for lipid content, since lipids are depleted in ^{13}C compared to protein and carbohydrates (DeNiro and Epstein 1977). Variability in lipid content can bias bulk tissue $\delta^{13}C$ values, and then cause dietary or habitat shifts to be incorrectly interpreted. We corrected *C. fluminea* muscle tissue data for lipid content using tissue C/N following the mass balance correction for fish muscle tissue proposed by Hoffman and Sutton (2010; Eq. 6), which uses estimates of $C:N_{protein}$ and $\Delta\delta^{13}C_{lipid}$ that are similar to those from the muscle tissue found for other fish (e.g. Sweeting et al. 2006) and taxonomic groups (e.g. shrimp and zooplankton; Fry et al. 2003, Smyntek et al. 2007). For the SIAR mixing model, we adjusted the $\delta^{13}C$ and $\delta^{15}N$ values for one trophic level. As no *C. fluminea* specific TEF values were available, we used TEF estimates from Post (2002; +0.4 ± 1.3 ‰ $\delta^{13}C$, +3.4 ± 1.0 ‰ $\delta^{15}N$).

4.3 Results

4.3.1 Environmental data

Stations 1-6 were brackish and stations 7-14 were within the tidal freshwater (TFW) portion of the estuary (Fig. 4.2). Saltwater intrusion was detected up to 12 km from the river mouth. Freshwater stations were well mixed.

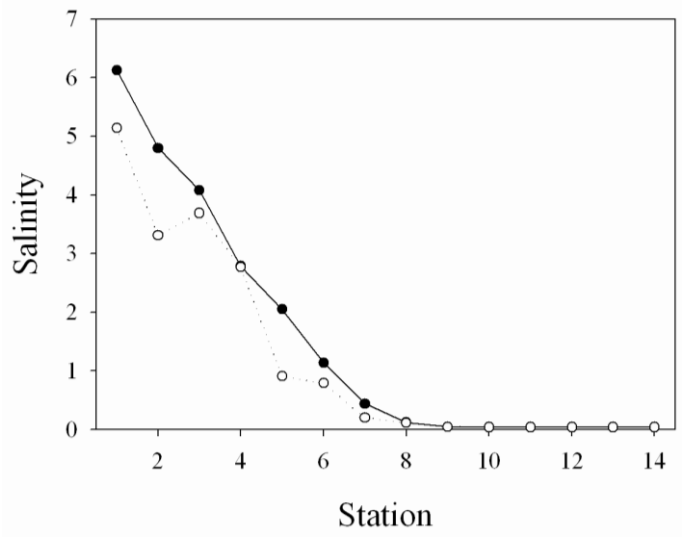

Fig. 4.2 Surface (open circles) and bottom (closed circles) salinity measured along the Minho River estuary, September 2010. The first and the last sampling stations were located at 8 km and 21 km away from the river mouth, respectively.

Chlorophyll *a* (Chl *a*) (average [± SD] of bottom and surface samples combined: 0.72 ± 0.23 µg L^{-1}) and particulate organic carbon (POC: average [± SD] of bottom and surface samples combined: 0.35 ± 0.02 mg L^{-1}) concentrations were low (Table 4.1).

Estimates of the phytoplankton contribution to the bulk POC pool in the brackish portion ranged from 4% to 47%, with an average (± SD) of 23.7 ± 16.6 % in bottom and 18.7 ± 5.3 % in surface water samples (Table 4.1). In the TFW, the contribution of phytoplankton to the bulk POC pool ranged from 5% to 36%, with an average (± SD) of 9.7 ± 8.9 % in bottom and 20.2 ± 22.5 % in surface water samples (Table 4.1).

Table 4.1 Mean water quality in the estuarine mixing zone of the Minho river (including salinity, chlorophyll *a* (Chl *a*), $\delta^{13}C$ value of total dissolved inorganic carbon ($\delta^{13}C_{DIC}$)), as well as the mean character of water column POM samples (including particulate organic carbon (POC) concentration (mg L^{-1}), POM carbon: nitrogen molar ratios (C/N), and phytoplankton fraction (F_{phyto}; %) in POM samples). Average $\delta^{13}C$ and $\delta^{15}N$ values are reported for the organic matter sources measured, including particulate organic matter (POM: POC, PN), phytoplankton (phyto), microphytobenthos (MPB) and sediment organic matter (SOM: SOC, SN). The stations located in either the tidal freshwater area (TFW; stations 5 to 14) or brackish (stations 1 to 4) and polyhaline estuary (marine; first 3 km away from the mouth) were pooled and the means calculated. Values in parentheses represent one standard deviation.

		Salinity	Chl *a* (µg.L^{-1})	$\delta^{13}C_{DIC}$ (‰)	POC (mg L^{-1})	$\delta^{13}C_{POC}$ (‰)	$\delta^{15}N_{PN}$ (‰)	C/N_{POM}	F_{phyto} (%)	$\delta^{13}C_{phyto}$ (‰)	$\delta^{13}C_{MPB}$ (‰)	$\delta^{15}N_{MPB}$ (‰)	$\delta^{13}C_{SOC}$ (‰)	$^{15}N_{SN}$ (‰)
Surface	**TFW**	0.07 (±0.06)	0.63 (±0.31)	-17.7 (±0.5)	0.32 (±0.09)	-28.6 (±1.0)	3.6 (±0.9)	10.1 (±0.6)	20.2 (±22.5)	-38.7 (±0.5)				
	Brackish	2.77 (±1.68)	1.00 (±1.31)	-13.3 (±2.1)	0.37 (±0.03)	-27.2 (±1.0)	5.8 (±1.0)	8.4 (±1.3)	18.7 (±5.3)	-34.3 (±2.1)				
	Marine	18.56 (±9.80)	1.25 (±0.63)	-4.6 (±2.3)	0.35 (±0.09)	-24.9 (±1.5)	3.7 (±0.9)	7.9 (±0.9)	30.4 (±20.8)	-25.6 (±2.3)				
Bottom	**TFW**	0.11 (±0.14)	0.80 (±1.17)	-17.9 (±0.7)	0.37 (±0.19)	-27.8 (±1.3)	4.9 (±0.9)	10.4 (±1.1)	9.7 (±8.9)	-38.9 (±0.7)	-24.0 (± 2.7)	7.3 (± 2.2)	-28.0 (± 0.8)	0.2 (± 2.5)
	Brackish	3.50 (±1.85)	0.46 (±0.23)	-11.7 (±2.4)	0.35 (±0.19)	-27.4 (±1.3)	5.3 (±0.7)	8.7 (±1.2)	23.7 (±16.6)	-32.7 (±2.4)	-18.7 (± 5.2)	7.8 (± 1.1)	-26.1 (± 1.5)	1.7 (± 0.3)
	Marine	31.74 (±2.24)	0.37 (±0.16)	-0.9 (±0.2)	0.35 (±0.29)	-20.2 (±1.3)	4.5 (±0.6)	8.8 (±0.4)	21.1 (±25.5)	-21.9 (±0.2)				

4.3.2 Organic matter sources stable isotope values

Microphytobenthos (MPB) $\delta^{13}C$ and $\delta^{15}N$ values were higher in brackish than in TFW stations. In the brackish portion, the average $\delta^{13}C_{MPB}$ (± SD) was -18.7 ± 5.2 ‰ and the average $\delta^{15}N_{MPB}$ (± SD) was 7.8 ± 1.1 ‰. In the TFW portion, the average $\delta^{13}C_{MPB}$ (± SD) was -24.0 ± 2.7 ‰ and the average $\delta^{15}N_{MPB}$ (± SD) was 7.3 ± 2.2 ‰ (Table 4.1).

Dissolved inorganic carbon (DIC) was ^{13}C-depleted in the TFW portion and increasingly ^{13}C-enriched toward the river mouth, following the mixing of marine and fresh water (Table 4.1). Based on $\delta^{13}C_{DIC}$ values, we estimated the average $\delta^{13}C_{phytoplankton}$ value (± SD) in the TFW portion as -38.9 ± 0.7‰ for bottom water samples and -38.7 ± 0.5 ‰ for surface water samples (i.e., $\delta^{13}C_{DIC}$-21‰; Table 4.1). In the brackish estuary, the average $\delta^{13}C_{phytoplankton}$ value (± SD) would be -32.7 ± 2.4 ‰ for bottom and -34.3 ± 2.1 ‰ for surface water samples (Table 4.1).

The $\delta^{13}C_{phytoplankton}$ estimates based on $\delta^{13}C_{POC}$ values were similar to those based on $\delta^{13}C_{DIC}$ values, corroborating our estimates. For TFW samples, the $\delta^{13}C_{phytoplankton}$ value (based on $\delta^{13}C_{POC}$ value) was -34.0‰ for bottom and -34.7‰ for surface water samples, which is about 4‰ higher than estimates based on the $\delta^{13}C_{DIC}$ value. For the marine end-member, $\delta^{13}C_{phytoplankton}$ value (based on $\delta^{13}C_{POC}$ value) for bottom water samples was -21.0‰, <1‰ higher than estimates based on the $\delta^{13}C_{DIC}$ value (-21.9 ± 0.2 ‰; Table 4.1). Therefore, we used the $\delta^{13}C_{phytoplankton}$ values estimated from the $\delta^{13}C_{DIC}$ values in the isotope mixing model. Notably, the estimate for marine surface water samples was lower (-36.1‰) than the $\delta^{13}C_{DIC}$-based estimate (-25.6 ± 2.3 ‰; Table 4.1). Most likely, more than two sources were contributing to POM in polyhaline surface stations, violating the fundamental assumption of the $\delta^{13}C_{POC}$-based approach.

For POM, the average $\delta^{13}C_{POC}$ and $\delta^{15}N_{PN}$ values were higher (up to 1.4‰ for $\delta^{13}C$ and 2.2‰ for $\delta^{15}N$) in the brackish than in the TFW portion of the estuary (Table 4.1). In the brackish estuary, the average of POM C/N (molar), 8.7 for bottom and 8.4 for surface samples, are between the C/N of terrestrial-derived OM (> 10) and marine phytoplankton (6.6, the Redfield ratio; Hedges et al. 1986, Hedges et al. 1997), indicating that brackish POM is a mixture of riverine and marine POM. In the TFW portion of the estuary, the average C/N of POM was >10, indicating a substantial contribution of terrestrial-derived OM to the POM pool (Table 4.1).

The average (± SD) $\delta^{13}C_{SOC}$ values were low at the head of the estuary (-28.0 ± 0.8 ‰) and progressively higher seaward (-26.1 ± 1.5 ‰; Table 4.1). The average (± SD) $\delta^{15}N_{SN}$ values were higher downstream, as well: 0.2 ± 0.5 ‰ in the TFW and 1.2 ± 0.3 ‰ in the brackish portion of the estuary (Table 4.1). The $\delta^{13}C_{SOC}$ values were slightly higher

than the $\delta^{13}C_{POC}$ values (+1.3‰ in the brackish portion, +0.2‰ in the TFW portion), and the $\delta^{15}N_{SN}$ values were much higher than $\delta^{15}N_{PN}$ values (+4.4‰ in the brackish estuary, +4.7‰ in the TFW).

4.3.3 *Corbicula fluminea* stable isotope values

The stable isotope ratios of *C. fluminea* varied with respect to salinity and size. With respect to salinity, the breakpoint identified by the segmented linear regression was 2.2 or 3.5 based on either $\delta^{13}C$ (Fig. 4.3A) or $\delta^{15}N$ values (Fig. 4.3B), respectively. Thus, the first group was composed of stations 1-4 (hereafter, lower estuary) and the second composed of stations 5-14, which include the TFW stations (7-14) and stations 5 and 6 (hereafter, upper estuary). *Corbicula fluminea* in the lower estuary were more enriched in both ^{13}C and ^{15}N than those in the upper estuary stations. In the lower estuary, $\delta^{13}C$ values ranged from -28.5‰ to -26.5‰ (average ± SD: -27.4 ± 0.4 ‰) and $\delta^{15}N$ values from 8.6‰ to 11.0‰ (average ± SD: 10.0 ± 0.8 ‰). In the upper estuary, $\delta^{13}C$ values oscilated between -29.2‰ and -26.6‰ (average ± SD: -28.1 ± 0.7 ‰) and $\delta^{15}N$ values from 6.3‰ to 9.6‰ (average ± SD: 7.8 ± 0.8 ‰). The factor salinity group was significant both for $\delta^{13}C$ ($F_{(1,98)}$= 3.99, $p< 0.05$) and $\delta^{15}N$ ($F_{(1,98)}$= 68.72, $p< 0.01$), and therefore, the two groups identified differed in their stable isotope values. Thus, a stable isotope food web model was developed for each salinity group.

The segmented linear regression did not identify a breakpoint with respect to size for either $\delta^{13}C$ or $\delta^{15}N$ values. However, there was a significant, positive relationship between $\delta^{13}C$ values and shell length from *C. fluminea* in the upper estuary group ($F_{(1,66)}$= 31.42, $p< 0.01$), and between $\delta^{15}N$ values and shell length in both the lower ($F_{(1,31)}$= 8.89, $p< 0.01$) and upper estuary ($F_{(1,66)}$= 17.62, $p< 0.01$) groups. Because we did not identify any breakpoint, a SIAR mixing model was not developed for different lengths. Moreover, the size distribution was not similar between salinity groups; *C. fluminea* from lower estuary had an average (± SD) shell length of 24.8 ± 2.9 mm and those from upper estuary had an average shell length of 18.6 ± 5.0 mm.

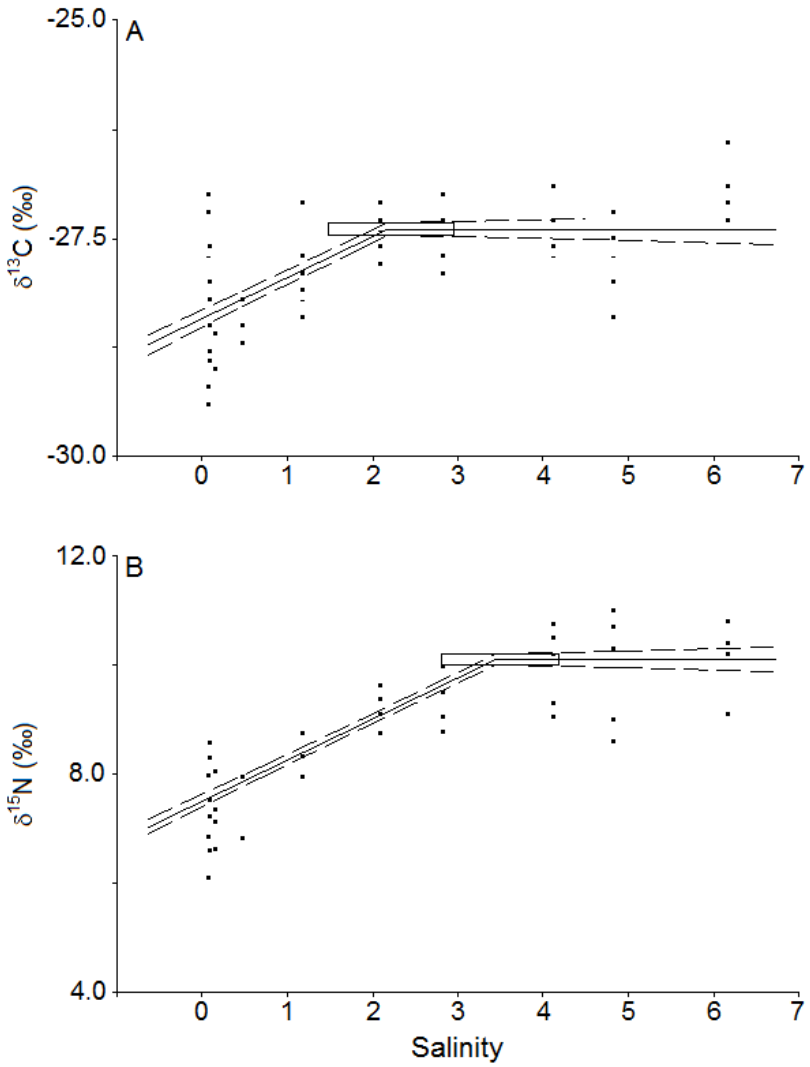

Fig. 4.3 Segmented linear regression for *Corbicula fluminea*'s $\delta^{13}C$ (A) and $\delta^{15}N$ (B) values in relation to salinity (relationship of type 4; Oosterbaan 1994); the 90% confidence belt and 90% confidence block of break-point are shown. Each point represents an individual *Corbicula fluminea*

4.3.4 SIAR mixing model

In the lower estuary group, *C. fluminea* $\delta^{13}C$ values were similar to $\delta^{13}C_{POC}$ values (Fig. 4.4 and 4.5); however, *C. fluminea* $\delta^{15}N$ values were slightly higher than would be expected if they were primarily consuming POM. This could be explained by consumption of marine phytoplankton or MPB or both (Fig. 4.4, Table 4.2). Although both sources have similar $\delta^{13}C$ and $\delta^{15}N$ values, marine phytoplankton is a less plausible source.

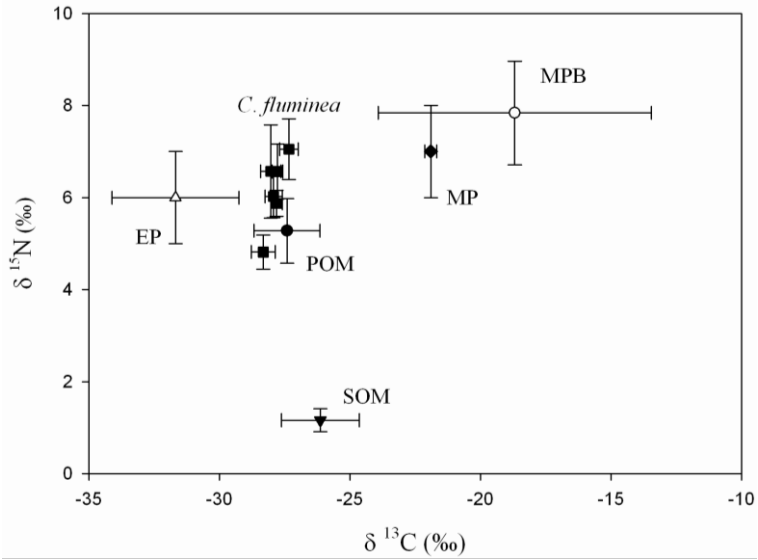

Fig. 4.4 Average $\delta^{13}C$ and $\delta^{15}N$ values of *Corbicula fluminea* adjusted for one trophic level fractionation (+0.4‰ $\delta^{13}C$, +3.4‰ for $\delta^{15}N$) and potential organic matter sources for lower estuary (stations 1-4). Error bars represent one standard deviation. The sources considered relevant for *Corbicula fluminea* (closed square; each represents a station-specific average) in this portion of the estuary were marine phytoplankton (MP; closed diamond), estuarine phytoplankton (EP; open triangle), microphytobenthos (MPB; open circle), sediment organic matter (SOM; closed triangle) and particulate organic matter (POM; closed circle).

In the stations closest to the river mouth (stations 1 and 2), salinity values can be higher than 20 during summer, depending on the tidal cycle. Salinities above 8 are lethal for adult clams (Mackie and Claudi 2010), although they can tolerate higher salinities for short periods (Ilarri and Sousa 2012b). Thus, it is still possible to find live clams in the stations closest to the river (1 and 2) during high tide. However, repeated fluctuations in salinity levels likely stress the clams. One common response to environmental stress in bivalves is valve closure (Shumway 1977, Kramer et al. 1989), and thus *C*. *fluminea* would not be able to consume marine phytoplankton during high tide, when it is available. For that reason, we excluded marine phytoplankton from the model.

Based on the SIAR mixing model (95% CI), phytoplankton contributed between 29.1% and 54.7%, and POM contributed between 19.1% and 56.1% to *C*. *fluminea* in the lower estuary (Fig. 4.6).

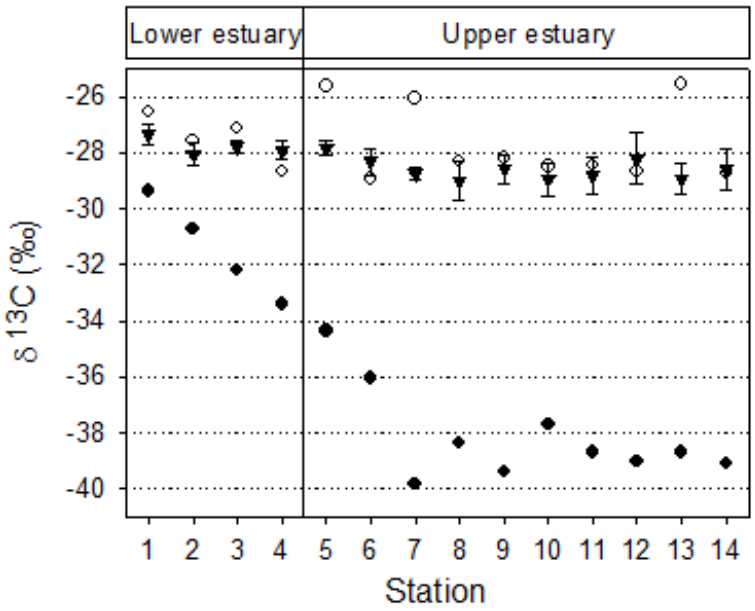

Fig. 4.5 Average $\delta^{13}C$ values of *Corbicula fluminea* (closed triangle) adjusted for one trophic level fractionation (+0.4‰ $\delta^{13}C$) and potential particulate organic matter sources ($\delta^{13}C_{phytoplankton}$ [estimated from $\delta^{13}C_{DIC}$]- closed circle; $\delta^{13}C_{POC}$- open circle) along the estuarine salinity mixing. Error bars represent one standard deviation.

Table 4.2 Average $\delta^{13}C$ (‰) and $\delta^{15}N$ (‰) (± SD) values used in the *Corbicula fluminea* SIAR mixing model for the organic matter sources used in either the upper estuary (UE) or lower estuary (LE) model. These values represent average bottom values for phytoplankton and particulate organic matter (POM), and also the average values for microphytobenthos (MPB) and sediment organic matter (SOM) pooled from each group of stations.

Source	$\delta^{13}C$	$\delta^{15}N$
UE phytoplankton	-38.5 (±0.7)	5.0 (±1.0)
LE phytoplankton	-32.0 (±2.4)	6.0 (±1.0)
UE POM	-27.8 (±1.3)	4.9 (±0.9)
LE POM	-27.4 (±1.3)	5.3 (±0.7)
UE MPB	-24.0 (±2.7)	7.3 (±2.2)
LE MPB	-18.7 (±5.2)	7.8 (±1.1)
UE SOM	-28.0 (±0.8)	0.2 (±2.5)
LE SOM	-26.1 (±1.5)	1.7(±0.3)

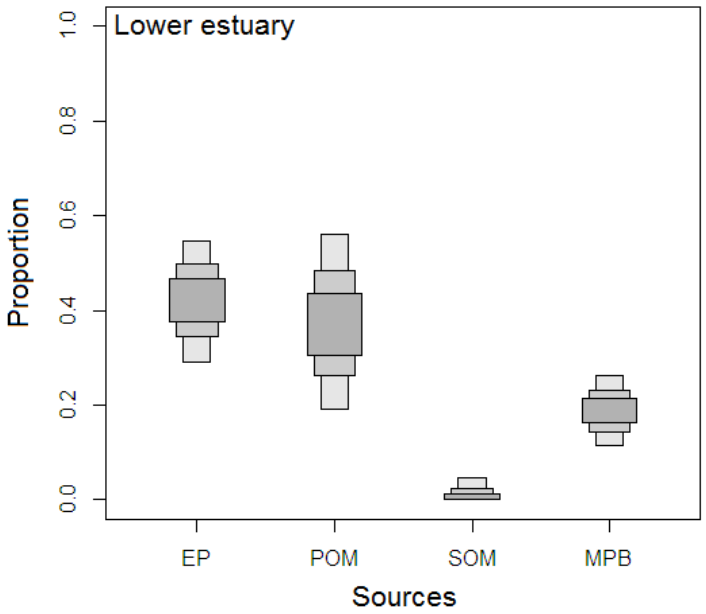

Fig. 4.6 Proportion of each food source to *Corbicula fluminea*'s biomass collected in the lower estuary (stations 1-4) based on the stable isotope mixing model. The food sources included in the model were estuarine phytoplankton (EP), particulate organic matter (POM), sediment organic matter (SOM) and microphytobenthos (MPB). Boxes indicate 50%, 75% and 95% Bayesian credibility interval

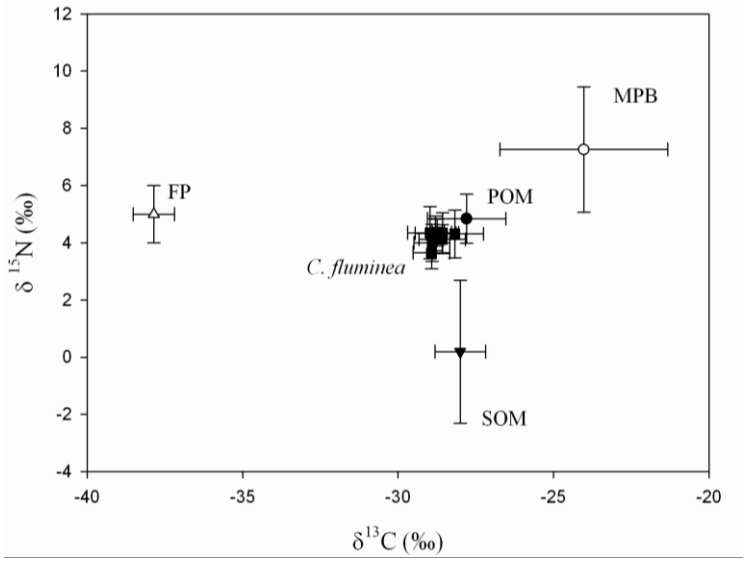

Fig. 4.7 Average $\delta^{13}C$ and $\delta^{15}N$ values of *Corbicula fluminea* adjusted for one trophic level fractionation (+0.4‰ $\delta^{13}C$, +3.4‰ for $\delta^{15}N$) and potential organic matter sources for the upper estuary (stations 5-14). Error bars represent one standard deviation. The sources considered relevant for *Corbicula fluminea* (closed square; each represents a station-specific average) in this portion of the estuary were freshwater phytoplankton (FP; open triangle), microphytobenthos (MPB; open circle), sediment organic matter (SOM; closed triangle) and particulate organic matter (POM; closed circle).

Corbicula fluminea $\delta^{13}C$ values in the upper estuary were similar to $\delta^{13}C_{POC}$ values, as well (Fig. 4.4 and 4.7, Table 4.2). Based on the results from the SIAR mixing model (95% CI), POM contributed between 40.9% and 64.4%, SOM contributed between 14.1 and 23.7%, and MPB contributed between 8.5 and 21.4% to *C. fluminea* biomass (Fig.

4.8). The contribution of phytoplankton to *C*. *fluminea* biomass in the TFW (10.6-16.4%) decreased ca. 20-40% in relation to the lower estuary (Fig. 4.6 and 4.8).

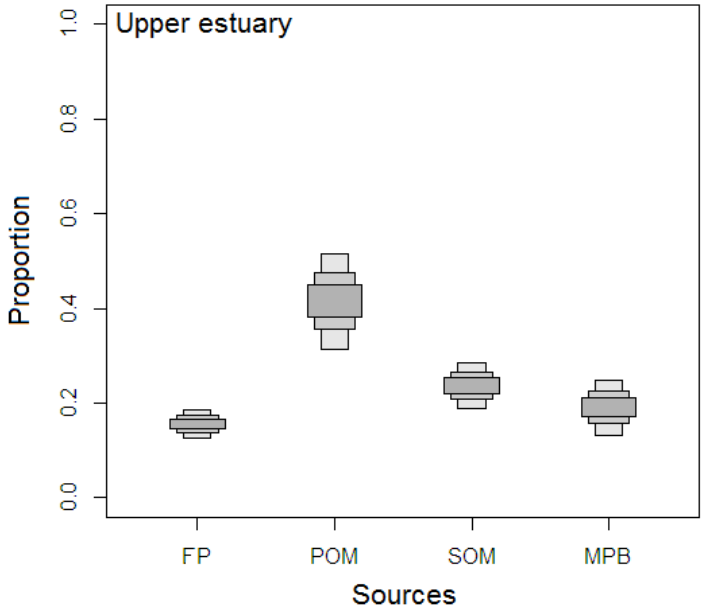

Fig. 4.8 Proportion of each food source to *Corbicula fluminea*'s biomass collected in the upper estuary (stations 5–14) based on the stable isotope mixing model. The food sources included in the model were freshwater phytoplankton (FP), particulate organic matter (POM), sediment organic matter (SOM) and microphytobenthos (MPB). Boxes indicate 50%, 75% and 95% Bayesian credibility intervals.

4.4 Discussion

A pronounced shift in the quality of OM available for *C*. *fluminea* in the Minho River estuary occurred along the estuarine mixing zone. In concert, stable isotope analysis, POM C/N ratios, and phytoplankton contribution estimates based on C: Chl *a* indicated that POM in freshwater stations was largely comprised of terrestrial-derived OM, a refractory food source, and was increasingly comprised of phytoplankton, a highly palatable food source, towards the polyhaline estuary. A similar shift in isotopic composition along the estuarine mixing zone was observed in *C*. *fluminea*, indicating a shift in the food sources used in response to the change in food quality. Accordingly, the SIAR mixing model attributed most *C. fluminea* biomass to terrestrial-derived OM in the TFW, with an increasing contribution from estuarine phytoplankton seaward. Notably, the stable isotope data indicates *C*. *fluminea* has the ability to consume and assimilate terrestrial-derived OM. Therefore, *C*. *fluminea* appears to link the estuary with the terrestrial ecosystem in the Minho River estuary benthic food web. The shifts in the

sources of OM consumed by *C*. *fluminea* along the estuarine mixing gradient and the possible effects of its feeding modes to the estuarine food web are discussed below.

4.4.1. Sources of organic matter supporting *C*. *fluminea* production in the upper estuary

Terrestrial-derived OM was estimated as the major contributor to *C*. *fluminea* biomass (50% to 64%) in the upper portion of the Minho River estuary. Terrestrial-derived OM is a prominent C source to rivers; terrestrial inputs are often equal to or larger than the autochthonous primary production (Caraco and Cole 2004). Yet, numerous studies have demonstrated that estuarine secondary production is primarily supported by autochthonous OM, especially phytoplankton (Deegan and Garritt 1997, Chanton and Lewis 2002). Nevertheless, there is growing evidence that terrestrial inputs can subsidize estuarine food webs, supporting up to 80% of invertebrate and fish biomass (Kasai and Nakata 2005, Hoffman et al. 2008).

In the Minho River TFW portion, the POM pool was comprised mostly of terrestrial-derived OM (C/N > 10) and the phytoplankton contribution to this pool was *ca*. 20% (surface). *C*. *fluminea* stable isotope values were similar to POM, which suggests that growth of this bivalve in the upper portion of the estuary was supported by terrestrial-derived OM, which was directly consumed by filter feeding. If phytoplankton were contributing greatly to *C*. *fluminea* biomass, we would expect to have observed both *C. fluminea* $\delta^{13}C$ and $\delta^{13}C_{phytoplankton}$ values follow a similar trend along the estuarine mixing zone (Fig. 4.5) and $\delta^{13}C$ values of *C*. *fluminea* would have been lower in the TFW portion, between -35‰ and -38‰ (Fig. 4.7), which was not the case. Moreover, the available environmental data indicate that phytoplankton standing stock was low and that the system was net-heterotrophic (i.e. low $\delta^{13}C_{DIC}$ values). The Chl *a* concentration was lower than 1 μg L^{-1} (0.74 μg L^{-1}, this study; also, 0.70 μg L^{-1} in 2009-2010, Brito et al. 2012) and based on C:Chl *a*, phytoplankton fraction in the POM was ≤ 20%. The latter estimate should be treated cautiously. Chl *a* concentration is often used to estimate phytoplankton biomass; the C:Chl *a* ratio typically ranges from 30 to 50 (e.g. Legendre et al. 1999). This ratio, however, varies within and among algal species (e.g. Chan 1980, Kruskopf and Flynn 2005, Putland and Iverson 2007), ranging from 5 to 345 mg C mg Chl a^{-1} (Putland and Iverson 2007). In a recent study, the effect of variation in the C:Chl *a* ratio on the proportion of algal C in POC was examined for different levels of Chl *a* concentrations (Marty and Planas 2008). The authors concluded that there is a positive relationship between the phytoplankton percentage in POC and the C:Chl *a* ratio, yet under low Chl *a* concentrations (0.6 μg L^{-1}), the contribution of phytoplankton would be low regardless of the C:Chl *a* ratio (Marty and Planas 2008). Therefore, even if the C:Chl *a* ratio used was

as high as 100, the contribution of phytoplankton to POC pool would remain close to 10%. Thus, *C*. *fluminea* appears to have been assimilating a relatively low quality, high flux food source (terrestrial inputs) in response to low phytoplankton availability (though rates of primary production were not measured).

In laboratory (18 clams per 18 L funnel) and field growth experiments with *C*. *fluminea*, clams were food limited at Chl *a* concentrations between 16 µg L^{-1} and 19 µg L^{-1} (Foe and Knight 1985), which are 22-26 times higher than the average Chl *a* concentration estimated, and 4-5 times higher than the peak concentration measured (3.6 µg L^{-1}, station 7). While it is difficult to compare clam densities and filtration rates measured in the laboratory with those measured in the field, these results suggest that *C*. *fluminea* may have been using alternative OM sources where phytoplankton availability was limited to maintain its high abundance in the Minho River estuary.

Although organic detritus has been identified in *C*. *fluminea* gut contents (Hill 1985 cited in Foe and Knight 1985), to our knowledge, this is the first study that quantifies the importance of terrestrial-derived OM as a food source to this species. Plant residues contain structural polysaccharides, such as cellulose and hemicellulose that are thought to be digested only by microorganisms (Sakamoto and Toyohara 2009). However, cellulase and hemicellulase activities have been found in the digestive organs of *Corbicula japonica* (Sakamoto et al. 2009, Niiyama and Toyohara 2011). We hypothesize that *C*. *fluminea* is using a similar biochemical process to digest a less palatable OM source (terrestrial-derived OM), and simultaneously catalyze vascular plant-derived refractory substances into assimilable OM.

Though terrestrial-derived OM was the main food source for *C*. *fluminea* in the upper portion of the estuary, sediment OM and MPB also contributed to their biomass. Filter feeding alone cannot balance *C*. *fluminea*'s energy budgets (Aldridge and McMahon, 1978), so pedal feeding is an important source of nutrition, especially in food-limited environments (Boltovskoy et al. 1995).

A critical assumption in the SIAR mixing model is that the OM source values measured were similar over the prior 2-3 months because the data collected during this study were obtained during a single sampling event. Although isotopic turnover rate studies of *C. fluminea* have not been conducted, it is likely that the isotopic turnover period of the *C*. *fluminea* population is about three months. For individuals, the isotopic turnover period depends on both somatic growth and metabolic turnover rates; in organisms that grow rapidly, somatic growth rates essentially determine the isotopic turnover period (del Rio et al. 2009). *C*. *fluminea* have rapid growth throughout their life and, owing to their rapid sexual maturity, have a population-level turnover time (in biomass) of 73-91 days (McMahon 2002). Moreover, previous studies have demonstrated

that stable isotope values of other bivalves *Dreissena polymorpha* (zebra mussel) and *Corbula amurensis* (Asian clam; formerly known as *Potamocorbula*) are spatially and temporally well aligned with environmental processes (e.g. watershed inputs, chemistry of river water; Fry 2002, Fry and Allen 2003). The same is likely true for *C*. *fluminea* owing to its rapid growth rate and similarlyshort life span (McMahon 2002, Thompson and Parchaso 2010). There are several features that we considered relevant when interpreting this data over the turnover period of *C*. *fluminea*. First, the sampling occurred at the end of the summer, during low flow conditions (below 25% percentile of the monthly averages along a hydrologic year; Confederación Hidrográfica del Miño-Sil, personal communication) that persisted over the summer months (July-September), a timeframe similar to the turnover period of *C*. *fluminea*. Second, the stable isotope values of terrestrial inputs are relatively constant (Cloern et al. 2002). Third, the low inflow variation associated with low flow conditions results in $\delta^{13}C_{DIC}$ values that are more stable (Striegl et al. 2001, Bade et al. 2004, Lehmann et al. 2004) and, therefore, $\delta^{13}C_{phytoplankton}$ values are likely similar throughout the low flow period. Thus, it is likely that the biogeochemical conditions in September were similar to the summer low flow biogeochemical conditions that preceded sampling.

4.4.2 Sources of organic matter sources supporting *C*. *fluminea* production in the lower estuary

In the brackish estuary, phytoplankton and POM were estimated to compose the majority of *C*. *fluminea*'s biomass, contributing between 29-55% and 19-56%, respectively. Although Chl *a* was higher in the brackish estuary than in the TFW portion, it was still within the range identified as limiting for *C*. *fluminea* growth (Foe and Knight 1985). Our estimates of the phytoplankton fraction in estuarine POM were around 20%. Therefore, positive selection for phytoplankton must have occurred prior to ingestion or during digestion, as the phytoplankton contribution to *C*. *fluminea* biomass was proportionally higher than the phytoplankton contribution to the bulk POM pool. Although *C*. *fluminea* is a generalist consumer, these clams prefer small-sized living and suspended particulate OM (Atkinson et al. 2011). The mechanisms involved in this selection, however, are not understood.

Corbicula fluminea also filtered and assimilated POM, which was a mixture of terrestrial-derived OM and marine POM, transported by surface freshwater outflow and bottom marine inflow, respectively.

4.4.3 Possible effects of *C*. *fluminea* in the estuarine food web

Corbicula fluminea filtered from the water column the majority (70-80%) of its food sources. The quality of these filtered food sources used by *C*. *fluminea* changed along the estuarine mixing zone, revealing that this invasive bivalve has the ability to use alternative and less labile OM sources (terrestrial and sediment OM) when phytoplankton is less abundant. If *C*. *fluminea* can utilize the refractory components of terrestrial detritus, it is possible that they may also act as initiators to break down plant cell wall structural polysaccharides. However, further studies are needed to clarify the presence of an enzymatic mechanism that enables *C*. *fluminea* to perform this function.

Corbicula fluminea can influence seston concentration (Leff et al. 1990) and particle size (Atkinson et al. 2011), as well as decrease Chl *a* concentrations by 20-70% (Cohen et al. 1984). Thus, the high filtration rates of *C*. *fluminea* (Strayer et al. 1999) and preference for small-sized living and suspended POM (Atkinson et al. 2011) may condition the availability of edible particles to other native suspension feeders. By removing suspended material from the water column at high rates, this species increases OM deposition into the sediments (Hakenkamp and Palmer 1999). This may increase sediment OM available to *C*. *fluminea*, as well as to other benthic organisms. In a recent study conducted in the Minho River estuary, a positive relationship was found between both the density and biomass of benthic invertebrates (chiromidae larvae, amphipods, and gastropods) and increasing densities of *C*. *fluminea* (Ilarri et al. 2012a). The authors concluded that the increase in habitat structure provided by shells and the increase in waste products (faeces and pseudofaeces) may play essential roles in this positive relationship (Ilarri et al. 2012a). Additionally, the stoichiometry of *C*. *fluminea* biodeposits from Ichawaynochaway Creek (USA) revealed that they represent a higher quality food source when compared to the biodeposits of a native bivalves (*Elliptio crassidens*) or seston (10-45 µm) (Atkinson et al. 2010) and thereby constitute a higher quality food source for benthic organisms.

Although *C. fluminea* filtered from the water column the majority of the food sources identified in this study, sediment OM and benthic periphyton did contribute between *ca*. 10-20% to their biomass, indicating *C*. *fluminea* was deposit feeding. This adaptation to access benthic carbon when water column food resources were apparently limiting is likely an additional factor explaining *C*. *fluminea*'s success in the Minho River estuary. Furthermore, this species has a broader diet breadth than is known for native unionids species (Vaughn and Hakenkamp 2001, Atkinson et al. 2010), which can be an advantage when competing with co-occurring native species for resources and habitat.

In conclusion, this study reveals that *C*. *fluminea* has the ability to adapt to environments with low food quality, not only because it deposit feeds, but also because it can filter and assimilate terrestrial-derived OM. This can be a competitive adaptation in systems with perennial low food quality such as the Minho River estuary. Moreover, its ability to directly (by consuming terrestrial inputs) and indirectly (by conditioning terrestrial OM for consumption by other benthic consumers in the form of biodeposits) couple the terrestrial ecosystem with estuarine benthic and pelagic environments constitutes a new identified process by which *C*. *fluminea* may alter food web flows in aquatic ecosystems. As an invader, the implication of this adaptive and flexible feeding behavior is that it likely facilitates its widespread success at establishing in new aquatic environments.

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Chapter 5

The role of aquatic and terrestrial material flows to an estuarine food web using stable isotopes: general conclusions

The main objective of this thesis was to contribute to the understanding of the processes that govern the exchange and consumption of different organic matter (OM) sources in estuarine food webs. To accomplish this objective, the Minho River estuary was used as a model ecosystem, to address four specific questions: 1) which factors govern the exchange and consumption of different OM sources within estuarine habitats; 2) what is the degree of connectivity within estuarine habitats and between the estuary and its end-members, and how ecosystem connectivity may influence estuarine food web dynamics; 3) which patterns and processes structure the benthic food web's spatial and temporal variability; and 4) how anthropogenic induced changes, as the introduction of non-indigenous species, may affect the flow of OM in estuarine food webs.

In order to characterize the estuarine food web dynamics, carbon and nitrogen stable isotope ratios were used. Stable isotopes have emerged as a useful tool to understand the structure and dynamics of estuarine food webs, because they provide time-integrated information about the trophic relationships and energy flow through food webs. During the last decades several approaches have been developed to improve data acquisition and analyses. The main approache used to analyze and interpret stable isotope data in the context of estuarine food webs is the qualitative (plot analysis) combined with statistical analyses. However, there is an increase in the use of quantitative approaches such as linear mixing models (e.g. IsoError, IsoSource, SIAR). The most commonly used quantitative approaches in the study of estuarine food webs are the IsoError (determined model) and IsoSource (indetermined model) mixing models. However, Bayesian mixing models seem to be the better approach to solve an indetermined system, not only because it allows to estimate a range of possible solutions based on statistical inferences, but also because uncertainty and variation of the parameters are included in those estimates. Thus, the best strategy should be to avoid the use of indetermined models, by constraining the number of possible sources, by increasing the number of tracers or use a priori information, such as data from stomach content analyses. Bad quality input data cannot generate good model results, so in order

to conduct a proper analyses of estuarine food webs, the end-members must be well characterized (i.e. sample all the possible sources, and spatial and temporal alignment between sources and consumers). Further, the interpretation of stable isotope data generally relies on several assumptions, such as that the isotopic composition of consumer's tissues equals the weighted average of the isotopic composition of its sources, and that trophic fractionation moves constantly through the food web. Because each approach has its strengths and weaknesses, the best one should start to correctly formulate the study's hypotheses and then use the most appropriated method to answer it, but always recognizing its limitations.

In the Minho River estuary, river discharge was a major factor governing the exchange and consumption of autochthonous and allochthonous OM sources. This estuary is particularly interesting for this purpose, because it is considered an oligotrophic ecosystem, and the current consensus for river-estuary complexes is that autochthonous OM, especially phytoplankton, are the main OM sources supporting consumers' productivity. There was a pronounced variability in the quality of OM used by primary consumers between different river discharge conditions. During typical low river discharge periods, autochthonous OM supported more than 50% of primary consumers' biomass, decreasing its importance during high river discharge periods (< 40%), probably because phytoplankton availability was substantially reduced (Chl *a* close to 1 µg L^{-1}). It was also possible to detect the effect of a major winter flood (2009-2010) on the estuarine food web during summer (low river discharge period). During this period, the availability of phytoplankton was low (Chl *a* < 1 µg L-1; phytoplankton fraction in the particulate OM pool (POM)< 20%), and the contribution of terrestrial-derived OM to the POM pool was high ($C:N_{POM}$> 10). The terrestrial signal was clear in the pelagic and benthic consumers, suggesting that primary consumers have the ability to adapt to low food quality conditions, by using less labile OM sources, such as terrestrial-derived OM or sediment OM (SOM). The ability of pelagic and benthic consumers to use terrestrial-derived OM, and to access both pelagic and benthic food sources, links the terrestrial ecosystem with the estuarine pelagic and benthic environments. The importance of terrestrial-derived OM to primary consumers in general increased towards the freshwater portion of the estuary, and phytoplankton contribution to primary consumers' biomass followed the opposite pattern. During typical low river discharge, the importance of marine OM increased in the brackish portion of the estuary. Thus, data indicates that estuarine habitats are inter-connected and connected with its' end-members, marine and terrestrial ecosystems.

The Minho River estuarine benthic food web dynamics provide evidences of spatial and temporal variability. Spatial variability was essentially related with the characteristics of the OM sources available in each sampling area, which was also influenced by the

proximity to other ecosystems (i.e. higher terrestrial influence in the tidal freshwater portion and higher marine influence in the brackish portion of the estuary). Temporal changes in the stable isotope ratios of consumers were driven essentially from changes occurring at the base of the food web, although there were also some evidences for a combination with changes in the resource use by consumers. River discharge was an important factor influencing the changes at the base of the estuarine food web, both spatially and temporally.

For the first time, the main OM sources supporting the production of the invasive bivalve *Corbicula fluminea* were identified. The study of *C*. *fluminea*'s functional response, in terms of both feeding behavior and food selectivity, is of particular interest in this ecosystem because this species represents more than 90% of the total benthic macrofauna biomass. A pronounced shift in the quality of OM available for *C*. *fluminea* in the Minho River estuary occurred along the estuarine mixing zone. The POM pool in the tidal freshwater stations (TFW) was largely comprised of terrestrial-derived OM, a refractory food source, and was increasingly comprised of a highly palatable food source, phytoplankton, towards the polyhaline estuary. A similar shift in isotopic composition along the estuarine mixing zone was observed in *C*. *fluminea*, indicating a shift in the food sources used in response to the change in food quality. Accordingly, the SIAR mixing model attributed most *C. fluminea* biomass to terrestrial-derived OM in the TFW (41-64%), with an increasing contribution from estuarine phytoplankton seawards (29-55%). Notably, the stable isotope data indicates that *C*. *fluminea* has the ability to consume and assimilate terrestrial-derived OM. If *C*. *fluminea* can utilize the refractory components of terrestrial detritus, it is possible that they may also act as initiators to break down plant cell wall structural polysaccharides. Although *C. fluminea* is mainly a suspension feeder, sediment OM and benthic periphyton were also assimilated (10-20%), indicating that *C*. *fluminea* was also deposit feeding. This adaptive and flexible feeding behavior might be an adaptation to access benthic carbon when water column food resources were apparently limiting. *Corbicula fluminea's* feeding flexibility might be an additional factor explaining its success in the Minho River estuary. Moreover, the ability of *C*. *fluminea* to directly (through the consumption of terrestrial-derived OM), and indirectly (by conditioning terrestrial OM for consumption by other benthic consumers in the form of biodeposits) couple the terrestrial ecosystem with estuarine benthic and pelagic environments, constitutes a new identified process by which *C*. *fluminea* may alter food web flows in aquatic ecosystems. As an invader, the implication of this adaptive and flexible feeding behavior is that it likely facilitates its widespread success at establishing in new aquatic environments.

The characterization of the Minho river estuarine food web, through the use of carbon and nitrogen stable isotope ratios of OM sources and pelagic and benthic consumers, provided evidences that pelagic and benthic food webs are highly connected with each other, and also with the terrestrial and marine ecosystems. Any anthropogenic activity that disrupts ecosystem connectivity will likely affect estuarine trophic dynamics. Yet, further research is compulsory to disclose the impact of disrupting ecosystem connectivity on estuarine productivity, in order to establish sound management and conservation plans.