Universidade do Algarve

Faculdade de Ciências do Mar e Ambiente

Isotopic tracking of sources in coastal systems: special emphasis to *Zostera noltii* (Horneman) food web

(Tese para a obtenção do grau de doutor no ramo de Ecologia, especialidade de Ecologia de Comunidades)

Maria Raquel de Assunção Gonçalves Machás

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Faro

(2007)

Aos meus avós

Só morrem, desaparecem de vez, as pessoas que não foram amadas. (Zélia Gattai)

AGRADECIMENTOS

Este trabalho foi financeiramente apoiado pelo Fundação para a Ciência e Tecnologia (scholarships Praxis XXI/BD/19579/99). O projecto "M&Ms - Monitoring and management of European seagrasses" (EVK3-CT-2000-00044) financiou a parte da investigação referente ao capítulo 3.

Ao longo destes anos foram muitos que contribuíram para a concretização desta dissertação. Por este motivo e com receio de omitir injustamente alguma destas pessoas, não os irei enumerar mas, apenas agradecer de um modo geral a todos, identidades e pessoas, indispensáveis à concretização desta longa etapa. Agradecimentos específicos encontram-se no final de cada artigo.

Um agradecimento especial aos investigadores da Universidade do Algarve e do Marine Biological Laboratory pela forma interessada e amiga com que sempre se disponibilizaram em ajudar. Um muito obrigada à Esmeralda Costa pelo apoio e incentivo na meta final.

Ao Professor Doutor Rui Santos pela oportunidade que me deu em trabalhar nesta área de investigação e pela forma empenhada com que se prontificou a ajudar na finalizar deste trabalho.

Ao Professor Bruce Peterson pela orientação e principalmente pela sua forma amiga com que sempre me apoiou e incentivou.

Aos meus pais por nunca terem deixado de acreditar em mim, estando sempre prontos em ajudar. Ao Filipe por estar ao meu lado e à nossa filha Sara, que me ensinou a relativizar a importância das coisas.

O meu muito obrigado a todos que o tornaram possível.

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FACULDADE: Faculdade de Ciências do Mar e Ambiente

ORIENTADOR: Doutor Rui Orlando Pimenta Santos

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DATA: Março 2007

TÍTULO DA TESE:

Isotopic tracking of sources in coastal systems: special emphasis to *Zostera noltii* (Horneman) food web.

ABSTRACT

The main objectives of this research were 1) to review the isotopic tracking of anthropogenic N sources 2) to trace the distribution of the urban effluent in Ria Formosa lagoon and to reveal its biological uptake and impact, 3) to assess the changes in stable isotope contents of *Zostera noltii* leaves during the early phases of decomposition and 4) to investigate the trophic interactions in *Z. noltii* meadows.

When evaluated with care, the $\delta^{15}N$ values can provide valuable information on qualitative and quantitative changes in the nitrogen status of aquatic systems. For this evaluation it is important to characterize the nutrient $\delta^{15}N$ values in the different compartment. Our study of the distribution of the urban effluent in Ria Formosa lagoon revealed that the seasonal variation of the Waste Water Treatment Works (WWTW) internal metabolism was reflected in the $\delta^{13}C_{DIC}$ and $\delta^{15}N_{NH4}$ of its effluent. The WWTW isotopic signal was most evident in the isotopic signature of the particulate organic matter, rather than in the sediment organic matter or in Z*ostera noltii*. The overall results show that the effluent discharge is likely to impact the clam cultivations areas that are as close as about 300 m from the WWTW.

The $\delta^{13}C$ and $\delta^{15}N$ values of *Zostera noltii* do not change during the breakdown of the biomass into finer particulate material. Stable isotope studies to assess the contribution of this species to secondary production can thus consider the natural $\delta^{13}C$ and $\delta^{15}N$ abundances of living tissues without any correction to account for decomposition. On the other hand, the use of $\delta^{34}S$ values of *Z. noltii* detritus should be done with caution due to the contamination of the samples with pyrite, which has a depleted $\delta^{34}S$ signal. The $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ values of primary producer, organic matter, detritus and consumers provided unique insights into trophic relationships within the *Z. noltii* meadows. δ-values indicate that animals, excluding the gastropod *Elysia* sp., are dependent to varying degrees upon seagrass and/or seagrass epiphytes and green algae for their C, N and S sources.

Key-words (máximo 6): Stable isotopes; *Zostera noltii*; nutrients; leaf decomposition; food-web Ria Formosa

TÍTULO DA TESE EM PORTUGUÊS:

Fluxos de matéria em sistemas costeiros através da análise de isótopos estáveis: especial ênfase para teias alimentares em prados de *Zostera noltii* (Horneman).

RESUMO

Os principais objectivos deste trabalho foram 1) fazer uma revisão sobre o uso da assinatura isotópica de azoto, $\delta^{15}N$, da matéria orgânica e inorgânica como traçador da carga de nutrientes de origem antropogénica em sistemas costeiros, 2) estudar a distribuição de efluentes urbanos na Ria Formosa e revelar o seu impacto biológico, 2) averiguar a variação das assinaturas isotópicas de *Zostera noltii* durante o processo de decomposição e 3) investigar as relações tróficas em pradarias de *Z. noltii*.

A análise de $\delta^{15}N$ é uma técnica que poderá ser bastante poderosa para a compreensão da origem e transferência de N nos ecossistemas costeiros. Para tal, é extremamente importante caracterizar os valores de $\delta^{15}N$ dos nutrientes nos diferentes compartimentos do sistema aquático. Na Ria Formosa, a variação sazonal do metabolismo interno da estação de tratamento de águas residuais (ETAR) reflectiu-se nos valores isotópicos de C e N do seu efluente. A incorporação deste sinal isotópico foi mais evidente na matéria orgânica particulada do que no sedimento ou em *Zostera noltii*. Os resultados totais mostram que a descarga do efluente na lagoa tem um impacte até uma distância de 500 a 600 metros da ETAR, podendo por isso influenciar as áreas de marisqueiro que estão localizadas a cerca de 300 m da ETAR.

Verificou-se que a assinatura de $\delta^{13}C$ e $\delta^{15}N$ de *Zostera noltii* não muda durante a decomposição, podendo assim realizar-se o estudo da contribuição desta espécie para a produção secundária através da análise da abundância natural destes isótopos em folhas vivas, sem ser necessário utilizar um factor de correcção. Por outro lado, os valores de $\delta^{34}S$ em detritos de *Z. noltii* devem ser utilizados cuidadosamente devido à contaminação das amostras com pirite (com um valor baixo de $\delta^{34}S$). Os valores isotópicos dos produtores primários, matéria orgânica, detritos e consumidores foram extremamente úteis na análise da estrutura trófica em pradaria de *Z. noltii* indicando que esta angióspermica marinha e/ou os seus epífitos e/ou as algas verdes são uma importante fonte de C, de N e de S para todos os organismos presentes, à excepção do Gastropoda *Elysia* sp.

Palavras-chave (máximo 6): Isótopos estáveis; *Zostera noltii*; nutrientes; decomposição foliar; teia alimentar; Ria Formosa

This thesis was based on the follow four papers, one published and three ready to submit to international scientific journals, which constitute the chapters 2, 3, 4 and 5 of the manuscript.

Machás R., Santos R., Peterson B. Isotopic tracking of N sources in aquatic environments – a review.

Machás R., Cabaço S., Kennedy H., Peterson B., Santos R. Effects of urban effluents in a temperate mesotidal lagoon.

Machás R., Santos R., Peterson B. 2006. Elemental and stable isotope composition of *Zostera noltii* (Horneman) leaves during the early phases of decay in a temperate mesotidal lagoon. Estuarine Costal and Shelf Science 66: 21-29.

Machás R., Peterson B., Santos R. 2006. Trophic interactions in a seagrass meadow (*Zostera noltii*): an isotopic approach.

Índice

Chapter 1 – General introduction

Importance and background of research theme

The high productivity of coastal ecosystems supports complex food webs that span terrestrial and aquatic environments including consumers with a variety of feeding strategies (Haines & Montague 1979, Sullivan & Moncreiff 1990). The importance of seagrasses in coastal and near shore environments and their contribution to the productivity of the world Ocean has become increasingly recognised over the last decades. These marine vascular plants are the physical and biological foundation for many costal marine systems.

Land-derived nutrient enrichment of shallow coastal systems may cause drastic changes in the ecosystem structure. Increasing nutrient loads result in the development of macroalgal blooms which replace the original seagrass areas (Cambridge & McComb 1984, Zaitsev 1992, Sfriso et al. 1992, Thybo-Christesen et al. 1993, Taylor et al. 1995, Valiela et al. 1997). This widespread and increasingly common phenomenon is accompanied by a series of fundamental and pervasive effects that propagate throughout the entire trophic web as seagrasses support a variety of consumers and are nurseries for juveniles of many fish species. One key management question, requiring both economic and ecological insight, is how to manage and conserve these systems in a sustainable way. A sound understanding of ecosystem function is required for successful management especially when active important fisheries are present.

Determining the fate of the carbon fixed by primary producers and analysing the trophic structure in communities is one of the most important considerations in understanding the ecosystems function. Establishing trophic relationships within communities is a problematic enterprise, particularly in seagrass ecosystems, as a consequence of the high number of primary producers, the strong degree of connectance and the elevated level of omnivory (Darnell 1958, Livingston 1982). In an examination of coastal food webs, it is important to note that seagrass detritus, more than living tissues, has been described as the major trophic path of energy flow (Zieman 1983). The fragmented organic material is difficult to identify and to quantify and the presence of this material in the gut of animals does not indicate the nutritive value of the detritus (Zieman et al. 1984, Valiela 1995).

Stable isotope methods as a tool to investigate several aspects of ecological research have increased tremendously in recent year (reviewed by Lajtha & Michener 1994, Peterson & Fry 1987) Analysis of the natural abundance of stable isotopes of carbon (conventionally expressed as $\delta^{13}C$ values), nitrogen ($\delta^{15}N$ values) and sulphur ($\delta^{34}S$ values) in organic matter producers and in consumers have each proven to be useful in describing the organic matter flow and food web relationships in coastal systems (Fry & Sherr 1984, Peterson et al. 1985, Peterson & Howarth 1987, Peterson 1999, Machás et al. 2003). There has also been rapid progress in the use of stable isotopes, mainly $\delta^{15}N$, to identify pollution sources by isotopic characterisation and quantification of end members (Heaton 1986, Macko & Ostrom 1994).

The basis for the use of stable isotope analysis in food web studies is that organisms retain or modify by known metabolic fractionations the stable isotope signals of the foods they assimilate (Currin et al. 1995, Peterson & Fry 1987). Thus, if sewage material is isotopically distinct from marine-derived material, plants and animals using sewage directly or indirectly as nutrient source can be distinguished isotopically from plants and animals feeding exclusively on marine-produced food sources. Several studies have reported that land-produced organic material is isotopically distinct from marine sources. These studies demonstrated that natural abundance stable isotope values can be used to trace the incorporation and movement of sewage in marine food webs (Fourqurean et al. 1997, Hansson 1997, McClelland et al. 1997, Rau et al. 1981, Spies et al. 1989). Because shifts in stable isotope values between living and detrital plant-derived material can occur (Benner et al. 1987, Cloern 2002, Currin et al. 1995), measurement of the changes in isotopic decomposition of detritus as it decays is required to refine our understanding of organic matter transfers in detrital food webs (Machás et al. 2003, Peterson 1999).

Despite the fact that *Zostera noltii* meadows are widespread along the intertidal coast of Western Europe, North-West Africa, Mediterranean Sea and Black Sea (North Atlantic and Meditteranean flora) (Den Hartog 1970, Heminga & Duarte 2000, Milchakova & Phillips 2003) little information exists on its trophic role and on the isotopic composition of *Z. noltii* meadows (Machás & Santos 1999, Machás et al. 2003).

Study site

Ria Formosa is a shallow mesotidal lagoon that extends for 55 km along the southern Portugal coastline. Fresh water input into the lagoon is low, and salinity remains close to 36 ppm except during sporadic and short periods of winter runoff (Falcão & Vale 1990). Intertidal benthic processes are most important to the system dynamics as the intertidal area is about 80% of the maximum inundated area at spring tide (Andrade 1990). Most of the intertidal is occupied by monospecific meadows of the seagrass *Zostera noltii*. The upper distribution of this species coincides with the lower edge of the salt marsh species *Spartina maritina* whereas its lower edge coincides with the edge of the subtidal habitat of the seagrass *Cymodocea nodosa*. The species production and leaf turnover is very high and sustained throughout the year. Blooms of green macroalgae of the genera *Enteromorpha* and *Ulva* develop in some areas of the lagoon, mostly in winter.

Stable isotope signatures of the main producers of Ria Formosa were characterised previously (Machás 1999, Machás & Santos 1999, Machás et al. 2003). Data indicated that the benthic macrophytes and not the phytoplankton are the main contributors to the particulate organic matter of the system. Phytoplankton may be important only in the outer zones near the inlets where oceanic influence is higher. Spatial patterns on the nitrogen stable isotope natural abundances (expressed as $\delta^{15}N$ values) of *Zostera noltii* leaves suggested an influence of isotopically heavy N from sewage (Machás et al. 2003). The major inputs of nutrients for the lagoon are the effluents of Waste Water Treatment Works (WWTW), the non-treated fish farms effluents and the fresh water inputs, which carry the fertilizers from agricultural areas around the lagoon. A preliminary survey was done in order to evaluate the impact of this nutrient in the lagoon. The results indicated that the urban effluent (samples collected near the WWTW) is the main nutrient source and that these nutrients were incorporated by sediment organic matter and by *Zostera noltii* (Fig.1).

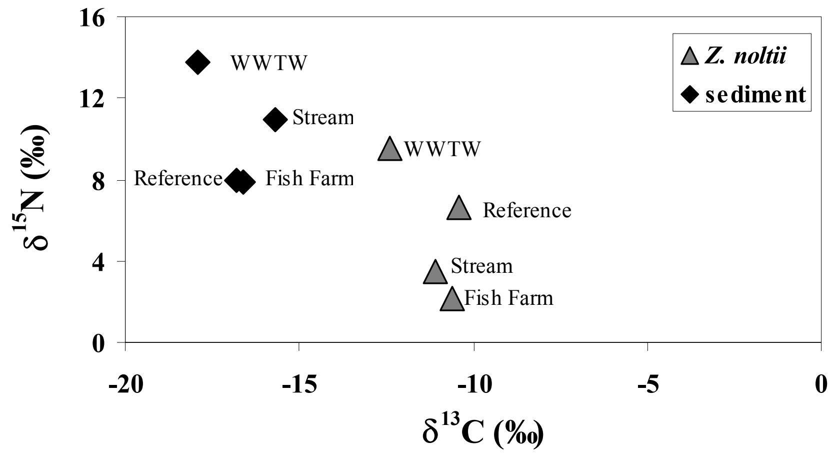


Figure 1. Carbon and nitrogen δ- values of *Zostera noltii* and sediment organic matter. Samples colleted in the Ramalhete channel (reference site) and close to the major inputs of nutrients to Ria Formosa lagoon: WWTW effluent, fish farm effluent and stream (Ludo stream).

Objectives

The four major objectives of this thesis were:

1) To review the isotopic tracking of anthropogenic N sources in aquatic environments,

2) To trace the distribution of the urban effluent in Ria Formosa lagoon and to reveal its biological uptake using the stable isotope signatures of organic matter and seagrasses.

3) To assess the changes in the elemental and stable isotope contents of *Zostera noltii* leaves during the early phases of decomposition,

4) To investigate the trophic interactions in Zostera *noltii* meadows using the stable isotope approach in conjunction with standard methods.

This information will help coastal habitat managers to detect incipient eutrophication while impacts are still relatively low and to make decisions about restoration.

Layout of the thesis

This thesis is developed in six main sections. The present chapter (Chapter 1) gives a general overview of the importance and background of the research theme, indicates the main objectives of the study and presents the structure of the thesis.

Chapter 2 presents an overall view of the $\delta^{15}N$ approach and the manner it can be employed in investigating the anthropogenic N sources in aquatic environments. General descriptions demonstrating that multiple tracers with stable isotope of carbon, sulphur and nitrogen can distinguish food resources are available in other reviews.

Chapter 3 presents and discusses the results concerning variables measured in the water column and sediment along the environmental gradient from a Waste Water Treatment Works (WWTW) to a main channel of Ria Formosa during both summer and winter. The environmental variables and $\delta^{13}C$ of dissolved inorganic carbon and $\delta^{15}N$ of ammonium values reveal the physical extent of the sewage influence into Ria Formosa, while the $\delta^{13}C$ and $\delta^{15}N$ values of *Zostera noltii* integrate variations in the environment over time and reflect the availability of nutrients to the seagrass. $\delta^{13}C$ and $\delta^{15}N$ values of particulate organic matter reflect the variability of the mixture of microalgae and other organic particles such as plant detritus suspended in the water column. $\delta^{13}C$ and $\delta^{15}N$ values of sediment organic matter reflect the presence of decomposed microalgae and plant material in the sediment.

Because shifts in stable isotope values between living and detrital plant-derived material can occur, chapter 4 assesses the changes in the elemental and stable isotope contents of *Zostera noltii* leaves during the early phase of decomposition, which is the most relevant in terms of the organic matter transferred to the food web.

Finally, chapter 5 focuses in the trophic interactions in a typical *Zostera noltii* meadow in the Ria Formosa lagoon. The stable isotope relationships among the community organisms were assessed at the same site several times during one year to eliminate the effects of spatial variation but to include the potential temporal variation of isotopic signatures. We used the stable isotope technique in combination with other standard approaches such as gut content analysis, behavioural observations and process rate measurements, to achieve the most robust conclusions.

Chapter 6 presents the general conclusions of this work

References

Andrade C.F. 1990. O ambiente de barreira da Ria Formosa Algarve-Portugal. PhD thesis, University of Lisboa, Lisboa.

Benner R., Fogel M.L., Sprague E.K., Hodson R.E. 1987. Depletion of ^{13}C in lignin and implications for stable carbon isotope studies. Nature 329: 708-710.

Cambridge M.L., McComb A.J. 1984. The loss of seagrasses in Cockburn Sound, Western Australia. 1. The time course and magnitude of seagrass decline in relation to industrial development. Aquatic Botany 20: 229-243.

Cloern J.E. 2002. Stable carbon and nitrogen isotope composition of aquatic and terrestrial plants of the San Francisco Bay estuarine system. Limnology and Oceanography 47: 713-729.

Currin C.A., Newell S.Y., Paerl H.W. 1995. The role of standing dead Spartina alterniflora and benthic microalgae in salt marsh food webs: considerations based on multiple stable isotope analysis. Marine Ecology Progress Series 121: 99-116.

Darnell R.M. 1958. Food habits of fishes and larger invertebrates of Lake Pontchartrain, Louisiana, an estuarine community. Publications Institute Marine Sciences. University of Texas 5: 353-416.

Den Hartog C. 1970. The Seagrasses of the World. Amsterdam, North Holland.

Falcão, M., Vale, C., 1990. Study of the Ria Formosa ecosystem: benthic nutrient remineralization and tidal variability of nutrients in the water. Hydrobiologia 207, 137-146.

Fourqurean J.W., Moore T.O., Fry B. 1997. Spatial and temporal variation in C: N: P ratios, $\delta^{15}N$ and $\delta^{13}C$ of eelgrass *Zostera marina* as indicators of ecosystem processes, Tomales Bay, California, USA. Marine Ecology Progress 157: 147-157.

Fry B., Sherr E.F. 1984. $\delta^{13}C$ Measurements as indicators of carbon flow in marine and freshwater ecosystems. Contributions in Marine Science 27: 13-47.

Haines E.B, Montague C.L. 1979. Food sources of estuarine invertebrates analyzed using $^{13}C/^{12}C$ Ratios. Ecology 60: 48-56.

Hansson S., Hobbie J.E., Elmgren R., Larsson U. 1997. The stable nitrogen isotope ratio as a marker of food-web interactions and fish migration. Ecology 78: 2249-2257.

Heaton T.H.E. 1986. Isotopic Studies of Nitrogen Pollution in the Hydrosphere and Atmosphere – a Review. Chemical Geology 59: 87-102.

Heminga M.A., Duarte C.M., 2000. Seagrass Ecology. Cambridge University Press, United Kingdom.

Lajtha K., Michener R.H. Stable Isotopes in Ecology and Environmental Science. Blackwell Scientific Publications, Oxford.

Livingston R.J. 1982. Trophic organization of fishes in acoastal seagrass system. Marine Ecology Progress Series 7: 1-12.

Machás R. (1999). Análise isotópica da cadeia alimentar da RiaFormosa: contribuição relative dos produtores primários. Master thesis, University of Coimbra, Coimbra.

Machás R., Santos R. 1999. Sources of organic matter in Ria Formosa revealed by stable isotope analysis. Acta Oecologica 20: 463-469.

Machás R., Santos R., Peterson B. 2003. Tracing the flow of organic matter from primary producers to filter feeders in Ria Formosa lagoon, southern Portugal. Estuaries 36: 846-856.

Macko S.A., Ostrom N.E. 1994. Pollution studies using stable isotopes. In: Lajtha K., Michener R.H. (eds). Stable Isotopes in Ecology and Environmental Science. Blackwell Scientific Publications, Oxford.

McClelland J.W., Valiela I., Michener RH 1997. Nitrogen-stable isotope signatures in estuarine food webs: a record of increasing urbanization in coastal watersheds. Limnology and Oceanography 42: 930-937.

Milchakova N.A, Phillips R.C. 2003. Black sea seagrasses. Marine Pollution Bulletin 46: 695-699.

Peterson B.J. 1999. Stable isotopes as tracers of organic matter input and transfer in benthic food webs. Acta Oecologica 20: 479-487.

Peterson B.J., Fry B. 1987. Stable isotopes in ecosystem studies. Annual Review of Ecology and Systematics 18: 293-320.

Peterson B.J., Howarth R.W. 1987. Sulfur, carbon, and nitrogen isotopes used to trace organic matter flow in the Salt-Marsh Estuaries of Sapelo Island, Georgia. Limnology and Oceanography 32: 1195-1213.

Peterson B.J., Howarth R.W., Garritt R.H. 1985. Multiple stable isotopes used to trace the flow of organic matter flow in estuarine food webs. Science 227: 1361-1363.

Rau G.H.; Sweeney, R.E.; Kaplan, I.R.; Mearns, A.J.;Young, D.R. (1981). Differences in animal ^{13}C, ^{15}N and D abundance between a polluted and an unpolluted coastal site: likely indicators of sewage uptake by a marine food web. Estuarine, Coastal and Shelf Science 13: 701-707.

Sfriso A., Pavoni B., Marcomini A., Orio A.A. 1992. Macroalgae, nutrient cycles, and pollutants in the lagoon of Venice. Estuaries: 15: 517-528.

Spies R.B., Kruger H., Ireland R. Rice Jr D.W.1989. Stable isotope ratios and contaminant concentrations in a sewage-discorted food web. Marine Ecology Progress Series 54: 157-170.

Sullivan M.J, Moncreiff C.A.1990. Edaphic algae are an important component of salt marsh food-webs: evidence from multiple stable isotope analyses. Marine Ecology Progress Series 62: 149-159.

Taylor D., Nixon S., Granger S., Buckley B. 1995. Nutriemt limitation and the eutrophication of coastal lagoons. Marine Ecology Progress Series 127: 235-244.

Thybo-Christesen M., Rasmussen M.B., Blackburn T.H. 1993. Nutrient fluxes and growth of *Cladophora sericea* in a shallow Danish bay. Marine Ecology Progress Series 100: 273-281.

Valiela I. 1995. Marine ecological processes. Springer-Verlag, New York.

Valiela I., McClelland J., Hauxwell J., Behr P.J., Hersh D., Foreman K. 1997. Macroalgal blooms in shallow estuaries: controls and ecophysiological and ecosystem consequences. Limnology and Oceanography 42: 1105-1118.

Zaitsev Y.P. 1992. Recent changes in the trophic structure of the Black Sea. Fisheries Oceanography 1: 180-189.

Zieman J.C. 1983. Coral reefs, seagrass beds and mangroves: their interaction in the coastal zones of the Caribbean. Unesco reports in marine science 23: 80-86.

Zieman J.C., Macko S.A., Mills A.L. 1984. Role of seagrasses and mangroves in estuarine food webs: temporal and spatial changes in stable isotope composition and amino acid content during decomposition. Bulletin of Marine Science 35: 380-392.

Chapter 2 – Isotopic tracking of anthropogenic N sources in aquatic environments – a review

Introduction

The nitrogen cycle is one of the most important of the Earth's element cycles and also probably the one most influenced by anthropogenic activity (Heaton 1986). The major sources of nitrogen to coastal watersheds include atmospheric contamination (Valiela & Bowen 2002, Russell et al. 1998), fertilizer runoff and wastewater disposal (Valiela et al. 1997, Vitousek et al. 1997). Nitrogen from these sources is carried to coastal waters via atmospheric deposition, sewage outfalls, river discharges, and groundwater flow (Valiela et al. 1997). Nitrogen loading has increased significantly worldwide in the latter half of the 20th century, contributing to eutrophication in nitrogen-limited coastal environments and dramatically altering their composition and function (Cloern 2001, Deegan et al. 2002, Galloway et al 1995, Galloway et al. 2004, Nixon 1995, Valiela et al. 1992, Vitousek et al. 1997).

Natural abundance of N stable isotopes ($\delta^{15}N$ values) can be used to distinguish the different sources of nutrient loads to coastal systems since the major sources of N to aquatic systems often have distinguishable $\delta^{15}N$ values. For example, groundwater nitrate derived from synthetic fertilizers, atmospheric deposition, and human and animal wastes typically have $\delta^{15}N$ values from: -3 to +2‰, +2 to +8‰, and +10 to +20‰, respectively (Kreitler et al. 1978). Inorganic nitrogen inputs to aquatic ecosystems may be assimilated into particulate organic matter and still reflect the origin of N (Cole et al. 2005, Yelenik et al. 1996, Voss & Struck 1997), as well as into sediment (Lake et al. 2001, Voss & Struck 1997, Voss et al.

2000), primary producers (Cole et al. 2005, Constanzo et al. 2001, Hobbie et al. 1990, McClelland et al. 1997, Rogers 1999, Savage & Elmgren 2004) and consumers (Cabana & Rasmussen 1996, Fry 1999, Lake et al. 2001, McClelland et al. 1997, Pruel et al. 2006, Rau et al. 1981, Spies et al. 1989, Wayland & Hobson 2001). Several studies demonstrate that nitrogen stable isotopes may be suitable indicators of anthropogenic delivery of nutrients to groundwater (Aravena et al. 1993, Kreither 1979, Kreither et al. 1978, Kreither & Browning 1983, Kroeger et al. 2006, McClelland et al. 1998a) and freshwater (Cabana & Rasmussen 1996, Hebert & Wassenaar 2001, Lake et al. 2001, Wayland & Hobson 2001), estuarine (Cole et al. 2004, Constanzo et al. 2001, Fry 1999, McClelland & Valiela 1998b, Voss & Struck 1997) and marine systems (Heikoop et al. 2000, Rish & Erdmann 2000, Rau et al. 1981, Spies et al. 1989). This paper reviews the overall generality of the $\delta^{15}N$ approach in aquatic environments and the manner in which it may be employed to track the N sources in aquatic environments. We used data from the literature to assess how well the $\delta^{15}N$ signals of sediment, particulate organic matter (POM), macrophytes and consumers reflect the relative amount of wastewater derived N load, the $\delta^{15}N$ signal of nutrients (nitrate, ammonium and dissolved inorganic nitrogen, DIN) in the water column and the groundwater $\delta^{15}N$ signature of DIN. Furthermore, we assessed how well the N isotopic signal of POM is passed on to suspension feeder consumers.

Isotopic fractionation

Many reactions of the N-cycle discriminate against the heavier isotope (fractionation) due to the faster processing of the ^{14}N than ^{15}N (Fry et al. 2003). Some processes result in little isotopic fractionation (e.g. N_2 fixation), while others leave residual N enriched in ^{15}N (Heaton 1986). The processes of nitrification, which converts ammonium to nitrite and nitrate, and volatilization, both leave residual ammonium enriched in ^{15}N. Likewise, the conversion of nitrate to nitrogen gas and nitrous oxide leaves residual nitrate enriched in ^{15}N (Mariotti et al. 1981, Macko & Ostrom 1994). In a closed system, the product gradually becomes less depleted in ^{15}N, so that when all the substrate is reacted the $\delta^{15}N$ of the product is identical to the $\delta^{15}N$ of the substrate at the start of the reaction (Owens 1987). The degree of fractionation in natural systems is difficult to predict from experiments because fractionation is affected by environmental conditions (Heaton 1986, Lund et al. 2000, Owens 1987). For example, Mariotti et al. (1982a) showed that the rate of supply of reductant in the form of reduced organic carbon compounds was temperature dependent and affected the degree of isotopic enrichment observed in denitrification.

Primary producers reflect the $\delta^{15}N$ of their inorganic N source plus a variable amount of fractionation during N uptake (Robinson et al. 1998). Their $\delta^{15}N$ signature is similar to the N source when N is in limited supply because the amount of fractionation depends on the concentration of N available to the enzymatic processes during N assimilation (Mariotti et al. 1982b, Pennock et al. 1996, Wada & Hattori 1978). For macrophytes there is generally a fractionation of -1‰ to -10‰, depending in nutrients concentrations and diffusion steps (Peterson & Fry 1987, Fogel & Cifuentes 1993) Consumers typically have a progressive enrichment of successive trophic levels by proximally 3.4 ‰ per trophic transfer (Post 2002). Post did not find significant differences in mean fractionation or in the variability in fractionation between carnivores and hervivores/ detritivores. In the metabolism of nitrogen, the light isotope is concentrated in nitrogenous excretion products while the heavy isotope is discriminated against and retained in body tissues (Peterson & Fry 1987, Peterson 1999).

Variation in the $\delta^{15}N$ values of nitrogen sources to aquatic environments

In nature, although there is a considerable range of $\delta^{15}N$ values within each system, the average values apparently increase from atmospheric to terrestrial, freshwater, estuarine and marine systems (Owens 1987). This is because the ultimate N source for terrestrial biomass is atmospheric N_2 (0‰) whereas the marine primary production is fuelled by nitrate that has a $\delta^{15}N$ value of approximately 5‰ (Peterson & Fry 1987). Terrestrial and fresh water materials are less distinct than marine organic matter.

Pristine estuarine gradients are characterized by increasing stable isotope values seaward. However, the gradients have been changed by human influences (Fry 2002). Examples include those of Voss et al. (2000), where the influence of eutrophication on the $\delta^{15}N$ values of sediment was found to be so dominant that it even overprinted the usually observed mixing gradient from terrestrial (low isotope values) to the marine environment (high isotope values). McClelland et al. (1997) showed that as the percentage of wastewater contributes to total N loading increases, the $\delta^{15}N$ values of estuarine biota increase.

The nitrogen derived from animal or sewage waste has very high $\delta^{15}N$ signature when compared with the other major sources of nitrogen. Nitrogen in excreted mainly in the form of urea, which when hydrolysed, produces a temporary rise in pH, a condition which favours the formation of ammonia which is easily lost by volatilization of ^{15}N depleted ammonia to the atmosphere. Most of the residual ammonium, now correspondingly enriched in ^{15}N, is subsequently converted to ^{15}N-enriched nitrate, which is more readily leached and dispersed by water (Heaton 1986). Also, a major fraction of sewage is derived from human foods of high trophic level (meats) and would therefore be expected to exhibit ^{15}N enrichment (Minagawa & Wada 1984). Primary producers from sewage treatment have high ^{15}N values (Wayland & Hobson 2001) while sewage-derived organic nitrogen, terrestrial in origin, is usually isotopically light compared to marine values (Rau et al. 1981, Rogers 1999, Spies et al. 1989, Tucker et al. 1999). The typical low $\delta^{15}N$ values associated with synthetic fertilizers result from the chemical fixation of atmospheric N_2 during manufacturing (Heaton 1986). The low $\delta^{15}N$ values of fertilizer facilitate the easy differentiation from sewage wastes.

Limitations of the approach

Analysis of the natural abundance of δ values in organic and inorganic material can be a very powerful tool in the resolution of the sources and pathways that a material travels in the environment if (1) the primary source of interest is isotopically distinct from the background source and (2) the isotopic signature of the source does not change as the material is transported and transformed in the environment or does so in a predictable manner (Macko & Ostrom 1994).

Some authors demonstrated that the first assumption is not always valid. For example, Jordan et al. (1997) showed that cold temperatures during winter can result in low nitrification rates that limit microbial N processing in wastewater and lead to low $\delta^{15}N$ values. The $\delta^{15}N$ values of anthropogenic sources can also be masked by natural biogeochemical and physical processes if within estuaries processes are creating high $\delta^{15}N$ values. Natural nitrification and denitrification within- estuary and in the sea can create high $\delta^{15}N$ signals in ammonium and nitrate (Brandes & Devol 1997, Cifuentes et al. 1989, Lindau et al. 1989, Mariotti et al. 1984, Horrigan et al. 1990). On the other hand, if the background system has high N concentration, the N sources of interest can be difficult to identify. Fry et al (2003) demonstrated that the nitrogen isotope approach can fail to detect N-loading under conditions of very high ammonium inputs from sewage loading because isotope fractionation increases at high ammonium concentrations, creating low $\delta^{15}N$ values in primary producers and masking the effect of adding ^{15}N enriched nutrients from sewage.

Despite the limitations of this approach, the $\delta^{15}N$ values can provide valuable information on qualitative and quantitative changes in the nitrogen status of aquatic systems (Owens 1987, Fry et al. 2003, Macko & Ostrom 1994, McClelland et al. 1997). The larger the isotopic difference between contributing sources, the easier it is to interpret isotopic results. In order to obtain the most accurate understanding at it is important to characterize the isotopic signatures in the different components of the aquatic system very well, including the potential seasonal variation in the $\delta^{15}N$ values of nutrients and biota.

Methods

The $\delta^{15}N$ values of sediment, POM, macrophytes and consumers were obtained from the literature reporting wastewater or groundwater gradients in aquatic environments. Whenever possible, macrophytes were divided into macroalgae and vascular plants. The N signature of these ecosystem components were related to the available information on the relative amount of wastewater load into the system as a percentage of total N load and with the $\delta^{15}N$ signal of nutrients (nitrate, ammonium and DIN). We also assessed the relationship between the $\delta^{15}N$ values of sestonic POM and suspension feeders. The sestonic POM samples include phytoplankton but can also contain significant amounts of bacteria, detritus and small zooplankton. Unfortunately we did not find any data on phytoplankton probably because of the difficulty in obtaining pure samples, even though new methods have recently been developed to assess the isotopic values of phytoplankton chlorophyll (Sachs et al. 1999, Sachs & Repeta 2000).

The significance of the relationships was explored using linear regression analysis. The test for the equality of regression coefficients (Zar 1996) was used to compare regression slopes. Regressions were considered statistically significant at $p < 0.05$.

Results and discussion

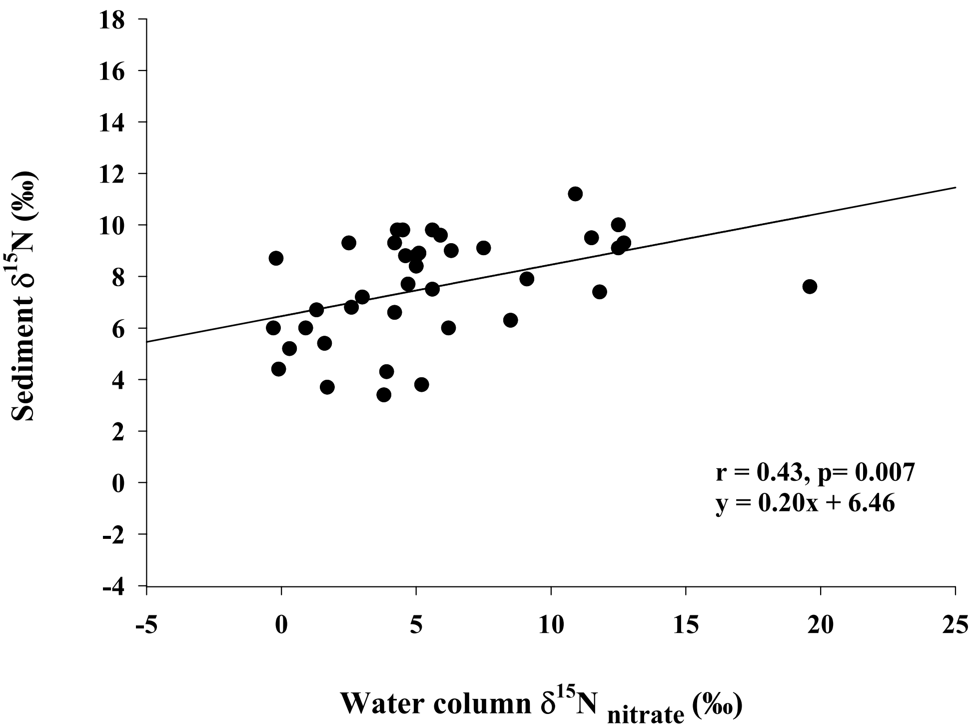


Fig. 1. Relationship between the $\delta^{15}N$ values of sediment and the $\delta^{15}N$ values of nitrate in the water column. Original data are presented in Table I (Annex I).

The $\delta^{15}N$ values of sediment increase significantly with the ^{15}N enrichment of the water column nitrate (Fig.1) indicating the potential of sediment samples to track N sources. Unfortunately, it was not possible to relate the $\delta^{15}N$ values of sediment with wastewater load due to the lack of available information. A positive relationship between sediment $\delta^{15}N$ values and nutrient load (DIN) was observed by Lake et al. (2001) in fresh ponds of Rhode Island

(Providence, USA) potentially confirming this pattern, but neither the ^{15}N values of DIN in the water or of the sources of DIN were taken into consideration. The sediment samples were taken from several freshwater sites affected by differing levels of anthropogenic activity that ranged from highly developed to rural but no direct measures of the amount or of the isotopic composition of wastewater inputs were performed.

Sediment organic matter (SOM) may not always be a suitable anthropogenic N tracer because it integrates the $\delta^{15}N$ signals of a variety of plant detritus, microphytobenthos and terrestrial particulate inputs of sewage particulates. Eventually, the organic matter from the anthropogenic source may not be isotopically distinct from the background source (Fry et al. 2003, Gartner et al. 2002, Owens 1987, Wayland & Hobson 2001). Waldron et al. (2001) did not find the sewage isotope signal to be strongly recorded in the sediment due both to the dilution caused by tidal movement and wind-induced wave action, and to benthic invertebrate grazing of particulate matter. It is also important to consider that shifts in the nitrogen isotope values of organic matter can occur during degradation (Benner et al. 1997, Cloern 2002, Currin et al. 1995). On the other hand, $\delta^{15}N$ shifts may not happen in the early phase of detritus decay as Machás et al (2006) showed for the seagrass *Zostera noltii*. In San Pedro Shelf area of Southern California, the $\delta^{15}N$ of POM of the sewage effluent was significantly different from the SOM of an undisturbed site. The $\delta^{15}N$ values of the sewage POM did not change during partial degradation and thus the distribution of $\delta^{15}N$ values in the sediment was a suitable tracer of anthropogenic N (Sweeney et al. 1980).

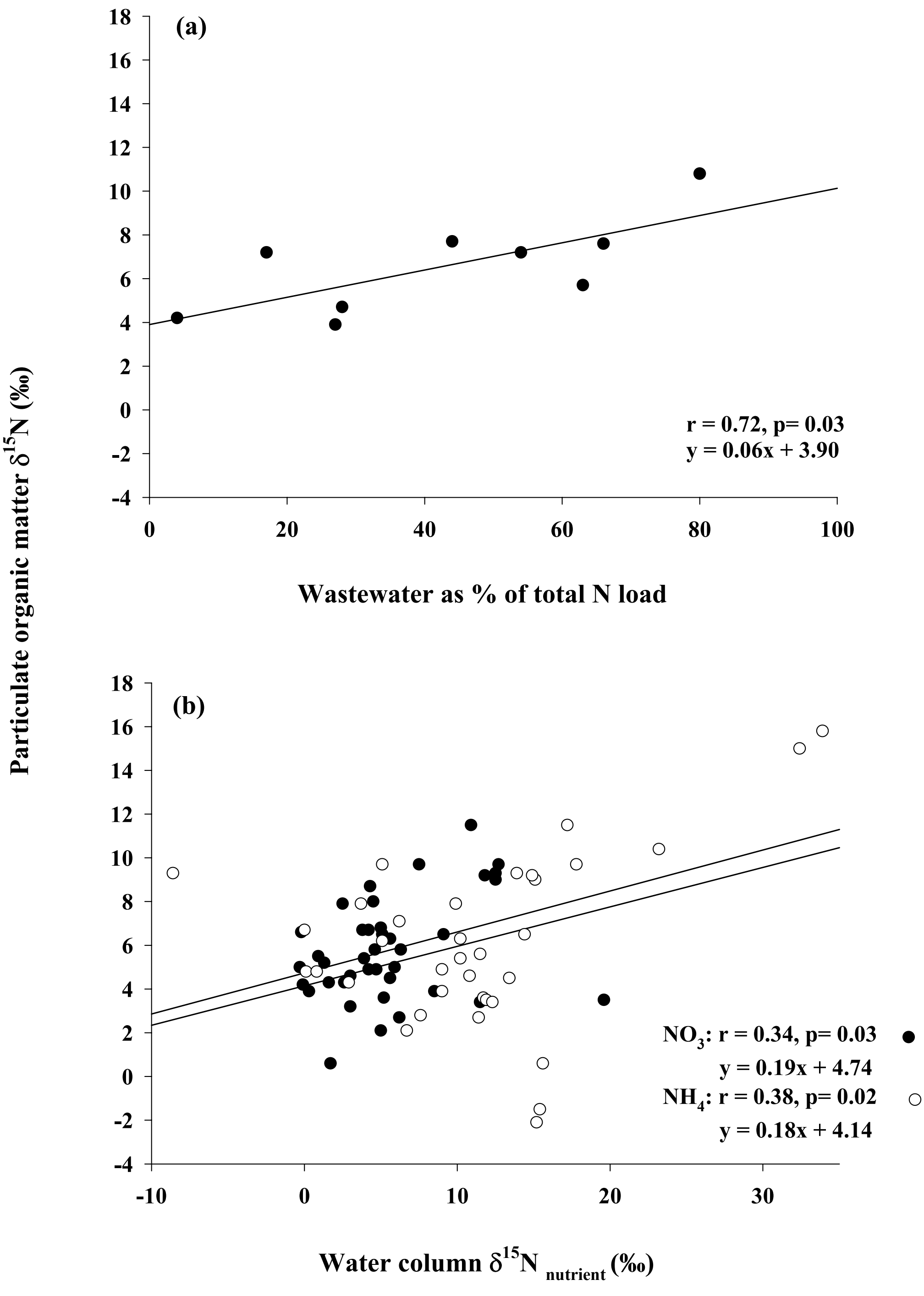


Fig. 2. Particulate organic matter as a tracer of anthropogenic N sources. (a) Relationship between $\delta^{15}N$ values of POM and wastewater N load (as the percentage of total N). (b) Relationship between $\delta^{15}N$ values of POM and $\delta^{15}N$ values of nitrate and ammonium in the water column. Original data are presented in Table I (Annex I).

The $\delta^{15}N$ values of POM are significantly related both to the wastewater load and to the $\delta^{15}N$ values of both nitrate and ammonium in the water column (Fig 2), which indicates that this parameter is a good N tracer in aquatic environments. A good example of this is given in Machás et al. (in prep.) where the influence of urban wastewater was traced along a gradient from the discharge of a Waste Water Treatment Works (WWTW) to an undisturbed site in the Ria Formosa lagoon, southern Portugal. The magnitude of the seasonal change of the $\delta^{15}N$ values of ammonium of the WWTW effluent was large enough to reverse the gradient of the $\delta^{15}N$ values of POM in Ria Formosa. In the summer, the $\delta^{15}N_{POM}$ values decreased from the WWTW to the undisturbed site due to the decreasing influence of isotopically heavy N from the WWTW. In the winter, the $\delta^{15}N_{POM}$ values showed the opposite trend because the WWTW $\delta^{15}N_{NH4}$ was lower than in the summer. This highlights the importance of characterizing the isotopic signatures in the different components of the aquatic system very well, including potential seasonal variations in the $\delta^{15}N$ values of nutrients.

The analysis of literature data showed significant linear relationships between the $\delta^{15}N$ values of macrophytes and wastewater N loads (Fig. 3a), revealing that macrophytes integrate variations in the nutrient environment over time and thus are suitable indicators of anthropogenic N sources. Different taxonomical groups (macroalgae and rooted macrophytes) did not show significant different slopes even though they have different nutrient uptake characteristics as Cole et al. (2005) pointed out.

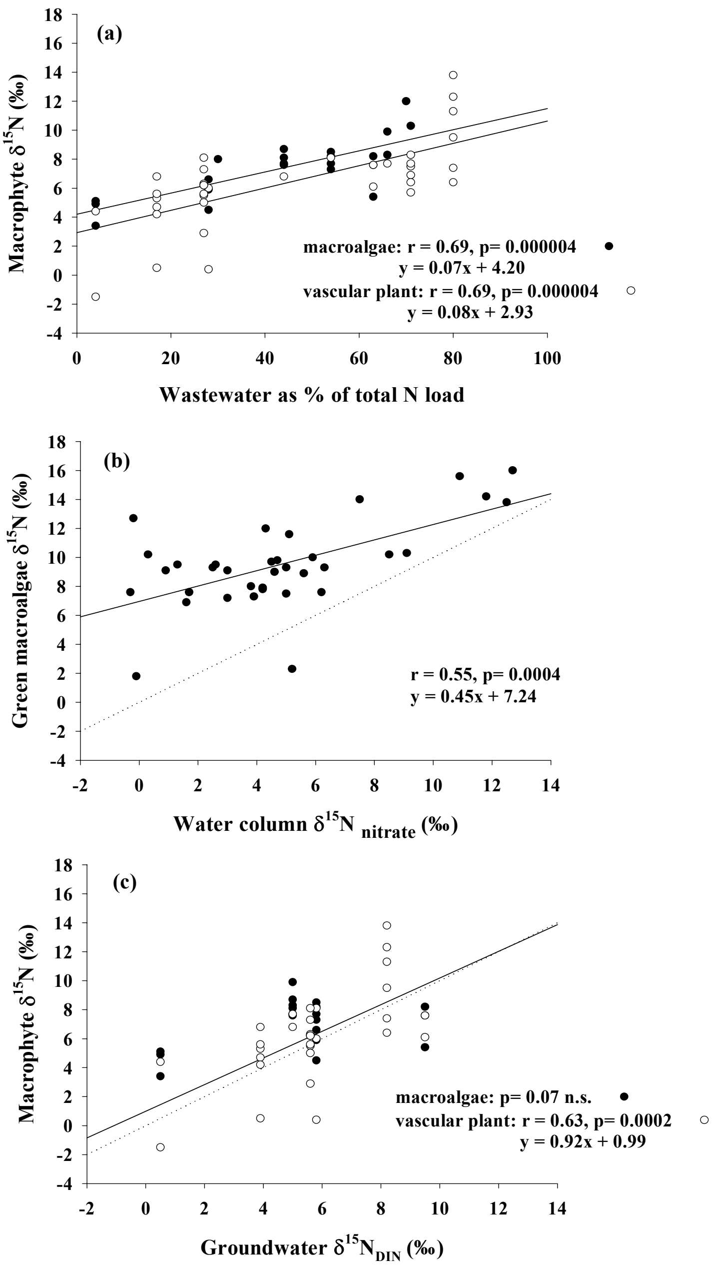


Fig. 3. Macrophytes as tracers of anthropogenic N sources. (a) Relationships between both the $\delta^{15}N$ values of macroalgae and vascular plants and the wastewater N load (as the percentage of total N). (b) Relationship between the $\delta^{15}N$ values of green macroalgae and the $\delta^{15}N$ values of nitrate in the water column. (c) Relationships between both the $\delta^{15}N$ values of macroalgae and vascular plants and the $\delta^{15}N$ values of dissolved inorganic nitrogen (DIN) in ground water. $\delta^{15}N$ values of DIN from Cole et al. (2005). Dotted 1:1 line represents a hypothetical situation of no isotopic fractionation between product and substrate. Original data are presented in Table I (Annex I).

The significant linear relationship between the $\delta^{15}N$ values of green macroalgae and the $\delta^{15}N$ values of water column nitrate suggest that these organisms can be used to track anthropogenic N sources (Fig. 3b). However, there were no significant relationships between green macroalgae and either ammonium or DIN in the water column. Green macroalgae had higher $\delta^{15}N$ values water column nitrate indicating that nitrate did not supply their entire N quota. Curiously, the difference between the $\delta^{15}N$ values of nitrate and green macroalgae was greater at lower nitrate $\delta^{15}N$ values and decreased when nitrate $\delta^{15}N$ values were higher. This may reflect a higher concentration of nitrates originating from wastewater in situations when the $\delta^{15}N$ values are higher. The fraction factor for nitrate uptake by algae is concentration dependent (Pennock et al. 1996) with higher fractionation occurring at higher nitrate concentrations.

The $\delta^{15}N$ values of rooted macrophytes and macroalgae also increased with the $\delta^{15}N$ values of groundwater DIN (Fig.3c). The regression was highly significant for rooted macrophytes whereas for macroalgae the p value was 0.07. This difference may reflect the smaller sample size for macroalgae or fact that rooted macrophytes have more access to groundwater nutrients through their roots whereas the macroalgae only have access to this source when the nutrients diffuse from the sediment to the water column. Rooted macrophytes seem to be better indicators than macroalgae of anthropogenic N sources that diffuse from the sediments.

Consumers with high $\delta^{15}N$ values were found in sites with higher wastewater N load (Fig. 4a) indicating that the $\delta^{15}N$ signatures of primary producers labelled with anthropogenic influence are passed on to consumers in the food webs of aquatic systems. Unfortunately, we did not find enough information to compare different group of organism as indicators of anthropogenic N sources. Care should be taken on the selection of organisms. For example, Pruell et al. (2006) concluded that sampling programs designed to determine long-term trends should consider species that do not show rapid fluctuations in isotope values such as the mud snail, *Nassarius obsoletus*. The N isotopic signature of a consumer may change seasonally in response to changing contribution of different primary producers in the diet (Waldron et al. 2001, McClelland & Valiela 1998b) or in the δ-values of the N source, or due to physiological changes in the species with season such as development stage and reproductive condition (Pruell et al. 2006). The opportunistic feeding behaviour of many species is detrimental to use them as a tracer. Some organisms move or migrate long distances and their isotopic composition will not reflect local nitrogen sources. The size of an organism is also an important factor to take into consideration because small organisms have fast nitrogen turnover rates and thus tend to show greater temporal variability in their $\delta^{15}N$ signature than larger organisms that integrate small scale time variations (Cabana & Rasmussen 1996).

The $\delta^{15}N$ values of suspension feeders increase significantly with the enrichment of the $\delta^{15}N$ values of POM confirming that the $\delta^{15}N$ signatures of primary producers are passed on to consumers (Fig. 4c). The fractionation of suspension feeders relative to POM was +5.3‰ ± 1.7. This enrichment was higher than 3.4‰, the mean trophic fractionation for $\delta^{15}N$ measured by Post (2002). This can be explained by the species selectivity of a ^{15}N enriched component of the POM, probably phytoplankton or by feeding on a mixture of phytoplankton and small zooplankton.

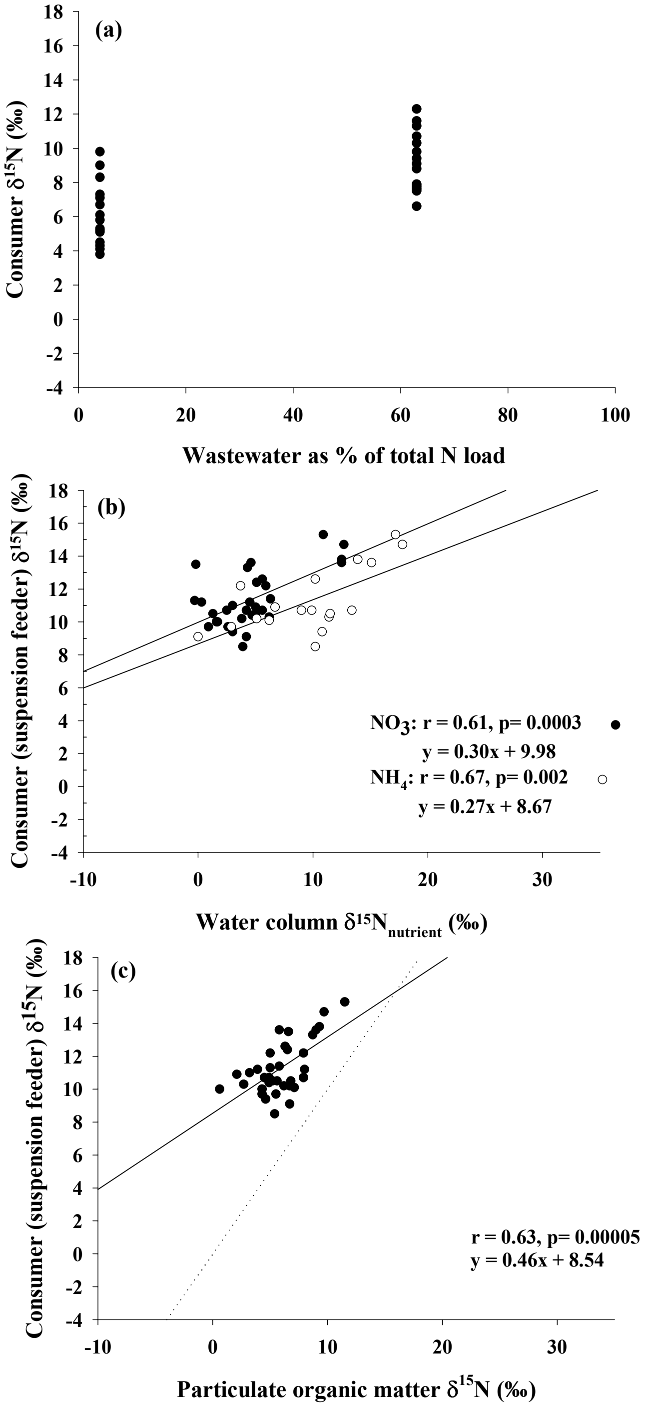


Fig. 4. Consumers as tracers of anthropogenic N sources. (a) Relationships between the $\delta^{15}N$ values of consumers and the wastewater N load (as the percentage of total N). (b) Relationships between the $\delta^{15}N$ values of suspension feeders and the $\delta^{15}N$ values of nitrate and ammonium in the water column. (c) Relationships between the $\delta^{15}N$ values of suspension feeders and the $\delta^{15}N$ values of particulate organic matter Dotted 1:1 line represents a hypothetical situation of no isotopic fractionation between product and substrate. Original data are presented in Table I (Annex I).

Conclusion

The $\delta^{15}N$ values of components of the estuarine ecosystem can provide valuable information on changes in the nitrogen status of aquatic systems. The larger the isotopic differences among contributing sources, the easier it is to interpret isotopic results. The best understanding can be achieved by characterizing the isotopic signatures in the different compartment of the aquatic system very well, including the potential seasonal variation in the $\delta^{15}N$ values of nutrients and biota. The analysis of data from the literature provided insights into the potential of sediment, POM, macrophytes and consumers samples to track anthropogenic N sources. However more studies that take into consideration the magnitude and $\delta^{15}N$ signal of nutrient sources (nitrate, ammonium, DIN, DON and PON) in relation to the N signature of ecosystem components values are needed.

References

Aravena R., Evans M.L., Cherry J.A. 1993. Stable isotopes of oxygen and nitrogen in source identification of nitrate from septic systems. Ground Water 31: 180-186.

Benner R., Biddanda B., Black B., McCarthy M. 1997. Abundance, size distribution, and stable carbon and nitrogen isotopic compositions of marine organic matter isolated by tangential-flow ultrafiltration. Marine Chemistry 57: 243-263.

Brandes J.A., Devol A.H. 1997. Isotopic fractionation of oxygen and nitrogen in coastal marine sediments. Geochimica et Cosmochimica Acta 61: 1793-1801.

Cabana G., Rasmussen J.B. 1996. Comparison of aquatic food chains using nitrogen isotopes. Proceedings of the National Academy of Sciences USA 93: 10844-10847.

Cifuentes L.A., Fogel M.L., Pennock J.R., Sharp J.H. 1989. Biogeochemical factors that influence the stable nitrogen isotope ratio of dissolved ammonium in the Delaware Estuary. Geochimica et Cosmochimica Acta. 53: 2713-2721.

Cole M.L., Kroeger K.D., McClelland J.W., Valiela I. 2005. Macrophytes as indicators of land-derived wastewater: application of a $\delta^{15}N$ method in aquatic systems. Water Resources Research. 41: 1-9.

Cole M.L., Valiela I., Kroeger K. D.,Tomasky G.L., Cebrián J., Wigand C., McKinney R.A., Grady S.P., Silva M.H.C. 2004. Assessment of a $\delta^{15}N$ isotopic method to indicate anthropogenic eutrophication in aquatic ecosystems. Journal of Environmental Quality 33: 124-132.

Constanzo S.D., O'Donohue M.J., Dennison W.C., Loneragan N.R.,Thomas M. 2001. A new approach for detecting and mapping sewage impacts. Marine Pollution Bulletin 42: 149-156.

Cloern J.E. 2001. Our evolving conceptual model of the coastal eutrophication problem. Marine Ecology Progress Series 210: 223-253.

Cloern J.E. 2002. Stable carbon and nitrogen isotope composition of aquatic and terrestrial plants of the San Francisco Bay estuarine system. Limnology and Oceanography 47: 713-729.

Currin C.A., Newell S.Y., Paerl H.W.. 1995. The role of standing dead *Spartina alterniflora* and benthic microalgae in salt marsh food webs: considerations based on multiple stable isotope analysis. Marine Ecology Progress Series 121: 99-116.

Deegan L.A., Wright A., Ayvazian S.G., Finn J.T., Golden H., Merson R.R., Harrison J. 2002. Nitrogen loading alters seagrass ecoystem structure and support of higher trophic levels. Aquatic Conservation: Marine and Freshwater Ecosystems 12: 193-212.

Fogel M.A., Cifuentes L.A. 1993. Isotopic fractionation during primary production. In Engel M.H., Macko S.A.(eds). Organic Geochemistry. Plenum, New York.

Fry B. 1999. Using stable isotopes to monitor watershed influences on aquatic trophodynamics. Canadian Journal of Fisheries and Aquatic Sciences 56: 2167-2171.

Fry B. 2002. Conservative mixing of stable isotopes across estuarine salinity gradients: a conceptual framework for monitoring watershed influences on downstream fisheries production. Estuaries 25: 264-271

Fry B., Gace A., McClelland J.W. 2003. Chemical indicators of anthropogenic nitrogen loading in four Pacific estuaries. Pacific Science 57:99-123.

Galloway J.N, Schesinger W.H., Levy II H., Michaels A., Schnoor J.L. 1995. Nitrogen fixation: anthropogenic enhancement-environmental response. Global Biogeochemical Cycles 9: 235-252.

Galloway J.N, Dentener F.J., Capone D.G., Boyer E.W., Howarth R.W., Seitzinger S.P., Asner G.P., Cleveland C.C., Green P.A., Holland E.A., Karl D.M., Michaels A.F., Porter J.H., Townsend A.R., Vorosmarty C.J. 2004. Nitrogen cycles: past, present, and future. Biogeochemistry 70: 153-226.

Gartner A., Lavery P., Smit A.J. 2002. Use of $\delta^{15}N$ signatures of different functional forms of macroalgae and filter-feeders to reveal temporal and spatial patterns in sewage dispersal. Marine Ecology Progress Series 235: 63-73.

Heaton T.H.E. 1986. Isotopic Studies of Nitrogen Pollution in the Hydrosphere and Atmosphere – a Review. Chemical Geology 59: 87-102.

Hebert C.E., Wassenaar L.I. 2001. Stable Nitrogen Isotopes in Waterfowl Feathers Agricultural land Use in Western Canada Environmental Science & Technology 35: 3482-3487.

Heikoop J.M., Risk M.J., Lazier A.V., Edinger E.N., Jompa J., Limmon G.V., Dunn J.J., Browne D.R., Schwarcz H.P. 2000. Nitrogen-15 signals of anthropogenic nutrient loading in reef corals. Marine Pollution 40: 628-636.

Hobbie J.E., Larsson U., Elmgren R., Fry B. 1990. Sewage derived ^{15}N in the Baltic traced in *Fucus*. EOS 71: 190.

Horrigan S.G., Montoya J.P., Nevins J.L., McCarthy J.J. 1990. Natural isotopic composition of dissolved inorganic nitrogen in the Chesapeake Bay. Estuarine, Coastal and Shelf Sciences 30: 393-410.

Jordan M.J., Nadelhoffer K.J., Fry, B. 1997. Nitrogen cycling in forest and grass ecosystems irrigated with ^{15}N-enriched wastewater. Ecological Applications 7: 864-881.

Kreitler C.W. 1979. Nitrogen-isotope ratio studies of soils and groundwater nitrate from alluvial fan aquifers in Texas. Journal of Hydrology 42: 147-170.

Kreitler C.W., Browning L.A. 1983. Nitrogen -isotope analysis of groundwater nitrate in carbonate aquifers: Natural sources versus human pollution. Journal of Hydrology 61: 285-301.

Kreitler C.W., Ragone S.E., Katz B.G. 1978. N15/N14 ratios of ground-water nitrate, Long Island, New York. Ground Water 16: 404-409.

Kroeger K. D., Cole M.L., York J.K., Valiela I. 2006. Nitrogen Loads to Estuaries from Waste Water Plumes: Modeling and Isotopic Approaches. Ground Water 44: 188-200.

Lake J.L., McKinney R.A., Osterman F.A., Pruell R.J., Kiddon J., Ryba S.A., Libby A.D. 2001. Stable nitrogen isotopes as indicators of anthropogenic activities in small freshwater systems. Canadian Journal of Fish and Aquatic Sciences. 58: 870-878.

Lindau C.W., Delaune R.D., Patrick W.H., Lambremont E.N. 1989. Assessment of stable nitrogen isotopes in fingerprinting surface water inorganic nitrogen sources. Water Air and Soil Pollution 48: 489-496.

Lund L.J, Horne A.J., Williams A.E. 2000. Estimating denitrificatin in a large constructed wetland using stable nitrogen isotope ratios. Ecological Applications 14: 67-76.

Machás R., Santos R., Peterson B. 2003. Tracing the flow of organic matter from primary producers to filter feeders in Ria Formosa lagoon, southern Portugal. Estuaries 26: 846-856.

Machás R. Santos R. Peterson B. 2006. Elemental and stable isotope composition of *Zostera noltii* (Horneman) leaves during the early phases of decay in a temperate mesotidal lagoon. Estuarine Costal and Shelf Science 66: 21-29.

Macko S.A., Ostrom N.E. 1994. Pollution studies using stable isotopes. In: Lajtha K., Michener R.H. (eds). Stable Isotopes in Ecology and Environmental Science. Blackwell Scientific Publications, Oxford.

Mariotti A., Germon J.C., Leclerc, A. 1982a. Nitrogen isotope fractionation associated with the $NO_2 \rightarrow N_2O$ step of denitrification in soils. Canadian Journal of Soil Science 62: 227-241.

Mariotti A., Lancelot C., Billen G. 1984. Natural isotopic composition of nitrogen as a tracer of origin for suspended organic matter in the Scheldt estuary. Geochimica et Cosmochimica Acta 48: 549-555.

Mariotti A., Champigny L., Amarger N., Moyse A. 1982b. Nitrogen isotope fractionation associated with nitrate reductase activity and uptake of NO_3^- by Pearl Millet. Plant Physiology 69: 880-884.

Mariotti A., Germon J.C., Hubert P., Kaiser P., Letolle R., Tardieux A., Tardieux P. 1981. Experimental determination of nitrogen kinetic isotope fractionation: some principles; illustration for the denitrification and nitrification processes. Plant Soil 62: 413-430.

McClelland J. W., Valiela I. 1998a. Linking nitrogen in estuarine producers to land-derived sources. Limnology and Oceanography 43:577-585

McClelland J.W., Valiela I. 1998b. Changes in food web structure under the influence of increased anthropogenic nitrogen inputs to estuaries. Marine Ecology Progress Series 168:259-271.

McClelland J. W., Valiela I., Michener R. H. 1997. Nitrogen stable isotope signatures in estuarine food webs: A record of increasing urbanization in coastal watersheds. Limnology and Oceanography 42:930-937.

Minagawa M., Wada E. 1984. Stepwise enrichment of ^{15}N along food chains: further evidence and the relation between $\delta^{15}N$ and animal age. Geochimica et Cosmochimica Acta 48: 1135-1140.

Nixon S.W. 1995. Coastal marine eutrophication: A definition, social causes, and future concerns. Ophelia 41: 199-219.

Owens N.J.P. 1987. ^{15}N in the marine environment. Advances in Marine Biology 24: 389-451.

Pennock J.R., Velinsky D.J., Ludlam J.M., Sharp J.H., Fogel M.L. 1996. Isotopic fractionation of ammonium and nitrate during uptake by *Skeletonema costatum*: Implications for $\delta^{15}N$ dynamics under bloom conditions. Limnology and Oceanography 41: 451-459.

Peterson B.J. 1999. Stable isotopes as tracers of organic matter input and transfer in bentic food webs. Acta Oecologica 20: 479-487

Peterson B.J., Fry B. 1987. Stable isotopes in ecosystem studies. Annual Review of Ecology Systematics 18: 293-320.

Post D.M. 2002. Using stable isotopes to estimate trophic position: models, methods, and assumptions. Ecology 83: 703-718.

Pruell R.J., Taplin B.K., Lake J.L., Jayaraman S. 2006. Nitrogen isotope ratios in estuarine biota collected along a nutrient in Narragansett Bay, Rhode Island, USA. Marine Pollution Bulletin 52: 612-620.

Rau G.H., Sweeney R.E., Kaplan I.R., Mearns A.J., Young D.R. 1981. Differences in animal ^{13}C, ^{15}N and D abundance between a polluted and an unpolluted coastal site: likely indicators of sewage uptake by a marine food web. Estuarine Coastal Shelf Science 13: 701-707.

Risk M.J., Erdmann M.V. 2000. Isotopic composition of nitrogen in stomatopod (Crustacea) tissues as an indicator of human sewage impacts on Indonesian Coral Reefs. Marine Pollution Bulletin 40: 50-58.

Robinson, D. Handley, L.L.Scrimgeour, C.M. 1998. A theory for 15N/14N fractionation in nitrate-grown vascular plants. Planta 205: 397-406.

Rogers K.M. 1999. Effects of sewage contamination on macro-algae and shellfish at Moa Point, New Zealand using carbon and nitrogen isotopes. New Zealand Journal of Marine and Freshwater Research 33: 181-188.

Russell K.M., Galloway J.N., Macko S.A., Moody J.L., Scudlark J.R. 1998. Sources of nitrogen in wet deposition to the Chessapeake Bay region. Atmospheric Enviromment 32: 2453-2465

Sachs J.P., Repeta D.J. 2000. The purification of chlorines from marine particles and sediments for nitrogen and carbon isotopic analysis. Organic Geochemistry 31: 317-329.

Sachs J.P., Repeta D.J., Goericke R. 1999. Nitrogen and carbon isotopic ratios of chlorophyll from marine phytoplankton. Geochimica et Cosmochimica Acta 63: 1431-1441.

Savage C., Elmgren R. 2004. Macroalgal (*Fucus vesiculosus*) $\delta^{15}N$ values trace decrease in sewage influence. Ecological Applications 14:517-526.

Schmitt R.J., Osenberg C.W. 1995. Detecting ecological impacts; concepts and applications in coastal habitats. Academic Press, San Diego, California.

Spies R.B, Kruger H., Ireland R., Rice Jr D.W. 1989. Stable isotope ratios and contaminant concentrations in a sewage-distorted food web. Marine Ecology Progress Series 54: 157-170.

Sweeney R.E., Kalil E.K., Kaplan, I.R. 1980. Characterisation of domestic and industrial sewage in southern California coastal sediments using nitrogen, carbon, sulphur and uranium tracers. Marine Environmental Research 3: 225-243.

Tucker J., Sheats N., Giblin A.E., Hopkinson C.S., Montoya J.P. 1999. Using stable isotopes to trace sewage-derived material through Boston Harbor and Massachusetts Bay. Marine Environmental Research 48: 353-375.

Valiela I., Bowen J.L. 2002. Nitrogen sources to watersheds and estuaries: role of land cover mosaics and losses within watersheds. Environmental Pollution 118: 239-248.

Valiela I., Collins G., Kremer J., Lajtha K., Geist M., Seely B., Brawley J., Sham C.H. 1997. Nitrogen loading from coastal watersheds to receiving estuaries: new method and application. Ecological Applications 7: 358-380.

Valiela I., Foreman K., LaMontagne M., Hersh D., Costa J., Peckol P., DeMeo Andreson, D'Avanzo C., Babione M., Sham C., Brawley J., Lajtha K. 1992. Couplings of watersheds and coastal waters: sources and consequences of nutrient enrichment in Waquoit Bay, Massachusetts. Estuaries 15: 443-457.

Vitousek P.E., Aber J.D., Howarth, R.W., Likens G.E., Matson P.A., Schindler.D., Schlesinger W.H., Tilman D.G. 1997. Human alteration on the global nitrogen cycle: sources and consequences. Ecological Applications 7: 737-750.

Voss M., Struck U. 1997. Stable nitrogen and carbon isotopes as indicator of eutrophication of the Oder River (Baltic Sea). Marine Chemistry 59: 35-49

Voss M., Larsen B., Leeivuori M., Vallius H. 2000. Stable isotope signals of eutrophication in Balthic Sea sediments. Journal of Marine Systems 25: 287-298.

Wada E., Hattoti A. 1978. Nitrogen isotope effects in the assimilation of inorganic nitrogenous compounds by marine diatoms. Geomicrobiology Journal 1: 85-101.

Waldron S., Tatner P., Jack I., Arnott C. 2001. The impact of sewage discharge in a marine embayment: a stable isotope reconnaissance. Estuarine, Coastal and Shelf Science 52: 111-115.

Wayland M., Hobson K.A. 2001. Stable carbon, nitrogen, and sulfur isotope ratios in riparian food webs on rivers receiving sewage and pulp-mill effluents. Canadian Journal of Zoology 79: 5-15.

Yelenik S., McClelland J., Feinstein N., Valiela I. 1996. Changes in N and C Stable Signatures of Particulate Organic Matter and Ribbed Mussels in Estuaries Subject to Different Nutrient Loading. Biological Bulletin 191: 329-330.

Zar J.H.1996. Biostatistical analysis. 3rd Ed., Prentice Hall International Editions, USA.

Chapter 3 – Effects of urban effluents in a temperate mesotidal lagoon

Introduction

Urbanization increases wastewater production, which in turn increases delivery of nutrients to groundwater, streams and coastal water (Valiela & Bowen 2002). Increasing inputs of nutrients to estuarine and near shore ecosystems is dramatically altering coastal habitats (Vitousek et al. 1997). In face of the complexity of the nitrogen cycle and of the relatively large spatial and temporal scales of interest, the identification and fate of nitrogen sources is difficult to fully resolve with traditional methods such as mass balance studies (Frazer et al. 1997). Most of the indexes developed to quantify the extent of eutrophication (Schmitt & Osenberg 1995) just provide information after there is alteration to the habitat. Methods that can detect increases in nutrient loads while impacts are still relatively low are required for coastal habitat management.

Analysis of the natural abundance of stable isotopes in inorganic and organic material provides a method to detect incipient eutrophication since anthropogenic nutrients are usually isotopically distinct from marine-derived material (Rau et al. 1981, Macko & Ostrom 1994, Fourqurean et al. 1997, McClelland et al. 1997, Fry 1999, Yamamuro et al. 2003). Primary producers using the anthropogenic nutrient pool reflect the stable isotopic composition of the pool and are therefore different in isotopic composition from producers using nutrients from natural sources.

The sensitivity of seagrasses to environmental conditions, the long life of individual seagrass organisms, and the sessile habit of these rooted plants make the analysis of stable isotopes in seagrass tissues a powerful indicator of conditions in the environment (Fourqurean et al. 1997, Yamamuro et al. 2003). Castro (2005) and Grice et al. (1996) reported higher $\delta^{15}N$ values in seagrasses from a more eutrophic site when compared to one with less anthropogenic influence. Ria Formosa lagoon (southern Portugal) is a shallow, mesotidal system dominated by saltmarsh and seagrass communities. The spatial pattern of the nitrogen stable isotope natural abundances (expressed as $\delta^{15}N$ values) of *Zostera noltii* leaves suggested an influence of isotopically heavy N from sewage in this system (Machás et al. 2003).

The aim of this study was to trace the distribution of the urban effluent in Ria Formosa lagoon and to understand its biological uptake and impact. A set of variables was measured in the water column and sediment along the environmental gradient from a Waste Water Treatment Works (WWTW) to a main tidal channel of Ria Formosa during both summer and winter: (1) environmental variables such as temperature, salinity, pH, redox potential (Eh) in sediments, nutrients, suspended particulate matter (SPM), particulate organic matter (POM), sediment organic matter (SOM) and water column chlorophyll a (Chla), (2) elemental contents of POM, sediment and *Zostera noltii* leaves and (3) carbon stable isotope values ($\delta^{13}C$ values) of total dissolved inorganic carbon (DIC), POM, SOM and *Z. noltii* leaves ($\delta^{13}C_{DIC}$, $\delta^{13}C_{POM}$, $\delta^{13}C_{SOM}$ and $\delta^{13}C_{Z.\ noltii}$, respectively), and $\delta^{15}N$ values of ammonium, POM, SOM and *Z. noltii* leaves ($\delta^{15}N_{NH4}$, $\delta^{15}N_{POM}$, $\delta^{15}N_{SOM}$ and $\delta^{15}N_{Z.\ noltii}$, respectively). The environmental variables and $\delta^{13}C_{DIC}$ and $\delta^{15}N_{NH4}$ values reveal the physical extent of the sewage influence into the lagoon, while the $\delta^{13}C_{Z.\ noltii}$ and $\delta^{15}N_{Z.noltii}$ values integrate variations in the environment over time and reflect the availability of DIC and nutrients to the seagrass. $\delta^{13}C_{POM}$ and $\delta^{15}N_{POM}$ reflect the variability of plankton and other organic particles such as plant detritus suspended in the water column. $\delta^{13}C_{SOM}$ and $\delta^{15}N_{SOM}$ represent microphytobenthos and other organic material in the sediment.

Materials and methods

Sampling design

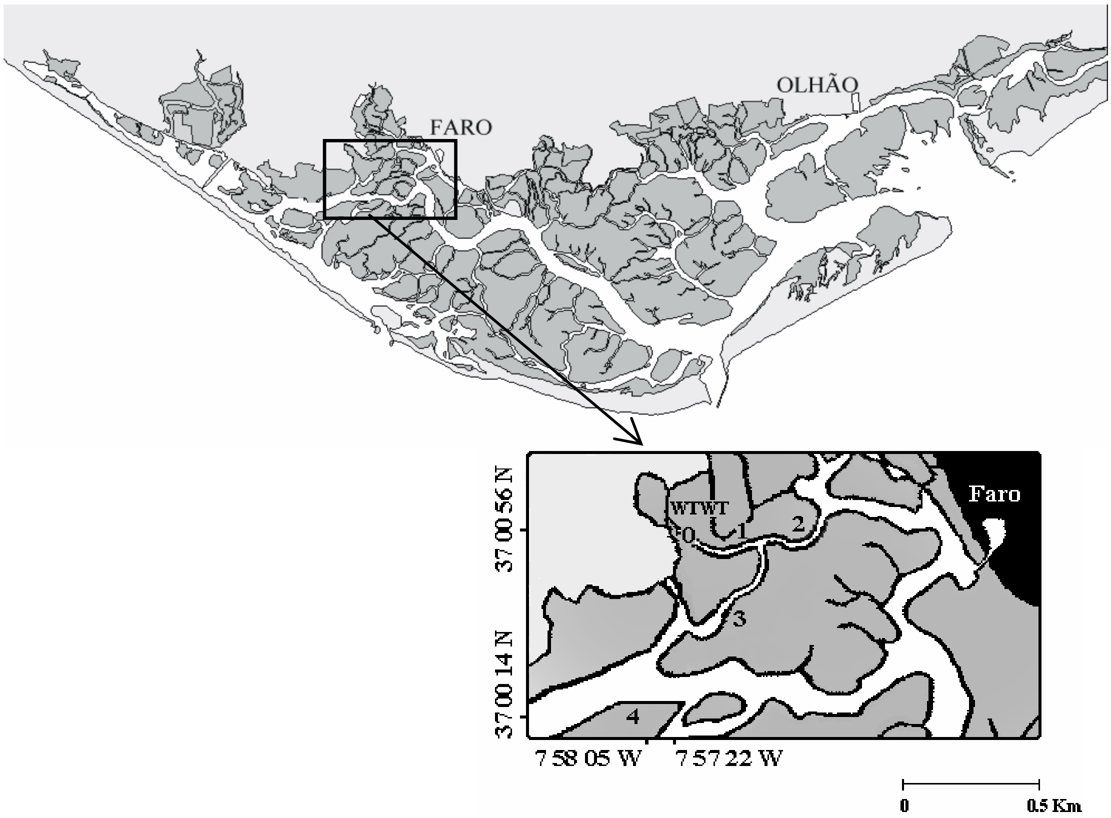


Fig 1. Map of Ria Formosa with location of sampling sites (station 0 to 4) along the environmental gradient from the WWTW to a main channel.

The Ria Formosa lagoon (Fig. 1) is a system of salt marshes, mudflats and channels, extending for about 55 km along the coast of southern Portugal, connected to the Atlantic Ocean through 6 inlets. Fresh water input into the lagoon is low, and salinity remains close to ocean water except during sporadic and short periods of winter run-off (Falcão & Vale 1990). Although the main navigation channel, the Faro channel, can reach 30 m in depth, the average depth of the lagoon is about 2 m. Andrade (1990) estimated a 50-75% exchange of the lagoon water with the ocean per tidal cycle. The total area of the lagoon is 84 km^2 with an exposed intertidal area of approximating 67 km^2 (Andrade 1990). The seagrass *Zostera noltii* dominates the lower intertidal, while the seagrass *Cymodocea nodosa* dominates the subtidal.

Blooms of green macroalgae of the genera *Enteromorpha* and *Ulva* develop in some mud flat areas of the lagoon, mostly in winter. Most of the fisheries production of Ria Formosa is accounted by the clam *Tapes decussatus* which is extensively cultivated in intertidal areas.

We performed this study along an environmental gradient from the WWTW (a 3ª sewage treatment plant) to a main channel of Ria Formosa (Ramalhete channel) in both summer and winter. WWTW has a12000 e.h. and is characterisedby a sewage flow of ca. 0.12 m^3s^{-1} of domestic sewage (CCDR Algarve). To evaluate the nutrient variation along the gradient, measurements of nitrate and ammonium concentrations in the water column at low-tide were taken. Four sampling sites (Fig. 1) were established in *Zostera noltii* meadows along the environmental gradient from the WWTW to the Ramalhete channel (station 1 to station 4). A sampling site (station 0) was also established at the outlet of the WWTW, where there was no *Z. noltii*.

The field campaigns were carried out in July 2001 and February 2002. The WWTW was characterized at the sewage outlet (station 0), by the following physical, chemical and biological variables: salinity, temperature, pH, concentrations of total DIC, nitrate+nitrite, ammonium and phosphate, particulate organic carbon (only in February), particulate nitrogen (only in February), Chla, SPM, POM, $\delta^{13}C_{DIC}$ and $\delta^{15}N_{NH4}$. Seston samples for analysis of $\delta^{13}C_{POM}$ and $\delta^{15}N_{POM}$ were also taken from this site.

The distribution of the urban effluent influence along the tidal creeks was described, from station 1 to station 4, through the assessment of the above variables plus those of sediment: salinity, temperature, pH, Eh, concentrations of nitrate+nitrite, ammonium, phosphate, organic carbon, organic nitrogen and organic matter. Sediment and *Zostera noltii* were sampled for elemental content analysis (C, N and P). Analysis of $\delta^{13}C$ and $\delta^{15}N$ values of SOM and *Z. noltii* leaves were also performed in stations 1 to 4.

Sampling was done at low tide, when the influence of the WWTW effluent is more apparent and its lateral extent is at a maximum. In general, triplicate samples were analyzed, except in the case of the $\delta^{15}N_{NH4}$ where only two samples were processed. The third one was not analysed because sample variation was very low. In the case of $\delta^{13}C_{DIC}$, $\delta^{13}C_{SOM}$ and $\delta^{15}N_{SOM}$ three samples were analyzed in the winter, but only one sample was analysed in the summer.

Sample preparation and analysis

Water samples for SPM, POM and Chla were filtered using glass fiber filters (GF/F). SPM and POM were analysed according to Strickland et al. (1968) APHA et al. (1985). Chl *a* was measured following Lorenzen (1967). Water salinity, temperature and pH were recorded *in situ* at each site (YSI 6000). The redox potential (Eh) was measured *in situ* with a metal electrode (Russell RL100).

In order to reduce the effect of horizontal patchiness on nutrient concentrations of sediment, a total of six minicores (3.5 cm diameter) were pooled for each sample (Rocha 1998). The minicores were sliced in the field to collect the first 5 cm of sediment corresponding to the *Zostera noltii* rhizosphere. The sediment samples were centrifuged (3000 rpm, 20 min., at in situ sediment temperature) and the supernatant water was filtered (Whatmann, 0.45 μm of porosity, 25 mm diameter) and frozen until analysis (storage time <1 month). Water column samples for nutrient analysis were filtered (Whatmann, 0.45 μm of porosity, 25 mm diameter) and frozen. Ammonium concentrations were measured using a spectrophotometric method (APHA et al. 1985). Nitrate+nitrite and phosphate were measured in a segmented-flow AutoAnalyzer system (Skalar, Sans Plus). All samples were processed at the Chemical Analytic Laboratory (LAQ), University of Algarve, Faro, Portugal.

Particulate organic C and N concentrations were determined with a Europa Roboprep C/N analyser, with a precision better than 5% (Science Laboratories, Bangor), while the content of organic C and N (% of dry weight) in the sediment and in the plant tissue was analysed using a Carlo-Erba, elemental analyser (EA1108). To measure the total P content in the plant tissue, dried and ground plant material was burned for one hour at 550°C, 0.2 M HCl was added to the residual ash and was extracted for one hour at 100°C. The liberated orthophosphate form was analysed using spectrophotometric analysis (Koroleff 1970). The determinations of C, N and P in the sediment and in the plant tissue were performed at the Freshwater Biological Laboratory- University of Copenhagen, Denmark. All elemental ratios were calculated on a mole:mole basis.

Samples for the determination of total DIC in seawater were filtered through glass fibre syringe filters (Whatmann 0.45 μm of porosity, 25 mm∅) into pre-poisoned ($HgCl_2$) 10 ml glass ampoules and were stored flame-sealed until analysis. The DIC was extracted from the sample by vacuum distillation as CO_2 gas after acidification with H_3PO_4 (85%) and was quantified manometrically. The CO_2 gas was then collected and its $\delta^{13}C_{DIC}$ was measured on the PDZ-Europa 20/20 mass spectrometer. Samples for $\delta^{15}N_{NH4}$ were prepared by the diffusion method (Holmes et al. 1998) and analysed using a continuous-flow isotope ratio mass spectrometer (PDZ Europa 20-20) with an elemental analyzer-GC combustion and purification system.

$\delta\,^{13}C_{Z.\ noltii}$ and $\delta\,^{15}N_{Z.\ noltii}$ analyses were made using the youngest leaf in each shoot, which reflects recent environmental conditions, whereas $\delta\,^{13}C_{SOM}$ and $\delta\,^{15}N_{SOM}$ were done using the fine fraction of the sediment (< 63 μm). Water samples for $\delta^{13}C_{POM}$ and $\delta\,^{15}N_{POM}$ analyses were filtered with a low-pressure vacuum pump until clogged using precombusted (500ºC; 4h) fiber glass filters (GF/F). Filters for S0M analysis were acid-fumed (concentrated HCl) overnight to remove carbonate material, dried at 40°C and then stored in a desiccator prior to analysis. For $\delta^{13}C_{Z. noltii}$, $\delta^{15}N_{Z. noltii}$, $\delta^{13}C_{SOM}$ and $\delta^{15}N_{SOM}$ analysis a subsample was weighted in precombusted silver boats (500ºC, 3hrs), and carbonate was removed through a combination of HCl (10%) additions and drying at 40ºC. The $\delta^{13}C$ and $\delta^{15}N$ values were determined on CO_2 and N_2 generated by vacuum combustion of samples in quartz tubes (Kennedy & Kennedy 1994) containing copper and pre-combusted copper oxide (910ºC, 3hrs). The CO_2 and H_2O were initially frozen, subsequently collected by vacuum distillation, and then measured on a PDZ-Europa 20/20 mass spectrometer. The N_2 was collected by adsorption onto silica gel in a liquid N_2 trap and then measured on a VG SIRA II IR mass spectrometer. The CO_2 was subsequently collected by vacuum distillation and was measured on a PDZ-Europa 20/20 mass spectrometer.

Results of the isotopic analysis are reported in δ notation i.e. $\delta^{13}\mathrm{C}$ or $\delta^{15}\mathrm{N}\ (‰) = 1000\left(\frac{R_{sample}}{R_{standard}} - 1\right)$, where R is the ratio ^{13}C: ^{12}C or ^{15}N: ^{14}N; Vienna Pee Dee Bellemnite (VPDB) and air were the reference standards for CO_2 and N_2, respectively. The external measurement precision of all the isotopic analyses was better than ±0.1‰ based on analyses of internal laboratory standards run concurrently with all the samples. Filters for $\delta^{15}N_{NH4}$ determinations were analysed at the Stable Isotope Laboratory, MBL, Woods Hole, USA. The remaining samples for $\delta^{13}C$ and $\delta^{15}N$ analyses were done by the Marine Science Laboratories- University of Wales, Bangor, UK.

Statistical analysis

The effects of space and time on variables were tested using 2-way ANOVA. Pairwise multiple comparison procedures (Tukey Test) revealed which site(s) differed from the others. Significant differences between summer and winter values of temperature, salinity, pH, Eh,

$\delta^{13}C_{DIC}$ and $\delta^{15}N_{NH4+}$ were tested with t-Tests. Pearson correlations were calculated to examine significant linear relationships between variables (environmental, elemental and stable isotopic compositions). In this case, summer and winter values were considered separately. Data were pooled whenever there were no seasonal differences. The relationships between the C and N isotopic values of POM, SOM and *Zostera noltii* and $\delta^{13}C_{DIC}$ and $\delta^{15}N_{NH4+}$, respectively, were tested with multiple linear regressions. The SigmaStat software package was used for data analysis. Relationships were considered significant at $p < 0.05$.

Results

Environmental gradient

The WWTW effluent (station 0) was characterized by a high organic content of SPM (POM/SPM > 0.8, Fig. 2.1 and 2.2) with higher algal component in the summer (Chla/POC = 0.012 ± 0.002, Fig. 2.1) than in the winter (Chla/POC = 0.007 ± 0.002, Fig. 2.1), low salinity (1 to 2 PSU, Fig. 2.2 and 2.3), very high concentrations of ammonium, particularly in winter (570.62 to 1658.67 μM, Fig. 3.1 and 3.2), moderate concentrations of nitrate+nitrite (9.23 to 19.39 μM, Fig. 3.3 and 3.4) and high concentrations of phosphates (125 to 48.88 μM, Fig. 3.5 and 3.6). The pH was high (8.6) in summer due to the effects of photosynthesis whereas in winter it was lower (7.7). The winter concentration of total DIC (6878.92 μM ± 92.84 , Fig. 3.6) was higher than in the summer (2166.91 μM, Fig. 3.5). The undisturbed water of Ria Formosa lagoon, represented in this study by the Ramalhete channel site (station 4), was generally dominated by inorganic particles comprising 80% of total SPM weight (Fig. 2.1 and 2.2). The algal component of the POC expressed as Chla/POC was much lower than at station 0, averaging about 0.002 (Fig. 2.1 and 2.2), and the salinity remained around 36 PSU (Fig. 3.1

and 3.2). The Ramalhete water showed low concentrations of ammonium (1.95 to 4.15 µM, Fig. 3.1 and 3.2), of nitrate+nitrite (0.51 to 0.62 µM , Fig. 3.3 and 3.4), of phosphates (0.034 to 0.45 µM, Fig. 3.5 and 3.6), and of total DIC (2.2 mM, Fig. 3.5 and 3.6) when compared to WWTW effluent.

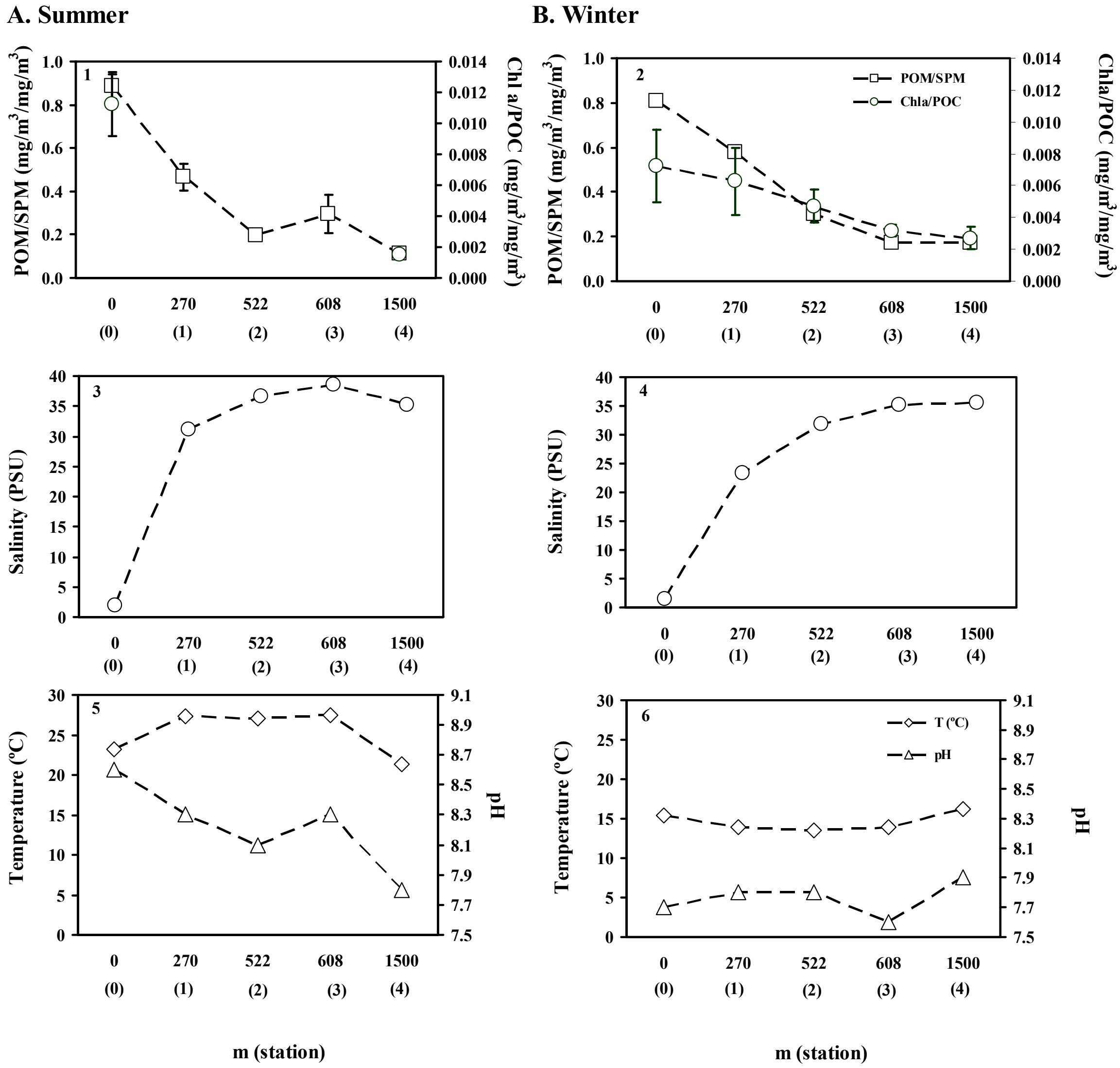


Fig. 2. Spatial distributions (plotted as station number and distance seaward from the WWTW outfall) of the ratio of particulate organic matter to suspended particulate matter (POM/SPM), chlorophyll a to POC (Chla/POC), salinity, temperature and pH. Distributions in the water column, at low tide, both in (A) summer and (B) winter. The summer data of Chl a/POC, from station 4, is from Machás et al (2003).

The POM/SPM and the Chla/POC ratio significantly decreased along the WWTW gradient, from station 0 to station 2, levelling to the other stations (Fig. 2.1 and 2.2). This is probably the result of the die-off and sedimentation of the fresh water plankton produced within the WWTW. The salinity increased to station 3 where it reached the normal lagoon levels (Fig. 2.3 and 2.4). The wastewater had higher algal biomass in the summer than in winter, in contrast with the Ramalhete channel, station 4, where the summer algal component of the POM was not different from winter (Fig. 2.1 and 2.2).

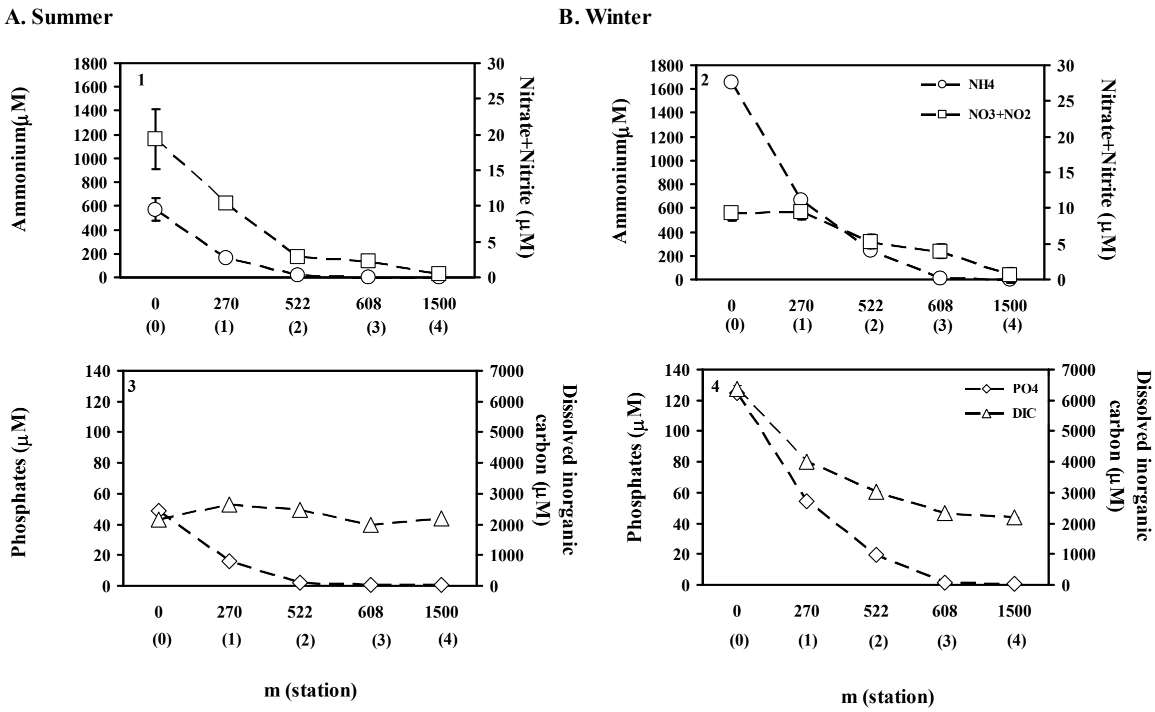


Fig. 3. Spatial distributions of nutrient concentrations in the water column, at low tide. Concentrations of nitrate+nitrite, ammonium, phosphates and total dissolved inorganic carbon (DIC), both in (A) summer and (B) winter.

The inorganic nutrient gradient, including DIN and DIC, was evident from the WWTW to station 2 in the summer and to station 3 in winter, with the exception that DIC summer values that did not vary significantly along the transect (Fig. 3). The water from the WWTW (station 0) showed lower concentrations of inorganic nutrients in summer than in winter, except for nitrate+nitrite, which were higher in summer. This was probably due to higher production of WWTW phytoplankton in the summer (summer [Chla] = 501.4 1 mg m^{-3} ± 40.; winter [Chla] = 247.7 6 mg m^{-3} ± 67.). The nutrient concentrations of the main channel water (station 4) did not vary significantly with season.

The sediment variables measured within the *Zostera noltii* meadows showed less pronounced gradients than those of the water column. Ammonium and phosphate concentrations in the sediment were generally low at station 1, peaked at station 2 and decreased onwards to station 4, except the summer values of phosphates that were highest in station 1 (Fig. 4). On the contrary, the lowest values of sediment redox potential were observed at station 2, increasing onwards to station 4 (Fig. 4). The lower nutrient values observed in the sediment of station 1 compared to station 2, and the higher values of redox potential at station 1 than station 2, were probably caused by the introduction of sediments from construction at the nearby airport coincident with the beginning of our sampling.

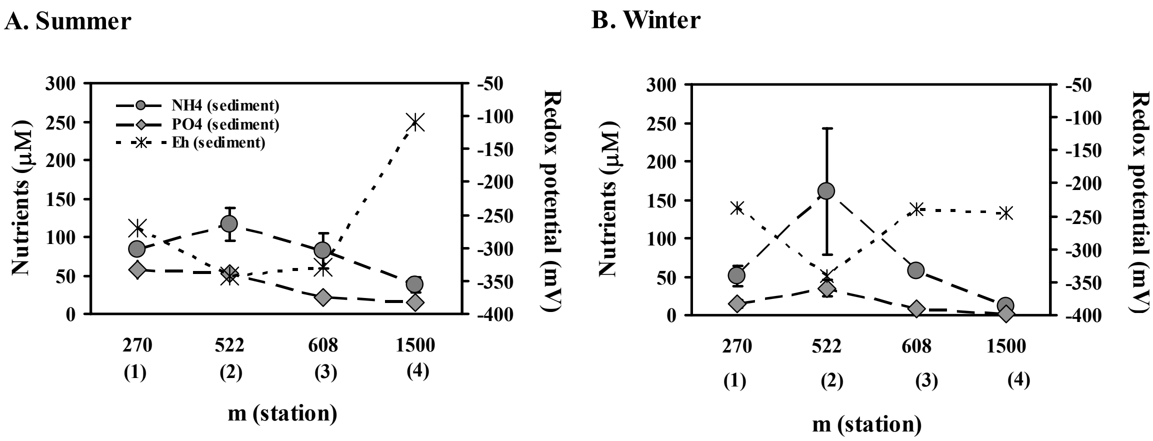


Fig. 4. Spatial distribution of nutrient (ammonium and phosphates) concentrations and redox potential in sediment pore water of *Zostera noltii* beds in (A) summer and (B) winter.

Elemental contents

In winter, the POC and PON concentrations in the water column decreased with distance from the WWTW (Fig. 5.1). These patterns are consistent with the decreasing content of freshwater phytoplankton in the POM (Fig. 2.2). The C/N ratio showed an opposite pattern, increasing from 5.8 ± 0.3 to 9.2 ± 0.5 (Fig. 5.2), showing the decreasing effect of freshwater phytoplankton with relatively low C/N on POM.

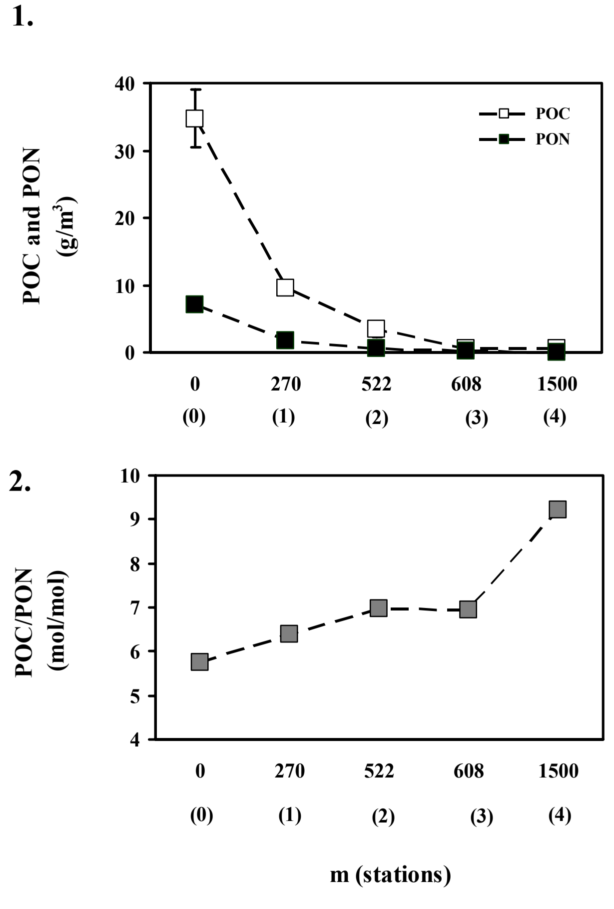


Fig. 5. Spatial distributions of the concentrations of (1) particulate organic C (POC) and particulate organic N (PON) and (2) ratio POC/PON along the environmental gradient from the WWTW to Ramalhete channel. Measurement taken in the water column, low tide, in the winter.

The C, N and P contents of the sediment did not reflect any clear gradient with WWTW distance (Fig. 6). Maximum values of C contents, 3.4 % ± 0.3, were observed in station 2 during winter (Fig. 6.1 and 6.2). The C/N and C/P ratios of sediment peaked at station 2 (Fig. 7) due to the increase of C elemental contents, probably caused by the sedimentation of the dead fresh water phytoplankton produced in the WWTW, rather than by a higher contribution of microphytobenthos to the sediment organic matter. The C/N ratio of sediment organic matter was higher (9.8 to 15.3, Fig. 7.2) than the C/N ratio of sestonic POM (5.8 to 9.3, Fig. 5.2).

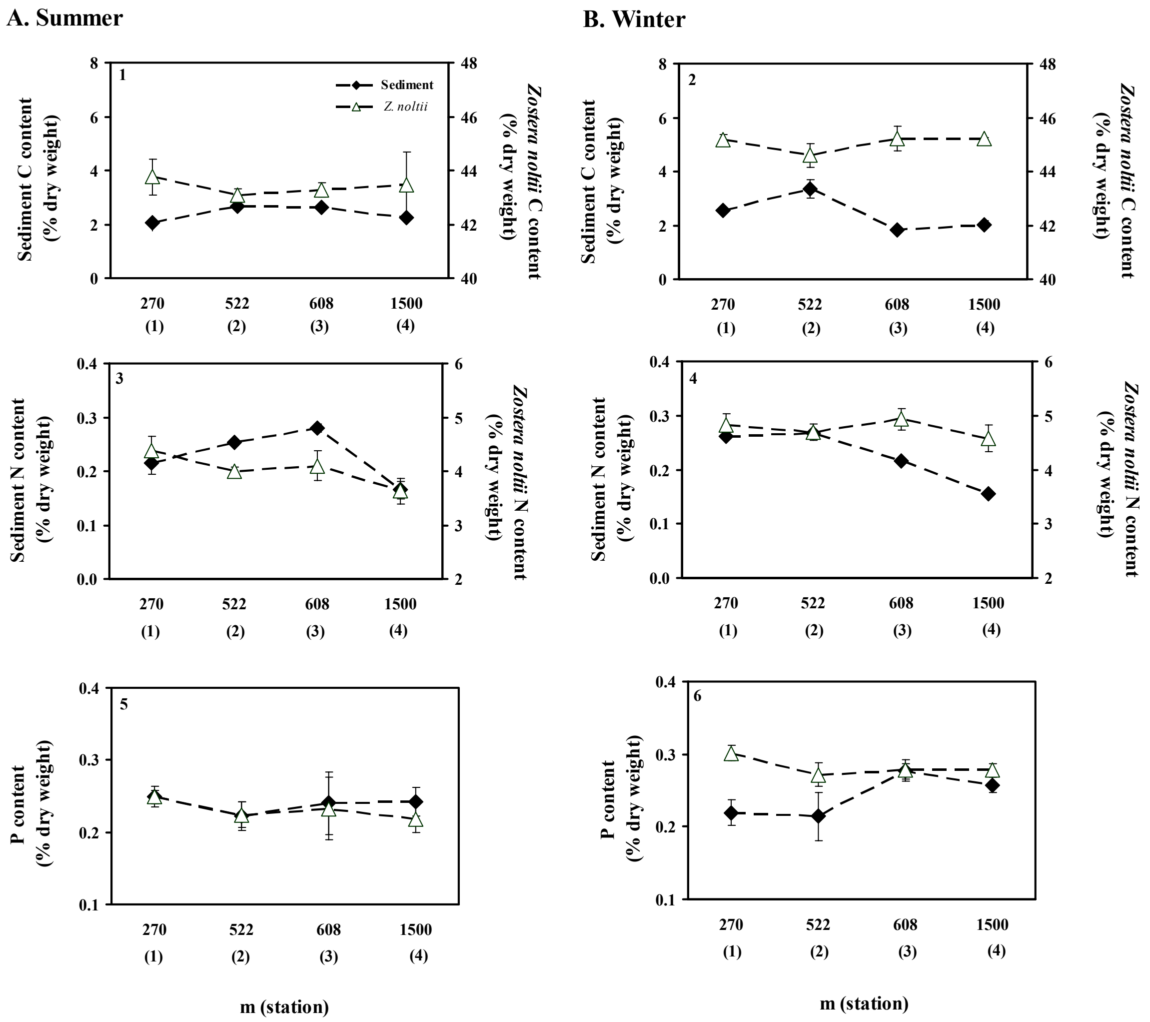


Fig. 6. Spatial distributions of C, N and P elemental composition of sediment and *Zostera noltii* leaves along the environmental gradient from the WWTW to Ramalhete channel both in (A) summer and (B) winter.

The C content of *Zostera noltii* leaves did not vary spatially but was slightly higher in winter than in summer (Fig. 6.1 and 6.2). The N content of *Z. noltii* leaves decreased along stations in the summer whereas in winter was relatively constant (Fig. 6.3 and 6.4). The maximum P content of *Z. noltii* was found at station 1 (Fig. 6.5 and 6.6), consistent with the high concentration of P in the water column (Fig. 3.5 and 3.6). Similarly to the C contents (Fig. 6.1 and 6.2), the N (Fig. 6.3 and 6.4) and P (Fig. 6.5 and 6.6) contents of *Z. noltii* were higher in the winter than in summer. The C/N and C/P ratios of *Z. noltii* increased from station 1 to station 4 (Figs. 7.1 and 7.3).

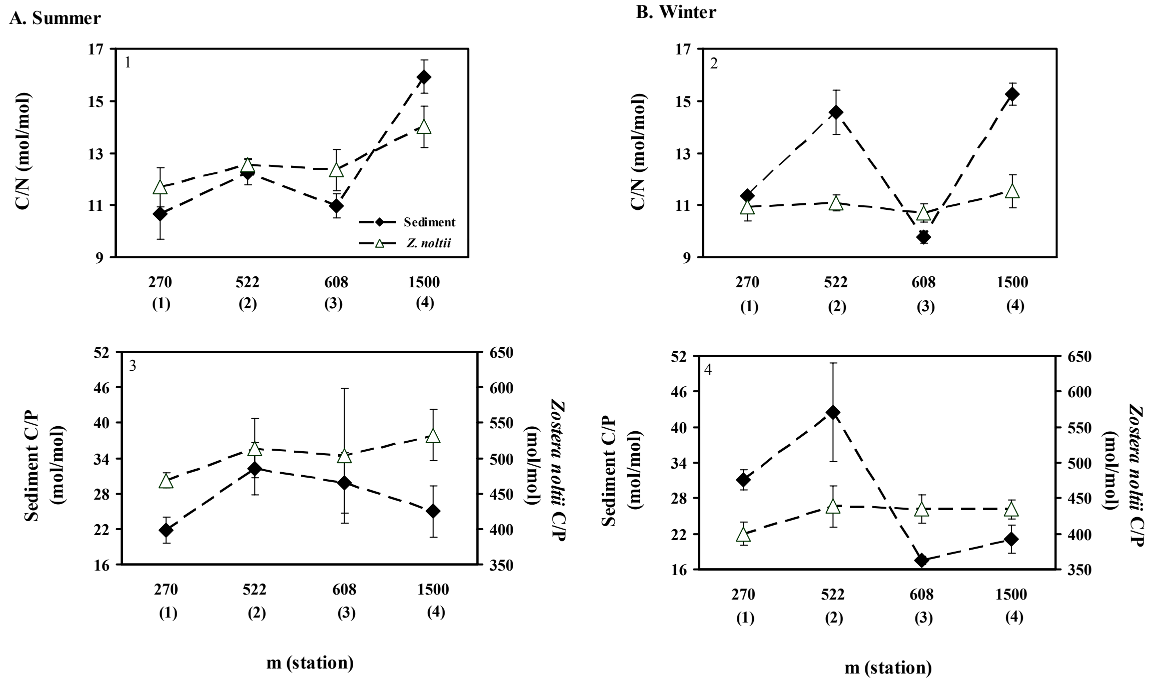


Fig. 7. Spatial distributions of the ratio C/N and C/P of sediment and *Zostera noltii* leaves along the environmental gradient from the WWTW to Ramalhete channel both in (A) summer and (B) winter.

Stable isotopic contents

The WWTW effluent determined the observed gradients of pH and DIC concentration. In the summer, the pH of the WWTW was high (Fig. 2.5) and the DIC concentration was typical of the seawater (no gradient, Fig. 3.3) while in winter the pH was low (Fig. 2.6) and DIC was very high (Fig. 3.4). Similarly, the $\delta^{13}C_{DIC}$ values of the WWTW effluent, which were much higher in summer than in winter, affected the $\delta^{13}C_{DIC}$ gradient (Fig. 8.1 and 8.2). It decreased along stations in the summer whereas in winter it showed the opposite spatial trend. The significant relationships observed between $\delta^{13}C_{DIC}$ and pH (Fig. 9.1 and 9.2) reflect the patterns determined by the WWTW effluent.

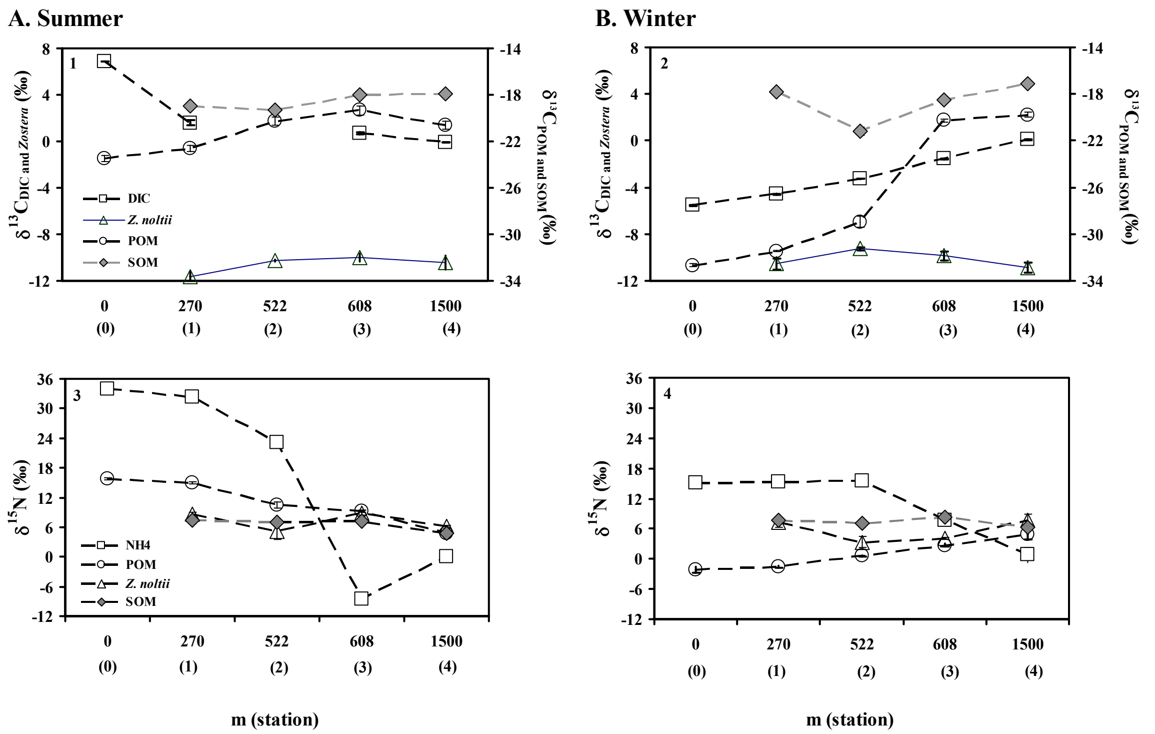


Fig. 8. Spatial distributions of stable isotopic composition of nutrients ($\delta^{13}C_{DIC}$, $\delta^{15}N_{NH4}$), POM ($\delta^{13}C_{POM}$, $\delta^{15}N_{POM}$) in the water column at low tide, SOM ($\delta^{13}C_{SOM}$, $\delta^{15}N_{SOM}$) and *Zostera noltii* leaves ($\delta^{13}C_{Z.\ noltii}$, $\delta^{15}N_{Z.\ noltii}$) along the environmental gradient from the WWTW to Ramalhete channel, both in (A) summer and (B) winter.

The influence of the urban effluent is also evident on the $\delta^{15}N_{NH4}$ gradient, which was more pronounced in the summer (Fig. 8.3 and 8.4). In summer, the $\delta^{15}N_{NH4}$ of the WWTW was enriched (Fig. 8.3), when the phytoplankton production was high (Fig. 2.1). The strong fractionation of ammonium within the WWTW determines the signal of the phytoplankton in the water column along the environmental gradient. This is supported by the positive relationship between $\delta^{15}N_{NH4}$ and the Chla/POC ratio shown in Figure 9.3. The positive relationship between $\delta^{15}N_{NH4}$ and the nitrate+nitrite concentrations suggest that high rates of nitrification contribute to the enrichment of $\delta^{15}N_{NH4}$ values (Fig. 9.4).

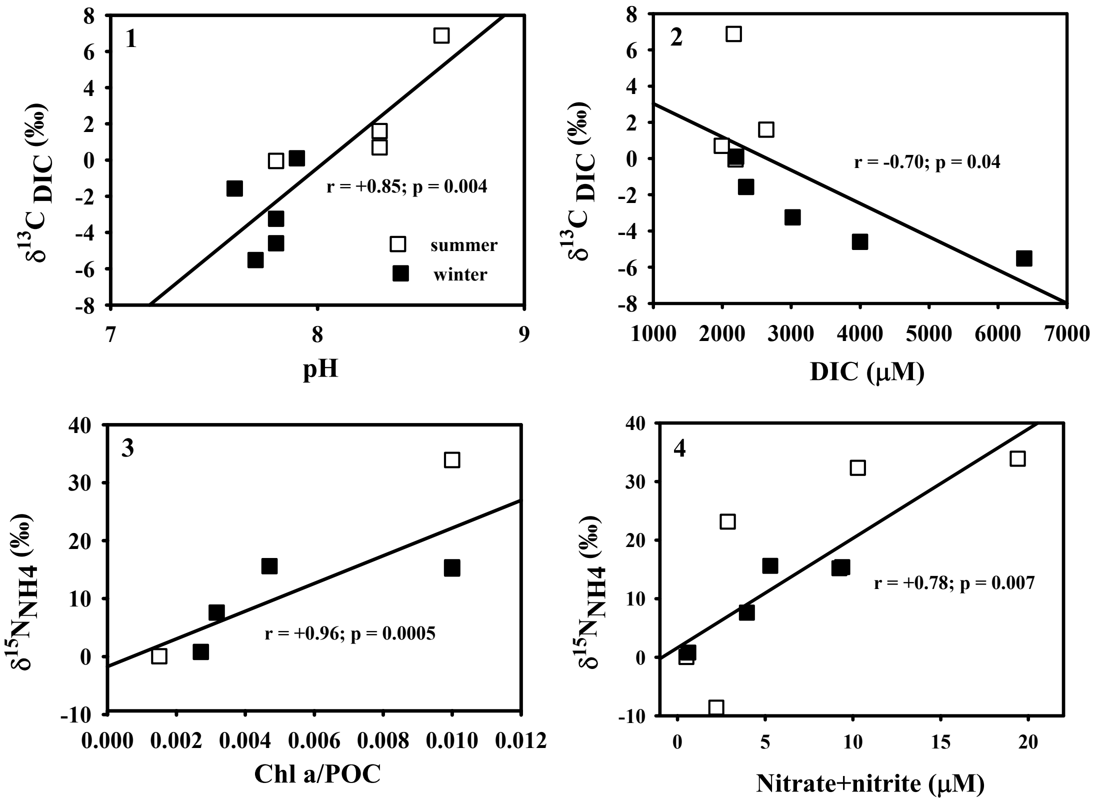


Fig. 9. Significant linear relationship between variables and stable isotopic composition of DIC and NH4 ($\delta^{13}C_{DIC}$, $\delta^{15}N_{NH4}$). Correlation coefficients (r) and significance of regressions (p) are shown.

Both the $\delta^{13}C_{DIC}$ and $\delta^{13}C_{POM}$ values of WWTW were significantly higher in the summer than in the winter (Figs. 8.1 and 8.2). Even though the $\delta^{13}C_{DIC}$ of WWTW was higher than Ria Formosa values in the summer while it was lower in winter, the $\delta^{13}C_{POM}$ of WWTW was always lower than main channel. This was due to the WWTW higher fractionation of $\delta^{13}C_{POM}$ in relation to $\delta^{13}C_{DIC}$ in the summer (Fig. 10). Consequently, the seasonal gradients observed in $\delta^{13}C_{DIC}$ values from the WWTW to the main channel, decreasing in the summer and increasing in winter, were not followed by the $\delta^{13}C_{POM}$ values in summer (Figs. 8.1 and 8.2). A significant relationship between $\delta^{13}C_{DIC}$ and $\delta^{13}C_{POM}$ was only observed in winter (Fig. 11.1) The increasing values of $\delta^{13}C_{POM}$ along the WWTW gradient (Fig. 8.1 and 8.2) reflect the decreasing effect of the WWTW phytoplankton, which is depleted in relation to Ria Formosa primary producers. The significant negative relationship observed in winter between the $\delta^{13}C_{POM}$ values and the phytoplankton content of POM, expressed by the Chla/POC ratio, supports this hypothesis (Fig. 11.2).

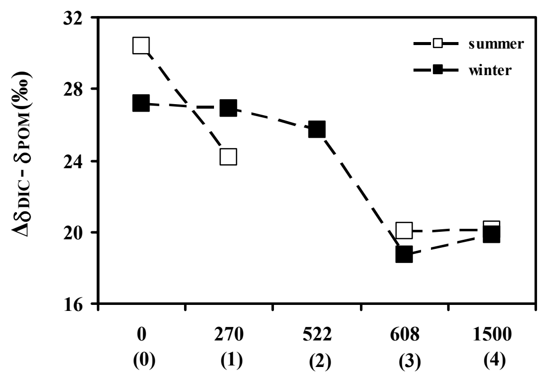
Both the $\delta^{15}N_{NH4}$ and the $\delta^{15}N_{POM}$ values of WWTW were higher in the summer than in the winter (Figs. 8.3 and 8.4). Even though the $\delta^{15}N_{NH4}$ of WWTW was higher than Ria Formosa values in both seasons, the $\delta^{15}N_{POM}$ of WWTW was only higher than main channel values in the summer. This was due to the lower winter differences between the WWTW and the lagoon $\delta^{15}N_{NH4}$ values. Consequently, the seasonal gradients observed in $\delta^{15}N_{NH4}$ values from the WWTW to the lagoon, decreasing in both seasons, were not followed by the $\delta^{15}N_{POM}$ values. In the summer $\delta^{15}N_{POM}$ decreased towards the lagoon, whereas it increased in the winter (Figs. 8.1 and 8.2). The significant negative relationship, observed in winter, between $\delta^{15}N_{POM}$ values and the Chla/POC ratio indicate the decreasing effect of the WWTW phytoplankton along the gradient (Fig. 10.4). The $\delta^{15}N_{POM}$ values of the main channel (station 4) did not vary significantly with season.

The $\delta^{13}C_{SOM}$ and $\delta^{15}N_{SOM}$ values were less variable than $\delta^{13}C_{POM}$ and $\delta^{15}N_{POM}$ values and no clear gradients were found (Fig. 8). As well, no clear gradients were observed in the $\delta^{13}C_{Z. noltii}$ and $\delta^{15}N_{Z. nolti}$ values. Only the $\delta^{13}C_{DIC}$ values were significantly correlated with the $\delta^{13}C_{POM}$ values.

Isotopic fractionation between nutrients and POM

The fractionation between the sources of carbon and the primary producers, i.e. the difference between the $\delta^{13}C_{DIC}$ and $\delta^{13}C_{POM}$ ($\Delta\delta_{DIC} - \delta_{POM}$) in the WWTW effluent was similar in summer (30‰) and winter (27‰) (Fig. 10.1). The fractionation between the N sources and the POM, $\Delta\delta_{NH4} - \delta_{POM}$, was also similar in summer (18‰) and winter (17‰) (Fig. 10.2). Both the C and N fractionation decrease from the WWTW to the lagoon. The zone where the fractionation decrease is abrupt (Fig. 10) represents a boundary between the zone under the influence of the WWTW fresh water phytoplankton and the lagoon.

1.



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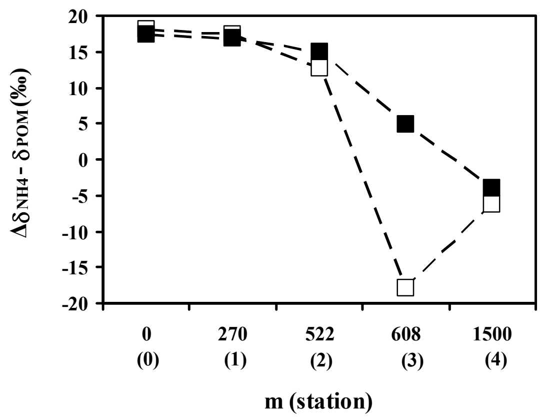

Fig. 10. Fractionation between the sources of nutrient and primary producer: (1) difference between the $\delta^{13}C_{DIC}$ and $\delta^{13}C_{POM}$ ($\Delta\delta_{DIC}$- δ_{POM}) and (2) difference between the $\delta^{15}N_{NH4}$ and $\delta^{15}N_{POM}$ ($\Delta\delta_{NH4}$- δ_{POM}).

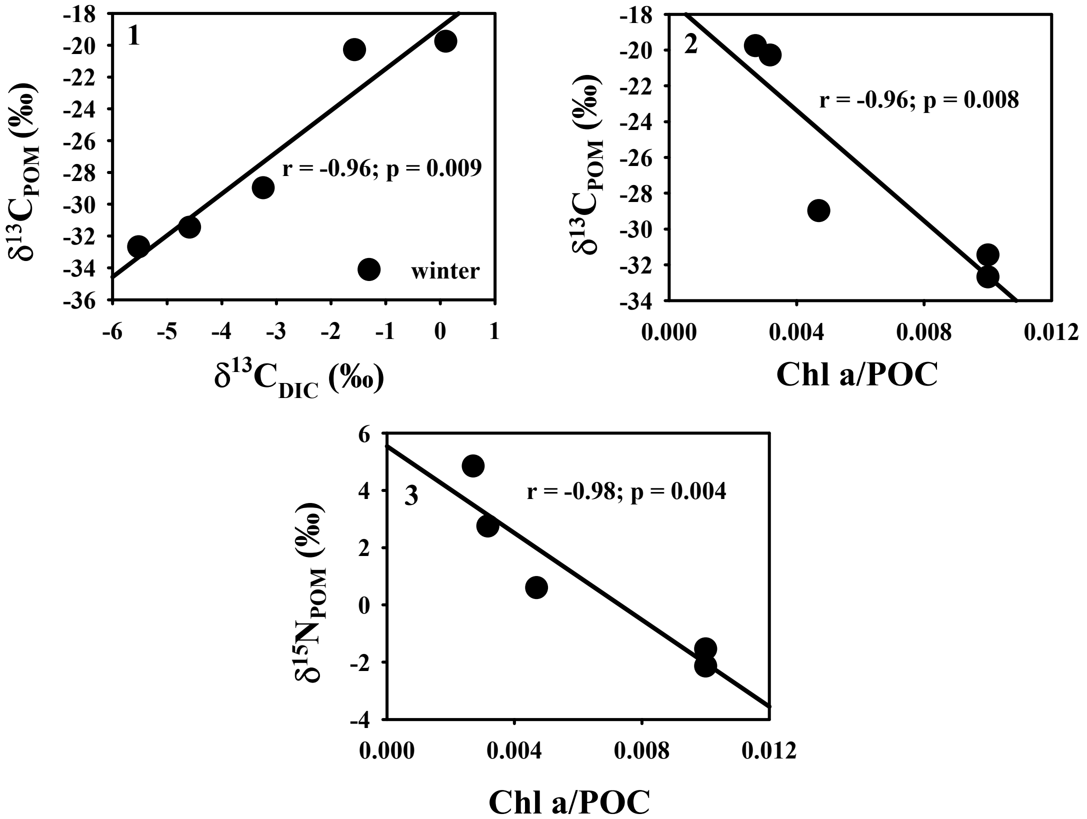

Fig. 11. Significant linear relationshipd between variables and stable isotopic composition of POM ($\delta^{13}C_{POM}$, $\delta^{15}N_{POM}$). Correlation coefficients (r) and significance of regressions are shown.

Discussion

Urban effluent characterization

The large seasonal differences observed in the WWTW effluent probably reflected the higher summer microalgae productivity. Higher primary production consumed the available CO_2 of the water shifting the inorganic carbon equilibrium and increasing the pH of the urban effluent (8.6 vs. 7.7). This process consumed the available ammonium and phosphate, which were about three times less in summer than in winter (Fig. 3). The higher concentrations of nitrate+nitrite observed in the summer (at day time) indicate that the high phytoplankton production probably oxygenated the water allowing higher rates of nitrification. On the other hand, the respiration processes are also expected to be higher in the summer than in winter due to the higher temperatures but the three times lower concentration of DIC in the summer than in winter indicate that this surplus production of CO_2 was likely consumed by high primary production.

The seasonal variation of the WWTW metabolism was reflected in the $^{13}C_{DIC}$ and $\delta^{15}N_{NH4}$ values observed in its effluent. In the summer, the WWTW $\delta^{13}C_{DIC}$ was enriched in ^{13}C due to the high rates of CO_2 consumption. The photosynthesis fractionation within the WWTW, as phytoplankton incorporates firstly the light stable isotope of C, contributed to the ^{13}C enrichment. As the pH was higher, there was more HCO_3^- available, which is ^{13}C enriched compared to CO_2 (Mook et al. 1974). These processes explain the observed seasonal differences in the $\delta^{13}C_{DIC}$ gradient from the WWTW to the main channel station, where values were always around 0 ‰, characteristic of seawater (Peterson 1999). In winter, the ^{13}C values of the WWTW effluent were lower than 0 and thus the $\delta^{13}C_{DIC}$ values increased from the WWTW to Ramalhete channel while in summer the opposite gradient was found (Fig. 8.1 and 8.2).

The maximum WWTW algal biomass in the summer was associated with lower concentrations of ammonium reflecting the nutrient uptake by the phytoplankton, higher rates of nitrification and probably also the high ammonium volatilization due to higher temperatures. Both high nitrification rates and high ammonium volatilization rates in the summer may contribute to the observed enrichment of $\delta^{15}N_{NH4}$ values. Faster processing of the ^{14}N than ^{15}N yields products enriched in the lighter isotope and leaves ammonium enriched in the heavy isotope (Mariotti et al. 1981, Macko & Ostrom 1994 and Fry et al. 2003). The $\delta^{15}N_{NH4}$ gradient, decreasing from the WWTW to the Ramalhete channel in both seasons, reflects the enriched signature of $\delta^{15}N_{NH4}$ originated from human metabolism. The magnitude of the change in WWTW $\delta^{15}N_{NH4}$ between summer (33.9‰) and winter (15.2 ± 0.3‰) was large enough to reverse the gradient of $\delta^{15}N_{POM}$ from the WWTW to the lagoon main channel (Fig. 8.3 and 8.4).

Biological uptake of urban effluent and extent of its influence

The WWTW C isotopic signal was most evident in the POM isotopic signature, $\delta^{13}C_{POM}$, rather than in the sediment organic matter or in *Zostera noltii*. The $\delta^{13}C_{POM}$ values of the WWTW essentially result of the C stable isotopic signal of the fresh water phytoplankton produced within the WWTW, reflecting the phytoplankton uptake of $\delta^{13}C_{DIC}$. They are consistently higher (summer = -23.5‰ ± 0.3; winter = -32.7‰ ± 0.1) than the $\delta^{13}C_{POM}$ main channel values (station 4; summer = -20.6‰ ± 0.4; winter = -19.8‰ ± 0.2), which probably result from a mixture of lagoon macrophytes with low contribution of phytoplankton (Machás et al. 2003). The $\delta^{13}C_{POM}$ summer values of the WWTW effluent was similar to sewage discharges reported elsewhere (-25.9‰ ± 1.1, Waldron et al. 2001; -22.7‰ ± 0.7, Van Dover et al. 1992).

In Ria Formosa, the $\delta^{15}N_{POM}$ values of the WWTW sewage (summer: 15.8‰ ± 0.2, winter: -2.1‰ ± 0.7) where more different from the reference site (summer: 4.8‰ ± 0.5, winter: 4.8‰ ± 1.0) than in other studies (Owens 1987: sewage 2.3 to 7‰, ocean 4.6 to 9.2‰; Gartner et al. 2002: sewage 9.16‰, ocean 7.1‰) indicating that $\delta^{15}N_{POM}$ is a suitable tracer of the urban effluent. On the other hand, sediment organic matter was not a suitable tracer of the urban effluent probably because the organic matter from the anthropogenic source was not isotopically distinct from the background source or the anthropogenic particles are masked by dilution with lagoon organic matter as reported by other authors (Fry et al. 2003, Gartner et al. 2002, Owens 1987, Wayland & Hobson 2001). Waldron et al. (2001) did not find the sewage isotope signal strongly recorded in the sediment due to the combined action of tidal movement, wind-induced, wave action and benthic invertebrates grazing of particulate matter. In the case of Ria Formosa, the organic matter from the WWTW source was isotopically distinct from the background source but no clear gradients were found for the $\delta^{13}C_{SOM}$ and $\delta^{15}N_{SOM}$ values (Fig. 8).

The higher C elemental (Fig. 6.1 and 6.2) and lower values of $\delta^{13}C$ (Fig. 8.1 and 8.2) found in the sediment organic matter at station 2, are probably due to the die-off and sedimentation of the fresh water plankton produced within the WWTW. The salinity gradient shows that the influence of fresh water does not reach station 2, which always show salinity values above 32 (Fig. 2.3 and 2.4). In station 2, the sediment C content was lower and the $\delta^{13}C_{SOM}$ values were ^{13}C enriched in summer than in winter in agreement with the DIC concentrations (Fig. 3.3 and 3.4) and isotopic values of the WWTW (Fig. 8.1 and 8.2). The C contents of sediment and $\delta^{13}C_{SOM}$ values reflected a high contribution of the high C content and ^{13}C depleted phytoplankton to SOM in this sampling site.

The suspension feeders that filter POM from the water column are likely to be highly affected by the urban effluent. Since filter feeders are a major commercial resource in Ria Formosa lagoon, the extend of the effluent is important to determine from a public health point of view. In fact, clam cultivations areas begin immediately after the first sampling site, about 300 m away from the WWTW. Our results indicate that the influence of the effluent discharge on the Ria Formosa lagoon extended to station 2 (522 m) and in some cases to station 3 (608 m). The studied variables, Chla/POM, POM/SPM, salinity (Fig. 2), inorganic nutrient concentrations (Fig. 3), δ values of POM, $\delta^{13}C_{DIC}$ and $\delta^{15}N_{NH4}$ (Fig. 8) support this conclusion as the values observed in stations 2 or 3 did not differ significantly from the undisturbed reference site (station 4). Because the ratio C/N of POM increases with decreasing contributions of phytoplankton to the organic matter (Cloern, 1996), the observed

POC/PON gradient (Fig. 5B) also indicates the decrease effect of the WWTW phytoplankton until station 2. It is clear that at station 4 there was no influence of the WWTW.

Yamamuro et al. (2003) suggested that $\delta^{15}N$ values in seagrasses can be a good tool to monitor time-integrated variations of DIN concentrations at a site, both in the water column and the interstitial water as both water and sediment may be nutrient sources to seagrasses. In this study, the $\delta^{15}N$ values of *Zostera noltii* were not significantly related either to nutrient concentration or to the $\delta^{15}N$ values of ammonia, suggesting than this species is not a good indicator of the WWTW influence. However, the annual average of $\delta^{15}N_{Z.\ noltii}$ collected from station 1 was significantly higher (7.9‰ ± 1.0) than other stations (station 2: 4.2‰ ± 1.6; station 3: 6.5‰ ± 2.6; station 4: 6.9‰ ± 1.1). Because the Ria Formosa water exchange during each tidal cycle is very high (50-75%), the influence of the WWTW nutrients is clearer at low tide, when *Z. noltii* are without water column influence. The WWTW influence will be more apparent in shallow and less dilution by ocean water sites of Ria Formosa. Isotopic composition of this species will indicate the dilution of the Ria Formosa water with the ocean.

Acknowledgements: We are grateful to all who contributed to this work. A special thanks to Ana Quaresma, Catarina Alves and José António Bentes for the valuable help in field work, to Carlos Rocha who kindly help to define sediment sampling. This research is a contribution to project "M&Ms - Monitoring and management of European seagrasses" (EVK3-CT-2000-00044) and it is part of the PhD thesis of Raquel Machás and Susana Cabaço, funded by the "Fundação para a Ciência e Technologic" (scholarships Praxis XXI/BD/19579/99 and SFRH/BD/2880/2000, respectively).

References

American Public Health Association (APHA), American Water Works Association and Water Environment Federation 1985. Standard methods for the examination of water and wastewater. Greenberg, Maryland.

Andrade C.F. 1990. O ambiente de barreira da Ria Formosa Algarve-Portugal. PhD thesis, University of Lisboa, Lisboa.

Castro P.C.O. 2005. Assessing key-habitat loss due to eutrophication in the Mondego and Mira estuaries. PhD thesis, University of Coimbra, Coimbra.

Cloern J.E. 1996. Phytoplankton bloom dynamics in coastal ecosystems: a review with some general lessons from sustained investigation of San Francisco Bay, California. Reviews of Geophysics 34: 127-168.

Falcão M., Vale C. 1990. Study of the Ria Formosa ecosystem: benthic nutrient remineralization and tidal variability of nutrients in the water. Hydrobiologia 207: 137-146.

Fourqurean J.W., Moore T.O., Fry B. 1997. Spatial and temporal variation in C:N:P ratios, $\delta^{15}N$, and $\delta^{13}C$ of eelgrass *Zostera marina* as indicators of ecosystem processes, Tomales Bay, California, USA. Marine Ecology Progress Series 157: 147-157.

Frazer T.K., Montoya J.P., Hoyer M.V., Bledsoe E.L. 1997. Movement of nitrogen in Florida's spring-fed rivers and their estuaries: using stable nitrogen isotopes as in situ tracers. International Council for the Exploration of the Sea (ICES) Meeting Papers (85th Statutory Meeting).

Fry B. 1999. Using stable isotopes to monitor watershed influences on aquatic trophodynamics. Canadian Journal of Fisheries and Aquatic Sciences 56: 2167-2171.

Fry B., Gace A., McClelland J.W. 2003. Chemical indicators of anthropogenic nitrogen loading in four Pacific estuaries. Pacific Science 57: 77-101.

Grice A.M., Loneragan N.R., Dennison W.C. 1996. Light intensity and the interactions between physiology, morphology and stable isotope ratios in five species of seagrass. Journal of Experimental Marine Biology and Ecology 195: 91-110.

Gartner A., Lavery P., Smit A.J. 2002. Use of $\delta^{15}N$ signatures of different functional forms of macroalgae and filter-feeders to reveal temporal and spatial patterns in sewage dispersal. Marine Ecology Progress Series 235: 63-73.

Holmes R.M., McClelland J.W., Sigman D.M., Fry B., Peterson B.J. 1998. Measuring $^{15}NH_4^+$ in marine, estuarine, and fresh waters: an adaptation of the ammonia diffusion method for samples with low ammonium concentration. Marine Chemistry 60: 235-243.

Kennedy H., Kennedy D.P. 1994. Simplified tube cracker for opening samples sealed in glass tubes while under vacuum. Analytical Proceedings Including Analytical Communications 31: 1-2.

Koroleff F. 1970 Determination of total phosphorus in natural waters by means of persulfate oxidation. Interlaboratory Report 3: 19-22.

Lorenzen C.J. 1967. Determination of chlrophyll and pheo-pigments: spectrophotometric equations. Limnology and Oceanography 12: 343-346.

Machás R., Santos R., Peterson B. 2003. Tracing the flow of organic matter from primary producers to filter feeders in Ria Formosa lagoon, southern Portugal. Estuaries 26: 846-856.

Macko S.A., Ostrom N.E. 1994. Pollution studies using stable isotopes. In: Lajtha K., Michener R.H. (eds). Stable Isotopes in Ecology and Environmental Science. Blackwell Scientific Publications, Oxford.

Mariotti A., Germon J.C., Hubert P., Kaiser P., Letolle R., Tardieux A., Tardieux P. 1981. Experimental determination of nitrogen kinetic isotope fractionation: some principles; illustration for the denitrification and nitrification processes. Plant Soil 62: 413-430.

McClelland J.W., Valiela I., Michener R.H. 1997. Nitrogen-stable isotope signatures in estruarine foods webs: A record of increasing urbanization in coastal watersheds. Limnology and Oceanography 42: 930-937.

Mook W.G., Bommerson J.C., Staverman W.H. 1974. Carbon isotope fractionation between dissolved bicarbonate and gaseous carbon dioxide Earth and Planetary Science Letters 22: 169-176.

Owens N.J.P. 1987. ^{15}N in the marine environment. Advances in Marine Biology 24: 389-451.

Peterson B.J. 1999. Stable isotopes as tracers of organic matter input and transfer in benthic food webs: A review. Acta Oecologica 20: 479-487.

Rau G.H., Sweeney R.E., Kaplan I.R., Mearns A.J., Young D.R. 1981. Differences in animal ^{13}C, ^{15}N and D abundance between a polluted and an unpolluted coastal site: likely indicators of sewage uptake by a marine food web. Estuarine, Coastal and Shelf Science 13: 701-707.

Rocha C. 1998. Rhythmic ammonium regeneration and flushing in intertidal sediments of the Sado estuary. Limnology and Oceanography 43: 823-831.

Schmitt R.J., Osenberg C.W. 1995. Detecting ecological impacts; concepts and applications in coastal habitats. Academic Press, San Diego, California.

Strickland J.D.H., Parsons T.R. 1968. A practical hand book of seawater analysis. Fisheries Research Board of Canada, Ottawa.

Valiela I., Bowen J.L. 2002. Nitrogen sources to watersheds and estuaries: role of land cover mosaics and losses within watersheds. Environmental Pollution 118: 239-248.

Van Dover C.L., Frey J.F.G., Garritt R.H., Starczak V.R. 1992. Stable isotope evidence for entry ofsewage-derived organic material into a deep-se food web. Nature 360: 153-155.

Vitousek P.E., Aber J.D., Howarth R.W., Likens G.E., Matson P.A., Schindler D., Schlesinger W.H., Tilman D.G. 1997. Human alteration on the global nitrogen cycle: sources and consequences. Ecological Applications 7: 737-750.

Waldron S., Tatner P., Jack I., Arnott C. 2001. The impact of sewage discharge in a marine embayment: a stable isotope reconnaissance. Estuarine, Coastal and Shelf Science 52: 111-115.

Wayland M., Hobson K.A. 2001. Stable carbon, nitrogen, and sulfur isotope ratios in riparian food webs on rivers receiving sewage and pulp-mill effluents. Canadian Journal of Zoology 79: 5-15.

Yamamuro M., Kayanne H., Yamano H. 2003. $\delta^{15}N$ seagrass leaves for monitoring anthropogenic nutrient increases in coral reef ecosystems. Marine Pollution Bulletin 46: 452-458.

Chapter 4 - Elemental and stable isotope composition of *Zostera noltii* (Horneman) leaves during the early phases of decay in a temperate mesotidal lagoon

Introduction

The importance of seagrasses in coastal and nearshore environments and their contribution to the productivity of the world's oceans has become increasingly recognized over the last decades. These marine vascular plants are the physical and biological foundation for many costal marine systems. Despite the difficulty of discerning their roles as food source, determining the fate of the carbon they fix and examining the trophic routes through which seagrasses production is transferred to higher trophic levels is crucial to understanding the food web of coastal ecosystems (Cebrián et al., 1997).

The detrital food web has long been inferred to be the major trophic path of energy flow in seagrass systems (Zieman, 1983). However, the fragmented organic material is difficult to identify and to quantify and the presence of this material in the gut of animals does not indicate the nutritive value of the detritus (Zieman et al., 1984; Valiela, 1995). The analysis of the natural abundance of stable isotopes of carbon ($\delta^{13}C$), nitrogen ($\delta^{15}N$), sulphur ($\delta^{34}S$) in organic matter is a relatively recent technique to assess the organic matter flow along the food webs of coastal systems (Fry and Sherr, 1984; Peterson et al., 1985; Peterson and Howarth, 1987). Because shifts in stable isotope values between living and detrital plant-derived material can occur (Benner et al., 1987; Currin et al., 1995; Cloern, 2002), measurement of the changes in isotopic decomposition of detritus as it decays is required to refine our understanding of organic matter transfers in detrital food webs (Peterson, 1999; Machás et al., 2003).

The decay of vascular plants in temperate salt marshes occurs in three phases: (1) an early phase lasting less than one month, with fast rates of weight loss due to the leaching of soluble compounds, (2) a slower phase lasting up to one year, including microbial degradation of organic matter and subsequent leaching of hydrolyzed substances and (3) a very slow decay phase of remaining refractory materials, which may last an additional year (Valiela et al., 1985). Decomposition of plant detritus is conducted by microorganisms and is dependent on the elemental content of the plant (Enríquez et al., 1993), on environmental factors like temperature, on the availability of both water and nutrients and on fragmentation by detritivores (Harrison, 1989). Bacterial assimilation of nutrients from the environment is thought to be necessary for the decomposition of plant material of low initial nutrient content, particularly nitrogen (Goldman et al., 1987). In this study, we investigated the initial N content of the seagrass *Zostera noltii* and whether decomposing seagrass was a source or sink of N to the environment.

In Ria Formosa lagoon, southern Portugal, (Fig. 1) most of the intertidal is occupied by monospecific meadows of the seagrass *Zostera noltii*. The upper distribution of this species aligns with the lower edge of the salt marsh species *Spartina maritina* whereas its lower edge aligns with the distribution of the subtidal seagrass *Cymodocea nodosa*. The species production and leaf turnover is very high and sustained throughout the year. Once the leaves of *Z. noltii* detach from the plant, they float, and are transported away with the water flow. A significant portion of the leaf wrack is accumulated in big masses on top of the *S. maritina* meadows.

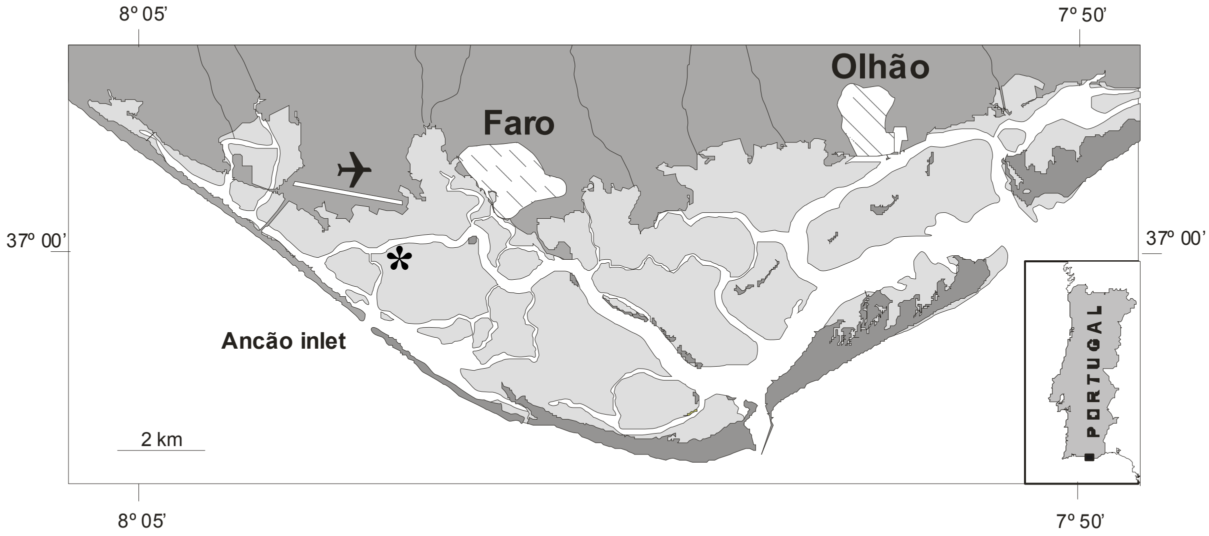

Fig. 1. Map of Ria Formosa. Asterisk shows the location of the sampling site (Ramalhete).

The aim of this study was to assess the changes in the elemental and stable isotope contents of *Zostera noltii* leaves during the early phases of decomposition, which is the most relevant in terms of the organic matter transferred to the food web. The changes in the biomass, the C, N and S elemental contents, the C:N ratios and the $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ values of the leaves were assessed using the litterbag method (Josselyn et al., 1986) in two different environments: submerged in a saltmarsh pond (Fig. 2A) and within a *Spartina maritina* meadow (Fig. 2B). In addition, the elemental and stable isotope composition of both the attached leaves of *Z. noltii* and the leaf wrack were compared.

Materials and methods

Sampling site

The Ria Formosa is a mesotidal system of salt marshes, mudflats and channels, extending for about 55 km along the coast of southern Portugal, connected permanently to the Atlantic Ocean through six inlets (Fig.1). The average depth of the lagoon is about 2 m and the fresh water input is low. The salinity remains close to 36 psu except during sporadic short periods of winter run-off (Falcão and Vale, 1990). The total area of the lagoon is 84 km^2, with tidal flats covering 80% of the total area (Andrade, pers. comm.). This study was conducted in the summer of 2000 at the Ramalhete tidal flat (Fig. 1), where *Zostera noltii* occupies a vertical zone of about 2 m in the intertidal.

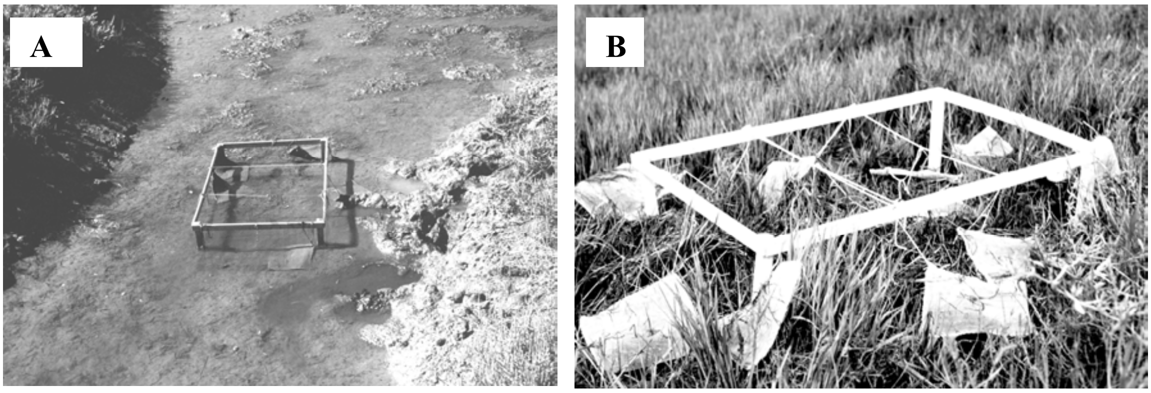

Fig. 2. Litterbag experiments under two different field conditions: (A) litterbags permanently submerged in a salt marsh pond, (B) high intertidal incubations.

Sampling design

Experiments using litterbags (15x20 cm, 1 mm mesh size) were conducted during 60 days, in two different field locations: (1) submerged in a salt marsh pond and (2) within a *Spartina maritina* meadow (high intertidal) (Figs. 2A and 2B, respectively). Living, healthy leaves were collected from *Zostera noltii* plants in September of 2000, cleaned of mud and epiphytic material, sorted, dried with filter paper and weighted. About 4-5 g wet weight (WW) of leaf material was placed inside six litterbags in each location. The bags were tied to a structure to avoid displacement (Fig. 2) in such a way that and they remained in contact with the sediment.

In each location, three bags were collected after 30 days of incubation and the remaining three bags were collected at the end of the experiment (60 days of incubation). The leaf material of each litterbag was separated from macroinvertebrates and gently rinsed with water to remove adhering sediments. The sample was then dried with filter paper to remove excess water, weighted (WW), dried at 60ºC in an oven until constant weight and reweighed (DW). At this point a subsample was separated for elemental and stable isotope analyses. The remaining material was then ashed at 450ºC during 4 hr and ash free dry weigh (AFDW) was determined. The AFDW provides a close approximation of the organic content of the litter (Wilson et al., 1986). The AFDW of the leaves at the initial time of incubation was obtained using a linear relationship between WW and AFDW obtained from twenty samples. An initial sample of living leaves was collected and analysed for elemental and stable isotope contents.

The decay rate of leafs within the litterbags was described by fitting a single negative exponential equation (Olson, 1963) to the weight data, using the following equation:

$\ln(\text{fraction of initial AFDW remaining}) = -k \text{ x } t$, where k ($d^{-1}$) corresponds to the decomposition rate and t (d) to the time in days elapsed since the beginning of the experiment.

To compare the elemental and stable isotope composition of attached leaves with the leaf wrack of *Zostera noltii* (with the exception of the samples for $\delta^{34}S$ analyses, where the initial values of the experiment were used) two samples of healthy, attached leaves, were taken from plants located in each of three levels of the vertical distribution of the species: the upper limit, the medium zone and the lower limit. The rational for sampling along the vertical distribution of the species is that the elemental and isotopic composition of the species may vary with exposure time. In fact, morphometric and physiological differences between higher and lower intertidal plants of *Z. noltii* have been described (Peralta et al., 2000; Alexandre et al., 2004; Silva and Santos, 2003). The *Z. noltii* wrack of the Ramalhete channel was sampled with both surface and bottom nets. We assumed that the leaves collected along the bottom were in a later stage of decomposition. At the laboratory, the *Z. noltii* leaf wrack was separated from the other macrophytes. Dried sub-samples of attached and leaf wrack were analysed for elemental and stable isotope composition. Other sub-samples were weighed (DW) and incinerated at 450ºC during 4 hr and reweighed to determine the inorganic content.

Elemental and stable isotopic analyses

The *Zostera noltii* sub-samples for elemental and stable isotopic analyses were cleaned more carefully than the sub-samples for biomass quantification in order to reduce the inorganic matter to a minimum. The sub-samples were cleaned of adhering sediment and epiphytic material with a razor blade, rinsed with deionised water and re-dried at 60ºC. Cleaned sub-samples were ground to a fine powder using a Wiley Mill and stored in glass vials inside a desiccator until sent off for analysis. Samples for $\delta^{13}C$ analysis were checked for contamination with carbonates by adding 10% HCl to a sub-sample of the ground sample. Carbonate contamination, could shift isotopic values in the positive direction. Sub-samples for $\delta^{34}S$ analysis were rinsed in deionised water to remove seawater sulphate. After grinding, samples were re-suspended in deionised water for 5 minutes, centrifuged, and the supernatant was discarded. This procedure was repeated 3 times. Then, sub-samples were re-dried at 60^{o}C. It was not possible to remove reduced inorganic S (pyrite) if present. Pyrite forms rapidly in salt marsh sediments (Giblin, 1988), being found in void cell spaces or attached to the walls of cortical cells of vascular plants.

The ratios of heavy to light stable isotopes (^{13}C:^{12}C; ^{34}S:^{32}S; and ^{15}N:^{14}N) are expressed in the δ notation which indicates the depletion (-) or the enrichment (+) of the heavy isotope compared to the lighter isotope relative to a standard according to the formula:

$$\delta^n X\ (‰) = 1000\left(\frac{\delta X_{\text{sample}}}{\delta X_{\text{standard}}} - 1\right)$$, where X = C, N or S and n = 13, 15 and 34 respectively.

Standards are C from Vienna Pee Dee Bellemnite (VPDB), N from the air and S from Esfalerite (IAEA NBS 123). Samples for $\delta^{13}C$ and $\delta^{15}N$ were analysed at the Stable Isotope Laboratory, Marine Biological Laboratory (MBL), Woods Hole (US), using a continuous-flow isotope ratio mass spectrometer (PDZ Europa 20-20) with an elemental analyzer-GC preparation purification system. Instrumental precision based on the SD of replicates of internal standards was ± 0.1‰ for $\delta^{13}C$ and ± 0.2‰ for $\delta^{15}N$. The $\delta^{34}S$ determinations were performed at the Stable Isotope Laboratory, ICAT, Lisboa - Portugal, using a continuous-flow isotope ratio mass spectrometer (VG ISOGAS SIRA II) with an elemental analyzer (EuroEA, Eurovector).

The percent of tissue C and N elemental contents were determined in conjunction with the $\delta^{13}C$ and $\delta^{15}N$ analysis, with a relative precision of 1%. The C:N ratios were calculated on a mole:mole basis. The S content of tissue was determined at Iso-Analytical Limited, Cheshire, UK.

Statistical analysis

The relationship between initial WW and AFDW was established by linear regression. The effects of the incubation site (submerged versus high intertidal) and time (days) on the organic matter loss (% AFWD), on the organic C and N content (% DW), on the C:N ratios and on the $\delta^{13}C$ and $\delta^{15}N$ composition (‰) of *Zostera noltii* were tested using 2-way ANOVA, followed by the Tukey test. The effects of time and field locations on the $\delta^{34}S$ data were not tested statistically due to the lack of replication (n=2).

Significant differences between both the elemental and stable isotope composition of attached leaves versus leaf wrack of *Zostera noltii* were tested with T-Tests. Pearson correlations were calculated to examine if the amount of the inorganic matter and the percent of S in the samples had quantitative relationships with $\delta^{34}S$ values. The SigmaStat software package was used for data analysis. Relationships were considered significant at $p < 0.05$.

Results

There were significant effects of sampling time (number of incubation days) on the biomass of *Zostera noltii* leaves remaining during the litterbag experiment (Fig. 3). The interactions between Time x Field location were also significant. The percent of litter remaining after 30 days of incubation was significantly less in the submerged (51%) than in the high intertidal incubations (61%), but after 60 days there were no significant differences. In the high intertidal incubations, significantly more litter remained after 30 days (61%) than after 60 days (40%) of incubation whereas in the submerged incubations no significant change was observed between 30 days (51%) and 60 days (46%) of incubation time (Fig. 3). The overall decay rate of *Z. noltii* leaf material throughout the whole experimental period was $k = 0.016 \pm 0.001\ d^{-1}$.

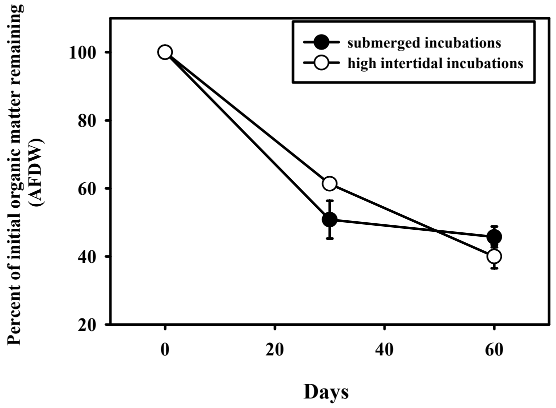

Figure 3. Decay of *Zostera noltii* leaf biomass (percent of initial organic matter AFDW remaining) during the litterbag incubations: vertical bars - standard deviation, n = 3.

The C and N content in the decomposing leaves of *Zostera noltii* decreased significantly with incubation time (Fig. 4A and B). The decay of the C and N content was of the same magnitude and thus the C:N molar ratio did not vary significantly during the experiment (Fig. 4C). The $\delta^{13}C$ and $\delta^{15}N$ values of the leaf litter did not vary significantly over time or between incubation sites (Fig. 5A, B and 6B). Even though the $\delta^{34}S$ values showed a decreasing trend during decomposition (Fig. 7B), the effects of time and field locations were not statistically significant due to the high variability of replicates. A higher percent of inorganic matter was found in the leaves with high S content

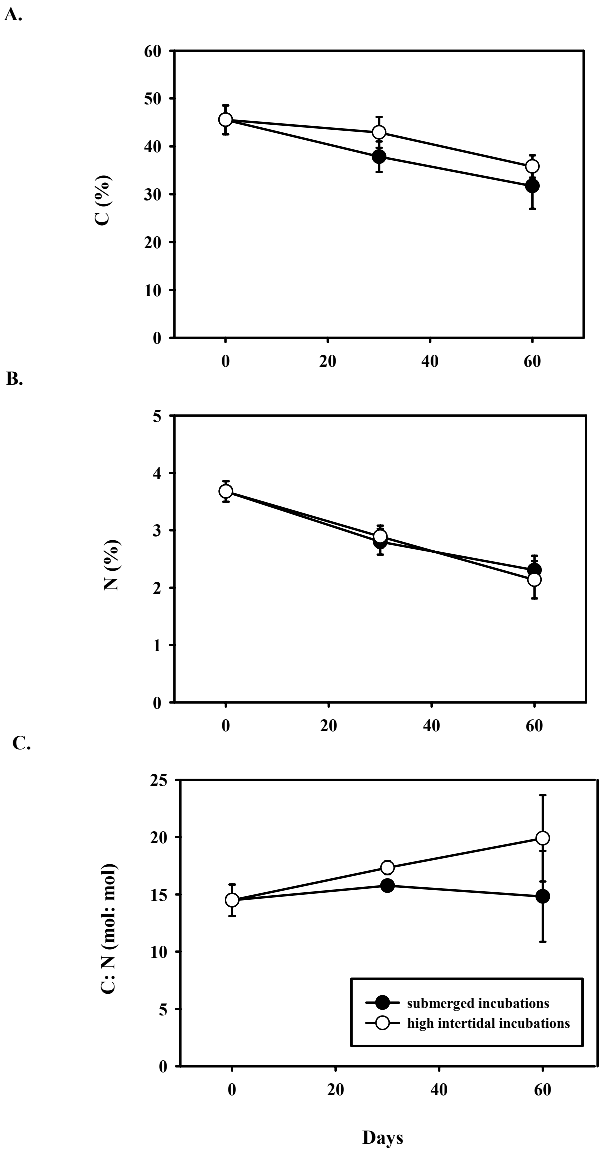

Figure 4. Variation of elemental contents of *Zostera noltii* leafs during the litterbag incubations. (A) Carbon content (%), (B) Nitrogen content (%) and (C) C:N ratios: vertical bars - standard deviation, n = 3.

and low $\delta^{34}S$ value, however, no significant correlations were found between the $\delta^{34}S$ and both the S content and the amount of inorganic matter.

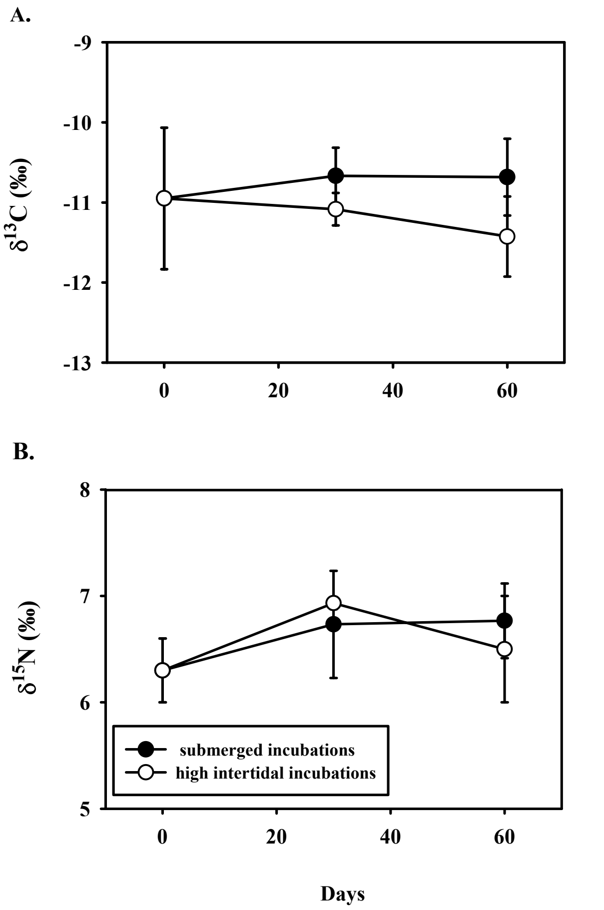

Figure 5. Variation of carbon and nitrogen stable isotope values of *Zostera noltii* leaves during the litterbag incubations. (A) Carbon δ-values (‰), (B) Nitrogen δ-values (‰): vertical bars – standard deviation, *n* = 3

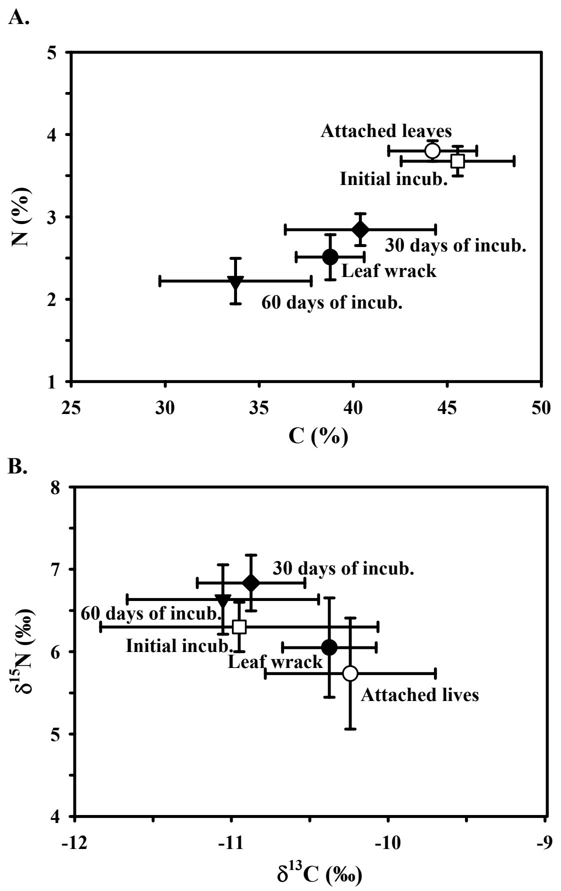

Figure 6. Elemental and stable isotope plots of leaf incubations, attached leaves and leaf wrack of *Zostera noltii*. (A) Carbon and nitrogen contents and (B) Carbon and nitrogen δ-values: bars – means ± standard deviation.

The C and N content of attached leaves of *Zostera noltii* was significantly higher than leaf wrack (Table I, Fig. 6A). In fact, the elemental contents of the leaf wrack were in the range of the leaf litter element contents after incubations. The C:N ratio of attached leaves of *Z. noltii* increased significantly from leaves to leaf wrack indicating that a large amount of structural C in relation to N remained after decomposition. The $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ values did not differ significantly between attached leaves and leaf wrack.

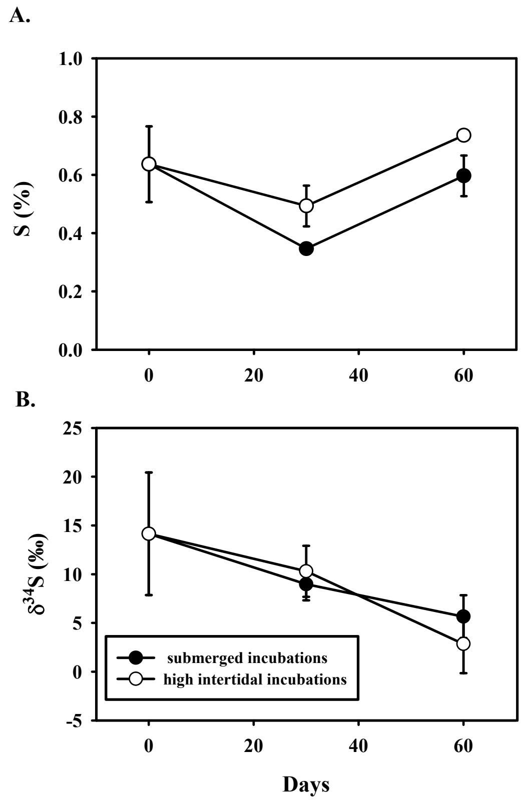

Figure 7. Variation of sulphur of *Zostera noltii* leafs during the litterbag incubations. (A) Sulphur contents (%) and (B) Sulphur stable isotope values (‰): vertical bars - standard deviation, *n* = 2.

Table I. Elemental contents and stable isotope values of attached leaves versus leaf wrack of *Zostera noltii*: n. sign.- not significant; sign.- significant. Level of significance was set at $p < 0.05$.* data from Machás et al (2003).

Type of sample	Site of sampling	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	$\delta^{34}S$ (‰)	C (%)	N (%)	S (%)	C: N (mol: mol)
Zostera noltii								
Attached leaves	Low intertidal	-10.0	5.7	15.9 *	44.8	3.9	-	13.5
	Low intertidal	-10.0	6.6	-	45.1	3.9	-	13.6
	Medium intertidal	-11.3	6.0	9.7	41.1	3.8	0.7	12.6
	Medium intertidal	-10.4	6.2	-	46.9	3.8	-	14.4
	High intertidal	-10.1	5.0	18.6	41.6	3.6	0.5	13.6
	High intertidal	-9.7	4.9	-	45.8	3.9	-	13.7
Group mean		**-10.2**	**5.7**	**14.2**	**44.2**	**3.8**	**0.6**	**13.6**
Standard deviation		**0.5**	**0.7**	**6.3**	**2.3**	**0.1**	**0.1**	**0.6**
Leaf wrack	Surface	-10.0	5.4	9.0	39.4	2.9	0.4	15.9
	Surface	-10.3	6.4	8.1	36.5	2.3	0.4	18.6
	Bottom	-10.5	6.7	6.7	38.4	2.5	0.5	17.9
	Bottom	-10.7	5.7	8.7	40.8	2.4	0.4	20.2
Group mean		**-10.4**	**6.1**	**8.1**	**38.8**	**2.5**	**0.4**	**18.1**
Standard deviation		**0.3**	**0.6**	**1.0**	**1.8**	**0.3**	**0.05**	**1.8**
T-Test (attached vs. leaf wrack)		n. sign.	n. sign.	n. sign.	sign.	sign.	n. sign.	sign.

Discussion

The decomposition experiment showed that the mass loss of the *Zostera noltii* leaves after 30 days was about 25% higher in submerged conditions than at the high intertidal, when plants are exposed to the air more than 6h in each 12h tidal cycle. Leaching of soluble compounds might have been reduced in the intertidal because dry material decomposes substantially slower than moist plant material (Enríquez et al., 1993). To support this hypothesis, the organic C and N contents of *Zostera noltii* leaves should have been significantly lower when submerged than at the intertidal, which they were not. As an alternative to differential leaching, the lower biomass remaining after 30 days of incubation in the submerged litterbags may reflect (1) higher consumption by invertebrates, and/or (2) the loss of fragmented biomass from the bags as a result of water movement. The observation that insect larvae and amphipods were found only in litterbags from intertidal incubations suggests that litter mass may have escaped from the submerged litterbags. Several studies have attempted to determine the effect of drying on seagrass decay. Drying either causes no change in biomass decay or reduces the decay rates (Harrison, 1989). Valiela et al. (1985) suggest that the litter quality (chemistry and species) may be the key factor setting decay rates.

The leaves of *Zostera noltii* in Ria Formosa lagoon showed a higher C content and a slight lower N content (45.6% and 3.7%, respectively, Fig. 6A and B) than the *Z. noltii* leaves from Palmones river estuary, southern Spain (37.6% and 3.9%, respectively, Peralta et al., 2000). The nitrogen content of *Z. noltii* is dependent on several factors such as the nutrient conditions where the plants were colleted, the age of leaves and the light availability (Pérez-Lloréns et al., 1991; Peralta et al., 2002). This species does not seem to be nitrogen limited in these systems as the reference N content for seagrass nitrogen limitation is about 1.8% (Duarte, 1990). On the other hand, the higher carbon content of *Z. noltii* at Ria Formosa may be related to the system respiration which determines the CO_2 availability for photosynthesis. Santos et al. (2004) observed high levels of respiration of the *Z. noltii* communities of Ria Formosa. The decay rate of *Z. noltii* leaves in Ria Formosa ($k = 0.016 \pm 0.001\ d^{-1}$) were similar to rates observed for this species by Cebrián et al., 1997 ($k = 0.015 \pm 0.003\ d^{-1}$) and were intermediate to decay rates of *Zostera marina* and *Cymodocea nodosa* ($k = 0.019 \pm 0.002\ d^{-1}$ and $k = 0.009 \pm 0.001\ d^{-1}$, respectively) at Cala Jonquet (northern Spanish Mediterranean Coast).

Different modes of decomposition were found for seagrasses compared to other vascular plants such as marsh grasses and mangroves (Zieman et al., 1984, Harrison, 1989). In contrast to other vascular plants where net immobilization of N is evident (Harrison, 1989), during the decay of seagrasses the N and other elements are gradually released. This agrees with the observations of this study that *Zostera noltii* leaves have undergone the early phases of decomposition with significant loss of both C and N content. A different behaviour of $\delta^{13}C$ and $\delta^{15}N$ values during decomposition was also found for other vascular plants (Zieman et al., 1984; Benner et al, 1987; Currin et al., 1995). While mangroves show little $\delta^{13}C$ change but marked reduction in $\delta^{15}N$ (Zieman et al., 1984) and the salt marsh species *Spartina alterniflora* showed declines in both $\delta^{13}C$ values (Benner et al, 1987) and $\delta^{15}N$ values (Currin et al., 1995), the seagrasses during the early stage of decomposition showed little change in $\delta^{13}C$ and $\delta^{15}N$ values (Zieman et al., 1984; this study). This contrast can be explained by species-specific differences in plant tissue and different microbial utilization of detritus. Since bacterial biomass has a $\delta^{13}C$ signature similar to that of the assimilated substrate (Coffin et al., 1990; Hullar et al., 1996), the changes in $\delta^{13}C$ values during plant decomposition may result from the differential decay of polysaccharides and lignin components of the original material. As lignin has low $\delta^{13}C$ values and it is decomposed very slowly, its relative abundance increases during decomposition causing the decrease in $\delta^{13}C$ values in plants with higher lignin content.

The presence of lignin in seagrasses, has been convincingly demonstrated (Opsahl and Benner, 1993; Klap et al., 2000). However, seagrass species with fast-growing leaves have lower concentrations of lignin than species with slow-growing leaves (Klumpp et al., 1989). Due to differences in lignin contents between species (Klap et al., 2000), we expected that only the ones with slow-growing leaves would show significant shifts in $\delta^{13}C$ values during decomposition. This is not the case for *Zostera noltii* because this species has one of the highest leaf turnover rates among seagrasses (Cebrián and Duarte, 1998); consequently, little change in $\delta^{13}C$ values is expected to occur during decomposition as it was observed in this study. Zieman et al. (1984) also did not find significant changes of isotopic values for the seagrass *Thalassia testudinum* during the early phases of decompositions probably because it is also a fast-growing species (Cebrián and Duarte, 1998).

Changes in $\delta^{15}N$ values have been reported during decomposition of vascular plants due to microbial immobilization of N from environmental sources (Zieman, 1984; Wilson et al., 1986; Fourqurean and Schrlau, 2003). The isotopic shift of $\delta^{15}N$ values depends on several factors: the initial N content (Zieman, 1984), the nature of the microbial community (Lehmann et al., 2002) and the isotopic composition of the DIN assimilated by the microbial community (Caraco et al., 1998). Since the mangroves and *Spartina* spp. contain little initial N, the growth of microbes may change the $\delta^{15}N$ signature during decomposition (Zieman et al., 1984; Wilson et al., 1986). However, the microbial metabolism during decomposition of seagrasses may rely largely on the endogenous N of the seagrass (Zieman et al., 1984) as was the case of *Zostera noltii* in Ria Formosa. During the early phases of decay the $\delta^{15}N$ values of this species did not vary probably due to the high N content of the starting material (3.7% DW ± 0.2). Changes in the $\delta^{15}N$ signature of *Thalassia testudinum* leaves during decomposition vary with the phase of decomposition. In the early phase of decomposition, the $\delta^{15}N$ values of this species did not vary (Zieman et al., 1984), while during the long-term decomposition these values decreased (Fourqurean and Schrlau, 2003). It may be that after an initial stage of decomposition, when endogenous N was consumed, the bacterial community relied largely on the immobilization of exogenous N.

The C and N contents of wrack are about 12% and 40% lower, respectively, than those for leaves. These results are in agreement with the results of the decomposition experiment (Fig. 6A) and with the results obtained for *Thalassia testudinum* after 40 days of litterbag incubations (Zieman et al., 1984). This suggests that leaf detritus of seagrasses undergo an early phase of decomposition with rapid weight loss due to leaching of soluble compounds. While the C and N elemental content of *Z. noltii* leaves decreased in the early phase of decomposition, the $\delta^{13}C$ and the $\delta^{15}N$ values reflected the signatures of the original material. The increase of C:N from attached leaves to leaf wrack of *Z. noltii* also indicates that microbial decomposers did not take up N from the environment. Balanced bacterial growth requires substrates with C:N ratio of 10.3 (Goldman et al., 1987). Bacteria are often supplied with plant detritus depleted in N relative to their requirement (Enríquez et al., 1993). Atkinson and Smith (1984) reported a ratio of 21.4 for marine macrophytes while Duarte (1990) reported a ratio of 20.4 for seagrasses. However, this was not the case of *Zostera noltii* as the C:N ratios of attached leaves and leaf wrack were respectively 13.6 ± 0.6 and 18.1 6 ± 1.8 (Table 1). This ratio was similar to that observed for this species (14.1) in Southwestern Netherlands by Pérez-Lloréns et al. (1991). This indicates that *Z. noltii* leaves are a good substrate for bacteria growth and that bacteria may not have to immobilize N from the environment in order to metabolize the organic material in the leaves.

The main reason for the observed variability of *Zostera noltii* $\delta^{34}S$ values (Fig. 4) may be the practical difficulties achieving an effective separation between organic detritus and reduced inorganic S forms such as pyrite. Pyrite has low $\delta^{34}S$ value (Peterson et al., 1986), which will lower the $\delta^{34}S$ signature of particulate matter (Machás et al., 2003). Sediments of the Ria Formosa have low concentrations of sulphides (Borum, pers. comm.), probably due to the high iron concentrations which favours pyrite formation. The higher percent of inorganic matter found in the *Z. noltii* leaves with high S content and low $\delta^{34}S$ value (Table 1), suggest a contamination with inorganic forms of reduced sulphur, such as pyrite.

In conclusion, our results indicate that the leaf detritus of the seagrass *Zostera noltii* in Ria Formosa lagoon are decomposed, in the early phase of the decay, by microorganisms without consumption of exogenous sources of N. As well, the $\delta^{13}C$ and $\delta^{15}N$ values of this species do not change during the breakdown of the biomass into finer particulate material. Stable isotope studies to assess the contribution of this species to secondary production can thus consider the natural $\delta^{13}C$ and $\delta^{15}N$ abundances of living tissues without any correction to account for decomposition effects. On the other hand, the use of $\delta^{34}S$ values of *Z. noltii* detritus should be done with caution. These values may be affected by the contamination of the samples with pyrite, which will decrease the $\delta^{34}S$ signal of tissues.

Acknowledgements: We are grateful to S. Cabaço, L. Mata and J. Silva for helping in the field work and to A. Engelen for the constructive comments on the manuscript. This study is part of the PhD thesis of R. Machás, funded by the FCT (scholarships Praxis XXI/BD/19579/99).

References

Atkinson, M.J., Smith, S.V., 1984. C:N:P ratios of benthic marine plants. Limnology and Oceanography 28, 568-574.

Alexandre, A., Silva, J., Santos, R., 2004. The maximum nitrate reductase activity of the seagrass *Zostera noltii* (Hornem.) varies along its vertical distribution. Journal of Experimental Marine Biology and Ecology 307, 127-135.

Benner, R., Fogel, M.L., Sprague, E.K., Hodson, R.E., 1987. Depletion of ^{13}C in lignin and implications for stable carbon isotope studies. Nature 329, 708-710.

Caraco, N.F., Lampman, G., Cole, J.J., Limburg, K.E., Pace, M.L., Fisscher, D., 1998. Microbial assimilation of DIN in a nitrogen rich estuary: implications for food quality and isotope studies. Marine Ecology Progress Series 167, 59-71.

Cebrián, J., Duarte, C. M. 1998. Patterns in leaf herbivory on seagrasses. Aquatic Botany 60, 67-82.

Cebrián, J., Duarte, C.M., Marbà, N., Enríques, S., 1997. Magnitude and fate of the production of four co-occurring Western Mediterranean seagrass species. Marine Ecology Progress Series 155, 29-44.

Coffin, R.B., Velinsky, D.J., Devereux, R., Price, W.A., Cifuentes, L.A., 1990. Stable carbon isotope analysis of nucleic acids to trace sources of dissolved substrates used by estuarine bacteria. Applied and Environmental Microbiology 56, 2012-2020.

Cloern, J.E., 2002. Stable carbon and nitrogen isotope composition of aquatic and terrestrial plants of the San Francisco Bay estuarine system. Limnology and Oceanography 47, 713-729.

Currin, C.A., Newell, S.Y., Paerl, H.W., 1995. The role of standing dead *Spartina alterniflora* and benthic microalgae in salt marsh food webs: considerations based on multiple stable isotope analysis. Marine Ecology Progress Series 21, 99-116.

Duarte, C.M., 1990. Seagrass nutrient content. Marine Ecology Progress Series 67, 201-207.

Enríquez, S., Duarte, C.M., Sand-Jensen, K., 1993. Patterns in decomposition rates among photosynthetic organisms: the importance of detritus C:N:P content. Oecologia 94, 457-471.

Falcão, M., Vale, C., 1990. Study of the Ria Formosa ecosystem: benthic nutrient remineralization and tidal variability of nutrients in the water. Hydrobiologia 207, 137-146.

Fourqurean, J.W., Schrlau, J.E., 2003. Changes in nutrient content and stable isotope ratios of C and N during decomposition of seagrasses and mangroves leaves along a nutrient availability gradient in Florida Bay, USA. Chemistry and Ecology 19, 373-390.

Fry, B., Sherr, E.F., 1984. $\delta^{13}C$ Measurements as indicators of carbon flow in marine and freshwater ecosystems. Contributions in Marine Science 27, 13-47.

Giblin, A.E., 1988. Pryrite formation in marshes during early diagenesis. Geomicrobiology Journal 6, 77-97.

Goldman, J.C.., Caron, D.A., Dennett, M.R., 1987. Regulation of gross growth efficiency and ammonium regeneration in bacteria by substrate C:N ratio. Limnology and Oceanography 32: 1239-1252.

Harrison, P.G., 1989. Detrital processing in seagrass systems: a review of factors affecting decay rates, remineralization and detritivory. Aquatic Botany 35, 263-288.

Hullar, M.A.J., Fry, B., Peterson, B.J., Wright, R.T., 1996. Microbial utilization of estuarine dissolved organic carbon - a stable isotope tracer approach tested by mass balance. Applied and Environmental Microbiology 62, 2489-2493.

Josselyn, M.N., Fonseca, M., Niesen, T., Larson, R., 1986. Biomass, production and decomposition of a deepwater seagrass, *Halophila decipiens* Ostenfeld. Aquatic Botany 25, 47-61.

Klap, V.C., Hemminga, M.A., Boon, J.J., 2000. Retention of lignin in seagrasses: angiosperms that returned to the sea. Marine Ecology Progress Series 194, 1-11.

Klumpp, D.W., Howard, R.K., Pollard, P.C., 1989. Trophodynamics and nutritional ecology of seagrass communities. In: Larkum, A.W.D., McComb, A.J., Shepherd S.A. (Eds.), Biology of Seagrasses, Elsevier, Amsterdam, pp. 394-457.

Lehmann, M.F., Bernasconi, S.M., Barbieri, A., McKenzie, J.A., 2002. Preservation of organic matter and alteration on its carbon and nitrogen isotope composition during simulated and in situ early diagenesis. Geochimica et Cosmochimica Acta 66, 3573-3584.

Machás, R., Santos, R., Peterson, B., 2003. Tracing the flow of organic matter from primary producers to filter feeders in Ria Formosa lagoon, southern Portugal. Estuaries 36, 846-856.

Opsahl, S., Benner, R., 1993. Decomposition of senescent blades of the seagrass *Halodule wrightii* in a subtropical lagoon. Marine Ecology Progress Series 94, 191-205.

Olson, J.S., 1963. Energy storage and the balance and decomposers in ecological systems. Ecology 44, 322-331.

Peralta, G., Pérez-Lloréns, J.L., Hernández, I., Brun, F., Vergara, 2002. Effects of light availability on growth, architecture and nutrient content of the seagrass *Zostera noltii* Hornem. Journal of Experimental Marine Biology and Ecology 269, 9-26.

Peralta, G., Pérez-Lloréns, J.L., Hernández, I., Brun, F., Vergara, J.J., Bartual, A., Gálvez, J.A.,García, C.M., 2000. Morphological and physiological differences between two morphotypes of *Zostera noltii* Hornem. From the south-western Iberian Peninsula. Helgoland Marine Research 54, 80-86.

Pérez-Lloréns, J.L., Muchtar, M., Niell, F.X., Nienhuis, P.H., 1991. Particulate organic carbon, nitrogen and phosphorus content in roots, rhizomes and differently aged leaves of *Zostera noltii* Hornem. in Oosterschelde Estuary (Southwestern Netherlands). Botanica Marina 34, 319-322.

Peterson, B.J., 1999. Stable isotopes as tracers of organic matter input and transfer in benthic food webs. Acta Oecologica 20, 479-487.

Peterson, B.J., Howarth, R.W., 1987. Sulfur, carbon, and nitrogen isotopes used to trace organic matter flow in the Salt-Marsh Estuaries of Sapelo Island, Georgia. Limnology and Oceonography 32, 1195-1213.

Peterson, B.J., Howarth, R.W., Garritt, R.H., 1985. Multiple stable isotopes used to trace the flow of organic matter flow in estuarine food webs. Science 227, 1361-1363.

Peterson, B.J., Howarth, R.W., Garritt, R.H., 1986. Sulfur and carbon isotopes as tracers of salt marsh organic matter flow. Ecology 67, 865-874.

Santos, R., Silva, J., Alexandre, A., Navarro, N., Barrón, C., Duarte, C.M., 2004. Ecosystem metabolism and carbon fluxes of a tidal-dominated coastal lagoon. Estuaries 27, 977-985.

Silva J., Santos, R., 2003. Daily variation patterns in seagrass photosynthesis along a vertical gradient. Marine Ecology Progress Series 257, 37-44.

Valiela, I., Teal, J.M., Allen, S.D., 1985. Decomposition in salt marsh ecosystems: the phases and major factors affecting disappearance of above-ground organic matter. Journal of Experimental Marine Biology and Ecology 89, 29-54.

Valiela, I., 1995. Marine ecological processes, 2nd Ed., Springer-Verlag, New York, pp. 247-274.

Wilson, J.O., Buchsbaum, R.,Valiela, I., Swain, T., 1986. Decomposition in salt marsh ecosystems: phenolic dynamics during decay of litter of *Spartina alternifora*. Marine Ecology Progress Series 29, 177-187.

Zieman, J.C., 1983. Coral reefs, seagrass beds and mangroves: their interaction in the coastal zones of the caribbean. Unesco reports in marine science 23, 80-86.

Zieman, J.C., Macko, S.A., Mills, A.L., 1984. Role of seagrasses and mangroves in estuarine food webs: temporal and spatial changes in stable isotope composition and amino acid content during decomposition. Bulletin of Marine Science 35, 380-392.

Chapter 5 – Trophic interactions in a seagrass meadow (*Zostera noltii*): an isotopic approach

Introduction

The high primary productivity of the seagrass system, including epiphytic and benthic algae, ensures an abundant supply of organic matter that can be used as the basis energy source for herbivore/grazer and detrital food webs (Heminga & Duarte 2000, Zieman 1983, Thayer et al. 1884). Despite the difficulty of discerning the organic matter and energy supply from one link to another in these complex systems (Valiela 1995, Peterson 1999), examining the trophic routes through which seagrasses production is transferred to higher trophic levels is crucial to understand their role in the food web of coastal ecosystems (Cebrián et al. 1997). Food webs capture the complexity of trophic interactions, but are time-consuming to construct, often subjective in their resolution and scope (Paine 1988).

Analysis of the natural abundance of stable isotopes of carbon (conventionally expressed as $\delta^{13}C$ values), nitrogen ($\delta^{15}N$ values) and sulphur ($\delta^{34}S$ values) in organic matter producers and in consumers have proven to be useful in describing the organic matter flow and food web relationships in coastal systems (Fry & Sherr 1984, Peterson et al. 1985, Peterson & Howarth 1987). Normally, a combination of this technique with other standard approaches such as gut content analysis, behavioural studies and process rate measurements, will ensure most robust conclusions. One advantage of stable isotope techniques is that they provide insights into both the food web and the trophic-level paradigms of food web ecology. An important rule for quality stable isotope tracer work is to know the δ-values of the sources of organic matter very well because any spatial or temporal variation will propagate through the trophic system and contribute to damped variations at higher trophic levels (Peterson 1999). Animals are similar in the isotopic composition of their diets for C and S, but average 3-4‰ higher than the $\delta^{15}N$ value of the diet (Michener & Schell 1994, Minaga & Wada 1984, Peterson & Fry 1987). As a consequence, the $\delta^{15}N$ values provide organic matter source as well as trophic information (Peterson 1999). The trophic position of a consumer can be estimated by: $\text{trophic position} = \left[\left(\delta^{15}\text{N consumer} - \delta^{15}\text{N base}\right)/3.4\right] + \lambda$ where 3.4 is the usual enrichment by trophic level and λ is the trophic position of the organism used to estimate $\delta^{15}N$ base (e.g. primary producers, $\lambda = 1$) (Cabana & Rasmussen 1996, Post 2002).

Several studies have been carried out in seagrass beds using natural abundance ratios of stable isotopes (Thayer et al. 1978, McConnaughey & McRoy 1979, Fry et al. 1987, Nichols et al. 1985, Stoner & Waite 1991, Loneragan et al. 1997, Moncreiff & Sullivan 2001, Vizzini et al. 2002, Jones et al. 2003). Although *Zostera noltii* is widespread along the intertidal coast of Western Europe, North-West Africa, Mediterranean Sea and Black Sea (North Atlantic and Meditteranean flora) (Den Hartog 1970, Heminga & Duarte 2000, Milchakova & Phillips 2003) little information exists on the isotopic composition of *Z. noltii* meadows and on its trophic role (Machás & Santos 1999, Machás et al. 2003).

Consequently, this work assesses the primary sources of organic matter for detritus sediment organic matter (SOM), suspended particulate organic matter (POM) and higher trophic levels in a typical *Zostera noltii* meadow in the Ria Formosa lagoon (southern Portugal) during one year. We investigated the relationships between: 1) $\delta^{13}C$ and $\delta^{15}N$ values of most abundant primary producers and detritus, SOM and POM and 2) $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ values of most abundant primary producers and most abundant benthic fauna. The food web relationships in this system were assessed using the stable isotopes values in conjunction with literature information about the diets of the macroconsumers. The trophic position of consumers was estimated using $\delta^{15}N$ values.

Methods

Sampling design

The Ria Formosa is a mesotidal lagoon, extending for about 55 km along the coast of southern Portugal. The total area of the lagoon is 84 km^2 with an exposed tidal flat area of about 67 km^2 (Andrade 1990).The seagrass *Zostera noltii* dominates the tidal flat below the level of the saltmarsh grass *Spartina maritima*. Blooms of green macroalgae of the genera *Enteromorpha* and *Ulva* may develop in this zone, mostly in winter. The field campaigns were carried out in the "Esteiro das Charradas" tidal flat (37º13´56´´N-08º03´21´´W), in a continuous meadow of *Z. noltii*, with an area of approximately 5000 m^2. The most abundance benthic primary producer in the "Esteiro das Charradas" is *Zostera noltii*, followed by green algae (mainly *Ulva* sp. with the presence of filamentous species, *Chadophora* sp., *Chaetomorpha* sp, *Enteromorpha* sp.) and the red alga *Gracilaria vermiculophylla.*

The benthic primary producers, detritus and sediment samples were collected monthly during one year, from January to December 2001, at low-tide, while the benthic consumers were sampled every season. The fraction of SOM and the chlorophyll *a* (Chla) concentration in the sediment were determined monthly to characterise the relative contribution of plant detritus and microalgae to SOM. Water samples for stable isotopic analysis of POM were collected in the winter of 2003, during high-tide.

To collect the most abundant benthic floral samples a total of 10 cores (squares 25 cm x 25 cm) were pooled for each sample. At least five samples of sites without *Zostera noltii* were taken and pooled into a single sample to collect vegetation detritus. In order to reduce the effect of horizontal patchiness on δ-values of SOM, Chla and organic matter concentrations in sediment from 6 minicores (3.5 cm diameter) were collected and pooled. The minicores were sliced in the field to collect the first 5 centimetres of sediment corresponding to the habitat of most of the macroconsumers. A portion of each sediment sample was used for stable isotopic analysis, as well as for Chla and SOM quantification. For consumers, as many microhabitats as possible were sampled within the system (within and outside *Z. noltii* canopy). Larger organisms were collected by hand along the *Zostera noltii* meadow. Smaller specimens were collected by sieving sediment and water through 500 and 1000 µm sieves *in situ* and placed in containers with water from the collection site. Fish was captured by seine net in the summer of 2002 (both at neap and spring tides). Juvenile life stages were selected for analysis because their tissue reflects recently metabolised food sources, rather than adults, which would have an integrated isotope signal from a variety of food sources consumed over a longer period. Macrophytes, detritus and larger consumers were frozen on return to the laboratory until subsequent processing.

Individuals of each species were pooled together for isotope analysis. This was done to minimise the variability associated with analyses of different individual organisms, to gain enough material, and to control analysis costs. In general, one composite sample was analysed, except in the case of δ-values of POM and Chla and SOM quantification where three samples were processed.

Sample preparation

After identification, macrophytes were cleaned of mud and epiphytic material. Prior to analysis, detritus samples were thawed and living material and animal detritus were carefully removed. After washed with deionized water, both type of samples were ground to a fine powder using a Wiley Mill, dried at 60^{o}C and stored inside desiccators in glass vials until sent for analysis. Samples for $\delta^{13}C$ analysis were checked for contamination with calcareous deposits or carbonates by adding 10% HCl to a sub-sample of the ground sample. Samples for $\delta^{34}S$ analysis were rinsed in deionised water to remove seawater sulphate. After grinding, samples for $\delta^{34}S$ were re-suspended in deionised water for 5 minutes, centrifuged, and the supernatant was discarded. This procedure was repeated 3 times. Afterwards, samples were re-dried at 60°C.

Epiphytes on *Zostera noltii* leaves were removed by carefully scraping a sample of pooled leaves. The epiphyte samples were concentrated by sieving the scrapings cautiously through a 100 µm sieve and subsequently through a 20 µm sieve, in order to remove sediment. The fraction remaining in both size sieves was washed with a gentle stream of deionised water and filtered separately with a low-pressure vacuum pump until clogged using precombusted (500ºC; 4 h) glass fiber filters (GF/F). The 100 µm sieve fraction was used for isotopic analysis of epiphytic algae. The diatom fraction was obtained by sieving with the 20 µm sieve. The filters with samples for $\delta^{13}C$ analysis were acidified to remove inorganic carbon. Deionised water was used to wash the filters with samples for $\delta^{34}S$ analysis in order to remove seawater sulphate. All filters were stored in glass vials and dried at 60ºC. A sub-sample of each fraction size was preserved in 4% buffered formalin and Lugol's solution respectively for species identification and cleanliness. With the exception of March, the 100 µm sieve fraction material did not contain enough material for $\delta^{34}S$ analysis.

Sediment samples were sieved through a 500 µm sieve to remove larger materials. Samples for $\delta^{13}C$ and $\delta^{15}N$ analysis were treated and analysed as described above for macrophytes and detritus. A sub-sample for Chla analysis was frozen (storage time <1 month). The Chla was extracted in 90% acetone for 15-20 h at 5ºC in the dark, and measured adapting the Lorenzen (1967) method. The volume of 100% acetone needed was calculated based on the water content of the sediment. The remaining sample was weighed wet (WW), dry weigh (DW) and ash-free dry weigh (AFDW). The percentage of SOM was calculated by the difference DW, AFDW in relation to DW. The contribution of benthic microalgae to SOM was calculated by the ratio Chla:SOM ($\mu g.g^{-1}$: $\mu g.g^{-1}$).

Water samples for sestonic POM isotope analysis were filtered with a low-pressure vacuum pump until clogged using precombusted (500ºC; 4 h) glass fiber filters (GF/F). Samples for $\delta^{13}C$ analysis were acidified to remove inorganic carbon and thus correspond to the $\delta^{13}C$ in POM, as opposed to $\delta^{34}S$ and $\delta^{15}N$ which also measure δ-values in inorganic matter.

The smaller animals were immediately sorted by taxonomic groups under a dissecting microscope and transferred to filtered seawater, enabling them to evacuate their gut. Larger organisms were dissected to isolate muscle tissue for analysis. The gut of fish was separated from muscle tissue under a dissecting microscope, fixed with buffered formalin (10%) and preserved in 70% ethanol, for identification of food items. Animals for isotope analysis were treated as described above.

Stable isotope analysis

The ratios of heavy to light stable isotopes (^{13}C:^{12}C; ^{15}N:^{14}N and ^{34}S:^{32}S) are expressed as $\delta^{n}X\ (‰) = 1000\left(\frac{\delta X_{sample}}{\delta X_{standard}} - 1\right)$, where X = C, N or S and n = 13, 15 and 34 respectively. Samples for $\delta^{13}C$ and $\delta^{15}N$ were analysed at the Stable Isotope Laboratory, at the Marine Biological Laboratory (MBL), (Woods Hole, US), using a continuous-flow isotope ratio mass spectrometer (PDZ Europa 20-20) with an elemental analyzer-GC preparation purification system. Instrumental precision based on the SD of replicates of internal standards was ± 0.1‰ for $\delta^{13}C$ and ± 0.2‰ for $\delta^{15}N$. Standards were C from Vienna Pee Dee Bellemnite (VPDB) and N from the air. The Stable Isotope Laboratory, at the University of Calgary (Alberta, Canada) processed the filters with diatoms for $\delta^{34}S$ and the remaining samples for $\delta^{34}S$ were analyzed at Iso-Analytical Limited (Cheshire, UK). The reference material used to $\delta^{34}S$ was barium sulphate (IAEA NBS 127). Silver sulphide (IAEA-S-1) and barium sulphate (Iso-Analytical R-025) were used for calibration and correction of the ^{18}O contribution to the SO^{+} ion beam. NBS 127, IAEA-S-1 and R-025 were also run as quality control check and analysis was undertaken by EA-IRMS.

Detritus and sediment samples were not analysed for $\delta^{34}S$ values because it was not possible to remove reduced inorganic S (pyrite), which would lower considerably the $\delta^{34}S$ values of samples (Peterson et al. 1986). SPM was not analysed for $\delta^{34}S$ due to the potential for contamination with suspended sediment containing inorganic reduced S (Machás et al. 2003).

Trophic position

Variation in the $\delta^{15}N$ values of primary producers at the base of the food web may produce variation in the $\delta^{15}N$ values within the same species of consumers. Thus, the application of the $\delta^{15}N$ values, as a time-integrated measure of variation of trophic level within a population of the same consumer species, has to account for the variation in the $\delta^{15}N$ values of primary producers by adjusting the $\delta^{15}N$ values of consumers to these reference values in order that their $\delta^{15}N$ values truly reflect variation in the trophic level and not variation in the $\delta^{15}N$ base (Cabana & Rasmussen 1996, Post 2002).

In this study, the trophic position of consumers was calculated as $\left[\left(\delta^{15}\mathrm{N\ consumer} - \mathrm{mean\ Z.\ noltii}\ \delta^{15}\mathrm{N}\right)/3.4\right]+1$ where 3.4 is the usual enrichment by trophic level and 1 is the trophic position of the organism used to estimate $\delta^{15}N$ (Cabana & Rasmussen 1996, Post 2002). Mean *Zostera noltii* value was based on one year of collection (12 samples) with the purpose of integrate the temporal variation of the same year of collection of the consumers.

Before comparing the δ-values of consumers with those of potential food sources, we corrected the $\delta^{15}N$ values for fractionation that occurs as organic matter passes from one trophic level to the next. This was done by subtracting 3.4‰ per trophic level from the measured $\delta^{15}N$ values of consumers. This value represents the mean trophic fractionation for $\delta^{15}N$ measured by Post (2002). Post did not find significant differences in mean fractionation nor in the variability in fractionation between carnivores and hervivores/ detritivores.

Statistics

Significant differences between seasons of organic matter and Chla in the sediment, ratio of Chla:SOM and stable isotope values of *Gracilaria vermiculophylla*, *Ulva* sp., *Zostera noltii*, *Z. noltii* epiphytes (fine and large fraction) and detritus were tested using one-way ANOVA. Three months of each season were considered replicates of that season. The STATISTICA software package was used for data analysis. Pairwise multiple comparison procedures (Tukey Test) revealed which group(s) differed from the others. Relationships were considered significant at $p<0.05$.

Results

Seasonal variation

Gracilaria vermiculophylla, *Ulva* sp., and *Zostera noltii* were present in all seasons whereas filamentous green algae were only present in winter and autumn. The seasonal variation of $\delta^{13}C$ signatures of primary producers was not significant except for *Zostera noltii*. Its lowest $\delta^{13}C$ value (-10.9‰ ± 0.5) was found in the summer, increasing continuously to autumn, winter and spring where it was highest (-8.1‰ ± 0.9) (Fig. 1a). As well, there were significant seasonal changes in the $\delta^{15}N$ values of *Z. noltii* and SOM (Fig. 2). The spring $\delta^{15}N$ values of *Z. noltii* (+5.4‰ ± 0.1) were significantly higher than winter (+3.1‰ ± 0.5), autumn (+3.4‰ ± 0.5) and summer values (+3.1‰ ± 0.5), whereas the $\delta^{15}N$ values of SOM were significantly higher in autumn (+6.5‰ ± 0.3) than in the winter (+5.3‰ ± 0.4) (Fig. 2). The annual mean of organic matter in the sediment was 10% ± 1.1. The maximum amounts of both SOM and Chla in the sediment were reached in autumn followed by winter values. The SOM autumn amounts (108 mg/g ± 5) were significantly higher than those of spring (97 mg/g ± 6), summer (95 mg/g ± 9) and winter (101 mg/g ± 3). The amounts of Chla in the sediment was only significantly higher in autumn (84.5 ± 19.1 µg/g) than in spring (62.7 µg/g ± 17.9) and summer (66.2 µg/g ± 19.5). However, the seasonal variation of the Chla:SOM ratio was not significant. This ratio was always very low (mean value of Chla:SOM = 0.001 ± 0.0004) indicating a low abundance of microphytobenthos. Concerning the $\delta^{34}S$ values (Fig. 3), the food sources only showed significant seasonal variation for *Ulva* sp., the values for summer (+16.1‰ ± 0.4) were slightly lower than the autumn (+17.8‰ ± 1.0) and winter values (+17.8‰ ± 0.4).

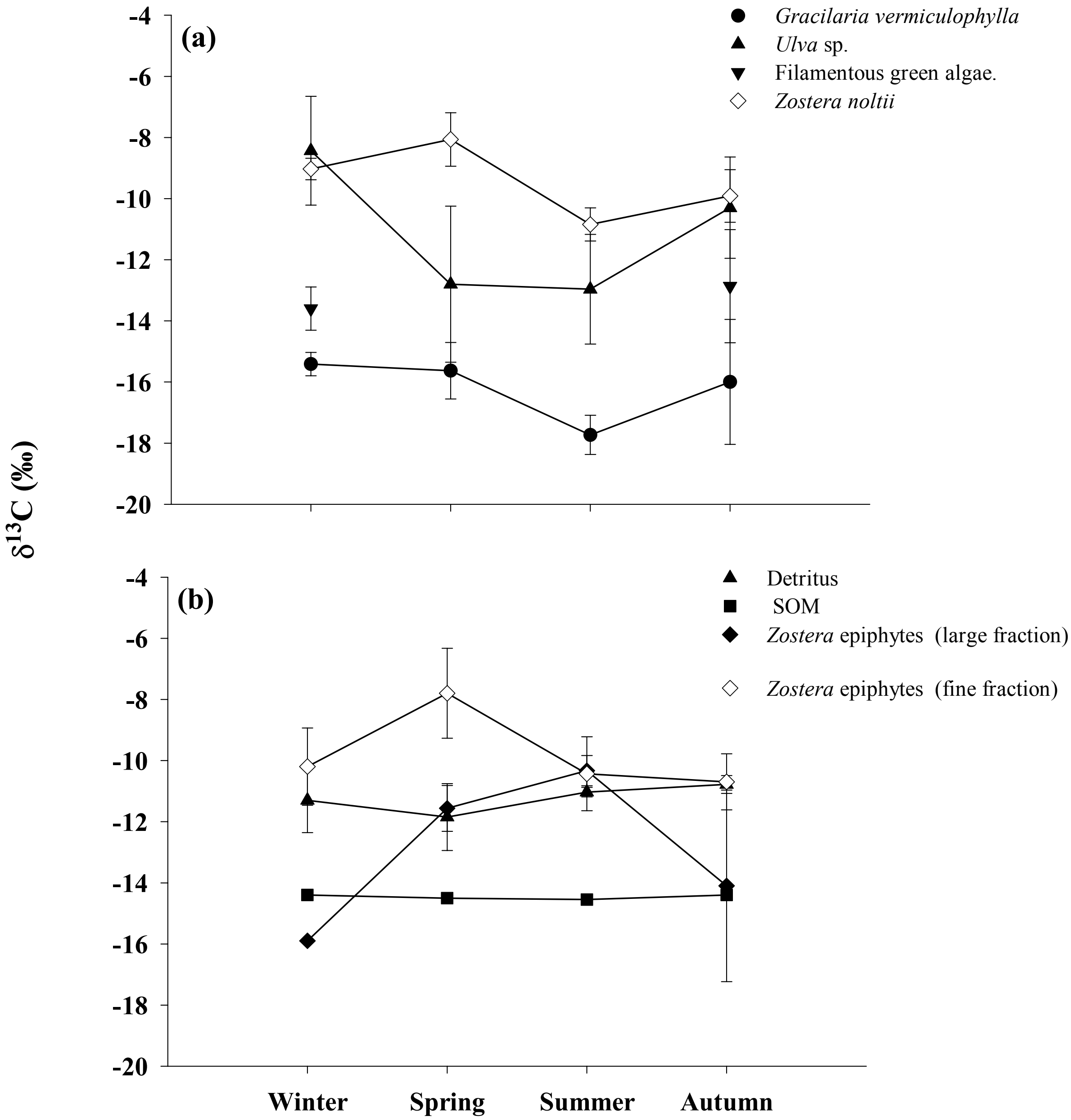

Fig. 1. Seasonal variation of $\delta^{13}C$ values of food sources in a *Zostera noltii* meadow. Bars are standard deviations of means.

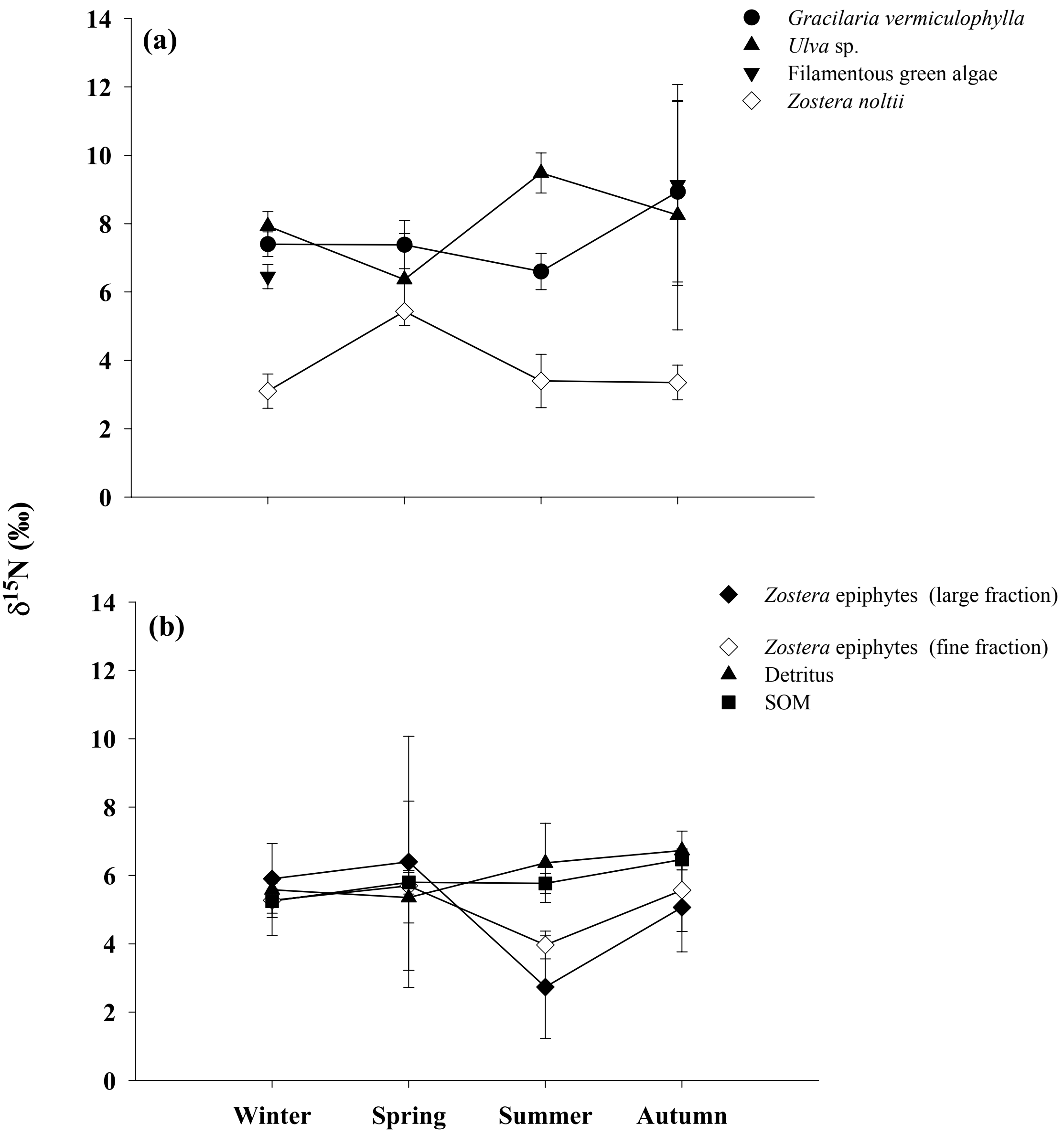

Fig. 2. Seasonal variation of $\delta^{15}N$ values of food sources in a *Zostera noltii* meadow. Bars are standard deviations of means.

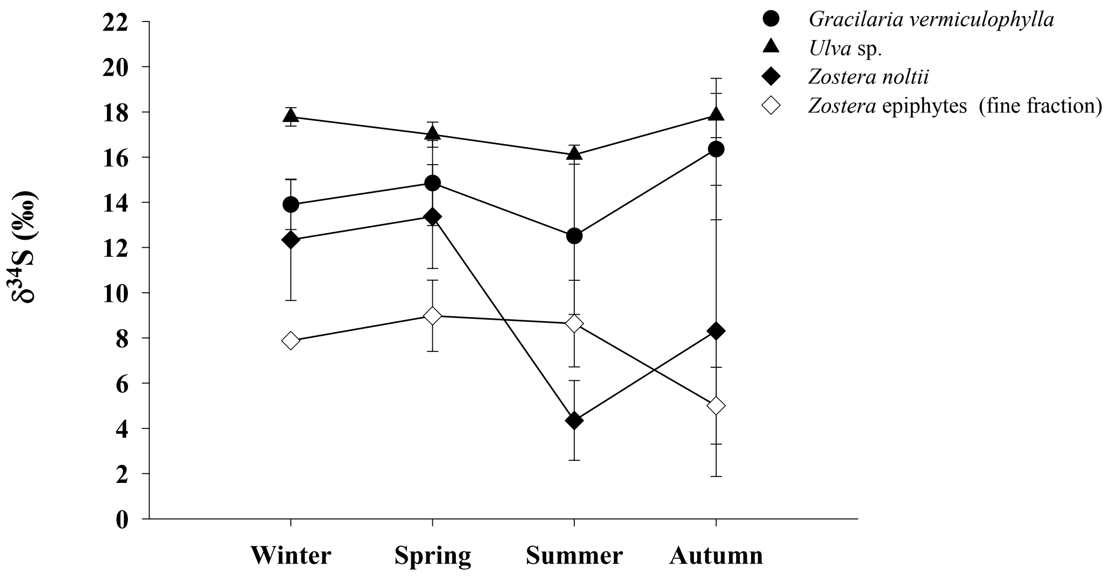

Fig 3. Seasonal variation of $\delta^{34}S$ values of food sources in a *Zostera noltii* meadow. Bars are standard deviations of means.

Figure 4 shows the δ-values of the consumers that were present in all seasons: the gastropods *Haminoea orbygniana*, *Cerithium vulgatum* and *Gibula umbilicalis*; the polychaets *Nereis* spp.; the amphipods; the decapods *Palaemon* spp. and the Ophiuroidea. The δ-values of organisms that were not found in the *Zostera noltii* meadow in all seasons are presented in Table 1. An unidentified actinarians, the gastropod *Nassarius pfeifferi* and the isopods were absent during summer. The decapod *Carcinus maenas* was only present in spring and summer.

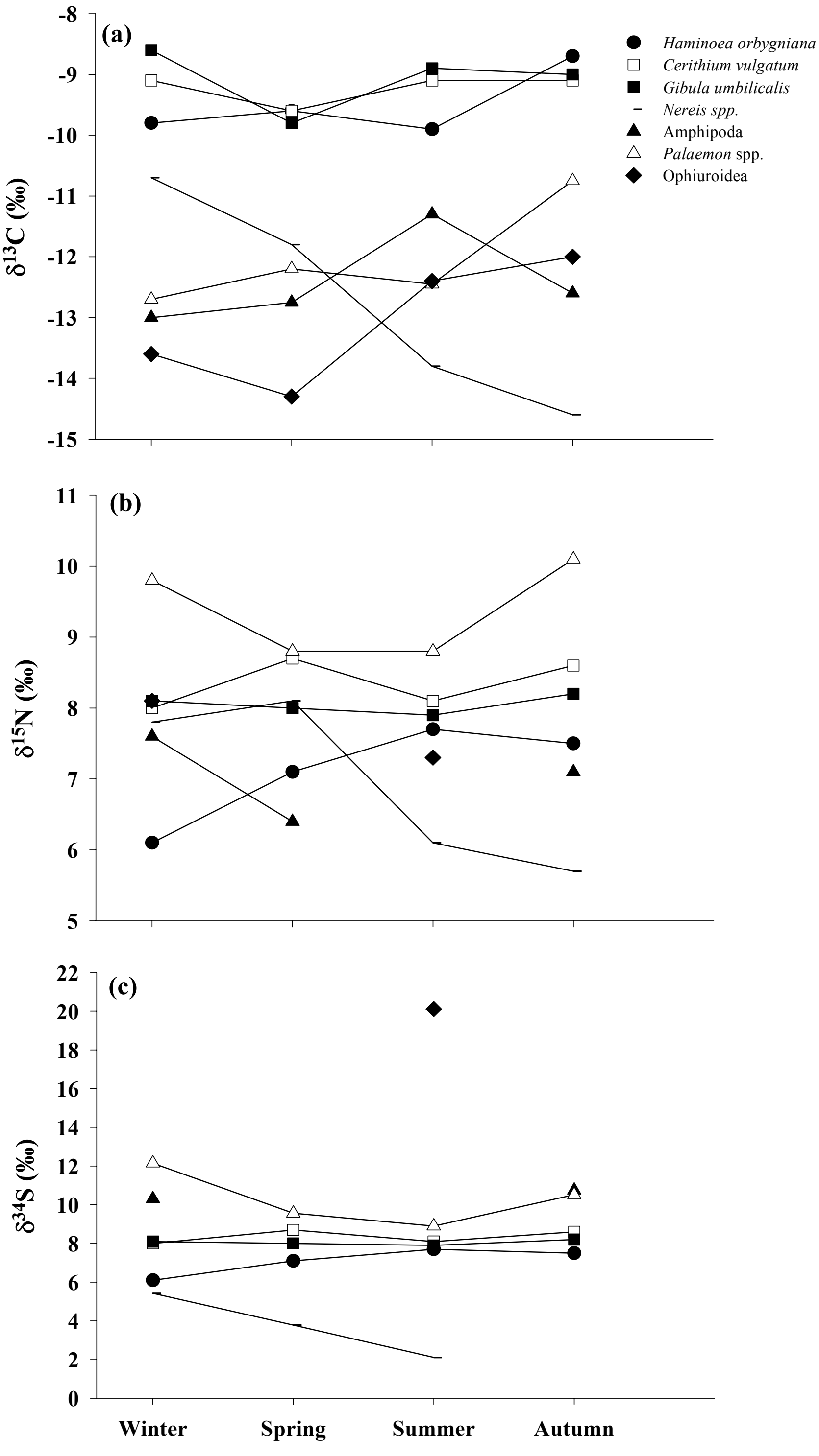

Fig. 4. Season of collection versus δ-values of the consumers that were present in the *Zostera noltii* meadow in all seasons. (a) season of collection versus $\delta^{13}C$ values, (b) season of collection versus $\delta^{15}N$ and (c) season of collection versus $\delta^{34}S$ values. Points missing in $\delta^{15}N$ and $\delta^{34}S$ values of some consumers indicate that the sample was not enough for this analysis.

Table 1. Identification and δ-values of consumer taxa that were not found in the *Zostera noltii* meadow in all seasons, with the exception of Pisces than only were sampled in summer.

Sample Taxa	Species Identification	Season	$\delta^{13}C$	$\delta^{15}N$	$\delta^{34}S$
Anthozoa	**Actinaria**	**Mean**	**-13.5 ± 0.7 (3)**	**+8.9 ± 2.3 (3)**	**+10.9 ± 1.3 (3)**
		winter	-13.0 (1)	+7.4 (1)	+11.5 (1)
		spring	-14.4 (1)	+7.8 (1)	+9.4 (1)
		summer	absent	absent	absent
Gastropoda		autumn	-14.0 (1)	+11.6 (1)	+11.7 (1)
	***Elysia* sp.**	**winter**	**-15.4 (1)**	**+10.1 (1)**	**+12.8 (1)**
	Nassarius pfeifferi	**Mean**	**-9.7 ± 0.1 (3)**	**+10.1 ± 0.2 (3)**	**+9.1 ± 0.2 (3)**
		winter	-9.6 (1)	+10.0 (1)	+8.9 (1)
		summer	absent	absent	absent
		spring	-9.8 (1)	+10.4 (1)	+9.0 (1)
		autumn	-9.6 (1)	+10.0 (1)	+9.4 (1)
Crustacea					
Isopoda		**Mean**	**-10.2 ± 0.5 (3)**	**+8.2 (1)**	**-**
	Idotea chelipes	winter	-9.7 (1)	+8.2 (1)	-
	N.I.	spring	-10.2 (1)	-	-
	-	summer	absent	absent	absent
	Idotea chelipes, Cyathura carinata	autumn	-10.8 (1)	-	-
Decapoda	***Carcinus maenas***	**Mean**	**-10.5 (2)**	**+10.7 ± 0.04 (2)**	**+6.4 ± 0.6 (2)**
		winter	absent	absent	absent
		spring	-10.5 (1)	+10.7 (1)	+6.9 (1)
		summer	-10.5 (1)	+10.7 (1)	+6.0 (1)
		autumn	absent	absent	absent
Pisces - Osteichthyes					
	Spaurus aurata	**summer**	-10.9 (1)	+11.1 (1)	+7.1 (1)
	Chelon labrosus	**summer**	-10.9 (1)	+9.5 (1)	+7.9 (1)

Table 2. Diet of abundant macrobenthic consumers associated to *Zostera noltii* meadows in Ria Formosa lagoon.

Species	Food Source	Data source	Reference
Anthozoa N.I	Suspension feeder (detritus, plankton), carnivore	Field observations	Sprung 1994
Gastropoda			
Haminoea orbygniana	Herbivourous, feeding mainly on diatoms	Gut contents and faecal pellets	Malaquias et al. 2004
Cerithium vulgatum	Microphytobenthos, detritus	Field observations	Sprung 1994
Gibula umbilicalis	Microphytobenthos, detritus	Field observations	Sprung 1994
	Small plants and plant detritus	-	Muzavor & Morenito 1999
Polychaeta			
Nereis diversicolor	Detritus, microphytobenthos, plankton, carnivorous, macrophytes	Field observations	Sprung 1994
Nereis caudata	Detritus, microphytobenthos, plankton, carnivorous, macrophytes	Field observations	Sprung 1994
Crustacea			
Amphipoda			
Melita palmata	Detritus, microphytobenthos, macroalgae, macrophytes	Field observations	Sprung 1994
Microdeutopus sp.	Detritus, microphytobenthos, macroalgae, macrophytes	Field observations	Sprung 1994
Isopoda			
Idotea chelipes	Macroalgae, macrophytes, detritus, microphytobenthos	Field observations	Sprung 1994
Cyathura carinata	Detritus, microphytobenthos	Field observations	Sprung 1994
Decapoda			
Carcinus maenas	Carnivorous	Field observations	Sprung 1994
Pisces - Osteichthyes			
Spaurus aurata	Mainly gastropods and bivalves	Gut contents	Pita et al. 2002
	Omnivore,diet based not only on animals but also on algae		Gamito 1994
	Molluscs, crustaceans and polychaetes	Gut contents	Andrade et al. 1996
	Gastropod fragments, somatic remaining portions N.I., scales, cral	Gut contents	This study
Chelon labrosus	Sediment, Foraminifera, Ostracoida, Insect larvae	Gut contents	This study
Lisa aurata	Sediment	Gut contents	This study

The fish species collected were *Sparus aurata* (Gilt-head sea bream), *Chelon labrosus* (Thicklip grey mullet) and *Lisa aurata* (Golden grey mullet). The results of the gut content identification are shown in Table 2 and correspond to the items found in three specimens of *S. aurata* and *C. labrosus*

Trophic position

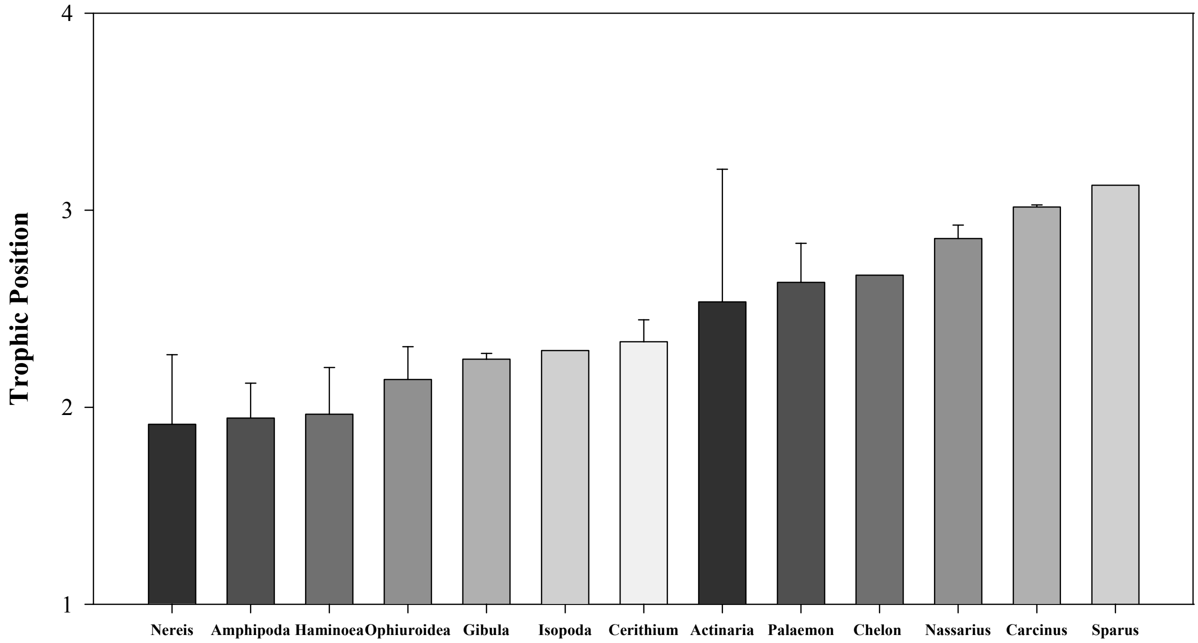

Figure 5. Trophic position of macroconsumers associated to *Zostera noltii*. Bars are standard deviation of an average of four samples corresponding to the four seasons.

The highest trophic position found in consumers associated with the *Z. noltii* community was occupied by the fish *Sparus aurata* (3.2), followed by the crab *Carcinus maenas* (3.0 ± 0.0.1). The gastropod *Nassarius pfeifferi* (2.9 ± 0.1) was distinct from the other gastropods (*Haminoea orbyniana*, 2.0 ± 0.2; *Gibula umbilicalis*, 2.2 ± 0.03, *Cerithium vulgatum*, 2.3 ± 0.1). *Nerei*s spp. occupied a lower trophic position than expected because this species may be carnivorous (Table 2) but can also feed on algae and detritus. The trophic position of actinarians specimens varied between 2.1 and 3.3. *Palaemon* spp. analysed in the spring and autumn showed lower trophic level (2.46) than on winter and autumn (2.8).

Relative contribution of primary producers to POM, SOM, detritus and consumers

The distinction between the red algae *Gracilaria vermiculophylla* and the green algae was possible because the $\delta^{13}C$ values of *G. vermiculophylla* were ^{13}C depleted. The $\delta^{15}N$ and $\delta^{34}S$ values of both groups of algae overlap almost at all the seasons (Fig. 6, 7, 8, 9). With the exception of the spring $\delta^{15}N$ values of the large fraction of *Zostera noltii* epiphytes (Fig. 7), green algae were isotopic distinct for the other primary producers due to the heavier $\delta^{15}N$ and $\delta^{34}S$ values. In the case of *Z. noltii*, excluding the winter δ-values (Fig. 6), the range of variation among the seagrass and the fine fraction of *Z. noltii* epiphytes (mainly diatoms) was low (Fig 7, 8, 9) suggesting difficulties in the assessment of their relative contribution to higher levels of the food web. With the exception of summer, the $\delta^{13}C$ values of the large fractions of *Z. noltii* epiphytes were more ^{13}C-depleted than *Z. noltii*. The species identification list of epiphytes on *Z. noltii* leaves (fine and large fraction together) is presented in Table I (Annex II).

Dual isotope plot of Fig. 6a indicate that the $\delta^{13}C$ and $\delta^{15}N$ signal of sestonic POM was not exclusively derived from primary producers in the *Zostera noltii* meadow. The $\delta^{13}C$ POM values were lower than all the primary producers. On the other hand, SOM and detritus were in the range of the primary producer (Fig. 6a). In all the seasons, the $\delta^{13}C$ values of SOM were lower than detritus and these were lower than *Z. noltii*.

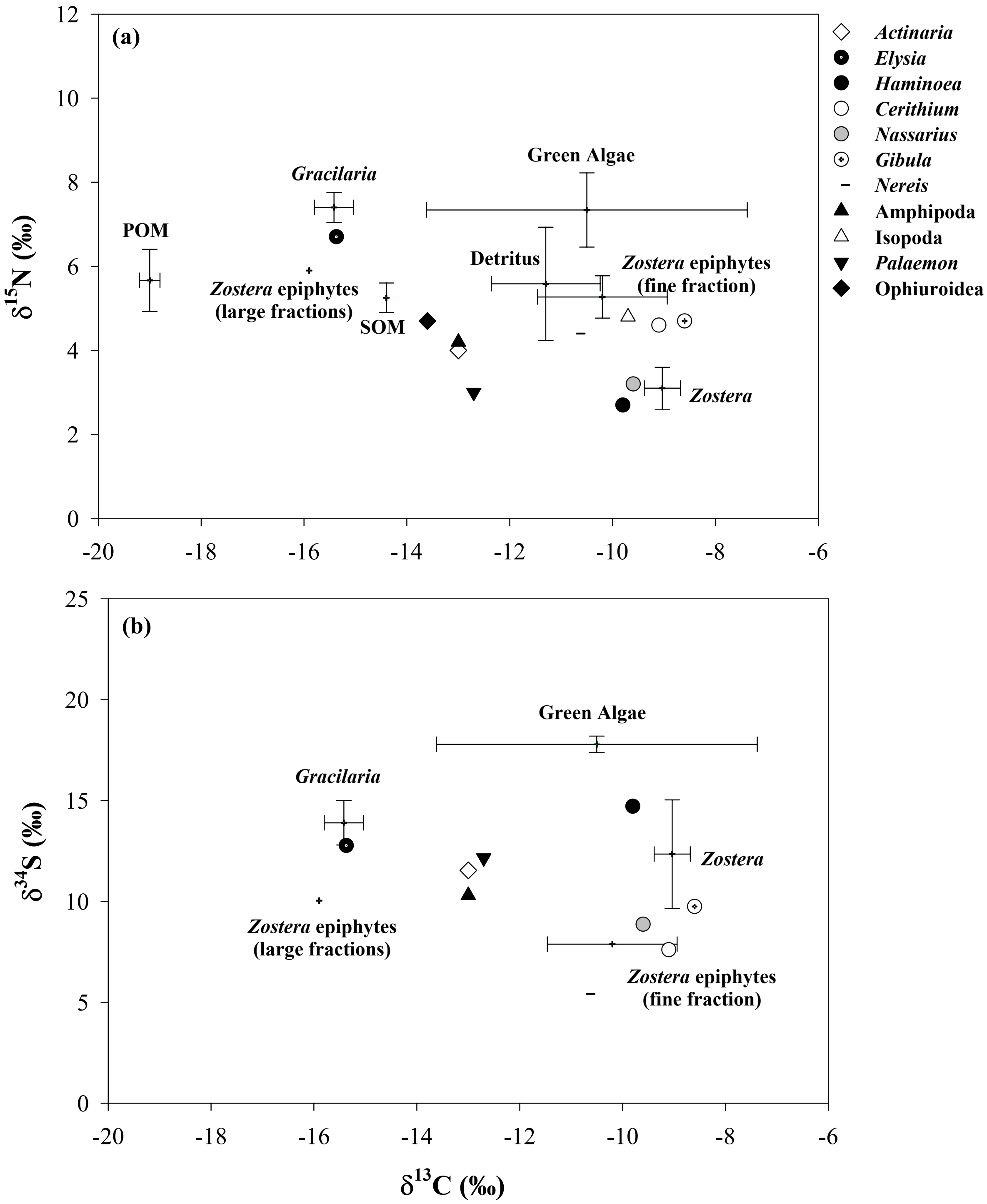

Fig. 6. *Zostera noltii* meadow multiple δ-values of food-sources vs. consumers colleted in winter. Bars are means ± standard deviation of three months of collection (3 samples) of the food sources. (a) $\delta^{13}C$ versus $\delta^{15}N$ values (b) $\delta^{13}C$ versus $\delta^{34}S$ values. $\delta^{15}N$ values with adjustment for trophic fractionation. 3.4‰ has been subtracted to the $\delta^{15}N$ values of trophic level 2, 6.8‰ has been subtracted from the $\delta^{15}N$ value of trophic level 3.

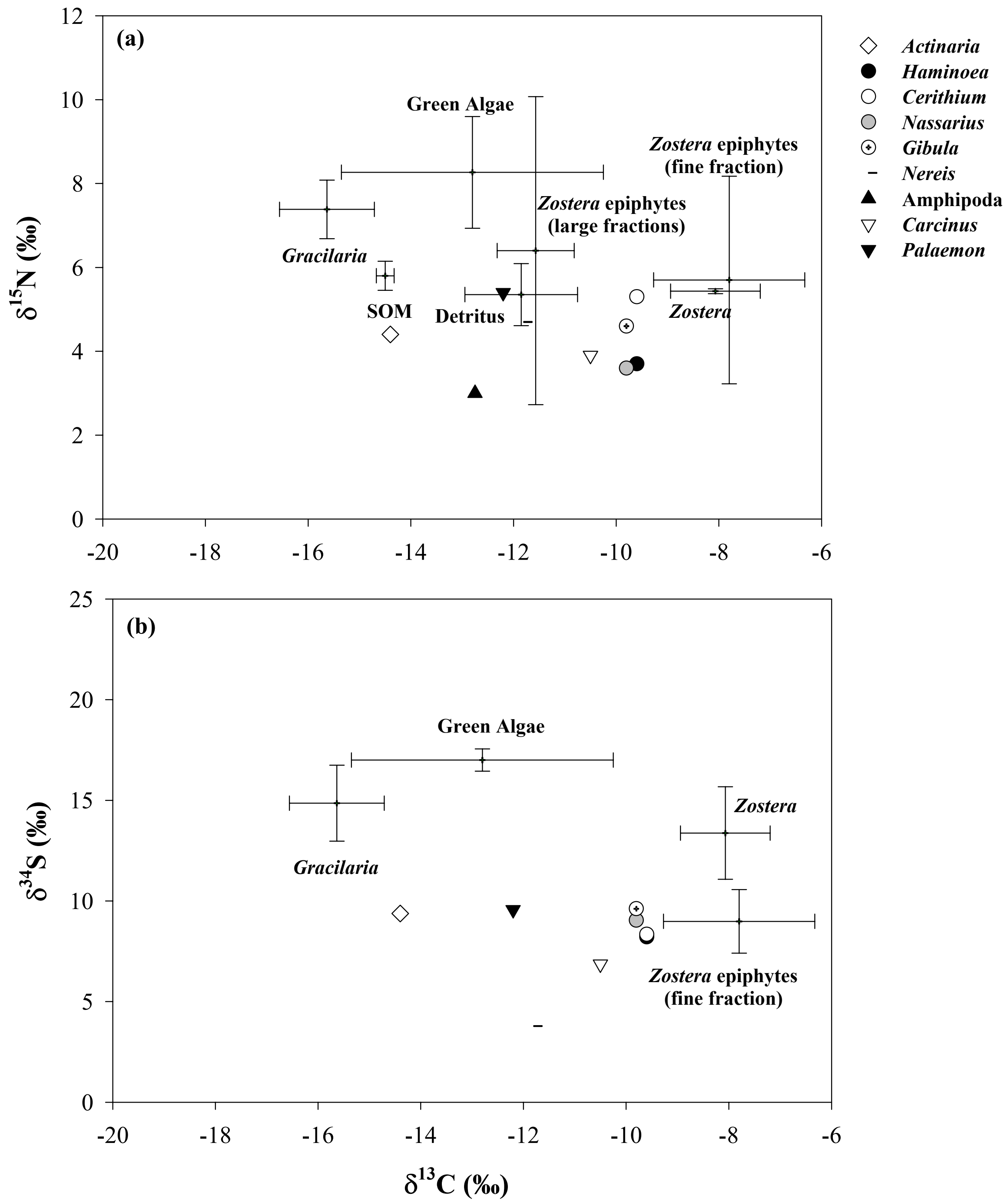

Fig. 7. *Zostera noltii* meadow multiple δ-values of food-sources vs. consumers colleted in spring. Bars are means ± standard deviation of three months of collection (3 samples) of the food sources. (a) $\delta^{13}C$ versus $\delta^{15}N$ values (b) $\delta^{13}C$ versus $\delta^{34}S$ values. $\delta^{15}N$ values with adjustment for trophic fractionation. 3.4‰ has been subtracted to the $\delta^{15}N$ values of trophic level 2, 6.8‰ has been subtracted from the $\delta^{15}N$ value of trophic level 3.

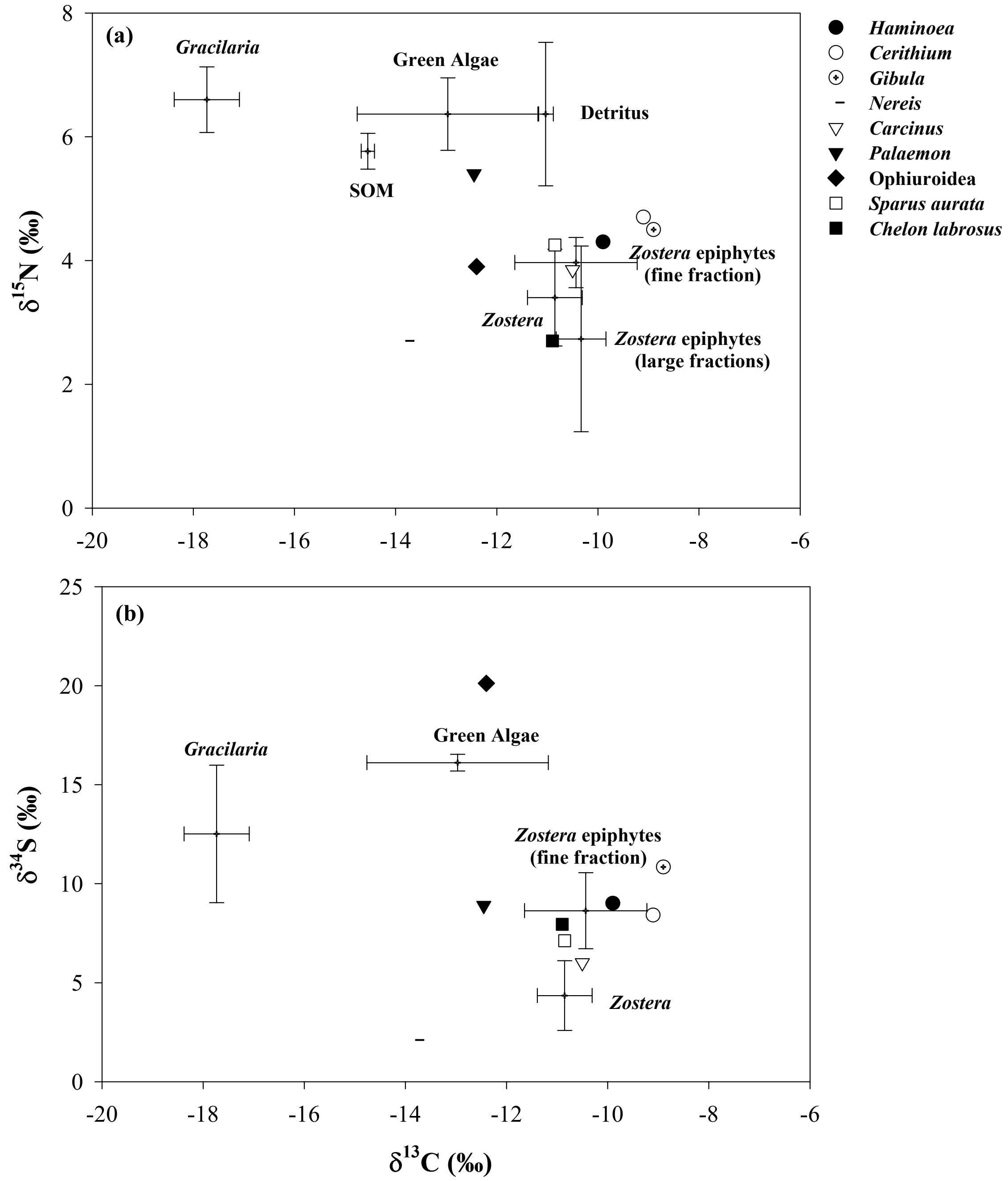

Fig. 8. *Zostera noltii* meadow multiple δ-values of food-sources vs. consumers colleted in summer. Bars are means ± standard deviation of three months of collection (3 samples) of the food sources. (a) $\delta^{13}C$ versus $\delta^{15}N$ values (b) $\delta^{13}C$ versus $\delta^{34}S$ values. $\delta^{15}N$ values with adjustment for trophic fractionation. 3.4‰ has been subtracted to the $\delta^{15}N$ values of trophic level 2, 6.8‰ has been subtracted from the $\delta^{15}N$ value of trophic level 3.

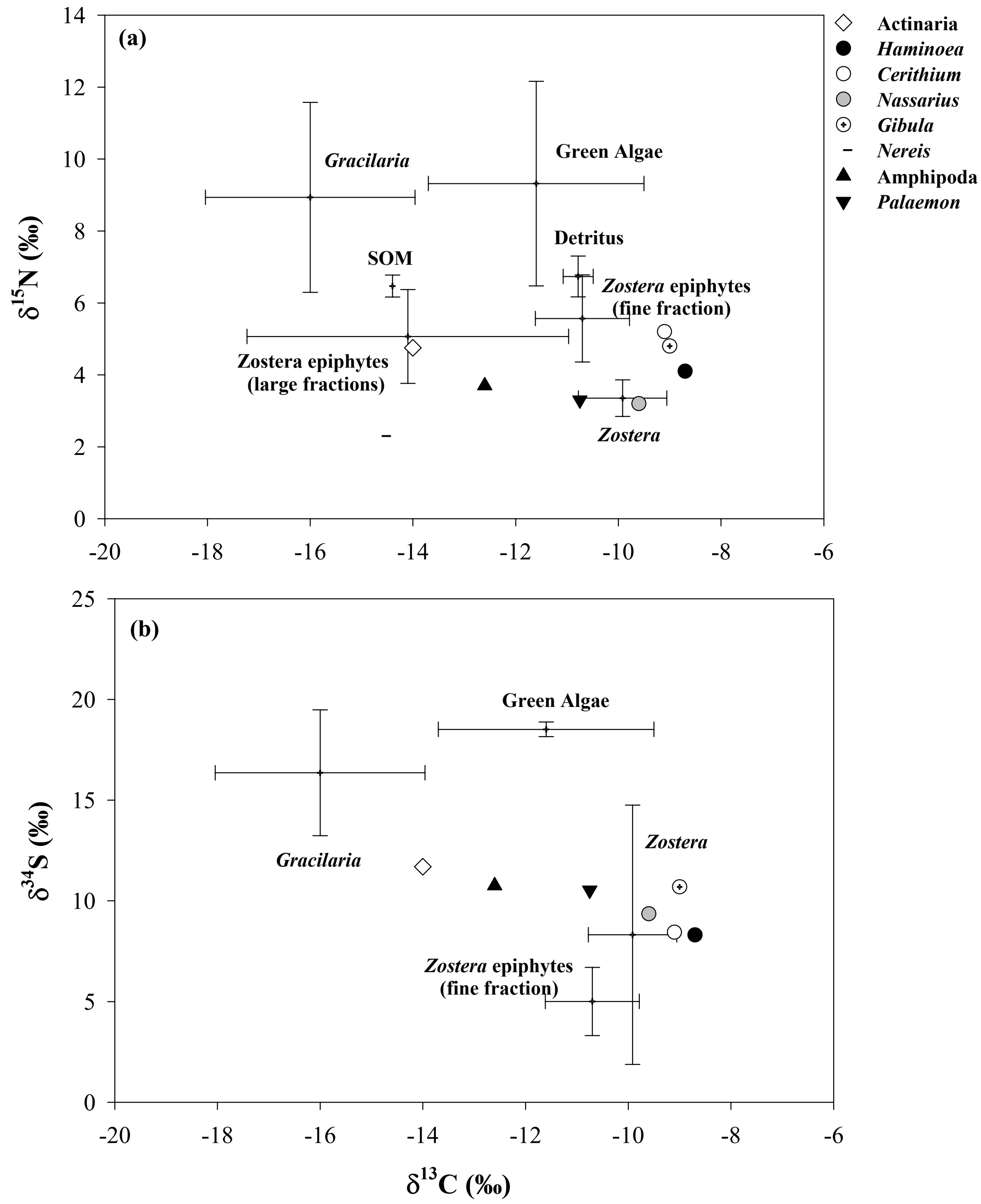

Fig. 9. *Zostera noltii* meadow multiple δ-values of food-sources vs. consumers colleted in autumn. Bars are means ± standard deviation of three months of collection (3 samples) of the food sources. (a) $\delta^{13}C$ versus $\delta^{15}N$ values (b) $\delta^{13}C$ versus $\delta^{34}S$ values. $\delta^{15}N$ values with adjustment for trophic fractionation. 3.4‰ has been subtracted to the $\delta^{15}N$ values of trophic level 2, 6.8‰ has been subtracted from the $\delta^{15}N$ value of trophic level 3.

The $\delta^{13}C$ value of the gastropod *Elysia* sp. was clearly distinct from all the other consumers, including the other gastropods. The $\delta^{13}C$, corrected $\delta^{15}N$, and $\delta^{34}S$ values were similar to those of *Gracilaria*, suggesting that it feeds on this red algae (Fig. 6). The others gastropods of trophic level 2 showed less distinct δ-values, being close to both *Zostera noltii* and fine fraction of *Z. noltii* epiphytes (Fig. 6, 7, 8, 9). However, in the winter the corrected $\delta^{15}N$ and $\delta^{34}S$ values of *Haminoea orbygniana* are nearer of those of *Z. noltii*, while the ones from *Cerithium vulgatum* are closest to the fine fraction of *Z. noltii* epiphytes (Fig. 6). In spring and summer the $\delta^{13}C$ and $\delta^{34}S$ values of *H. orbygniana*, *C. vulgatum* and *Gibula umbilicalis* are close to fine fraction of *Z. noltii* epiphytes (Fig. 7b and 8b) while in autumn were similar to those of *Z. noltii* (Fig. 9b). In the winter, the $\delta^{13}C$ and $\delta^{15}N$ values of isopods (the samples of this group were not enough to $\delta^{34}S$ determinations and $\delta^{15}N$ values in the other seasons) were close to fine fraction of *Z. noltii* epiphytes (Fig. 6a).

The seasonal variation of $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ values of *Nereis spp*. (Fig. 4) was high indicating seasonal differences of the relative contribution of food sources. In winter the δ-values are close to those of the fine fraction of *Zostera noltii* epiphytes and detritus (Fig. 6), in spring to those of the large fraction of *Z. noltii* epiphytes and detritus (Fig. 7), in summer and autumn are out of the range of food sources analysed in this study (Figs 8 and 9). The amphipods showed $\delta^{13}C$ and $\delta^{15}N$ values close to the large fraction of *Z. noltii* epiphytes, with the exception of winter δ-values, whereas only one replicate sample of the large fraction of *Z. noltii* epiphytes was analysed.

Ophiuroids (brittle star), only analyzed in the winter for $\delta^{13}C$ and $\delta^{15}N$ and in the summer for $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ values (Fig. 6 and 8), had intermediate $\delta^{15}N$ values between those of green algae and *Zostera noltii* but $\delta^{34}S$ value close to green algae (Fig. 8). The trophic position 2 is occupied by actinarians in winter and spring and in spring and summer by

Palaemon spp. Both had intermediate δ–values between the depleted and enriched primary producers. (Fig.6, 7 and 8).

With the exception of *Nassarius pfeifferi* that had $\delta^{13}C$ values similar to those other gastropods, the consumers of trophic level 3, *Carcinus maenas*, *Sparus aurata* and *Chelon labrosous* had intermediate $\delta^{13}C$ values between the ^{13}C depleted consumer and the rest of trophic level 2, suggesting a mixed diet (Fig. 8) in agreement with information on Table 2.

Discussion

Variability of δ- values of primary producers

The success of the stable isotope approach is based upon the ability to distinguish the food resources isotopically. Stable isotope values of seagrasses and macroalgae have been shown to vary seasonally, owing to seasonality in source δ-values, light intensity and temperature (Fry et al. 1987, Grice et al.1996). The $\delta^{13}C$ values of the primary producers analysed in this study ranged from -9.5‰ ± 1.2 in *Zostera noltii* to -16.2‰ ± 1.4 in *Gracilaria vermiculophylla*).

Seasonal variations of the $\delta^{15}N$ values of *Zostera noltii*, which is the only rooted primary producer in this study, are probably due to factors other than temporal changes in the $\delta^{15}N$ values of DIN in the water column, because only this species showed seasonal variations of $\delta^{15}N$ values. These values may indicate that biogeochemical processes, like denitrification, cause temporal changes in the isotopic composition of the dissolved inorganic (DIN) source in interstitial water.

The primary producers most enriched in ^{15}N and ^{34}S are the green macroalgae : +8.0‰ ± 2.1 and 17.4‰ ± 1.0 respectively and *G. vermiculophylla*: +7.6‰ ± 1.5 and 14.4‰ ± 2.6, respectively. The isotopic difference between seawater sulphate and sulphides makes sulphur useful in distinguishing pelagic vs. benthic producers (Peterson & Howarth 1987) with values close to +20‰ indicating use of sulphate from seawater and the lowest values indicating use of porewater sulphides.

Relative contribution of primary producers to POM, SOM and detritus

Dual isotope plot of Fig. 6 shows that the POM signal results from a mixture of macrophytes with a high contribution of ^{13}C depleted plants outside the seagrass meadow. The salt marsh species *Sarcocornia perenis* and the red seaweed *Bostrychia scorpiodes* may have a high POM influence because they are the most ^{13}C-depleted primary producers of Ria Formosa (-30.7‰ and -25.6‰ ± 1.2 respectively, Machás et al. 2003). The phytoplankton fraction of POM was very low (Chla:POM = 0.001 ± 0.0004, Machás unpublished data) in agreement with previous results for Ria Formosa (Machás et al. 2003).

The depleted $\delta^{13}C$ values of SOM and detritus relative to those of *Zostera noltii* could be explained by a shift in stable isotope values between living and detrital *Z. noltii*-derived material. However, unlike other vascular plants rich in lignin, which degrades gradually and causes a decrease of the $\delta^{13}C$ values during decay (Benner et al. 1987), the $\delta^{13}C$ values of *Z. noltii* do not decrease during decomposition (Machás et al. 2006). A large influence of $\delta^{13}C$ values of benthic microalgae in the sediment is also unlikely since the contribution of benthic microalgae to SOM was always very low (mean value of Chla:SOM = 0.001 ± 0.0004). Figures 6 to 9 shows that the SOM and detritus signal might result from a mixture of several primary producers. The $\delta^{13}C$ values of SOM were lower than those of detritus probably due to some influence of the POM signal (Fig. 6). The contribution of green algae to SOM and detritus might be higher in summer than in the remaining seasons possibly as a result of higher temperatures and degradation of green algae. Live filamentous green algae were only present in the *Z. noltii* meadow in autumn and winter.

Trophic relationships

Some species were not present on the *Zostera noltii* meadow during summer, when the crab *Carcinus maenas* is normally abundant (Sprung 1994), which could be a sign of the species mortality caused by this predator. Apparently, this may be true for isopods and actinarians although not for the gastropods *Nassarius pfeifferi*, since all the analysed specimens occupied a trophic position similar to *C. maenas* (Fig. 5). The comparison of trophic position with δ-values indicate that *Haminoea orbygniana*, *Cerithium vulgathum*, *Gibula umbilicalis*, *Nereis* spp. and amphipods are all potential prey to *C. maenas*. While some of these species may not be easily accessible to *C. maenas*, it is not very selective and can consume a variety of invertebrate species and all types of decaying material (Ropes 1969).

Our trophic position calculations for *Sparus aurata* (3.2) are in agreement with Gamito & Erzini (2005) by the means of a top-down modelling approach (3.1). The trophic position of the gastropods *Nassarius pfeifferi* (2.9 ± 0.1) indicate a carnivorous behaviour similar to what is observed in Ria Formosa (Afonso unpublished data). The trophic position of *Nereis* spp. (1.9‰ ± 0.4) indicate that this specie does not have a carnivorous behaviour like is normally mentioned (Sprung, 1994). These results are in conformity with the indication that *Nereis* spp. feeds predominantly on benthic diatoms by a ^{15}N enrichment study (Hughes personal communication). In this study the δ-values of benthic microalgae were not analysed, but in the summer and autumn the $\delta^{13}C$ (-13.8‰ and -14.6‰ respectively) and the corrected $\delta^{15}N$ values (+2.7‰ and +2.3‰ respectively) of *Nereis* spp. are similar to those of literature values for benthic microalgae ($\delta^{13}C$: -14.9‰ ± 3.1 and $\delta^{15}N$: +2.2‰ ± 2.1, Currin et al. 1995). The δ^{34} values of *Nereis* spp., are also in the range of literature values for benthic microalgae (+9.9‰ ± 6.8, Currin et al. 1995).

Matsura & Wada (1994) found similar $\delta^{13}C$ values for the brittle star (Ophiuroidea) and *Halodule wrigtii*, suggesting that they feed on seagrass. However, in Ria Formosa the $\delta^{13}C$ values of ophiuroids were more depleted in ^{13}C than those of *Zostera noltii* indicating that this seagrass can not be the main food resource. The relation between δ-values of ophiuroids and the measured food sources can not explain the diet of this organism (Fig. 6 and 8).

Laboratory experiment with amphipods indicates that these organisms had $\delta^{13}C$ values similar to those of their detrital seagrass fragment (Fry et al. 1987). However, our $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ values indicate that at the *Zostera noltii* meadows they may preferentially feed on the large fraction of *Z. noltii* epiphytes.

According to Fry & Parker (1979) and Vizzini et al. (2002), seagrasses and others benthic plants contributed significantly to the diets of shrimp. In this study, the δ-values and trophic position of the shrimps, *Palaemon* spp. and *Hippolyte longirostris* indicate that, in addition to a mixture of benthic primary producer, these species can be carnivorous.

Consumption of *Zostera marina* by *Idotea chelipes* was reported by Nienhuis & Groenendijk (1986) using a field-based bioenergetic study and laboratory experiments. However, our $\delta^{13}C$ and $\delta^{15}N$ values (Fig. 6) suggest that isopods (mostly *I. chelipes*) feed mainly on diatom epiphytes of *Z. noltii* leaves in contrast to the field observations of Sprung (1994).

The species of diatoms on *Zostera noltii* leaves, identified in this study (Table I from Annex II), were in agreement with the species observed by Malaquias et al. (2004) in

Haminoea orbyniana gut and faecal pellet contents. The above authors suggested that *H. orbygniana* feeds mainly in diatoms. In this study, the range of variation of δ-values among *Z. noltii* and the fine fraction of *Z. noltii* epiphytes (mainly diatoms), in spring, summer and autumn seasons was low (Figs. 7 to 9), suggesting difficulties in the assessment of their relative contribution to this species diet. On the other hand, in the winter, when the amount of epiphytes on *Z. noltii* leaves is low (0.01 % g DW ± 0.004, Machás unpublished data), the δ-values indicate that *H. orbygniana* assimilate predominantly *Z. noltii* leaves with a lower contribution of epiphytic diatom (Fig. 6). The winter δ-values are in conformity with Garcia et al. (1991) that point out that *Haminoea* spp. are able to feed on seagrass leaves. Future studies should focus in ^{15}N enrichment to, differentially, label the two potential food sources and quantify the relative contribution of each source (see for instance Winning et al., 1999).

Our study can not explain the observation of *Z. noltii* leaves with bite marks from herbivores. This phenomenon is not common and the fish *Sarpa salpa* and the waterfowl *Anas penelope* are potential herbivorous since that is unlikely that any of the Ria Formosa species in our samples have made the bite marks. Regrettably we did not capture *S. salpa* but other studies related to $\delta^{13}C$ values of this species with the seagrass and epiphyte material signals (Havelange et al. 1997, Lepoint et al. 2000). Tomas et al. (2005), based on measurements of bite marks, estimated that, as much as 70% of the production of seagrasses (*Posidonia oceanica*) in some areas of Mediterranean Sea, was consumed by *S. salpa*. Through field-based bionergethic study and field experiment, Jacobs et al. (1981) reported that *Z. noltii* is consumed by *A. penelope* indicating that this waterfowl feeds on this seagrass species.

Acknowledgements: We are grateful to all who contributed to this work. A special thanks to A. Malaquias, K. Erzini and S. Cabaço for the precious help in field work, and E. Berecibar,

M. Cristo, M. Machado, J. Ribeiro for specific groups species identification. This study is part of the PhD thesis of R. Machás funded by the Fundação para a Ciência e Tecnologia (scholarships Praxis XXI/BD/19579/99).

References

Andrade C.F. 1990. O ambiente de barreira da Ria Formosa Algarve-Portugal. PhD thesis, University of Lisboa, Lisboa.

Andrade P.J., Erzine K., Palma J. 1996. Gastric evacuation and feeding in the gilthead sea bream reared under semi-intensive conditions Aquaculture International 4: 129-141.

Benner R., Fogel M.L., Sprague E.K., Hodson R.E. 1987. Depletion of ^{13}C in lignin and implications for stable carbon isotope studies. Nature 329: 708-710.

Cabana G., Rasmussen J.B. 1996. Comparison of aquatic food chains using nitrogen isotopes. Proceedings of the National Academy of Sciences USA 93: 10844-10847.

Cebrián J., Duarte C.M., Marbà N., Enríques S. 1997. Magnitude and fate of the production of four cooccurring Western Mediterranean seagrass species. Marine Ecology Progress Series 155: 29-44.

Currin C.A., Newell S.Y., Paerl H.W. 1995. The role of standing dead *Spartina alterniflora* and benthic microalgae in salt marsh food webs: considerations based on multiple stable isotope analysis. Marine Ecology Progress Series 21: 99-116.

Den Hartog C. 1970. The Seagrasses of the World. Amsterdam, North Holland.

Fry B., Parker P.L. 1979. Animal diet Animal diet in Texas seagrass meadows: $\delta^{13}C$ evidence for the importance of benthic plants Estuarine and Coastal Marine Science 8: 499-509.

Fry B., Sherr E.F. 1984. $\delta^{13}C$ Measurements as indicators of carbon flow in marine and freshwater ecosystems. Contributions in Marine Science 27: 13-47.

Fry B., Macko S.A., Zieman, J.C. 1987. Review of stable isotopic investigations of food webs in seagrass meadows. In: Durako M.J., Phillips R.C, Lewis, R.R. (eds). Proceedings of a Symposium on Subtropical-Tropical Seagrasses, Southeast United States. Florida Marine Research Publications 42: 189-209.

Gamito S. 1994. The benthic ecology of some Ria Formosa lagoons, with reference to the potential for production of the gilthead seabream (*Sparus aurata* L.). PhD thesis, University of Algarve, Faro.

Gamito S., Erzini K. 2005. Trophic food web and ecosystem attributes of a water reservoir of the Ria Formosa. Ecological Modelling18: 1509-520.

García G.D., Perez-Hurtado A., García-Gómez J.C. 1991. *Haminoea lempladoi*, anew species of cephalaspidean opisthobranchfrom the AtlanthicIberiancoast. Journal of Molluscan Studies57: 395-399.

Grice A.M., Loneragan N.R., Dennison W.C. 1996. Light intensity and the interactions between physiology, morphology and stable isotope ratios in five species of seagrass. Journal of Experimental Marine Biology and Ecology 195: 91-110.

Havelange S., Lepoint G., Dauby P., Bouquegneau J.M. 1997. Feeding of the sparid fish Sarpa salpa in a seagrass ecosystem:dietand carbon flux. Marine Ecology 18: 289-297.

Heminga M.A., Duarte C.M., 2000. Seagrass Ecology. Cambridge University Press, United Kingdom.

Jacobs R.P.W., den Hartog M.C., Braster B.F., Carriere F.C. 1981. Grazing of the seagrass *Zostera noltii* by birds at Terschelling (Dutch Wadden Sea). Aquatic Botany 10:241-259.

Jones W.B. Cifuentes L.A., Kaldy J.E.2003. Stable carbon isotope evidence for coupling between sedimentary bacteria and seagrasses in a sub-tropical lagoon. Marine Ecology Progress Series 255: 15-25.

Lepoint G., Nyssen F., Gobert S., Dauby P., Bouquegneau J.M. 2000. Relative impact of a seagrass bed and its adjacent epilithic algal community in consumer diets. Marine Biology 136: 513-518.

Loneragan N.R., Bunn S.E., Kellaway D.M. 1997. Are mangroves and seagrasses sources of organic carbon for penaeid prawns in a tropical Australian estuary? A multiple stable-isotope study. Marine Biology 130: 289-300.

Lorenzen C.J. 1967. Determination of chlrophyll and pheo-pigments: spectrophotometric equations. Limnology and Oceanography 12: 343-346.

Machás R., Santos R. 1999. Sources of organic matter in Ria Formosa revealed by stable isotope analysis. Acta Oecologica 20: 463-469.

Machás R., Santos R., Peterson B. 2003. Tracing the flow of organic matter from primary producers to filter feeders in Ria Formosa lagoon, southern Portugal. Estuaries 26: 846-856.

Machás R. Santos R. Peterson B. 2006. Elemental and stable isotope composition of *Zostera noltii* (Horneman) leaves during the early phases of decay in a temperate mesotidal lagoon. Estuarine Costal and Shelf Science 66: 21-29.

Malaquias M.A.E., Condinho S., Cervera J.L., Sprung M. 2004. Diet and feeding biology of *Haminoea orbygniana* (Mollusca: Gastropoda: Cephalaspidea). Journal of Marine Biological Association of the United Kingdom 84:4522/1-6.

MatsuuraY., Wada E. 1994. Carbon and nitrogen stable isotope ratios in marine organic matters of the coastal ecosystem in Ubatuba, southern Brazil. Ciência e Cultura (Journal of the Brazilian Association for the Advancement of Science) 46: 141-146.

McConnaughey T., McRoy C.P. 1979. ^{13}C label identifies eelgrass (*Zostera marina*) carbon in an Alaskan estuarine food web. Marine Biology 53: 263-269.

Michener R.H, Schell D.M. 1994. Stable isotope ratios as traces in marine aquactic food webs. In: Lajtha K., Michener R.H. (eds.). Stable Isotopes in Ecology and Environmental Science. Blackwell Scientific Publications, Oxford.

Milchakova N.A, Phillips R.C. 2003. Black sea seagrasses. Marine Pollution Bulletin 46: 695-699.

Minagawa M., Wada E. 1984. Stepwise enrichment of ^{15}N along food chains: further evidence and the relation between $\delta^{15}N$ and animal age. Geochimica et Cosmochimica Acta 48: 1135-1140.

Moncreiff C.A., Sullivan M.J. 2001. Trophic importance of epiphytic algae in subtropical seagrass beds: evidence from multiple stable isotope analyses. Marine Ecology Progress Séries 215: 93-106.

Muzavor S., Morenito P.M. 1999. Roteiro ecológico da Ria Formosa. Vol. IV – Moluscos Gastrópodos. Universidade do Algarve, Faro.

Nichols P.D., Klumpp D.W., Johns R.B. 1985. A study of food chains in seagrass communities III. Stable carbon isotope ratios. Australian Journal of Marine and Freshwater Research 36: 683-690.

Nienhaus P.H., Groenendijk A.M. 1986. Consumption of eelgrass (*Zostera marina*) by birds and invertebrates: an annual budget. Marine Ecology Progress Series 29: 29-35.

Paine R.T. 1988. Food webs. Road maps of interactions or grist for theoretical development? Ecology 69: 38: 159-166.

Peterson B.J. 1999. Stable isotopes as tracers of organic matter input and transfer in benthic food webs. Acta Oecologica 20: 479-487.

Peterson B.J., Fry B. 1987. Stable isotopes in ecosystem studies. Annual Review of Ecology and Systematics 18: 293-320.

Peterson B.J., Howarth R.W. 1987. Sulfur, carbon, and nitrogen isotopes used to trace organic matter flow in the Salt-Marsh Estuaries of Sapelo Island, Georgia. Limnology and Oceanography 32: 1195-1213.

Peterson B.J., Howarth R.W., Garritt R.H. 1985. Multiple stable isotopes used to trace the flow of organic matter flow in estuarine food webs. Science 227: 1361-1363.

Peterson B.J., Howarth R.W., Garritt R.H. 1986. Sulfur and carbon isotopes as tracers of salt marsh organic matter flow. Ecology 67: 865-874.

Pita C. Gamito S. Erzini K. 2002. Feeding habits of the gilthead seabream (*Sparus aurata*) from the Ria Formosa (southern Portugal) as compared to the black seabream (*Spondyliosoma cantharus*) and the annular seabream (*Diplodus annularis*). Journal of Applied Ichthyology 18: 81-86.

Post D.M. 2002. Using stable isotopes to estimate trophic position: models, methods, and assumptions: Ecology 83: 703-718.

Ropes J.W. 1969. The feeding habitats of the green crab, *Carcinus maenas* (L.). Fishery Bulletin, US Fish and Wildlife service 67: 183-203.

Sprung M. 1994. Macrobenthic secondary production in the intertidal zone of the Ria Formosa - a lagoon in southern Portugal. Estuarine, Coastal and Shelf Science 38: 539-558.

Stoner A.W., Waite J.M. 1991. Trophic biology of *Strombus Gigas* in nursery habitats: diets and food sources in seagrass meadows. Journal of Molluscan Studies 57: 451-460.

Thayer G.W., Parker P.L., LaCroix M.W., Fry B. 1978. The stable carbon isotope ratio of some components of an eelgrass, *Zostera marina* bed. Oecologia 35: 1-12.

Thayer G., Bjorndal K., Ogden J., Williams S., Zieman J. 1984. Role of larger herbivores in seagrass communities. Estuaries 7: 351-376.

Tomas F., Turon X., Romero J. 2005. Seasonal and small-scale variability of hervivorypressureon the temperate seagrass *Posidonia oceanica*. Marine Ecology Progress Series 201: 95-107.

Valiela I., 1995. Marine ecological processes, 2nd Ed., Springer-Verlag, New York.

Vizzini S., Sarà G., Michener R.H., Mazzola A. 2002. The role and contribution of the seagrass Posidonia oceanica (L.) Delile organic matter for secondary consumers as revealed by carbon and nitrogen stable analysis. Acta Oecologica 23: 277-285.

Winning M.A., Connolly R.M., Loneragan N.R., Bunn S.E. 1999. ^{15}N enrichment as a method of separating signatures of seagrass and its epiphytes for food web analysis. Marine Ecology Progress Series189: 289-294.

Zieman J.C. 1983. Coral reefs, seagrass beds and mangroves: their interaction in the coastal zones of the Caribbean. Unesco reports in marine science 23: 80-86.

Chapter 6 – General conclusions

The main conclusions of this study are:

The $\delta^{15}N$ values of ecosystem components can provide valuable information on qualitative and quantitative changes in the nitrogen status of aquatic systems. The larger the difference in isotopic composition among contributing sources, the easier it is to interpret isotopic results. A careful characterization of the isotopic signatures in different compartments of the aquatic system, including the potential seasonal variation in the $\delta^{15}N$ values of nutrients and biota, is required to achieve the most secure understanding.

The large seasonal differences observed in the Waste Water Treatment Works (WWTW) effluent reflected the higher summer microalgae productivity, which consumed the available CO_2 in the water shifting the inorganic carbon equilibrium and increasing the pH of the urban effluent. Ammonium and phosphate concentrations during summer were about three times less than in winter due to higher uptake while nitrate+nitrite concentrations were higher during summer probably due to higher rates of nitrification. The seasonal variation of the WWTW internal metabolism was reflected in the $\delta^{13}C_{DIC}$, and $\delta^{15}N_{NH4}$ of its effluent, which were higher in summer than in winter.

In both seasons, the $\delta^{13}C_{POM}$ produced in the WWTW was depleted in relation to values found in the Ria Formosa tidal channels and thus the $\delta^{13}C_{POM}$ values increased with distance from the WWTW. The depleted signal of the WWTW $\delta^{13}C_{POM}$ reflected the signal of the freshwater phytoplankton uptake of $\delta^{13}C_{DIC}$. The WWTW values of $\delta^{13}C_{POM}$ were always higher than the Ria Formosa values for POM that probably reflect a mixture of lagoon macrophytes with a low contribution of phytoplankton. The magnitude of the seasonal change in WWTW $\delta^{15}N_{NH4}$ was large enough to reverse the gradient of $\delta^{15}N_{POM}$ from the outfall to the tidal channels of the lagoon. In summer, the $\delta^{15}N_{POM}$ values decreased from the WWTW to the main channel whereas in winter the $\delta^{15}N_{POM}$ values showed the opposite pattern.

The WWTW $\delta^{13}C_{DIC}$ signal was most evident in $\delta^{13}C_{POM}$ isotopic signature, rather than in the sediment organic matter ($\delta^{13}C_{SOM}$) or in *Zostera noltii* ($\delta^{13}C_{Z.\ nolti}$). The C contents of sediment and $\delta^{13}C_{SOM}$ values reflected the important contribution of both the high C content and the ^{13}C depleted phytoplankton to SOM, particularly in station 2 (522m distant to the WWTW) due to the die-off and sedimentation of the fresh water plankton produced within the WWTW. No clear gradients were observed in the δ-values of *Z. noltii* because this species are without water column influence during low tide, when the influence of the WWTW nutrients is clearer. The overall results show that the measurable effects of the effluent discharge on the Ria Formosa extended 500 to 600 meters from the outfall. Filter feeding clams are a major commercial resource in Ria Formosa lagoon and the urban effluent is likely to impact clam cultivation areas that are as close as 300 m from the WWTW.

Contrary to other vascular plants rich in ^{13}C-depleted lignin, which degrades slowly, and thus causes a decrease in the $\delta^{13}C$ values of detritus during decay, the $\delta^{13}C$ values of *Zostera noltii* leaves did not vary significantly over time or between incubation sites. The $\delta^{15}N$ values of leaves did not increase as has been reported for vascular plants with high C: N ratio. This suggests that during the early phase of the *Z. noltii* decomposition the community of decomposers relied mostly on the endogenous nitrogen of the plants, not needing to immobilize exogenous N or assimilated exogenous ^{15}N of similar isotopic composition to the leaves.

Stable isotope studies to assess the contribution of *Zostera noltii* to secondary production at these sites can thus consider the natural $\delta^{13}C$ and $\delta^{15}N$ abundances of living tissues without any correction to account for decomposition effects. On the other hand, the use of $\delta^{34}S$ values of *Z. noltii* detritus should be done with caution. These values may be affected by the contamination of the samples with pyrite from sediments, which will decrease the $\delta^{34}S$ signal of tissues.

The $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ values of primary producer, organic matter, detritus and consumers provided unique insights into trophic relationship of *Zostera noltii* meadows. It appears from this approach that animals, excluding the gastropod *Elysia* sp., are dependent to varying degrees upon seagrass and/or seagrass epiphytes and green algae for their C, N and S sources. *Elysia* sp. showed distinct isotope values from other consumers, suggesting that they feed on the red algae *Gracilaria vermiculophylla*. The range of variation between *Z. noltii* and epiphytic diatoms was low suggesting difficulties in the assessment of their relative contribution to higher levels of the food web. However, in the winter the δ-values indicate that the gastropod *Haminoea orbyniana* assimilate predominantly cell of *Z. noltii* leaves while the gastropod *Cerithium vulgatum* depends on epiphytic diatoms. Future studies should consider using experimental addition of ^{15}N- enriched inorganic N to differentially label epiphytes and *Z. noltii* leaves in order to quantify the relative contribution of each source.

Annex I

Table I. Sites selected, percentage of land derived N load that is contributed by wastewater, $\delta^{15}N$ values of nutrients in the water column, sediment, particulate organic matter (POM), macrophytes and consumers, macrophytes and consumers identification and nitrate: ammonium concentration ratio in the water column nutrient. The $\delta^{15}N_{DIN}$ values were calculated by the concentration weighted of $\delta^{15}N_{NO3}$ and $\delta^{15}N_{NH4,}$ using the NO_3: NH_4 ratio. B- Bivalvia, C – Crustacean, F- Fish, H – Holothurian, P – Polychaete.

		Water column						Macrophyte			Consumer			
Site	Wastewater as a percentage of total N load	$\delta^{15}N_{NO3}$	$\delta^{15}N_{NH4}$ (‰)	$\delta^{15}N_{DIN}$	NO_3: NH_4 (µM: µM)	Sediment $\delta^{15}N$ (‰)	POM $\delta^{15}N$ (‰)	Group	Specie	$\delta^{15}N$ (‰)	Nutrition type	Specie	$\delta^{15}N$ (‰)	Source
Padilla Bay (WA), USA	-	4.2	0.0	2.6	62: 31	9.3	6.7	green macroalgae	-	7.9	suspension feeders	barnacles and some mussels	9.1	Fry et al. (2003)
		3.0	10.8	6.6	48: 48	-	4.6	green macroalgae	-	7.2	suspension feeders	barnacles and some mussels	9.4	Fry et al. (2003)
		4.5	-	-	46: 46	9.8	8.0	green macroalgae	-	9.7	suspension feeders	barnacles and some mussels	11.2	Fry et al. (2003)
		4.6	-	-	47: 53	8.8	5.8	green macroalgae	-	9.0	suspension feeders	barnacles and some mussels	13.6	Fry et al. (2003)
		5.2	11.7	9.7	30: 70	3.8	3.6	green macroalgae	-	2.3	-	-	-	Fry et al. (2003)
		6.2	11.4	9.2	38: 60	6.0	2.7	green macroalgae	-	7.6	suspension feeders	barnacles and some mussels	10.3	Fry et al. (2003)
		5.6	10.2	8.0	43: 55	9.8	6.3	green macroalgae	-	8.9	suspension feeders	barnacles and some mussels	12.6	Fry et al. (2003)
		8.5	9.0	8.4	68: 29	6.3	3.9	green macroalgae	-	10.2	-	-	-	Fry et al. (2003)
		5.9	-	-	43:52	9.6	5.0	green macroalgae	-	10.0	suspension feeders	barnacles and some mussels	12.2	Fry et al. (2003)
		4.2	9.0	6.8	46: 54	6.6	4.9	green macroalgae	-	7.8	suspension feeders	barnacles and some mussels	10.7	Fry et al. (2003)
		4.7	-	-	69: 31	7.7	4.9	green macroalgae	-	9.8	suspension feeders	barnacles and some mussels	10.4	Fry et al. (2003)
		5.0	-	-	64: 36	8.8	6.8	green macroalgae	-	7.5	suspension feeders	barnacles and some mussels	10.5	Fry et al. (2003)
South Slough (OR), USA	-	3.8	-	-	59:41	3.4	6.7	green macroalgae	-	8.0	suspension feeders	barnacles and some mussels	10.2	Fry et al. (2003)
		3.9	10.2	6.9	46: 50	4.3	5.4	green macroalgae	-	7.3	suspension feeders	barnacles and some mussels	8.5	Fry et al. (2003)
		1.7	-	-	36:62	3.7	0.6	green macroalgae	-	7.6	suspension feeders	barnacles and some mussels	10.0	Fry et al. (2003)
		1.6	-	-	79: 21	5.4	4.3	green macroalgae	-	6.9	suspension feeders	barnacles and some mussels	10.0	Fry et al. (2003)
		2.6	2.9	2.6	72: 25	6.8	4.3	green macroalgae	-	9.5	suspension feeders	barnacles and some mussels	9.7	Fry et al. (2003)
		1.3	-	-	26: 74	6.7	5.2	green macroalgae	-	9.5	suspension feeders	barnacles and some mussels	10.5	Fry et al. (2003)
		0.9	-	-	24: 73	6.0	5.5	green macroalgae	-	9.1	suspension feeders	barnacles and some mussels	9.7	Fry et al. (2003)
		0.3	-	-	73: 27	5.2	3.9	green macroalgae	-	10.2	suspension feeders	barnacles and some mussels	11.2	Fry et al. (2003)
		-	-	-	40: 60	3.8	2.4	-	-	-	-	-	-	Fry et al. (2003)
		3.0	-	-	28: 67	7.2	3.2	green macroalgae	-	9.1	-	-	-	Fry et al. (2003)
		-0.3	-	-	50: 50	6.0	5.0	green macroalgae	-	7.6	-	-	-	Fry et al. (2003)
		-0.1	-	-	30: 60	4.4	4.2	green macroalgae	-	1.8	-	-	-	Fry et al. (2003)
Elkhorn Slough (CA), USA	-	5.0	6.7	5.2	86: 14	8.4	2.1	green macroalgae	-	9.3	suspension feeders	barnacles and some mussels	10.9	Fry et al. (2003)
		6.3	-	-	84: 13	9.0	5.8	green macroalgae	-	9.3	suspension feeders	barnacles and some mussels	11.4	Fry et al. (2003)
		12.7	17.8	12.6	96: 2	9.3	9.7	green macroalgae	-	16.0	suspension feeders	barnacles and some mussels	14.7	Fry et al. (2003)
		12.5	15.1	12.4	97: 2	10.0	9.0	green macroalgae	-	13.8	suspension feeders	barnacles and some mussels	13.6	Fry et al. (2003)
		7.5	5.1	5.0	3: 93	9.1	9.7	green macroalgae	-	14.0	-	-	-	Fry et al. (2003)
		5.1	-	-	56:40	8.9	6.5	green macroalgae	-	11.6	suspension feeders	barnacles and some mussels	12.4	Fry et al. (2003)
		-0.2	-	-	62: 34	8.7	6.6	green macroalgae	-	12.7	suspension feeders	barnacles and some mussels	13.5	Fry et al. (2003)
		4.3	-	-	67: 29	9.8	8.7	green macroalgae	-	12.0	suspension feeders	barnacles and some mussels	13.3	Fry et al. (2003)
		10.9	17.2	11.0	87: 9	11.2	11.5	green macroalgae	-	15.6	suspension feeders	barnacles and some mussels	15.3	Fry et al. (2003)
		11.8	14.9	12.5	41: 51	7.4	9.2	green macroalgae	-	14.2	-	-	-	Fry et al. (2003)
		9.1	14.4	12.1	32:64	7.9	6.5	green macroalgae	-	10.3	-	-	-	Fry et al. (2003)

Table I. (Continuation).

Tijuana Estuary (CA), USA	-	2.5	9.9	7.8	17: 75	9.3	7.9	green macroalgae	-	9.3	suspension feeders	barnacles and some mussels	10.7	Fry et al. (2003)
		-	6.2	-	6: 94	10.0	7.1	green macroalgae	-	10.2	suspension feeders	barnacles and some mussels	10.1	Fry et al. (2003)
		12.5	13.9	12.4	59: 35	9.1	9.3	green macroalgae	-	14.1	suspension feeders	barnacles and some mussels	13.8	Fry et al. (2003)
		-	3.7	-	33: 50	9.1	7.9	green macroalgae	-	13.3	suspension feeders	barnacles and some mussels	12.2	Fry et al. (2003)
		-	5.1	-	17: 90	8.1	6.2	green macroalgae	-	12.9	suspension feeders	barnacles and some mussels	10.2	Fry et al. (2003)
		-	11.5	-	6: 92	7.8	5.6	green macroalgae	-	11.9	suspension feeders	barnacles and some mussels	10.5	Fry et al. (2003)
		5.6	13.4	12.6	6: 92	7.5	4.5	green macroalgae	-	11.8	suspension feeders	barnacles and some mussels	10.7	Fry et al. (2003)
		19.6	11.9	12.1	4: 95	7.6	3.5	-	-	-	-	-	-	Fry et al. (2003)
		11.5	12.3	12.0	14: 84	9.5	3.4	green macroalgae	-	6.0	-	-	-	Fry et al. (2003)
Beenyup WWTW effluent, Western Australia	-	-	-	25.3	-	-	9.2	-	-	-	-	-	-	Gartner et al. (2002)
Ocean Reef, Western Australia	-	-	-	6.7	-	-	7.0	-	-	-	-	-	-	Gartner et al. (2002)
		-	15.2	-	1: 99	-	-2.1	-	-	-	-	-	-	Machás (unpublished data, 2006)
Himmerfjärden bay, Sweden	70	-	-	-	-	-	-	brown macroalgae	*Fucus vesiculosus*	12.0	-	-	-	Savage & Elmgren (2004)
	30	-	-	-	-	-	-	brown macroalgae	*Fucus vesiculosus*	8.0	-	-	-	Savage & Elmgren (2004)
WWTW effluent, Southern Portugal	-	-	33.9	-	3: 97	-	15.8	-	-	-	-	-	-	Machás (unpublished data, 2006)
Ria Formosa, Southern Portugal	-	-	32.4	-	6: 94	7.4	15.0	seagrass	*Zostera noltii*	8.5	-	-	-	Machás (unpublished data, 2006)
		-	23.2	-	15: 85	7.1	10.4	seagrass	*Zostera noltii*	5.1	-	-	-	Machás (unpublished data, 2006)
		-	-8.6	-	53: 47	7.2	9.3	seagrass	*Zostera noltii*	8.9	-	-	-	Machás (unpublished data, 2006)
		-	0.1	-	11: 89	4.8	4.8	seagrass	*Zostera noltii*	6.1	-	-	-	Machás (unpublished data, 2006)
		-	15.4	-	1: 99	7.6	-1.5	seagrass	*Zostera noltii*	7.2	-	-	-	Machás (unpublished data, 2006)
		-	15.6	-	2: 98	7.0	0.6	seagrass	*Zostera noltii*	3.3	-	-	-	Machás (unpublished data, 2006)
		-	7.6	-	30: 70	8.3	2.8	seagrass	*Zostera noltii*	4.1	-	-	-	Machás (unpublished data, 2006)
		-	0.8	-	24: 76	6.3	4.8	seagrass	*Zostera noltii*	7.8	-	-	-	Machás (unpublished data, 2006)
Quashnet River, Cape Cod (Ma), USA	28	-	-	-	-	-	4.7	green macroalgae	*Cladophora vagabunda*	4.5	-	-	-	McClelland et al.(1997); McClelland & Valiela.(1998a)
								green macroalgae	*Enteromorpha sp.*	6.6	-	-	-	McClelland et al.(1997); McClelland & Valiela.(1998a,b)
								red macroalgae	*Gracilaria tikvahiae*	5.9	-	-	-	McClelland et al. (1997), McClelland & Valiela (1998a)
								seagrass	*Zostera marina*	0.4	-	-	-	McClelland & Valiela et al. (1998a)
								marsh plant	*Spartina alterniflora*	6.0	-	-	-	McClelland et al. (1997), McClelland & Valiela (1998a)
Childs River, Cape Cod (Ma), USA	63	-	-	-	-	-	5.7	green macroalgae	*Cladophora vagabunda*	5.4	suspension feeders	*Geukensia demissa* (B)	9.1	McClelland et al.(1997); McClelland & Valiela.(1998a,b)
								green macroalgae	*Enteromorpha sp.*	8.2	suspension feeders	*Mya arenaria* (B)	7.9	McClelland et al.(1997); McClelland & Valiela.(1998a,b)
								red macroalgae	*Gracilaria tikvahiae*	7.6	deposit/detritus feeders	*Leitoscoloplos fragilis (* P)	7.5	McClelland et al.(1997); McClelland & Valiela.(1998a,b)
								seagrass	*Zostera marina*	6.1	deposit/detritus feeders	*Palaemonetes vulgaris* (C)	8.8	McClelland & Valiela et al. (1998a,b)
								marsh plant	*Spartina alterniflora*	7.6	deposit/detritus feeders	*Leptosynapta sp.* (H)	9.4	McClelland et al.(1997); McClelland & Valiela.(1998a,b)
								-	-	-	deposit/detritus feeders	*Sclerodactyla briareus* (H)	10.3	McClelland & Valiela et al. (1998a,b)
								-	-	-	herbivores	*Cyprinodon variegatus* (F)	9.8	McClelland & Valiela et al. (1998a,b)
								-	-	-	herbivores	*Cymadusa compta* (C)	7.6	McClelland & Valiela et al. (1998a,b)
								-	-	-	herbivores	*Gammarus mucronatus* (C)	7.8	McClelland & Valiela et al. (1998a,b)
								-	-	-	herbivores	*Erichsonella filiformis* (C)	7.7	McClelland & Valiela et al. (1998a,b)
								-	-	-	herbivores	*Microdeutopus gryllotalpa* (C)	6.6	McClelland & Valiela et al. (1998a,b)
								-	-	-	carnivorous	*Menidia menidia* (F)	11.6	McClelland & Valiela et al. (1998a,b)
								-	-	-	carnivorous	*Gasterosteus aculeatus* (F)	12.3	McClelland & Valiela et al. (1998a,b)
								-	-	-	carnivorous	*Fundulus heteroclitus* (F)	11.3	McClelland & Valiela et al. (1998a,b)
								-	-	-	carnivorous	*Podarke obscura* (P)	10.7	McClelland & Valiela et al. (1998a,b)

Table I. (Continuation).

Sage Lot Pond, Cape Cod (Ma), USA	4	-	-	-	-	-	4.2	green macroalgae	*Cladophora vagabunda*	3.4	suspension feeders	*Geukensia demissa* (B)	7.3	McClelland et al.(1997); McClelland & Valiela.(1998a,b)
								green macroalgae	*Enteromorpha sp.*	4.9	suspension feeders	*Mya arenaria* (B)	5.3	McClelland et al.(1997); McClelland & Valiela.(1998a,b)
								red macroalgae	*Gracilaria tikvahiae*	5.1	deposit/detritus feeders	*Leitoscoloplos fragilis (* P)	4.5	McClelland et al.(1997); McClelland & Valiela.(1998a,b)
								seagrass	*Zostera marina*	-1.5	deposit/detritus feeders	*Palaemonetes vulgaris* (C)	5.8	McClelland & Valiela et al. (1998a,b)
								marsh plant	*Spartina alterniflora*	4.4	deposit/detritus feeders	*Leptosynapta sp.* (H)	6.1	McClelland et al.(1997); McClelland & Valiela.(1998a,b)
								-	-	-	deposit/detritus feeders	*Sclerodactyla briareus* (H)	6.7	McClelland & Valiela et al. (1998a,b)
								-	-	-	herbivores	*Cyprinodon variegatus* (F)	5.2	McClelland & Valiela et al. (1998a,b)
								-	-	-	herbivores	*Cymadusa compta* (C)	4.1	McClelland & Valiela et al. (1998a,b)
								-	-	-	herbivores	*Gammarus mucronatus* (C)	5.1	McClelland & Valiela et al. (1998a,b)
								-	-	-	herbivores	*Erichsonella filiformis* (C)	4.3	McClelland & Valiela et al. (1998a,b)
								-	-	-	herbivores	*Microdeutopus gryllotalpa* (C)	3.8	McClelland & Valiela et al. (1998a,b)
								-	-	-	carnivorous	*Menidia menidia* (F)	9.8	McClelland & Valiela et al. (1998a,b)
								-	-	-	carnivorous	*Gasterosteus aculeatus* (F)	9	McClelland & Valiela et al. (1998a,b)
								-	-	-	carnivorous	*Fundulus heteroclitus* (F)	8.3	McClelland & Valiela et al. (1998a,b)
								-	-	-	carnivorous	*Podarke obscura* (P)	7.1	McClelland & Valiela et al. (1998a,b)
Mashpee River, Cape Cod (MA),USA	44	-	-	-	-	-	7.7	green macroalgae	*Enteromorpha sp.*	7.7	-	-	-	Cole et al. (2005)
								green macroalgae	*Ulva lactuca*	8.1	-	-	-	Cole et al. (2005)
								brown macroalgae	*Sargassum filipendula*	8.7	-	-	-	Cole et al. (2005)
								red macroalgae	*Gracilaria tikvahiae*	7.6	-	-	-	Cole et al. (2005)
								marsh plant	*Spartina alterniflora*	6.8	-	-	-	Cole et al. (2005)
Great Pond, Cape Cod (MA,USA	66	-	-	-	-	-	7.6	green macroalgae	*Enteromorpha sp.*	9.9	-	-	-	Cole et al. (2005)
								red macroalgae	*Gracilaria tikvahiae*	8.3	-	-	-	Cole et al. (2005)
								marsh plant	*Spartina alterniflora*	7.7	-	-	-	Cole et al. (2005)
Green Pond, Cape Cod (MA,USA	54	-	-	-	-	-	7.2	green macroalgae	*Enteromorpha sp.*	7.3	-	-	-	Cole et al. (2005)
								green macroalgae	*Ulva lactuca*	8.2	-	-	-	Cole et al. (2005)
								brown macroalgae	*Sargassum filipendula*	7.7	-	-	-	Cole et al. (2005)
								red macroalgae	*Gracilaria tikvahiae*	8.5	-	-	-	Cole et al. (2005)
								marsh plant	*Spartina alterniflora*	8.1	-	-	-	Cole et al. (2005)
Oyster Pond, Cape Cod (Ma), USA	71	-	-	-	-	-	-	green macroalgae	*Vaucheria spp.*	10.3	-	-	-	Cole et al. (2004)
								vascular plant	*Elatine americana*	7.5	-	-	-	Cole et al. (2004)
								vascular plant	*Eleocharis sp.*	8.3	-	-	-	Cole et al. (2004)
								vascular plant	*Lemna spp.*	6.9	-	-	-	Cole et al. (2004)
								vascular plant	*Myriophillum spp.*	5.7	-	-	-	Cole et al. (2004)
								vascular plant	*Najas sp.*	6.4	-	-	-	Cole et al. (2004)
								vascular plant	*Potamogeton spp.*	7.7	-	-	-	Cole et al. (2004)
Ashumet Pond, Cape Cod (MA,USA	80	-	-	-	-	-	10.8	vascular plant	*Callitriche palustris*	6.4	-	-	-	Cole et al. (2005)
								vascular plant	*Elatine americana*	11.3	-	-	-	Cole et al. (2005)
								vascular plant	*Eleocharis sp.*	7.4	-	-	-	Cole et al. (2005)
								vascular plant	*Gratiola lutea*	9.5	-	-	-	Cole et al. (2005)
								vascular plant	*Ludwigia sp.*	12.3	-	-	-	Cole et al. (2005)
								vascular plant	*Potamogeton sp.*	13.8	-	-	-	Cole et al. (2005)
Coonamessett Pond, Cape Cod (MA,USA	17	-	-	-	-	-	7.2	vascular plant	*Callitriche palustris*	4.7	-	-	-	Cole et al. (2005)
								vascular plant	*Elatine americana*	6.8	-	-	-	Cole et al. (2005)
								vascular plant	*Eleocharis sp.*	5.3	-	-	-	Cole et al. (2005)
								vascular plant	*Eriocaulon sp.*	5.6	-	-	-	Cole et al. (2005)
								vascular plant	*Gratiola lutea*	4.2	-	-	-	Cole et al. (2005)
								vascular plant	*Polygonum sp.*	0.5	-	-	-	Cole et al. (2005)
Miacomet Pond, Nantucket Island (Ma), USA	27	-	-	-	-	-	3.9	vascular plant	*Callitriche palustris*	7.3	-	-	-	Cole et al. (2005)
								vascular plant	*Ceratophyllum sp.*	8.1	-	-	-	Cole et al. (2005)
								vascular plant	*Elatine americana*	6.3	-	-	-	Cole et al. (2005)
								vascular plant	*Eriocaulon sp.*	6.2	-	-	-	Cole et al. (2005)
								vascular plant	*Najas sp.*	6.2	-	-	-	Cole et al. (2005)
								vascular plant	*Potamogeton perfoliatus*	5.5	-	-	-	Cole et al. (2005)
								vascular plant	*Ruppia maritima*	2.9	-	-	-	Cole et al. (2005)
								vascular plant	*Typha latifolia*	5.6	-	-	-	Cole et al. (2005)
								vascular plant	*Vallisneria americana*	5.0	-	-	-	Cole et al. (2005)

Annex II

Table I. Identifications of epiphytes on *Zostera noltii* leaves colleted from January of 2001 to December 2002 and analysed for δ-values.

Epiphytes fraction	Sample composition
January	
20 µm < epiphytes < 100 µm	diatoms - *Cocconeis* sp. (mainly specie), *Grammatophora unduleta*, *Fracillaria* sp., *Bacilaria paxillifer*, *Nitzschia* sp.
February	
20 µm < epiphytes < 100 µm	diatoms - *Cocconeis* sp. (mainly specie), *Grammatophora unduleta*, *Nitzschia* sp., *Fracillaria* sp., *Navicula* sp., *Nitzschia longissima*
March	
20 µm < epiphytes < 100 µm	diatoms - *Cocconeis* sp. (mainly specie), *Navicula* sp., *Grammatophora unduleta*, *Fracillaria* sp., *Nitzschia* sp., *Amphipleura* sp., *Diploneis* sp.
100 µm < epiphytes	red algae calcareous- *Sahlingia* sp. detritus, vegetal debris
April	
20 µm < epiphytes < 100 µm	diatoms - *Cocconeis* sp. (mainly specie), *Navicula* sp., *Grammatophora unduleta*, *Fracillaria* sp., *Nitzschia* sp., *Nitzschia distans*
100 µm < epiphytes	Rhodphyta - *Ceramium* sp. calcareous- *Sahlingia* sp., *Hydrolithon* sp. Diatoms - *Navicula* sp. (aggregated to *Ceramiun sp.*) Cyanophyta - *Calotrix* (low occurrence) Vegetal debris - (low occurrence)
May	
20 µm < epiphytes < 100 µm	diatoms - without identification
100 µm < epiphytes	Calcareous red algae - *Hydrolithon* sp., Specie N.I lentil pardas microscopic algae - Myrionema Hidropolipos - Thecaphora
June	
20 µm < epiphytes < 100 µm	diatoms - *Cocconeis* sp. (mainly specie), *Fracillaria* sp., *Nitzschia* sp., *Navicula* sp., *Grammatophora unduleta*, *Baccillaria paxillipa* Cyanophyta - one specie (l occurrence)
100 µm < epiphytes	Calcareous red algae - *Sahlingia* sp., *Hydrolithon* sp. Diatoms - *Navicula* sp. Cyanophyta - *Calotrix*, *Anabaina sp.* Vegetal debris

Table I. (continued).

Epiphytes fraction	Sample composition
July	
20 µm < epiphytes < 100 µm	diatoms - *Cocconeis* sp. (mainly specie), *Fracillaria* sp., *Nitzschia* sp., *Navicula* sp., *Grammatophora unduleta*, *Baccillaria paxillipa*
	Cyanophyta - one specie (l occurrence)
	N.I - 1 occurrence
100 µm < epiphytes	Calcareous red algae - *Sahlingia* sp., *Hydrolithon* sp. (low occurrence)
	Diatoms -(high occurrence aggregated to vegetal detritus)
	Cyanophyta - *Calotrix* (high occurrence)
	Vegetal debris
August	
20 µm < epiphytes < 100 µm	diatoms - *Cocconeis* sp. (mainly specie), *Fracillaria* sp., *Nitzschia* sp., *Baccillaria paxillipa*
	Cyanophyta - three specie (l occurrence)
	N.I - 2 occurrences
100 µm < epiphytes	Diatoms - (aggregated to vegetal detritus)
	Cyanophyta - *Calotrix*
	Vegetal debris
September	
20 µm < epiphytes < 100 µm	diatoms - *Cocconeis* sp. (mainly specie), *Fraccillaria* sp., *Nitzschia* sp., *Baccillaria paxillipa, Navicula* sp., *Amphipleura* sp.
	N.I - 1 occurrence
100 µm < epiphytes	Calcareous red algae - *Sahlingia* sp.
	Diatoms - aggregated to vegetal detritus)
	Cyanophyta - *Calotrix*, N.I. (high occurrence, aggregated to vegetal detritus)
	Vegetal debris
October	
20 µm < epiphytes < 100 µm	diatoms - *Cocconeis* sp. (mainly specie), *Fracillaria* sp., *Baccillaria paxillipa*, *Navicula* sp., *Amphipleura* sp.
100 µm < epiphytes	Chlorophyta - *Enteromorpha* sp.
	filament red algae - N.I.
	Calcareous red algae - *Sahlingia* sp.
	Diatoms -(aggregated to vegetal detritus)
	Cyanophyta - *Calotrix, Anabaina, Spirolina,* N.I. (high occurrence aggregated to vegetal detritus)
	Vegetal debris (high occurrence)
November	
20 µm < epiphytes < 100 µm	diatoms - *Cocconeis* sp. (mainly specie), *Grammatophora unduleta*, *Fraccillaria* sp., *Amphipleura* sp., *Nitzschia* sp.
	N.I - 1 occurrence
100 µm < epiphytes	Calcareous red algae - *Sahlingia* sp.; *Hydrolithon* sp.
	Hidropolipos - *Orthopyxis caliculata* or *Clytia johnstoni*
	Diatoms -*Amphipleura* sp. dominant species (aggregated to vegetal detritus)
	Cyanophyta - *Calotrix* (low occurrence)
	Vegetal debris (high occurrence, dominant)
December	
20 µm < epiphytes < 100 µm	diatoms - *Cocconeis* sp. (mainly specie), *Navicula* sp., *Fracillaria* sp., *Nitzschia* sp., *Grammatophora unduleta*
	N.I - 2 occurrences
100 µm < epiphytes	Rhodphyta - *Ceramium* sp.
	Calcareous red algae - *Sahlingia* sp.
	Diatoms - (high occurrence)
	Cyanophyta
	Vegetal debris
	Postures