



## Fatty acid profiles indicate the habitat of mud snails *Hydrobia ulvae* within the same estuary: Mudflats vs. seagrass meadows

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### ABSTRACT

Mud snails *Hydrobia ulvae* occupy different habitats in complex estuarine ecosystems. In order to determine if fatty acid profiles displayed by mud snails can be used to identify the habitat that they occupy within the same estuary, fatty acids of *H. ulvae* from one mudflat and one seagrass meadow in the Ria de Aveiro (Portugal) were analyzed and compared to those displayed by microphytobenthos (MPB), the green leaves (epiphyte-free) of *Zostera noltii*, as well as those exhibited by the epiphytic community colonizing this seagrass. MPB and epiphytic diatom-dominated samples displayed characteristic fatty acids, such as 16:1n-7 and 20:5n-3, while 18:2n-6 and 18:3n-3 were the dominant fatty acids in the green leaves of *Z. noltii*. Significant differences between the fatty acid profiles of *H. ulvae* specimens sampled in the mudflat and the seagrass meadow could be identified, with those from the mudflat displaying higher levels of fatty acids known to be characteristic of MPB. This result points towards the well known existence of grazing activity on MPB by mud snails. The fatty acid profiles displayed by *H. ulvae* inhabiting the seagrass meadows show no evidence of direct bioaccumulation of the two most abundant polyunsaturated fatty acids of *Z. noltii* (18:2n-6 and 18:3n-3) in the mud snails, which probably indicates that either these compounds can be metabolized to produce energy, used as precursors for the synthesis of essential fatty acids, or that the snails do not consume seagrass leaves at all. Moreover, the fatty acid profiles of mud snails inhabiting the seagrass meadows revealed the existence of substantial inputs from microalgae, suggesting that the epiphytic community colonizing the leaves of *Z. noltii* displays an important role on the diet of these organisms. This assumption is supported by the high levels of 20:5n-3 and 22:6n-3 recorded in mud snails sampled from seagrass meadows. In conclusion, fatty acid analyses of *H. ulvae* can be successfully used to identify the habitat occupied by these organisms within the same estuary (e.g. mudflats and seagrass meadows) and reveal the existence of contrasting dietary regimes.

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### 1. Introduction

Estuaries contain a spatially complex diversity of habitats, which contribute to a highly dynamic food environment. Grazing-benthic food chains are common in intertidal habitats, representing and regulating the flow of nutrients and energy from the bottom and through up the estuarine ecosystem (Carlier et al., 2007). The mud snail *Hydrobia ulvae* (Pennant) is an important primary consumer in temperate European estuarine ecosystems (Newell, 1965; Riera, 2010) and can be found abundantly in a variety of intertidal habitats, from mudflats to seagrass meadows (Newell, 1965; Lillebø et al., 1999; Riera, 2010). The population structure of these mud

snails is influenced by large-scale movements of individuals (Haubois et al., 2002, 2004), which results in the snails experiencing a wide range of habitats and allows them to explore several food sources. Benthic primary productivity in estuarine habitats is generally dominated by microphytobenthos (MPB) production (Underwood and Kromkamp, 1999), even though these environments may also contain a multiplicity of other primary producers (e.g. macroalgae, macrophytes). Muddy sediments usually include digestible and nutritive food sources, such as benthic microalgae or bacterial communities, while seagrass meadows are dominated by less digestible marine vascular plants (Pascal et al., 2008). The existence of a complex trophic link between *H. ulvae* and MPB in mudflats has already been indicated (Fenchel et al., 1975; Morrissey, 1988; Haubois et al., 2002), with mud snails density and sediment chlorophyll-*a* content playing a major role on *H. ulvae* ingestion

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rates (Blanchard et al., 2000; Haubois et al., 2005). *Zostera noltii* is a widely distributed seagrass in estuarine ecosystems (Duarte, 1989; Plus et al., 2001), forming highly structured intertidal habitats and providing trophic resources and refuge areas. These marine plants have an important ecological function on areas inhabited by *H. ulvae*, as their meadows provide a greater environmental stability (Lillebø et al., 1999; Cardoso et al., 2008). Its direct role in the dietary regime of estuarine invertebrates is somehow limited, as recognized for marine vascular plants in general (Kharlamenko et al., 2001). However, *Z. noltii*'s epiphytic community constitutes an important food source, as epiphytes are potentially important primary producers for herbivores in seagrass meadows (Lebreton et al., 2009).

Fatty acids have been widely used as qualitative markers to confirm and trace trophic relationships in marine and estuarine environments (Auel et al., 2002; Dalsgaard et al., 2003; Phillips et al., 2003; Shin et al., 2008). These compounds display great structural diversity and substantial taxonomic specificity. In general, marine primary producers have characteristic fatty acids and these are transferred into the storage lipids of higher trophic organism with unchanged or recognizable forms (Dalsgaard et al., 2003; Shin et al., 2008). The identification of characteristic fatty acid patterns at different trophic levels allows researchers to trace the relationship between primary producers, primary consumers and/or higher trophic organisms (Biandolino et al., 2008). The present work tests if fatty acid profiles can be used to identify the habitat (mudflat vs. seagrass meadows) occupied by *H. ulvae* in the Ria de Aveiro, Portugal, and if they can reveal the existence of contrasting dietary regimes when these gastropods colonize different habitats within the same estuary.

## 2. Material and methods

### 2.1. Sampling

*Hydrobia ulvae* specimens were collected in the Ria de Aveiro, a shallow coastal lagoon located on the north west coast of Portugal (Dias et al., 2000). This coastal lagoon has a very complex geometry and is characterized by the existence of significant intertidal zones, namely mudflats and salt marshes, distributed along narrow channels (Dias et al., 2000). The sampling sites were a seagrass meadow located in the east margin of the Canal de Mira at Gafanha da Encarnação (40°38'N, 8°44'W) and a mudflat in the west margin of the Canal de Ílhavo, near Vista Alegre VA (40°35'N, 8°41'W). All *H. ulvae* specimens were randomly collected in an area corresponding to 10 m<sup>2</sup>. Snails were immediately separated from the sediment by sieving (still in the field) and were carefully scraped from the leaves in seagrass meadow. Mud snails collected in the mudflat were either buried or crawling over the mud, while in the seagrass meadow only the specimens located over the green leaves of *Z. noltii* were sampled. Both samples were rapidly transported to the laboratory, with mud snails always being kept submerged in water collected at each sampling location. *Hydrobia ulvae* from both sampling sites were further separated according to shell height (the maximum distance from apex to aperture), which was determined under a stereoscopic microscope to the nearest 0.1 mm (Mouritsen and Thomas Jensen, 1994; Grudemo and Johannesson, 1999). Only specimens corresponding to adult size (>4 mm) were selected for the present study (Haubois et al., 2002), with six samples of ca. 50 individuals each being prepared for fatty acid analysis. Snail guts were not removed, as during the time elapsed from sampling until freezing all ingested material was egested by the snails (through fecal pellets). At the time of freezing, no fecal pellets were recorded from any of the selected specimens, which indicated that the amount of ingested food still present in the gut was negligible.

MPB samples were collected on the mudflat using sediment corers (Ø 6.8 mm). Later, in the laboratory, benthic microalgae were separated from the mud using the lens tissue method (Eaton and Moss, 1966): four pieces of lens tissue were placed on the air-exposed surface of the sediment during the expected time of diurnal low tide; the upper two pieces of lens tissue were removed after approximately 1 h; benthic microalgae which had migrated through the lens tissue were resuspended on artificial sea water (prepared using freshwater purified by a reverse osmosis unit and mixed with Pro-Reef<sup>®</sup> salt produced by Tropic Marine<sup>®</sup>). Three replicate samples were used for determination of the relative abundance of major taxonomic groups (fixed in 1% v/v formaldehyde) by counting a minimum of 400 cells, while other six samples were concentrated for fatty acid analysis. Six samples of *Z. noltii* were collected on the seagrass meadow referred above for fatty acid analysis. Only the green leaves of *Z. noltii* were considered for the present study, as the majority of mud snails observed in the seagrass meadow were clustered in their surface. All green leaves were carefully cleared of epiphytes or other particles by rinsing the leaf blade with artificial sea water (see above for preparation details) and carefully scraping their surface with a razor blade (Kharlamenko et al., 2001). Green leaves were also cut into small pieces to enable a more efficient fatty acid extraction. Nine samples of the epiphytic community colonizing *Z. noltii* were collected by scraping the surface of their green leaves, with six samples being used for fatty acid analysis and the other three for the determination of the relative abundance of major taxonomic groups. This determination followed the same procedure described above for MPB samples. All collected samples (snails, MPB, green leaves of *Z. noltii* and *Z. noltii* epiphytes) were weighed and freeze-dried prior to fatty acid analysis.

### 2.2. Fatty acid analysis

Fatty acid extraction and preparation of methyl esters were carried out according to Lepage and Roy (1986) modified by Cohen et al. (1988). Freeze-dried samples (100 mg) were transmethylated with 5 ml of methanol/acetyl chloride (95:5 v/v). The mixture was sealed in a light-protected Teflon-lined vial under nitrogen atmosphere and heated at 80 °C for 1 h. The vial contents were then cooled, diluted with 1 ml water and extracted with 2 ml of n-heptane. The heptane layer was dried over Na<sub>2</sub>SO<sub>4</sub>, evaporated to dryness under a nitrogen atmosphere and redissolved in heptane, which contained the methyl esters. The methyl esters were then analyzed by gas–liquid chromatography, on a VARIAN 3800 gas–liquid chromatograph (Palo Alto, USA), equipped with a flame ionization detector. Separation was carried out on a 0.32 mm × 30 m fused silica capillary column (film 0.32 µm) Supelcowax 10 (SUPELCO, Bellefonte PA, USA) with helium as carrier gas at a flow rate of 1.3 ml min<sup>-1</sup>. The column temperature was programmed at an initial temperature of 200 °C for 10 min, then increased at 4 °C min<sup>-1</sup>–240 °C and held there for 16 min. Injector and detector temperatures were 250 and 280 °C, respectively, and split ratio was 1:100. Peak identification was carried out using known standards (Nu-Chek-Prep, Elysian, USA). Peak areas were determined using Varian software.

### 2.3. Data analysis

The percentage of individual fatty acids and fatty acid ratios present in mud snails sampled from the mudflat and the seagrass meadow, as well as in food sources (MPB, green leaves of *Z. noltii* and epiphyte community) was compared using the Student's *t*-test and ANOVA, respectively. Both analyses were performed using STATISTICA v8 (StatSoft Inc., USA). Samples were also analyzed

using multidimensional scaling (MDS) ordination on the Bray–Curtis similarity index, described by Clarke and Gorley (2006). The MDS stress value indicates the level of an appropriate representation of the multidimensional distances. Data were fourth root transformed prior to analysis and in order to validate our interpretation of the MDS we performed an analysis of similarity (ANOSIM), built on a simple non-parametric permutation procedure, and applied to the similarity matrix underlying the ordination of the samples. This procedure allows the identification of differences in the fatty acid profiles displayed by mud snails from both habitats and by MPB, green leaves of *Z. noltii* and *Z. noltii* epiphytes. Similarity percentages (SIMPER) were also explored to examine the similarity within the fatty acids of: (1) *H. ulvae* from both sampling sites; (2) MPB; (3) the green leaves of *Z. noltii*; and (4) the epiphyte community of *Z. noltii*. All multivariate analyses were performed using PRIMER v6 with PERMANOVA add-on (Primer-E Ltd., Plymouth, UK).

### 3. Results

#### 3.1. Taxonomic composition of MPB and of *Z. noltii* epiphytic community

The microphytobenthic assemblage from the mudflat was clearly dominated by diatoms (74.2 ± 3.3%). The relative abundance of euglenophytes was 23.2 ± 1.6%, while cyanobacteria and other benthic microalgae accounted for less than 5% of the microphytobenthic assemblage. The epiphytic algae community of the green leaves of *Z. noltii* was dominated by diatoms (91.2 ± 1.3%), with other benthic microalgae and cyanobacteria also being observed (3.5 ± 0.2 and 5.3 ± 1.2%, respectively).

#### 3.2. Fatty acid composition of food sources

MPB and green leaves of *Z. noltii* exhibited substantial variations in their fatty acid profiles, while the epiphytic algae of *Z. noltii* showed several similarities with the profile found for MPB (Table 1). The main differences in MPB fatty acid profiles, when compared with the green leaves of *Z. noltii*, were a significantly higher content

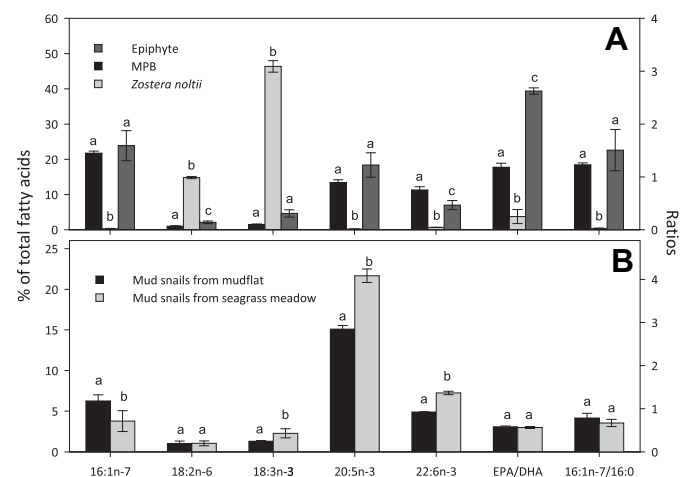
of 16:1n-7 (palmitoleate), 20:5n-3 (eicosapentaenoic acid, EPA) and 22:6n-3 (docosahexaenoic acid, DHA) and a significantly lower content of 18:2n-6 (linoleic acid, LA) and 18:3n-3 (alpha-linolenic acid, ALA) (Tukey's test,  $P < 0.05$ , Fig. 1A). The same significant differences were found for 16:1n-7, ALA and EPA when epiphytes were compared with the green leaves of *Z. noltii*. Moreover, the epiphytic community also exhibited significant differences in the levels of LA and DHA when compared with MPB and *Z. noltii* (Tukey's test,  $P < 0.05$ , Fig. 1A). Together, the fatty acids 16:1n-7, EPA and DHA comprised 46.44 ± 0.98% and 49.25 ± 1.05% of the total pool of fatty acids recorded for MPB and for the epiphytes of *Z. noltii* (respectively), while the sum of LA and ALA represented 74.19 ± 1.74% of the total pool of fatty acids detected in the green leaves of *Z. noltii*. Saturated fatty acids (SFAs) were predominant in both MPB and *Z. noltii*, with their percentage of total SFAs (29.5 ± 1.5 and 30.17 ± 1.9%, respectively) not being significantly different (Tukey's test,  $P > 0.05$ , Fig. 2A). The epiphytes from the green leaves of the seagrass showed a significantly lower content of SFAs when compared with both MPB and *Z. noltii* (Tukey's test,  $P < 0.05$ , Fig. 2A). Monounsaturated fatty acids (MUFAs) varied from 3.05 ± 0.9% in *Z. noltii* to 37.56 ± 0.5% in MPB samples, showing a statistically significant difference (Tukey's test,  $P < 0.05$ , Fig. 2A). A significant difference was also found between *Z. noltii* and its epiphytic community (Tukey's test,  $P < 0.05$ , Fig. 2A). Significant differences were also recorded between the percentage of polyunsaturated fatty acids (PUFAs) present in MPB and in the green leaves of *Z. noltii*, as well as in their epiphytes (Tukey's test,  $P < 0.05$ , Fig. 2A). Both MPB and *Z. noltii* epiphytes showed significantly higher levels of highly unsaturated fatty acids (HUFAs) when compared with *Z. noltii* (Tukey's test,  $P < 0.05$ , Fig. 2A). PUFAs displayed higher values in *Z. noltii* samples, when compared with those from MPB and *Z. noltii* epiphytes, while HUFAs displayed an inverse pattern, with higher levels being recorded in MPB and in the samples of *Z. noltii* epiphytes.

A significant difference in the fatty acid profiles of MPB, green leaves of *Z. noltii* and their epiphytes was also shown by the analysis of similarity (ANOSIM,  $R = 1$ ,  $P = 0.001$ ) and by the MDS plot (stress value = 0.01, representative of a clear separation) (Fig. 3A). Although the food sources available at both habitats occupied

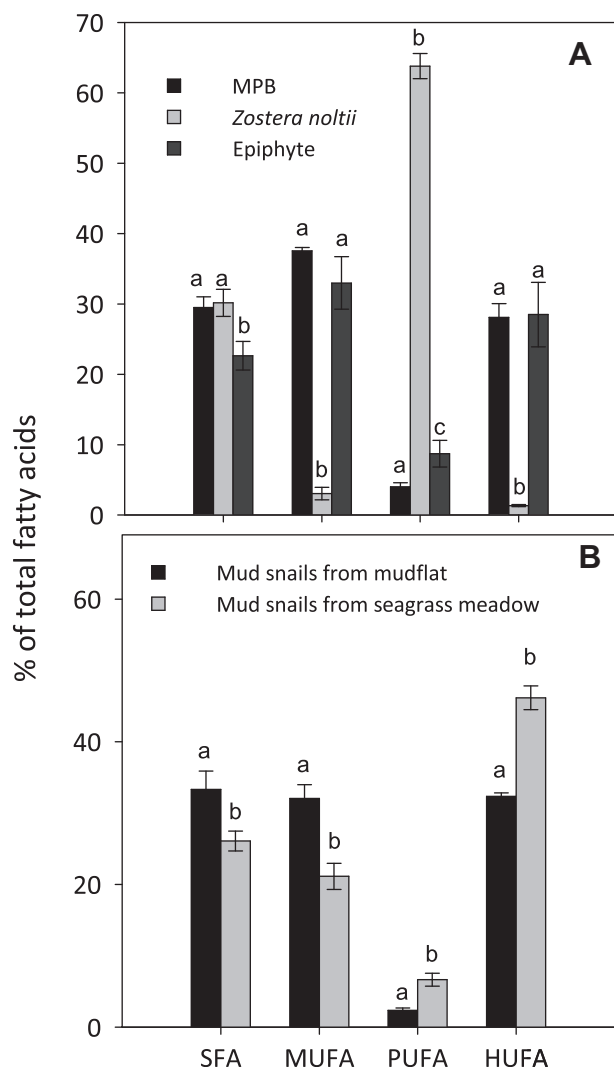
**Table 1**  
Fatty acid composition (% of total fatty acids) of MPB, green leaves (epiphyte-free) of *Zostera noltii* and epiphytes of *Z. noltii* from a mudflat and a seagrass meadow (respectively) in Ria de Aveiro, Portugal (mean ± SD,  $n = 6$ ).

Fatty acid	MPB	<i>Z. noltii</i>	Epiphytes of <i>Z. noltii</i>
14:0	3.42 ± 0.33 <sup>a</sup>	0.80 ± 0.23 <sup>b</sup>	4.92 ± 0.41
16:0	17.68 ± 0.97 <sup>a</sup>	12.99 ± 1.74 <sup>b</sup>	16.07 ± 1.53 <sup>a</sup>
16:1n-7	21.75 ± 0.58 <sup>a</sup>	0.24 ± 0.15 <sup>b</sup>	23.87 ± 4.27 <sup>a</sup>
16:3n-4	1.65 ± 0.28 <sup>a</sup>	2.28 ± 0.15 <sup>b</sup>	1.22 ± 0.49 <sup>a</sup>
17:0	2.75 ± 0.16 <sup>a</sup>	0.51 ± 0.12 <sup>a</sup>	n.d.
18:0	2.31 ± 0.10 <sup>a</sup>	2.64 ± 0.11 <sup>b</sup>	0.81 ± 0.07 <sup>c</sup>
18:1n-9	2.89 ± 0.07 <sup>a</sup>	0.62 ± 0.05 <sup>b</sup>	4.65 ± 1.13 <sup>c</sup>
18:1n-7	3.04 ± 0.28 <sup>a</sup>	1.17 ± 0.03 <sup>b</sup>	3.22 ± 0.30 <sup>a</sup>
18:2n-6	0.85 ± 0.43 <sup>a</sup>	14.82 ± 0.30 <sup>b</sup>	2.12 ± 0.38 <sup>c</sup>
18:3n-3	1.51 ± 0.14 <sup>a</sup>	46.38 ± 1.65 <sup>b</sup>	4.65 ± 1.05 <sup>a</sup>
18:4n-3	1.36 ± 0.02 <sup>a</sup>	n.d.	2.59 ± 0.26 <sup>a</sup>
20:0	n.d.	2.65 ± 0.77	n.d.
20:1n-9	2.68 ± 0.21	n.d.	1.25 ± 0.52
20:4n-6	2.06 ± 0.43 <sup>a</sup>	0.49 ± 0.12 <sup>b</sup>	0.52 ± 0.05 <sup>b</sup>
20:5n-3	13.37 ± 0.81 <sup>a</sup>	0.17 ± 0.09 <sup>b</sup>	18.39 ± 3.46 <sup>a</sup>
22:0	n.d.	4.86 ± 0.06	n.d.
22:6n-3	11.32 ± 0.89 <sup>a</sup>	0.69 ± 0.03 <sup>b</sup>	7.00 ± 1.29 <sup>c</sup>
24:0	2.89 ± 0.45 <sup>a</sup>	5.71 ± 0.48 <sup>b</sup>	n.d.
24:1n-9	7.21 ± 0.54 <sup>a</sup>	1.03 ± 0.72 <sup>b</sup>	n.d.
Others	1.27	1.96	8.73

Note: Only the fatty acids whose content exceeds at least 1% in MPB, *Z. noltii* or epiphytes of *Z. noltii* are displayed. n.d. = not detected. Different letters within the same fatty acid (row) represent significant differences (Tukey's test,  $P < 0.05$ ).



**Fig. 1.** Content of specific individual fatty acid markers (% of total FAs) and ratios in both microalgae communities (MPB and epiphytes of *Z. noltii*) and in green leaves (epiphyte-free) of *Zostera noltii* (*Zostera*) (A) and in *Hydrobia ulvae* individuals from the mudflat or the seagrass meadow (B). Values are mean ± SD ( $n = 6$ ). Different letters within the same fatty acid or ratio represent significant differences (Tukey's test,  $P < 0.05$  in plot A and Student's  $t$ -test,  $P < 0.01$  in plot B).

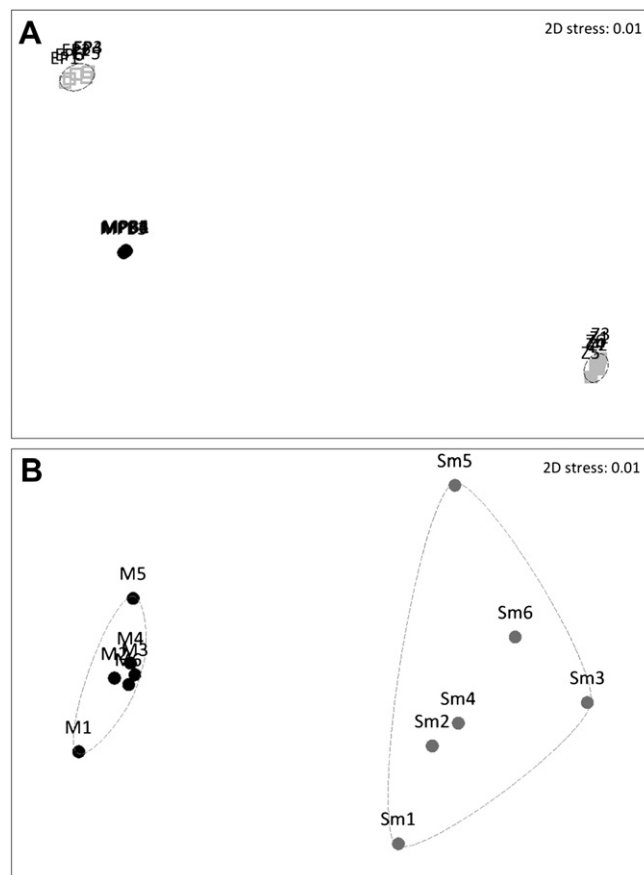


**Fig. 2.** Content of major classes of FAs in the profile of both microalgae communities (MPB and epiphytes of *Z. noltii*) and in the green leaves (epiphyte-free) of *Zostera noltii* (A) and in *Hydrobia ulvae* individuals from the mudflat or the seagrass meadow (B). Values are mean  $\pm$  SD ( $n = 6$ ). Different letters within the same fatty acid class means significant differences (Tukey's test,  $P < 0.05$  in plot A and Student's  $t$ -test,  $P < 0.001$  in plot B). SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids; HUFA – highly unsaturated fatty acids.

different regions on the MDS plot, samples from MPB and *Z. noltii* epiphytes are displayed closer to each other.

### 3.3. Fatty acid composition of mud snails

The fatty acid composition (% of total fatty acids) of the mud snail *H. ulvae* from both estuarine habitats is listed in Table 2. All specimens of *H. ulvae* exhibited high levels of EPA and 20:0, with EPA showing statistically significant differences between sites ( $t = 17.08$ ,  $P < 0.001$ ). Mud snails colonizing the mudflat displayed significantly higher levels of 20:1n-9 (eicosenoic acid) ( $t = 6.80$ ,  $P < 0.001$ ) and 20:4n-6 (arachidonic acid, AA) ( $t = -4.26$ ,  $P < 0.01$ ) than those inhabiting the seagrass meadow. However, individuals from the seagrass meadow displayed significantly higher levels of 22:5n-3 (docosapentaenoic acid, DPA) ( $t = -17.08$ ,  $P < 0.001$ ). The SFAs and MUFAs were significantly higher in mud snails from the mudflat (SFAs:  $t = -6.05$ ,  $P < 0.001$ , MUFAs:  $t = 10.04$ ,  $P < 0.001$ , Fig. 2B). The major SFAs registered, in increasing order, were 16:0 and 18:0. MUFAs varied between  $32.03 \pm 1.9\%$  and  $21.13 \pm 1.8\%$  of



**Fig. 3.** MDS plot of the fatty acid profiles of microphytobenthos (MPB), green leaves (epiphyte-free) of *Zostera noltii* (Z) and epiphytes (EP) (A) and of *Hydrobia ulvae* individuals from the mudflat (M) or the seagrass meadow (Sm) (B). Dotted lines represent 95% of similarity (SIMPER). Dark and gray colors represent the mudflat and seagrass meadow habitat, respectively, on both plots (A and B).

total fatty acids in mud snails living on the mudflat and the seagrass meadow, respectively. PUFAs were characterized by lower levels than MUFAs, varying from  $2.34 \pm 0.3\%$  (mudflat) to  $6.65 \pm 0.9\%$  (*Z. noltii* meadow), while the percentage of HUFAs reached  $46.14 \pm 1.7\%$  in mud snails from the seagrass meadow and  $32.32 \pm 0.5\%$  in those from the mudflat. Both groups of unsaturated fatty acids exhibited statistically significant differences between habitats, being significantly lower in *H. ulvae* individuals from the mudflat (PUFAs:  $t = -10.86$ ,  $P < 0.001$  and HUFAs:  $t = -19.44$ ,  $P < 0.001$ , Fig. 2B). The mean values of *iso*- and *anteiso* branched fatty acids, such as *iso*- and *anteiso* 17:0, characteristic of marine bacteria, were always very low or not detectable. The level of 18:1n-7 (vaccenic acid) was always significantly higher in mud snails inhabiting *Zostera* meadows ( $4.67 \pm 0.88\%$ ) than in those occurring on the mudflat ( $5.95 \pm 0.22\%$ ) ( $t = 3.43$ ,  $P < 0.01$ ).

The content of fatty acids considered to be good trophic markers was compared between sites and also between MPB and green leaves of *Z. noltii*, as well as between these and *Z. noltii* epiphytes. The percentage of fatty acids known to be characteristic of diatoms (16:1n-7 and EPA) was significantly higher in MPB and also in samples from *Z. noltii* epiphytes (Fig. 1A), while significant levels of LA and ALA were normally found on samples from the green leaves of *Z. noltii* (see Section 3.2.). Distinct differences between sampling areas were detected for the fatty acids characteristic of both microalgae communities (benthic and epiphytic: EPA:  $t = 17.06$ ,  $P < 0.001$  and 16:1n-7:  $t = -4.02$ ,  $P < 0.01$ ) (Fig. 1B). Regardless of the sampled habitat, mud snails always displayed lower levels of



**Table 2**

Fatty acid composition (% of total fatty acids) of *Hydrobia ulvae* from a mudflat and a seagrass meadow in Ria de Aveiro, Portugal (mean  $\pm$  SD,  $n = 6$ ).

Fatty acid	<i>H. ulvae</i> mudflat	<i>H. ulvae</i> seagrass meadow
16:0	8.14 $\pm$ 1.76 <sup>a</sup>	5.68 $\pm$ 1.85 <sup>b</sup>
16:1 $n$ -7	6.25 $\pm$ 0.75 <sup>a</sup>	3.78 $\pm$ 1.28 <sup>b</sup>
17:0	2.92 $\pm$ 0.23	n.d.
18:0	8.64 $\pm$ 1.05 <sup>a</sup>	5.35 $\pm$ 1.17 <sup>b</sup>
18:1 $n$ -7	5.94 $\pm$ 0.22 <sup>a</sup>	4.67 $\pm$ 0.88 <sup>b</sup>
18:2 $n$ -6	1.04 $\pm$ 0.29 <sup>a</sup>	1.05 $\pm$ 0.30 <sup>a</sup>
18:3 $n$ -3	1.30 $\pm$ 0.09 <sup>a</sup>	2.28 $\pm$ 0.56 <sup>b</sup>
20:0	10.07 $\pm$ 3.78 <sup>a</sup>	11.52 $\pm$ 3.28 <sup>a</sup>
20:1 $n$ -9	8.97 $\pm$ 0.30 <sup>a</sup>	7.24 $\pm$ 0.55 <sup>b</sup>
20:2 $n$ -6	n.d.	1.71 $\pm$ 0.04
20:3 $n$ -6	n.d.	1.60 $\pm$ 0.63
20:4 $n$ -6	6.35 $\pm$ 0.76 <sup>a</sup>	7.87 $\pm$ 0.43 <sup>b</sup>
20:5 $n$ -3	15.10 $\pm$ 0.44 <sup>a</sup>	21.67 $\pm$ 0.84 <sup>b</sup>
22:1 $n$ -9	4.68 $\pm$ 1.78 <sup>a</sup>	1.88 $\pm$ 0.19 <sup>b</sup>
22:5 $n$ -3	5.96 $\pm$ 0.03 <sup>a</sup>	8.37 $\pm$ 0.89 <sup>b</sup>
22:6 $n$ -3	4.90 $\pm$ 0.02 <sup>a</sup>	7.24 $\pm$ 0.21 <sup>b</sup>
24:0	1.83 $\pm$ 0.06 <sup>a</sup>	3.09 $\pm$ 0.30 <sup>b</sup>
24:1 $n$ -9	4.97 $\pm$ 0.16 <sup>a</sup>	2.61 $\pm$ 0.87 <sup>b</sup>
Others	2.91	2.38

Note: Only fatty acids whose content exceeds at least 1% in *Hydrobia ulvae* from mudflat or seagrass meadow are displayed. n.d. – not detected. Different letters within the same fatty acid (row) represent significant differences (Student's  $t$ -test,  $P < 0.05$ ).

fatty acids known to be characteristic of *Z. noltii* (Fig. 1B). The statistical analysis on the contribution of ALA to the total fatty acids of *H. ulvae* between sites did not show a significant difference between the mudflat and the seagrass meadow ( $t = 0.09$ ,  $P = 0.931$ ). On the other hand, LA was significantly higher in mud snails from the seagrass meadow ( $t = 4.24$ ,  $P < 0.01$ ). Mud snails from both locations exhibited higher levels of palmitic acid than 16:1 $n$ -7, contributing to low 16:1 $n$ -7/16:0 ratios. Both 16:1 $n$ -7/16:0 and EPA/DHA ratios did not differ significantly between sampled habitats (16:1 $n$ -7/16:0:  $t = 2.03$ ,  $P = 0.07$  and EPA/DHA:  $t = -1.38$ ,  $P = 0.197$ ) (Fig. 1B). The fatty acid 22:6 $n$ -3 was recorded in significantly different levels in *H. ulvae* collected in both habitats ( $t = 27.12$ ,  $P < 0.001$ ).

Considering the whole fatty acid profile of sampled mud snails, there was a significant difference in those inhabiting the mudflat and those from the seagrass meadow (ANOSIM,  $R = 0.967$ ,  $P = 0.002$ ). A high degree of within group similarity was displayed for each of the estuarine habitats (SIMPER: 99% and 97% similarity, for mudflat and seagrass meadow, respectively). This is reinforced by the MDS analysis, which also showed a high degree of within group similarity and a distinct separation between *H. ulvae* individuals from each habitat (Fig. 3B). The stress value for the multidimensional scaling was 0.01, which is representative of a clear separation between sites.

## 4. Discussion

### 4.1. Fatty acid composition of food sources

Marine and estuarine primary producers biosynthesise an important structural diversity of fatty acids (Dalsgaard et al., 2003), as demonstrated by the diversity of fatty acids recorded in the present work in both microalgae communities (MPB and epiphytes) and in the green leaves of *Z. noltii*. The existence of specific fatty acids or ratios associated with particular groups of primary producers allows us to use them as fatty acid markers along trophic links. The main primary producers in the benthic intertidal community of the Ria de Aveiro (Portugal), MPB and *Z. noltii*, exhibited marked differences in their fatty acid profiles. Moreover, the epiphytic community of the seagrass *Z. noltii* also showed

a distinct fatty acid profile, namely when compared with *Z. noltii*. This aspect was emphasized by the distinct distribution exhibited by the samples through the application of the multidimensional scaling analysis. The main fatty acids found in MPB and in *Z. noltii* epiphytes profiles were the SFA 16:0, the MUFA 16:1 $n$ -7 and the HUFAs EPA, which are known to be typically associated with microphytobenthic assemblages (Kharlamenko et al., 2008) and the epiphytic community of *Zostera* spp. (Kharlamenko et al., 2001; Jaschinski et al., 2008). The fatty acids 16:1 $n$ -7 and EPA are biosynthesized by diatoms (Behrens and Kyle, 1996; Volkman et al., 1998; Kharlamenko et al., 2001; Dalsgaard et al., 2003) and have been frequently used as benthic diatom markers in food chains studies (Kharlamenko et al., 1995; Bachok et al., 2003). The relevance of diatoms in the taxonomic composition of MPB and epiphytes of *Z. noltii* was highlighted in the present study by the ratio 16:1 $n$ -7/16:0 being greater than 1, as this is commonly considered as an indicator of diatoms dominance (Napolitano et al., 1997; Kharlamenko et al., 2001).

Although the levels and ratios of fatty acids recorded in both benthic and epiphytic microalgal samples generally agree with the taxonomic composition determined in this study (ca. 74% and 91% of diatoms), the content of DHA is more difficult to explain. This highly unsaturated fatty acid is a typical marker of dinoflagellates (Kharlamenko et al., 2001; Bachok et al., 2003; Dalsgaard et al., 2003). However, this group of microalgae was not recorded in the taxonomic analysis of MPB or *Z. noltii* epiphytes. HUFAs, such as EPA or DHA, are often biosynthesized *de novo* by primary producers (Veloza et al., 2006) and Moreno et al. (1979) have already shown that the diatom *Phaeodactylum tricornutum* is able to synthesize DHA *de novo*. This ability displayed by some diatoms can explain the high levels of DHA recorded especially in MPB.

The fatty acid profiles of *Z. noltii* displayed high levels of specific fatty acids, such as LA and ALA, which are frequently associated with the green leaves of these marine plants (Canuel et al., 1997; Kharlamenko et al., 2001; Sanina et al., 2008). Our data agree well with the results published by Kharlamenko et al. (2001), who showed that the sum of LA, ALA and palmitic acid comprised up to 80% of the total fatty acids in the green leaves of the seagrass *Zostera marina*. These fatty acids are highly relevant in trophic relationships, as both LA and ALA can be used as precursors for the synthesis of essential fatty acids by heterotrophic organisms (Dalsgaard et al., 2003; Veloza et al., 2006).

### 4.2. Fatty acid composition of *H. ulvae*

The fatty acid profiles of mud snails showed an extensive contribution of SFAs, MUFAs and HUFAs, either in specimens collected in the mudflat or the seagrass meadow, with PUFAs being the less abundant group of fatty acids present in *H. ulvae* from both locations. The comparison of mud snail fatty acid profiles among sites indicates that diatom-dominated MPB communities were clearly the major food source consumed by *H. ulvae* in the mudflat. The assumption that MPB assemblages represent an important nutritional component in *H. ulvae* diet has already been suggested in several studies (Fenchel et al., 1975; Blanchard et al., 2000; Haubois et al., 2005). However, to our knowledge, the present work is the first study providing analytical evidence of this trophic relationship based on fatty acid profiles.

Several gastropod species are known to feed by grazing the leaves of seagrasses (Stephenson et al., 1986; Kharlamenko et al., 2001), even though our study provided no evidence of direct bioaccumulation of fatty acids known to be characteristic signatures of *Z. noltii* in the mud snails. The hypothesis of bioconversion by *H. ulvae*, with precursors such as ALA, characteristic of *Z. noltii*, being elongated and desaturated to EPA or DHA, cannot be

discarded, as it has already been shown for other organisms (Dalsgaard et al., 2003; Veloza et al., 2006). Nevertheless, bioconversion is usually characterized to be a slow process and commonly does not meet the metabolic demands of consumers (Veloza et al., 2006), being unlikely that bioconversion can explain the significant levels of EPA and DHA recorded for *H. ulvae* sampled in the seagrass meadow. Moreover, the absence of evidence pointing towards a direct bioaccumulation of specific fatty acids from *Z. noltii* on mud snails also suggests that they are unable to incorporate such compounds or that the mud snails did not eat the leaves at all. The fatty acid profiles recorded should reflect the voluntary or involuntary ingestion of components of the epiphytic community associated with the seagrass (Philippart, 1995; Kharlamenko et al., 2001). The high levels of EPA measured in the mud snails from this habitat should thus be connected to the ingestion of the epiphytic diatoms, as supported by the fatty acid profile of epiphytes from *Z. noltii*.

The values observed for the EPA/DHA ratio in mud snails from both habitats are characteristic of marine invertebrates, namely molluscs, and provide evidence of which group of primary producers was the main food source of surveyed specimens (Biandolino et al., 2008). The present study showed that it was not possible to separate specimens from both sites according to EPA/DHA ratios. The specimens of *H. ulvae* collected from the mudflat displayed higher EPA/DHA ratios than the ones from the seagrass meadow, indicating the higher contribution of MPB to the levels of EPA recorded in the mud snails from this habitat.

In general, *H. ulvae* specimens collected from both habitats contained reduced levels of fatty acids known to be characteristic of marine or sediment bacteria, such as *iso*- or *anteiso* 17:0 (Kharlamenko et al., 2001). Nevertheless, significant levels of vaccenic acid, often associated with bacteria inhabiting marine sediments, were found on mud snails not only from the mudflat, but also from the seagrass meadow. Considering that neither MPB nor *Z. noltii* samples contained significant levels of these fatty acids, it seems that these gastropods ingest (voluntarily or involuntarily) significant numbers of bacteria during their feeding activity. The role that bacteria may play in the feeding ecology of marine organisms has already been clearly demonstrated (Kharlamenko et al., 1995; Pascal et al., 2008). Gastropods inhabiting estuarine habitats can ingest bacteria by grazing upon the bacteria-rich epiphyte community (Kirchman et al., 1984) (e.g. covering the leaves of seagrass) or simply from assimilated sediment (Pascal et al., 2008).

#### 4.3. Fatty acid as markers to differentiate estuarine habitats

A “perfect trophic marker” can be described as a compound that is not selectively processed during food uptake, is metabolically stable and contributes to a transfer from one trophic level to the next in a qualitative manner (Dalsgaard et al., 2003). In several situations, fatty acid markers are incorporated into higher trophic levels in a conservative way, providing useful information on trophic relationships (Dalsgaard et al., 2003; Iverson, 2009). Fatty acid markers are used to provide information on the dietary intake leading to the sequestering of stored lipids over time (Auel et al., 2002; Dalsgaard et al., 2003), contrasting with traditional analyses of simply monitoring gut content. However, this approach also has some limitations, as no individual fatty acid can be unequivocally assigned to a given species, and its dynamic is always linked with the metabolic and reproductive condition of the consumer (Dalsgaard et al., 2003). Despite such limitations, the fatty acid profiles exhibited by *H. ulvae* in the present study allowed us to clearly differentiate the estuarine habitats that they were occupying at the time of sampling. Within the same estuary, small-sized

motile organisms which are able to perform large-scale spatial movements (Haubois et al., 2002, 2004) and display a considerable trophic plasticity (Riera, 2010) (such as mud snails) can still present diagnosing fatty acid signatures. This feature allowed us to link the fatty acid profiles of *H. ulvae* to the main energy sources present in each sampled habitat and identify the existence of contrasting dietary regimes. The use of fatty acid profiles thus increases our understanding of the role played by motility in the trophic ecology of estuarine organisms at a small spatial scale.

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