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Fatty acid profiling reveals seasonal and spatial shifts in zooplankton diet in a temperate estuary

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ABSTRACT

Fatty acids composition of copepod and cladoceran species and their possible food sources was investigated in the Mondego estuary (southern Europe) in order to explain the seasonal variation of the small copepods *Acartia clausi*, *Acartia tonsa*, *Copidodiaptomus numidicus*, *Temora longicornis* and the freshwater cladoceran *Daphnia longispina*. A total of 12 zooplankton species (7 marine, 2 estuarine and 3 freshwater species) were studied. A multivariate analysis revealed a clear seasonal distribution of zooplankton species in terms of fatty acids composition and abundance, with winter and spring zooplankton species showing maximal concentrations and diversity of total fatty acids. These findings underline the role of lipids as storage during the colder seasons in a highly variable environment like an estuary. Estuarine and freshwater species showed a more diverse array of saturated and unsaturated fatty acids rather than marine species, except for *Centropages typicus*. Fatty acids markers of trophic position indicated the presence of two trophic levels: copepod species were primarily omnivorous, whereas cladocerans showed to be herbivorous. Our results suggest that feeding patterns of plankton change spatially and temporally, reflecting the shifts in dominance between diatoms and flagellates as well as between dinoflagellates/diatoms and small animals.

1. Introduction

Estuaries are transition zones between rivers and the sea and differ from both in terms of biotic (e.g. predation, competition) and abiotic conditions (e.g. temperature, salinity, food quantity and quality), showing more fluctuations than in marine or freshwater systems (David et al., 2005; Isari et al., 2007). Plankton (and mainly zooplankton) is known to be particularly sensitive to these variations because it is strongly influenced by climatic features and changes in hydrological conditions (Beaugrand et al., 2000; Ara, 2001). Copepods are the dominant group of mesozooplankton and play a key role in the food web as they form a link between primary producers and secondary consumers (Richmond et al., 2007; Guschina and Harwood, 2009). There are important differences between both zooplankton guilds, especially regarding their impact on the lower trophic levels, either directly via feeding or indirectly by influencing nutrient cycling (Hessen and Lyche, 1991; DeMott, 1995).

Studies on food web dynamics may provide important information to understand organisms' baseline ecology, predict community-level consequences of abiotic and biotic changes and characterize trophic interactions. Traditional studies on food web dynamics have used gut content analysis and direct field observations to elucidate various aspects of population dynamics and community structure. While a great deal of information may be gleaned, these approaches are labour intensive, logistically difficult and often ambiguous with regard to what was consumed and what was assimilated (Kelly and Scheibling, 2012). More recently, stable isotopes and lipid biomarkers (fatty acid analysis) have been used to identify specific food web relationships as they provide time-integrated information on an organism's assimilated diet (El-Sabaawi et al., 2009; Van den Meersche et al., 2009; Allan et al., 2010; Kelly and Scheibling, 2012). Stable isotopes are useful in assessing the sources of carbon in food web. However it is sometimes an ambiguous tracer of food source, particularly where the differences of different carbon sources are small (Van den Meersche et al., 2009). Fatty acid analysis is more specific to dietary sources

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than stable isotope ratios, alleviating some of the ambiguities that can result from using stable isotopes alone. Furthermore, their biological specificity and the fact that they are transferred from primary producers to higher trophic levels without change, make fatty acids suitable as biomarkers (Alfaro et al., 2006).

Fatty acids (FA) are one of the most important molecules transferred across the plant-animal interface in aquatic food webs (Dalsgaard et al., 2003; Allan et al., 2010). Moreover, the so-called essential compounds like highly unsaturated fatty acids (HUFA) (e.g. eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA)), play a key role in the health and function of all animals at all trophic levels, including plankton invertebrates, fish and humans. These HUFAs cannot be synthesized *de novo*, or at least not in sufficient amounts (Wacker and Von Elert, 2001; Arts et al., 2009). Furthermore, lipid components are very sensitive to stressors and environmental changes (Arts et al., 2009). Thus, large-scale processes such as eutrophication and global warming may act either independently, or together, leading to an overall decrease in HUFA production in aquatic ecosystems.

In recent decades the interest in fatty acid composition of aquatic organisms has increased. Knowledge on biochemical composition of copepod and cladoceran communities has become important to understand their physiological functions, metabolism and nutritive value, as this is very relevant for the energy transfer in aquatic ecosystems and secondary production.

Marine zooplankton, mainly marine copepod species, is a well-documented group due to their use as live feed for commercial species, especially marine fish (Dalsgaard et al., 2003; Arts et al., 2009; Perumal et al., 2010). However, the link between fatty acid profiles and the seasonal variation of plankton species in the field is still poorly known.

The present study was conducted in the Mondego estuary (Portugal), a typical Southern-European estuary that is well documented in terms of the effects of eutrophication on benthic communities (Pardal et al., 2000; Cardoso et al., 2004; Verdelhos et al., 2005), and on zooplankton distribution (Primo et al., 2009; Gonçalves et al., 2010a, b; 2012a, b; Falcão et al., 2011). Despite the extensive literature on the Mondego benthic and pelagic ecology, a more functional approach to trace spatio-temporal changes in energy transfer in the food web is lacking.

Therefore, it remains unclear how structural changes in species composition are linked to functional changes in species or in species' response to environmental changes. Up-to-date tracing techniques such as fatty acid profiling can contribute to answer these questions. Moreover, a better knowledge on the ecosystem functioning is crucial in order to predict the potential impact of future environmental changes. Hence a first attempt was made to determine and compare the fatty acids composition of 12 zooplankton species (7 marine, 2 estuarine and 3 freshwater species) collected at different stations and different seasons. This study focused on the characterization of zooplankton species fatty acid (FA) composition in an estuary in order to 1) interrogate dietary preferences of zooplankton species in relation to potential food sources and to 2) analyse spatial and temporal (seasonal) patterns in these FA profiles. The central hypothesis to test that the FA profile of zooplankton remains constant throughout the year and does not differ in different sites along an estuarine gradient.

2. Materials and methods

2.1. Study site

The Mondego estuary is a small mesotidal system covering an area of 8.6 km^2 along the West Atlantic coast of Portugal (40° 08′ N, 8° 50′ W) (Fig. 1). It comprises two channels, the so-called north and south arms, separated by the Murraceira island at about 7 km from the shore and joining again near the mouth. These represent different hydrological characteristics with the north arm being deeper (4–8 m during high tide, tidal range about 2–3 m), has a low residence time (<1 day) and is the location of the commercial harbour and the main navigation channel. The south arm is more shallow (2–4 m deep, during high tide), has higher residence times (2–8 days) and the water circulation is mainly dependent on the tides and on the freshwater input from a small tributary, the Pranto

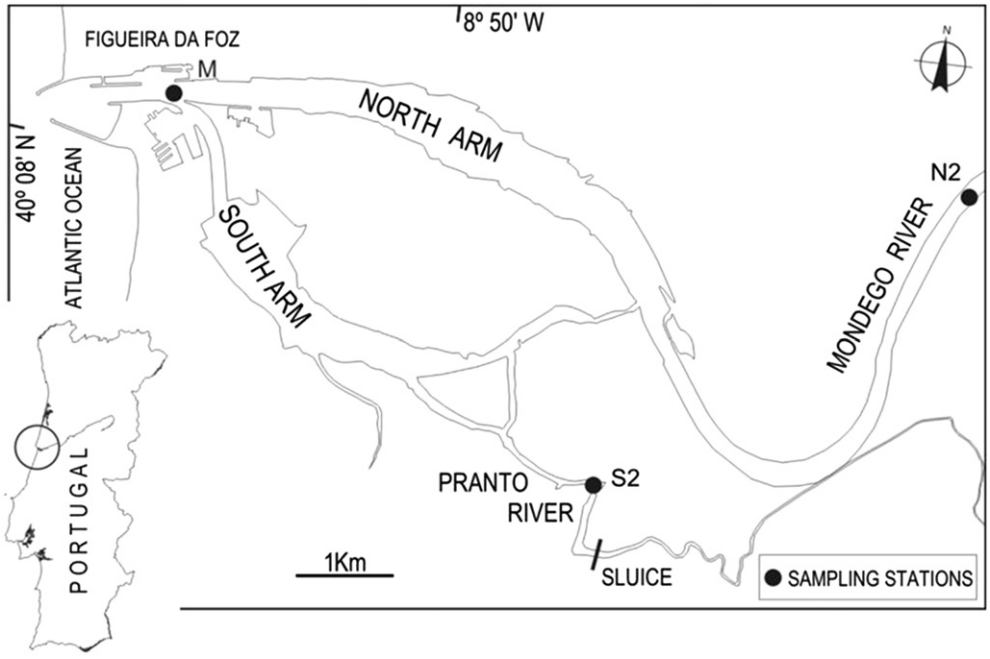

Fig. 1. Map of the Mondego estuary, located along the west coast of Portugal. Sampling stations are indicated (M – mouth station; S2 – southern arm station; N2 – northern arm station).

River. Freshwater discharge of this river is controlled by a sluice according to the water needs of the Mondego valley rice fields.

2.2. Sample collection and laboratory analyses

In the Mondego estuary, seasonal sampling (Winter – January; Spring – April; Summer –July; and Autumn – October) of zooplankton was performed, during 2010, at three stations (St M – mouth station; St N2 – station of the north arm; St S2 – station of the south arm) (Fig. 1). As seasons are clearly delineated in the study area, four sampling actions should cover any sharp seasonal pattern in the data. Samples were collected by horizontal subsurface tows (bongo net: mesh size 335 μm, mouth diameter: 0.5 m), equipped with a Hydro-Bios flowmeter. Zooplankton samples were brought to the laboratory, sorted alive at species level, concentrated on GF/F Whatman filters (25 mm diameter) and stored frozen at −80 °C in eppendorfs. For each species, 3 replicates containing 60 individuals each were prepared. Water samples were collected and filtered on GF/F Whatman filters (25 mm diameter) in each sampling station, at each sampling occasion (season) in order to quantify potential food sources (Fig. 2).

The extraction of total lipids of copepods and cladocerans and methylation to fatty acid methyl esters (FAMEs) for FA analysis was achieved by a modified one-step derivatisation method after Abdulkadir and Tsuchiya (2008). The boron trifluoride-methanol reagent was replaced by a 2.5% H_2SO_4-methanol solution since BF_3-methanol can cause artefacts or loss of PUFAs (Eder, 1995). The fatty acid Methylnonadecanoate C19:0 was added as an internal standard for the quantification (Fluka 74208). Samples were centrifuged (eppendorf Centrifuge 5810R) and vacuum dried (Rapid Vap LABCONCO). The FAMEs thus obtained were analysed using a Hewlet Packard 6890N GC with a mass spectrometer (HP 5973). All samples were run in splitless mode, with a 5 μL injection per run, at an injector temperature of 250 °C, using a HP88 column (60 m × 25 mm i.d., Df = 0.20; Agilent J & W; Agilent Co., USA) with He flow rate of 1.3 ml min^{-1}. The oven temperature was programmed at 50 °C for 2 min, followed by a ramp at 25 °C min^{-1} to 75 °C, then a second ramp at 2 °C min^{-1} to 230 °C with a final 4 min hold.

FAMEs were identified by comparison with the retention times and mass spectra of authentic standards and available ion spectra in WILEY mass spectral libraries, and analysed with the software Agilent MSD Productivity ChemStation. Quantification of individual FAMEs was accomplished by the use of external standards (Supelco™ 37 Component FAME Mix, Supelco # 47885, Sigma–Aldrich Inc., USA). The quantification function of each FAME was obtained by linear regression applied to the chromatographic peak areas and corresponding known concentrations of the standards (ranging from 5 to 250 μg ml^{-1}).

2.3. Data analysis

The FA profiles of zooplankton were reported for each season and sampling station, over a spatio-temporal scale, by determining

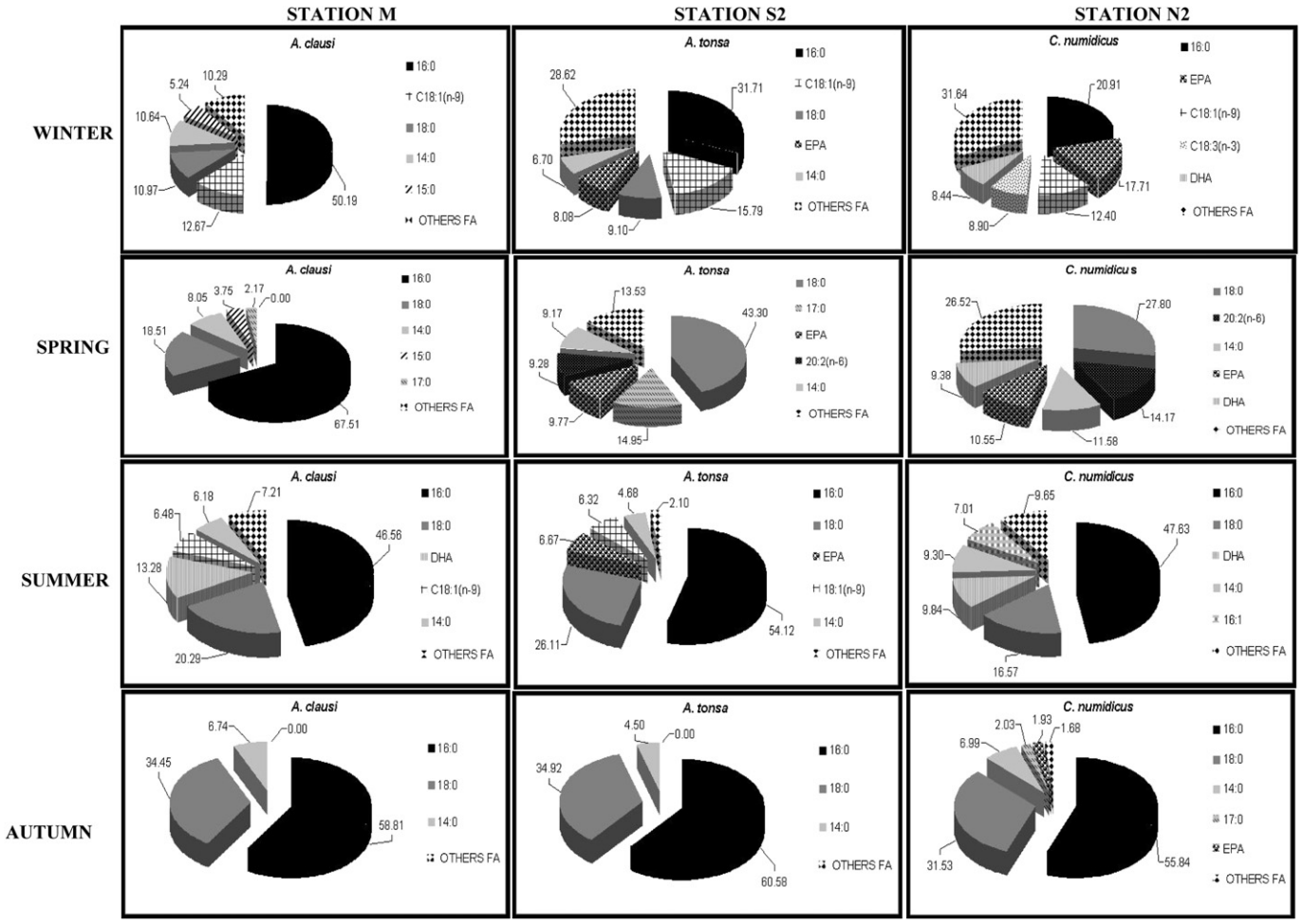

Fig. 2. Relative fatty acid composition (in %) of the 5 most abundant fatty acids in the 3 indicator species (*A. clausi*, *A. tonsa* and *C. numidicus*) of each sampling station (seasonal characterization).

their total (mg ind^{-1}) or relative (%) concentrations. Multivariate statistical analyses were carried out using PRIMER-6 software (Clarke and Gorley, 2006) in order to examine the variation in FA composition through non-metric multidimensional scaling (n-MDS) plots. The data were converted into similarity triangular matrices using a Bray–Curtis resemblance measure (Clarke and Warwick, 2001). One-way analysis of similarity (ANOSIM) was used to test differences in fatty acid profiles across the spatial and temporal factors (sampling station and season). A cluster analysis was conducted to assess the degree of similarity between FA samples, using the PRIMER statistical package (Clarke and Warwick, 2001). The contribution of individual FAs to similarities and dissimilarities within and between sample groups were tested using similarity percentage analysis routine (SIMPER). A Principal Component Analysis (PCA) was conducted on fatty acid trophic markers in order to highlight any seasonal, spatial or interspecific pattern of the zooplankton diet, using CANOCO version 4.5 (ter Braak and Smilauer, 1998).

2.4. Fatty acid trophic markers (FATMS)

Fatty acid ratios were calculated and used as biomarkers based on El-Sabaawi et al. (2009) to inspect whether animal, bacteria or algae class ratios were maintained in the lipid extracts of zooplankton species thus reflecting their trophic position and dietary quality. Typically, carnivorous zooplankton show higher quantities of polar lipids (rich in PUFA) than herbivorous crustaceans. Thus, the ratio PUFA (sum of all polyunsaturated fatty acids)/SFA (sum of all saturated fatty acids) denote carnivory in copepods (Cripps and Atkinson, 2000). Another index to determine the degree of carnivory was the ratio DHA/EPA (docosahexaenoic acid to eicosapentaenoic acid, 22:6n-3/20:5n-3) (Dalsgaard et al., 2003). DHA is highly conserved in food webs as it is an important component of polar lipids (Scott et al., 2002). Thus, the ratio DHA/EPA should increase towards higher trophic levels. Besides the use of DHA/EPA to determine carnivory, this ratio may also reflects the proportion of dinoflagellates and diatoms in the diets of omnivorous and herbivorous organisms as DHA is often dominant in dinoflagellates, whereas EPA is mainly found in diatoms (Dalsgaard et al., 2003). The proportion of all diatom markers (D = 16PUFA + 16:1n-7 + 20:5n-3) to all flagellate markers (F = 18PUFA + 18:2n-6+22:6n-3), D/F, was also used to distinguish between diatom and dinoflagellate-based diet (El-Sabaawi et al., 2009). High proportions of 18:2n-6 denote the presence of terrestrial detritus or green algae in zooplankton dietary (Dalsgaard et al., 2003). As bacteria biosynthesize large amounts of *iso* and ante-*iso* branched chains containing 15–17 carbons, the sum 15:0 + 17:0 was used to detect the presence of bacteria in the consumer's diet (Parkes, 1987; Vestal and White, 1989; Rajendran et al., 1994).

3. Results

3.1. Water samples analysis

Water samples were mainly composed of saturated fatty acids (SFA) as e.g. 16:0 and 18:0. SFA were found in highest concentrations (<0.08 mg ml^{-1}). C_{18} PUFAs (polyunsaturated fatty acids) were only found in very low concentrations (<0.01 mg ml^{-1}) in autumn samples collected in the southern arm station (St S2). Winter samples showed the highest levels of total SFA.

3.2. Zooplankton fatty acids composition – general patterns

Species for FA analysis were chosen based on their indicator value (based on densities) within each sampling station. Zooplankton species showed higher FA concentrations in winter and spring than in summer and autumn (Table 1). Indeed, in species that occurred all year round in the same sampling station (see Table 2), a sharp increase in FA concentration was observed from autumn to winter. In general, saturated and unsaturated fatty acids were predominant in estuarine and freshwater copepods than in the marine copepod species. The only exception to this finding was *Centropages typicus* since this species showed a similar fatty acid composition as the estuarine and freshwater species with high amounts of HUFA (5.77%). A similar pattern was observed in the cladoceran *Daphnia longispina* showing a higher diversity and

Table 1
Total fatty acid (FA) concentration (mean ± standard error, in mg/ind) extracted from each species in different seasons. The number below the line indicates the total number of FAs.

		Winter	Spring	Summer	Autumn
Marine species	*A. clausi*	0.02 ± 0.02 n = 12	0.01 ± 0.03 n = 5	0.01 ± 0.01 n = 7	0.01 ± 0.01 n = 3
	E. velox	–	–	–	0.02 ± 0.01 n = 11
	T. longicornis	–	–	0.01 ± 0.00 n = 11	0.01 ± 0.01 n = 7
	C. typicus	–	0.13 ± 0.07 n = 13	–	–
	E. nordmanni	–	–	0.01 ± 0.01 n = 10	–
	P. polyphemoides	–	–	0.01 ± 0.01 n = 10	
	E. nordmanni + *P. avirostris*	–	–	–	0.03 ± 0.04 n = 6
Estuarine species	*A. tonsa*	0.04 ± 0.01 n = 21	0.02 ± 0.02 n = 9	0.01 ± 0.01 n = 7	0.03 ± 0.04 n = 9
	C. aquae dulcis	–	0.01 ± 0.02 n = 7	0.01 ± 0.01 n = 10	–
Freshwater species	*C. numidicus*	0.03 ± 0.01 n = 35	0.05 ± 0.03 n = 11	0.01 ± 0.01 n = 8	0.01 ± 0.01 n = 6
	A. robustus	–	0.09 ± 0.04 n = 13	–	–
	D. longispina	0.05 ± 0.02 n = 46	0.04 ± 0.03 n = 10	–	0.01 ± 0.01 n = 7

Table 2
Relative fatty acid (FA) concentration (%) in all species at each station.

		A. clausi			*E. velox*			*T. longicornis*			*C. typicus*			*E. nordmanni*			*P. polyphemoides*			*E. nordmanni* + *P. avirostris*			*A. tonsa*			*C. aquae dulcis*			*C. numidicus*			*A. robustus*			*D. longispina*		
	Station	M	S2	N2	M	S2	N2	M	S2	N2	M	S2	N2	M	S2	N2	M	S2	N2	M	S2	N2	M	S2	N2	M	S2	N2	M	S2	N2	M	S2	N2	M	S2	N2	M	S2	N2
SFA	14:0	0.34	–	–	–	–	0.06	0.12	–	–	0.81	–	–	0.05	–	–	0.04	–	–	0.10	–	–	–	0.41	0.08	–	0.13	0.03	0.00	–	0.77	–	–	0.22	0.00	0.03	0.14			
	15:0	0.14	–	–	–	–	0.01	0.01	–	–	0.10	–	–	0.01	–	–	0.01	–	–	0.02	–	–	–	0.13	0.01	–	0.05	0.01	0.00	–	0.15	–	–	0.12	0.00	0.22	0.19			
	16:0	2.13	–	–	–	–	0.54	0.58	–	–	2.59	–	–	0.25	–	–	0.32	–	–	0.75	–	–	–	2.39	1.06	–	1.19	0.19	0.81	–	2.01	–	–	2.23	1.13	2.46	1.20			
	17:0	0.06	–	–	–	–	0.02	0.04	–	–	0.09	–	–	0.01	–	–	0.01	–	–	0.03	–	–	–	0.09	0.03	–	0.03	0.01	0.04	–	0.16	–	–	0.10	0.08	0.17	0.14			
	18:0	0.63	–	–	–	–	0.32	0.20	–	–	0.69	–	–	0.08	–	–	0.08	–	–	0.78	–	–	–	0.82	1.48	–	0.48	0.05	0.19	–	0.93	–	–	0.70	0.50	0.80	0.66			
	20:0	0.01	–	–	–	–	0.00	0.00	–	–	0.04	–	–	0.00	–	–	0.00	–	–	0.00	–	–	–	0.02	0.00	–	0.00	0.00	0.01	–	0.02	–	–	0.05	0.02	0.00	0.01			
	22:0	0.01	–	–	–	–	0.00	0.00	–	–	0.00	–	–	0.00	–	–	0.00	–	–	0.00	–	–	–	0.01	0.00	–	0.00	0.00	0.00	–	0.02	–	–	0.00	0.02	0.04	0.00			
	24:0	0.00	–	–	–	–	0.00	0.00	–	–	0.00	–	–	0.00	–	–	0.00	–	–	0.00	–	–	–	0.01	0.00	–	0.00	0.00	0.00	–	0.02	–	–	0.00	0.02	0.04	0.01			
MUFA	16:1	0.05	–	–	–	–	0.03	0.02	–	–	0.95	–	–	0.03	–	–	0.03	–	–	0.00	–	–	–	0.41	0.00	–	0.20	0.02	0.60	–	1.53	–	–	0.47	0.29	2.12	1.42			
	17:1	0.03	–	–	–	–	0.00	0.00	–	–	0.00	–	–	0.00	–	–	0.00	–	–	0.00	–	–	–	0.02	0.00	–	0.00	0.00	0.00	–	0.05	–	–	0.00	0.04	0.00	0.02			
	18:1(n- 9)	0.23	–	–	–	–	0.03	0.01	–	–	0.95	–	–	0.02	–	–	0.03	–	–	0.00	–	–	–	0.77	0.00	–	0.18	0.00	0.32	–	1.07	–	–	1.40	0.39	0.47	1.71			
	18:1(n-9)	0.03	–	–	–	–	0.02	0.02	–	–	0.00	–	–	0.03	–	–	0.06	–	–	0.00	–	–	–	0.03	0.00	–	0.00	0.00	0.00	–	0.08	–	–	0.00	0.10	1.66	0.00			
	20:1(n-9)	0.00	–	–	–	–	0.00	0.00	–	–	0.00	–	–	0.00	–	–	0.00	–	–	0.00	–	–	–	0.02	0.00	–	0.00	0.00	0.00	–	0.02	–	–	0.09	0.00	0.03	0.00			
	22:1(n-9)	0.00	–	–	–	–	0.00	0.00	–	–	0.00	–	–	0.00	–	–	0.00	–	–	0.00	–	–	–	0.01	0.00	–	0.00	0.00	0.00	–	0.00	–	–	0.00	0.00	0.00	0.00			
	24:1(n-6)	0.00	–	–	–	–	0.00	0.00	–	–	0.21	–	–	0.00	–	–	0.00	–	–	0.00	–	–	–	0.02	0.00	–	0.00	0.00	0.00	–	0.04	–	–	0.00	0.00	0.00	0.00			
PUFA	18:2(n-6)	0.00	–	–	–	–	0.00	0.01	–	–	0.00	–	–	0.00	–	–	0.00	–	–	0.00	–	–	–	0.05	0.00	–	0.00	0.00	0.00	–	0.02	–	–	0.00	0.02	0.00	0.00			
	18:2(n-6)	0.02	–	–	–	–	0.01	0.00	–	–	0.40	–	–	0.00	–	–	0.00	–	–	0.02	–	–	–	0.14	0.04	–	0.00	0.00	0.12	–	0.45	–	–	0.62	0.06	0.69	0.40			
	18:3(n-3)	0.00	–	–	–	–	0.00	0.00	–	–	0.26	–	–	0.00	–	–	0.00	–	–	0.00	–	–	–	0.18	0.00	–	0.00	0.00	0.12	–	0.69	–	–	1.03	0.02	0.10	0.67			
	18:3(n-6)	0.00	–	–	–	–	0.00	0.00	–	–	0.00	–	–	0.00	–	–	0.00	–	–	0.00	–	–	–	0.01	0.00	–	0.00	0.00	0.01	–	0.03	–	–	0.00	0.00	0.17	0.04			
	20:2(n-6)	0.00	–	–	–	–	0.00	0.00	–	–	0.00	–	–	0.00	–	–	0.00	–	–	0.00	–	–	–	0.02	0.00	–	0.00	0.00	0.00	–	0.02	–	–	0.00	0.00	0.00	0.01			
	20:3(n-6)	0.00	–	–	–	–	0.00	0.00	–	–	0.00	–	–	0.00	–	–	0.00	–	–	0.00	–	–	–	0.00	0.00	–	0.00	0.00	0.00	–	0.00	–	–	0.00	0.00	0.02	0.00			
HUFA	ARA - 20: 4(n-6)	0.00	–	–	–	–	0.00	0.00	–	–	0.00	–	–	0.00	–	–	0.00	–	–	0.00	–	–	–	0.03	0.00	–	0.00	0.00	0.03	–	0.01	–	–	0.00	0.00	0.51	0.16			
	EPA - 20: 5 (n-3)	0.06	–	–	–	–	0.08	0.10	–	–	2.14	–	–	0.11	–	–	0.14	–	–	0.00	–	–	–	0.51	0.00	–	0.00	0.00	0.10	–	1.51	–	–	0.48	0.01	0.05	0.98			
	DHA - C22: 6(n-3)	0.06	–	–	–	–	0.11	0.16	–	–	3.63	–	–	0.07	–	–	0.11	–	–	0.00	–	–	–	0.18	0.00	–	0.00	0.00	0.00	–	0.90	–	–	1.14	0.00	0.07	0.00			
	n	**14**	–	–	–	–	**11**	**11**	–	–	**13**	–	–	**10**	–	–	**10**	–	–	**6**	–	–	–	**23**	**6**	–	**7**	**6**	**13**	–	**22**	–	–	**13**	**14**	**18**	**16**			

concentration of total FA (SFA = 7.86%; MUFA = 8.25%; PUFA = 2.2% and HUFA = 1.78%), while the marine cladoceran species showed a lower share of fatty acids (<1%) (Table 2). Furthermore, the SFA were mainly composed of mixtures of 14:0; 16:0 and 18:0 and represented about 35% of the total lipid composition. Copepod species showed higher quantities of n-3 PUFA than cladoceran species, whereas *D. longispina* accumulated more ARA (eicosatetraenoic acid, 20:4n-6) (Table 2).

Fig. 1 showed that saturated fatty acids (14:0, 16:0, 18:0) mainly dominated the FA profile of *Acartia clausi*. The SFA 15:0 and 17:0 as indicators of bacterial feeding (Dalsgaard et al., 2003; El-Sabaawi et al., 2009) were found in small amounts (<5.3%) in winter and spring. The monounsaturated fatty acid (MUFA) content was higher in winter than in summer, whereas the highly unsaturated fatty acid DHA recorded the highest value in summer (13.28%). The fatty acid composition of *Acartia tonsa* was dominated by the same MUFA and SFA as *A. clausi* plus 17:0 (14.95%) in spring. EPA was found in all seasons, except for autumn, whereas the polyunsaturated fatty acid 20:2(n-6) represented less than 10% in spring. The calanoid copepod *Copidodiaptomus numidicus* was the indicator species with the highest concentrations of FA. The SFA and MUFA composition of *C. numidicus* resembled that of *A. tonsa*. The polyunsaturated fatty acids 20:2(n-6), 18:3(n-3), DHA and EPA were found in a specific season, or along the year but showed a large seasonal variation. The huge seasonal variability in FA composition was noticeable for the three indicator species (*A. clausi*, *A. tonsa* and *C. numidicus*). In winter and spring the three species showed the highest richness of fatty acids, followed by summer. In autumn the SFA 14:0, 16:0 and 18:0 were the main FAs present in the three species. Indeed, autumn was the season where species revealed the poorest FA composition, with *C. numidicus* showing a slightly higher richness in FA composition than both *Acartia* species. In addition, *C. numidicus* was the species with the highest quantities of FA, mainly MUFA and PUFA, with EPA and DHA being part of the top 5 FAs across the year.

3.3. Multivariate analysis of the zooplankton assemblages

Cluster analysis (Fig. 3) separated zooplankton species into two groups: Group I included only samples from winter and spring, consisting of species with higher concentrations and diversity of FA. Group II represented species with lower FA levels and lower FA diversity. This group was subdivided in two smaller groups: group IIA representing summer and autumn samples, whereas in group IIB species from the four seasons were pooled. The species from Group I were grouped based on their higher concentrations of 16:0, 16:1, 18:0, 18:1(n-9), 18:2(n-6) and EPA, while the species of Group II were characterized by higher concentrations of 14:0, 16:0 and 18:0 (see Table 2). The species *Acartia tonsa*, *Copidodiaptomus numidicus* and *Daphnia longispina* presented a high affinity with species of both groups due to the specificity of FA profiles that characterized both groups (I and II). The results of multivariate analysis showed a clear significant difference in the FA composition of the zooplankton community, defining two major groups (Fig. 4).

The n-MDS plot (Fig. 4) revealed a clear seasonal distribution of the sampled species based on FA composition and concentration (stress = 0.05). Group A represented the winter and spring samples, whereas in group B all species occurring in summer and autumn were found. ANOSIM analysis indicated a clear separation of the groups defined ($R = 0.505$; $p = 0.001$). Regarding pairwise differences, almost all seasons were significantly different ($p < 0.05$) and present a high *R*-value, indicating a good segregation of groups (winter/summer: $R = 0.928$; $p = 0.001$; winter/autumn: $R = 0.668$; $p = 0.002$; spring/summer: $R = 0.806$; $p = 0.001$; spring/autumn: $R = 0.474$; $p = 0.004$). However, non-significant differences were also found between winter-spring ($R = -0.023$; $p = 0.476$) and summer-autumn ($R = 0.142$; $p = 0.068$). Since some of these species occurred in all seasons, differences between groups appeared to be mainly the result of the variations in FA composition and concentration. SIMPER analysis showed that in winter/spring 7 FA (in decreasing order of importance: 16:0; 18:0; 18:1(n-9); 16:1; EPA; 14:0; 18:2(n-6)) explained 80% of the group similarity, whereas in

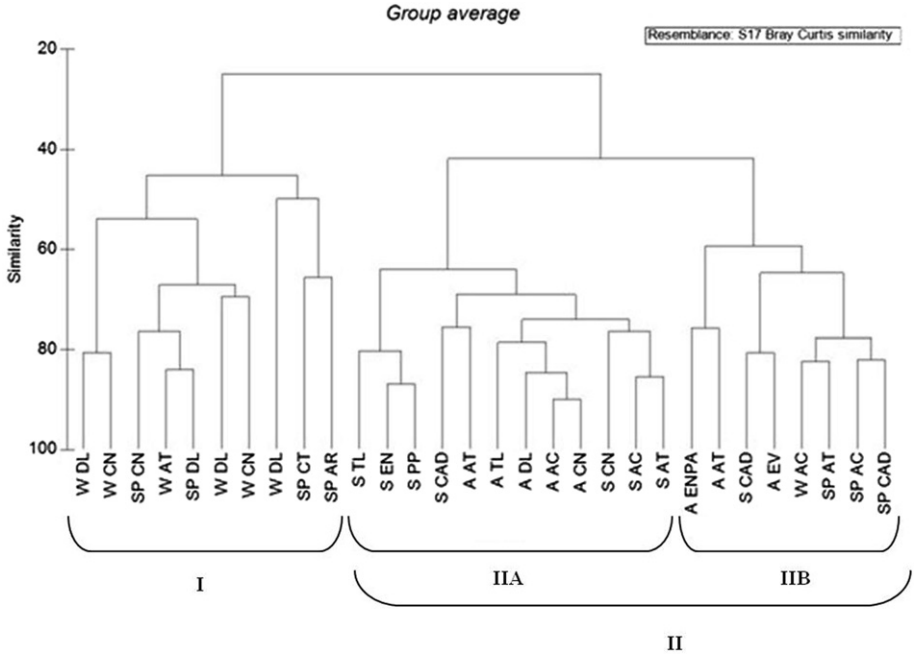

Fig. 3. Cladogram (cluster analysis) grouping zooplankton species based on their total fatty acid composition in different seasons and sampling stations. The two groups (and subgroups) are indicated by roman numbers.

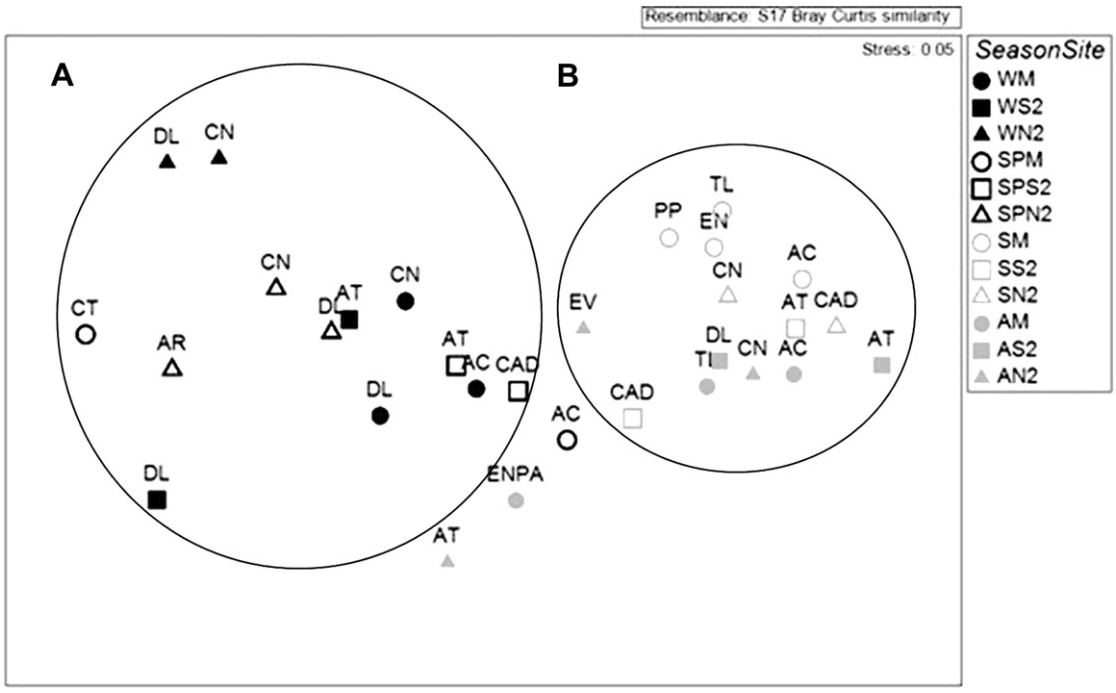

Fig. 4. Two-dimensional non-metric MDS ordination plot of fatty acid composition of zooplankton species at the Mondego estuary. Black symbols represent winter and spring sampling species; grey symbols represent summer and autumn sampling species at three stations (mouth station - St M; station of the south arm - St S2 and station of the north arm - St N2) of the estuary. A and B are the different groups defined in the MDS. AC – *Acartia clausi*; AT – *Acartia tonsa*; CT – *Centropages typicus*; TL – *Temora longicornis*; EV – *Eurytemora velox*; AR – *Acanthocyclops robustus*; CN – *Copidodiaptomus numidicus*; CAD – *Calanipeda aquae dulcis*; DL – *Daphnia longispina*; EN – *Evadne nordmanni*; PP – *Podon polyphemoides*; ENPA – *Evadne nordmanni* + *Penilia avirostris*.

summer/autumn 3 FA (in decreasing order of importance: 16:0, 18:0, 14:0) explained 59.2% of the similarity (Table 3). Furthermore, 72.1% of the dissimilarity between groups was explained by the contribution of the following FA, in decreasing order of importance: 16:0; 16:1; 18:1(n-9); 18:0; EPA, DHA; 18:3(n-3); 14:0; 18:2(n-6).

3.4. Dietary fatty acid biomarkers

Apart from a few exceptions, the PCA biplot (Fig. 5) showed that copepod species collected in spring are related to a biomarker for carnivory (PUFA/SFA), whereas species collected in autumn and summer show a low correlation with the fatty acid (FA) markers of bacteria and terrestrial detritus/presence of green algae (18:2n-6) and 15:0 + 17:0. The first PCA axis (eigenvalue = 0.521) showed to be mainly defined by FATMS of carnivory, bacteria and terrestrial detritus or green algae (PUFA/SFA; 15:0 + 17:0; 18:2n-6), whereas the second axis was more related to carnivory (DHA/EPA). FATMS of herbivory (*D*/*F*) appear on the negative side of the second axis (Fig. 5A).

The Cladoceran species did not show any high correlation with FATMS of carnivory (Fig. 5B). The first axis of the PCA (Fig. 5B, eigenvalue = 0.560) showed to be mainly related with FATMS of

Table 3
Results of SIMPER analysis showing average similarity within the samples groups and average dissimilarity between samples groups, according to MDS analysis.

MDS groups	Similarity	Fatty acid	Av. % abund.		Av. Sim.	Sim/SD	Contrib. %	Cum. %
W + SP	50.81	16:0	28		19.23	1.30	37.85	37.85
		18:0	9		7.21	2.61	14.19	52.04
		18:1(n-9)	13		7.09	1.62	13.96	66.00
		16:1	14		6.22	1.31	12.24	78.24
		EPA	10		2.61	0.71	5.13	83.37
		14:0	4		1.98	0.85	3.89	87.26
		18:2(n-6)	5		1.57	0.93	3.09	90.35
S + A	59.18	16:0	9		34.78	3.18	58.78	58.78
		18:0	6		14.68	2.26	24.80	83.58
		14:0	1		4.40	2.59	7.44	91.02
	Dissimilarity		W + SP	S + A	Av. Diss.	Diss/SD		
W + SP & S + A	72.11	16:0	28	9	18.25	2.04	25.31	25.31
		16:1	14	0	10.07	1.51	13.96	39.28
		18:1(n-9)	13	0	9.95	1.97	13.80	53.08
		18:0	9	6	6.42	1.31	8.91	61.98
		EPA	10	1	6.13	1.08	8.50	70.49
		DHA	10	1	4.72	0.68	6.54	77.03
		18:3(n-3)	5	0	3.54	0.96	4.91	81.94
		14:0	4	1	3.16	1.48	4.38	86.32
		18:2(n-6)	5	0	3.06	1.37	4.25	90.57

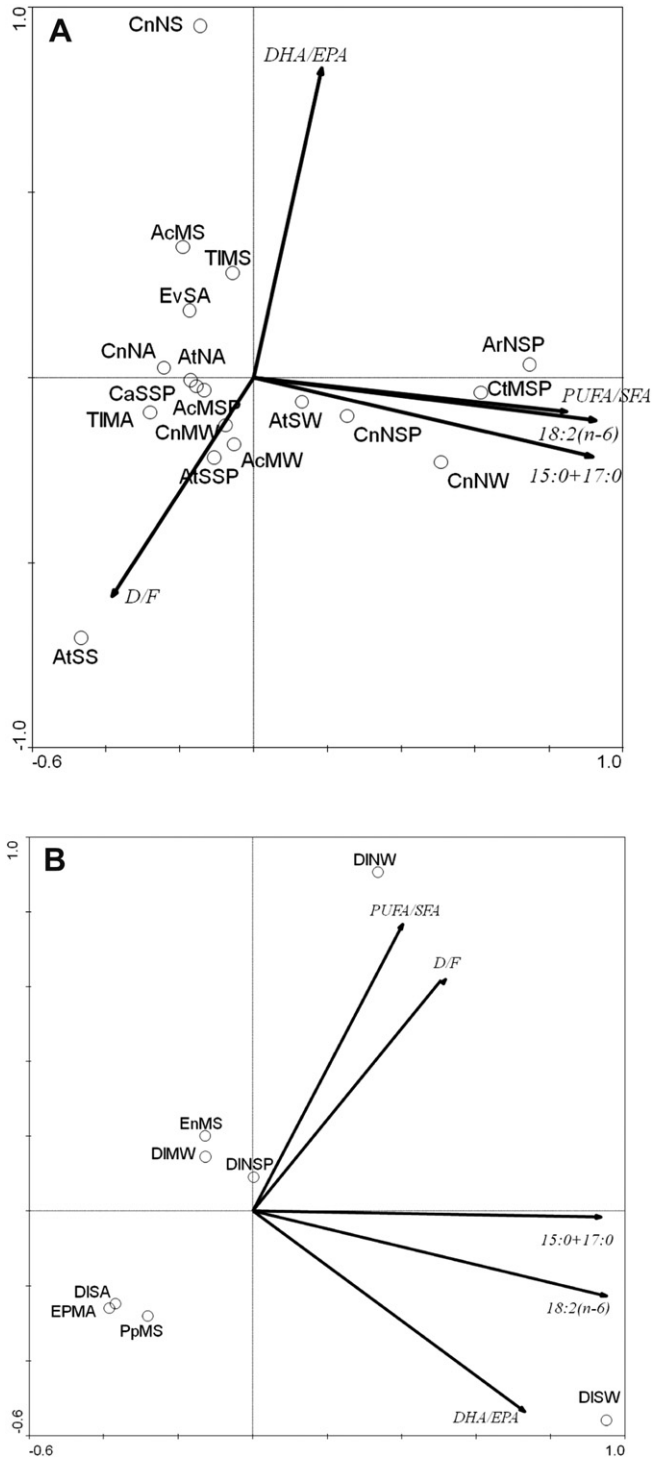

Fig. 5. Principal component analysis (PCA) of fatty acid trophic markers (FATMS) for copepods (A) and cladocerans (B) collected in winter, spring, summer and autumn at three stations (mouth station - St M; station of the south arm - St S2 and station of the north arm - St N2) of the estuary. CnNS – *Copidodiaptomus numidicus* St N2 Summer; CnNA – *C. numidicus* St N2 Autumn; CnMW – *C. numidicus* St M Winter; CnNSP – *C. numidicus* St N2 Spring; CnNW – *C. numidicus* St N2 Winter; AcMS – *Acartia clausi* St M Summer; AcMSP – *A. clausi* St M Spring; AcMW – *A. clausi* St M Winter; AtNA – *Acartia tonsa* St N2 Autumn; AtSSP – *A. tonsa* St S2 Spring; AtSW – *A. tonsa* St S2 Winter; TlMS – *Temora longicornis* St M Summer; TlMA - *T. longicornis* St M Autumn; EvSA – *Eurytemora velox* St S2 Autumn; CaSSP – *Calanipeda aquae dulcis* St S2 Spring; ArNSP – *Acanthocyclops robustus* St N2 Spring; CtMSP – *Centropages typicus* St M Spring; DlNW – *Daphnia longispina* St N2 Winter; DlMW – *D. longispina* St M Winter; DlNSP – *D. longispina* St N2 Spring; DISA – *D. longispina* St S2 Autumn; DlSW – *D. longispina* St S2 Winter; EPMA – *Evadne nordmanni* + *Penilia avirostris* St M Autumn; PpMS – *Podon polyphemoides* St M Summer; EnMS – *Evadne nordmanni* St M Summer.

bacteria, terrestrial detritus or green algae and presence of dinoflagellates/diatoms (15:0 + 17:0; 18:2n-6; DHA/EPA); the second axis was mostly defined related with fatty acid markers of carnivory and consumption of diatoms/flagellates (PUFA/SFA and *D*/*F*). The first two axis of PCA accounted for 74.5% and 82.8% of the total variance of copepod and cladoceran species' diet, respectively. Two fatty acid markers of herbivory (DHA/EPA; *D*/*F*) are significantly correlated with *Daphnia longispina* collected in winter at St S2 and St N2.

These data are corroborated by ratios of FATMS (see Table 4). The majority of small copepods are omnivorous feeding on small animals and consuming diatoms or flagellates, which is evidenced by the increase of DHA/EPA and the decline or increase of *D*/*F*, respectively (Table 4). The dietary quality varies during the year which seems to be dependent on food availability in a specific period of the year. On the other hand, the ratio DHA/EPA and *D*/*F* both pointed at the increased diatom consumption by cladoceran species (Table 4) and DHA/EPA displayed an opposite pattern for *Daphnia longispina* in winter at station S2. There were no observable differences in the proportion of bacterial and green algae markers among the different species as their contribution to the diet was in general low.

In terms of spatial variability, at the mouth station, during the warmer months, the decline of *D*/*F* reflects the change in dominance from diatoms to flagellates, whereas in winter and autumn an opposite trend was observed. At the station of the southern arm (St S2), copepods mainly consumed diatoms in summer and spring months, while flagellates were the main food item in the other months. Based on these biomarkers, *Acartia tonsa* showed diverse dietary feeding including also small animals. At the station of the north arm (St N2), in winter and spring months, mainly diatoms and small animals were consumed by copepods. An exception was observed for *Acanthocyclops robustus* as its FA profile mainly reflected a diet composed of flagellates rather than diatoms Table 4).

4. Discussion

In the present study, fatty acid profiling allowed us to interrogate seasonal and spatial changes in functional (feeding) behaviour of zooplankton species in the Mondego estuary. In the winter and spring, the species showed the highest concentration and diversity of fatty acids, pointing at the high availability of food quality and the storage behaviour of the zooplankton. These high FA concentration and diversity in winter were followed by lower concentrations in species collected in spring and summer. Autumn had the lowest diversity of fatty acids although species collected in autumn showed a higher concentration of fatty acids than in summer. This seasonal pattern in FA profiles is probably largely linked to the seasonal fluxes in availability of potential food sources. Moreover, the FA composition of a particular species varies between seasons but also among stations, within the same season. For instance, *Daphnia longispina* showed different lipid composition from station to station, during the same season, but also among seasons. This could point at different food sources that are available in the stations or it may be due to different feeding behaviour related to the environmental conditions in the stations. Although the data do not allow disentangling both alternatives, some species show more variability and thus flexibility in their food uptake. In most species, the seasonal differences govern the feeding response and strategy while in other cases (e.g. *D. longispina*) it is the spatial pattern that is of main importance.

Also combined spatial and seasonal differences were found as species in the south arm station (St S2) showed higher concentrations of FAs in winter and spring, while in summer and autumn

Table 4
Seasonal interspecific differences in fatty acid trophic markers (FATMS) for copepods and cladocerans from the Mondego estuary.

	Marine Copepod species									Estuarine Copepod species							Freshwater Copepod species							Marine cladoceran species						Freshwater cladoceran species					
	A. clausi			*E. velox*		*T. longicornis*		*C. typicus*		*A. tonsa*						*C. aquae dulcis*	*C. numidicus*						*A. robustus*	*E. nordmanni*		*P. polyphemoides*		*E. nordmanni + P. avirostris*	*D. longispina*					
Station	M			S2		M		M		S2			N2			S2	M	N2		S		N2	M		M	M		M	S2		N2		
Season	W	SP	S	A	S	M	A	SP		SP	S	A	S	SP	A	SP	W	W	SP	S	A	SP	S	S	A	A		W	W	A	W	SP	
DHA/EPA	0.00	0.00	2.17	1.36	2.08	0.29	1.69	0.62	0.00	0.06	0.00	0.00	0.00	0.00	0.00	0.00	0.48	0.66	6.02	0.00	2.39	0.67	0.82	0.00		0.00	2.39	0.00	0.00	0.00				
18:2(n-6)	0.01	0.00	0.00	0.00	0.00	0.00	0.10	0.03	0.01	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.05	0.06	0.00	0.00	0.15	0.00	0.00	0.00		0.02	0.17	0.00	0.05	0.04				
15:0 + 17:0	0.03	0.02	0.00	0.01	0.01	0.00	0.04	0.03	0.02	0.00	0.01	0.02	0.00	0.00	0.00	0.02	0.01	0.04	0.03	0.00	0.00	0.00	0.00	0.01		0.02	0.08	0.02	0.05	0.03				
PUFA/SFA	0.04	0.00	0.26	0.20	0.65	0.05	1.49	0.45	0.17	0.08	0.01	0.00	0.36	1.96	0.79	0.15	0.02	0.95	0.47	0.53	0.01		0.07	0.49	0.04	2.78	0.33						
D/F	1.99	0.00	0.46	0.88	0.53	3.50	0.66	0.79	3.44	15.81	0.00	0.00	1.92	1.35	1.03	0.88	0.00	0.28	1.86	0.00			1.63	1.25	0.00	1.77	1.28						

higher concentrations were found in the marine station at the mouth of the estuary (St M). This shows that seasonal feeding by zooplankton species is less clear in the marine part than in other study sites of the estuary in winter and spring. This also suggests that the prey is less abundant and/or less diverse in FA composition during these seasons. This may explain the relatively low percentage of mono and polyunsaturated fatty acids in marine zooplankton species in contrast to the estuarine and freshwater species. The same patterns were found when focussing on the 5 most abundant FA in each indicator species. The marine copepod species *Acartia clausi* was mainly characterized by SFA, whereas the estuarine and freshwater species (*Acartia tonsa* and *Copidodiaptomus numidicus*, respectively) contained high quantities of MUFA, PUFA and HUFA. The higher amounts of n-3 HUFA in copepod species than in the cladoceran species may be explained by their ability to adjust their n-3HUFA to temperature variations, whereas cladocerans cannot modify their n-3 PUFA content (Farkas, 1979). These variations and low levels of some fatty acids components in zooplankton species can also partly be linked to the poor fatty acid composition of water samples which highlights the low contribution of water contents to food webs in the estuary studied.

The present study also highlights taxon-specific seasonal feeding patterns and provides a first attempt to infer about feeding strategies of different planktonic species. Copepods typically showed to feed on small animals and diatoms or flagellates according to food availability, pointing at omnivory. On the other hand, cladoceran species fed mainly on diatom species, showing an herbivorous feeding behaviour as efficient filter feeders. Indeed, Bacillariophyceae (diatoms) and Dynophyta (dinoflagellates) are the main groups of phytoplankton in the Mondego estuary, showing highest abundances in summer and spring (Vieira et al., 2002) and cladocerans seem to respond well to that. Bacteria, diatoms, flagellates and small animals are generally reported as part of the diet of copepod and cladoceran species (Dalsgaard et al., 2003; Samchyshyna, 2008; Arts et al., 2009; El-Sabaawi et al., 2009). Consequently, zooplankton organisms are typically classified as herbivores or omnivores, according to their diet in a particular site and/or season. Although classified as omnivores (Dalsgaard et al., 2003; Arts et al., 2009), copepods are known to actively select and catch their food particles based on particle size and nutritive value (Tackx et al., 1989; Adrian and Schneider-Olt, 1999). According to DeMott (1988) and Cotonnec et al. (2001) the food selection by copepods, for instance in the case of phytoplankton cells, depends on the algal group and the grazer species and can be defined by a selective index – ranging from weakly, intermediate to highly selective - according to the discriminatory behaviour. Still, the major fatty acid component of the copepod is closely related to the selected food particles as the FA profile is a reflection of the consumed food ('you are what you eat' principle). However, one should keep in mind that the selection of a certain food source also depends on the availability of this food source. Therefore, this selective index can largely depend on the study site and on the time of the year. For instance, Cotonnec et al. (2001) referred *Temora longicornis* to be more selective than *Acartia clausi*, whereas DeMott (1988) found *T. longicornis* to be weakly selective and *A. clausi* intermediate. Our results suggest a similar selectivity by both species, showing no large difference in FA composition. However, it is not always clear from FA profiles which food sources contributed most to the final FA pool of the consumer. As stated above it will depend on the food availability and in addition, it can be expected that the FA content of various food items are incorporated differently by the consumer. The calanoid *Calanipeda aquae dulcis* was the only copepod species that did not revealed any significant difference among the trophic markers studied and neither a large diversity in fatty acids composition was

found. Indeed, SFA, within the range C_{10}–C_{18} are the dominant fatty acids in this freshwater copepod species' composition, plus small quantities of MUFA, whereas no PUFA was detected. Thus, our findings suggest that *C. aquae dulcis* may ingest bacteria, incorporating the FA commonly biosynthesized by bacteria (Dalsgaard et al., 2003).

Samchyshyna (2008) reported that despite of their classification as herbivores, filterfeeders or carnivores, some calanoids also include bacteria and detritus into their diet. Furthermore, in Chesapeake Bay, some copepod species had the ability to ingest suspended detritus and sediments with the associated microfauna (Roman et al., 2001).

Small cladocerans have the ability to feed on a wide range of particle types and sizes, showing unselective food preferences (Geller and Müller, 1981; Gophen and Geller, 1984). It is well documented that Cladocera such as *Daphnia* can filter a heterogeneous mixture of bacteria, algae and organic detritus that constitute an adequate source of food that could vary in the relative proportions of the components (Arnold, 1971). The increase of DHA/EPA in *Daphnia longispina* observed in winter at the station of the south arm could be related to a higher amount of organic detritus filtered from the water. Of course, species also take combinations of these different food sources but the different grazing behaviour of cladocerans and copepods may have important and contrasting impacts on the FA profile of the mesozooplankton itself as well as of the phytoplankton community, affecting, at last, the zooplankton growth. Indeed, the concentration of essential PUFAs can limit zooplankton growth as they are unable to synthesize these fatty acids at significant rates (Bell and Tocher, 2009; Perumal et al., 2010). Some copepods such as *Acartia clausi* and *Temora longicornis* fed on younger stages of copepods, phytoplankton and detritus (Kattner et al., 1981). However, the species ingested by these or other copepods may be composed of different fatty acids composition which may explain the spatio-temporal variations between copepod species.

In addition to species-specific FA profiles that may reflect different food utilisation, Mclusky and Elliot (2004) stated that turbidity and tidal currents can limit phytoplankton production and thus food availability for zooplankton species in estuaries. Furthermore, David et al. (2005) found in the Gironde estuary (France), the temporal variations of some copepod species were indirectly controlled by the high suspended particulate matter (SPM) levels, and therefore turbidity. Thus, these two features are potentially responsible for a lower primary production and have severe consequences for consumers like copepods. According to our previous work (Gonçalves et al., 2010b) there is a close correlation between SPM, associated with other environmental parameters, and zooplankton community structure. Our findings may suggest that these environmental conditions may not be nutritionally favourable for some zooplankton species, whereas other species have the ability to prosper and fed within these conditions.

Zooplankton is one of the main subsystems in water bodies. It regulates the cycling of nutrients and sedimentation and serves as a link in the energy transfer from phytoplankton to the highest trophic levels. Any changes in the composition and functioning of the zooplankton community affect the state of the whole community (David et al., 2006). Spatiotemporal variation and habitat types are among the most important factors affecting patterns of species abundance, composition and size structure of estuarine plankton (Gonçalves et al., 2010a,b). In the Mondego estuary, zooplankton assemblages are mainly influenced by hydrological circulation pattern and direct and indirect human impacts that occur in each arm of the estuary (Gonçalves et al., 2010a, b; 2012a, b). As in other marine coastal areas copepods dominate the mesozooplankton in the Mondego estuary. The stability of the system is based on its ability to deal with these natural variable conditions (Lobry et al., 2008). Differences in the hydrodynamic of both arms of the Mondego estuary maintain the estuary in a non-climax state, being the origin of the resilience of the estuarine system to natural and anthropogenic perturbations (Dolbeth et al., 2007; Lobry et al., 2008). Further studies are required to assess the extent to which the structure of communities translates into the trophic functioning of the subsystem. Thus, a central theory of the stability of estuarine ecosystems may be achieved.

5. Conclusion

Our work is a first attempt to give an overview of fatty acids composition of zooplankton species present in a temperate shallow estuarine system in southern Europe. Since zooplankton forms an essential link between primary producers and higher trophic levels (Dalsgaard et al., 2003), explaining the functional role of the first trophic levels will be a pivotal contribution to understand trophic relationships in the Mondego estuary. As lipids are sensitive and good indicators of stress (Arts et al., 2009) and seasonality (present work), they constitute a valuable tool to monitor natural variability in ecosystem functioning i.e. energy fluxes in the present context. Moreover, the use of trophic biomarkers such as FA allows to disentangle the effects of this natural variability from the effects of potential threats (e.g. climate change, eutrophication, contaminants or invasive species).

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