Vol. 497: 93–103, 2014
doi: 10.3354/meps10569

MARINE ECOLOGY PROGRESS SERIES
Mar Ecol Prog Ser

Published February 5

Simultaneous analysis of $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ ratios uncovers food web relationships and the trophic importance of epiphytes in an eelgrass *Zostera marina* community

Agnes Mittermayr*, Thomas Hansen, Ulrich Sommer

GEOMAR Helmholtz Centre for Ocean Research Kiel, Düsternbooker Weg 20, 24105 Kiel, Germany

ABSTRACT: Simultaneous analysis of carbon, nitrogen and sulphur stable isotope ratios was applied in this pilot study to examine the food web of a *Zostera marina* L. system in the western Baltic Sea. Samples of 3 potential food sources: eelgrass, epiphytic algae and seston, as well as 69 consumer species were collected during the growing season of *Z. marina* from March to September 2011. The measured $\delta^{13}C$ values of epiphytes were close to $\delta^{13}C$ values of eelgrass, impeding a clear distinction of those 2 carbon sources, whereas seston $\delta^{13}C$ values were clearly different. This frequently encountered problem was solved by the additional use of $\delta^{34}S$, which resulted in easily distinguishable values for sediment and seawater derived sulphur. The combination of $\delta^{34}S$ and $\delta^{13}C$ values made a separation of carbon sources possible and enabled the allocation of potential food sources to consumers and a description of their trophic relationships. The results of stable isotope ratio analysis of this eelgrass community strongly indicate a food web based on epiphyte and seston production. $\delta^{15}N$ values show a food web consisting of large numbers of generalists and a high degree of omnivory amongst the consumer species analysed. This implies an occupation of every trophic position possible, which is supported by an even distribution of $\delta^{15}N$ values. Previously described eelgrass food webs may have to be re-evaluated by considering sulphur stable isotope ratios in order to provide a clear picture on primary carbon sources.

KEY WORDS: Stable isotopes · Food web · Seagrass · Epiphytes · Trophic level

INTRODUCTION

Seagrass meadows are widely distributed in coastal zones throughout the world and are one of the most productive autotrophic communities on earth (Hemminga & Duarte 2000). They provide nurseries for economically important fish and shellfish, improve water quality by taking up dissolved nutrients from the water column, and stabilize the sediment (Williams & Heck 2001, Orth et al. 2006). While there is no doubt about the important role of seagrass as a habitat for animals (Douglass et al. 2010 and references therein), its role as a nutritional source for invertebrates, which in turn are food sources for many higher trophic level species, is still controversial. Seagrasses in temperate systems have been found to make little or no contribution to macroinvertebrate diet; instead, mesograzers feed primarily on attached epiphytic algae (Valentine & Duffy 2006, Ouisse et al. 2012). According to Nelson (1997), fresh eelgrass (*Zostera marina*) is not a favoured food source. Instead, its epiphytes are the preferred food source for many animals in seagrass communities. In contrast, Reynolds et al. (2012), Connolly et al. (2005) and Kharlamenko et al. (2001) demonstrated the importance of seagrass

*Corresponding author: amittermayr@geomar.de

production to diets of a variety of smaller invertebrates living in seagrass meadows. These contradictions may be a result of inherent limitations in the methods used, such as direct observation, gut content analysis and feeding experiments, which provide only a snapshot in time and do not necessarily capture the long-term behaviour of consumers.

Therefore, the use of techniques to track feeding relationships via biomass composition has gained importance. Stable isotopes give time-integrated information and the use of stable isotope ratio analysis has proved to be successful in tracing food webs. $\delta^{13}C$ and $\delta^{15}N$ analyses are standard in food web studies and their use is well documented (review by Grey 2006). $\delta^{13}C$ values have been mainly used to identify the primary carbon sources of food webs, while $\delta^{15}N$ values are used to determine the trophic level. This application of stable carbon and nitrogen isotopes is possible because of their different fractionation factors: fractionation of ^{13}C during the trophic transfer is weak: 0.5 ± 0.5‰ (mean ± SD; France & Peters 1997, Jaschinski et al. 2011b), whereas ^{15}N is fractionated heavily: approx. 3 to 4‰ (Peterson & Fry 1987, Vander Zanden & Rasmussen 1999). More recent studies (McCutchan et al. 2003, Nordström et al. 2009) show a fractionation of 2.4 ± 0.5‰ for the first trophic step, followed by a larger 3.4 ± 0.5‰ for the second step and the following carnivores.

The frequently observed similarity between $\delta^{13}C$ values of seagrass and epiphytes has impeded a distinction between these 2 carbon sources (Connolly et al. 2005, Jaschinski et al. 2008a). The comparison of sulphur isotope ratios offers a solution to this frequently encountered problem. Epiphytes obtain sulphur mostly from seawater sulphate while seagrass leaves (Kharlamenko et al. 2001, Moncreiff & Sullivan 2001) at least partially obtain their sulphur from the interstitial waters of the sediment. The $\delta^{34}S$ stable isotope value of sulphate in the water column is 21‰ (Grey & Deines 2005), compared to $\delta^{34}S$ values of 1‰ of reduced sulphur (H_2S^-) derived from depleted sediment pore water (Hansen et al. 2009). The trophic shift for sulphur is assumed to be negligible (McCutchan et al. 2003, Michener & Kaufman 2007). It is generally assumed that producers that mainly utilize seawater sulphates (e.g. phytoplankton) tend to be enriched in ^{34}S and producers that gain the necessary sulphur from sediments (e.g. seagrass) are depleted in ^{34}S (Kharlamenko et al. 2001, Michener & Kaufman 2007). Based on this, a distinction between the sulphur isotopic signature of seagrass and its epiphytes is possible.

The goal of this pilot study was to resolve the trophic structure and feeding relationships of a *Zostera marina* food web and to quantify the contribution of primary carbon sources to the diet of consumers, ranging from herbivores and filter feeders to carnivorous invertebrates and fish, including both pelagic and benthic organisms. Even though the potential of $\delta^{34}S$ stable isotope analyses in coastal marine ecosystems is known, the extensive methodological problems have rarely been tackled. The simultaneous triple stable isotope analysis of C, N and S developed by Hansen et al. (2009) is a unique and comprehensive approach to reveal carbon fluxes and food web structures of marine systems which are characterized by benthic-pelagic coupling, which we employ here for the first time.

MATERIALS AND METHODS

Study area and sample collection

The collection site (Fig. 1) was an eelgrass *Zostera marina* meadow alongside Falckenstein Beach in the inner Kiel Fjord, Germany (54° 21′ N, 10° 9′ E). The Kiel Fjord is located in the Kiel Bight and part of the western Baltic Sea. The eelgrass meadow covers an area of 23 ha and is interrupted by small, unvegetated patches (Bobsien 2006). During the sampling period (March to September 2011) salinity in this area ranged between 13 and 18, depending on season, discharge rates (wastewater and rivers) and prevailing winds. The sediment is sandy (grain sizes 0.5–1 mm: 42%, >1 mm: 51%) (Jaschinski et al. 2011a) and organic matter (e.g. epipsammic microalgae) content is low (<1%) (Jaschinski et al. 2008a). The studied seagrass system is typical of a well-flushed eelgrass meadow, where eelgrass detritus does not accumulate. Frequent video and diving observations by members of the GEOMAR Helmholtz Centre for Ocean Research Kiel have shown that decomposed eelgrass gets flushed out to deeper deposition zones. Astronomical tides are negligible in the Kiel Fjord, although storm events can cause changes in sea-level.

Sample collection

Samples of seston, zooplankton, eelgrass, attached epiphytes, macrozoobenthic organisms and fish species were analysed in this study. Since phyto-

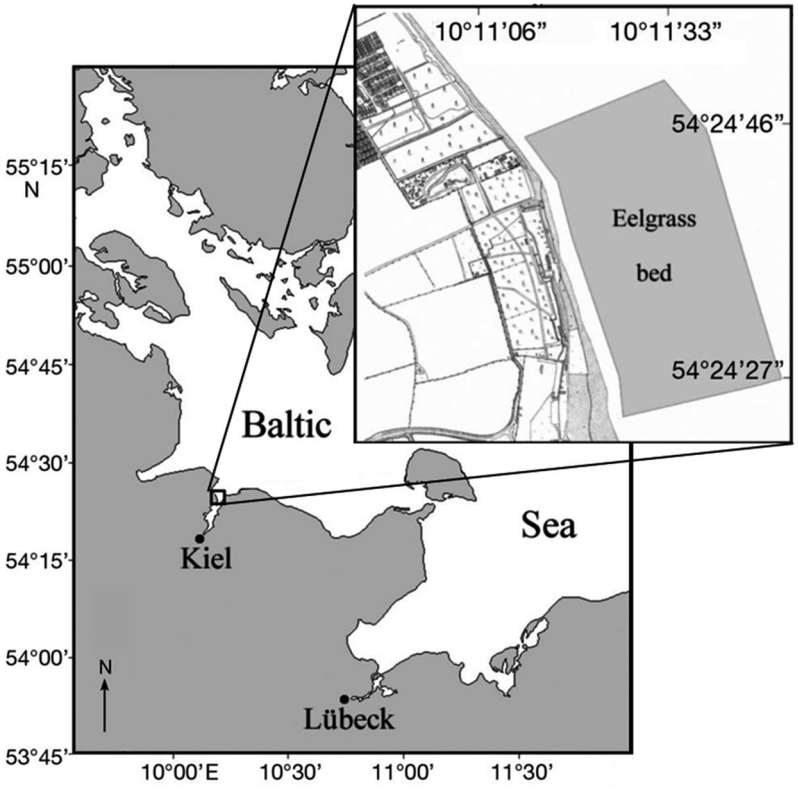

Fig. 1. Study area for investigation of food web relations in an eelgrass *Zostera marina* community in the outer Kiel Fjord, Baltic Sea, Germany (Bobsien 2006)

plankton cannot be separated reliably from similar sized heterotrophic or detrital particulate organic carbon for stable isotope analysis, seston was treated as a proxy for phytoplankton, even though they are not identical and seston also contains bacteria and protozoa. Samples were collected biweekly from March to September 2011. All phytoplankton samples were collected in 3 m water depth. Zooplankton was sampled by vertical plankton net hauls (mesh size 150 µm) from a depth of 3 m. Eelgrass, attached epiphytes and macrozoobenthos were collected with a Van Veen grab sampler (opening: 1000 cm^2) from depths of 0.5 to 10.8 m. Minimum numbers of vertebrates (fish) required for sufficient statistical power were seined manually with a beach seine with a mesh size of 0.5 cm (length: 8 m, height: 1 m). The sampled animals and plants were collected in plastic containers with water from the sampling site and transported to the laboratory for identification and further processing. A total of 73 species were identified over the entire sampling season.

Sample processing

In the laboratory, epiphytes were carefully scraped off eelgrass blades into distilled water with plastic scrapers and desiccated in small watch glasses. Seston samples were filtered onto 0.8 µm cellulose acetate filters (Sartorius) and carefully scraped off into distilled water with plastic cell scrapers and desiccated in small watch glasses. This procedure bypassed the use of GF/F filters (Ouisse et al. 2012) and further minimized the amount of sample needed for analysis. Zooplankton samples were transferred directly into tin cups (3.2 × 4 mm, Hekatech) filled with distilled water. All consumer species were kept in filtered seawater overnight to ensure gut evacuation before being thoroughly rinsed in distilled water and fixed in liquid nitrogen. For bivalves and fish only muscle tissue was analysed, while other species were processed as whole organisms.

All samples were dried to constant weight (60°C, 48 h) and animal and plant samples were ground with an agate mortar and pestle as fine as possible and then stored in airtight glass vials. Shells of gastropods, bivalves and Asteroidae were removed as completely as possible. Acidification of samples was omitted because the effects of acid washing on $\delta^{15}N$ are still controversial. Bunn et al. (1995) showed changes in stable isotope signature, particularly in $\delta^{15}N$ after acid washing, while Bosley & Wainright (1999) found no significant effects on $\delta^{15}N$ or $\delta^{13}C$ when analysing a decapod species. According to Carabel et al. (2006), acid washing is not necessary for seaweeds and fish muscle tissue, while a decrease in $\delta^{15}N$ in organisms including carbonated structures was found after acid washing. According to Jaschinski et al. (2008b), samples should only be acidified if absolutely necessary because nitrogen in exoskeletons originates from the diet and may be relevant in the determination of food sources. Therefore acidifying subsamples for $\delta^{13}C$ analysis was also not considered, as the simultaneous analysis of $\delta^{13}C$, $\delta^{15}N$ and $\delta^{34}S$ ratios was at the core of this study.

Subsamples were weighed in tin cups at 0.05 mg for animal and 0.25 mg for plant material. To facili-

tate complete combustion, ~0.25 mg vanadium pentoxide (V_2O_5) was added to every sample (Hansen et al. 2009). Individuals with too little dry weight were pooled across sampling events.

Stable isotope analysis

A total of 723 samples were analysed. $\delta^{15}N$, $\delta^{13}C$ and $\delta^{34}S$ ratios of all samples were measured simultaneously. Samples were combusted in an elemental analyser system (NA 110, Thermo) connected to a temperature controlled gas chromatography oven (SRI 9300, SRI Instruments), connected to an isotope ratio mass spectrometer (DeltaPlus Advantage, Thermo Fisher Scientific) as described by Hansen et al. (2009). $\delta^{15}N$, $\delta^{13}C$ and $\delta^{34}S$ ratios were calculated as:

$$\delta X\,(‰) = [(R_{sample}/R_{standard}) - 1] \times 1000 \quad (1)$$

where X is $\delta^{15}N$, $\delta^{13}C$ or $\delta^{34}S$ and $R = {}^{15}N/{}^{14}N$, ${}^{13}C/{}^{12}C$ or ${}^{34}S/{}^{32}S$. N_2, CO_2 and SO_2 gases were used as reference gases and were calibrated against International Atomic Energy Agency (IAEA) reference standards (S1, S2, NBS123, NBS127, NBS600). Cadmiumsulphide (CdS), 2,5-bis[5-(tert-butyl)-benzoxazol-2-yl) thiophene (BBOT), acetanilide and hay powder were used as internal standards after every sixth sample to test if the analytical setup was working properly. The overall precision for hay was ±0.47‰ for $\delta^{15}N$ and $\delta^{34}S$ and ±0.12‰ for $\delta^{13}C$. To determine nutrient sources, the mixing model MixSIR (Semmens & Moore 2008) was used. MixSIR is a graphical user interface (GUI) built on MATLAB that employs an algorithm based on a Bayesian framework to determine the probability distributions for proportional contributions of each source to the mixture (Semmens & Moore 2008). This model allows for allocation of different fractionation values ±SD for each element and source respectively and thus accounts for uncertainty in isotope values when estimating contributions of sources. A 0.5 ± 0.5‰ (mean ± SD) fractionation increase was chosen for $\delta^{13}C$, and 2.4 ± 1.1 and 3.4 ± 1.1‰ fractionation increases in $\delta^{15}N$ for the first and following trophic level transfers respectively. Fractionation for $\delta^{34}S$ was assumed to be zero (Peterson & Fry 1987, Michener & Kaufman 2007).

Seston, epiphytes and *Zostera marina* were used as ultimate carbon sources for all consumers. As the carbon of these primary producers travels to the top of the food web, it passes through herbivorous, omnivorous and carnivorous consumers alike. The trophic position of these consumers was calculated as:

$$TL = 1 + \Sigma(TL_S \times C_S) \quad (2)$$

where TL is the trophic level of the consumer, TL_S is the trophic level of the source and C_S is the contribution of the source to the food mix. This equation avoids direct usage of the trophic enrichment factor as in the Hobson & Welch (1992) equation. Instead the trophic enrichment is embedded in MixSIR.

Statistical analysis

Differences in stable isotope signatures were tested using multivariate and univariate methods. A non-metric multidimensional scaling (nMDS) and a 1-way analysis of similarity (ANOSIM) were conducted using the PRIMER 5 software package (Clarke & Warwick 2001). The nMDS test was used to determine similarities among species belonging to the same families using a data matrix with all 3 stable isotope ratios. The test of similarity was performed for the following pairs: *Mya arenaria* and *M. truncata*, *Mytilus edulis* and *M. galloprovinicialis*, *Pomatoschistus microps* and *P. minutus*, *Syngnathus typhle* and *S. rostellatus* and all genera of Maxillopoda collected. The nMDS stress values were between 0 and 0.09, which represents a good 2-dimensional ordination with no real prospect of misinterpretation (Clarke & Warwick 2001). Additionally a 1-way ANOVA was conducted (using 'R' statistical computing software Version 2.12.0), which showed no significant statistical differences ($p > 0.17$) between species belonging to the groups identified by the nMDS. Therefore, we used the following collective categories for further analysis: *Mya* spp., *Mytilus* spp., Gobiidae, Syngnathidae and zooplankton.

The nMDS test was also used to determine similarities between *Zostera marina* roots and rhizomes. Fig. 2 shows a non-clustered plot with evenly scattered data (stress value: 0.07). Therefore, *Z. marina* roots and rhizomes were treated as one group of *Z. marina* below-ground biomass. Below- and above-ground biomass were treated as 2 separate source groups as they represent 2 distinct food sources for infaunal and epibenthic fauna respectively.

The ANOSIM was used to test for seasonal differences in all 3 stable isotope ratio compositions within a species or higher taxon. Values from each sampling date were used. ANOSIM tests for dissimilarities and produces an R-value and a p-value. The R-value is scaled from −1 to 1. $R < 0.25$ indicate groups (sampling dates) that are barely different, $R > 0.5$ suggests groups are clearly different but may be overlapping and $R > 0.75$ indicates well-separated groups (Ja-

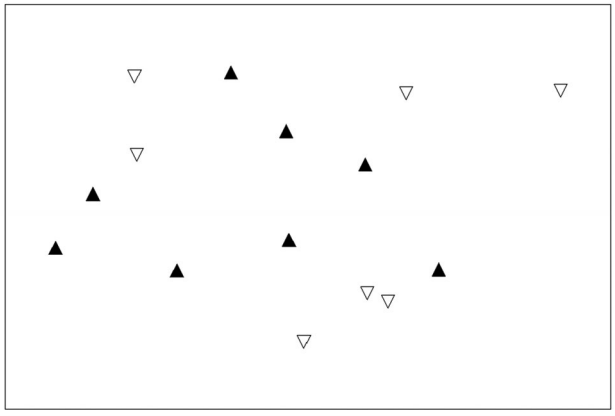

Fig. 2. Non-metric multidimensional scaling (nMDS) plot. Positioning of *Zostera marina* roots (▽) and rhizomes (▲) is based on a similarity matrix calculated with $\delta^{15}N$, $\delta^{13}C$ and $\delta^{34}S$ values. Axes are dimensionless and proximity (nearness in space between any 2 points) indicates that samples are similar in isotopic makeup (stress value = 0.07)

schinski et al. 2011a). Negative R values indicate high variation between replicates (Clarke & Warwick 2001). The p-value is similar to that of ANOVA with $p < 0.05$ indicating significance of the corresponding R-value. Groups of species or higher taxa showing no significant seasonality (e.g. high p-value, low R-value) were pooled before running MixSIR. ANOVA was used to evaluate C:N ratios.

RESULTS

Primary producer groups

Zostera marina below-ground biomass showed highly significant seasonal differences in $\delta^{15}N$ values (global R = 0.79, $p < 0.01$) across the entire sampling period (Fig. 3). *Z. marina* leaves also exhibited significant seasonality in $\delta^{15}N$ values (global R = 0.83, p = 0.02) and highly significant seasonality in $\delta^{13}C$ values

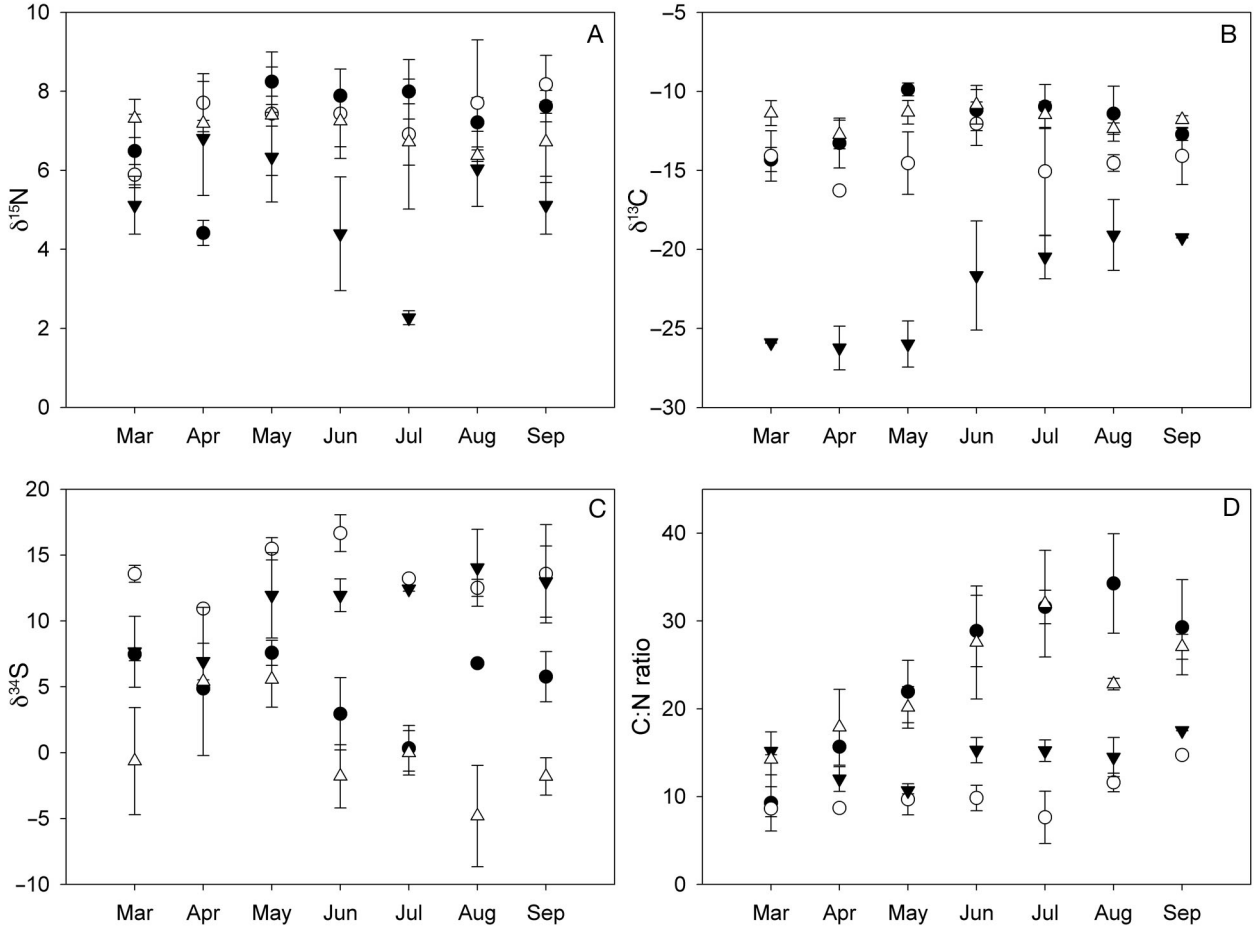

Fig. 3. Seasonal variation (mean ± SD) of the stable isotopes (A) $\delta^{15}N$, (B) $\delta^{13}C$ and (C) $\delta^{34}S$, and of (D) C:N ratios in (●) *Zostera marina* leaves, (△) *Z. marina* below ground biomass, (▼) seston and (○) epiphytes

(global R = 0.76, p < 0.01). Molar C:N ratios (Fig. 3) in leaves increased from 9.28 ± 0.49 (mean ± SD) on the first sampling day in March, to 35.01 ± 5.63 in the first week of August before starting to decline. A similar, but not significant, increase in molar C:N ratios (Fig. 3) was found in *Z. marina* below-ground biomass.

Seston samples showed no significant seasonality although a slight increase in all 3 stable isotope ratios was detected. Molar C:N ratios started to increase in August, correlating with a nearly 5-fold increase of carbon content from August to the end of September while particulate sulphur and nitrogen remained constant across the sampling period (Fig. 3). Similar observations were made for epiphytes: increasing C:N ratios starting in August and constant particulate sulphur and nitrogen values. Stable isotope ratios remained constant, molar C:N ratios started to increase in July, coinciding with an increase in carbon content, which doubled until the end of the sampling period, and contents of S and N remained stable.

ANOSIM showed highly significant separation of *Zostera marina* from seston (global R = 0.72, p < 0.01) and from epiphytes (global R = 0.55, p < 0.01).

Consumer groups

Stable isotope ratios of zooplankton were significantly similar among sampling dates (global R = 0.22, p < 0.01). Significantly higher C:N ratios were detected during June and July, when carbon concentration peaked at 8.26 ± 3.65 µg before decreasing to 1.2 ± 0.12 µg of carbon per individual over the course of 6 wk.

Stable isotope ratios in bivalves showed no significant seasonal changes. C:N ratios, however, changed significantly in *Cerastoderma edule* (r^2 = 0.85, p < 0.01) and *Mytilus* spp. (r^2 = 0.77, p < 0.01) over the course of seasons. Elevated C:N ratios coincided with maxima of the carbon biomass of these species. No seasonal effects could be found in other bivalve species because the required numbers of specimens could not be obtained over the sampling period to detect them. Similar observations were made for *Asterias rubens* and *Littorina littorea*; these species showed no seasonality in stable isotope ratios but elevated C:N ratios coinciding with maximal carbon biomass by the end of the sampling period.

In our study community, *Littorina littorea* had the highest biomass of all animal species per area. The 4 other gastropod species sampled (see Table S1 in the Supplement at www.int-res.com/articles/suppl/m497p093_supp.pdf) were of no quantitative importance. *L. littorea*, usually considered an epiphyte grazer, was mainly found on shells of *Mytilus* spp. covered by barnacles (*Amphibalanus improvisus*). The model results showed that *L. littorea* obtains ~55% of its carbon from *A. improvisus* and the remaining ~45% originate from cirripedia larvae. A distinction between adult barnacles and their larvae was possible as larval $\delta^{34}S$ values (20.19‰) are more pelagic-influenced than than those of adults (17.5‰) and larvae also show lower $\delta^{15}N$ values (larvae: 6.88‰, adults: 8.65‰). Epiphytes were of very low importance for *L. littorea* (less than 1% contribution to the food mix).

Idotea balthica showed significant seasonality in stable isotope ratios (global R = 0.21, p = 0.01), which can be traced back to a slight but steady increase in $\delta^{13}C$ and $\delta^{34}S$ values over the sampling period. Significant differences (r^2= 0.65, p = 0.01) could also be detected in molar C:N ratios, which increased towards the end of the sampling period. Other Malacostraca, e.g. *Praunus flexuosus* and *Gammarus locusta*, showed a similar change in stable isotope ratios (global R = 0.68, p < 0.01 and global R = 0.73, p < 0.01 respectively). C:N ratios also increased during this time (r^2 = 0.69, p = 0.01 and r^2 = 0.61, p < 0.01 respectively).

All polychaete species exhibited R-values below 0 (–0.04 to –0.11), when testing for similarity over time using stable isotope ratios, which indicates high variation between replicates (Chapman & Underwood 1999). In contrast, molar C:N ratios, of *Nephthys hombergii* and *Nereis pelagica* showed highly significant (r^2= 0.48, p < 0.01 and r^2 = 0.62, p < 0.01 respectively) peaks at the end of August which can be ascribed to an increase in carbon biomass while nitrogen biomass remained stable.

Fish generally showed a small increase in $\delta^{15}N$ values with progressing seasons, albeit not significant. C:N ratios of *Syngnathus* spp. and *Nerophis ophidion* significantly increased over the sampling period (r^2 = 0.82, p < 0.01 and r^2 = 0.82, p < 0.01 respectively).

Diet composition estimated by the mixing model

The mixing model was applied to consumer species and their respective food sources shown in Fig. 4. Seston comprised 100% of zooplankton diet (Fig. 4, Table 1). *Idotea balthica* showed the strongest dependence on epiphytes as a primary carbon source (52 to 62% contribution), with planktonic sources accounting for most of the remainder (36 to 46%). Suspension feeders such as Anthozoa and Porifera

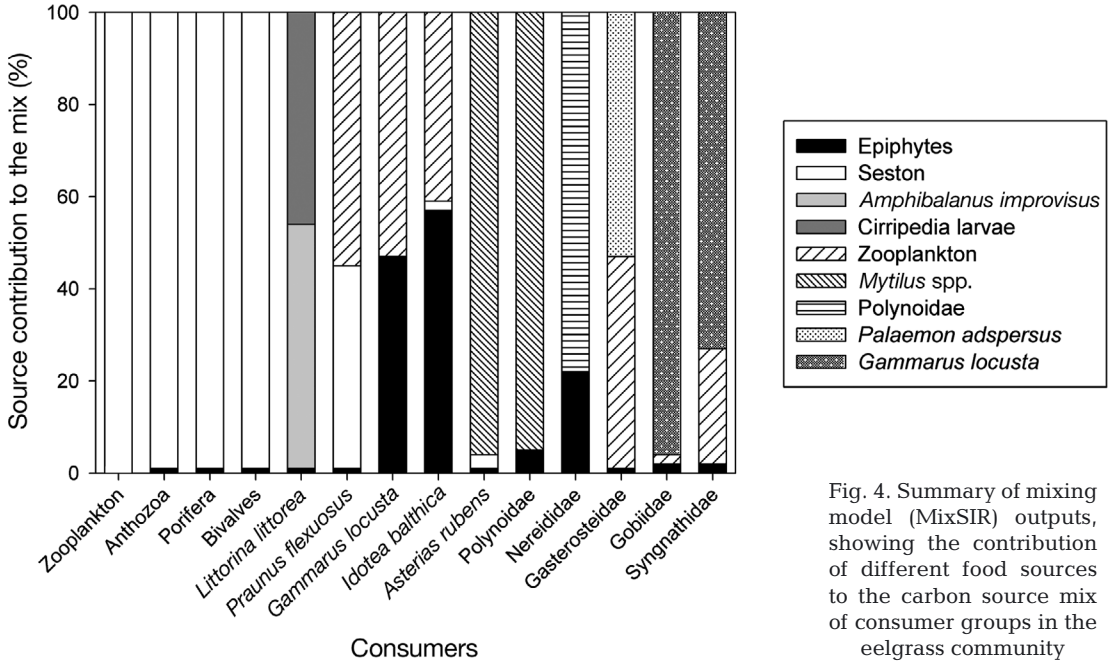

Fig. 4. Summary of mixing model (MixSIR) outputs, showing the contribution of different food sources to the carbon source mix of consumer groups in the eelgrass community

mainly fed on seston, and the epiphyte contribution was <5% in both cases (Table 1). Food sources determined for bivalves, which were dominated by *Mytilus* spp., showed no significant differences between species, and comprised 99% seston. Malacostraca fed partially on zooplankton, but there were also significant contributions by ephiphytes to the diet of *Gammarus locusta*, and by seston to the diets of *Praunus flexuosus* and *Crangon crangon. Asterias rubens* fed almost exclusively on *Mytilus* spp. (Table 1). Model outputs for polychaetes indicated other invertebrates as the main food source. When testing species-specific models, bivalves emerged to be the main food source of Polynoidae, which in turn, were the main food source of Nereididae. Fish depended on Malacostraca for roughly two-thirds of their diet and zooplankton for the remaining third (Table 1) in general, but Gasterosteidae specifically preferred *Palaemon adspersus,* whereas Gobiidae and Syngnathidae preferred *G. locusta* to any other Malacostraca species. The trophic level was calculated for all major groups (Table 1).

DISCUSSION

Primary producer carbon sources

$\delta^{15}N$, $\delta^{13}C$ and $\delta^{34}S$ values of primary producers and consumers (Fig. 5) of this eelgrass bed strongly support the hypothesis that the local food web is mainly based on epiphytes and seston. *Zostera marina* appears to be of little to no importance as a food source.

Stable isotope analyses are increasingly used to determine the contribution of different primary producers to the nutrition of higher trophic levels. The importance of seagrass and other marine angiosperms as a food resource has been discussed for years (Moncreiff & Sullivan 2001, Connolly et al. 2005), but could never be quantified as the frequently occurring similarity in stable isotope values of seagrass and epiphytes impeded a distinction between these 2 carbon sources (Connolly et al. 2005, Jaschinski et al. 2008a). Our study also found no significant differences between the $\delta^{13}C$ values of *Zostera marina* and epiphytes, but the issue could be resolved by the additional analysis of sulphur stable isotopes.

The present study suggests that seston and epiphytes are equally important carbon sources for higher trophic levels of this subtidal eelgrass community. According to the mixing model (MixSIR) employed, seston was the only carbon source (100%) for zooplankton. Thus, seston and epiphytes are the major carbon sources of this system. No parts of *Zostera marina* were of nutritional importance for any trophic level. Considering all potential food sources, herbivores on trophic levels below 2.4, rely on seston for 99.25% of food uptake (Table 1). Omni-

Table 1. Trophic levels and probable food sources of major consumer groups according to MixSIR model output

Consumer	Trophic level	Food source	Contribution to the mix (%)	Probability
Zooplankton	2	Epiphytes	0	1
		Seston	100	1
Anthozoa	2	Epiphytes	0–3	1
		Seston	99–100	1
Porifera	2	Epiphytes	0–4	1
		Seston	99–100	1
Bivalves	2	Epiphytes	0–3	1
		Seston	97–100	1
Idotea balthica	2.4	Epiphytes	52–62	1
		Zooplankton	36–46	1
		Seston	0–10	1
Gammarus locusta	2.5	Epiphytes	42–52	1
		Zooplankton	47–57	0.99
Praunus flexuosus	2.6	Epiphytes	0–6	1
		Seston	39–49	0.89
		Zooplankton	50–60	0.9
Littorina littorea	3	Epiphytes	0–2	1
		Amphibalanus improvisus	53–57	1
		Cirripedia larvae	42–46	1
Asterias rubens	3	Epiphytes	0–6	0.97
		Seston	0–6	1
		Mytilus spp.	91–100	0.97
Polynoidae	3	Epiphytes	0–6	0.75
		Mytilus spp.	86–96	0.85
Nereididae	3.6	Epiphytes	17–27	1
		Polynoidae	74–84	1
Gasterosteidae	3.5	Epiphytes	0–6	0.95
		Palaemon adspersus	35–45	0.73
		Zooplankton	40–50	0.84
Gobiidae	3.5	Epiphytes	0–6	0.91
		Gammarus locusta	85–95	0.76
		Zooplankton	0–6	0.9
Syngnathidae	3.3	Epiphytes	0–6	0.94
		Gammarus locusta	54–64	0.73
		Zooplankton	20–30	0.79

vores on trophic levels higher than 2.4 show a seston:epiphyte ratio of 1:2.4 in their food sources.

Even though seston via zooplankton is the more significant original carbon source for most organisms overall, some species, (e.g. *Gammarus locusta* and *Idotea balthica*) rely heavily on epiphytes (~50 and ~60% respectively). *Littorina littorea*, a species usually considered an epiphyte grazer, was found to rely on adult and larval barnacles as a food source in this study, which is confirmed by Buschbaum (2000). Given the anatomical and behavioural constraints of *L. littorina*, the high proportion of cirripedia larvae in the food mix can be explained by *L. littorina* feeding on them after settlement or sedimentation to the bottom. Epiphytes were of very low importance for *L. littorea* (less than 1% contribution to the food mix).

Malacostraca were abundant in this system (see Table S1 in the Supplement) and were mainly represented by *Gammarus locusta*, *Praunus flexuosus* and *Idotea balthica*. Malacostraca diet depended equally on zooplankton and seston and also included a small amount of epiphytes. When running the model at the species level, carbon sources of *P. flexuosus* were similar to those of Malacostraca as a whole (~55% zooplankton and ~45% seston). A previous study by Lehtiniemi & Nordström (2008) described similar percentages (42% plant and 50% animal material) based on stomach content analysis. However, *G. locusta* and *I. balthica* showed higher percentages of epiphytes in their diets (50 and 60% respectively), with zooplankton accounting for the remainder of probable carbon sources. Deposit and filter feeders cannot feed directly on epiphytes, but detached and suspended epiphytes may play an important role. Field observations indicate that *G. locusta* and *I. balthica* were found on or very close to *Zostera marina* leaves. No feeding scars were found on eelgrass leaves and the applied mixing model showed no evidence of *Z. marina* as a potential carbon source. This is in good agreement with Hootsmans & Vermaat (1985), who described *Idotea* sp. grazing on eelgrass only when epiphyte levels were low, which was not the case during the sampling period. $\delta^{15}N$ values put Malacostraca at TL 2.5 (Table 1).

The main carbon source of *Asterias rubens* and Polynoidae in this study was *Mytilus* spp., with both taxa depending on the bivalve for more than 90% of their carbon uptake. *Asterias rubens* has been described to clear whole patches of *Mytilus* spp. (Saier 2001), which supports the mainly homogenous diet identified here. Polynoidae have been described as true omnivores in other systems (Plyuscheva et al. 2010) but our mixing model showed that ~90% of the

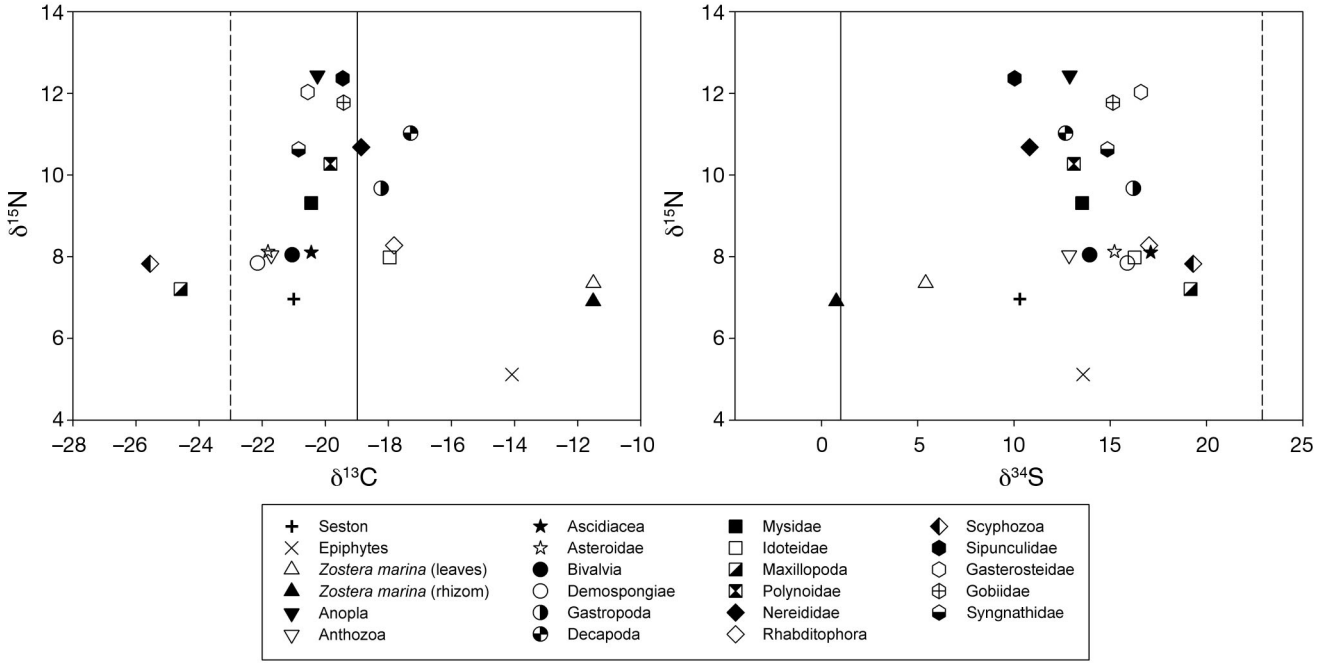

Fig. 5. Stable isotope values of (A) $\delta^{15}N$ vs. $\delta^{13}C$, (B) $\delta^{15}N$ vs. $\delta^{34}S$ for principal elements of the eelgrass community, including reference lines for $\delta^{34}S$ and $\delta^{13}C$ of sediment (solid line) and seawater (dashed line). Values are means calculated from samples collected between March and September 2011. For reasons of clarity, error bars are omitted in this figure (but see Table S1 in the Supplement at www.int-res.com/articles/suppl/m497p093_supp.pdf)

carbon taken up originated from *Mytilus* spp. Polynoidae in turn, represent ~80% of carbon sources for Nereididae, the remaining ~20% being from epiphytes.

The direct contribution of epiphyte carbon to the diet of fish was less than ~10%, whereas Malacostraca and zooplankton contributed ~69 and ~28% respectively. The separate analysis of fish species showed clear preferences for a certain prey species, e.g. Gasterosteidae preferred *Palaemon adspersus* (~49%) over any other Malacostraca species (less than ~5%) and, Gobiidae and Syngnathidae favoured *Gammarus locusta* (~94 and ~61% respectively). Similar observations were made by Bobsien (2006), where gut content analyses revealed a diet mainly consisting of decapods, amphipods and copepods in varying amounts.

In addition to the relative abundances, digestibility, quality and chemical defences of primary producers can influence selective grazing. Epiphytes, dominated by benthic diatoms in this system (Jaschinski et al. 2008a), are considered to be a highly nutritious food source (Creach et al. 1997). In contrast, *Zostera marina* leaves are nutritionally poor, lacking nitrogen compared to epiphytes (C:N = 25 and 10 respectively) and containing phenolic compounds which are known to impede herbivory (Harrison 1982). Furthermore, eelgrass contains lignin which increases the proportion of indigestible material. Therefore, we conclude that *Z. marina* in the Kiel Bight mainly provides habitat, shelter and substrate for epiphyte growth, but food is primarily supplied by epiphytes. Thus this system depends on eelgrass as an ecosystem engineer, but not as a food source. This agrees with previous conclusions based on different methodology (Connolly et al. 2005, Jaschinski et al. 2011a).

Food web structure

The suspension feeding bivalves in this system, predominantly *Mytilus* spp., occupied a low trophic level (TL = 2) in the Kiel Bight and relied on seston as their main carbon source. These mussels are preyed upon by *Asterias rubens* (TL = 3), Polynoidae (TL = 3) and *Carcinus maenas* (TL = 4). *C. maenas* has a wide range of potential food items, including polychaetes, small crustaceans and epiphytes, resulting in a higher trophic level. Polynoidae are preyed upon by carnivorous polychaetes, such as Nereididae (TL = 3.5), which also feed on epiphytes. All sampled fish species were carnivores. Gasterosteus aculeatus (TL = 3.5) and Syngnathidae (TL = 3.3) were the most common species sampled in this eelgrass system.

By avoiding the use of a single trophic enrichment factor (Hobson & Welch 1992) in our trophic level calculations and instead employing the output of the mixing model, the results are a better fit for the studied food web. As has been pointed out by Mancinelli (2012), the enrichment factor is the most important parameter in isotope mixing models, as it can considerably affect their output. Caut et al. (2009) suggest that errors should be incorporated in mixing models to strengthen their output. MixSIR allows for different trophic enrichment factors ±SD for every food source, which eliminates the potential source of error when using one trophic enrichment factor averaged over all primary producers of a system. Calculated trophic levels did not cluster near integer values. Instead, they were continuously distributed, which indicates widespread omnivory in this community. This conclusion agrees with the hypothesis of France et al. (1998), that consumer species in aquatic systems make use of every possible trophic position.

In this study we did not sample epipsammic microalgae which are known to play a significant role in seagrass food webs. Expanding the scope of sampling to include these resources in future studies would aid in assessing the connection between eelgrass epifaunal food webs and their associated soft-bottom food webs (Williams & Heck 2001). The Falckenstein eelgrass meadow is a well-flushed system where detritus does not accumulate. The absence of detritus prevented stable isotope analysis and the inclusion as a possible food source in the mixing model. Even though detritus is of no significant importance in the studied system, other seagrass communities may depend on it.

In summary, this pilot study highlights the importance of epiphytic microalgae for the carbon flux in the Falckenstein eelgrass system. The trophic contribution of live *Zostera marina* proves to be minimal, its most essential function being provision of habitat, shelter and substrate. This agrees with past research indicating that epiphytic algae may be the primary food sources within this community, as opposed to seagrasses and their detrital material which are of minor importance (Moncreiff & Sullivan 2001 and references therein) The Falckenstein eelgrass bed was characterised by a very high degree of omnivory and many generalist feeders. The system can be classified as a 'seagrass detrital system' (Valentine & Duffy 2006), which is defined as being dominated by small invertebrate mesograzers preferentially feeding on epiphytes. The simultaneous triple stable isotope analysis of C, N and S, as developed by Hansen et al. (2009), was a successful tool for the study of carbon flux and food web structure.

Acknowledgements. We acknowledge the financial support from the German Research Foundation (SO 145/28-1). The comments of S. E. Fox and 5 anonymous reviewers significantly improved an early version of our manuscript.

LITERATURE CITED

Bobsien IC (2006) The role of small fish species in eelgrass food webs of the Baltic Sea. PhD thesis, University of Kiel

Bosley KL, Wainright SC (1999) Effects of preservatives and acidification on the stable isotope ratios (^{15}N:^{14}N, ^{13}C:^{12}C) of two species of marine animals. Can J Fish Aquat Sci 56:2181–2185

Bunn SE, Loneragan NR, Kempster MA (1995) Effects of acid washing on stable isotope ratios of C and N in penaeid shrimp and seagrass: implications for food-web studies using multiple stable isotopes. Limnol Oceanogr 40:622–625

Buschbaum C (2000) Direct and indirect effects of *Littorina littorea* (L.) on barnacles growing on mussel beds in the Wadden Sea. Hydrobiologia 440:119–128

Carabel S, Godínez-Domínguez E, Verísmo P, Fernández L, Freire J (2006) An assessment of sample processing methods for stable isotope analyses of marine food webs. J Exp Mar Biol Ecol 336:254–261

Caut S, Angulo E, Courchamp F (2009) Variation in discrimination factors ($\Delta^{15}N$ and $\Delta^{13}C$): the effect of diet isotopic values and applications for diet reconstrution. J Appl Ecol 46:443–453

Chapman MG, Underwood AJ (1999) Ecological patterns in multivariate assemblages: information and interpretation of negative values in ANOSIM tests. Mar Ecol Prog Ser 180:257–265

Clarke K, Warwick R (2001) Change in marine communities: an approach to statistical analysis and interpretation. PRIMER-E, Plymouth

Connolly RM, Hindell JS, Gorman D (2005) Seagrass and epiphytic algae support nutrition of a fisheries species, *Sillago schomburgkii*, in adjacent intertidal habitats. Mar Ecol Prog Ser 286:69–79

Creach V, Schricke MT, Bertru G, Mariotti A (1997) Stable isotopes and gut analyses to determine feeding relationships in saltmarsh macroconsumers. Estuar Coast Shelf Sci 44:599–611

Douglass JG, France KE, Richardson JP, Duffy JE (2010) Seasonal and interannual change in a Chesapeake Bay eelgrass community: insights into biotic and abiotic control of community structure. Limnol Oceanogr 55: 1499–1520

France RL, Peters RH (1997) Ecosystem differences in the trophic enrichment of ^{13}C in aquatic food webs. Can J Fish Aquat Sci 54:1255–1258

France R, Chandler M, Peters R (1998) Mapping trophic continua of benthic foodwebs: body size-$\delta^{15}N$ relationships. Mar Ecol Prog Ser 174:301–306

Grey J (2006) The use of stable isotope analyses in freshwater ecology: current awareness. Polish J Ecol 54:563–584

Grey J, Deines P (2005) Differential assimilation of methanotrophic and chemoautotrophic bacteria by lake chironomid larvae. Aquat Microb Ecol 40:61–66

Hansen T, Burmeister A, Sommer U (2009) Simultaneous $\delta^{15}N$, $\delta^{13}C$ and $\delta^{34}S$ measurements of low-biomass samples using a technically advanced high sensitivity elemental analyzer connected to an isotope ratio mass spec-

trometer. Rapid Commun Mass Spectrom 23:3387–3393
Harrison PG (1982) Control of microbial growth and of amphipod grazing by water-soluble compunds from leaves of *Zostera marina*. Mar Biol 67:225–230
Hemminga MA, Duarte CM (2000) Seagrass ecology. Cambridge University Press, Cambridge
Hobson KA, Welch HE (1992) Determination of trophic relationships within a high Arctic marine food web using $\delta^{13}C$ and $\delta^{15}N$ analysis. Mar Ecol Prog Ser 84:9–18
Hootsmans MJM, Vermaat JE (1985) The effect of periphyton-grazing by three epifaunals species on the growth of *Zostera marina* L. under experimental conditions. Aquat Bot 22:83–88
Jaschinski S, Brepohl DC, Sommer U (2008a) Carbon sources and trophic structure in an eelgrass *Zostera marina* bed, based on stable isotope and fatty acid analyses. Mar Ecol Prog Ser 358:103–114
Jaschinski S, Hansen T, Sommer U (2008b) Effects of acidification in multiple isotope analysis. Limnol Oceanogr Methods 6:12–15
Jaschinski S, Brepohl DC, Sommer U (2011a) Seasonal variation in carbon sources of mesograzers and small predators in an eelgrass community: stable isotope and fatty acid analyses. Mar Ecol Prog Ser 431:69–82
Jaschinski S, Brepohl DC, Sommer U (2011b) The trophic importance of epiphytic algae in a freshwater macrophyte system (*Potamogeton perfoliatus* L.): stable isotope and fatty acid analysis. Aquat Sci 73:91–101
Kharlamenko VI, Kiyashko SI, Imbs AB, Vyshkvartzev DI (2001) Identification of food sources of invertebrates from the seagrass *Zostera marina* community using carbon and sulfur stable isotope ratio and fatty acid analyses. Mar Ecol Prog Ser 220:103–117
Lehtiniemi M, Nordström H (2008) Feeding differences among common littoral mysids, *Neomysis integer*, *Praunus flexuosus* and *P. inermis*. Hydrobiologia 614: 309–320
Mancinelli G (2012) On the trophic ecology of Gammaridae (Crustacea: Amphipoda) in coastal waters: a European-scale analysis of stable isotopes data. Estuar Coast Shelf Sci 114:130–139
McCutchan JH, Lewis WM, Kendall C, McGrath CC (2003) Variation in trophic shift for stable isotope ratios of carbon, nitrogen, and sulfur. Oikos 102:378–390
Michener RH, Kaufman L (2007) Stable isotope ratios as tracers in marine food webs: an update. In: Michener R, Lajtha K (eds) Stable isotopes in ecology and environmental science. Blackwell, Oxford, p 238–282
Moncreiff CA, Sullivan MJ (2001) Trophic importance of epiphytic algae in subtropical seagrass beds: evidence from multiple stable isotope analyses. Mar Ecol Prog Ser 215:93–106
Nelson TA (1997) Interannual variance in a subtidal eelgrass community. Aquat Bot 56:245–252
Nordström M, Aarnio K, Bonsdorff E (2009) Temporal variability of a benthic food web: patterns and processes in a low-diversity system. Mar Ecol Prog Ser 378:13–26
Orth RJ, Carruthers TJB, Dennison WC, Duarte CM and others (2006) A global crisis for seagrass ecosystems. Bioscience 56:987–996
Ouisse V, Riera P, Migne A, Leroux C, Davoult D (2012) Food web analysis in intertidal *Zostera marina* and *Zostera noltii* communities in winter and summer. Mar Biol 159:165–175
Peterson BJ, Fry B (1987) Stable isotopes in ecosystem studies. Annu Rev Ecol Syst 18:293–320
Plyuscheva M, Martin D, Britayev T (2010) Diet analyses of the scale-worms *Lepidonotus squamatus* and *Harmothoe imbricata* (Polychaeta, Polynoidae) in the White Sea. Mar Biol Res 6:271–281
Reynolds LK, Carr LA, Boyer KE (2012) A non-native amphipod consumes eelgrass inflorescences in San Francisco Bay. Mar Ecol Prog Ser 451:107–118
Saier B (2001) Direct and indirect effects of seastars *Asterias rubens* on mussel beds (*Mytilus edulis*) in the Wadden Sea. J Sea Res 46:29–42
Semmens BX, Moore JW (2008) MixSIR: a Bayesian stable isotope mixing model, Version 1.0. http://conserver.iugocafe.org/user/brice.semmens/MixSIR
Valentine JF, Duffy JE (2006) The central role of grazing in seagrass ecology. In: Larkum AWD, Orth RJ, Duarte CM (eds) Seagrasses: biology, ecology and conservation. Springer, Dordrecht, p 463–501
Vander Zanden MJ, Rasmussen JB (1999) Primary consumer $\delta^{13}C$ and $\delta^{15}N$ and the trophic position of aquatic consumers. Ecology 80:1395–1404
Williams SL, Heck KL (2001) Seagrass community ecology. In: Bertness MD, Gaines SD, Hay M (eds) Marine community ecology. Sinauer Associates, Sunderland, MA, p 317–338

Editorial responsibility: Matthias Seaman, Oldendorf/Luhe, Germany

Submitted: February 19, 2013; Accepted: September 20, 2013
Proofs received from author(s): December 19, 2013