Dietary and seasonal variability in trophic relations at the base of the North Sea pelagic food web revealed by stable isotope and fatty acid analysis

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Published version available in Journal of Sea Research, Volume 141, November 2018, Pages 61-70
https://doi.org/10.1016/j.seares.2018.08.004

ABSTRACT

A two-dimensional biomarker approach including fatty acids and stable isotopes of seston and copepods was applied to examine how the variability at the base of the food web affects trophic interactions between primary producers and copepod consumers over a sampling period of two years. We investigated how the composition of the seston affected feeding behaviour by analysing the fatty acid and stable isotope signals of the copepods *Calanus helgolandicus*, *Acartia* spp., *Centropages* spp. and *Temora longicornis* at Helgoland Roads, North Sea. Our results indicate that the relative contributions of autotrophic and heterotrophic fractions in the seston determined the stable isotope signal of the seston and hence the $\delta^{15}N$ of copepods. Our findings show that the combination of stable isotope and fatty acid analyses provides an ideal tool to address the complexity of trophic relations in planktonic food-webs and to define relative trophic position and feeding preferences of e.g. copepods. Defining accurate baselines from bulk seston samples containing a mixture of auto- and heterotroph protist communities still remains a challenge when defining lower food-web dynamics in natural plankton communities.

Keywords: planktonic food web; baseline variation; copepod feeding; lower food-web dynamics; seston

INTRODUCTION

Despite decades of research, consumer-producer interactions in the pelagic zone are still not entirely understood. There are several reasons for this. On the producer side, there are many organisms that are at least partly heterotrophic, and on the consumer side, there is large variation in diets between and within species. Especially copepods, which form an important link between primary producers and higher consumers, require further study, as the trophic position of copepods plays a major role in shaping aquatic food webs (Hairston and Hairston, 1993). Most copepods are omnivores feeding on a wide range of dietary items, such as diatoms, flagellates and ciliates (Kleppel, 1993). However, copepods are able to feed selectively (Fileman et al., 2007; Irigoien et al., 2000; Paffenhöfer, 1988) and thus they are capable of switching between dietary items of different quality, even within species (Meunier et al., 2016). This switch by copepods from feeding lower in the food web, as herbivores, to carnivory has consequences for lower levels in the food web and for consumers at higher trophic levels. As such, the trophic flexibility of copepods affects the structure of entire marine food webs. Therefore, the objective of the present study was to establish the role of different copepod species in the planktonic food web by using a combined tracer approach combining stable isotope and fatty acid data to investigate seasonal patterns and shifts in trophic positions of major North Sea copepod species.

The interactions in the marine pelagic food web are complex and subject to a great variety of influences. Particularly at the base of the food web the interactions between primary producers and consumers are characterized by a great variability in food quantity (e.g. Sommer, 1996; Wiltshire et al., 2008) and quality (e.g.Boersma et al., 2008; Klausmeier et al., 2004; Malzahn et al., 2007; Schoo et al., 2012). Strong seasonal changes in the availability and composition of microalgae occur due to high peaks in productivity during blooms. During the spring bloom, for example, phytoplankton biomasses reach a peak, which is usually followed by a rapid increase in zooplankton abundance. As the increase of phytoplankton biomass during the bloom causes a depletion of nutrients available in the seawater, the quality (in terms of nutrient stoichiometry) of the phytoplankton decreases over the

course of the bloom. At the same time, increasing numbers of micro- and mesozooplankton exert high grazing pressure on phytoplankton and reduce its biomass substantially. This change in prey quality (nutrient stoichiometry), composition and quantity at the base of the pelagic food webs has been shown to not only affect the herbivores directly feeding on microalgae, but also potentially those secondary consumers that feed on the herbivores (Malzahn and Boersma, 2009; Malzahn et al., 2010; Schoo et al., 2010; Schoo et al., 2014).

As food sources have distinct biochemical compositions that can become incorporated into the consumers' body, and tracers such as stable isotopes and fatty acids integrate the diet over a longer period of time (days to weeks in small ectotherms, e.g. *Acartia tonsa* (Tiselius and Fransson, 2016; Vander Zanden et al., 2015), tracer approaches are an effective way to investigate trophic interactions (Aberle et al., 2010; El-Sabaawi et al., 2009; Richoux and Froneman, 2009). As such they have allowed for detailed reconstructions of food sources and trophodynamic interactions (Dalsgaard et al., 2003; Kurten et al., 2013; Peterson and Fry, 1987; Ponsard and Arditi, 2000). Stable isotopes are commonly used in ecological studies to deduce trophic position and dietary source (El-Sabaawi et al., 2013; Post, 2002; Vander Zanden and Rasmussen, 2001). As a rule, the $\delta^{15}N$ signal is used to infer the trophic position of an organism, as the percentage of ^{15}N relative to ^{14}N in the tissue increases progressively and predictably with increasing trophic position of the consumer. $\delta^{15}N$ fractionates with trophic level on average around 3.4‰ (Minagawa and Wada, 1984), however, the values observed in aquatic animals may vary from 2.3‰ to 4.5‰ (McCutchan et al., 2003). Carbon stable isotopes are used to infer the carbon dietary source (Fry, 2006; Minagawa and Wada, 1984), as the carbon source and the different enzymes involved in carbon fixation show distinct fractionation, leading to different $\delta^{13}C$ values. Trophic enrichment, however, is not static and it varies both between different consumer species (Aberle et al., 2005; Gutierrez-Rodriguez et al., 2014; Post, 2002; Vander Zanden and Rasmussen, 2001), as well as within species as a result of changing food qualities (Vander Zanden and Rasmussen, 2001), and differences in specificity of different metabolic processes (Aberle and Malzahn, 2007; Gorokhova and Hansson, 1999; Ponsard and Averbuch, 1999).

Fatty acid markers commonly used in trophic studies can be either single fatty acids, associated with a particular type of organism, or a ratio of fatty acids. Certain primary producers contain very specific fatty acids, which can be used to characterize them. As fatty acids are often incorporated by their consumers without being modified, they can be used to trace dietary sources. Palmitoleic acid (16:1ω7), for example, is a diatom fatty acid marker (Lee et al., 2006). The ratio of 22:6ω3 (Docosahexaenoic acid, DHA) to 20:5ω3 (Eicosapentaenoic acid, EPA) is used to assess the proportion of dinoflagellates to diatoms in the diet, because dinoflagellates contain high amounts of DHA, while diatoms are rich in EPA (Budge and Parrish, 1998; Dalsgaard et al., 2003; El-Sabaawi et al., 2010). A high ratio of DHA to EPA could also indicate a carnivorous diet (El-Sabaawi et al., 2009). High amounts of 18:1ω9 relative to 18:1ω7 have been shown to indicate carnivory in copepods and other crustaceans (Nyssen et al., 2005; Schmidt et al., 2003; Stevens et al., 2004a). Since carnivorous copepods contain larger amounts of polyunsaturated fatty acids (PUFA) than herbivorous copepods, the ratio of PUFA to saturated fatty acids (SFA) can be used to identify the degree of carnivory (Stevens et al., 2004b). However, because some of the fatty acids, such as DHA and some polar fatty acids, are sometimes preferentially retained by certain copepods, this can obfuscate the dietary signature of primary producers (Dalsgaard et al., 2003; El-Sabaawi et al., 2009). Additionally, some fatty acids can be metabolised and transformed by the consumers (Budge and Parrish, 1998). Assertions about the trophic position of consumers based solely on fatty acids, without precise knowledge of that particular consumer's metabolism and physiology, are therefore problematic.

While both fatty acid and stable isotope analysis have their limitations, the combination of these techniques may provide a more powerful tool to determine trophic interactions in complex food webs (Gaillard et al., 2017; Perga et al., 2006; Petursdottir et al., 2012; van der Bank et al., 2011) . The advantage of this combined tracer approach is mainly attributed to the fact that FAs are more specific to dietary source than stable carbon isotopes, particularly when differences in $\delta^{13}C$ of different carbon sources are small (El-Sabaawi et al., 2009). Combining both techniques has thus a high potential to enable investigations of seasonal changes in trophic relations and dietary variability in the plankton

in detail. Hence, in this study we used these two markers to investigate inter- and intra-species variation in key copepod species in the Southern North Sea. Further, by estimating the proportion of autotrophs vs. heterotrophs in the seston fraction, we aimed to refine the estimate of baseline stable isotope signals. Given the finding by previous authors (e.g. Kleppel, 1993) that different copepod species have different diets, we investigated the trophic positions of four dominant copepod species in the North Sea over the course of two years.

MATERIALS AND METHODS

The rocky island of Helgoland is situated in the Southern North Sea, German Bight, about 70 km from the mainland. The long-term sampling station Helgoland Roads is located between the main island and the sand dune island (54°11' N, 7°54'E). Due to strong tidal currents and the shallow depth, the water column is well mixed (Hickel, 1998). Surface water samples for the analysis of seston composition, stable isotope signature, fatty acid content and nutrient concentrations as well as zooplankton samples were gently taken with buckets by the RV Aade at Helgoland Roads between January 2007 and December 2008.

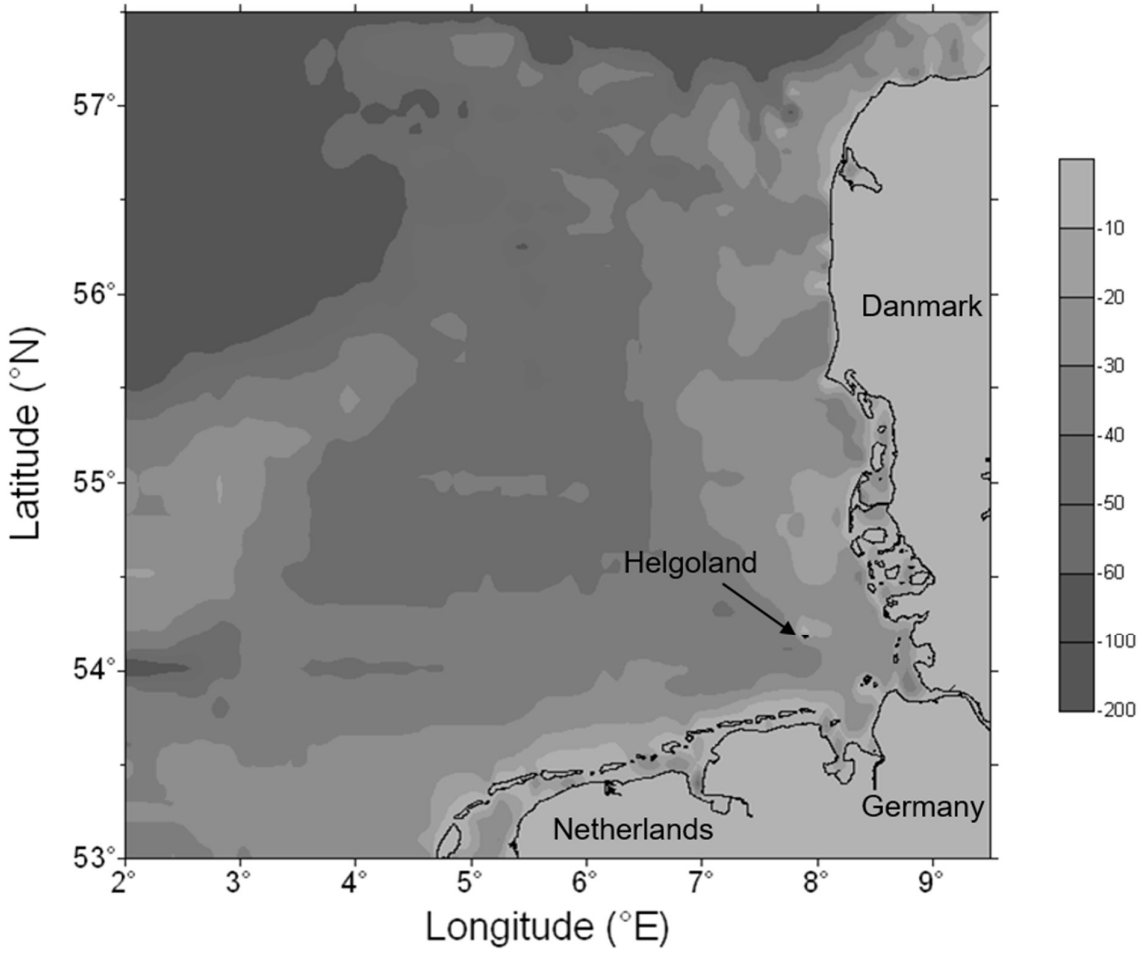


Figure 1: Location of the study site (Helgoland, North Sea)

Sampling focused on the base of the food web, represented by the seston (particulate organic matter) and mesozooplankton consumers, represented by copepods. To provide a baseline relevant to the feeding of the primary consumers seston samples were collected at the same time as the zooplankton. Nutrient content of the seawater was measured as part of the Helgoland long-term data series (Wiltshire et al., 2008). For the determination of the seston stable isotope signature, surface water from Helgoland Roads was pre-screened with a 200 µm sieve to remove larger organisms and filtered onto pre-combusted glass fibre filters (GF/C). The filters were examined under a dissecting microscope to remove any mesozooplankton or large particles and dried at 60°C. In addition to the samples for stable isotope analysis, filters were taken for fatty acid analysis of the seston in the same manner. However, seston material for fatty acid analyses was freeze-dried prior to analysis.

Phytoplankton carbon concentrations were obtained from the Helgoland Roads long-term monitoring program (Wiltshire et al., 2008). Samples of surface water for the determination of microzooplankton were preserved with acid Lugol's solution (2% final concentration), and the organisms identified to species level as described by Löder et al. (2010). Many of the dinoflagellates in the plankton are considered to be mixotrophs and able to take up particles via phagotrophy, even if they contain chloroplasts. Hence, for our division of heterotrophic versus autotrophic components in the plankton they were assigned to the microzooplankton (Löder et al., 2010). Biovolume of microzooplankton was calculated from the measurement of cell dimensions using geometrical formula according to Hillebrand et al. (1999) and subsequent conversion to carbon content was done after Putt and Stoecker (1989) and Menden-Deuer and Lessard (2000).

Zooplankton samples were obtained by oblique net hauls (mesh size 180 µm and 500 µm). Animals were sorted shortly after collection. Four copepod taxa were sampled: *Calanus helgolandicus*, *Temora longicornis*, *Centropages* spp. and *Acartia* spp. (mainly *A. clausi*). Copepod samples were taken for the analysis of stable isotopes and fatty acids.

Fatty acid analysis

Seston was extracted for the analysis of fatty acids by filtering pre-screened surface water samples through pre-combusted GF/F filters (Whatman). Three replicate filters were taken on each sampling occasion. The filters were placed in reaction tubes and frozen at -80°C. Copepods for the fatty acid analysis were sorted into reaction tubes and frozen at -80°C until further analysis. The fatty acids of seston and copepods were measured as fatty acid methyl esters (FAMEs). Lipids extraction followed modified methods described by Folch (1957) and Bligh and Dyer (1959). Fatty acid samples were extracted in Dichloromethane:methanol (2:1 vol:vol) using an ultrasound bath for 30 min. After centrifugation, water-soluble fractions were removed by washing with 0.88% KCl buffer. Thereafter, the aqueous phase was removed and the organic remainder evaporated using nitrogen gas. Esterification was achieved using methanolic-sulphuric acid at 70°C for 75 min. FAMEs were washed

from the methanolic sulphuric acid using n-Hexane, excess n-Hexane evaporated using nitrogen and FAMEs analysed using a Varian CP 8400 gas chromatograph equipped with a DB-225 column (J&W Scientific, 30 m length, 0.25 mm ID, 0.25 µm film). 1 µL aliquots of samples were injected using a split less mode. FAMEs were quantified using calibrations set up for each fatty acid separately and a known amount of C 23:0 was added at the first step of the preparation as an internal standard. More detailed information on injector temperature, column oven set-up and carrier gases are described in Malzahn et al. (2010). A known amount of C23:0 was used as an internal standard to calculate fatty acid concentration.

In this study, we focussed on fatty acids as trophic markers in the lipid fractions and did not account for wax esters and fatty alcohols although a considerable amount of these can be found especially in calanoid copepods (Kattner et al., 2007; Kattner and Krause, 1989; Lee et al., 2006).

The tracer fatty acids and fatty acid trophic markers (FATM) used here are summarized in Table 1.

Table 1: Fatty acid biomarkers and fatty acid trophic markers used in this study. Abbreviations: PUFA= sum of polyunsaturated fatty acids; SFA = sum of saturated fatty acids; D= sum of diatom markers; F= sum of dinoflagellate markers.

Marker	Diet	Reference
16:1ω7	Diatom	Lee et al., 2006
18:1ω7	Bacteria or *de novo* synthesis	Stevens et al., 2004b
18:1ω9	Carnivory	Graeve et al., 1994
18:1ω9/18:1ω7	Carnivory	Stevens et al., 2004a Nyssen et al., 2005
18:4ω3	Dinoflagellates	Lee et al., 2006
20:5ω3 (EPA)	Diatoms	Dalsgaard et al., 2003
12:6ω3 (DHA)	Dinoflagellates	Budge and Parish, 1998
DHA/EPA	Dinoflagellates / Diatoms Carnivory	Budge and Parish, 1998 Dalsgaard et al., 2003
PUFA/SFA	Carnivory	Stevens et al., 2004b
D/F	Diatoms / Flagellates	Dalsgaard et al., 2003 El-Sabaawi et al., 2009

Stable isotope analysis

Copepods for stable isotope analysis were rinsed in distilled water and dried in tin capsules. Depending on the size (biomass) of the copepods each tin cup contained between 3 and 30 individuals to meet the analytical requirements for the isotope analysis.

Stable isotope analysis of the samples was performed in two laboratories, at the GEOMAR in Kiel, Germany, and at the UC Davis Stable Isotope Facility in Davis, California, USA. At the GEOMAR in Kiel the samples were analysed by using an isotope ratio mass spectrometer (Thermofinnigan EA 1110 CHNS). Samples at UC Davis Stable Isotope Facility were analyzed using a PDZ Europa ANCA-GSL elemental analyser interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). The standards used were PeeDee belemnite for C and atmospheric nitrogen for N. During measurements, the ratio of the $^{13}C/^{12}C$ and the ratio of the $^{15}N/^{14}N$ stable isotopes were determined. Isotopic abundances are expressed in δ notation in parts per thousand (‰): $\delta = ((R_{sample} / R_{standard})-1) *1000$, where R is the ratio of the heavier isotope to the lighter isotope, i.e. $^{13}C/^{12}C$ or $^{15}N/^{14}N$. Trophic fractionation of stable isotopes is described as the difference of the δ values among food sources, namely the seston, (A) and consumer (B) using Δ notation, where $\Delta = \delta_B - \delta_A$. A positive Δ value indicates an enrichment of the heavier stable isotope in the consumer B.

Apart from detritus and inorganic material, the seston samples consist of autotroph fractions (e.g. diatoms, phytoflagellates) and heterotroph fractions (e.g. ciliates, mixo-/heterotrophic dinoflagellates). To estimate the $\delta^{15}N$ signal of these different fractions in the seston, we used the following equation:

$$\delta^{15}N_{seston} = C_{autotroph} * \delta^{15}N_{autotroph} + C_{heterotroph} * (\delta^{15}N_{autotroph} + 2.2)$$

where $C_{autotroph}$ is the carbon biomass of the autotrophs expressed as fraction of total seston carbon biomass and $C_{heterotroph}$ is the fraction of the heterotrophic biomass, estimated from the microzooplankton counts, thus $C_{autotroph} + C_{heterotroph} = 1$. We assumed a 2.2‰ trophic fractionation between the autotrophic and the heterotrophic fractions of the seston. This level of fractionation between two trophic levels is generally accepted for invertebrates (McCutchan et al., 2003). In this manner, the theoretical $\delta^{15}N$ signals of the autotroph and the heterotroph fractions of the seston were calculated and used to compute the delta signals of both fractions.

Statistical analyses

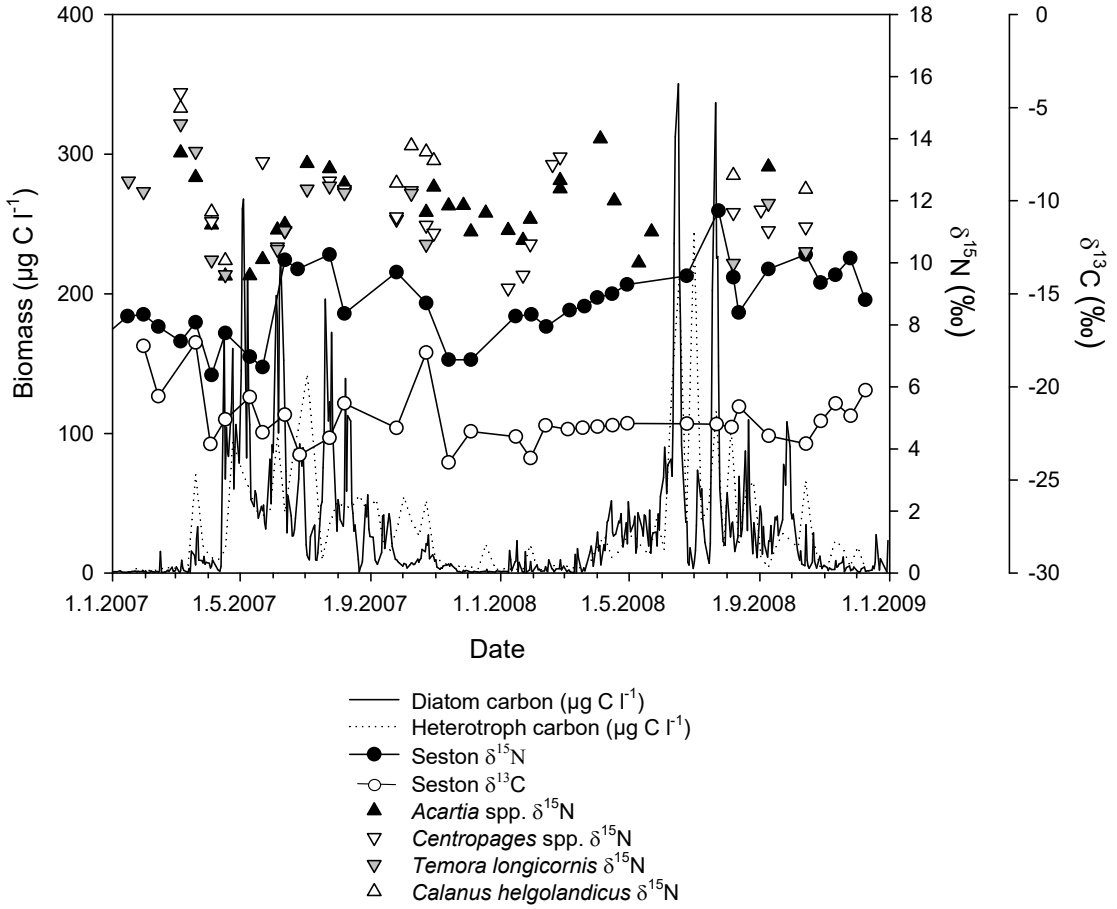
Correlations between seston fatty acids and copepod fatty acids as well as $\delta^{15}N$ of the copepods and their fatty acid markers were conducted using linear regression analyses.

Linear regressions were also performed for: (1) $\delta^{15}N$ signals of autotroph and heterotroph fractions, (2) $\delta^{15}N$ of the seston and the biomass of the heterotrophic organisms as well as (3) between the fatty acids from the seston and the $\delta^{13}C$ signal.

RESULTS

Seston

The spring bloom in 2007 was dominated mainly by diatoms (Figure 2). The diatom bloom developed rapidly from mid-April onwards and diatom biomass reached a maximum of 270 µg C L^{-1} in early May. The diatom bloom was instantaneously followed by a bloom of microzooplankton dominated by ciliates. Throughout the rest of the year, the microzooplankton was dominated by mixo- and heterotrophic dinoflagellates reaching a maximum of about 140 µg C L^{-1} in July. Total biomass then decreased to about 100 µg C L^{-1} for the remainder of the summer and declined further following a short secondary bloom in October. During the winter months the biomass remained low at around 20-30 µg C L^{-1}. The spring bloom of 2008 occurred later than in the previous year, with a higher peak diatom biomass (335 µg C L^{-1}) recorded only in June. The microzooplankton peak biomass of 240 µg C L^{-1} was reached in July.



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233 *Figure 2: δ15N (‰) and δ13C (‰) of the seston and δ15N (‰) of the four copepod species as well as carbon biomass (µg l-1) of*
234 *diatoms and heterotrophic microzooplankton at Helgoland Roads from January 2007 to December 2008. Note the two different axes*
235 *for δ15N (‰) and δ13C (‰).*

236

237 The $\delta^{15}N$ stable isotope signal of the seston ranged from 6.3‰ in spring 2007 to 11.7‰ in summer
238 2008 (Figure 2). The $\delta^{15}N$ of the seston decreased from 8‰ in winter to 6‰ at the start of the spring
239 bloom. Following the diatom bloom peak the $\delta^{15}N$ increased again, reaching values of 10‰ in July.
240 This corresponded to the period of the highest heterotrophic biomass. The $\delta^{15}N$ decreased during the
241 winter months, with decreasing seston biomass. The $\delta^{15}N$ signal of the seston increased again rapidly
242 in February of 2008 and continued to increase until the summer. A drop in the $\delta^{15}N$ stable isotope
243 values was observed in August 2008, followed by an increase during an autumn bloom of diatoms
244 and mixo-/heterotrophic dinoflagellates in October (Figure 2).

245 There was a significant positive correlation between the $\delta^{15}N$ of the seston and the biomass of the
246 heterotrophic organisms (linear regression analysis, $r^2 = 0.21$, $p<0.01$), indicating an influence of the
247 heterotrophic organisms on the seston $\delta^{15}N$ stable isotope signal. No correlation was found between
248 the $\delta^{15}N$ signature of the seston and the diatom biomass ($r^2= 0.04$, $p>0.05$).
249 The $\delta^{13}C$ signal of the seston showed a range from -17 to -24‰. A steep change in the signal from -17
250 to -23‰ was observed in early spring 2007. The seston signal showed strong variations during the
251 summer before a sharp increase in November 2007. The $\delta^{13}C$ was not significantly correlated to the
252 biomass of the diatoms or the heterotrophs.
253 The $\delta^{15}N$ signals for autotroph and heterotroph fractions showed a strong linear correlation between
254 the total signal (measured $\delta^{15}N$) and the computed $\delta^{15}N$ signal of the two fractions ($r^2=0.18$, $p<0.05$,
255 and $r^2=0.20$, $p<0.001$ for diatoms and the heterotrophic fraction, respectively) (Figure 3). Thus, the
256 primary driver of the $\delta^{15}N$ signal of the total seston is the relative proportion of heterotrophic
257 organisms, combined with the total available living biomass.

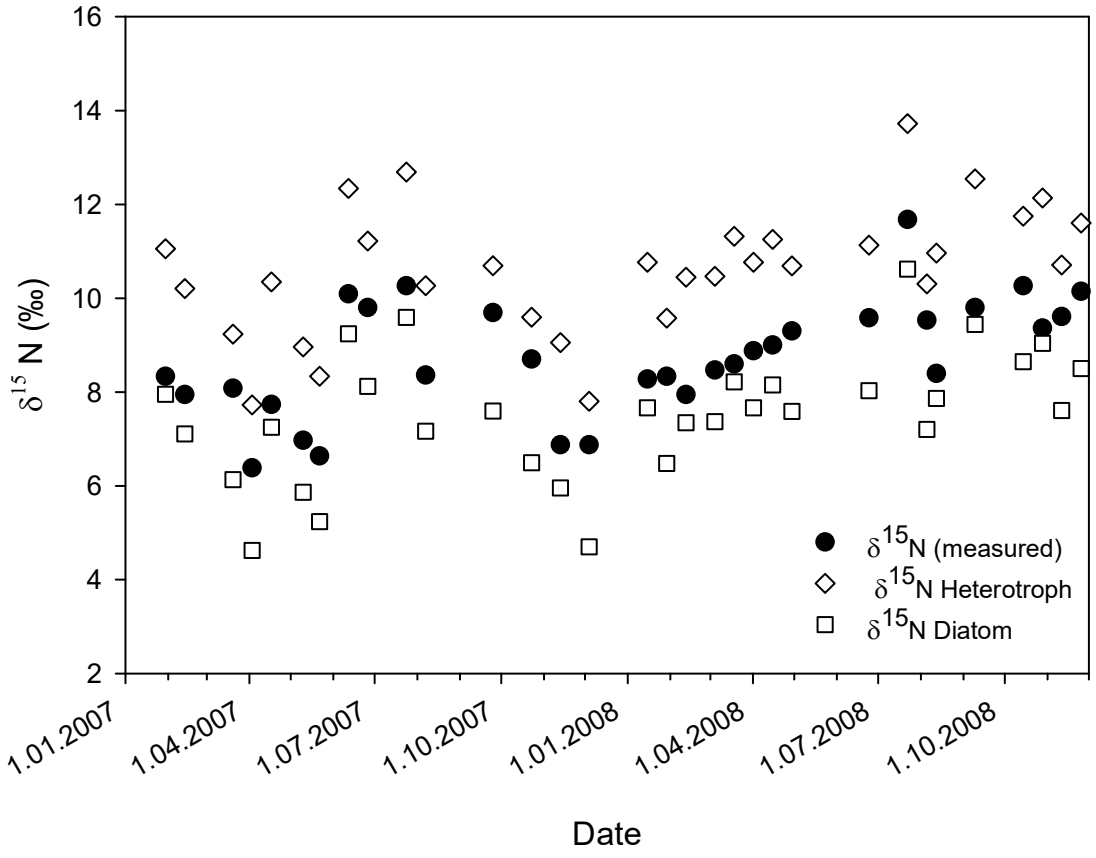
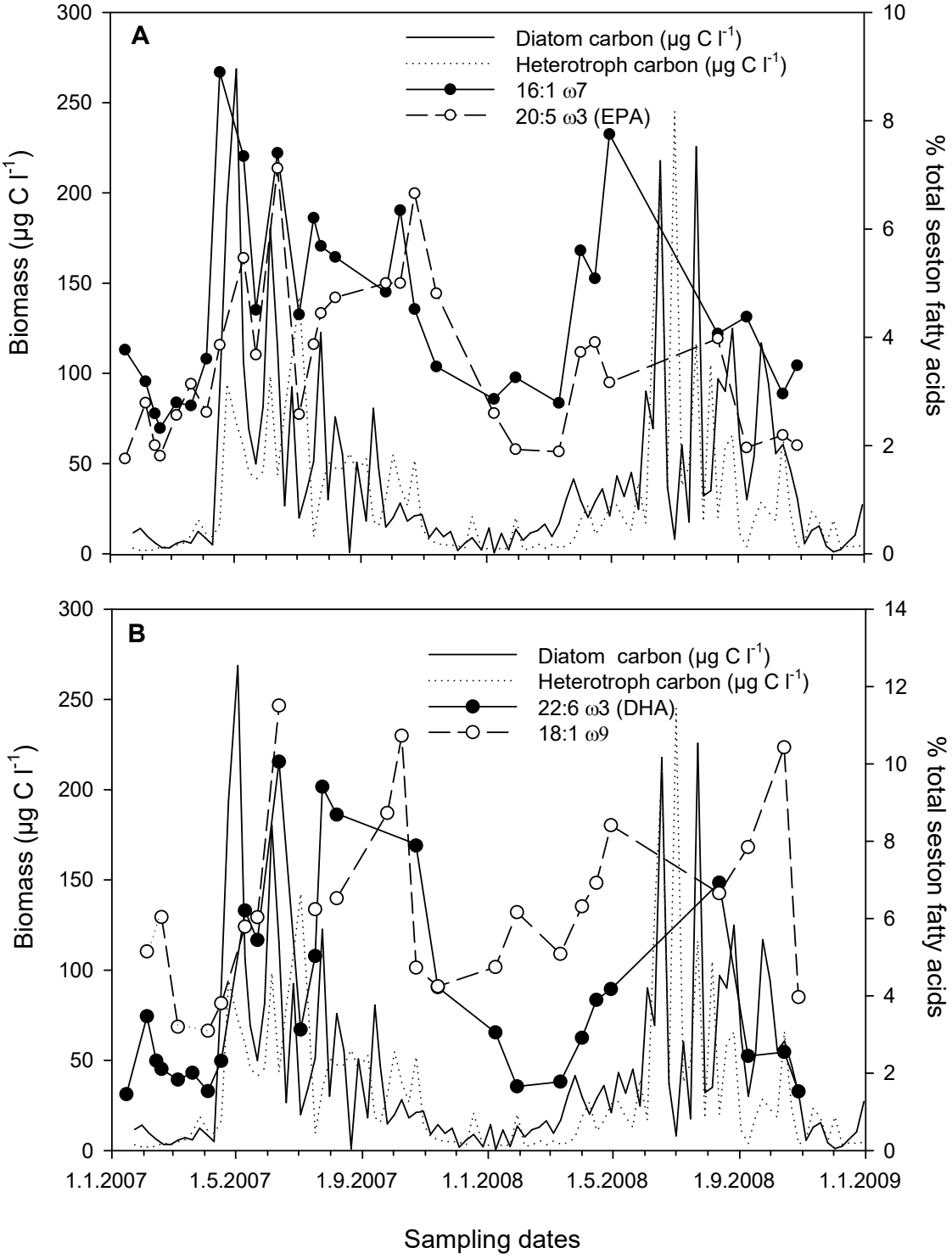


Figure 3: Seston $\delta^{15}N$ (‰) and calculated $\delta^{15}N$ for diatom and heterotroph fractions.

The fatty acid content of the seston changed according to the seston composition (Figure 4). There was a strong seasonal change in the relative amounts of certain fatty acids. During the diatom bloom in May 2007 high amounts of eicosapentanoic acid (20:5 ω3, EPA), prevalent in diatoms, were recorded (Figure 4A). Concurrently to the increase in heterotrophic biomass in June 2007 increased amounts of the dinoflagellate tracer fatty acids 18:1 ω9 and 22:6 ω3 (docosahexaenoic acid, DHA) were measured (Figure 4B). Throughout summer and autumn the concentration of 18:1 ω9 remained high in the seston, while 22:6 ω3 (DHA) displayed a second peak in late summer. The dominant fatty acids during the winter months were again those associated with heterotrophic organisms, in particular 18:1 ω9.



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272 *Figure 4: Seasonal variability of diatom fatty acid markers (A) and dinoflagellate fatty acid markers (B) overlaid on diatom and*
273 *microzooplankton biomass.*

The $\delta^{15}N$ signal of the seston correlated with 18:1 ω7 (linear regression analysis: $r^2= 0.19$, $p<0.05$), 18:1 ω9 ($r^2= 0.48$, $p<0.001$) and the diatom-specific fatty acid 18:4 ω3 ($r^2= 0.27$, $p<0.01$). No significant correlations between the fatty acids from the seston and the $\delta^{13}C$ signal were found.

Copepods

The $\delta^{15}N$ signature of the copepods showed strong seasonal fluctuations (Figure 2). The $\delta^{15}N$ signals ranged from 9‰ to 15‰. Overall, the highest average $\delta^{15}N$ throughout the sampling period was recorded in *Calanus helgolandicus*, followed by *Centropages* spp. and *Acartia* spp., while the lowest $\delta^{15}N$ was observed in *Temora longicornis* (Figure 2 & Figure 5).

The trophic fractionation of the copepods relative to the seston was calculated and expressed as $\Delta\delta^{15}N$ of the copepods. This value also showed a wide range over the time sampled, from as low as 1‰ to 8‰, with strong differences between species and seasons. Generally, the $\Delta\delta^{15}N$ of the copepods was highest in winter, declined with the onset of the spring bloom and reached its lowest level in early summer. This pattern displays the opposite trajectory to the diatom biomass and could indicate an increased feeding on autotrophic organisms during the spring bloom. The $\Delta\delta^{15}N$ of most copepods increased again in July and remained elevated through the autumn. The highest difference in trophic enrichment between species was observed in autumn, where the $\Delta\delta^{15}N$ values ranged from 1.8‰ to 6.4‰. In *Acartia* spp. the lowest enrichment coincided with the spring bloom, indicating that this copepod species fed on a herbivorous diet during that particular time. Enrichment was higher in late autumn and winter, when the diatom biomass was lowest. A similar pattern was observed in *C. helgolandicus. T. longicornis* showed a high level of enrichment in spring and late summer, while the highest level of enrichment for *Centropages spp.* was recorded in July and August. *Centropages* spp. displayed the highest increase in $\Delta\delta^{15}N$ in the winter with values rising from 0.9‰ in January to 5.5‰ in late February.

The $\delta^{13}C$ of copepods showed strong fluctuations (Figure 2). The highest $\delta^{13}C$ signals were recorded in May 2007 around the time of the diatom spring bloom. The $\delta^{13}C$ signal of *Acartia* spp. varied from -23 to -18‰. The highest $\delta^{13}C$ signals for this copepod were observed in May 2007 and September 2008. The lowest values (-23‰) were found in early March 2007, with another strong decrease in the spring of 2008. A very similar pattern was observed for the $\delta^{13}C$ of *T. longicornis* and *Centropages* spp.. The $\delta^{13}C$ for *C. helgolandicus* was slightly lower, i.e. less enriched, than that of the other copepods throughout the sampling period (Figure 2 & Figure 5).

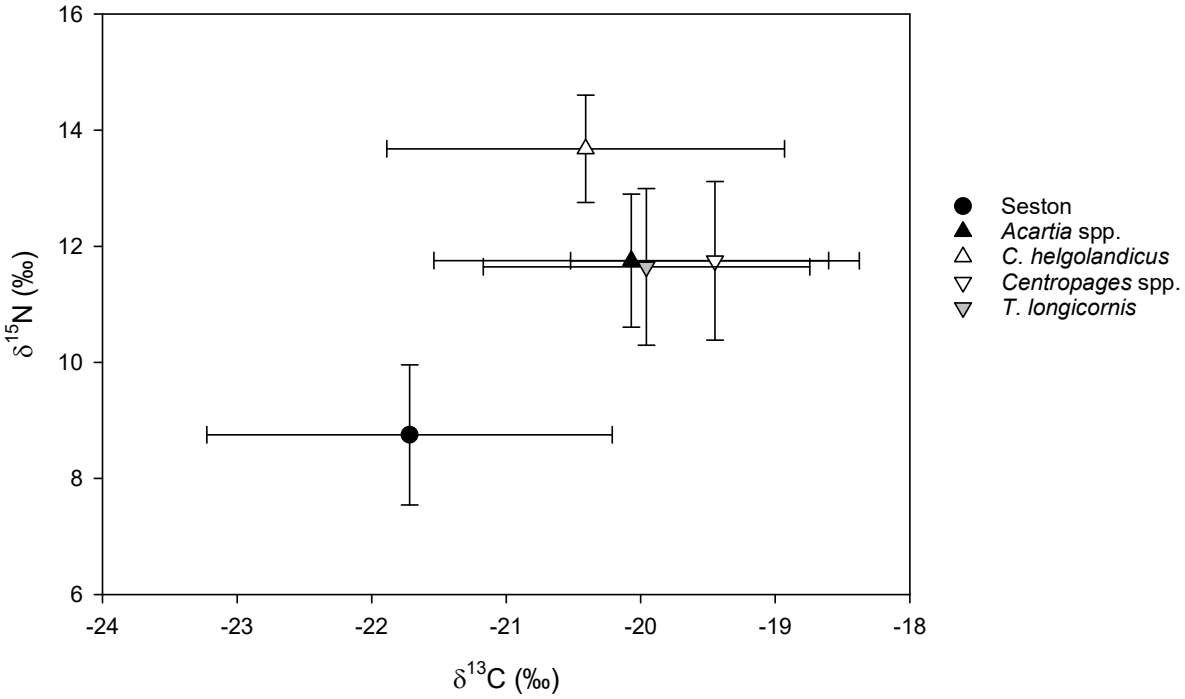

Figure 5: Isotope biplot of $\delta^{15}N$ (‰) and $\delta^{13}C$ (‰) of seston and zooplankton collected at Helgoland Roads from 2007-2008. Shown are means and standard deviations.

*Table 2: Correlations between seston fatty acids and copepod fatty acids. * denotes p<0.05, ** denotes p<0.01, n.s. identifies no significant correlation.*

Fatty acid	*Acartia* spp.	*T. longicornis*	*Centropages* spp.	*C. helgolandicus*
18:1 ω7	**	n.s.	n.s.	n.s.
18:1 ω9/18:1 ω7	*	**	n.s.	n.s.
18:4 ω3	n.s.	*	*	*

20:5 ω3 (EPA)	**	*	n.s.	**
22:6 ω3 (DHA)	*	*	**	*
DHA/EPA	n.s.	n.s.	n.s.	n.s.
PUFA/SFA	**	**	n.s.	n.s.
D/F	*	**	**	**

312

313 The fatty acid content of the four copepod species sampled was correlated with some specific fatty
314 acid markers in the seston (see Table 2). *Acartia* spp. showed significant correlations with the diatom
315 fatty acid 20:5 ω3 (EPA), and the dinoflagellate fatty acid 22:6 ω3 (DHA). The fatty acid signature
316 of *T. longicornis* was strongly correlated to the FATM 18:1 ω9/18:1 ω7 and PUFA/SFA, both
317 indicators of carnivory. Fatty acids in *Centropages* spp. were significantly correlated to the fatty acids
318 18:4 ω3 and DHA, which are associated with dinoflagellates, in the seston. *C. helgolandicus* showed
319 significant correlations with the diatom fatty acids (16:1 ω7 and EPA) and to the dinoflagellate fatty
320 acids (18:4 ω3 and DHA), indicating that *Calanus* fed on a mixed diet.

321

322 *Combined tracer approach: Stable isotopes and fatty acids*

323 Some strong correlations between the $\delta^{15}N$ of the copepods and their fatty acid markers, i.e. the fatty
324 acids incorporated by the copepods were observed. The $\delta^{15}N$ of *Acartia* spp. correlated significantly
325 with two fatty acid markers for diatoms (16:1ω7 and D/F). There was also a strong correlation to the
326 carnivory marker DHA/EPA in *Acartia* spp. *Centropages* spp. displayed the strongest correlations
327 between $\delta^{15}N$ and fatty acid markers for carnivory, such as DHA/EPA and PUFA/SFA. No
328 correlations were found between the $\delta^{15}N$ of *T. longicornis* or *C. helgolandicus* and the fatty acid
329 markers. Significant correlations between the $\delta^{13}C$ signal and FATMs were only observed for *T.*
330 *longicornis*.

331 To investigate whether the combination of stable isotope data and fatty acid markers is useful in
332 determining the trophic position of consumers the $\delta^{15}N$ values were plotted against fatty acid trophic

markers (Figure 6). The relative positions of the copepods on the plot give an indication of the dietary preference and the resulting trophic position. By using the combined FA and SI approaches we could depict a distinct trophic position of *C. helgolandicus* compared to other copepod species, showing the highest $\delta^{15}N$ values, almost one trophic level above that of the other copepods, and also the highest concentration of the carnivory markers PUFA/SFA (Figure 6 B) and 18:1 ω9/18:1 ω7 (Figure 6 C). In terms of the ratio of diatoms to dinoflagellates in the diet, however, *C. helgolandicus* showed a rather balanced diet (Figure 6 D). This stresses the outstanding trophic role of *C. helgolandicus* when compared to other North Sea copepods. In contrast, the other three copepods examined in this study show similar $\delta^{15}N$ values, but have slightly different fatty acid profiles. The fatty acid composition of *T. longicornis* reveals a preference for dinoflagellates, indicated by the high D/F ratio (Figure 6 D). Confounding this is the relatively low DHA/EPA ratio observed, which indicates a larger amount of diatoms (EPA) relative to dinoflagellates (DHA) in the diet of this copepod. *Centropages spp.* on the other hand contained a relatively high ratio of DHA/EPA, indicating a preference for dinoflagellates, and a comparatively low amount of D/F (Figure 6 A). Both the fatty acid spectrum and the $\delta^{15}N$ values of *Acartia spp.* indicate the omnivorous diet of this copepod, not exhibiting any clear feeding preference.

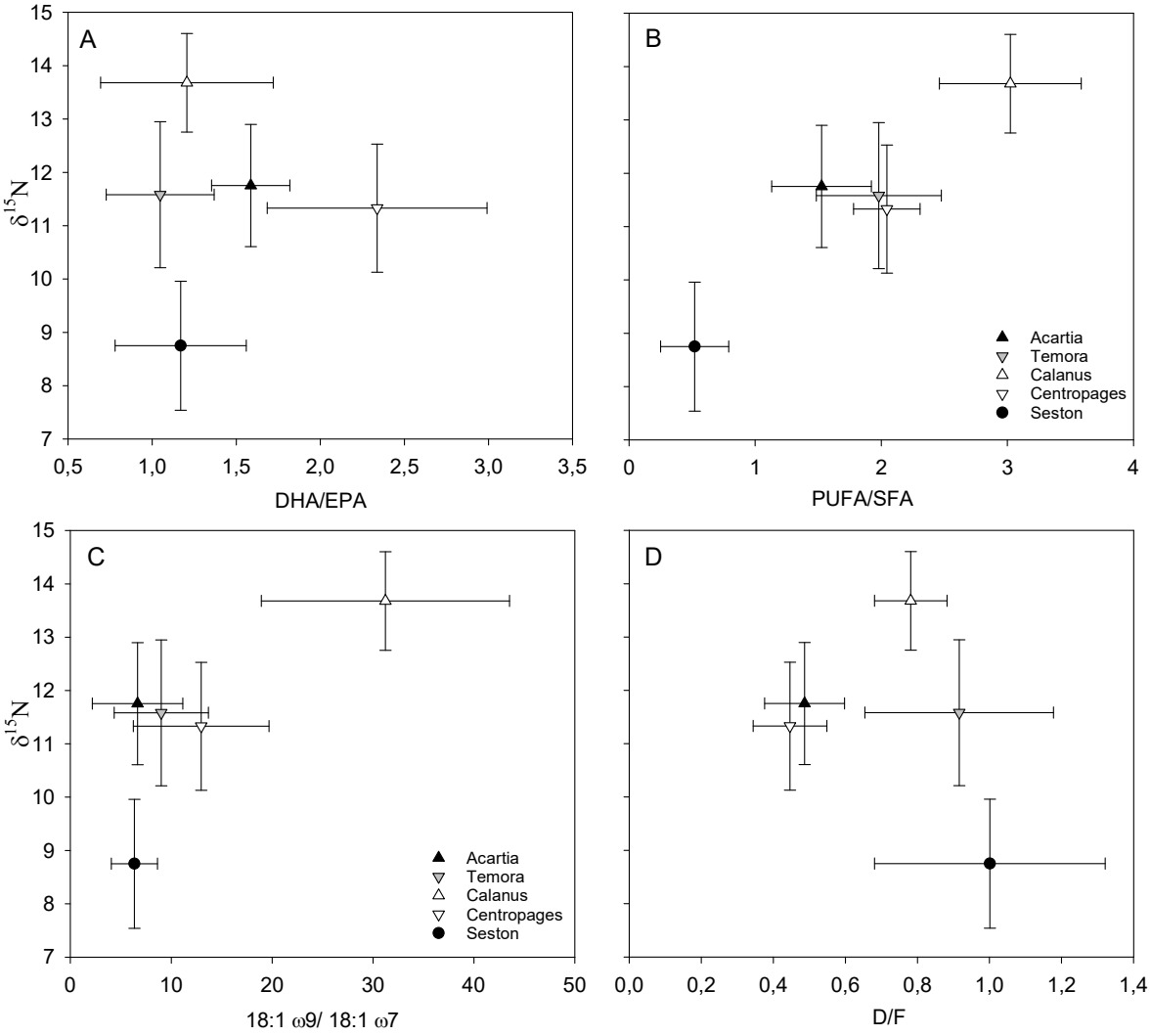

Figure 6**:** $\delta^{15}N$ (‰) and concentration of different fatty acid biomarkers (A) DHA/EPA, (B) PUFA/SFA, (C) 18:1ω9/18:1ω7 and (D) D/F expressed as % of total fatty acids for four species of copepods. Mean values for one year. Error bars indicate standard deviation.

DISCUSSION

Due to their pivotal role in the marine food web, the feeding of copepods has important consequences both for lower and higher trophic levels. Copepod grazing can exert a top-down control on primary producers and their survival and food quality greatly affects their consumers.

Disentangling the trophic linkages in a complex multi-trophic system requires the establishment of an appropriate baseline against which the variations of the higher trophic levels can be gauged. However, obtaining a reliable baseline for food web studies is a challenge.

362 Stable isotopes of particulate organic matter (POM) are typically used as a proxy for primary
363 producers in studies aiming to elucidate consumer diets. This is problematic since the isolation of
364 pure primary producers from the plankton is impossible and filtration results in bulk seston samples
365 containing a mixture of phytoplankton, mixo- and heterotrophic flagellates, ciliates, bacteria and
366 detritus, each with different trophic positions and isotope signals. Even size fractionation does not
367 alleviate this problem, as there are no natural size-borders separating primary producers from primary
368 consumers. Although in the present study we had detailed data on the composition and temporal
369 patterns of the autotrophic and mixo-heterotrophic organisms present at the base of the food web, the
370 seston isotope signal did not entirely match the composition of the known fractions from our data.
371 The seasonal variability in seston stable isotope signatures is commonly attributed to shifts in the
372 species composition, with higher $\delta^{15}N$ signals usually related to a higher amount of heterotrophic
373 organisms (Aberle et al., 2010; Agurto, 2007). This pattern was visible in our data, with the main
374 drivers of this signal seeming to be the mixo- and heterotrophic fraction. The range of the $\delta^{15}N$ of the
375 seston, i.e. at the base of the food web, measured over the sampling period was larger than the 2-5‰
376 difference normally attributed to a one step difference in trophic levels within food webs (Post, 2002).
377 The stable isotope signature of phytoplankton is known to be influenced by a variety of factors, such
378 as the CO_2 concentration, temperature, salinity, nutrient availability species, and cell size (Aberle and
379 Malzahn, 2007; Burkhardt et al., 1999; Needoba et al., 2003). The enrichment of $\delta^{15}N$ therefore varies
380 greatly within and between phytoplankton taxa and seasons (Vuorio et al., 2006). Furthermore, the
381 nitrogen source and content of the algae can affect the fractionation and enrichment of $\delta^{15}N$ in the
382 consumers (e.g.Jones et al., 2004; Vanderklift and Ponsard, 2003; Vuorio et al., 2006). The
383 enrichment of $\delta^{15}N$ between primary producers and their consumers can as a consequence range from
384 0‰ to 8‰ (Schmidt et al., 2003), a range which is similar to the results observed in this present study.
385 This further complicates the description of trophic linkages based entirely on stable isotope data.
386 One of the other major problems underlying this approach is the vast array of potential food sources
387 in complex ecosystems such as the marine ecosystem studied here. Additionally, consumers tend to

feed on more than one food source and change their feeding strategy in relation to the food availability. The signal of e.g. the different diatom species, as well as that of the organisms making up the microzooplankton, may have varied greatly due to interspecific differences in fractionation (Aberle and Malzahn, 2007; Needoba et al., 2003).

Recent studies have used compound specific isotope analysis (CSIA) to investigate trophic linkages in marine food webs (e.g. Chikaraishi et al., 2014; Reiffarth et al., 2016). This technique measures the stable isotopes of biomarkers such as fatty acids or some amino acids (CSIA-AA) in the consumer and thereby determines its trophic level. While this method bypasses some of the potential issues of variable isotopic baselines it remains very labour- and cost-intense and analytically demanding. In addition, CSIA has some lingering issues, notably an underestimation of trophic positions based on CSIA-AA in the field (Decima et al., 2013) .

Combining stable isotope and fatty acid data

While the $\delta^{15}N$ signal shows the trophic level an organism feeds on, the $\delta^{13}C$ signal is habitually used to infer the dietary source of carbon. In our study, the $\delta^{13}C$ of the different copepod species were within similar ranges thus not allowing for food source differentiation based on stable carbon isotopes only. Herein lies the advantage of combined stable isotope and fatty acid analysis as with the help of the fatty acid composition we were able to trace the actual dietary preferences of the copepods (Dalsgaard et al., 2003; El-Sabaawi et al., 2009; Rossi et al., 2006; Stevens et al., 2004a). The fatty acid composition of the copepods helped strengthen and further elucidate the trophic linkages and food preferences between these consumers and their prey.

Acartia spp., *Centropages* spp. and *Temora longicornis* shared a similar $\delta^{15}N$ signature, which is in line with observations by Agurto (2007) and Aberle et al. (2010), and could therefore be assumed to feed on the same dietary items. A closer look at the fatty acid markers, however, showed some slight differences in feeding preference. Both *T. longicornis* and *Acartia* spp. only show relatively low amounts of carnivorous fatty acid markers and biomarkers indicate an omnivorous diet. *Centropages* spp. was richer in the carnivorous marker DHA/EPA than *T. longicornis* and *Acartia* spp., indicating

414 a higher proportion of heterotrophic dinoflagellates in the diet and hence a carnivorous tendency.
415 Previous studies have reported that while *Centropages* is considered an omnivorous copepod, it
416 selectively feeds on large motile prey, including ciliates and dinoflagellates, particularly at times of
417 high dinoflagellate biomass (Calbet et al., 2007; Saage et al., 2009). In the case of this copepod, the
418 fatty acid signatures presented in this study show selective feeding on microzooplankton invisible
419 from the stable carbon isotope signal. *Temora longicornis* is also known to be an omnivorously
420 feeding copepod, whose trophic position is highly variable throughout the year and shows great
421 flexibility in its feeding behaviour (Dam and Lopes, 2003; Gentsch et al., 2009). The fatty acid
422 markers found in *T. longicornis* reflect a flexible and omnivorous diet; the levels of the dietary fatty
423 acid markers DHA/EPA and the ratio of D/F in *T. longicornis* closely echo those of the seston. In a
424 recent study *T. longicornis* has been shown to feed selectively depending on temperature, preferring
425 autotrophic prey under warmer conditions and selectively feeding on heterotrophic organisms under
426 lower temperatures (Boersma et al., 2016). Higher δ_{15}N found in *T. longicornis* in winter might hence
427 not only reflect a passive feeding behaviour following the higher share of heterotrophic organisms in
428 the plankton, but also the temperature related selectivity for heterotrophic prey at colder temperatures
429 described by Boersma et al (2016).
430 While the annual mean δ^{15}N of *Calanus* spp. was higher than that of the other copepods sampled,
431 indicating feeding on a higher trophic level and a more carnivorous diet, the fatty acid biomarkers
432 showed that the diet also contained diatoms. *Calanus* is known to be omnivorous, feeding on both
433 dinoflagellates and diatoms (Harris et al., 2000; Meyer-Harms et al., 1999), although some studies
434 have shown *C. helgolandicus* to have a slight preference for diatoms (Irigoien et al., 2000). As
435 *Calanus* are known to occasionally feed selectively based on the size of the food particles (Frost,
436 1972), the relatively larger size of some diatoms could explain the marked presence of these
437 organisms in their diet. This was highlighted in the fatty acid composition of the *Calanus* in the
438 present study, while the trophic level based on stable isotope data alone would have indicated a strong
439 preference for heterotrophic prey.

In conclusion, combining the stable isotope and fatty acid biomarker approach to investigate food web interactions and trophic linkages has proven to be a powerful tool, disentangling the relative trophic position and feeding preferences of copepods at Helgoland Roads. This combination is particularly valid since seston stable isotope signals display such an amount of unexplained variance. Finding a proper baseline for stable isotope studies on plankton communities is still a major challenge for further research.

ACKNOWLEDGEMENTS

Special thanks to the crew of R.V. Aade for collecting samples in all seasons and and at all weather conditions. Gunnar Gerdts and Antje Wichels provided valuable input at different phases of the experimental design, analysis, and writing of the manuscript. We are grateful to S. Peters for the diatom counts, to K. Carstens for the analysis of the nutrient data and to B. Oppermann for the fatty acid analysis. We thank Thomas Hansen for stable isotope analysis and helpful discussions, and Ulrich Sommer, Birte Matthiessen and Jamileh Javidpour for their hospitality and scientific support at GEOMAR. K.L.S. was funded by the German Science Foundation (DFG AB 289/2-1). This study is part of the AWI Food Web Project

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