1   Dietary and seasonal variability in trophic relations at the base of the North Sea

2   pelagic food web revealed by stable isotope and fatty acid analysis

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17 **ABSTRACT**

18 A two-dimensional biomarker approach including fatty acids and stable isotopes of seston and

19 copepods was applied to examine how the variability at the base of the food web affects trophic

20 interactions between primary producers and copepod consumers over a sampling period of two years.

21 We investigated how the composition of the seston affected feeding behaviour by analysing the fatty

22 acid and stable isotope signals of the copepods *Calanus helgolandicus*, *Acartia* spp., *Centropages*

23 spp. and *Temora longicornis* at Helgoland Roads, North Sea. Our results indicate that the relative

24 contributions of autotrophic and heterotrophic fractions in the seston determined the stable isotope

25 signal of the seston and hence the $\delta^{15}N$ of copepods. Our findings show that the combination of stable

26 isotope and fatty acid analyses provides an ideal tool to address the complexity of trophic relations in

27 planktonic food-webs and to define relative trophic position and feeding preferences of e.g. copepods.

28 Defining accurate baselines from bulk seston samples containing a mixture of auto- and heterotroph

29 protist communities still remains a challenge when defining lower food-web dynamics in natural

30 plankton communities.

31

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

33 seston

INTRODUCTION

34

35   Despite decades of research, consumer-producer interactions in the pelagic zone are still not entirely

36   understood. There are several reasons for this. On the producer side, there are many organisms that

37   are at least partly heterotrophic, and on the consumer side, there is large variation in diets between

38   and within species. Especially copepods, which form an important link between primary producers

39   and higher consumers, require further study, as the trophic position of copepods plays a major role in

40   shaping aquatic food webs (Hairston and Hairston, 1993). Most copepods are omnivores feeding on

41   a wide range of dietary items, such as diatoms, flagellates and ciliates (Kleppel, 1993). However,

42   copepods are able to feed selectively (Fileman et al., 2007; Irigoien et al., 2000; Paffenhöfer, 1988)

43   and thus they are capable of switching between dietary items of different quality, even within species

44   (Meunier et al., 2016). This switch by copepods from feeding lower in the food web, as herbivores,

45   to carnivory has consequences for lower levels in the food web and for consumers at higher trophic

46   levels. As such, the trophic flexibility of copepods affects the structure of entire marine food webs.

47   Therefore, the objective of the present study was to establish the role of different copepod species in

48   the planktonic food web by using a combined tracer approach combining stable isotope and fatty acid

49   data to investigate seasonal patterns and shifts in trophic positions of major North Sea copepod

50   species.

51   The interactions in the marine pelagic food web are complex and subject to a great variety of

52   influences. Particularly at the base of the food web the interactions between primary producers and

53   consumers are characterized by a great variability in food quantity (e.g. Sommer, 1996; Wiltshire et

54   al., 2008) and quality (e.g.Boersma et al., 2008; Klausmeier et al., 2004; Malzahn et al., 2007; Schoo

55   et al., 2012). Strong seasonal changes in the availability and composition of microalgae occur due to

56   high peaks in productivity during blooms. During the spring bloom, for example, phytoplankton

57   biomasses reach a peak, which is usually followed by a rapid increase in zooplankton abundance. As

58   the increase of phytoplankton biomass during the bloom causes a depletion of nutrients available in

59   the seawater, the quality (in terms of nutrient stoichiometry) of the phytoplankton decreases over the

60　course of the bloom. At the same time, increasing numbers of micro- and mesozooplankton exert high

61　grazing pressure on phytoplankton and reduce its biomass substantially. This change in prey quality

62　(nutrient stoichiometry), composition and quantity at the base of the pelagic food webs has been

63　shown to not only affect the herbivores directly feeding on microalgae, but also potentially those

64　secondary consumers that feed on the herbivores (Malzahn and Boersma, 2009; Malzahn et al., 2010;

65　Schoo et al., 2010; Schoo et al., 2014).

66　As food sources have distinct biochemical compositions that can become incorporated into the

67　consumers' body, and tracers such as stable isotopes and fatty acids integrate the diet over a longer

68　period of time (days to weeks in small ectotherms, e.g. *Acartia tonsa* (Tiselius and Fransson, 2016;

69　Vander Zanden et al., 2015), tracer approaches are an effective way to investigate trophic interactions

70　(Aberle et al., 2010; El-Sabaawi et al., 2009; Richoux and Froneman, 2009). As such they have

71　allowed for detailed reconstructions of food sources and trophodynamic interactions (Dalsgaard et

72　al., 2003; Kurten et al., 2013; Peterson and Fry, 1987; Ponsard and Arditi, 2000). Stable isotopes are

73　commonly used in ecological studies to deduce trophic position and dietary source (El-Sabaawi et al.,

74　2013; Post, 2002; Vander Zanden and Rasmussen, 2001). As a rule, the $\delta^{15}N$ signal is used to infer

75　the trophic position of an organism, as the percentage of ^{15}N relative to ^{14}N in the tissue increases

76　progressively and predictably with increasing trophic position of the consumer. $\delta^{15}N$ fractionates with

77　trophic level on average around 3.4‰ (Minagawa and Wada, 1984), however, the values observed in

78　aquatic animals may vary from 2.3‰ to 4.5‰ (McCutchan et al., 2003). Carbon stable isotopes are

79　used to infer the carbon dietary source (Fry, 2006; Minagawa and Wada, 1984), as the carbon source

80　and the different enzymes involved in carbon fixation show distinct fractionation, leading to different

81　$\delta^{13}C$ values. Trophic enrichment, however, is not static and it varies both between different consumer

82　species (Aberle et al., 2005; Gutierrez-Rodriguez et al., 2014; Post, 2002; Vander Zanden and

83　Rasmussen, 2001), as well as within species as a result of changing food qualities (Vander Zanden

84　and Rasmussen, 2001), and differences in specificity of different metabolic processes (Aberle and

85　Malzahn, 2007; Gorokhova and Hansson, 1999; Ponsard and Averbuch, 1999).

86　Fatty acid markers commonly used in trophic studies can be either single fatty acids, associated with

87　a particular type of organism, or a ratio of fatty acids. Certain primary producers contain very specific

88　fatty acids, which can be used to characterize them. As fatty acids are often incorporated by their

89　consumers without being modified, they can be used to trace dietary sources. Palmitoleic acid

90　(16:1ω7), for example, is a diatom fatty acid marker (Lee et al., 2006). The ratio of 22:6ω3

91　(Docosahexaenoic acid, DHA) to 20:5ω3 (Eicosapentaenoic acid, EPA) is used to assess the

92　proportion of dinoflagellates to diatoms in the diet, because dinoflagellates contain high amounts of

93　DHA, while diatoms are rich in EPA (Budge and Parrish, 1998; Dalsgaard et al., 2003; El-Sabaawi

94　et al., 2010). A high ratio of DHA to EPA could also indicate a carnivorous diet (El-Sabaawi et al.,

95　2009). High amounts of 18:1ω9 relative to 18:1ω7 have been shown to indicate carnivory in copepods

96　and other crustaceans (Nyssen et al., 2005; Schmidt et al., 2003; Stevens et al., 2004a). Since

97　carnivorous copepods contain larger amounts of polyunsaturated fatty acids (PUFA) than herbivorous

98　copepods, the ratio of PUFA to saturated fatty acids (SFA) can be used to identify the degree of

99　carnivory (Stevens et al., 2004b). However, because some of the fatty acids, such as DHA and some

100　polar fatty acids, are sometimes preferentially retained by certain copepods, this can obfuscate the

101　dietary signature of primary producers (Dalsgaard et al., 2003; El-Sabaawi et al., 2009). Additionally,

102　some fatty acids can be metabolised and transformed by the consumers (Budge and Parrish, 1998).

103　Assertions about the trophic position of consumers based solely on fatty acids, without precise

104　knowledge of that particular consumer's metabolism and physiology, are therefore problematic.

105　While both fatty acid and stable isotope analysis have their limitations, the combination of these

106　techniques may provide a more powerful tool to determine trophic interactions in complex food webs

107　(Gaillard et al., 2017; Perga et al., 2006; Petursdottir et al., 2012; van der Bank et al., 2011) . The

108　advantage of this combined tracer approach is mainly attributed to the fact that FAs are more specific

109　to dietary source than stable carbon isotopes, particularly when differences in δ^{13}C of different carbon

110　sources are small (El-Sabaawi et al., 2009). Combining both techniques has thus a high potential to

111　enable investigations of seasonal changes in trophic relations and dietary variability in the plankton

112 in detail. Hence, in this study we used these two markers to investigate inter- and intra-species

113 variation in key copepod species in the Southern North Sea. Further, by estimating the proportion of

114 autotrophs vs. heterotrophs in the seston fraction, we aimed to refine the estimate of baseline stable

115 isotope signals. Given the finding by previous authors (e.g. Kleppel, 1993) that different copepod

116 species have different diets, we investigated the trophic positions of four dominant copepod species

117 in the North Sea over the course of two years.

118

119 **MATERIALS AND METHODS**

120 The rocky island of Helgoland is situated in the Southern North Sea, German Bight, about 70 km

121 from the mainland. The long-term sampling station Helgoland Roads is located between the main

122 island and the sand dune island (54°11' N, 7°54'E). Due to strong tidal currents and the shallow

123 depth, the water column is well mixed (Hickel, 1998). Surface water samples for the analysis of seston

124 composition, stable isotope signature, fatty acid content and nutrient concentrations as well as

125 zooplankton samples were gently taken with buckets by the RV Aade at Helgoland Roads between

126 January 2007 and December 2008.

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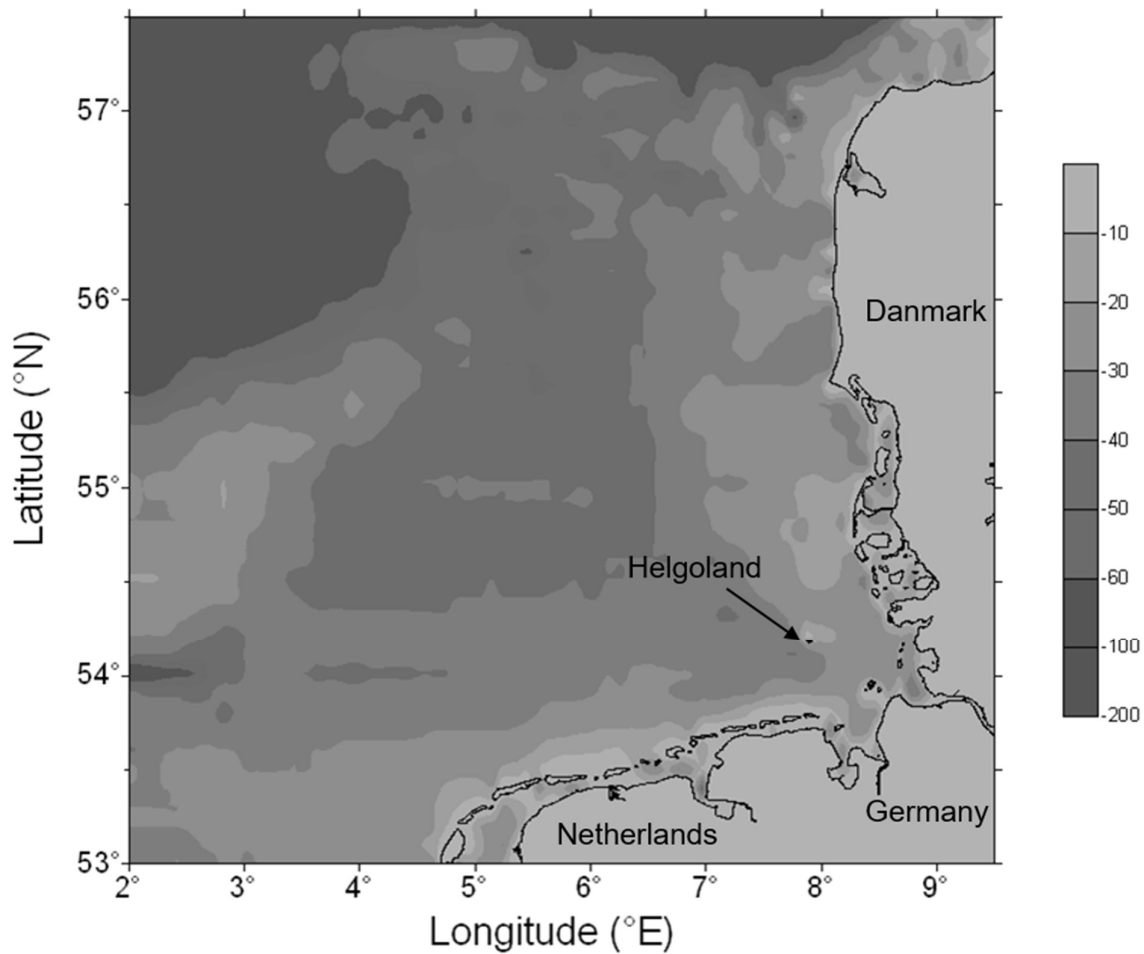
Sampling focused on the base of the food web, represented by the seston (particulate organic matter) and mesozooplankton consumers, represented by copepods. To provide a baseline relevant to the feeding of the primary consumers seston samples were collected at the same time as the zooplankton. Nutrient 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.

141 Phytoplankton carbon concentrations were obtained from the Helgoland Roads long-term monitoring

142 program (Wiltshire et al., 2008). Samples of surface water for the determination of microzooplankton

143 were preserved with acid Lugol's solution (2% final concentration), and the organisms identified to

144 species level as described by Löder et al. (2010). Many of the dinoflagellates in the plankton are

145 considered to be mixotrophs and able to take up particles via phagotrophy, even if they contain

146 chloroplasts. Hence, for our division of heterotrophic versus autotrophic components in the plankton

147 they were assigned to the microzooplankton (Löder et al., 2010). Biovolume of microzooplankton

148 was calculated from the measurement of cell dimensions using geometrical formula according to

149 Hillebrand et al. (1999) and subsequent conversion to carbon content was done after Putt and Stoecker

150 (1989) and Menden-Deuer and Lessard (2000).

151 Zooplankton samples were obtained by oblique net hauls (mesh size 180 μm and 500 μm). Animals

152 were sorted shortly after collection. Four copepod taxa were sampled: *Calanus helgolandicus*,

153 *Temora longicornis*, *Centropages* spp. and *Acartia* spp. (mainly *A. clausi*). Copepod samples were

154 taken for the analysis of stable isotopes and fatty acids.

155

Fatty acid analysis

157 Seston was extracted for the analysis of fatty acids by filtering pre-screened surface water samples

158 through pre-combusted GF/F filters (Whatman). Three replicate filters were taken on each sampling

159 occasion. The filters were placed in reaction tubes and frozen at -80°C. Copepods for the fatty acid

160 analysis were sorted into reaction tubes and frozen at -80°C until further analysis. The fatty acids of

161 seston and copepods were measured as fatty acid methyl esters (FAMEs). Lipids extraction followed

162 modified methods described by Folch (1957) and Bligh and Dyer (1959). Fatty acid samples were

163 extracted in Dichloromethane:methanol (2:1 vol:vol) using an ultrasound bath for 30 min. After

164 centrifugation, water-soluble fractions were removed by washing with 0.88% KCl buffer. Thereafter,

165 the aqueous phase was removed and the organic remainder evaporated using nitrogen gas.

166 Esterification was achieved using methanolic-sulphuric acid at 70°C for 75 min. FAMEs were washed

167 from the methanolic sulphuric acid using n-Hexane, excess n-Hexane evaporated using nitrogen and

168 FAMEs analysed using a Varian CP 8400 gas chromatograph equipped with a DB-225 column (J&W

169 Scientific, 30 m length, 0.25 mm ID, 0.25 μm film). 1 μL aliquots of samples were injected using a

170 split less mode. FAMEs were quantified using calibrations set up for each fatty acid separately and a

171 known amount of C 23:0 was added at the first step of the preparation as an internal standard. More

172 detailed information on injector temperature, column oven set-up and carrier gases are described in

173 Malzahn et al. (2010). A known amount of C23:0 was used as an internal standard to calculate fatty

174 acid concentration.

175 In this study, we focussed on fatty acids as trophic markers in the lipid fractions and did not account

176 for wax esters and fatty alcohols although a considerable amount of these can be found especially in

177 calanoid copepods (Kattner et al., 2007; Kattner and Krause, 1989; Lee et al., 2006).

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

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

181

Marker	Diet	Reference
$16:1\omega7$	Diatom	Lee et al., 2006
$18:1\omega7$	Bacteria or *de novo* synthesis	Stevens et al., 2004b
$18:1\omega9$	Carnivory	Graeve et al., 1994
$18:1\omega9/18:1\omega7$	Carnivory	Stevens et al., 2004a
		Nyssen et al., 2005
$18:4\omega3$	Dinoflagellates	Lee et al., 2006
$20:5\omega3$ (EPA)	Diatoms	Dalsgaard et al., 2003
$12:6\omega3$ (DHA)	Dinoflagellates	Budge and Parish, 1998
DHA/EPA	Dinoflagellates / Diatoms	Budge and Parish, 1998
	Carnivory	Dalsgaard et al., 2003
PUFA/SFA	Carnivory	Stevens et al., 2004b
D/F	Diatoms / Flagellates	Dalsgaard et al., 2003
		El-Sabaawi et al., 2009

182
183 **Stable isotope analysis**

184 Copepods for stable isotope analysis were rinsed in distilled water and dried in tin capsules.

185 Depending on the size (biomass) of the copepods each tin cup contained between 3 and 30 individuals

186 to meet the analytical requirements for the isotope analysis.

187 Stable isotope analysis of the samples was performed in two laboratories, at the GEOMAR in Kiel,

188 Germany, and at the UC Davis Stable Isotope Facility in Davis, California, USA. At the GEOMAR

189 in Kiel the samples were analysed by using an isotope ratio mass spectrometer (Thermofinnigan EA

190 1110 CHNS). Samples at UC Davis Stable Isotope Facility were analyzed using a PDZ Europa

191 ANCA-GSL elemental analyser interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer

192 (Sercon Ltd., Cheshire, UK). The standards used were PeeDee belemnite for C and atmospheric

193 nitrogen for N. During measurements, the ratio of the $^{13}C/^{12}C$ and the ratio of the $^{15}N/^{14}N$ stable

194 isotopes were determined. Isotopic abundances are expressed in δ notation in parts per thousand (‰):

195 $\delta = ((R_{sample} / R_{standard})-1) *1000$, where R is the ratio of the heavier isotope to the lighter isotope, i.e.

196 $^{13}C/^{12}C$ or $^{15}N/^{14}N$. Trophic fractionation of stable isotopes is described as the difference of the δ

197 values among food sources, namely the seston, (A) and consumer (B) using Δ notation, where

198 $\Delta = \delta_B - \delta_A$. A positive Δ value indicates an enrichment of the heavier stable isotope in the consumer

199 B.

200 Apart from detritus and inorganic material, the seston samples consist of autotroph fractions (e.g.

201 diatoms, phytoflagellates) and heterotroph fractions (e.g. ciliates, mixo-/heterotrophic

202 dinoflagellates). To estimate the $\delta^{15}N$ signal of these different fractions in the seston, we used the

203 following equation:

204 $\delta^{15}N_{seston} = C_{autotroph} * \delta^{15}N_{autotroph} + C_{heterotroph} * (\delta^{15}N_{autotroph} + 2.2)$

205 where $C_{autotroph}$ is the carbon biomass of the autotrophs expressed as fraction of total seston carbon

206 biomass and $C_{heterotroph}$ is the fraction of the heterotrophic biomass, estimated from the

207 microzooplankton counts, thus $C_{autotroph}+ C_{heterotroph} = 1$. We assumed a 2.2‰ trophic fractionation

208 between the autotrophic and the heterotrophic fractions of the seston. This level of fractionation

209 between two trophic levels is generally accepted for invertebrates (McCutchan et al., 2003). In this

210 manner, the theoretical $\delta^{15}N$ signals of the autotroph and the heterotroph fractions of the seston were

211 calculated and used to compute the delta signals of both fractions.

212

213 **Statistical analyses**

214 Correlations between seston fatty acids and copepod fatty acids as well as $\delta^{15}N$ of the copepods and

215 their fatty acid markers were conducted using linear regression analyses.

216 Linear regressions were also performed for: (1) $\delta^{15}N$ signals of autotroph and heterotroph fractions,

217 (2) $\delta^{15}N$ of the seston and the biomass of the heterotrophic organisms as well as (3) between the fatty

218 acids from the seston and the $\delta^{13}C$ signal.

219

220 **RESULTS**

221 *Seston*

222 The spring bloom in 2007 was dominated mainly by diatoms (Figure 2). The diatom bloom developed

223 rapidly from mid-April onwards and diatom biomass reached a maximum of 270 µg C L^{-1} in early

224 May. The diatom bloom was instantaneously followed by a bloom of microzooplankton dominated

225 by ciliates. Throughout the rest of the year, the microzooplankton was dominated by mixo- and

226 heterotrophic dinoflagellates reaching a maximum of about 140 µg C L^{-1} in July. Total biomass then

227 decreased to about 100 µg C L^{-1} for the remainder of the summer and declined further following a

228 short secondary bloom in October. During the winter months the biomass remained low at around 20-

229 30 µg C L^{-1}. The spring bloom of 2008 occurred later than in the previous year, with a higher peak

230 diatom biomass (335 µg C L^{-1}) recorded only in June. The microzooplankton peak biomass of 240

231 µg C L^{-1} was reached in July.
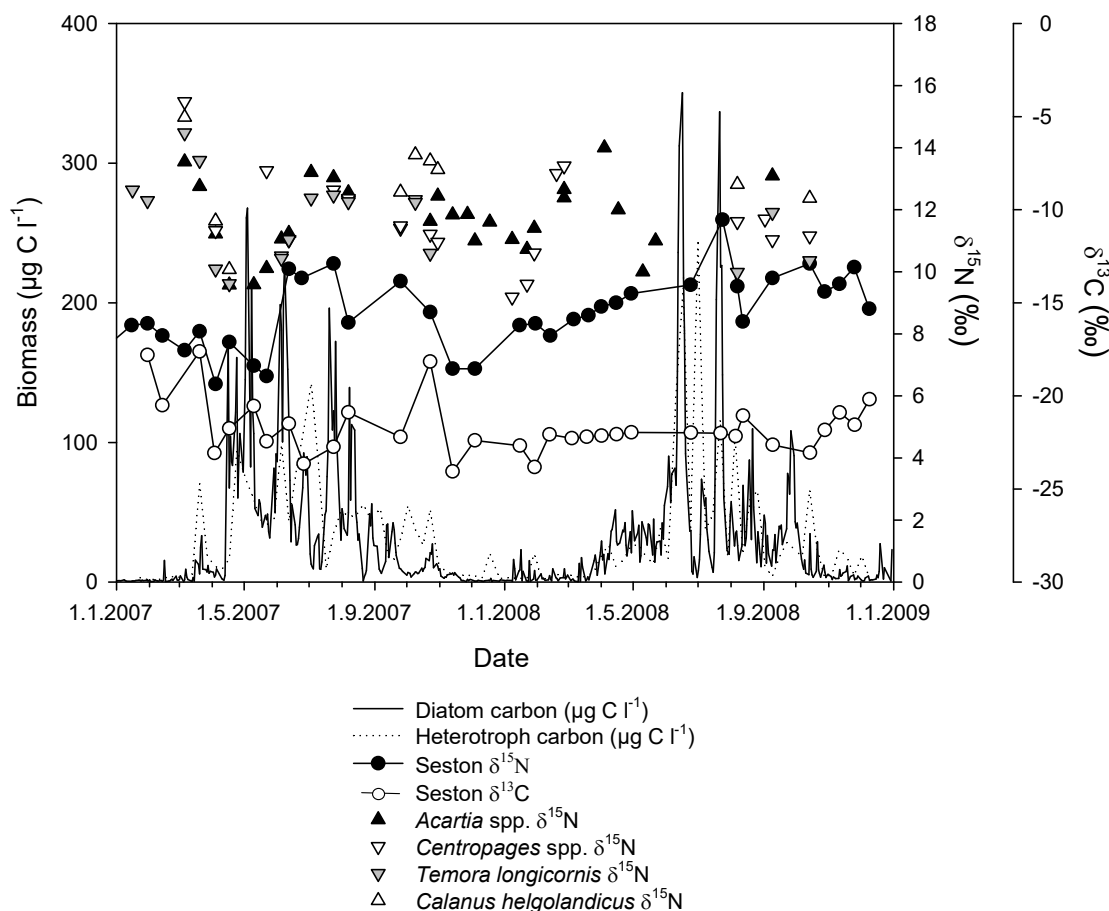
Figure 2: *δ15N (‰) and δ13C (‰) of the seston and δ15N (‰) of the four copepod species as well as carbon biomass (µg l-1) of diatoms and heterotrophic microzooplankton at Helgoland Roads from January 2007 to December 2008. Note the two different axes for δ15N (‰) and δ13C (‰).*

The $\delta^{15}N$ stable isotope signal of the seston ranged from 6.3‰ in spring 2007 to 11.7‰ in summer 2008 (Figure 2). The $\delta^{15}N$ of the seston decreased from 8‰ in winter to 6‰ at the start of the spring bloom. Following the diatom bloom peak the $\delta^{15}N$ increased again, reaching values of 10‰ in July. This corresponded to the period of the highest heterotrophic biomass. The $\delta^{15}N$ decreased during the winter months, with decreasing seston biomass. The $\delta^{15}N$ signal of the seston increased again rapidly in February of 2008 and continued to increase until the summer. A drop in the $\delta^{15}N$ stable isotope values was observed in August 2008, followed by an increase during an autumn bloom of diatoms 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.
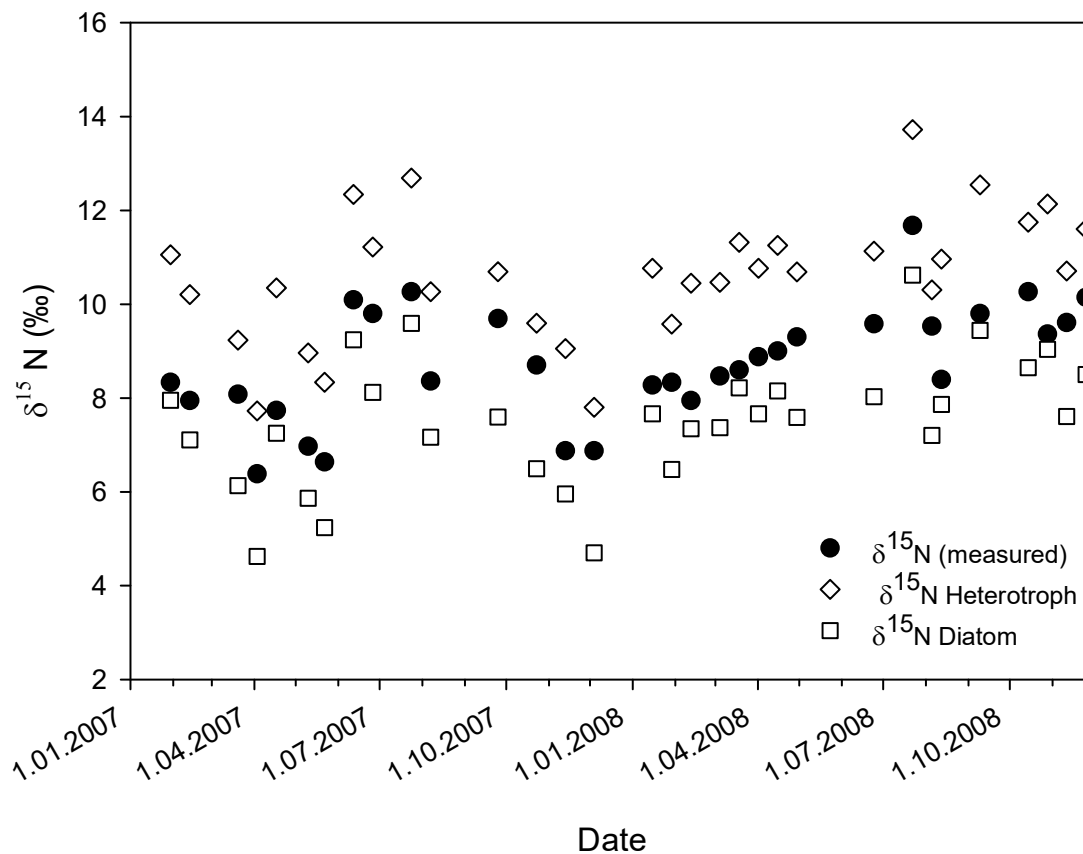
259   *Figure 3: Seston $\delta^{15}N$ (‰) and calculated $\delta^{15}N$ for diatom and heterotroph fractions.*

260

261   The fatty acid content of the seston changed according to the seston composition (Figure 4). There

262   was a strong seasonal change in the relative amounts of certain fatty acids. During the diatom bloom

263   in May 2007 high amounts of eicosapentanoic acid (20:5 ω3, EPA), prevalent in diatoms, were

264   recorded (Figure 4A). Concurrently to the increase in heterotrophic biomass in June 2007 increased

265   amounts of the dinoflagellate tracer fatty acids 18:1 ω9 and 22:6 ω3 (docosahexaenoic acid, DHA)

266   were measured (Figure 4B). Throughout summer and autumn the concentration of 18:1 ω9 remained

267   high in the seston, while 22:6 ω3 (DHA) displayed a second peak in late summer. The dominant fatty

268   acids during the winter months were again those associated with heterotrophic organisms, in

269   particular 18:1 ω9.

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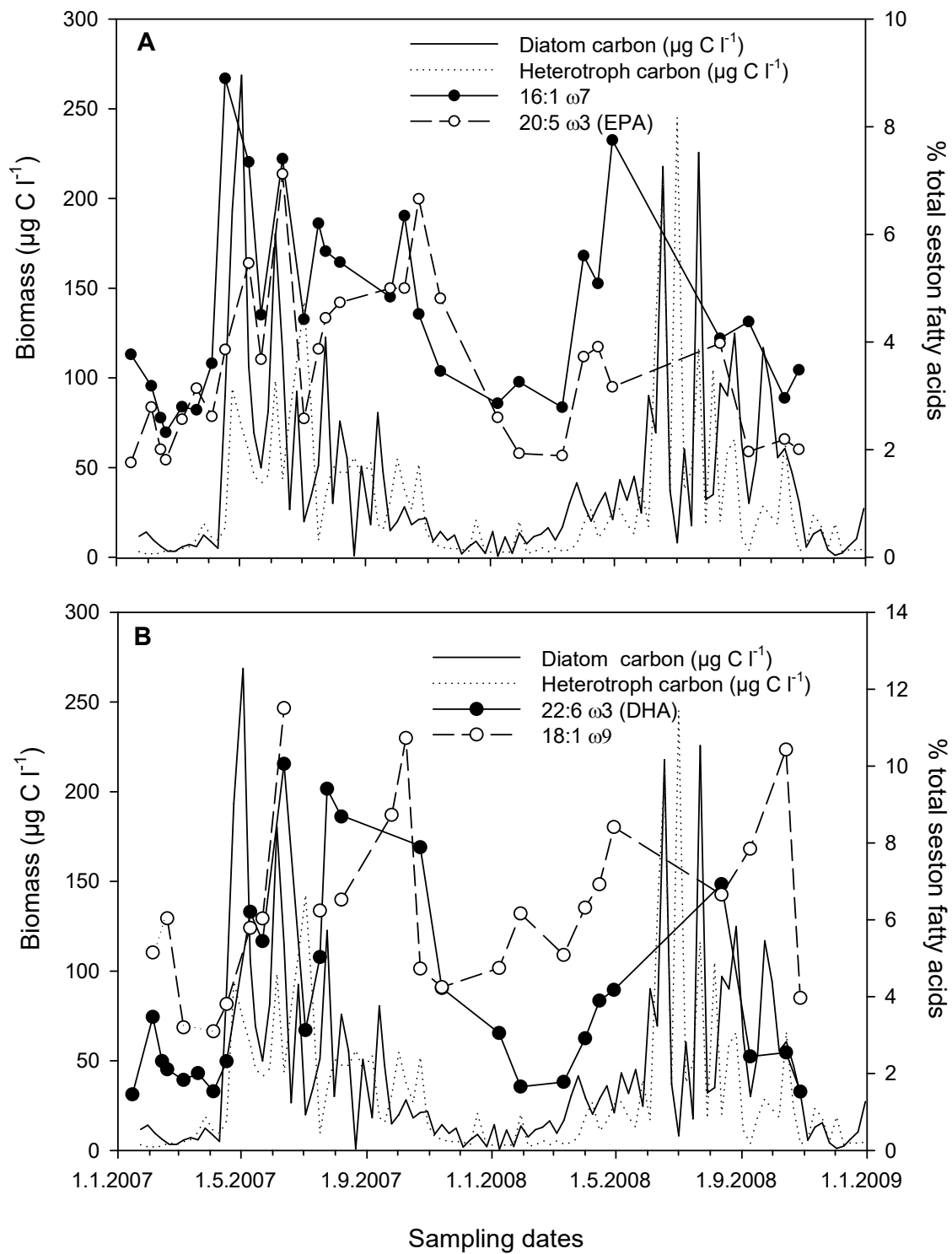
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272　　*Figure 4: Seasonal variability of diatom fatty acid markers (A) and dinoflagellate fatty acid markers (B) overlaid on diatom and*
273　　*microzooplankton biomass.*

274

275 The δ^{15}N signal of the seston correlated with 18:1 ω7 (linear regression analysis: r^2= 0.19, p<0.05),

276 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

277 significant correlations between the fatty acids from the seston and the δ^{13}C signal were found.

278

279 *Copepods*

280 The δ^{15}N signature of the copepods showed strong seasonal fluctuations (Figure 2). The δ^{15}N signals

281 ranged from 9‰ to 15‰. Overall, the highest average δ^{15}N throughout the sampling period was

282 recorded in *Calanus helgolandicus*, followed by *Centropages* spp. and *Acartia* spp., while the lowest

283 δ^{15}N was observed in *Temora longicornis* (Figure 2 & Figure 5).

284 The trophic fractionation of the copepods relative to the seston was calculated and expressed as $\Delta\delta^{15}$N

285 of the copepods. This value also showed a wide range over the time sampled, from as low as 1‰ to

286 8‰, with strong differences between species and seasons. Generally, the $\Delta\delta^{15}$N of the copepods was

287 highest in winter, declined with the onset of the spring bloom and reached its lowest level in early

288 summer. This pattern displays the opposite trajectory to the diatom biomass and could indicate an

289 increased feeding on autotrophic organisms during the spring bloom. The $\Delta\delta^{15}$N of most copepods

290 increased again in July and remained elevated through the autumn. The highest difference in trophic

291 enrichment between species was observed in autumn, where the $\Delta\delta^{15}$N values ranged from 1.8‰ to

292 6.4‰. In *Acartia* spp. the lowest enrichment coincided with the spring bloom, indicating that this

293 copepod species fed on a herbivorous diet during that particular time. Enrichment was higher in late

294 autumn and winter, when the diatom biomass was lowest. A similar pattern was observed in *C.*

295 *helgolandicus. T. longicornis* showed a high level of enrichment in spring and late summer, while the

296 highest level of enrichment for *Centropages spp.* was recorded in July and August. *Centropages* spp.

297 displayed the highest increase in $\Delta\delta^{15}$N in the winter with values rising from 0.9‰ in January to 5.5‰

298 in late February.

299 The δ¹³C of copepods showed strong fluctuations (Figure 2). The highest δ¹³C signals were recorded

300 in May 2007 around the time of the diatom spring bloom. The δ¹³C signal of *Acartia* spp. varied from

301 -23 to -18‰. The highest δ¹³C signals for this copepod were observed in May 2007 and September

302 2008. The lowest values (-23‰) were found in early March 2007, with another strong decrease in the

303 spring of 2008. A very similar pattern was observed for the δ¹³C of *T. longicornis* and *Centropages*

304 spp.. The δ¹³C for *C. helgolandicus* was slightly lower, i.e. less enriched, than that of the other

305 copepods throughout the sampling period (Figure 2 & Figure 5).
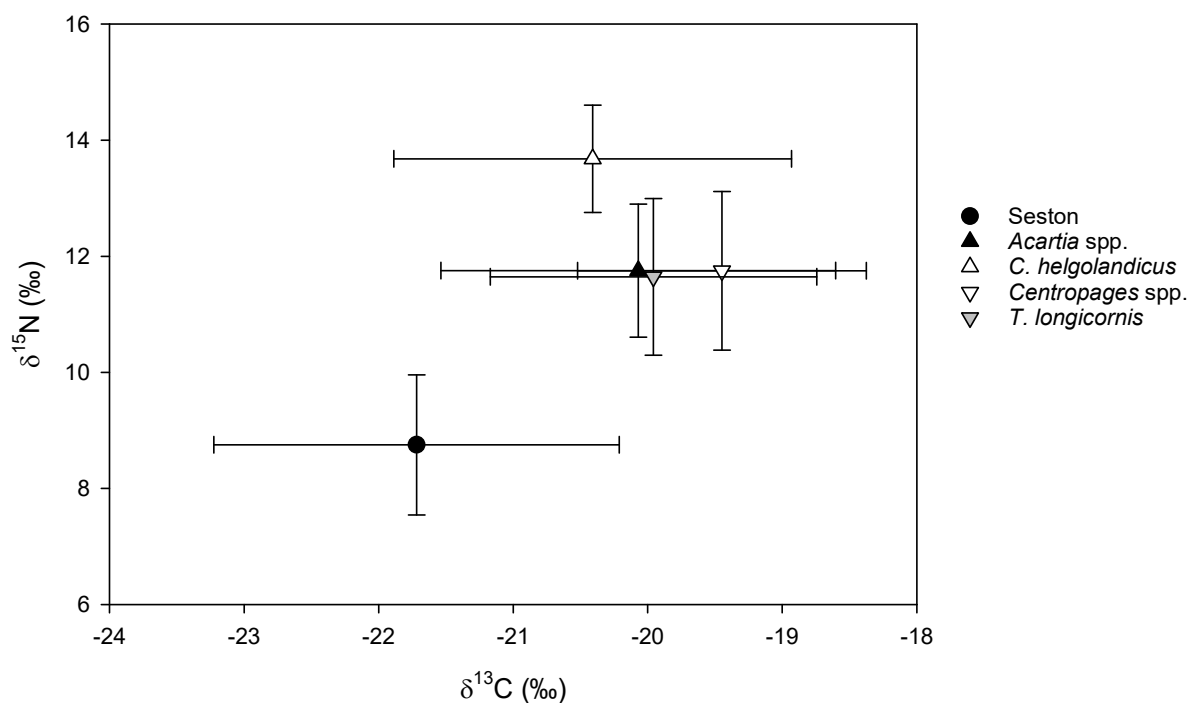
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307 *Figure 5: Isotope biplot of δ¹⁵N (‰) and δ¹³C (‰) of seston and zooplankton collected at Helgoland Roads from 2007-2008. Shown*
308 *are means and standard deviations.*

309
310 *Table 2: Correlations between seston fatty acids and copepod fatty acids. * denotes p<0.05, ** denotes p<0.01, n.s. identifies no*
311 *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

333   markers (Figure 6). The relative positions of the copepods on the plot give an indication of the dietary

334   preference and the resulting trophic position. By using the combined FA and SI approaches we could

335   depict a distinct trophic position of *C. helgolandicus* compared to other copepod species, showing the

336   highest δ^{15}N values, almost one trophic level above that of the other copepods, and also the highest

337   concentration of the carnivory markers PUFA/SFA (Figure 6 B) and 18:1 ω9/18:1 ω7 (Figure 6 C).

338   In terms of the ratio of diatoms to dinoflagellates in the diet, however, *C. helgolandicus* showed a

339   rather balanced diet (Figure 6 D). This stresses the outstanding trophic role of *C. helgolandicus* when

340   compared to other North Sea copepods. In contrast, the other three copepods examined in this study

341   show similar δ^{15}N values, but have slightly different fatty acid profiles. The fatty acid composition

342   of *T. longicornis* reveals a preference for dinoflagellates, indicated by the high D/F ratio (Figure 6

343   D). Confounding this is the relatively low DHA/EPA ratio observed, which indicates a larger amount

344   of diatoms (EPA) relative to dinoflagellates (DHA) in the diet of this copepod. *Centropages spp.* on

345   the other hand contained a relatively high ratio of DHA/EPA, indicating a preference for

346   dinoflagellates, and a comparatively low amount of D/F (Figure 6 A). Both the fatty acid spectrum

347   and the δ^{15}N values of *Acartia spp.* indicate the omnivorous diet of this copepod, not exhibiting any

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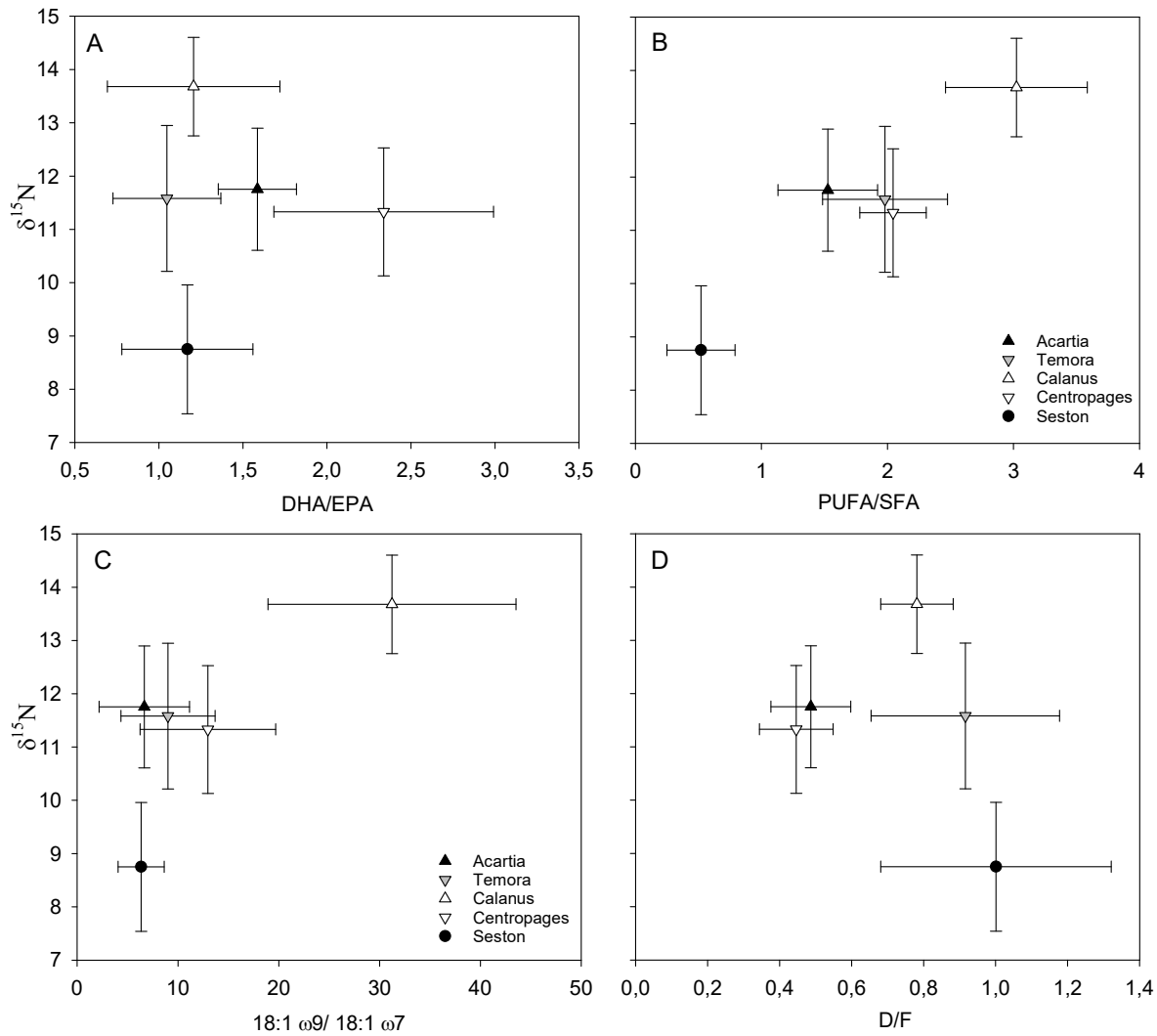
Figure 6: δ^{15}N (‰) and concentration of different fatty acid biomarkers (A) DHA/EPA, (B) PUFA/SFA, (C) 18:1ω9/18:1ω7 and (D) D/F expressed as % of total fatty acids for four species of copepods. Mean values for one year. Error bars indicate standard deviation.

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 δ^{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 δ^{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 δ^{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 δ^{15}N in the

382 consumers (e.g.Jones et al., 2004; Vanderklift and Ponsard, 2003; Vuorio et al., 2006). The

383 enrichment of δ^{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

388 feed on more than one food source and change their feeding strategy in relation to the food

389 availability. The signal of e.g. the different diatom species, as well as that of the organisms making

390 up the microzooplankton, may have varied greatly due to interspecific differences in fractionation

391 (Aberle and Malzahn, 2007; Needoba et al., 2003).

392 Recent studies have used compound specific isotope analysis (CSIA) to investigate trophic linkages

393 in marine food webs (e.g. Chikaraishi et al., 2014; Reiffarth et al., 2016). This technique measures

394 the stable isotopes of biomarkers such as fatty acids or some amino acids (CSIA-AA) in the consumer

395 and thereby determines its trophic level. While this method bypasses some of the potential issues of

396 variable isotopic baselines it remains very labour- and cost-intense and analytically demanding. In

397 addition, CSIA has some lingering issues, notably an underestimation of trophic positions based on

398 CSIA-AA in the field (Decima et al., 2013) .

399 *Combining stable isotope and fatty acid data*

400 While the δ^{15}N signal shows the trophic level an organism feeds on, the δ^{13}C signal is habitually used

401 to infer the dietary source of carbon. In our study, the δ^{13}C of the different copepod species were

402 within similar ranges thus not allowing for food source differentiation based on stable carbon isotopes

403 only. Herein lies the advantage of combined stable isotope and fatty acid analysis as with the help of

404 the fatty acid composition we were able to trace the actual dietary preferences of the copepods

405 (Dalsgaard et al., 2003; El-Sabaawi et al., 2009; Rossi et al., 2006; Stevens et al., 2004a). The fatty

406 acid composition of the copepods helped strengthen and further elucidate the trophic linkages and

407 food preferences between these consumers and their prey.

408 *Acartia* spp., *Centropages* spp. and *Temora longicornis* shared a similar δ^{15}N signature, which is in

409 line with observations by Agurto (2007) and Aberle et al. (2010), and could therefore be assumed to

410 feed on the same dietary items. A closer look at the fatty acid markers, however, showed some slight

411 differences in feeding preference. Both *T. longicornis* and *Acartia* spp. only show relatively low

412 amounts of carnivorous fatty acid markers and biomarkers indicate an omnivorous diet. *Centropages*

413 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.

440 In conclusion, combining the stable isotope and fatty acid biomarker approach to investigate food

441 web interactions and trophic linkages has proven to be a powerful tool, disentangling the relative

442 trophic position and feeding preferences of copepods at Helgoland Roads. This combination is

443 particularly valid since seston stable isotope signals display such an amount of unexplained variance.

444 Finding a proper baseline for stable isotope studies on plankton communities is still a major challenge

445 for further research.

446

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