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Seasonal and spatial fatty acid profiling of the calanoid copepods *Temora longicornis* and *Acartia clausi* linked to environmental stressors in the North Sea

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ABSTRACT

The Belgian part of the North Sea (BPNS) is subjected to multiple environmental stressors. The impact of these stressors includes the modulation of fatty acid (FA) composition of the zooplankton. This study recorded temporal and spatial patterns of the FA profiles of two dominant calanoid copepods within the BPNS: *Temora longicornis* (Müller, 1785) and *Acartia clausi* (Giesbrecht, 1889). By means of distance-based linear modelling and by applying multi model inference to generalized additive models, environmental stressors were linked to patterns of the FA profiles of these species. The FA profiles of *A. clausi* and *T. longicornis* showed distinct intraspecific, spatial and temporal differences within the BPNS. Temperature and algal food quality (marked by the ratio of silicate concentration to dissolved inorganic nitrogen concentration, SiO_4/DIN) were the most important drivers of seasonal fluctuations in the DHA/EPA ratio of both species. DHA/EPA ratio can be used as marker for stress in copepods in the BPNS in order to have a quick indication of food quality changes at the basis of the food web.

1. Introduction

The Belgian part of the North Sea (BPNS) is characterized by shallow waters with pronounced seasonal variability of abiotic factors such as light and temperature (Otto et al., 1990; Sündermann and Pohlmann, 2011). This seasonal variability is challenging for marine organisms inhabiting these waters, as the changing abiotic factors also lead to large fluctuations in food availability and quality (e.g. Muylaert et al., 2006; Otto et al., 1990; Sündermann and Pohlmann, 2011). In addition to seasonal variability, sea water temperature, salinity, and pH are being altered due to climate change (IPCC, 2014; Wiltshire and Manly, 2004), while vast amounts of nutrients and pollutants have entered the marine ecosystem (Airoldi & Beck 2007; Weis, 2014). Pollutant-related adverse effects were previously found in the BPNS resulting from the presence of complex mixtures of micropollutants (Janssen et al., 2010). The combination of these environmental stressors may instigate changes in a whole range of ecological interactions with potentially vast repercussions for overall ecosystem functioning and stability (Crain et al., 2008).

Very fast responses to the changing marine environment are found in the zooplankton, consisting of passively drifting small organisms and fish larvae (Taylor et al., 2002). At the base of the marine pelagic food web, they form the plant-animal interface and any change in the structure of this community is expected to propagate to higher trophic levels (Hays et al., 2005; Taylor et al., 2002). Therefore, understanding and quantifying the potentially adverse effects of multiple stressor conditions on the zooplankton is of primary importance. The impacts of multiple stressors are not only observed in altered biomasses and productivity of the species discerned (Crain et al., 2008), but also lead to fluctuations within the fatty acid (FA) composition of the zooplankton (Brett et al., 2009; Farkas and Herodek 1964; Jeffries, 1970; Norrbin et al., 1990). Importantly, climate change might alter the FA production of phytoplankton (Litzow et al., 2006) which is expected to cascade to the zooplankton and to higher trophic levels (Brett et al., 2009). FA are important trophic markers in marine ecosystems providing several applications for resolving ecosystem dynamic processes (Dalsgaard et al., 2003). In contrast to classical approaches, which often provide a snapshot of complex and highly variable interactions, FA allow to

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expand the temporal window of resolution of key processes and allow us to investigate more subtle effects of environmental stressors on zooplanktonic organisms (Dalsgaard et al., 2003). Essential fatty acids (EFAs) are polyunsaturated fatty acids with the first double bond in the n-3 position, which represent critical nutritional components but cannot be synthesized in sufficient quantities by animals and must be obtained through the diet (Parrish, 2009). Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are well known EFAs that play an essential role in aquatic food webs (Parrish, 2009), making the study of changes or fluctuations in the concentrations of these FA particularly important. EPA can drive reproduction and somatic growth (e.g. in macrozooplankton such as *Daphnia* ssp., Müller-Navarra et al., 2000; Kainz et al., 2004; Taipale et al., 2013). DHA enhances membrane fluidity due its high number of double bonds (Guo et al., 2016), and appears to be the most important FA for the reproductive success and development of copepods and fish (Shields et al., 1999; Arendt et al., 2005; Taipale et al., 2013).

Our study aims to record temporal and spatial patterns within the FA profiles of two dominant calanoid copepods occurring within the BPNS: *Temora longicornis* (Müller, 1785) and *Acartia clausi* (Giesbrecht, 1889), and to identify FA that might serve as markers indicating stress responses for these species. *T. longicornis* and *A. clausi* are two of the most abundant pelagic copepods within the BPNS (together comprising up to 66% of total zooplankton densities (Van Ginderdeuren et al., 2014)). Both species have high metabolic requirements, low energy reserves (low lipid content) and a short generation time, investing most of the available energy directly into growth and reproduction (Evjemo and Olsen, 1997; Helland et al., 2003; Kattner et al., 1981; Mayzaud et al., 1992). Both species are known to be omnivorous, feeding on phytoplankton, microzooplankton, copepod eggs and nauplii (Dam and Lopes, 2003; Kleppel, 1993; Mauchline, 1998; Wiadnyana et al., 1989). We therefore aim to investigate whether any potential FA markers in these species can be indicative in bulk zooplankton samples as well, providing us with a user-friendly tool to assess the overall health state of the ecosystem.

2. Material and methods

2.1. Sampling calanoid copepods

Zooplankton and water samples were collected every month from February 2015 to February 2016 at four stations in the Belgian part of the North Sea (BPNS) and at two stations within the Belgian harbours of Nieuwpoort (station NP1) and Zeebrugge (station ZB1). Within the BPNS two nearshore and two offshore stations were selected: the nearshore stations were station 700 located close to the harbour of Zeebrugge, situated near the mouth of the Scheldt river, and station 120 close to the harbour of Nieuwpoort. The two offshore stations were station ZG02 on the Flemish banks in the western part of the BPNS, and station 780 which is situated within the Zeeland banks in the eastern part of the BPNS (Fig. 1). At each sampling station, conductivity, temperature and depth profiles (CTD) data were collected with a Seabird 19plusV2 CTD (Flanders Marine Institute, 2017a). Water samples were taken with Teflon-coated Niskin bottles at a depth of 3 m and were analysed for nutrient and pigment concentrations by the LifeWatch observatory as part of the Flemish contribution to the LifeWatch ESFRI by Flanders Marine Institute (Flanders Marine Institute, 2017b).

Zooplankton samples were collected as in Van Ginderdeuren et al. (2014) with a WP2 zooplankton net (70 cm diameter, 200 μm mesh size) fitted with a flow meter that was towed in an oblique haul from bottom to surface at each station. Five replicate zooplankton samples were taken at each sampling event: one replicate for FA analysis of *T. longicornis* and *A. clausi*, one for FA analysis of bulk zooplankton samples and three replicates to quantify the zooplankton population densities. For the FA analysis, zooplankton was rinsed with milli-Q water and stored at −80 °C. In the laboratory, samples were thawed for species identification under a stereoscopic microscope (Leica MZ 10), keeping the temperature of the samples as low as possible to keep the FA stable. *A. clausi* and *T. longicornis* individuals were identified and transferred to glass vials and stored at −80 °C prior to FA extraction and identification. About 260 individuals of different sex and developmental stages were pooled ad random per sample to acquire sufficient material for FA extractions. An overview of the samples containing sufficient material for FA extractions and the obtained FA concentrations is provided in Table S4 to Table S9. The extra zooplankton samples that were collected for the analysis of the FA profile of bulk zooplankton samples were rinsed with milli-Q water, stored at −80 °C and were analysed for FA profiles (see further) without prior sorting. The triplicate zooplankton samples used for calculation of population densities of the species were fixed and stored in a 4% formaldehyde solution. Calanoid copepods (Crustacea, Copepoda, Calanoida) were identified in the lab to the lowest taxonomic level possible using a stereomicroscope (Leica MZ 10) to identify the abundances of the different taxonomic groups.

2.2. Fatty acid profiling

FA extractions were performed according to the protocol developed by Abdulkadir and Tsuchiya (2008) and modified as in De Troch et al. (2012). Hereby the boron trifluoride-methanol reagent was replaced by a 2.5% H_2SO_4-methanol solution, reducing the odds of artifacts or loss of PUFA (Eder, 1995; De Troch et al., 2012). The FA methyl esters (FAMEs) were concentrated to 200 mL hexane in order to obtain sufficient concentrations to reach the detection limits of the gas chromatograph. 19:0 FAME (Fluka 74208) was added as internal standard. FAMEs (1 μl) were injected and analysed using a Hewlett Packard 6890N gas chromatograph coupled to a HP 5973 mass spectrometer as in De Troch et al. (2012). The FAMEs were identified using the software MSD ChemStation (Agilent Technologies), comparing the FAMEs retention times and mass spectra to those of authentic standards and mass spectral libraries (WILEY, NITS05). Quantification of individual FAME was accomplished by the use of external standards (Supelco #47885, Sigma-Aldrich). The quantification of each individual FAME was obtained by linear regression of the chromatographic peak areas and corresponding known concentrations of the standards (ranging from 5 to 250 μg mL^{-1}, De Troch et al., 2012).

Shorthand FA notations of the form A:BωX were used, where A represents the number of carbon atoms, B gives the number of double bonds, and X gives the position of the double bond closest to the terminal methyl group. The FA included in the present study are 14:0, 15:0, 16:0, 16:1ω7, 18:0, 18:1ω9, 18:2ω6, 20:0, 18:3ω6, 18:3ω3, 20:5ω3 (EPA), 24:0 and 22:6ω3 (DHA). This selection was based on the measurements above the quantification limit of gas chromatograph and the relevance of FA as trophic marker (Dalsgaard et al., 2003).

2.3. Environmental variables

Nutrient sampling was performed as part of the LifeWatch Sampling Surveys conducted by the Flanders Marine Institute (VLIZ). In total, 200 mL seawater was filtered with cellulose-acetate filter for residual water for nutrient analysis. Nutrients were analysed using a QuAAtro39 Continuous Segmented Flow Analyzer (SEAL Analytical GmbH, Norderstedt, Germany): water samples and reagents were continuously pumped through a heated chemistry manifold (37 °C), resulting in the development of a specific colour with an intensity proportional to the concentration of the specific nutrient. The instrument is equipped with high-resolution digital spectrophotometers (used to measure the intensity of the colour), allowing the analysis of each nutrient at a rate of about 60 samples each hour. The following nutrients were measured; total oxidizable nitrogen (TON), nitrite (NO_2^-), phosphate (PO_4^{3-}), silica (Si) and ammonium (NH_4^+). Nitrate (NO_3^-) was derived by evaluating the reduction efficiency of the NO_3^- to NO_2^- conversions

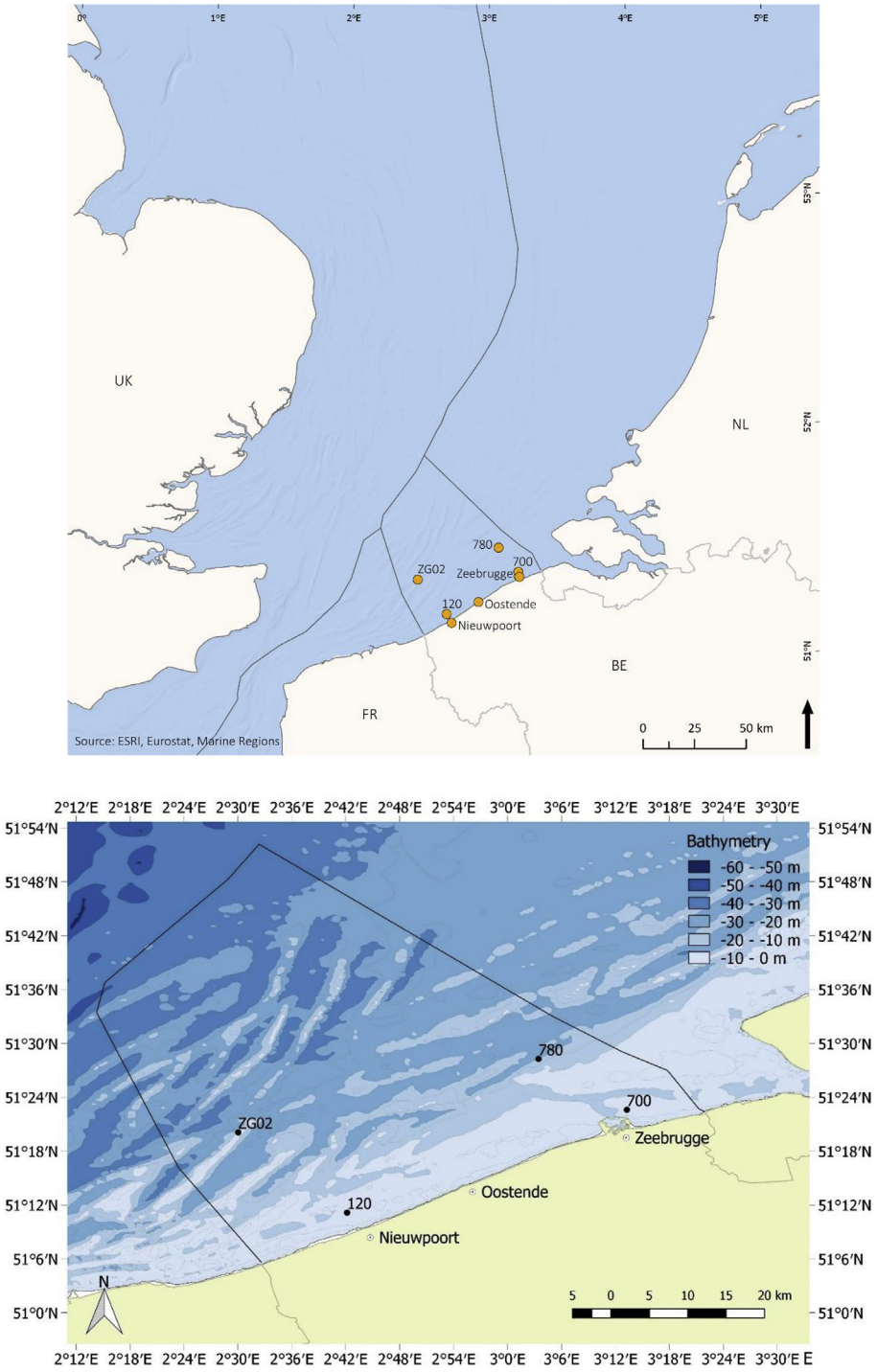

Fig. 1. Sampling locations in the Belgian part of the North Sea. Station 120: 51° 11′ 1″ N, 2° 42′ 07″ E, station 700: 51° 22′ 6″ N, 3° 12′ 2″ E, station ZG02: 51° 20′ 0″ N, 2° 30′ 0″ E, station 780: 51° 28′ 27″ N, 3° 3′ 48″ E, station Nieuwpoort: 51° 08′ 43″ N, 2° 44′ 13″ E, station Oostende: 51° 14′ 12″ N, 2° 55′ 23″ E, station Zeebrugge 51° 20′ 47″ N, 3° 12′ 26″ E.

and then subtracting NO_2^- from TON. Dissolved inorganic nitrogen (DIN) was quantified as the sum of NO_2^-, NO_3^- and NH_4^+.

Polychlorinated biphenyls (PCB) and polycyclic aromatic hydrocarbons (PAH) were quantified for each sampling station in samples of 5 L seawater (obtained with Niskin bottles, see 2.1), of which 4 L were retained for the analyses. Freely dissolved concentrations of the PAHs and PCBs were quantified according to the methodology described in Everaert et al. (2016). Internal standards (deuterated analogues of parent PAH compounds (VWR) and PCB congeners 14, 112, 143, 155 and 204 (Sigma-Aldrich)) were added. Volumes were extracted three times with dichloromethane, the extract was subsequently dried on Na2SO4 and concentrated to about 0.2 mL using a rotary evaporator with N2 cooling. Anthracene-d10 (VWR) was added as recovery standard. The extracts were concentrated and analysed with a gas chromatography–mass spectrometer (GC–MS, Thermoquest, Austin, Texas, USA). The extracts were injected into the GC–MS with a 30 m * 0.25 mm DB5-ms cross-linked fused silica capillary (0.25 μm film thickness). The carrier gas was helium (99.999%) at a linear flow of 1 mL min^{-1}. Splitless injection at an injection temperature of 230 °C with a split valve being opened to purge the injector 1 min after

injection was performed. Column initial temperature was kept at 55 °C during injection and its temperature increased at 15 °C min^{-1} to 310 °C which was held for 6 min. Via a transfer line heated to 310 °C, the GC column was directly coupled to the ion source of the MS mass spectrometer. The quadruple MS operated in the selected ion monitoring electron ionization mode with the ion source at 250 °C. All solvents used were of purity suitable for organic residue analysis.

2.4. Data analysis

2.4.1. Species-specific, spatial and temporal patterns in FA composition

All statistical analyses were performed on relative FA data (i.e. percentages of the total FAs identified with the applied methods). Samples were classified into different seasons. March, April and May samples were considered as spring samples. June, July and August samples were considered summer samples. Samples taken in September, October and November were autumn samples, and those from December, January and February were our so-called winter samples. Nonparametric univariate and multivariate analyses were performed using PRIMER 6 (version 6.1.11, Clarke and Gorley, 2006) to explore underlying patterns in the FA data. Analysis of similarities (ANOSIM, 9999 permutations) was used to test for differences between the FA composition of *T. longicornis*, *A. clausi* and the bulk samples, as in Werbrouck et al. (2016a). Also spatial and seasonal differences in the relative FA composition of each species were tested. In combination with each ANOSIM analysis, a PERMDISP test was performed to check for the homogeneity of variance. PERMDISP is a powerful test for homogeneity (Anderson and Walsh, 2013), which should be taken into account when drawing conclusions from ANOSIM tests, since differences in the homogeneity of variances between groups might interfere with the results of the test. In case of significant differences between groups, similarity percentage (SIMPER) analysis was performed to reveal the FA contributing most to the observed differences.

Two-way PERMANOVA tests were used to assess spatial and seasonal differences in total concentrations of mono unsaturated fatty acids (MUFA), poly unsaturated fatty acids (PUFA) and saturated fatty acids (SAFA). Differences in the relative percentage of these FA classes between species (PERMANOVA with species as fixed factor, season and station as random factors) and within each species (PERMANOVA with season and station as fixed factors) were tested as well.

2.4.2. Link between FA patterns and environmental variables

Correlations between the patterns in relative FA composition and patterns in environmental variables were tested for each species by performing distance-based linear modelling (DISTLM). Prior to DISTLM analysis, environmental variables were normalized by application of the $\log_{10}(x+1)$ transformation. Forward stepwise model construction was used in combination with the AICc selection criterion. AICc is the Akaike information criterion with a correction for finite sample sizes. Following Happel et al. (2017), Euclidian distances were used both for the environmental data and the relative FA concentration data.

As we expected distinct spatial and seasonal patterns in both *T. longicornis* and *A. clausi* to vary along a temporal and seasonal scale (with a higher relative abundance of *T. longicornis* in February to May, as in Van Ginderdeuren et al. 2014), we investigated the relative importance of different environmental variables to the DHA/EPA ratio by means of multi model inference (MMI) on generalized additive models (GAM) as in Deschutter et al. (2017). GAMs were fitted in R using the package mgcv (Mixed GAM Computation Vehicle with Automatic Smoothness Estimation), and smoothers were inferred from the data using the same package (Wood and Wood, 2016).

The environmental variables included water temperature (temp), salinity (sal), the ratio of dissolved inorganic nitrogen concentration over silicate concentration (DIN/SiO_4), chlorophyll *a* concentration (chl *a*) and the sum of polychlorinated biphenyls (PCB) and polycyclic aromatic hydrocarbons (PAH) concentrations (sumtox, Table S21). Assuming nonpolar narcosis to be the main mode of action for both groups of toxicants (McCarty and Mackay, 1993), the latter were first multiplied with their corresponding octanol/water partition coefficient (K_{ow}) and converted to molar concentration (w_m = molecular weight (g mol^{-1})) (Eq. (1)) as in Deschutter et al. (2017) and Everaert et al. (2015). Since individual outlier data could disproportionately influence the smoothers and result in non-representative models, outlier data points (such as high DIN/SiO_4 ratios in March) were excluded from the model fits.

$$sumtox = \sum_i ((conc_i \times K_{ow,i})/w_m) \quad (1)$$

3. Results

3.1. Demographic characteristics of T. longicornis and A. clausi

Depending on the sampling location, peak densities occurred from April until May for *T. longicornis*, with maximum densities of 2449 ± 622 ind m^{-3} (Fig. S1, April 2015, station 700), and from May until July for *A. clausi,* with densities up to 593 ± 87 ind m^{-3} (Fig. S1, May 2015, station 700). Reproduction occurred mainly in spring, with maximal copepodite (stage I to III) densities of 1287 ± 611 ind m^{-3} in April (Fig. S1, Station 700) for *T. longicornis* and of 446 ± 247 ind m^{-3} in July for *A. clausi* (Fig. S1, Station ZB1).

3.2. Species-specific, spatial and temporal patterns in fatty acid composition

3.2.1. FA profiling of bulk samples: are they representative for the zooplankton community?

FA profiling of bulk samples is not representative for the zooplankton community. The relative FA composition differed between *T. longicornis* and *A. clausi* (ANOSIM, p = 0.01) and between *A. clausi* and the bulk samples (p = 0.01, Table S1, Fig. 2). No difference in the relative FA composition was found between *T. longicornis* and the bulk samples. We found no difference in homogeneity of the variances between the two species and the bulk samples (PERMDISP, p = 0.71). As species-specific responses of the FA composition were found, bulk

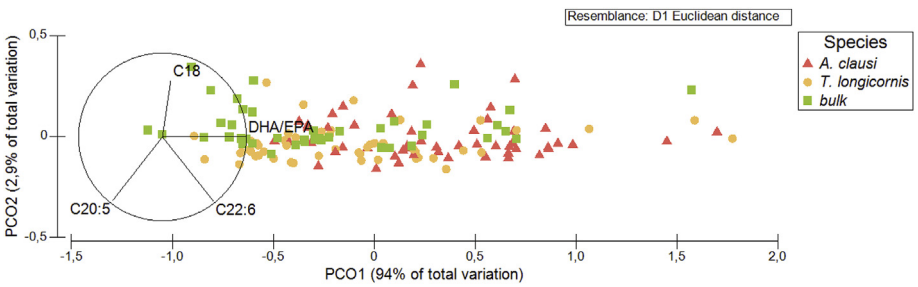

Fig. 2. Principle coordinates analysis of relative fatty acids (% of total fatty acids) of *A. clausi*, *T. longicornis* and bulk samples from February 2015 to February 2016 at 6 (*T. longicornis* and A. clausi) and 5 (bulk) stations within the Belgian Part of the North Sea.

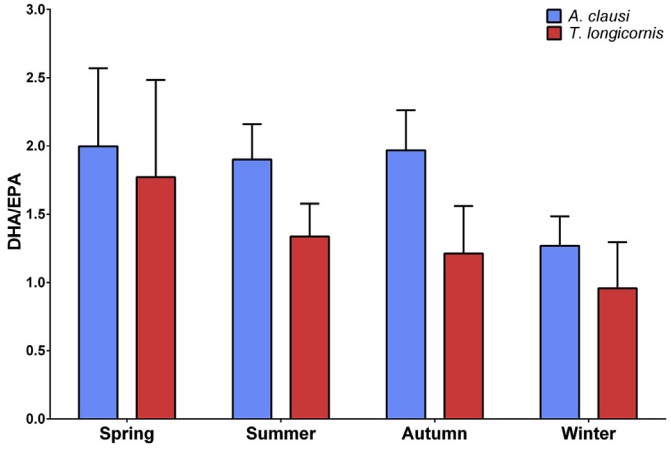

Fig. 3. DHA/EPA ratio of *A. clausi* and *T. longicornis*, averaged per season and over six sampling stations in the Belgian Part of the North Sea. The DHA/EPA ratio of both species for individual months and sampling stations is indicated in Fig. S3. Error bars denote the standard error on the mean DHA/EPA ratio. Two outliers (*T. longicornis* summer samples with DHA/EPA ratio of 156 and 23 respectively) were not taken into account.

samples cannot be used to draw conclusions about the entire zooplankton community.

3.2.2. FA composition of *A. clausi* versus *T. longicornis*

The DHA/EPA ratio explained 94.4% of the difference between *A. clausi* and *T. longicornis* (Table S2), showing distinct species-specific and temporal patterns. Both *A. clausi* and *T. longicornis* showed the highest DHA/EPA ratios in spring (1.99 and 1.77 respectively, Fig. 3). In summer and autumn, the DHA/EPA ratio of *T. longicornis* (1.34 and 1.19 respectively) was lower than that of *A. clausi* (1.87 and 1.91 respectively; $p < 0.01$, Fig. 3). In general, the seasonal variation in the DHA/EPA ratio was less pronounced in *A. clausi,* whose DHA/EPA ratio stayed at values equal or larger than 1, whereas it ranged between 0.6 and 1 for *T. longicornis* at offshore stations in autumn and winter (Fig. S2). A higher relative concentration of DHA, 16:0 and 18:0 in *A. clausi* together with a higher relative concentration of EPA and 16:1ω7 in *T. longicornis* also contributed to the differences in FA composition between both species (Table S3, Fig. 4). Even though spatial variability was found in the relative percentage of SAFA, overall relative proportions of SAFA were lower in *T. longicornis* than in *A. clausi* (33.6 ± 9.3% and 38.0 ± 11.5%, resp.; $p = 0.04$). Relative MUFA concentrations were higher in *T. longicornis* than in *A. clausi* (10.75 ± 4.06% and 7.72 ± 4.24%, resp.; $p < 0.01$), despite its spatial and seasonal interactive effects ($p < 0.01$). No differences in the overall relative proportion of PUFA were found between the species. Relative concentrations of SAFA, MUFA, PUFA and all individual fatty acids per species, month and sampling station are indicated in Tables S4–S9.

3.2.3. Spatial and seasonal patterns of *T. longicornis* FA composition

T. longicornis individuals from station 780 had a different relative FA composition in comparison to individuals captured at all the other stations, with the exception of station 700. The FA composition of specimens from Nieuwpoort harbor significantly differed from those at other stations, except for the Zeebrugge harbor (Table S10). In addition to differences in FA composition, the FA composition of specimens from station 780 differed from all other stations in the homogeneity of variance (Table S11, P(perm) < 0.05). *T. longicornis* individuals from station 780 contained higher relative concentrations of EPA and DHA and lower relative concentrations of 16:0 than the other stations (Table S12). By contrast, the individuals from Nieuwpoort contained less EPA and DHA and more 16:0, 18:0 and 18:1ω9 than the other stations (Table S14). In addition to differences on a spatial scale, seasonal patterns were found in the FA concentration profile of *T. longicornis*. Winter FA concentrations of this species differed from the FA concentrations in spring and summer (Table S13) as lower DHA concentrations were found in winter together with higher concentrations of EPA, 18:0 and 16:0 (Table S14, Fig. 4). These differential concentrations of DHA, EPA, 18:0 and 16:0 per season together explained 88% of the differences between winter and spring, and 84% of the differences between winter and summer (Table S14). Furthermore, the patterns found in the FA composition of *T. longicornis* are translated to the larger FA classes. The higher EPA and DHA concentrations at station 780 lead to a lower relative percentage of SAFA in this station (Table S15). No effects of seasonality or location were found for MUFA and PUFA relative percentages for *T. longicornis*. The DHA/EPA ratio on the other hand was lower in winter than in spring ($p < 0.01$) and summer ($p < 0.01$) and was lower in autumn than in spring ($p = 0.04$, Table S15, Fig. 3).

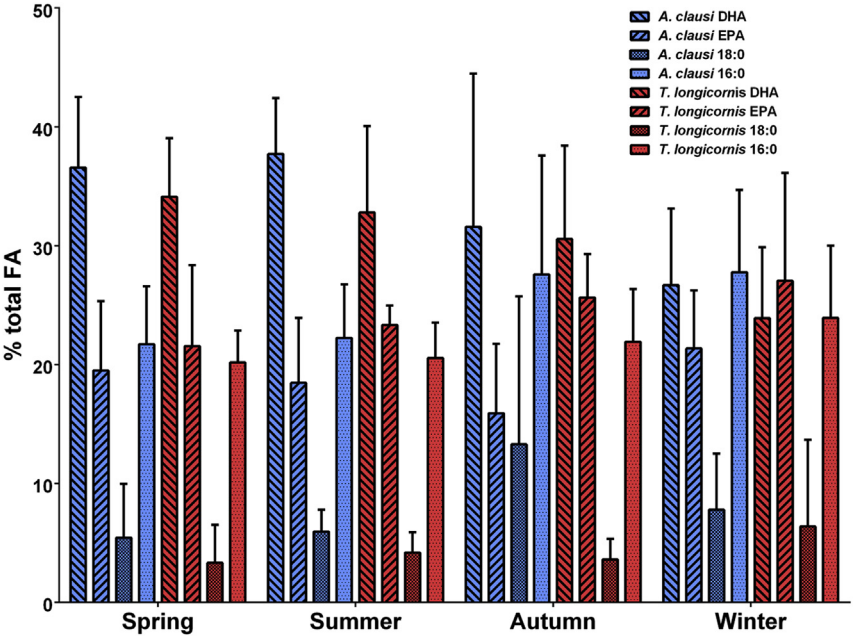

Fig. 4. Relative concentrations of DHA, EPA, 18:0 and 16:0 (% of total fatty acids) in *A. clausi* and *T. longicornis*, averaged per season and over six sampling stations in the Belgian Part of the North Sea. Error bars denote the standard error. Two outliers (*T. longicornis* summer samples with relative EPA concentrations 1.5% and 0.3% respectively) were not taken into account The relative concentrations of all fatty acids for individual months and sampling stations are indicated in Tables S4–S9.

3.2.4. Spatial and seasonal patterns of A. clausi FA composition

No clear spatial patterns in relative FA composition were found for *A. clausi.,* as only differences were found between Nieuwpoort harbor and station ZG02 and 780 (Table S16). These differences were due to lower EPA, DHA and higher 18:0, 16:0 concentrations in Nieuwpoort harbor (Table S17). A difference in dispersion ($P = 0.03$) was also found between station 780 and Nieuwpoort harbor (Table S18). Regarding seasonality, we found that the FA profiles of *A. clausi* in winter differed from those measured in other seasons (Table S16). This seasonal pattern can be mainly explained due to a smaller proportion of DHA, in combination with a larger proportion of EPA and 16:0 (Fig. 4). The latter is confirmed in our statistical analyses as DHA, EPA and 16:0 together explained 75%, 83% and 74% of the difference between winter and spring, between winter and summer and between winter and autumn, respectively (Table S19). In autumn, a dispersion effect in comparison with the other seasons was found (Table S18). Furthermore, we found the relative percentage of SAFA to be higher at Nieuwpoort harbor than at the other stations, except for Zeebrugge harbor (Table S20). Interactions between season and station were found for the MUFA relative percentage, but no clear trend could be found (Table S20). No effects of seasonality or location on PUFA relative percentages were found.

3.2.5. Link between FA patterns and environmental variables

Forward stepwise modelling using the AICc criterion (DISTLM) revealed that DIN/SiO_4 explained 11.7% of the total variation ($p < 0.01$) in the relative FA composition of *T. longicornis*, while salinity explained 13.9% of the total variation ($p < 0.01$). None of the other environmental variables showed a correlation with the relative FA composition patterns. The best model to explain the variation in the FA composition of *T. longicornis* included DIN/SiO_4 and salinity, which together were able to explain 20.6% of the total variation in the FA data. Note that none of individual or combinations of environmental variables were able to explain the variation in the relative FA composition for *A. clausi.* The best model containing both DIN/SiO_4 and temperature explained only 10.3% of the variation in the FA data.

The DHA/EPA ratio showed distinct species-specific, spatial and temporal patterns (see 3.2.2 to 3.2.4). MMI on GAM showed that temperature and DIN/Si04 ratio were the most important drivers of the DHA/EPA ratio of both *A. clausi* (relative importance of respectively 0.45 ± 0.13 and 0.45 ± 0.06) and *T. longicornis* (relative importance of respectively 0.23 ± 0.14 and 0.74 ± 0.43, Fig. 5). Temperatures above 9 °C had a positive effect on the DHA/EPA ratio of both species (with a maximum of 18 °C for *T. longicornis*), explaining the lower DHA/EPA ratios in wintertime (Fig. 6). Increasing DIN/SiO_4 ratios led to decreasing DHA/EPA ratios for both species (Fig. 6). The sum of toxicants in the water had a small effect on the DHA/EPA ratio of *T. longicornis* (relative importance of 0.02 ± 0.03), while salinity had a small effect on the DHA/EPA ratio of *A. clausi* (0.10 ± 0.04). Chl *a* concentration did not seem to affect the DHA/EPA ratio of neither of the species (Fig. 5). The model containing temperature, DIN/SiO_4 and the sum of toxicants in the water was able to explain 30.4% of the variation in the DHA/EPA ratio of *T. longicornis*, while the model containing temperature, DIN/SiO_4 and salinity was able to explain 59.7% of the variation in the DHA/EPA ratio of *A. clausi.* Environmental variables which were included in the GAM (temperature, salinity, chl. a, DIN/SiO_4 and the sum of toxicants in the water) are shown in Fig. 7. PO_4^2, SiO_4 and DIN are presented in Fig. S3.

4. Discussion

Our aim was to test if the FA profiles of two dominant calanoid copepods in the BPNS (*T. longicornis* and *A. clausi*) were predictable from environmental variables, identify FA that could serve as markers for stress, and test whether FA markers in these species can be indicative of bulk zooplankton. We found pronounced seasonal patterns

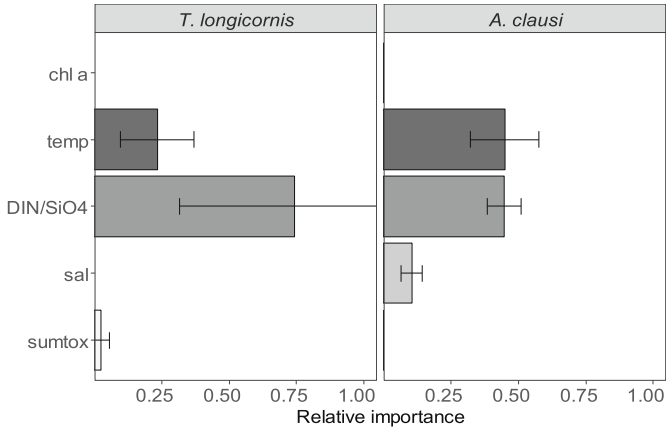

Fig. 5. Average relative importance (+/− SE) of different stressors for the DHA/EPA ratio of *Temora longicornis* and *Acartia clausi* in the BPNS, averaged over generalized additive models with multi model inference, excluding outliers and with ER < 2. ER is the Evidence Ratio of the models, which represents the fit of the model divided by the fit of the best model. Chl. a is the chlorophyll *a* concentration, temp is temperature, DIN/SIO_4 the ratio of dissolved organic nitrogen concentration to silicate concentration, sal is salinity, and sumtox is the sum of toxicants in the water.

in environmental variables, spatial differences in the timing and extent of these seasonal fluctuations corresponding to those reported by Muylaert et al. (2006). Temperature, the DIN/SiO_4 ratio, salinity and the sum of toxicants in the water were the environmental variables predicting FA profiles, and the DHA/EPA ratio was the FA marker responding most sensitively to environmental fluctuations and explaining most of the FA differences *in T. longicornis* and *A. clausi*. Since *A. clausi* and *T. longicornis* comprise up to 66% of total zooplankton densities (Van Ginderdeuren et al., 2014), but the FA composition of bulk zooplankton only resembled that of *T. longicornis* and was different from *A. clausi* (Table S1, Fig. 2), we can conclude that the FA signal of bulk zooplankton samples is not representative for the entire community. Different zooplankton classes will respond differently to external drivers such as food availability and temperature (e.g. Richardson, 2008; Breteler and Gonzalez, 1988) and will display different intrinsic mechanisms to cope with stressful situations, leading to differences in their FA concentrations (e.g. Dalsgaard et al., 2003; Scott et al., 2002; Kattner and Krause, 1989).

DHA and EPA are both considered to be essential fatty acids for copepods, with egg production rates of *T. longicornis* depending on the EPA ingested from their diet (Jónasdóttir et al., 2009), and nauplii hatching as well as egg production being driven by DHA (Arendt et al., 2005; Evjemo et al., 2008; Jónasdóttir et al., 2009). The DHA/EPA ratios found in the present study correspond to those reported in the literature (Evjemo and Olsen, 1997; Evjemo et al., 2008). The ratio showed clear spatial and seasonal patterns, with a maximum in spring and a decline in autumn and winter, which was more pronounced in *T. longicornis* than in *A. clausi*, indicating a high potential to be a relevant marker for food quality and stress in copepods of the BPNS. The DHA/EPA ratio is used as a marker for trophic position and also for the proportion of diatoms to flagellates in the diet (El-Sabaawi et al., 2009), and has been shown to be of importance as a food quality indicator for copepod reproduction (Støttrup and Jensen, 1990; Jónasdóttir, 1994; Arendt et al., 2005). The DHA/EPA ratio is also an index of carnivory, next to the proportion of polyunsaturated fatty acids (PUFA) to saturated fatty acids (SAFA) (El-Sabaawi et al., 2009), as well as an important component of polar lipids, and is highly conserved in marine foodwebs (Scott et al., 2002). The DHA/EPA ratio is expected to change as a response to changes in food availability. The main input of DHA and EPA comes from primary producers, with their ratio reflecting the relative proportions of dinoflagellate to diatoms in the diets of

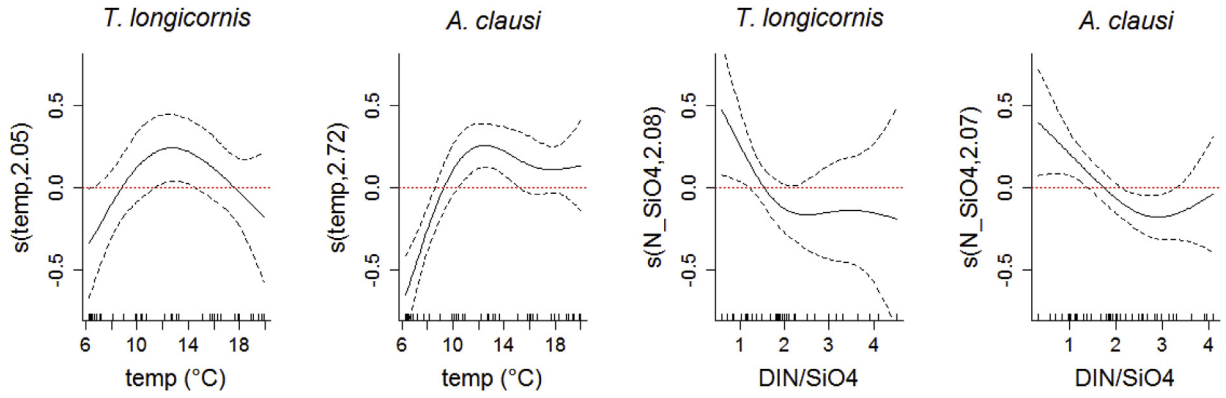

Fig. 6. Smoothers of temperature and DIN/SiO_4 for the DHA/EPA ratio of *T. longicornis* (model fitted without outliers, and including temperature, DIN/SiO4 and the sum of toxicants in the water (sumtox)) and *A. clausi* (model fitted without outliers, and including temperature, DIN/SiO_4 and salinity). Values > 0 indicate a positive effect of the environmental variable on the DHA/EPA ratio of the species, values < 0 indicate a negative effect. Vertical tick marks at the x-axis indicate measurement points.

herbivorous and omnivorous copepods: dinoflagellates are rich in DHA, whereas diatoms are particularly rich in EPA (Viso and Marty, 1993; Taipale et al. 2013), although ciliates have also been shown to be an EPA-rich food source for *A. clausi* (Dutz and Peters, 2008). As a consequence, changes in community composition of the phytoplankton will be reflected in the DHA/EPA ratio. Environmental conditions also affect the trophic transfer of EPA and DHA differently. For instance, dietary resources have been found to fully compensate for the temperature effects on total lipid and EPA content in the copepods, but no such counterweigh was observed for the DHA dynamics, with heat stress lowering the DHA concentration in copepods regardless of the resources available, implying negative effects for higher trophic levels (Werbrouck et al., 2016b). Together with the outcome of the present study, these insights point at the DHA/EPA ratio as a good indicator for the response of copepods to changing environmental conditions.

4.1. Extrinsic factors

The BPNS is characterized by shallow, well-mixed waters that are subjected to high seasonal variability of both abiotic factors such as light and temperature (Fig. 7), and biotic factors such as food availability and quality (e.g. Muylaert et al., 2006; Otto et al., 1990; Sündermann and Pohlmann, 2011). Copepods, who are year round present in the BPNS (Van Ginderdeuren et al., 2014), have to cope with these distinct changes in environmental conditions. *T. longicornis* and *A. clausi* can reach very high population densities in the BPNS, with a strong seasonal cycling (Deschutter et al., 2017). Within the BPNS, the cycling in the concentration of chl *a* (Fig. 7) marks the cycle of increasing and decreasing concentrations of phytoplankton cells, which are an important food source for most copepod species. The presence of harmful blooms of the inedible algae *Phaeocystis globasa* is however known to disturb the relation between the chl *a* concentration and the amount of edible algae present for the copepods (Arendt et al., 2005; Muylaert et al., 2006). Changes in the nutrient composition (more specifically an increasing DIN/SiO_4 ratio, Fig. 7) determine the transition from the spring bloom of diatoms, which are considered an excellent food source amongst all algal classes due to their high EPA content (Taipale et al., 2013), to the bloom of the harmful, inedible

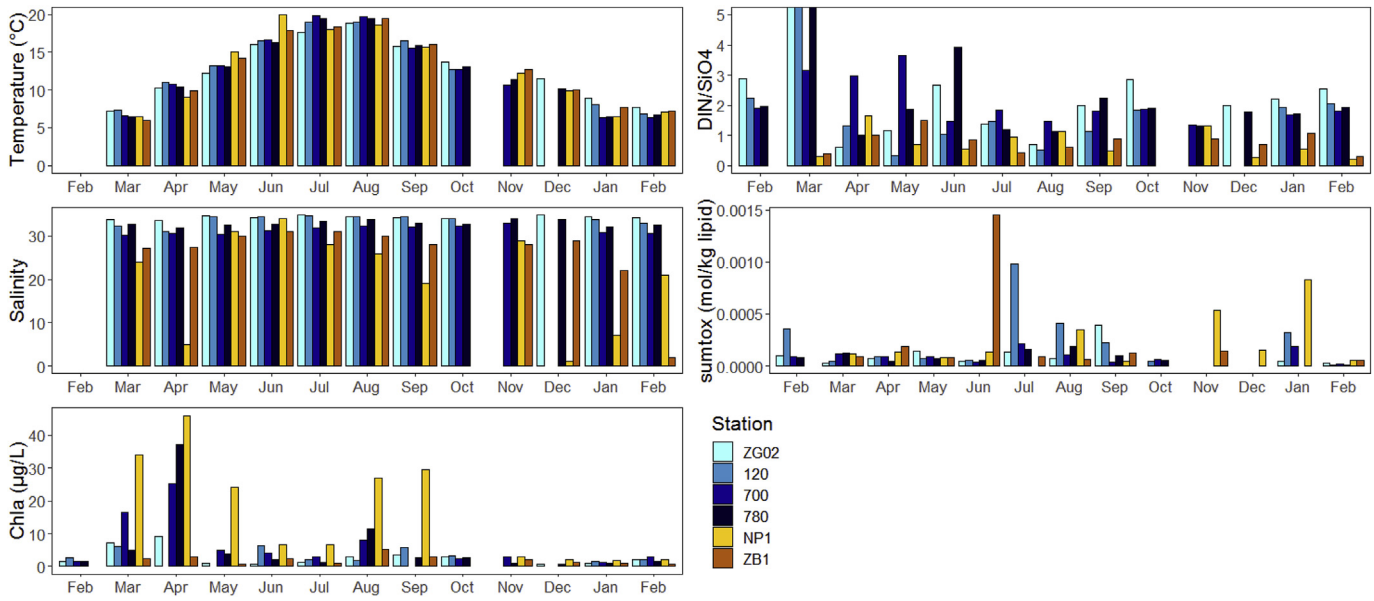

Fig. 7. Environmental variables measured at different stations in the Belgian Part of the North Sea from February 2015 to February 2016. Top left: water temperature (°C), top right: the DIN/SiO_4 ratio, middle left: salinity, middle right: the sum of toxicants (POP - persistent organic pollutants) in the water (sumtox, mol POP kg lipid weight^{-1}), bottom left: Chlorophyll *a* concentration (chl a, µg L^{-1}). For the sake of visibility, the Y-axis for the DIN/SiO_4 ratio is limited to 5. Samples exceeding these values were stations ZG02, 120 and 780 in March (DIN/SiO_4 ratios of 13.8, 9.3 and 28 respectively).

algae *P. globosa,* leading to large fluctuations in food quality for the copepods (Arendt et al., 2005). The DIN/SiO_4 ratio reaches its peak in March, with the onset of a diatom bloom once there is sufficient sunlight, which depletes the SiO_4 pool. Shortly after, both a small flagellate and a pronounced *P. globolosa* colony bloom start to develop with increasing temperatures, subsequently causing DIN/SiO_4 ratios to decline again (Fig. 7, Arndt et al., 2011). DIN/SiO_4 are commonly used as tool reflecting diatom production (Everaert et al., 2018; Gilpin et al., 2004; Suikkanen et al., 2007). Higher DIN/SiO_4 values indicate less favorable conditions for diatoms, favoring the development of *P. globosa* blooms and resulting in a lower food quality for the copepods. This explains the negative correlation between the DIN/SiO_4 ratio and the DHA/EPA ratio of the copepods (Fig. 6). The importance of this cycling in food quality for the copepods is reflected in the high importance of DIN/SiO_4 in the GAM predicting the DHA/EPA ratio of *T. longicornis* and *A. clausi* (Fig. 5).

Despite the disturbed relation between the chl *a* concentration and the amount of edible algae present for the copepods, we did assume that lower chl *a* concentrations in wintertime would affect the DHA/EPA ratios of *T. longicornis* and *A. clausi.* Both species show lower DHA/EPA ratios in winter in comparison with spring and summer, but as no effects of chl *a* on the DHA/EPA ratio was found, other factors might play a more important role here.

Next to food quantity and quality, environmental stressors will affect the FA composition of the copepods (Litzow et al., 2006; Werbrouck et al., 2016a). The optimal temperature for *T. longicornis* and *A. clausi* lies between 14 °C and 16 °C (Chinnery and Williams, 2004; Halsband et al., 2002). The temperature fluctuations in the BPNS (Fig. 7) are likely to alter he DHA/EPA ratio of both *T. longicornis* and *A. clausi,* as suggested in the importance of temperature in the model output (Figs. 5 and 6). Copepods are known to increase their DHA content when exposed to lower temperatures, allowing many species to overwinter in an active stage (Brett et al., 2009). Jeffries (1970) on the other hand, reported higher DHA content in *Acartia* spp. during warmer seasons, which was probably the result of high densities of DHA–rich dinoflagellates serving as copepod food during summer. We also found higher DHA contents for both species in spring and summer, indicating that a DHA-rich food source must be present within the BPNS even during the warm seasons. This could correspond to small flagellate summer blooms reported in the literature (Arndt et al., 2011), with increasing DHA/EPA ratios indicating an increased consumption of dinoflagellates (Kürten et al., 2013). Long term changes in sea water temperature due to climate change are likely to influence the fatty acid composition of these copepods as well, by causing further shifts in phytoplankton community composition (Lagring et al., 2018). Water temperature and chlorophyll *a* concentration are also the main drivers of *A. clausi* population density (Everaert et al., 2018). Next to these structural changes, shift in FA composition will imply also functional changes that are relevant for the functioning of the ecosystem in terms of energy flow in the food web. Copepod nauplii are an important food item for fish larvae and *T. longicornis* is known to be one of the most important food items for adult pelagic fish in the BPNS as well (amongst others herring (*Clupea harengus*), sprat (*Sprattus sprattus*), mackerel (*Scomber scombrus*), and horse mackerel (*Trachurus trachurus*) (Van Ginderdeuren et al., 2013)). Any changes in the FA composition of these copepods will be propagated to higher trophic levels, possibly affecting the health of secondary consumers or their nutritional value for human consumers (Tocher, 2010).

The measured PAH concentrations remained below the EQS set in the context of the Water Framework Directive (WFD; 2000/60/EC) by the European commission (EC, 2008) and the Flemish government (Vlaamse regering, 2010), while PCB concentrations often exceeded the EQS values during our study (Table S21, Fig. S4). Although a previous study found probable effects of these toxicant concentrations on *T. longicornis* and *A. clausi* densities (Deschutter et al. 2017), they did not affect their fatty acid profiles. These contrasting findings indicate that the examined toxicants are likely to affect the eggs or the naupliar stages of the copepods, as these might be the most sensitive stages in the reproductive cycle (Mohammed, 2013). A more thorough investigation of the environmental effects on the embryonic development and juvenile stages of the copepods is therefore recommended.

While the GAM were able to explain 30.4% and 59.7% of the variation in the DHA/EPA ratio of *T. longicornis* and *A. clausi*, the DISTLM models were able to explain 20.6% and 10.3% of the variation in their FA composition. A large part of the variation in the FA composition of the copepod species is hence left unexplained, as the mixed effect of the multiple external factors potentially affecting the FA profile of the copepods is still difficult to unravel. Our knowledge on the effect of multiple stressors (chemical pollution, increasing temperatures, low pH) on copepods is recently rapidly increasing (Deschutter et al., 2017; Everaert et al., 2018, Foo and Byrne, 2017; Zervoudaki et al., 2014). Models taking into account interactions between variables and indirect effects through species interactions need to be developed to enhance our understanding of the effect of environmental stress on the FA profiles of these omnipresent copepod species within the BPNS. Therefore, a valid and robust alternative to the regression-based models is the construction of mechanistic models as in Everaert et al. (2015). In their approach, Everaert et al. (2015) integrated 21 years of data in a nutrient-phytoplankton-zooplankton model to quantify the relative contribution of four classical drivers of phytoplankton growth (light and nutrient availability, temperature and zooplankton grazing) and one persistent organic pollutant induced growth limitation term to marine phytoplankton biomass dynamics. Even though they are very data intensive, mechanistic models are able to capture the effects of changing environmental drivers and the links between different trophic levels in a detailed way (Everaert et al., 2015). However, note that in the context of the present research, not enough data were available in order to properly parameterize a mechanistic model.

The present study focused on environmental parameters and algal food quality as drivers of copepod fatty acid profiles. One of the major factors to shape the FA profiles of the copepod species under study is the presence of high seston nutritional quality (Dutz and Peters, 2008). Next to their role for FA, the presence of large ciliates is also particularly important for the recruitment of marine zooplankton populations. For instance, there are strong indications that the spring recruitment of *A. clausi* in the North Sea is related to the successional occurrence of large ciliates (Dutz and Peters, 2008). Koski et al. (2010) found that seasonal differences in body size (likely controlled by temperature) and food quality (EPA:DHA ratio) could control copepod egg production. Microzooplankton are key components of marine foodwebs, and also represent a potential food source for omnivorous copepods such as *T. longicornis* and *A. clausi*. Microzooplankton are also important contributors to mesozooplankton diet, especially in oligotrophic areas, although the strength of the mesozooplankton–microzooplankton link is traditionally overlooked in plankton studies (Calbet, 2008). They occupy a key position in marine food webs as major consumers of primary production (Calbet and Landry, 2004) and as intermediaries between primary producers and copepods (Gifford, 1991; Calbet and Saiz, 2005). It is therefore very plausible that microzooplankton also play an important role in the zooplankton community under study.

4.2. Intrinsic factors

Despite their similar diet, *T. longicornis* and *A. clausi* have different life history strategies allowing them to cope with seasonal changes in temperature and food supply (Halsband and Hirche, 2001; Wesche et al., 2007). Several studies performed in the Southern North Sea (at the German Bight) found that *A. clausi* has an overwintering strategy based on reproductively inactive females which is decoupled from environmental changes during autumn and winter, while *T. longicornis* continuously reproduces throughout the year, allowing them to respond fast to increasing food levels (Gentsch et al., 2008; Halsband and

Hirche, 2001; Wesche et al., 2007). In the BPNS however, small quantities of *A. clausi* copepodites (stage I-III) were found throughout the winter period (copepodite density averaged over the winter months over all stations: 7 ± 8 ind m^{-3}), indicating that some reproduction continued during the winter months as well. This is probably due to the slightly warmer winter temperatures in the BPNS (minimal temperature during our study: 6.3 °C) in comparison with the German Bight (minimal temperature of −0.5 °C) (Halsband and Hirche, 2001). *T. longicornis* however showed higher copepodite (stage I-III) densities throughout the winter months (32 ± 92 ind m^{-3}), indicating that reproduction still plays a more important role in the overwintering strategy of this species within the BPNS.

The difference in FA composition between *A. clausi* and *T. longicornis* reflects their different ecological characteristics and preferences. As mentioned earlier, continuous reproduction allows *T. longicornis* to respond faster to increased food levels, so when the diatom bloom starts this species is able to take optimal advantage of this sudden increase in food levels (Arendt et al., 2005; Halsband and Hirche, 2001; Gentsch et al., 2008). This is confirmed by our copepodite density data, showing peak reproduction of *T. longicornis* in April, while *A. clausi* only reaches its reproduction peak in July (Deschutter et al., 2017, Fig. S1). The relative proportion of EPA and 16:1ω7 was higher in *T. longicornis* than in *A. clausi*. Both FA are markers for diatom consumption (Brett et al., 2009; Kürten et al., 2013), suggesting that diatoms contribute more to the diet of *T. longicornis* than to the one of *A. clausi*. Gentsch et al. (2008) performed feeding experiments confirming the flexible diet of *T. longicornis* as this species showed omnivorous feeding during late winter and early spring, switching to a more herbivorous feeding mode during the phytoplankton bloom in spring. *A. clausi* on the other hand contained a higher relative proportion of DHA, which is a marker for dinoflagellate consumption (Arts et al., 2009). The higher relative percentage of SAFAs in *A. clausi*, together with a lower relative percentage of MUFAs also suggest that *A. clausi* is taking less advantage of the MUFA-rich phytoplankton bloom in the BPNS than *T. longicornis*. Within the BPNS, 11 dinoflagellate species are known to occur, but they comprise only a small fraction of the total number of algal cells representing > 5% of total cell numbers in only four out of 50 samples (Muylaert et al., 2006). Even though a considerable overlap in the FA composition of the major phytoplankton groups withdraws us from strong conclusions here, *A. clausi* seems to be selectively feeding on dinoflagellates within the BPNS.

5. Conclusion

The fatty acid profiles of *A. clausi* and *T. longicornis* show intraspecific, spatial and temporal differences within the BPNS, which can be assigned for a large part to differences in the DHA/EPA ratio. As different species show distinct differences in their FA composition, and in spatial and temporal fluctuations in this FA composition, bulk zooplankton FA signals are not representative for the entire zooplankton community. The DHA/EPA ratio showed high potential to be a relevant marker for temperature, salinity, food quality and chemical stress in copepods in the BPNS. Temperature and algal food quality (marked by the DIN/SiO_4 ratio), were the most important drivers of seasonal fluctuations in the DHA/EPA ratio of both *A. clausi* and *T. longicornis*. Seasonal and long term patterns in FA concentrations of zooplankton species should be included for an improved understanding of multiple stressor conditions to the base of the marine food web.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marenvres.2018.12.008.

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