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Discussions

Structural and functional responses of harpacticoid copepods to anoxia in the Northern Adriatic: an experimental approach

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Abstract

Combined in situ and laboratory studies were conducted to document the effects of anoxia on the structure and functioning of meiobenthic communities, with special focus on harpacticoid copepods. In a first step, anoxia was created artificially by means of an underwater chamber at 24 m depth in the Northern Adriatic, Gulf of Trieste (Mediterranean). Nematodes were found as most abundant taxon, followed by harpacticoid copepods. While nematode densities were not affected by treatment (anoxia/normoxia) or sediment depth, these factors had a significant impact on copepod abundances. Harpacticoid copepod family diversity, in contrast, was not affected by anoxic conditions, only by depth. Ectinosomatidae and Cletodidae were most abundant in both normoxic and anoxic samples.

The functional response of harpacticoid copepods to anoxia was studied in a laboratory tracer experiment by adding ^{13}C pre-labelled diatoms to sediment cores in order to test (1) if there is a difference in food uptake by copepods under normoxic and anoxic conditions and (2) whether initial (normoxia) feeding of harpacticoid copepods on diatoms results in a better survival of copepods in subsequent anoxic conditions. Independent of the addition of diatoms, there was a higher survival rate in normoxia than anoxia. The supply of additional food did not result in a higher survival rate of copepods in anoxia, which might be explained by the presence of a nutritionally better food source and/or a lack of starvation before adding the diatoms. However, there was a reduced grazing pressure by copepods on diatoms in anoxic conditions. This resulted in a modified fatty acid composition of the sediment. We concluded that anoxia not only impacts the survival of consumers (direct effect) but also of primary producers (indirect effect), with important implications for the recovery phase.

1 Introduction

The dissolved oxygen concentration in bottom waters of many shallow coastal seas and estuaries is decreasing, often leading to so-called "dead zones", areas with too little oxygen to support most marine life (Rabalais et al., 2002; Gray et al., 2002). With world-wide more than 400 systems recognized, covering a total area of ca. 245 300 km^2 (Diaz and Rosenberg, 2008), hypoxia (defined here as DO levels ≤ 2 mL L^{-1}) and anoxia (no oxygen) are among the top-list of emerging environmental challenges (UNEP, 2004; Rabalais et al., 2010). Hypoxia caused by eutrophication is now considered to be amongst the most pressing water pollution problems in the world (Wu, 2002). Water column stratification – the isolation of bottom water from exchange with oxygen-rich surface water – is another main cause of hypoxia (Diaz, 2001).

In the Northern Adriatic, seasonal oxygen depletions, often associated with massive marine snow events, have also been noted periodically for centuries (Crema et al., 1991). In the second half of the 20th century their frequency and severity, however, have markedly increased due to high anthropogenic input of nutrients (Justić et al., 1987; Druon et al., 2004). Studies on the effects of hypoxia/anoxia here have mainly focused on benthic macrofauna organisms (i.e. Stefanon and Boldrin, 1982; Stachow-itsch, 1984; Hrs-Brenko et al., 1994; Šimunović et al., 1999; Occhipinti-Ambrogi et al., 2002). Little research was done to detect and quantify stress induced by oxygen de-pletion on meiofauna (Metazoa passing through a 1 mm sieve but retained on a 38 μm sieve)(but see Barmawidjaja and Van Der Zwaan, 1995; Travizi, 2000), and information on species- and/or community-level responses (including sensitivity and tolerance) to low DO is mainly provided by aquaculture impact studies (e.g. D'Agaro and Lanari, 2006; Grego et al., 2009; Zhou et al., 2012). While aquaculture, however, represents a rather long-term impact (weeks to months) for the benthic fauna, the course of oxygen depletion in the Northern Adriatic often takes place within a few days (Stachowitsch, 1984). The potential short-term impacts (direct and indirect effects) on the meiofauna compartment might be different and are still underdocumented. Besides differences in

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terms of causes and timeframe of anoxia, the ecology and the contribution to ecosystem functioning of the organisms under study are pivotal to evaluate the final impact of anoxia. These parameters will determine the survivors after anoxia and will thus ultimately govern the recovery of the ecosystem. In contrast to several studies on the effects of anoxia on the structural diversity of biotic communities, little is known about their functional response to anoxia, e.g. in terms of their feeding ecology.

The present study focusses on both the structural and functional responses of meiobenthic organisms to hypoxia/anoxia. Because of their small size, high abundances, short generation times and their close association with the sediment, meiobenthic organisms form an appropriate tool to determine the effects of perturbations in aquatic ecosystems (Kennedy and Jacoby, 1999). More specifically, the present study targeted harpacticoid copepods as they represent, after nematodes, the second most abundant meiofauna group (Giere, 2009) and are known to respond rapidly to hypoxic/anoxic conditions (Moodley et al., 1997; Modig and Ólafsson 1998). Moreover, harpacticoid copepods form an essential link between primary producers and higher trophic levels (Giere, 2009). Consequently, any impact of low oxygen levels on this group in terms of lower density/diversity will have an important, cascading effect on the energy flow of the overall ecosystem.

To obtain detailed information on shifts in abundance, diversity, community composition and distribution of harpacticoid copepod species after short-term hypoxia/anoxia (i.e. days), an in situ experiment was performed. This field experiment was designed to better understand the impact of anoxia in the Northern Adriatic by gaining detailed information on the survivors, as this is crucial to interpret post-disturbance community patterns and in the resumption of overall ecosystem functioning. Although copepods have generally been found to be very sensitive to low oxygen levels (e.g. Modig and Ólafsson, 1998; Travizi, 2000) it is important to know which copepod families are most sensitive. Since many hypoxic/anoxic systems are eutrophication-promoted (Diaz, 2001), the functional response of copepods to additional food supply (using ^{13}C pre-labelled diatoms) was studied in the laboratory. In particular, we tested for

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differences in diatom uptake by copepods under normoxic and anoxic conditions and whether initial feeding (i.e. before the onset of anoxia) on high-quality food like diatoms resulted into a better survival of and a differential response of the consumers (copepods) to anoxic conditions. The use of trophic tracers, for example, allows to test the effect of additional food sources over the short term (stable isotopes, see review by Boecklen et al., 2011), while fatty acid (FA) profiling (see review by Kelly and Scheibling, 2012) provides insight into changes in the long run. The combination of both techniques will allow to measure carbon assimilation and contribution of provided diatoms to the energy level in the consumers under oxic vs. hypoxic conditions. Here we used FA profiling to check the quality of the food present in the sediment in the control vs. the treatments; subsequently, stable isotope analyses were used to trace the food uptake of copepods in normoxic and anoxic conditions. Data from this experiment will contribute to our knowledge on the energy flow between primary producers and consumers in low DO conditions, which is a main issue in the overall functioning of benthic communities in impacted sites.

2 Material and methods

2.1 Study area

The effect of anoxia on meiobenthic organisms, with special focus on harpacticoid copepods, was studied in August 2009 (in situ experiment) and August 2010 (laboratory experiment) in the Gulf of Trieste, Northern Adriatic Sea (Mediterranean). The experimental site is located on a sublitoral soft bottom at 24 m depth, near the oceanographic buoy of the Marine Biology Station, Piran (Slovenia) about 2 km offshore ($45°32'55.68''$ N, $13°33'1.89''$ E) in order to avoid any impact of fishery activities.

2.2 In situ experimental set-up and sampling

Anoxia was experimentally induced in situ at a depth of 24 m in the period 8–12 August 2009 with a plexiglas underwater device (50 × 50 × 50 cm), originally referred to as EAGU (Experimental Anoxia Generating Unit) and described in detail in Stachowitsch et al. (2007). The chamber, originally designed to document macrofauna behaviour during low DO conditions (see also Riedel et al., 2013), is equipped with a digital camera (Canon EOS 30D; images taken in 6-min intervals), underwater flashes and a sensor array (DO, hydrogen sulphide H_2S). The sensors were positioned 2 cm above the sediment and values were recorded in 1-min intervals. Bottom water pH and temperature was measured with a WTW TA 197 pH sensor at the beginning and end of the deployment.

The deployment was terminated on day 5 and triplicate sediment PVC cores (length 20 cm, inner diameter = 4.6 cm, surface = 16.6 cm^2) were taken from the anoxic sediment inside the chamber by scuba divers. Additionally, normoxic samples (3 replicates) were taken at ca. 4–5 m distance from the chamber. In the laboratory, the sediment cores were immediately sliced into 5 depth layers: 0–0.5, 0.5–1, 1–1.5, 1.5–2 and 2–3 cm and stored in 4 % formaldehyde (final concentration).

Meiobenthic organisms were extracted by washing the sediment on a 1000 μm sieve mounted over a 38 μm sieve. The sediment retrieved (38 μm sieve) was centrifuged at 3000 rpm during 10 min with Ludox solution (specific density of 1.18 g cm^{-3}) (Heip et al., 1985). This step was repeated 3 times to ensure that all meiofauna was extracted. Finally, meiofauna samples were stained with Rose Bengal and preserved in 4 % formaldehyde (final concentration).

2.3 Identification of harpacticoid copepods

Higher meiofauna taxa were counted under a binocular microscope (cnidarians were not taken into account). Copepods were picked out and stored in 75 % ethanol. For the identification of harpacticoid copepods, all or a minimum of 100 copepods were

sorted at random from the extracted samples. From the anoxic and normoxic samples, 3 replicates per layer were identified, yielding a total of about 900 copepods for identification. Picked individuals were mounted on glycerine slides. In order to avoid flattening the specimens and to allow rotation of the animals for better observation, a small piece of a cover glass was placed in the glycerine before closing the slide with a cover glass. Harpacticoid copepods were identified at family level using a Leitz laborlux 12 microscope equipped with a drawing tube. Identification keys included Boxshall and Hasley (2004) and Lang (1948, 1965).

2.4 Laboratory experimental set-up

To test for the effect of available food (here ^{13}C pre-labelled diatoms) on the response of copepods to anoxia, a lab experiment was set up.

The cores were taken by scuba divers on 3 August 2010 at the same location as the field samples from 2009 (see before). Overall, 24 sediment cores (same dimensions as for field samples) were retrieved and immediately transported to a thermostatic chamber at $17 \pm 1\,°C$ and stored in complete darkness. The cores were provided with oxygen through an aeration system (tubes connected to the cores) to give a constant air flow in the water column. Air tubes were placed in the water column just above the sediment such as not to disrupt the sediment of the cores (Fig. 1). Four additional sediment cores (further referred to as cores taken at T_0) were collected to estimate the field harpacticoid copepod community composition at the start of the experiment.

The effect of feeding before the onset of anoxia was tested using the epipelic pennate diatom *Seminavis robusta* as additional food source (see also De Troch et al., 2007). The clone involved in the current experimental work is referred to as F2-44 in the diatom culture collection of the Laboratory of Protistology and Aquatic Ecology, Ghent University, Belgium (clones from which the lineage was started were isolated from a sample collected in November 2000 from the "Veerse Meer", a brackish water lake in Zeeland, The Netherlands). By the time of setting the experiment, the diatom cells were 51–56 µm ($n = 20$) in length.

To trace the diatom uptake by copepods, the cells were labelled with the stable isotope ^{13}C by adding 5 mL of a NaH[13]CO$_3$ stock solution (336 mg NaH[13]CO$_3$ in 100 mL milliQ H$_2$O sodium bicarbonate, ^{13}C, 99 %, Cambridge Isotope Laboratories, Inc.) per 100 mL f/2 growth medium (Guillard, 1975).

The diatom cultures were grown in flat tissue bottles (175 cm^2 surface) in a climate room at 16–18°C with a 12:12 h light-dark and 25–50 μmol photons m^{-2} × s^{-1} light regime. The labeling technique resulted in isotope signatures (δ^{13}C) of −17.29‰ and 8949.51‰ for untreated and ^{13}C enriched cultures, respectively. Prior to the experiment, the labelled medium was replaced by autoclaved artificial seawater (salinity: 32) without any additional nutrients (no f/2 growth medium added).

To estimate the density of diatom cells in culture, the cells were homogeneously suspended by shaking the bottles in which cultures were grown and 50 μL of the suspension was transferred into a well of a 96-well plate. After cell settlement, their number was counted under a Zeiss Axiovert 40°C inverted microscope (Zeiss Gruppe, Jena, Germany), allowing to estimate the available amount (densities) to apply in the experimental cores. At the start of the experiment (day 1) 1.25×10^6 fresh diatom cells were pipetted into the experimental units, corresponding to about 7.5×10^5 10 cm^{-2}. The experiment was conducted in complete darkness to avoid additional diatom growth.

2.5 Experimental design

The experimental design consisted of 6 treatments with 4 replicates each (Fig. 2):

1. T_1N: Cores incubated for 3 days (T_1) in normoxic conditions (N), (control for T_1ND).

2. T_1ND: cores incubated for 3 days in normoxic conditions with ^{13}C enriched diatoms (D) (1.25×10^6 cells/core). This treatment allowed to check for difference in copepod abundance between treatments with and without extra diatoms.

3. T_2N: cores collected at day 9 of the experiment (T_2), i.e. 6 days after T_1 in normoxic conditions, without extra diatoms. This treatment serves as a control for anoxia without diatoms (treatment T_2A).

4. T$_2$ND: Ccores in normoxic conditions, enriched with ^{13}C diatoms (1.25×10^6 cells/core), collected at day 9 (T_2) of the experiment, i.e. 6 days after T_1. This treatment serves as a control for anoxia with diatoms (treatment T_2AD).

5. T_2A: cores made anoxic at day 3 (T_1) of the experiment and collected at day 10, i.e. after 7 days in anoxic conditions (T_2), without extra diatoms. This treatment is a control for anoxic treatment with extra diatoms (T_2AD).

6. T_2AD: cores enriched with diatoms, made anoxic at day 3 of the experiment and collected at day 10, i.e. after 7 days in anoxic conditions.

Anoxia was created by sealing the core with a thick layer of paraffin oil (density: $0.8 \, \mathrm{g \, cm^{-3}}$) in order to prevent oxygen diffusion between the seawater and the atmosphere (technique successfully used by Justin and Armstrong, 1983; Stokes, 2009). The cores were sliced into two layers: 0–1 cm and 1–3 cm. Meiofauna was extracted by means of decantation with filtered seawater. The sample was put in 5 liters of seawater and stirred, with meiofauna remaining suspended. The supernatant was collected over a 38 µm sieve; this was repeated 5 times in order to collect all meiofauna from the sediment (Vincx, 1996). The survival rate was estimated by manually removing dead specimens from the sample; live specimens were preserved at −20°C prior to further stable isotope analysis.

2.6 Stable isotopes, fatty acids and pigment analysis

Copepods were picked out, washed several times with milliQ water, placed in tin capsules (8×5 mm, Elemental Microanalysis Limited) within 2 h (to avoid label leakage) and desiccated overnight at 60°C. For each replicate, δ^{13}C values and copepod

biomass (total carbon) were measured with a continuous flow isotope ratio mass spectrometer (type Europa Integra) at the UC Davis Stable Isotope Facility (University of California, USA).

Prior to meiofauna extraction, two aliquots of the sediment were taken from each core, one for FA analysis and one for pigment analysis. Hydrolysis of total lipid extracts of sediment (on average 30 mg per sample) and methylation to fatty acid methyl esters (FAME) was achieved by a modified one-step derivatisation method after Abdulkadir and Tsuchiya (2008). The boron trifluoride-methanol reagent was replaced by a 2.5 % H_2SO_4-methanol solution since BF_3-methanol can cause artifacts or loss of PUFA (Eder, 1995). The FA methylnonadecanoate C19:0 (Fluka 74208) was added as an internal standard to allow quantification. Samples were centrifuged (Eppendorf Centrifuge 5810R) and vacuum dried (Rapid Vap LABCONCO). The FAMEs obtained were analysed using a gas chromatograph (Hewlet Packard 6890N) coupled to a mass spectrometer (HP 5973). All samples were run in splitless mode (1 µL injector per run, injector temperature 250 °C), using a HP88 column (60 m × 25 mm, i.d. Df = 0.20; Agilent J&W; Agilent Co., USA) with He flow rate of 1.3 mL min^{-1}. The initial oven temperature was 50 °C for 2 min, followed by a ramp at 25 °C min^{-1} to 175 °C and a final ramp at 2 °C min^{-1} to 230 °C with a 4-min hold. The FAME were identified by comparison with the retention times and mass spectra of authentic standards and mass spectral libraries (WILEY, NITS05), and analysed with the software MSD ChemStation (Agilent Technologies).

Quantification of individual FAME was accomplished by using external standards (SupelcoTM 37 Component FAME Mix, Supelco no. 7885, Sigma-Aldrich Inc., USA). The quantification 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}).

Pigments were extracted according to the protocol of Wright and Jeffrey (1997) for chlorophylls and carotenoids of marine phytoplankton. The sediment samples were lyophilized and weighted. 2 mL acetone 90 % was added as extraction fluid, followed

by sonication of the samples (30 s in red light) to avoid degradation of pigments. Then 1.5 mL of the overlying fluid was taken with a syringe containing a filter (Syringe Alltech HPLC 13mm 0.2 μm PFTE). The first 0.5 mL was considered waste, while the last 1 mL sample was captured in a dark vial immediately closed afterwards. The vials (extract + standards + blank samples) were then placed in the auto sampler of the HPLC (Gilson Macherey-Nagel column C18 250/4.6 nucleodur 5 μm).

2.7 Data treatment

2.7.1 In situ experiment

Meiofauna counts were standardised to densities 10 cm^{-2}. The diversity indices of Hill (1973) were calculated as N_0 = number of taxa or families; N_1 = exp. (H'), with H' as the Shannon-Wiener diversity index (log$_e$ based); N_{inf} = the reciprocal of the proportional abundance of the most common taxa or family. Community structure was analysed through clustering and Multidimensional Scaling (MDS) based on Bray-Curtis similarity calculations. SIMPER (SIMilarity PERcentage) was carried out and differences amongst identified groups were tested with a two-way analysis of variance (two-way ANOSIM) with treatment (anoxia/normoxia) and sediment depth as main factors. Calculations and multivariate analyses were performed using the PRIMER 6 software package (version 6.1.10) (Clarke and Warwick, 2001).

2.7.2 Lab experiment

Standardisation of $\delta^{13}C$ values was done following Middelburg et al. (2000).Incorporation of ^{13}C is reflected as excess (above background) ^{13}C and expressed as total uptake (I) in mg ^{13}C ind.$^{-1}$, calculated as the product of excess ^{13}C (E) and individual biomass (organic carbon). E is the difference between the fraction ^{13}C of the control ($F_{control}$) and the sample (F_{sample}) : $E = F_{sample} - F_{control}$, where $F = {}^{13}C/({}^{13}C + {}^{12}C) = R/(R+1)$. The carbon isotope ratio (R) was derived from the

measured $\delta^{13}C$ values as $R = (\delta^{13}C/1000 + 1) \times R\text{VPDB}$ with $R\text{VPDB} = 0.0112372$ as $\delta^{13}C$ is expressed relative to Vienna Pee Dee Belemnite (VPDB). This total uptake was further standardised and expressed per unit carbon of copepod.

Differences in uptake were tested by means of two-way analyses of variance (two-way ANOVA) with treatment (anoxia/normoxia) and sediment depth as main factors using the software Statistica 7.0. Prior to all ANOVAs, the Cochran's C-test was used to check the assumption of homoscedasticity.

3 Results

3.1 In situ experiment – structural responses

3.1.1 Sensor data

Dissolved oxygen concentration decreased exponentially from initial $5.51\,\text{mL}\,\text{L}^{-1}$ ($= 7.87\,\text{mg}\,\text{L}^{-1}$) to $0\,\text{mL}\,\text{L}^{-1}$ within 48 h and anoxic conditions persisted until the end of the deployment on day 5. H_2S started to increase soon after onset of anoxia, with final values reaching $\sim 29\,\mu\text{M}$. The temperature in the chamber remained constant (average $20\,^\circ\text{C}$), the bottom water pH dropped from initial 8.1 to a minimum of 7.6. The salinity was 38.

3.1.2 Meiofauna densities and community composition

Two-way ANOVA showed that sediment depth was a determining factor for the overall meiofauna density (decreasing abundance with depth; $p = 0.002$), while the different oxygen treatments (normoxia/anoxia) interestingly showed no effect ($p = 0.05$, Fig. 3A). Nematodes were the dominant meiofauna taxon and reached the highest relative abundance $\pm$ stdev in the 1–1.5 cm layer in normoxia ($97.8 \pm 3.5\,\%$) and 1.5–2 cm depth in anoxia ($97.0 \pm 3.7\,\%$). However, no significant effect of treatment (anoxia/normoxia) or

depth layer was found ($p = 0.19$ for treatment, $p = 0.13$ for depth) for nematodes. Copepods were the second most abundant taxon, reaching a maximum relative abundance in the top sediment layers (0–0.5 cm, normoxia $38.1 \pm 3.1\%$, anoxia $31.2 \pm 10.6\%$, respectively). In contrast to nematodes, there was a significant effect of both treatment ($p = 0.03$) and depth ($p < 0.001$) on the relative copepod contribution (Fig. 3b). Kinorhyncha represented the third most abundant taxon with $8.3 \pm 1.3\%$ at 0.5–1 cm depth (normoxia) and $12.6 \pm 6.8\%$ in the top 0–0.5 cm layer (anoxia). The percentage proportion of Kinorhyncha differed significantly with depth ($p < 0.001$) but there was no effect of treatment ($p = 0.84$).

Remaining taxa present in the normoxic samples included (in order of decreasing abundance) Polychaeta, Ostracoda, Acarina, Bivalvia, Amphipoda, Mysidacea, Turbellaria and Insecta. The anoxic samples contained Polychaeta, Ostracoda, Acarina and Turbellaria. The total contribution of these taxa was significantly different for both factors, treatment ($p = 0.04$) and sediment depth ($p = 0.004$).

On the MDS plot on the relative meiofauna composition (stress = 0.05, Fig. 4a), the two surface layers (0–1 cm) were grouped separately from the deeper layers (1–3 cm), which points to a strong effect of the sediment depth and no clear effect of anoxia on relative meiofauna composition. This separation was further confirmed by ANOSIM ($R = 0.508$, $p = 0.001$). SIMPER analysis showed an average similarity in taxa contribution of 69.9% in the surface layers (0–1 cm) and 82.8% in the deeper layers (1–3 cm). Nematodes (80.4%, 96.4%, respectively in 0–1 cm and 1–3 cm) and copepods (13.6% in 0–1 cm) contributed most to the dissimilarity between surface and the deeper layers.

3.1.3 Harpacticoid copepod composition

The relative abundance of the harpacticoid copepod families in normoxic samples (Fig. 5a) showed a dominance of Ectinosomatidae (overall average: $40.1 \pm 23.8\%$) followed by Cletodidae ($28.2 \pm 2.1\%$) and Miraciidae ($20.1 \pm 16.9\%$). The remaining families accounted for $11.6 \pm 9.2\%$ of the total relative abundance and included, in

order of decreasing abundance: Laophontidae, Longipediidae, Thalestridae, Tisbidae and Ameiridae. Except for the family Tisbidae ($p = 0.06$), the relative abundance of all families differed significantly with depth (all $p \leq 0.01$).

In anoxic samples (Fig. 5b), Cletodidae showed the highest relative abundance ($47.0 \pm 22.4\%$) followed by Ectinosomatidae ($37.0 \pm 24.4\%$) and Laophontidae ($6.3 \pm 7.9\%$). The remaining families ($9.7 \pm 9.6\%$) were, in order of decreasing abundance: Miraciidae, Ameiridae, Thalestridae, Longipediidae and Tisbidae. Moreover, Cletodidae represented up to $61.7 \pm 37.5\%$ and Ectinosomatidae showed a maximum relative contribution of $52.8 \pm 41.1\%$ (Fig. 5b). Relative abundance differed significantly with sediment depth in Ectinosomatidae ($p < 0.01$), Cletodidae ($p < 0.001$), Thalestridae ($p < 0.01$) and Laophontidae ($p < 0.1 = 0.12$, Fig. 4b) of the relative copepod families composition revealed a high similarity between the top layers (0–0.5 cm) of normoxic and anoxic samples, while the deeper layers showed a higher variability (i.e. sample points are more spread). The difference in relative family composition between surface (0–1 cm) and deeper sediment layers (1–3 cm) was supported by ANOSIM ($R = 0.719$, $p = 0.001$).

SIMPER analysis showed an average similarity in family composition of 53.2 % in the surface sediment layers (0–1 cm) and 46.7 % in the deeper layers (1–3 cm). Ectinosomatidae (44.9 % and 41.5 % in 0–1 cm and 1–3 cm, respectively), Cletodidae (42.4 %, 42.2 %) and Miraciidae (6.7 %, 13.6 %) were the copepod families that contributed most to the dissimilarity between surface and deeper sediment layers.

3.1.4 Diversity patterns

Independent of the treatment, the meiofauna in the surface layers (0–0.5, 0.5–1 cm) showed the highest diversity (Table 1a). Overall, the Hill diversity indices (Table 1a) revealed no significant difference in meiofauna diversity between the normoxic and anoxic samples (two-way ANOVA, $p = 0.65$). However, there is a significant decrease of diversity with increasing sediment depth (two-way ANOVA, $p = 0.02$), indicated by the number of taxa (N_0) in normoxia and, to a much lesser extent, in anoxia. The decreasing

N_{inf} (Table 1a) values in deeper sediment layers illustrate that the dominance level increased with depth.

For harpacticoid copepods, no significant difference in diversity (N_0, number of families, Table 1b) was found between normoxic and anoxic samples (two-way ANOVA, $p = 0.47$). Sediment depth, however, had a significant effect, resulting in a decrease in copepod diversity with increasing depth (two-way ANOVA, $p < 0.0001$).

3.2 Laboratory experiment – functional responses

The initial harpacticoid copepod community at T_0 was dominated by Cletodidae ($37.8 \pm 12.1\%$), followed by Laophontidae ($18.3 \pm 2.4\%$) and Miraciidae ($17.1 \pm 6.3\%$). The remaining families represented $26.8 \pm 4.5\%$ and included, in order of decreasing abundance: Longipediidae, Ectinosomatidae, Ameiridae, Thalestridae, Tisbidae and Tetragonicipitidae.

The induction of anoxia was successful as oxygen levels in the overlying water decreased significantly (one-way ANOVA, $p < 0.00001$) from initial 6.6 ± 0.2 mg L^{-1} (T_1N) and 6.4 ± 0.06 mg L^{-1} (T_2N) to 0.58 ± 0.29 mg L^{-1} (T_2A) after 7 days of closure of the core, independent of the addition of extra diatoms or not.

Chlorophyll a (Chl a) concentrations were measured at time T_1 and T_2 and ranged between 0 and 90 µg g^{-1}. The addition of diatoms had a highly significant effect on Chl a values ($p < 0.0001$). Samples without additional diatoms had ¡ 5 µg g^{-1} Chl a while treatments with extra diatoms had > 35 µg g^{-1} Chl a. There was no significant difference in Chl a between the different time intervals, T_1 and T_2, (one-way ANOVA, $p = 0.35$) because of the high variance in Chl a concentrations in treatments with additional diatoms (83.9 ± 52.2 µg g^{-1} (T_1ND), 69.8 ± 33.3 µg g^{-1} (T_2ND), 36.6 ± 24.0 µg g^{-1} (T_2AD)). Chlorophyll $c2$ (Chl $c2$) showed similar patterns with max. 3.5 ± 4.2 µg g^{-1} (T_1N) in treatments without diatoms and up to 8.7 ± 7.2 µg g^{-1} after adding diatoms (T_2ND). In terms of carotenoids, the concentration of fucoxanthin ranged between 1.3 ± 0.1 µg g^{-1} and 2.0 ± 0.4 µg g^{-1} without additional diatoms and

between $18.2 \pm 19.2\,\mu g\,g^{-1}$ (T_2AD) and $39.7 \pm 24.8\,\mu g\,g^{-1}$ (T_2ND) in treatments with extra diatoms.

3.2.1 Survival rate

The survival rate of harpacticoid copepods was significantly higher in normoxic conditions ($93.2 \pm 3.7\,\%$) than in anoxic conditions ($73.6 \pm 16.2\,\%$) at T_2 (two-way ANOVA, $p = 0.005$). Additional food supply tended to have a negative effect on copepod survival as the average survival rate for treatments with extra diatoms was $79.9 \pm 14.5\,\%$ in contrast to $86.9 \pm 16.1\,\%$ in treatments without extra diatoms. Due to the high variance, this effect was, however ,not significant (two-way ANOVA, $p = 0.2$). More specifically, the anoxic treatment with extra diatoms (T_2AD) had a lower survival rate ($66.9 \pm 5.8\,\%$) than the one with diatoms ($80.3 \pm 21.5\,\%$, T_2A). In combination with the effect of the anoxic condition, the former (T_2AD) had a significantly lower survival rate than the oxic treatments with or without extra diatoms (T_2ND: $93.0 \pm 2.0\,\%$, T_2N : $93.5 \pm 5.3\,\%$) (post-hoc Tukey HSD, $p < 0.01$). It is worth noting that there was no effect of food addition on the survival rate in the oxic treatments (T_2N, T_2ND).

3.2.2 Diatom uptake as indicated by biomarkers

The total FA concentration in the sediment was highest in treatments with additional diatoms (note different scales in Fig. 6a, b), with the highest values in T_1ND ($307.6 \pm 42.0\,\mu g\,mL^{-1}$) and T_2AD ($966.1 \pm 272.2\,\mu g\,mL^{-1}$). The unsaturated FA C16:1ω7, for example, was most abundant in these treatments, and linoleic acid C18:2ω6 was found only in samples with additional diatoms. Independent of the additional food supply, the anoxic samples showed a higher total FA concentration than the normoxic treatments (Fig. 6a, b). Especially the relative contribution of C16:1ω7 increased in the anoxic conditions, in particular when diatoms were added (T_2A, T_2AD, Fig. 6c, d).

Before the onset of the anoxia, the copepods were fed for 3 days with labelled diatoms, which resulted in the increase of their $\Delta\delta^{13}C \pm$ stdev from -22.4 ± 1.4‰ (T_1N) to 276.9 ± 96.4‰ (T_1ND). In the normoxic treatments, a significant increase of $\Delta\delta^{13}C$ from T_1ND (276.9 ± 96.4‰) to T_2ND (952.2 ± 907.2‰) was recorded (one-way ANOVA, $p = 0.03$), indicating continuous feeding in normoxia (Fig. 7). In the anoxic treatments, food uptake ceased, with $\Delta\delta^{13}C$ values showing no significant difference between T_1ND (276.9 ± 96.4‰) and T_2AD (115.7 ± 43.0‰) (one-way ANOVA, $p = 0.16$). Consequently, the $\Delta\delta^{13}C$ value differed significantly between normoxic and anoxic treatment (one-way ANOVA, $p = 0.014$).

Standardisation towards total uptake (I) and uptake per unit carbon showed similar patterns (not illustrated).

4 Discussion

4.1 In situ experiment

In the present field experiment, total meiofauna densities were not significantly affected by anoxic conditions. This is in contrast with previous studies that showed a significant decrease of meiofauna densities due to anoxia (Moodley at al., 1997; Travizi, 2000). While studies on macrofauna revealed a peak in mortality at the transition from severe hypoxia to anoxia (Riedel et al., 2012), meiofauna – in general being more tolerant (Moodley et al., 1997) – may still be alive. Van Colen et al. (2009) created hypoxic conditions in a tidal mudflat for 40 days. While no macrobenthos survived, nematode diversity and abundances, for example, changed but no complete mortality occurred. The strong resistance of nematodes to anoxia and the fact that they are the dominant meiofauna group can explain the lack of a clear effect of anoxia on total meiofauna density.

Another and probably reliable explanation is the short duration of the anoxic/sulphidic phase (i.e. 5 days). In contrast to long-term impact studies, e.g. from aquaculture

impact (see Grego et al., 2009), the present study aimed to analyse short-term effects since this is often the case in the Northern Adriatic (Stachowitsch, 1984). The present study concluded that only copepods showed a short-term anoxia response while nematodes, kinorhynchs and other meiofauna taxa were not affected. In addition to the duration of anoxia, also the way of creating anoxia can be pivotal in the interpretation of the results. The way the benthic underwater chamber created anoxia could also explain the lack of response by meiofauna. By sealing a $50 \times 50 \times 50$ cm volume off from the surrounding environment (Stachowitsch et al., 2007), also the food supply was cut-off. As such, the field experiment mimics the situation where water column stratification is the main cause of hypoxia, i.e. the isolation of bottom water from oxygen-rich surface water (Diaz, 2001). In this set-up, however, eutrophication as an important factor in creating anoxia (Gray et al., 2002) was neglected. Moreover, there was a total cut-off of the food supply for the enclosed fauna. In view of their large biomass and energy storage, macrofauna can generally cope with low to no-food supply for longer periods (Ott, 1981). Moreover, a 5-days experiment/cut-off is negligible compared to weeks of strong stratification. The response of meiofauna is less well-known and we can merely speculate that their low energy demands may be crucial to overcome food restrictions. In that context, the lab experiment (see further) could contribute to our understanding of the role of food for the response of meiofauna to anoxia because it tested specifically for the effect of food level (normal or elevated).

In contrast to the lack of a clear effect of anoxia on meiofauna, it was mainly the sediment depth that determined meiofauna densities and community composition. This corresponds with other meiofauna studies where the upper few centimetres of the sediment harboured more meiofauna than the deeper horizons (see review by Giere, 2009). As there was no significant difference in overall density between the natural (normoxic) and the treated cores (anoxia), we conclude that the impacted meiofauna community corresponded to a 'natural, pristine' meiofauna community. Note that the meiofauna counts for this study were based on rose bengal-coloured individuals and may therefore be an overestimation of the actual number of life animals (see Grego et al., 2013a).

Nematodes are known to dominate in terms of abundance and biomass in most meiofauna samples (Giere, 2009) and were also the most abundant taxon in this study, independent of the treatment (normoxia and anoxia), followed by copepods. Our results showed that nematode densities were not affected by depth and treatment, whereas copepods were less abundant in anoxic conditions and in the deeper sediment layers. Wetzel et al. (2001) summarized that tolerance of meiofauna to anoxia is probably related to exposure history, phylogenetic constraints and lifestyle. Low oxygen demand and a high surface: volume ratio and anaerobic metabolism enable some species to survive hypoxia/anoxia for extended times.

Studies within a given habitat typically concluded that of all permanent meiobenthic taxa, foraminiferans (see also Langlet et al., 2013) and nematodes are best adapted to hypoxia/anoxia and that crustaceans (e.g. copepods) are the least tolerant (see review by Wetzel et al., 2001). The generally high tolerance of nematodes to low oxygen concentrations (see also Moodley et al., 2000) often results in an increase in their relative abundance. One of the mechanisms to escape from temporal anoxia is to migrate from hypoxic sediments into the water column and to settle again to the sediment with the return of normoxic conditions (Wetzel et al., 2001). Harpacticoid copepods, in contrast, represent the meiobenthic taxon most sensitive to low DO levels (Hicks and Coull, 1983; Modig and Ólafsson, 1998; Travizi, 2000), which might explain their restriction to the well-oxygenated top sediment layers.

A similar trend was found for meiofauna diversity: it decreased with depth, independent of the treatment (anoxia vs. normoxia). The top sediment layers (0–0.5, 0.5–1 cm) were also characterized by the highest meiofauna taxa diversity. According to Wu (2002), species diversity drops in anoxic conditions, but in our experiment we found mainly an effect of sediment depth.

At lower taxonomic level, the only effect of anoxia was seen in the harpacticoid copepods' family composition. The families Ectinosomatidae and Cletodidae dominated in anoxic conditions, while the abundance of other families, including Longipediidae and Miraciidae, decreased rapidly in anoxic conditions. Very little is known so far about

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differential resistance of harpacticoid copepod families to anoxia (by see Grego et al., 2013b). Overall, harpacticoid copepods have a high sensitivity to low oxygen conditions (see before), except for so-called worm-like copepod species belonging to the families Paramesochridae and Leptastacidae, thought to be able to survive in anoxic conditions (Hicks and Coull, 1983). The diversity of harpacticoid copepod families differed significantly with depth. Again, as for meiofauna taxa, depth was found to be the most important factor structuring the harpacticoid copepod community.

4.2 Lab experiment

The lab experiment was conducted to get a better insight into the functional responses of harpacticoid copepods to anoxia. The use of pre-labelled diatoms (i.e. *Seminavis robusta)*, as a typical food source for many meiofaunal taxa (Giere, 2009), proved to be successful in previous studies on harpacticoid copepods and their contribution to the energy flow in benthic ecosystems (e.g. De Troch et al., 2006, 2007). The attractiveness of meiofauna to diatoms can mainly be explained by the high content of polyunsaturated fatty acids (PUFA) and the FA C16:1ω7 (Coull, 1999; De Troch et al., 2012). First we wanted to test for a functional response in terms of food uptake by harpacticoid copepods in anoxic conditions. Previous studies indicated that anoxia negatively affected feeding behaviour of planktonic calanoid copepods, *Acartia tonsa* (Sedlacek and Marcus, 2005) and of the juvenile blue crabs *Callinectes* spp. (Das and Stickle, 1993). Widdows et al. (1989), for example, explained the reduced food uptake by oyster larvae (*Crassostrea virginica*) in anoxia as a way to save energy because digestion and absorption of food accounted for about 17 % of total metabolic energy expenditure. Studies on harpacticoid copepods showed that organisms became quiescent in order to survive unstable niches (Vopel et al., 1996). The same was observed for planktonic copepods, which entered a form of stasis, shutting down metabolic activity to minimize the energy expense of respiration (Kiørboe et al., 1985). This could explain why the copepods survived low DO conditions but showed no feeding in the anoxic treatments. They even leaked some of the heavy carbon (^{13}C) into the environment.

As expected, copepods in normoxic conditions had a 1.25 times higher survival rate than copepods in anoxic conditions. Furthermore, we hypothesized that an additional high-quality food source before the start of anoxic conditions might result in a higher survival rate during anoxia. Diet and level of food intake influence oxidative stress (Monaghan et al., 2009), and dietary intake influences the availability of exogenous antioxidants. Reduced caloric intake is known to be associated with reduced oxidative damage (Masoro, 2006). Souza et al. (2010), for example, found that certain calanoid copepods species survived better when they received additional food at the onset of a stress condition (i.e. ultraviolet radiation-induced oxidative stress). In contrast, in our experiment additional food did not yield any higher survival rate in anoxic conditions. One explanation might be the different types of stress involved in a mesocosm. However, also Sedlacek and Marcus (2005) found that the reduced egg production of the calanoid copepod *Acartia tonsa* as a response to anoxia could not be mitigated by extra food (dinoflagellates).

Another explanation is that we did not reach complete anoxia in the sediment cores in the laboratory (DO was measured in the overlying water), although low oxygen concentrations in the water column can lead to anoxic conditions in the sediments (Diaz, 2001). Alternatively, as decomposition of organic matter (here diatoms) typically results into low oxygen concentrations (Gray et al., 2002), the cores with additional diatoms had lower DO concentrations and consequently lower survival rates than those without diatoms. The latter is not the case in the present study as there was no significant difference in oxygen level in cores with or without extra diatoms. In both cases the oxygen level was as low as $0.58 \pm 0.29\,\text{mg L}^{-1}$ ($= 0.41 \pm 0.20\,\text{mL L}^{-1}$). This oxygen level can be ranked as medium severe hypoxia because it is just $\leq 0.5\,\text{mL L}^{-1}$ (Riedel, 2013), whereas in the field experiment it dropped down to $0\,\text{mL L}^{-1}$.

Finally, it is possible that there was already a high amount of initial food present in the sediment and any addition of extra diatoms would not imply any significant difference between both treatments at the onset of anoxia. The contrary, however, was true because the Chl *a* concentrations increased significantly in the treatments with

additional diatoms. Moreover, the FA composition of the sediments without additional diatoms did not show the presence of any other good food source, as FA concentrations in these cores were relatively low. The increase of the FA 16:1ω7, that is typical for diatoms, in the treatment with extra diatoms underlined this. More interestingly, however, was the considerable increase of FA in anoxic conditions, independent of the treatment (additional diatoms or not). This can be explained by a reduced grazing pressure by copepods on diatoms in anoxic conditions. Thus, anoxia does not only impact the survival of consumers (direct effect) but also of primary producers (indirect effect). Moreover, the reduced grazing pressure and the increased diatom density might have important consequences for the recovery phase after anoxia. Van Colen et al. (2012) stressed that a full recovery of ecosystem functioning after hypoxia implies a complete recovery of organism-sediment interactions. The latter mainly points to bioturbating (macro)organisms. Nonetheless, the present study underlines the role of the players at the basis of the food web, i.e. the primary producers and grazers on them, as being essential for the recovery phase.

5 Conclusions

In contrast with what we expected, total meiofauna community density was not affected by anoxic conditions, mainly because of the dominance of highly tolerant nematodes. Sediment depth seemed to be the most important factor structuring meiofauna communities in our in situexperiment. The lack of effect of anoxia on total meiofauna composition could be due to too short incubation time of the benthic underwater chamber for the effect to be shown on the most abundant meiofauna group, the nematodes. Deployments for longer time intervals (i.e. 1 month, 2 months, 1 yr) have been done but focused on foraminiferans (Langlet et al., this volume) and sediment geochemistry (Koron et al., 2013; Metzger et al., 2013). Moreover, novel approaches such as the use of vital stains, may allow a more accurate assessment of alive/dead individuals following short-term impact by hypoxia and anoxia (i.e. see Grego et al., 2013a; a for copepods

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and nematodes). Harpacticoid copepods were the most sensitive meiofauna taxa to low oxygen concentrations. While densities of harpacticoid copepods were impacted by both sediment depth and anoxia, family diversity was affected by depth only. Stable isotope analysis showed that initial feeding with diatoms did not result in a higher survival rate under anoxic conditions. In fact, copepods stopped feeding under anoxic conditions, yielding increased primary producer levels. Accordingly, this reduced grazing response has important implications for the recovery phase.

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Table 1. Average Hill's diversity indices ($\pm$ stdev) for (A) meiofauna taxa and (B) copepod family composition. N_0 = number of taxa. N_{inf} = dominance index, $H'(\log_e) = \Sigma\ P_i^2\ \log(P_i)$; N = Normoxia, A = Anoxia; sediment depth layers indicated in cm. Averaged values over all depth layers.

	Depth (cm)	N_0	N_{inf}	$H'(\log_e)$	Depth (cm)	N_0	N_{inf}	$H'(\log_e)$
(A)	N 0–0.5	6.3 ± 0.6	1.9 ± 0.1	1.1 ± 0.1	A 0–0.5	5.3 ± 1.0	1.6 ± 0.4	0.9 ± 0.3
	N 0.5–1	4.3 ± 1.5	1.2 ± 0.1	0.5 ± 0.3	A 0.5–1	4.3 ± 0.6	1.2 ± 0.1	0.5 ± 0.2
	N 1–1.5	4.0 ± 0.0	1.1 ± 0.0	0.2 ± 0.3	A 1–1.5	5.0 ± 0.0	1.1 ± 0.0	0.4 ± 0.0
	N 1.5–2	3.7 ± 0.6	1.0 ± 0.0	0.2 ± 0.0	A 1.5–2	4.3 ± 0.6	1.0 ± 0.0	0.2 ± 0.2
	N 2–3	4.3 ± 0.6	1.0 ± 0.0	0.2 ± 0.0	A 2–3	4.7 ± 1.2	1.1 ± 0.0	0.2 ± 0.1
	N overall	4.5 ± 1.2	1.2 ± 0.4	0.4 ± 0.3	A overall	4.7 ± 0.7	1.2 ± 0.2	0.4 ± 0.2
(B)	N 0–0.5	7.7 ± 0.6	2.6 ± 0.5	1.5 ± 0.1	A 0–0.5	6.0 ± 0.0	2.0 ± 0.2	1.1 ± 0.1
	N 0.5–1	3.7 ± 1.5	1.6 ± 0.4	0.9 ± 0.4	A 0.5–1	4.3 ± 0.6	1.8 ± 0.4	1.0 ± 0.1
	N 1–1.5	3.7 ± 1.2	2.2 ± 0.5	1.1 ± 0.2	A 1–1.5	3.0 ± 0.0	2.1 ± 0.2	1.0 ± 0.1
	N 1.5–2	2.0 ± 1.0	1.4 ± 0.4	0.5 ± 0.5	A 1.5–2	2.3 ± 1.2	1.6 ± 0.5	0.7 ± 0.6
	N 2–3	3.0 ± 1.0	2.3 ± 0.6	1.0 ± 0.3	A 2–3	2.3 ± 1.2	1.7 ± 0.6	0.7 ± 0.6
	N overall	4.0 ± 2.2	2.0 ± 0.6	1.0 ± 0.4	A overall	3.6 ± 1.6	1.8 ± 0.4	0.9 ± 0.4

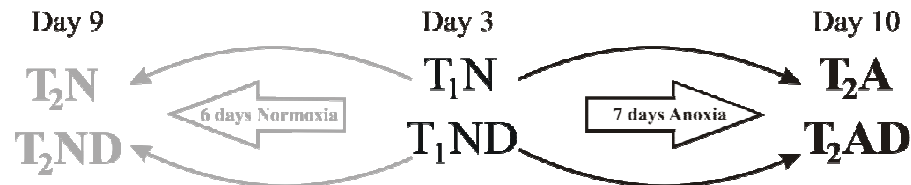**Fig. 1.** Experimental set up of the lab experiment before induction of anoxia (T_1).

Fig. 2. Experimental design: T_1: 3 days, T_2: after 6 or 7 days (i.e. 9 or 10 days in total), N = Normoxia, A = Anoxia, D = Diatoms added.
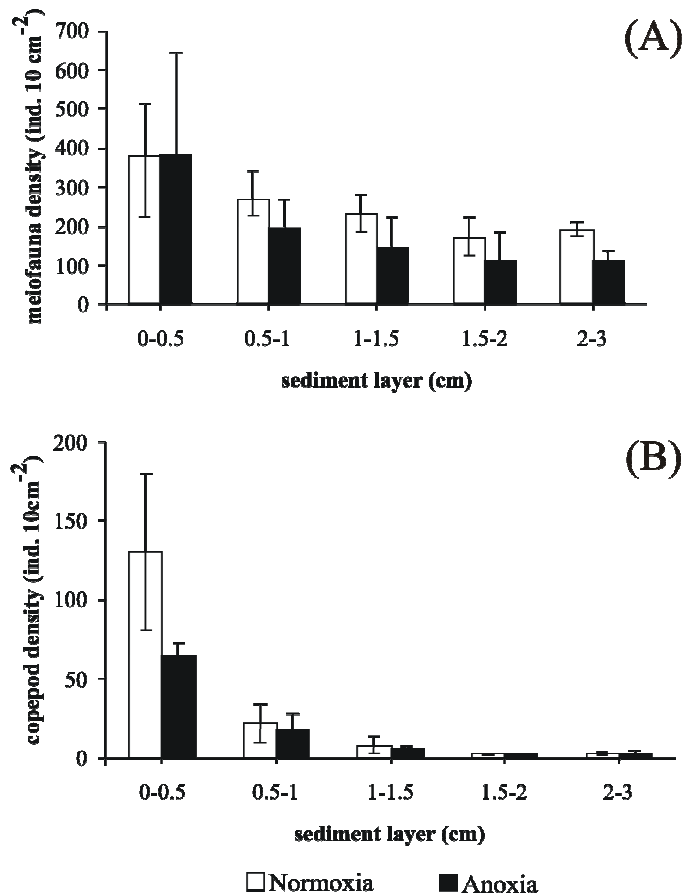
Fig. 3. Average (± STDEV) total densities of **(A)** meiofauna and **(B)** copepods in normoxic and anoxic field conditions.

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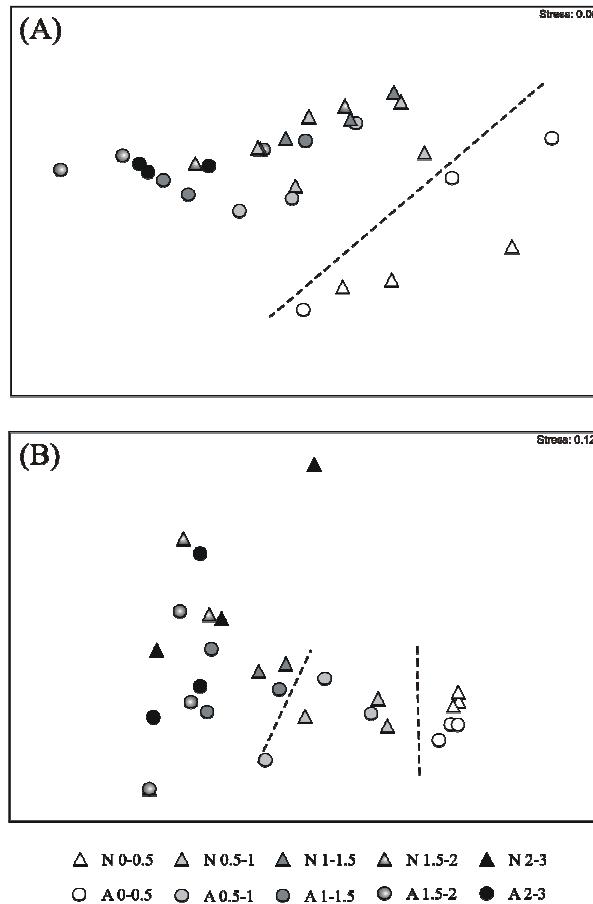
Fig. 4. MDS of relative **(A)** meiofauna and **(B)** copepod community composition based on Bray-Curtis similarity: Normoxia (N) and Anoxia (A), sediment depth layers indicated in cm.
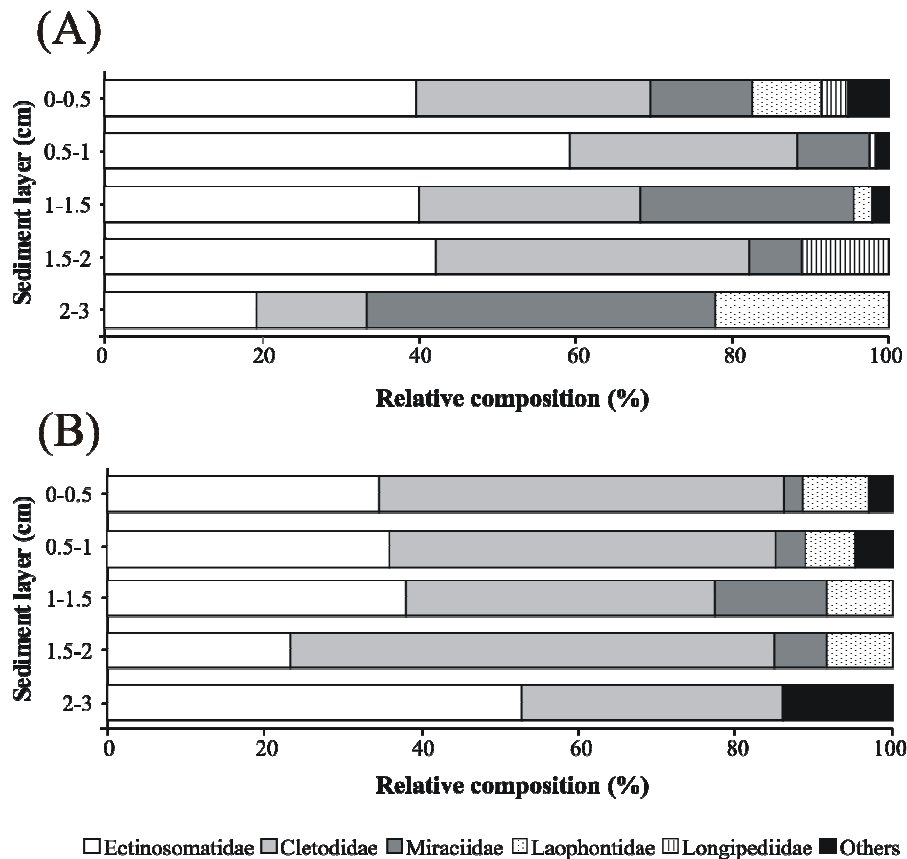
Fig. 5. Relative composition of the harpacticoid copepod families: vertical distribution in **(A)** normoxic and **(B)** anoxic conditions.
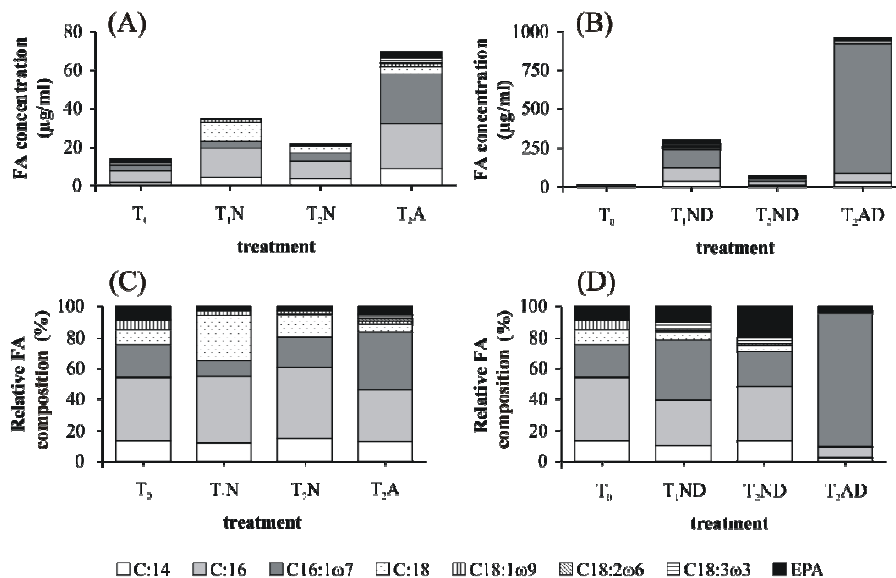
Fig. 6. Absolute and relative fatty acid composition of the sediment (0–3 cm) for **(A)**, **(C)** treatments without extra diatoms and for **(B)**, **(D)** treatments with diatoms.
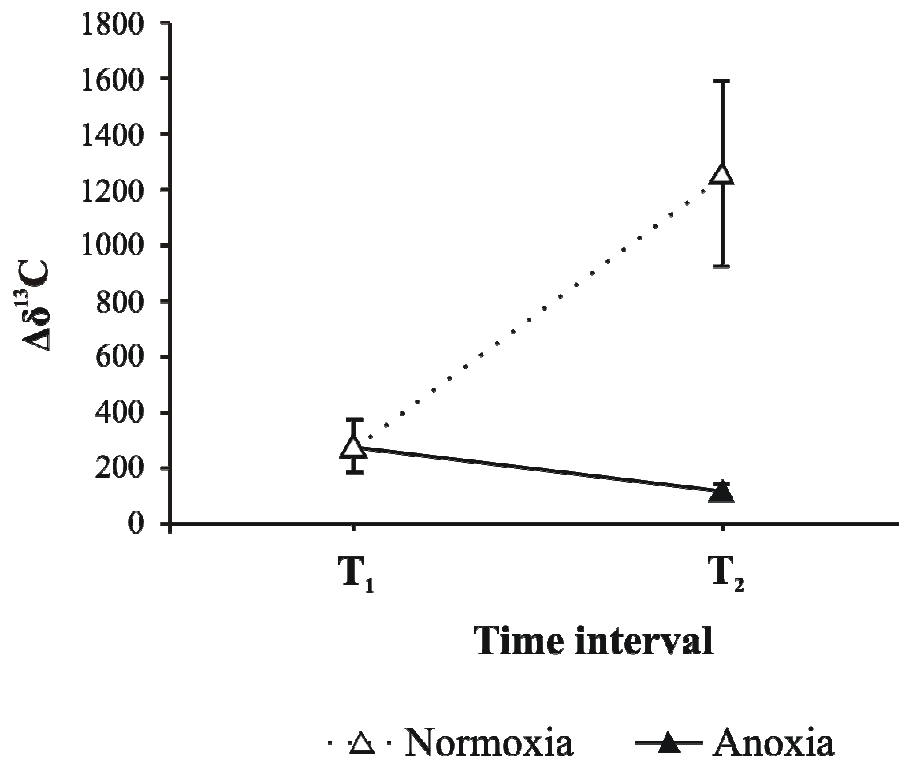
Fig. 7. Average (± STDEV) diatom uptake by harpacticoid copepods under normoxic and anoxic conditions at two time intervals (T_1, T_2) expressed as **(A)** specific uptake ($\Delta\delta^{13}$C), **(B)** total uptake per individual copepod and **(C)** total uptake per unit copepod carbon. T_2 represents 6 or 7 additional days in normoxic or anoxic conditions, respectively.