**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 in 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 13C 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; this special issue). With worldwide more than 400 systems recognized, covering a total area of ca. 245,300 km2 (Diaz and Rosenberg, 2008), hypoxia (defined here as DO levels ≤ 2ml 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 (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; Stachowitsch, 1984; Occhipinti-Ambrogi et al., 2002). Little research was done to detect and quantify stress induced by oxygen depletion 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.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 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 13C pre-labelled diatoms) was studied in the laboratory. In particular, we tested for 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* from 8-12 August2009 with a plexiglas underwater device (50x50x50 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., this volume), is equipped with a digital camera (Canon EOS 30D; images taken in 6-min intervals), underwater flashes and a sensor array (DO, hydrogen sulphide H2S). 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 cm2) were taken from the anoxic sediment inside the chamber by scuba divers. One day before the the end of the deployment (i.e. at day 4) 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 minutes 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 13C 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±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 T0) 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 13C by adding 5 ml of a NaH13CO3 stock solution (336 mg NaH13CO3 in 100 ml milliQ H2O sodium bicarbonate, 13C, 99%, Cambridge Isotope Laboratories, Inc.) per 100 ml f/2 growth medium (Guillard 1975).

The diatom cultures were grown in flat tissue bottles (175 cm2 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 (δ13C) of -17.29‰ for untreated and 8949.51‰ in the 13C enriched cultures. 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 40C 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 x 106 fresh diatom cells were pipetted into the experimental units, corresponding to about 7.5 x 105 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) T1N: Cores incubated for 3 days (T1) in normoxic conditions (N), (control for T1ND).

(2) T1ND: Cores incubated for 3 days in normoxic conditions with 13C enriched diatoms (D) (1.25 x 106 cells/core). This treatment allowed to check for difference in copepod abundance between treatments with and without extra diatoms.

(3) T2N: Cores collected at day 9 of the experiment (T2), i.e. 6 days after T1 in normoxic conditions, without extra diatoms. This treatment serves as a control for anoxia without diatoms (treatment T2A).

(4) T2ND: Cores in normoxic conditions, enriched with 13C diatoms (1.25 x 106 cells/core), collected at day 9 (T2) of the experiment, i.e. 6 days after T1. This treatment serves as a control for anoxia with diatoms (treatment T2AD).

(5) T2A: Cores made anoxic at day 3 (T1)of the experiment and collected at day 10, i.e. after 7 days in anoxic conditions (T2), without extra diatoms. This treatment is a control for anoxic treatment with extra diatoms (T2AD).

(6) T2AD: 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.

Short-term anoxia was created by sealing the core with a thick layer of paraffin oil (density: 0.8 g cm-3) in order to prevent oxygen diffusion between the seawater and the atmosphere (technique successfully used by Justin and Armstrong, 1983).

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). Centrifugation with ludox was not applied as we targeted live copepods for the stable isotope analysis. Furthermore, ludox could impact the final 13C signal through its osmotic effect on components of low molecular weight. 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. To detect a reliable 13C/12C ratios in the tissue of the harpacticoids, a minimum of 15 µg C per samples is required. Therefore we used all live copepods from the 0-1 cm layer. There was insufficient biomass of copepods in the deeper sediment layer.

**2.6 Stable isotopes, fatty acids and pigment analysis**

Copepods were picked out, washed several times with milliQ water, placed in tin capsules (8x5mm, Elemental Microanalysis Limited) within 2 h (to avoid label leakage) and desiccated overnight at 60°C. For each replicate, δ13C 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 μg 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% H2SO4-methanol solution since BF3-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 ºC), using a HP88 column (60m×25mm 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 # 47885, 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 cm2. The diversity indices of Hill (Hill 1973) were calculated as N0= number of taxa or families; N1= exp (H’), with H’ as the Shannon-Wiener diversity index (loge based); Ninf= the reciprocal of the proportional abundance of the most common taxa or family. Community structure was analysed through 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**

Stable isotope ratios are expressed relative to the conventional standards (Vienna Pee Dee Belemnite - VPDB for carbon) in units of parts per thousand, according to the formula:

δX= (Rsample/Rstandard -1) x 103 ‰

where X is 13C and R the ratio of 13C/12C.

Incorporation of 13C was expressed a specific uptake i.e. the difference between the 13C fraction of the control (i.e., based on the natural signature of organisms that did not feed on labelled diatoms) and the sample ∆δ13C= δ13Csample - δ13Ccontrol) where δ13C is expressed relative to VPDB standard).

Further standardisation of δ13C values was done following Middelburg et al. (2000)*.* Incorporation of 13C is reflected as excess (above background) 13C and expressed as total uptake (*I*) in mg 13C ind.-1, calculated as the product of excess 13C (*E*) and individual biomass (organic carbon). *E* is the difference between the fraction 13C of the control (*F*control) and the sample (*F*sample): *E*= *F*sample - *F*control, where *F*= 13C/(13C+12C)= *R*/(*R* + 1). The carbon isotope ratio (*R*) was derived from the measured δ13C values as *R*= (*δ*13C/1000 + 1) x *R*VPDB with *R*VPDB= 0.0112372 as *δ*13C 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 ml/l (= 7.87 mg/l) to 0 ml/l within 48 h and anoxic conditions persisted until the end of the deployment on day 5. H2S started to increase soon after onset of anoxia, from 0 µM to final values reaching ~ 29 μM. The temperature in the chamber remained constant (average 20°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**

Overall, a two-way ANOVA on the total meiofauna densities (ind./10cm2) showed a significant effect of sediment depth (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±stdev in the 1-1.5 cm layer in normoxia (97.8±3.5%) and 1.5-2 cm depth in anoxia (97.0±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±3.1%, anoxia 31.2±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). In the latter case, the difference between normoxia and anoxia was only significant for the top sediment layer (0-0.5cm, post-hoc Tukey HSD, p=0.003). Kinorhyncha represented the third most abundant taxon with 8.3±1.3% at 0.5-1 cm depth (normoxia) and 12.6±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. For all these taxa, there was both a treatment (p=0.04) and a sediment depth (p=0.004) effect.

On the MDS plot on the relative meiofauna composition (stress=0.05, Fig. 4A), the top sedimentlayer (0-0.5 cm) grouped separately from the deeper layers (0.5--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=0.651, p=0.001). A two-way crossed SIMPER analysis showed an average similarity in taxa contribution of 72.4% in the surface layers (0-0.5 cm) and 79.8% in the deeper layers (0.5-3 cm). Nematodes (60.0%, 95.4%, respectively in 0-0.5 cm and 0.5-3 cm) and copepods (29.5% in 0-0.5 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±23.8%) followed by Cletodidae (28.2±2.1%) and Miraciidae (20.1±16.9%). The remaining families accounted for 11.6±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≤0.01).

In anoxic samples (Fig. 5B), Cletodidae showed the highest relative abundance (47.0±22.4%) followed by Ectinosomatidae (37.0±24.4%) and Laophontidae (6.3±7.9%). The remaining families (9.7±9.6%) were, in order of decreasing abundance: Miraciidae, Ameiridae, Thalestridae, Longipediidae and Tisbidae. Moreover, Cletodidae represented up to 61.7±37.5% and Ectinosomatidae showed a maximum relative contribution of 52.8±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).

The MDS plot (stress=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 (0-0.5, 0.5-1 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 (0-0.5, 0.5-1 cm) and 46.7% in the deeper layers (1-3 cm). Ectinosomatidae (44.9% and 41.5% in 0-0.5, 0.5-1 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 (N0) in normoxia and, to a much lesser extent, in anoxia. The decreasing Ninf (Table 1A) values in deeper sediment layers illustrate that the dominance level increased with depth.

For harpacticoid copepods, no significant difference in diversity (N0, 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 T0 was dominated by Cletodidae (37.8±12.1%), followed by Laophontidae (18.3±2.4%) and Miraciidae (17.1±6.3%). The remaining families represented 26.8±4.5% and included, in order of decreasing abundance: Longipediidae, Ectinosomatidae, Ameiridae, Thalestridae, Tisbidae and Tetragonicipitidae. Copepod family diversity was within the range of the diversity levels recorded for the field experiment (Table 1): N0= 6.8 ±1.0, Ninf = 3 ±0.8, H’ (loge) = 1.7 ± 1.0. The higher Ninf is explained by the lower level of dominance of Cletodidae in comparison to their contribution in the field experiment (collected a year earlier).The induction of anoxia yielded a significant decrease of the oxygen levels in the overlying water (one-way ANOVA, p<0.00001) from initial 6.6 ± 0.2 mg/l (T1N) and 6.4 ± 0.06 mg/l (T2N) to 0.58 ± 0.29 mg/l (T2A) after 7 days of closure of the core, independent of the addition of extra diatoms or not.

Chlorophyll a (Chla) concentrations were measured at time T1 and T2 and ranged between 0 and 90 μg/g. The addition of diatoms had a highly significant effect on Chl *a* values (p<0.0001) as samples without additional diatoms had <5 μg/g Chl *a* while treatments with extra diatoms had >35 μg/g Chl *a*. Because of the high variance in Chl *a* concentrations in treatments with additional diatoms (83.9 ± 52.2 µg/g (T1ND), 69.8 ± 33.3 µg/g (T2ND), 36.6 ± 24.0 µg/g (T2AD)), there was no significant difference in Chla between the different time intervals, T1 and T2, (one-way ANOVA, p=0.35). Another pigment, Chlorophyll *c2* (Chl *c2*), showed similar patterns as Chl *a* with max. 3.5 ± 4.2 µg/g (T1N) in treatments without diatoms and up to 8.7 ± 7.2 µg/g after adding diatoms (T2ND). In terms of carotenoids, the concentration of fucoxanthin ranged between 1.3 ± 0.1 µg/g and 2.0 ± 0.4 µg/g without additional diatoms and between 18.2 ±19.2 µg/g (T2AD) and 39.7 ± 24.8 µg/g (T2ND) in treatments with extra diatoms.

**3.2.1 Survival rate**

The survival rate of harpacticoid copepods was significantly higher in normoxic conditions (93.2 ± 3.7%) than in anoxic conditions (73.6 ± 16.2%) at T2 (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±14.5% in contrast to 86.9±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 (T2AD) had a lower survival rate (66.9±5.8%) than the one without extra diatoms (80.3±21.5%, T2A). In combination with the effect of the anoxic condition, the former (T2AD) had a significantly lower survival rate than the oxic treatments with or without extra diatoms (T2ND: 93.0±2.0%, T2N: 93.5±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 (T2N, T2ND).

**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 T1ND (307.6±42.0 µg/ml) and T2AD (966.1±272.2 µg/ml). 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 (T2A, T2AD, 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 δ13C±stdev from -22.4±1.4‰ (T1N) to 276.9±192.8‰ (T1ND) (Δδ13C=299.2±192.8‰) . In the normoxic treatments, a significant increase of Δδ13C from T1ND (299.2±192.8‰) to T2ND (1281.5±667.6‰) was recorded (one-way ANOVA, p=0.03), indicating continuous feeding in normoxia (Fig. 7). In the anoxic treatments, food uptake ceased, with Δδ13C values showing no significant difference between T1ND (299.2±192.8‰) and T2AD (138.6±43.0‰) (one-way ANOVA, p=0.16). Consequently, the Δδ13C value differed significantly between normoxic and anoxic treatment (one-way ANOVA, p=0.014).

**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) – decreased in density but 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 50x50x50 cm volume off from the surrounding environment (Stachowitsch et al., 2007), 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). As this set-up caused a total cut-off of the food supply, eutrophication as an important factor in creating anoxia (Gray et al., 2002) was however neglected. 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., this volume).

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. This difference in response can be due to phylogenetic constraints and lifestyle (Wetzel et al., 2001). Typically, a low oxygen demand in combination with a high surface:volume ratio 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., this volume) 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.

At lower taxonomic level, there was a clear effect of anoxia on 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 differential resistance of harpacticoid copepod families to anoxia (but 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.

* 1. **4.2 Lab experiment**

The lab experiment was conducted to get a better insight into the functional responses of harpacticoid copepods to anoxia. The initial copepod community (at T0) differed slight from the one reported in the field experiment (see before, collected a year before). The community was dominated by the family Cletodidae, Laophontidae and Miraciidae but a lower share of Ectinosomatidae was found while they dominated in the normoxic cores of the field experiment. This can be explained by interannual variability in the benthic communities. However, the samples collected the year afterwards by Grego et al (2013b, this volume) were also dominated by the family Cletodidae. Cletodidae were also found to dominate in the anoxic cores of the field experiment in the present study. Next to interannual variability, the underestimation of the family Ectinosomatidae can also be due to the extraction via decantation and not by menas of centrifugation with Ludox (see field experiment). Since we wanted to use the live copepods, i.e. the individuals that survived the lab experimental treatment, the use of Ludox was not an option. Species of the family Ectinosomatidae are often rather small and closely associated with sediment grains, it is plausible that they were not sufficiently extracted by the decantation method. In spite of these small differences in copepod family composition, there were no major changes in the overall diversity as the average number of copepod families in the T0 cors (6.8 ±1.0) falls within the ranges reported for the normoxic (7.7 ± 0.6) and anoxic cores (6.0 ± 0.0) of the field experiment.

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 (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 (13C) 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 in low oxygen concentrations (Gray et al., 2002), the cores with additional diatoms (T2AD) were expected to have lower DO concentrations and consequently lower survival rates than those without diatoms. This was however 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 ± 0.29 mg/l (= 0.41 ± 0.20 ml/l). This oxygen level can be ranked as medium severe hypoxia because it is just ≤0.5 ml/l (Riedel, unpubl. data), whereas in the field experiment it dropped down to 0 ml/l.

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.

1. **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 situ* experiment. 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 year) have been done but focused on foraminiferans (Langlet et al., this volume) and sediment geochemistry (Koron et al., this volume; Metzger et al., this volume). 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, for copepods 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.

**Acknowledgements**

The research was conducted in the framework of the Austrian Science Fund (FWF) project P21542-B17 entitled 'Low dissolved oxygen events in the Northern Adriatic: *in situ* experimental insights into benthic responses before, during and post-anoxia'. Additional financial support was received from the Marine Biology Section at the Ghent University. The first author is a postdoctoral researcher at the Ghent University (BOF-GOA 01GA1911W). Special thanks to Ir. Dirk Van Gansbeke (Marine Biology, UGent) for the fatty acid analyses. We thank M. Stachowitsch, M. Zuschin, I. Gallmetzer and A. Haselmair for technical and diving support. Special thanks go to the director and staff of the Marine Biology Station (MBS) in Piran, Slovenia, for their hospitality and support during our work. The authors wish to thank the three referees for their valuable remarks that helped us to improve an earlier version of the manuscript.

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Table 1.Average Hill’s diversity indices (± stdev) for (A) meiofauna taxa and (B) copepod family composition in the field experiment. N0 = number of taxa. Ninf = dominance index, H’(loge)= Σ Pi2 log(Pi); N= Normoxia, A= Anoxia; sediment depth layers indicated in cm. Averaged values (± stdev) over all depth layers.

(A)

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Depth (cm) | **N0** | **Ninf** | **H'(loge)** | **N0** | **Ninf** | **H'(loge)** |
|  | **Normoxia** | | | **Anoxia** | | |
| **0-0.5** | 6.3±0.6 | 1.9±0.1 | 1.1±0.1 | 5.3±1.0 | 1.6±0.4 | 0.9±0.3 |
| **0.5-1** | 4.3±1.5 | 1.2±0.1 | 0.5±0.3 | 4.3±0.6 | 1.2±0.1 | 0.5±0.2 |
| **1-1.5** | 4.0±0.0 | 1.1±0.0 | 0.2±0.3 | 5.0±0.0 | 1.1±0.0 | 0.4±0.0 |
| **1.5-2** | 3.7±0.6 | 1.0±0.0 | 0.2±0.0 | 4.3±0.6 | 1.0±0.0 | 0.2±0.2 |
| **2-3** | 4.3±0.6 | 1.0±0.0 | 0.2±0.0 | 4.7±1.2 | 1.1±0.0 | 0.2±0.1 |
| **overall** | 4.5±1.2 | 1.2±0.4 | 0.4±0.3 | 4.7±0.7 | 1.2±0.2 | 0.4±0.2 |

(B)

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Depth (cm) | **N0** | **Ninf** | **H'(loge)** | **N0** | **Ninf** | **H'(loge)** |
|  | **Normoxia** | | | **Anoxia** | | |
| **0-0.5** | 7.7±0.6 | 2.6±0.5 | 1.5±0.1 | 6.0±0.0 | 2.0±0.2 | 1.1±0.1 |
| **0.5-1** | 3.7±1.5 | 1.6±0.4 | 0.9±0.4 | 4.3±0.6 | 1.8±0.4 | 1.0±0.1 |
| **1-1.5** | 3.7±1.2 | 2.2±0.5 | 1.1±0.2 | 3.0±0.0 | 2.1±0.2 | 1.0±0.1 |
| **1.5-2** | 2.0±1.0 | 1.4±0.4 | 0.5±0.5 | 2.3±1.2 | 1.6±0.5 | 0.7±0.6 |
| **2-3** | 3.0±1.0 | 2.3±0.6 | 1.0±0.3 | 2.3±1.2 | 1.7±0.6 | 0.7±0.6 |
| **overall** | 4.0±2.2 | 2.0±0.6 | 1.0±0.4 | 3.6±1.6 | 1.8±0.4 | 0.9±0.4 |

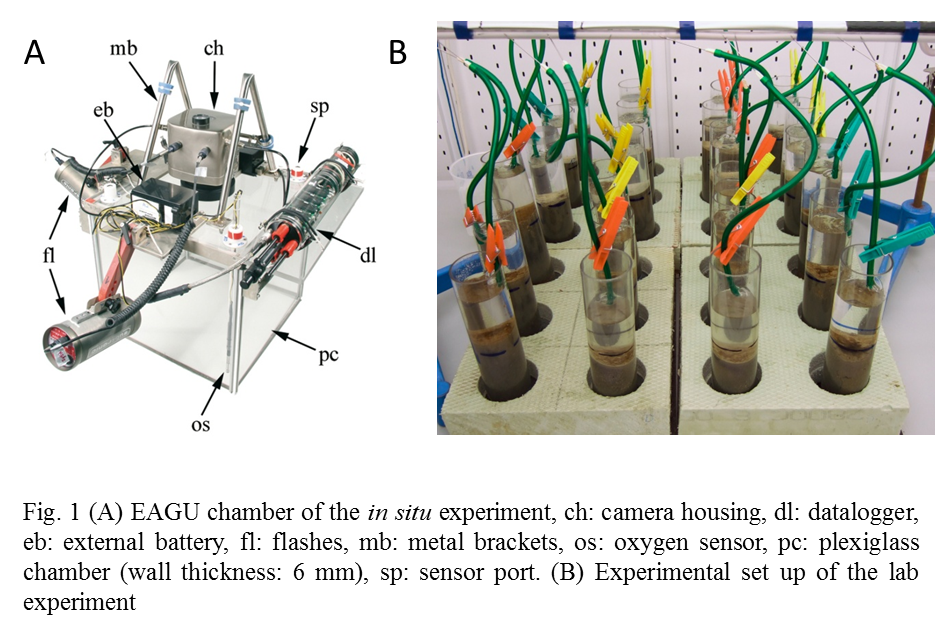


Figure 1. Experimental set up of the lab experiment before induction of anoxia (T1)

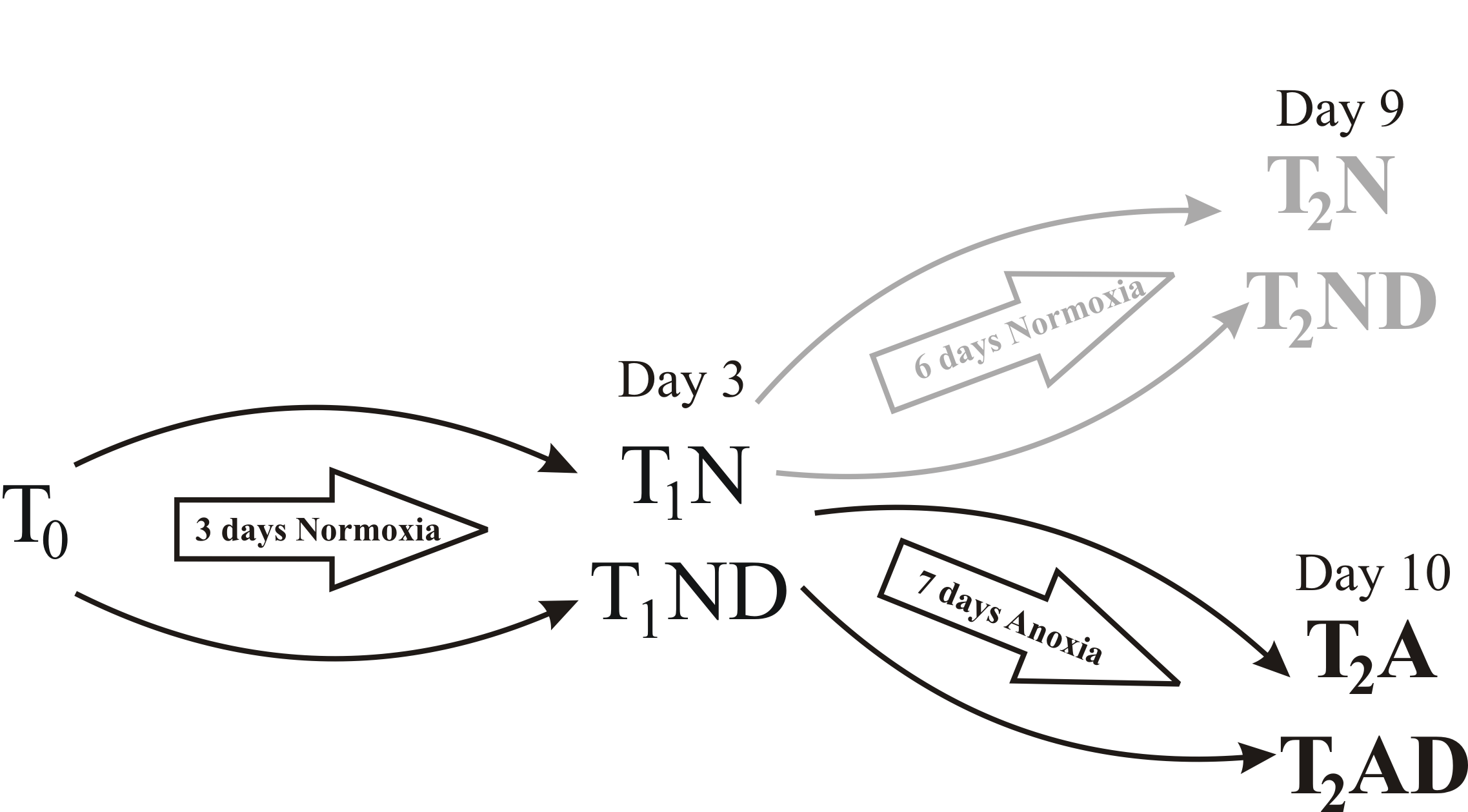


Figure 2. Experimental design: T0: start of the experiment, T1: 3 days, T2: after 6 or 7 days (i.e. 9 or 10 days in total), N: Normoxia, A: Anoxia, D: Diatoms added

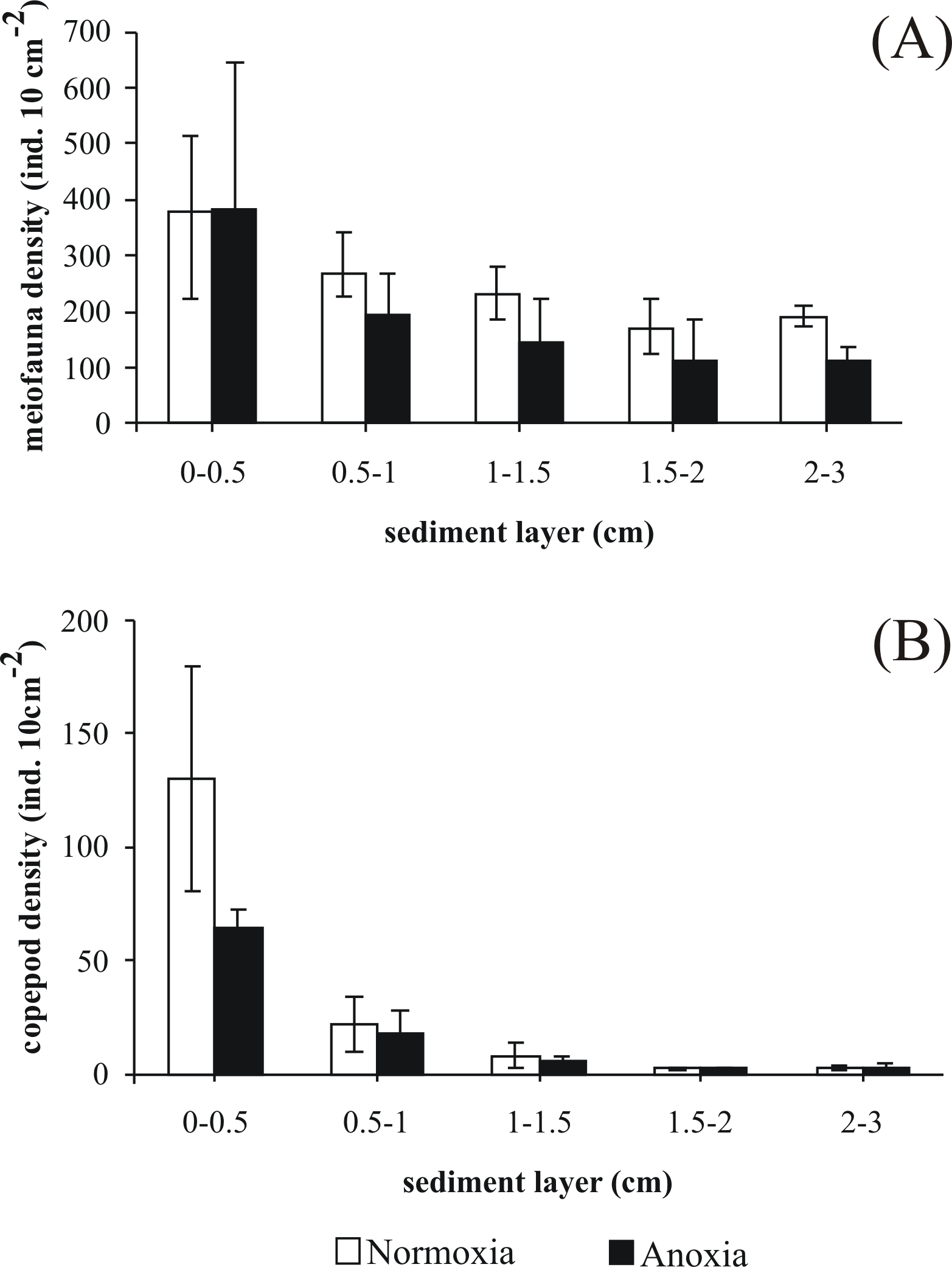


Figure 3. Average (± STDEV) total densities of (A) meiofauna and (B) copepods in normoxic and anoxic field conditions.

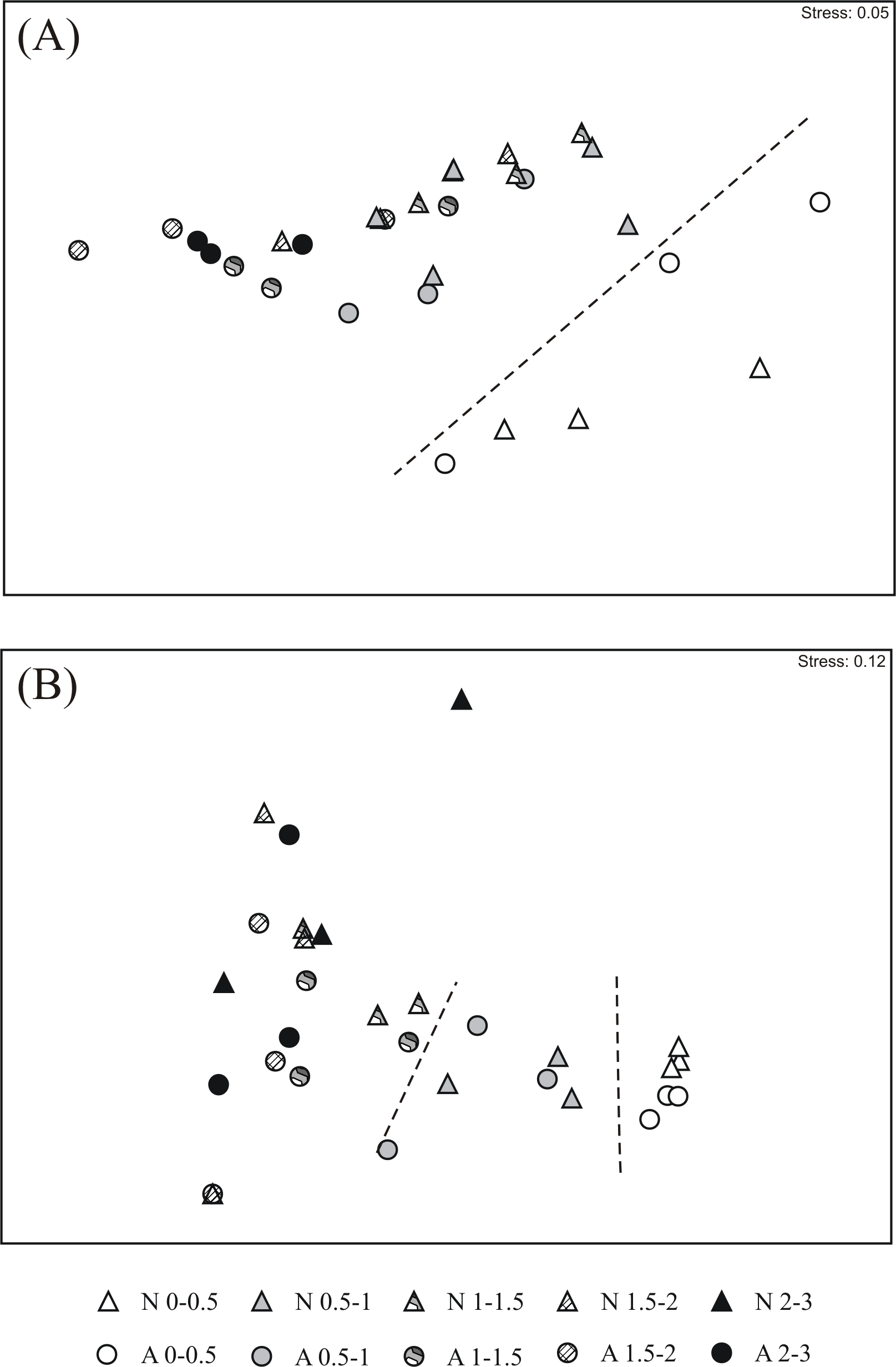


Figure 4. MDS of relative (A) meiofauna and (B) copepod community composition based on Bray-Curtis similarity: normoxia (N) and anoxia (A), sediment depth layers indicated in cm.

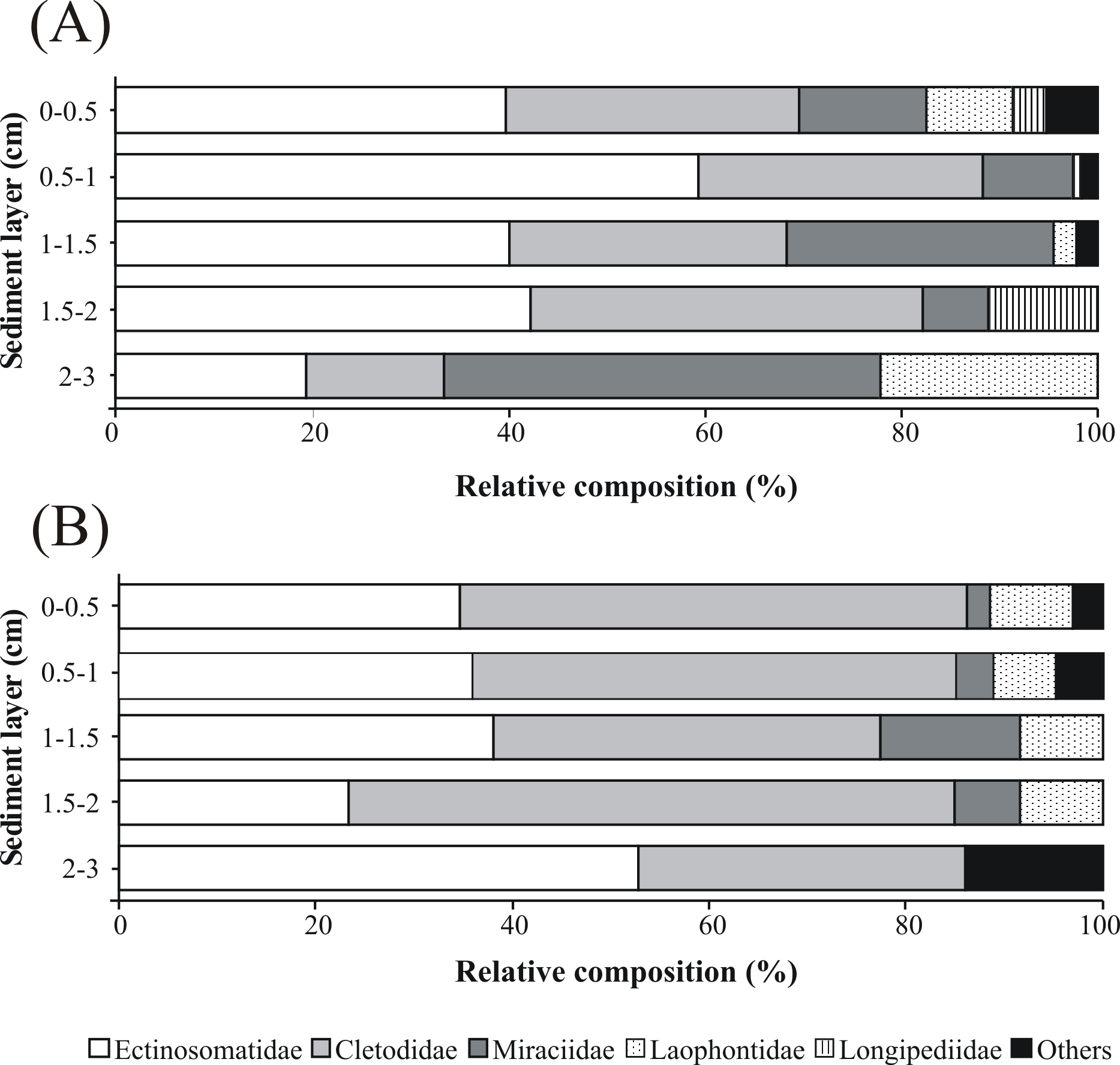


Figure 5. Relative composition of the harpacticoid copepod families: vertical distribution in (A) normoxic and (B) anoxic conditions.

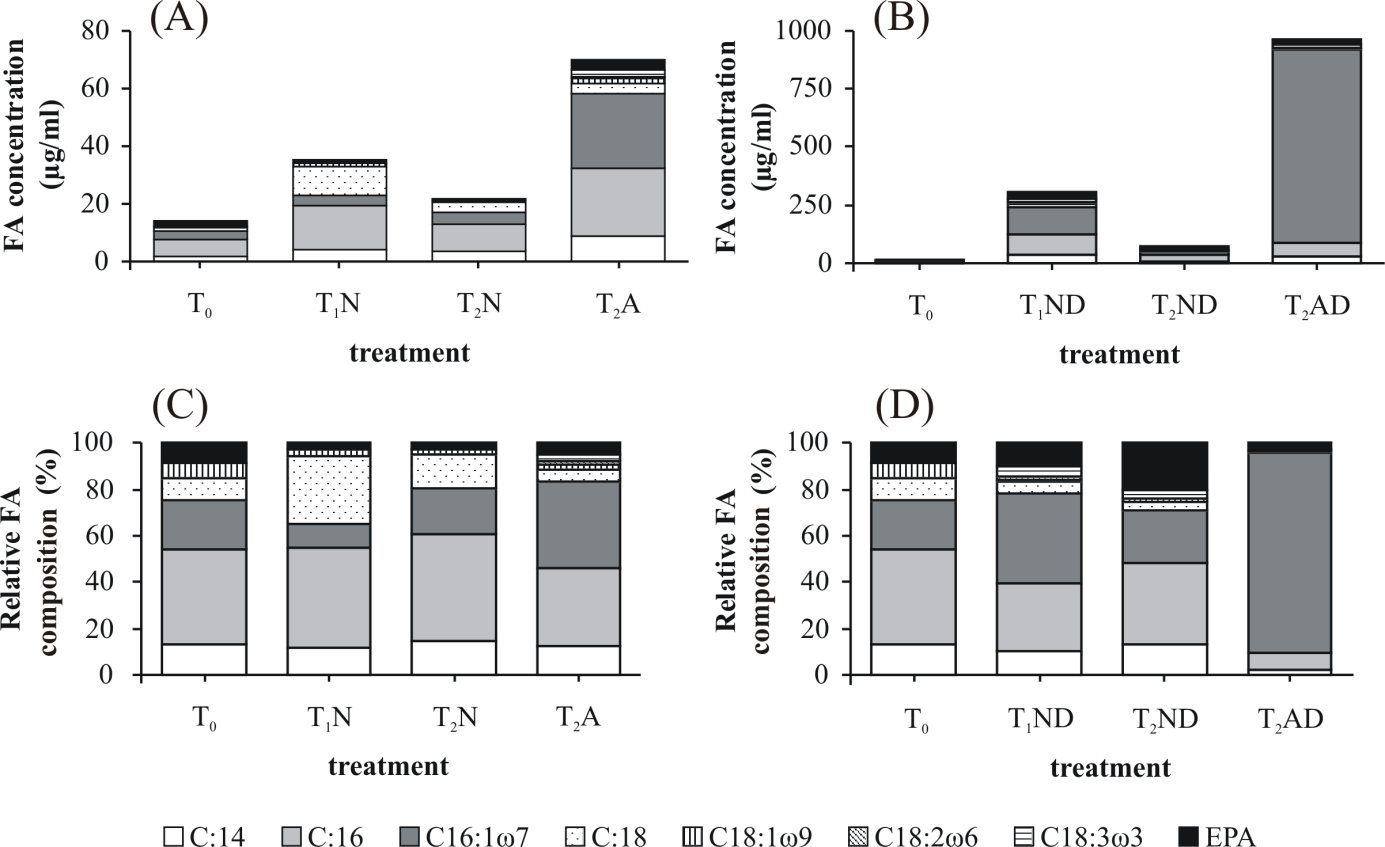


Figure 6. Absolute and relative fatty acid composition of the sediment (0-3 cm) for treatments without extra diatoms (A, C) and for treatments with diatoms (B, D).

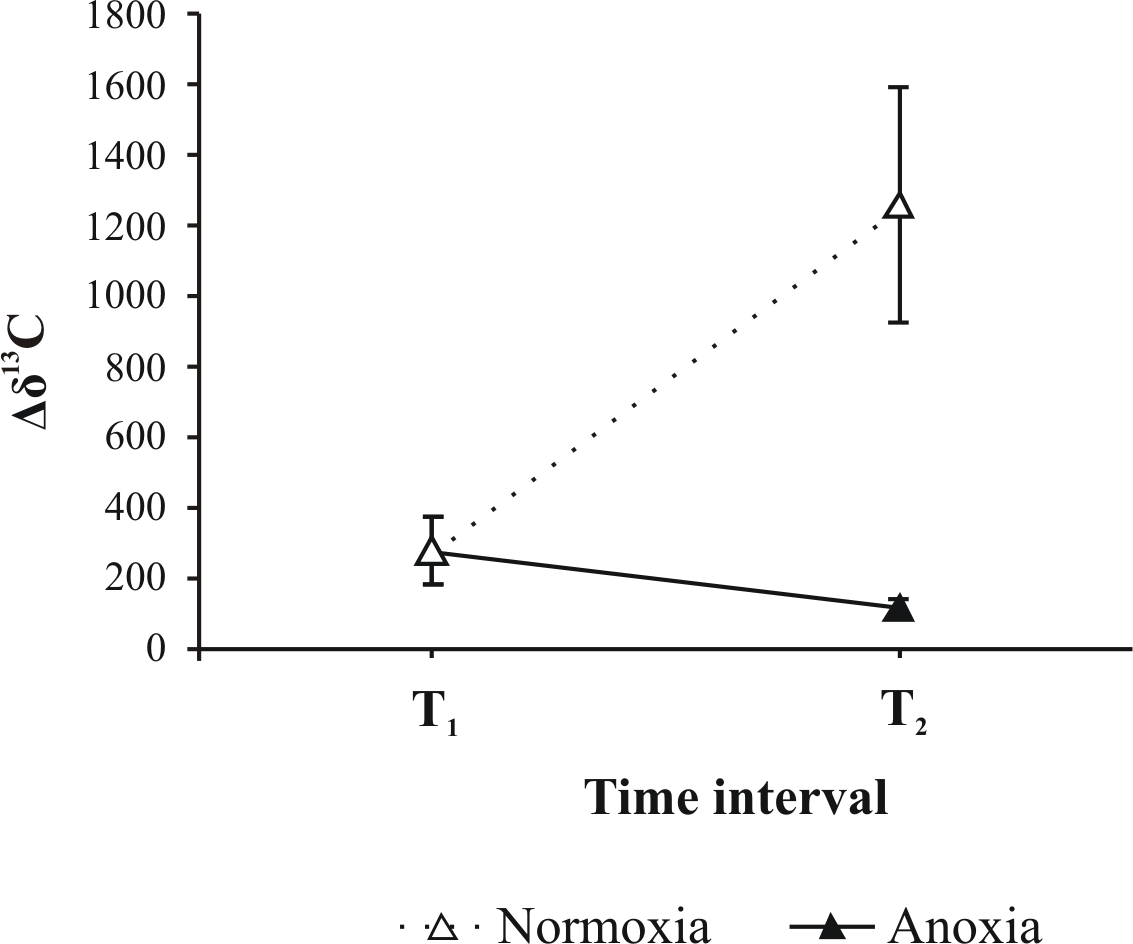


Figure 7. Average (± STDEV) diatom uptake by harpacticoid copepods under normoxic and anoxic conditions at two time intervals (T1, T2) expressed as specific uptake (Δδ13C). T2 represents 6 or 7 additional days in normoxic or anoxic conditions, respectively.