



Effect of reoxygenation and *Marenzelleria* spp. bioturbation on Baltic Sea sediment metabolism

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ABSTRACT: Nutrient reduction and the improvement of bottom water oxygen concentrations are thought to be key factors in the recovery of eutrophic aquatic ecosystems. The effects of reoxygenation and bioturbation of natural hypoxic sediments in the Baltic Sea were studied using a mesocosm experiment. Anoxic sediment box cores were collected from 100 m depth in Kanholmsfjärden (Stockholm Archipelago) and maintained in flow-through mesocosms with 3 treatments: (1) hypoxic: supplied with hypoxic water; (2) normoxic: supplied with oxic water; and (3) *Marenzelleria*: supplied with oxic water and the polychaete *Marenzelleria* spp. (2000 ind. m⁻²). After a 7 wk long conditioning period, net fluxes of dissolved O₂, CH₄, Fe²⁺, Mn²⁺, NH₄⁺, NO₂⁻, NO₃⁻, PO₄³⁻ and H₄SiO₄, and rates of nitrate ammonification (DNRA), denitrification and anammox were determined. Phosphate was taken up by the sediment in all treatments, and the uptake was highest in the normoxic treatment with *Marenzelleria*. Normoxic conditions stimulated the denitrification rate by a factor of 5. Denitrification efficiency was highest under normoxia (50%), intermediate in bioturbated sediments (16%), and very low in hypoxic sediments (4%). The shift from hypoxic to normoxic conditions resulted in a significantly higher retention of NH₄⁺, H₄SiO₄ and Mn²⁺ in the sediment, but the bioturbation by *Marenzelleria* reversed this effect. Results from our study suggest that bioturbation by *Marenzelleria* stimulates the exchange of solutes between sediment and bottom water through irrigation and enhances bacterial sulfate reduction in the burrow walls. The latter may have a toxic effect on nitrifying bacteria, which, in turn, suppresses denitrification rates.

KEY WORDS: Hypoxia · Macrofauna · Mesocosm · Denitrification · Dissimilatory nitrate reduction to ammonium · DNRA · Benthic Flux · Baltic Sea

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INTRODUCTION

Large zones with anoxic or hypoxic sediments are common in many eutrophic coastal areas with stratified waters or low water circulation (Rabalais et al. 2010). Hypoxia (O₂ < 60 μM) can be transient and local, with minor consequences at the ecosystem level, but it can also persist for long periods over large areas and can alter the structure of aquatic communities and energy transfer processes (Diaz &

Rosenberg 2008). Vast areas of the Baltic Sea suffer from bottom water hypoxia, mainly due to cultural eutrophication, the long residence time and thermal stratification of water masses (Conley et al. 2009). In sediments under hypoxic waters faunal aerobic respiration processes are suppressed and anaerobic mineralization pathways of organic carbon such as bacterial sulfate reduction, methanogenesis, and reduction of manganese and iron oxyhydroxides prevail. The dominance of anaerobic bacterial pro-

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cesses also profoundly alters the pathways and stoichiometry of nutrient cycling. For example, the lack of oxygen and high concentrations of sulfide inhibit nitrification within the sediment (Burgin & Hamilton 2007), suppressing denitrification rates and resulting in low N_2 loss rates. High iron reduction rates and the formation of iron sulfides, in turn, stimulate the phosphate loss from anoxic sediments (Reed et al. 2011). Changes in benthic oxygenation therefore not only alter rates and pathways of carbon mineralization, but also change the rates and stoichiometry of recycled nitrogen and phosphorus, which can have significant implications for primary producer communities and contribute to shifts from calcareous or diatom-dominated to cyanobacterial bloom-dominated primary production (Elmgren 2001, Vahtera et al. 2007).

Bottom water hypoxia or anoxia affect the macrofaunal community and reduce sediment bioirrigation and bioturbation (Gray et al. 2002, Karlson et al. 2002, Glud 2008, Gambi et al. 2009). The bioturbation activities of benthic macrofauna, i.e. the biological mixing and transport of particles and solutes in the upper sediment layer, are known to profoundly affect essential ecosystem functions of benthic ecosystems, including biogeochemical cycling, carbon storage and various chemical and microbial processes, as well as physical characteristics such as porosity and sediment surface topography (Kristensen et al. 1992, Aller & Aller 1998, Schiffrers et al. 2011). Bioirrigating benthic macrofauna enhance the oxygenation of sediments and increase the availability of electron acceptors such as oxygen, NO_3^- , Mn and Fe oxides. Bioturbation also leads to a more efficient decomposition of buried organic matter, which renders refractory organic matter more labile and decreases the rates of anaerobic carbon mineralization processes (Kristensen & Kostka 2005). In many sediments of the Baltic Sea the abundance of burrowing macrofauna has decreased as a consequence of spreading hypoxia and sulfidic conditions (Gray et al. 2002). During the last 2 decades, however, invading polychaete species of the genus *Marenzelleria* have colonized large sediment areas of the Baltic Sea, at densities ranging from several 100 to several 1000 ind. m^{-2} , making it one of the dominating macrofaunal species of the Baltic Sea (Zettler et al. 2002).

The new *Marenzelleria* spp. consist of 3 morphologically similar coexisting sibling species only distinguishable using genetic tools (Blank et al. 2008). The ecology and bioturbation mode of the 3 species are still largely unknown. Recent studies have, however, shown that *Marenzelleria* spp. is able to dig

deep burrows down to ca. 20 cm depth (Zettler et al. 1995) and that they are highly efficient bioirrigators (Hedman et al. 2011, Kristensen et al. 2011). Based on modeling data, Norkko et al. (2012) suggested that the colonization of the Baltic Sea sediment by *Marenzelleria* spp. could improve the quality of the benthic system through enhanced oxygenation and phosphorus retention in the sediment and thus counteract eutrophication.

We collected intact sediment box cores from a 100 m anoxic fjord within the Stockholm archipelago and carried out a long-term mesocosm experiment in order to examine the effects of (1) reoxygenation from hypoxic (<60 μM dissolved oxygen) to normoxic conditions (~300 μM dissolved oxygen) and (2) bioturbation by the polychaete *Marenzelleria* spp. on biogeochemical processes and benthic nutrient fluxes.

MATERIALS AND METHODS

Setup of mesocosms

Sediments (12 box cores, 20 × 20 × 30 cm high) were collected on June 7, 2011 at 105 m depth in Kanholmsfjärden (Stockholm archipelago, 59° 20.1814' N, 018° 46.2680' E), using a Jonasson-Olausson box corer (Fig. 1). Kanholmsfjärden has year-round anoxic or hypoxic bottom waters, and the sediment is typically black and sulfidic, with a porosity of 0.9. Total carbon content ($mmol g^{-1}$ dry weight) and molar C/N ratios of Kanholmsfjärden sediment in June 2011 were 5.7 and 9.7 from 0 to 3 cm depth, 5.6 and 10.6 from 3 to 6 cm, 5.1 and 10.0 from 6 to 10 cm, respectively (Ekeröth et al. unpubl. data). Bottom water was collected using a modified Niskin water sampler to measure temperature (4.7°C), salinity (8) and dissolved oxygen (<5 μM) using a digital multi-meter and the Winkler method for the latter. On board, sediments from each box core were transferred to Plexiglas mesocosms (20 × 20 × 50 cm high) and sealed with a bottom and a lid. The mesocosms were transported to the Askö Laboratory, Stockholm University Marine Research Center, filled to the top with hypoxic seawater and left for acclimatization during 48 d in a dark climate room at 5°C. The water column of the mesocosms was kept hypoxic (ca. 20 μM) during the whole conditioning phase.

After the conditioning phase, the mesocosms were immersed in a water bath with circulating, natural seawater to keep the temperature constant at 5°C. The mesocosms were then connected to peristaltic pumps and continuously circulated (ca. 15 $ml min^{-1}$)

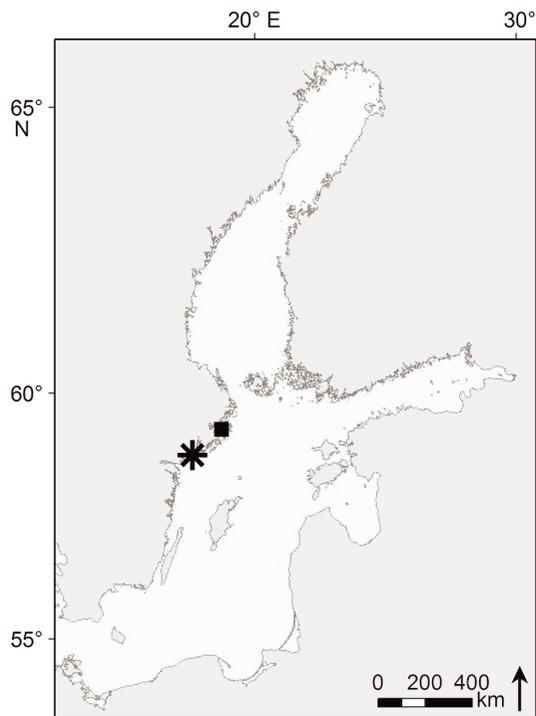


Fig. 1. The Baltic Sea, showing where the box cores and *Marenzelleria* spp. were collected (■), and the location of Stockholm University Marine Research Center at Askö, where the experiment was performed (✱)

with sand-filtered natural seawater pumped from 20 m depth. Of the mesocosms, 8 were supplied with oxic ($\sim 300 \mu\text{M O}_2$) and 4 with hypoxic seawater ($\sim 50 \mu\text{M O}_2$). The seawater was first circulated into 3 water towers (1.8 m high, 30 cm inner diameter). Of the towers, 1 was used to supply the mesocosms with oxic and the other 2, connected in series, were used to supply the mesocosms with hypoxic seawater. Hypoxic O_2 concentrations were maintained by bubbling the incoming tower with N_2 . The O_2 concentration was kept constant by using an optical O_2 sensor (dTRANS O2 01, JUMO) in the outgoing tower, connected to a digital unit that regulated the amount of N_2 bubbling in the incoming tower. The water inside all mesocosms was mixed by magnetic stirrers at 60 rpm. The mesocosms were supplied in flow-through conditions with either oxic ($n = 8$) or hypoxic water ($n = 4$) over 26 d, in order to start the reoxygenation of the surface sediment, after which 80 ind. of *Marenzelleria* spp., corresponding to a density of 2000 ind. m^{-2} were added to 4 of the oxic mesocosms. The polychaetes were collected on September 9, 2011 in Kanholmsfjärden ($59^\circ 20.3701' \text{N}$, $018^\circ 45.3815' \text{E}$) near the location where the box cores were taken, but in oxic waters at a shallower depth (55 m; Fig. 1). Following addition of the polychaetes, the mesocosms were kept

in flow-through conditions for another 48 d until the end of the experiment. In total, the experiment included 3 different treatments with 4 replicate mesocosms each: (1) hypoxic water (HY), (2) normoxic water (NO) and (3) normoxic and bioturbated, i.e. normoxic with *Marenzelleria* spp. (NOB).

Collection of intact sediment cores from mesocosms and flux measurements

After 48 d, sediments from each mesocosm were subsampled by inserting Plexiglas tubes (25 cm length, 3.6 cm inner diameter, $n = 3$) in each mesocosm. The cores were capped with rubber stoppers, gently extracted and then capped below. A total of 36 cores (12 replicates for each treatment) were collected; all cores were adjusted to contain 12 cm of sediment and 10 cm of water phase. Cores from the same treatment were then transferred to a temperature-controlled room at 5°C and placed in incubation buckets containing water taken from each respective mesocosm. The cores were submerged, the top cap was removed and stirrer bars, driven by an external magnet at 60 rpm, were inserted to the water phase of each core. The cores were then maintained for 6 h before the incubation started. Water in the buckets containing the NO and NOB cores was bubbled with air and mixed with aquarium pumps, while the bucket with HY cores was bubbled with a mixture of air and N_2 , maintaining an O_2 concentration of $\sim 50 \mu\text{M}$.

At the beginning of the incubation, water samples (ca. 50 ml) were collected from the buckets ($n = 5$ for each tank) with plastic syringes, and all cores were closed with rubber stoppers and stirred. An incubation period of 8 h was selected in order to keep the final O_2 concentration to within 20% of the initial value. At the end of the incubation an additional water sample (ca. 50 ml) was collected from each core. Immediately after water sampling, 1 aliquot was transferred to 7.7 ml Exetainers (Labco Scientific) and poisoned with $50 \mu\text{l}$ of 7 M ZnCl_2 for CH_4 analysis; 1 aliquot was filtered (GF/F glass-fiber filters) and stored in plastic vials for dissolved NH_4^+ , NO_3^- , NO_2^- and H_4SiO_4 determination; 1 aliquot was filtered and transferred into glass vials for phosphate determination and 1 aliquot was filtered, transferred into glass vials and acidified with $50 \mu\text{l}$ of concentrated HNO_3^- for dissolved Mn and Fe analyses. O_2 concentrations before and after the incubation were measured directly in the tanks and in each core using a polarized pre-calibrated microelectrode (OX-500, Unisense). Samples for nutrients were frozen at -80°C and analyzed within 2 wk.

Net fluxes ($\mu\text{mol m}^{-2} \text{h}^{-1}$) of all measured solutes across the sediment–water interface were calculated according to the following equation:

$$\text{flux} = \frac{(C_f - C_i) \times V}{t \times A} \quad (1)$$

where C_f and C_i are the final and initial concentrations of the target solute (μM), V is the volume of the water phase in the core (l), t is the incubation time (h) and A is the surface of the core (m^2).

After the incubation for solute flux measurement, a second incubation was conducted to determine N_2 production via denitrification, and rates of anaerobic ammonium oxidation (anammox) and dissimilatory nitrate reduction to ammonium (DNRA). In order to simultaneously detect and quantify these 3 processes, ^{15}N -labeling experiments following the revised isotope pairing technique were carried out (Risgaard-Petersen et al. 2003). The 36 sediment cores were left submerged and uncapped, and preincubated again for 6 h following the procedure described above. After this period, each core was amended with labeled sodium nitrate ($\text{Na}^{15}\text{NO}_3$, 99 atomic%) to final concentrations of 5, 10, 25 and 50 μM $^{15}\text{NO}_3^-$ (3 replicates for each concentration). The sediment cores were then sealed again with rubber stoppers and incubated in the dark for 8 h with the stirring system on. At the end of the incubation all the cores were gently mixed to slurry. Subsamples of the slurries were collected with plastic syringes protected with a plastic net and Viton tubing to avoid collection of *Marenzelleria* individuals, and transferred into 7.7 ml Exetainers to which 100 μl 7 M ZnCl_2 was added to stop bacterial activity. An additional 30 ml of each slurry was transferred into each 50 ml centrifuge tube from the cores incubated with 25 and 50 μM $^{15}\text{NO}_3^-$ concentrations ($n = 6$ for each treatment), and immediately frozen at -80°C for later determination of the $^{15}\text{NH}_4^+$ fraction in the ammonium pool. All the remaining slurries from NOB cores were finally sieved through a 500 μm mesh net to recover all polychaetes and determine their density and biomass. Wet weight (WW) and dry weights (DW) were measured using a laboratory balance (MS105, Mettler Toledo).

Oxygen consumption by *Marenzelleria*

A variable number (1 to 3) of *Marenzelleria* spp. individuals, together with water filtered through GF/F glass-fiber filters, were collected from NOB mesocosms and placed in 7.7 ml Exetainers ($n = 10$),

taking care to avoid the inclusion of air bubbles. A Clark-type oxygen microelectrode was used for measuring O_2 concentrations every 2 h for an incubation period of 10 h, during which the vials were maintained at the temperature of the sediment incubation. Oxygen concentrations measured after 4 h of incubation were discarded as they decreased by $>20\%$ of the initial values. A micrometer manipulator was used to introduce the electrode in the vials in order to avoid that the sample would warm up during the measurement. At the end of the incubation, polychaetes from each Exetainer were recovered, and dried at 60°C to constant weight. Oxygen consumption was then calculated as a function of DW (g).

Oxygen, sulfide and pH microelectrode profiles

For each treatment (HY, NO and NOB), 3 to 6 microprofiles of dissolved oxygen, H_2S and pH were measured in order to determine the depth distribution of dissolved oxygen, total dissolved sulfide and pH. Profiles were determined simultaneously directly inside the mesocosms, using a manual micro-manipulator (Unisense) and microsensors with a tip diameter of 50 μm (OX-50, H_2S -50, pH-50; Unisense). O_2 microelectrodes were calibrated each day at O_2 saturation and under anoxic conditions using 2-point calibrations according to the manufacturer's recommendation. H_2S microelectrodes were calibrated daily in fresh Na_2S solutions that were prepared every day with washed and cleaned Na_2S crystals in anoxic water and calibrated using the methylene blue method of Cline (1969). pH microelectrodes were calibrated with pH standards of 4 and 7. Total dissolved sulfide was calculated from the measurement pairs of H_2S and pH for each depth using the first and second dissociation constants of $\text{H}_2\text{S}/\text{HS}^-$ and $\text{HS}^-/\text{S}^{2-}$.

Laboratory analyses and rate calculations

Inorganic nutrients (NH_4^+ , NO_3^- , NO_2^- , total dissolved inorganic PO_4^{3-} and H_4SiO_4) were determined spectrophotometrically on a segmented flow nutrient analyzer system (ALPKEM, Flow Solution IV). Precision was ± 0.036 μM for NH_4^+ , ± 0.021 μM for NO_3^- , ± 0.014 μM for NO_2^- , ± 0.016 for PO_4^{3-} and ± 0.036 for H_4SiO_4 . Fe^{2+} and Mn^{2+} concentrations were determined using a Varian AA240FS fast sequential atomic absorption spectrometer. The precision of the metal analysis was ± 0.3 μM . CH_4 was determined by

headspace analysis with a Fisons 9000 gas chromatograph equipped with a flame ionization detector (precision was ± 1 ppb).

In order to calculate DNRA rates, the total ammonium pool in the slurries was extracted with 2 M KCl. An aliquot of the extract was analyzed spectrophotometrically, while another aliquot was treated with a hypobromite-iodine solution to oxidize ammonium to N_2 (Risgaard-Petersen et al. 1995). The abundance of $^{29}N_2$ and $^{30}N_2$ of the N_2 obtained from the oxidation of NH_4^+ with hypobromite-iodine and of the N_2 pool produced by denitrification were determined on an isotope ratio mass spectrometer (Delta V Advantage, Thermo Scientific) coupled via an open-split interface (Conflo IV, Thermo Scientific) to a separation inlet system equipped with a freeze trap, a reduction oven and a packed gas chromatography column (Holtappels et al. 2011).

Co-occurrence of denitrification and anammox was evaluated by plotting the calculated $^{28}N_2$ production as a function of the added ^{15}N -nitrate. The calculation of *in situ* denitrification rates, nitrification coupled denitrification (D_n) and water column nitrate-driven denitrification (D_w) followed the rationale and the equations reported in Nielsen (1992). Rates of DNRA were calculated following Risgaard-Petersen & Rysgaard (1995), and *in situ* rates distinguished between DNRA based on nitrate diffusion from the water column (DNRA_w) and DNRA coupled to sediment nitrification (DNRA_n).

Statistical analyses

Differences between net solute fluxes calculated for each treatment (HY, NO and NOB) were tested using 1-way analysis of variance (ANOVA). Most measured rates were not normally distributed, even after data transformation, and the non-parametric Kruskal-Wallis test was used. The parametric test was used only for D_w rates and PO_4^{3-} fluxes that fulfilled the ANOVA assumptions. Pairwise post hoc comparisons between the 3 treatments (HY, NO and NOB) were done with the Tukey test. The slopes of the regression between $p^{29}N_2$ and $p^{30}N_2$ and the amount of added $^{15}NO_3^-$ in the 3 treatments were compared by means of the analysis of covariance (ANCOVA). Correlations between macrofaunal biomass and solute fluxes were tested using Pearson's correlation coefficient (r), with significant differences set at $p < 0.05$. Statistical analyses were performed with R statistical package. Mean values are reported with associated standard errors.

RESULTS

Respiration-related fluxes, denitrification and DNRA rates

The main physicochemical parameters measured in the mesocosms before the incubation began and full statistical results are reported in Appendix 1 (Tables A1 to A4). Solute fluxes measured in the HY and NO treatments had a similar variability (mean coefficients of variation [CV] for all calculated fluxes of 0.71 and 0.70). Fluxes in NOB sediments were more variable (mean CV = 1.52), which can be attributed to the variable number of polychaetes in each core. *Marenzelleria* individuals in the cores varied between 0 and 7, with an average density of 2041 ± 545 ind. m^{-2} , corresponding to an average dry weight (DW) of 7.67 ± 2.50 g DW m^{-2} .

In the HY treatments dissolved O_2 penetrated to ~ 0.45 mm, while in the NO and NOB treatments O_2 penetration was ~ 2 and 2.5 mm, respectively (Fig. 2). Total sediment oxygen uptake was lower in the HY (-61 ± 11 $\mu\text{mol } m^{-2} \text{ h}^{-1}$) than in the NO treatment (-209 ± 17 $\mu\text{mol } O_2 \text{ m}^{-2} \text{ h}^{-1}$) (Fig. 3). Bioirrigation by *Marenzelleria* significantly increased benthic respiration (-441 ± 64 $\mu\text{mol } O_2 \text{ m}^{-2} \text{ h}^{-1}$) (Kruskal-Wallis ANOVA, $H_{2,33} = 29.13$, $p < 0.001$) (Fig. 3). In the NOB treatment there was a highly significant and positive linear correlation between sediment oxygen demand and the dry weight of *Marenzelleria* ($y = -22.21x - 271.02$, $r = 0.86$, $p < 0.001$). The respiration of the polychaetes alone, calculated from the incubation of *Marenzelleria* individuals in filtered water, was 20.7 $\mu\text{mol } O_2 \text{ g}^{-1} \text{ DW } \text{h}^{-1}$. Multiplication by the average biomass of *Marenzelleria* in NOB cores yielded a corresponding rate of 158.7 ± 51.8 $\mu\text{mol } m^{-2} \text{ h}^{-1}$, a contribution of about 36% to total sediment respiration. Surface sediment contribution to total respiration (47%) was considered equivalent to that measured in NO cores. The contribution of burrow structures (17%) was calculated by subtraction of *Marenzelleria* respiration and NO core respiration from total benthic respiration measured in NOB cores.

Dissolved sulfide was detectable at 0.5, 2.8 and 2.0 mm depth in the HY, NO and NOB treatments, respectively (Fig. 2). The depth of sulfide appearance overlapped with the presence of *Marenzelleria*, indicating their sulfide tolerance (Schneider 1996). From visual inspections it was evident that the polychaetes burrowed down to 7–8 cm in the sediment. Net fluxes of dissolved iron and methane were low and insignificantly different in the 3 treatments (Kruskal-Wallis ANOVA, $H_{2,33} = 5.30$ and 2.02, respectively, $p > 0.05$)

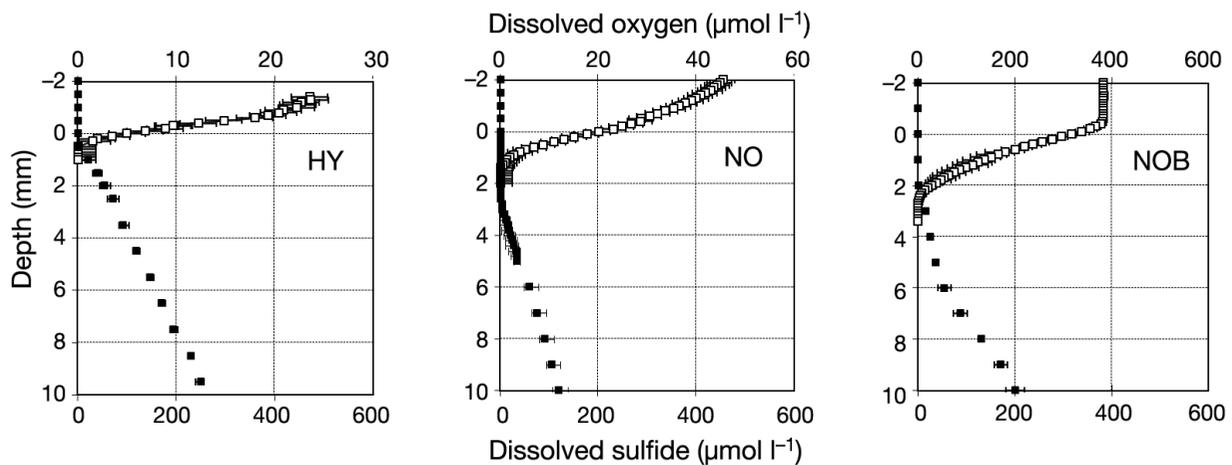


Fig. 2. Microelectrode profiles of dissolved oxygen (□) and total dissolved sulfide (■) for the hypoxic (HY), normoxic (NO) and normoxic-bioturbated (NOB) treatments. Means \pm SD, $n = 6$

(Fig. 3). There were positive correlations between these fluxes and the biomass of *Marenzelleria* ($y = 0.50x - 5.8$, $r = 0.92$, $p < 0.001$ and $y = 0.04x - 0.01$, $r = 0.68$, $p < 0.05$). Mn^{2+} was always released to the water

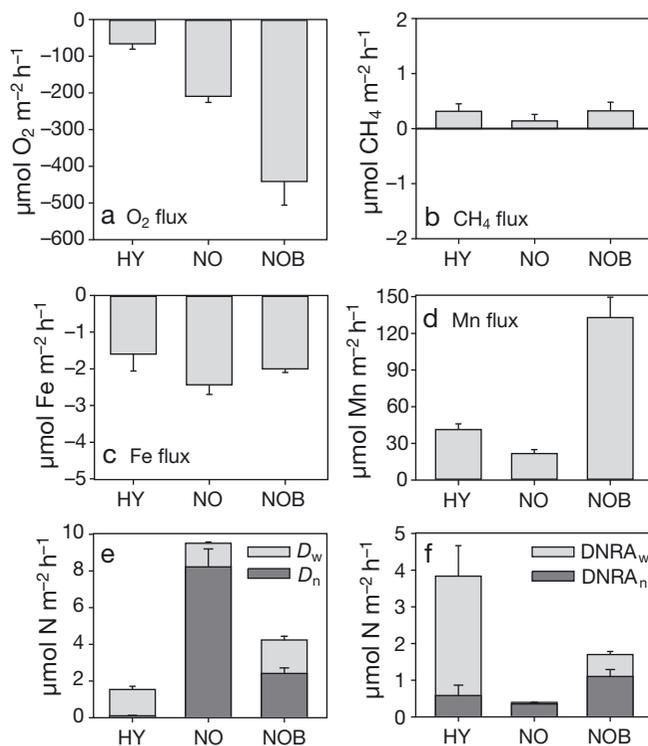


Fig. 3. Net fluxes of (a) oxygen, (b) methane, and dissolved (c) iron and (d) manganese at the sediment–water interface; (e) rates of nitrification-coupled (D_n) and of water column nitrate denitrification (D_w); (f) rates of dissimilatory nitrate reduction to ammonium coupled to nitrification (DNRA_n) and fueled by water column nitrate (DNRA_w) measured in intact sediment cores. Mean \pm SE, $n = 12$; positive fluxes are from the sediment to the water column

column. The highest flux was measured in the NOB treatment ($133 \pm 17 \mu\text{mol m}^{-2} \text{ h}^{-1}$) followed by the hypoxic ($41 \pm 5 \mu\text{mol m}^{-2} \text{ h}^{-1}$) and normoxic treatments ($22 \pm 3 \mu\text{mol m}^{-2} \text{ h}^{-1}$) (Kruskal-Wallis ANOVA, $H_{2,33} = 26.02$, $p < 0.001$, Tukey, $p < 0.05$) (Fig. 3). Evidently, bioturbation by the polychaete stimulated the release of Mn^{2+} , as suggested by the significant positive correlation between Mn^{2+} efflux and *Marenzelleria* biomass ($y = 5.96x + 87.18$, $r = 0.90$, $p < 0.001$).

There was no relationship between genuine N_2 production and the amount of added ^{15}N -nitrate, suggesting that anammox activity was insignificant (Risgaard-Petersen et al. 2003). Rates of denitrification were low in all treatments ($< 10 \mu\text{mol N m}^{-2} \text{ h}^{-1}$) compared to sediment oxygen uptake and net Mn^{2+} release (Fig. 3). Differences between the treatments were largely due to variations in the amount of D_n . The contribution of D_n to total denitrification was highest in NO (87%), intermediate in NOB (57%) and lowest in HY (7%) (8.2 ± 1.0 , 2.4 ± 0.3 , $0.1 \pm 0.0 \mu\text{mol m}^{-2} \text{ h}^{-1}$, respectively) (Fig. 3). *Marenzelleria* biomass and D_n were not correlated ($r = 0.09$, $p = 0.78$). D_w was comparable in the HY, NO and NOB treatments (1-way ANOVA, $F_{2,33} = 2.84$, $p = 0.07$). There was a positive correlation between the polychaete biomass and D_w , but the overall effect was quantitatively small ($y = 0.07x + 1.32$, $r = 0.80$, $p < 0.01$).

The combined analysis of $^{29}\text{N}_2$ and $^{30}\text{N}_2$ production relative to added $^{15}\text{NO}_3^-$ highlights some differences concerning nitrification and denitrification activity in the 3 experimental conditions (Fig. 4). Overall, there was a positive and significant correlation between the production (p) of $^{29}\text{N}_2$ and $^{30}\text{N}_2$ and the concentration of added $^{15}\text{NO}_3^-$ in all treatments, with HY as the only exception (t -test, $p > 0.05$). In NO, the slopes of the linear regression between $p^{29}\text{N}_2$ and $p^{30}\text{N}_2$ and the

$^{15}\text{NO}_3^-$ concentration were not statistically different (ANCOVA, $p = 0.735$). In NOB, the production of $^{30}\text{N}_2$ was more stimulated than the production of $^{29}\text{N}_2$ at increasing $^{15}\text{NO}_3^-$ concentrations (ANCOVA, $p < 0.001$).

DNRA was detected in all treatments, but rates were generally $< 4 \mu\text{mol N m}^{-2} \text{h}^{-1}$ (Fig. 3). Differences between the 3 treatments were significant and inversely proportional to denitrification rates: highest rates were found in the HY treatment, while lowest rates were measured in the NO treatment (Kruskal-Wallis ANOVA, $H_{2,15} = 10.25$, $p < 0.01$, Tukey, $p < 0.05$) (Fig. 3). There was no significant correlation between animal biomass and DNRA ($r = 0.42$, $p = 0.41$).

Nutrient fluxes

Soluble reactive phosphorus (SRP) fluxes were low and negative ($-1 < x < 0 \mu\text{mol SRP m}^{-2} \text{h}^{-1}$), indicating uptake of phosphorus by the sediment (Fig. 5). The uptake was stronger in the NOB treatment (1-way ANOVA, $F_{2,33} = 3.70$, $p < 0.05$, Tukey, $p < 0.05$). NOB treatment showed the highest dissolved silica fluxes ($60.3 \pm 5.4 \mu\text{mol H}_4\text{SiO}_4 \text{m}^{-2} \text{h}^{-1}$), followed by the HY treatment ($49.6 \pm 2.6 \mu\text{mol H}_4\text{SiO}_4 \text{m}^{-2} \text{h}^{-1}$) and the NO treatment ($40.0 \pm 1.3 \mu\text{mol H}_4\text{SiO}_4 \text{m}^{-2} \text{h}^{-1}$) (Kruskal-Wallis ANOVA, $H_{2,33} = 13.92$, $p < 0.001$, Tukey, $p < 0.05$). There was also a positive correlation between the biomass of *Marenzelleria* and the flux of dissolved silica ($y = 1.62x + 47.89$, $r = 0.75$, $p < 0.01$).

Sediments from all treatments were net sources of dissolved inorganic nitrogen (DIN). Measured fluxes for the NO, NOB and HY treatments were 9.4 ± 1.1 , 31.7 ± 5.4 and $40.6 \pm 2.6 \mu\text{mol DIN m}^{-2} \text{h}^{-1}$, respectively (Fig. 5). In the HY treatment, DIN fluxes were almost entirely comprised of ammonium release ($39 \pm 2.0 \mu\text{mol NH}_4^+ \text{m}^{-2} \text{h}^{-1}$), whereas the nitrite and nitrate fluxes were negligible. In the NOB treatment, the ammonium flux was only ~30% of the DIN flux ($11.7 \pm 3.9 \mu\text{mol NH}_4^+ \text{m}^{-2} \text{h}^{-1}$). In the NO treatment, the DIN flux was entirely represented by nitrate efflux, while the ammonium flux was slightly negative ($-2.3 \pm 0.3 \mu\text{mol NH}_4^+ \text{m}^{-2} \text{h}^{-1}$). Of the 3 species comprising DIN, only the ammonium flux was positively correlated with *Marenzelleria* biomass ($y = 1.32x + 1.54$, $r = 0.85$, $p < 0.001$).

The denitrification efficiency (DE) was calculated from the ratio of the inorganic nitrogen fluxes and the N_2 production via denitrification. This is the amount of mineralized nitrogen that is permanently removed from the sediment as N_2 . DE was highest in the NO treatment (50%) and decreased significantly in NOB (16%) and HY (4%) treatments.

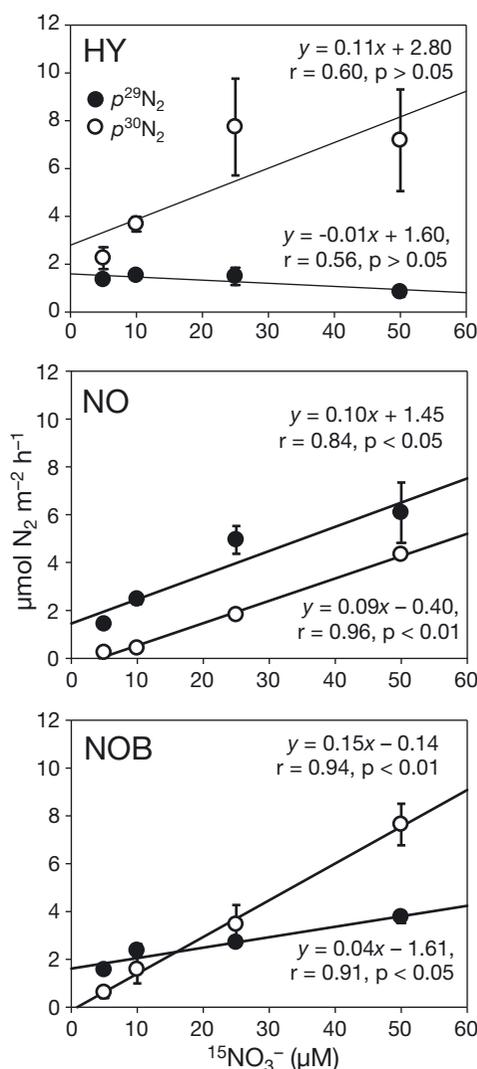


Fig. 4. Production (p) of $^{29}\text{N}_2$ and $^{30}\text{N}_2$ as a function of the concentration of $^{15}\text{NO}_3^-$ in the core water phase. In the hypoxic treatment (HY), the slopes of the linear regressions between $p^{29}\text{N}_2$ and $p^{30}\text{N}_2$ and $[^{15}\text{NO}_3^-]$ were not significantly different from zero; in the normoxic treatment (NO), they were > 0 and parallel, while in the normoxic-bioturbate treatment (NOB), $p^{30}\text{N}_2$ was more stimulated than $p^{29}\text{N}_2$ by increasing $^{15}\text{NO}_3^-$ concentrations

DISCUSSION

Experimental design

Previous experiments of the effects of bioturbation and bioirrigation have been conducted using sieved fauna-free sediments with short conditioning periods (Bartoli et al. 2000, 2009, Hietanen et al. 2007) with the main purpose of reducing background variability. The disadvantage of this approach is that sediment-sieving changes the grain size distribution,

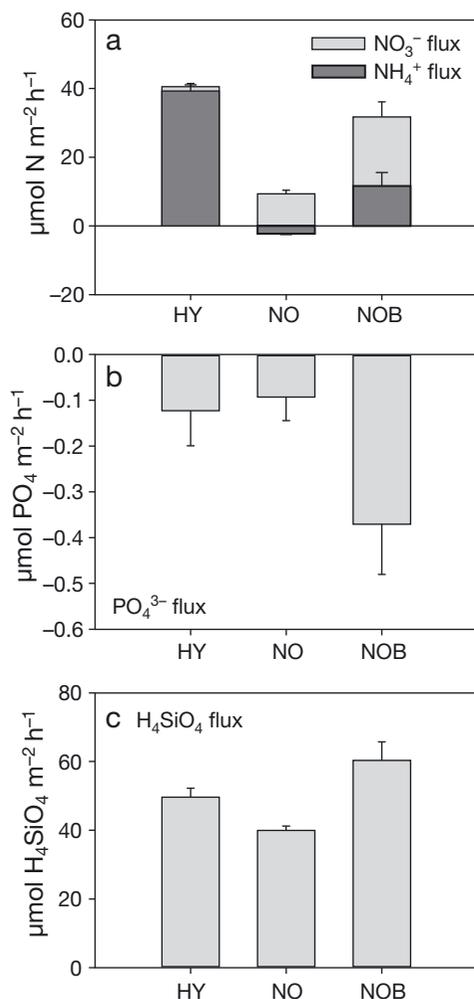


Fig. 5. Net fluxes of (a) ammonium and nitrate, (b) reactive phosphorus and (c) silica measured in intact sediment cores. Means \pm SD, $n = 12$; positive fluxes are from the sediment to the water column

reduces bacterial biomass and changes the microbial community composition by favoring fast-growing bacteria (Langezaal et al. 2003, Stocum & Plante 2006). Furthermore, in the case of muddy sediment, animal burrows are poorly consolidated (Karlson et al. 2005).

In the present experiment, sediments that were free of macrofauna were collected from a deep anoxic site and transferred into mesocosms with minimal disturbance, except for replacement of overlying anoxic with normoxic bottom water (NO) and the addition of macrofauna (NOB). The NOB treatment underwent a relatively long conditioning period after addition of macrofauna (48 d), allowing the consolidation of new burrow structures, their colonization by microbial communities and the establishment of steep gradients that characterize bioturbated sedi-

ments. Since the conditioning time was long, it is likely that *Marenzelleria* individuals were not actively burrowing during our sampling and incubation procedures, and the number of individuals recovered in the cores at the end of incubations reflected the patchy distribution of *Marenzelleria* spp. We thus believe that the rates we report are not affected by density-dependent features or by edge-effects associated with elevated numbers of burrowing organisms on a limited sediment volume. In this respect, indirect evidence is provided by the linearity of most measured process rates at different animal densities.

Contrasting respiration and nutrient fluxes in the HY, NO and NOB treatments

The most conspicuous characteristic of our experiments was that the change from hypoxic to normoxic conditions stimulated sediment oxygen consumption rates by a factor of 3.4 and nitrification-coupled denitrification by a factor of >6 , while the Mn^{2+} efflux decreased by 50% and the efflux of ammonium was stopped. Normoxia apparently enhanced aerobic respiration rates and O_2 -consuming chemoautotrophic processes, such as nitrification, and the oxidation of reduced compounds, such as NH_4^+ , Mn^{2+} , Fe^{2+} and dissolved sulfide. The decrease in the Mn^{2+} efflux from the HY to the NO treatment was stoichiometrically equivalent to consumption of $20 \mu\text{mol O}_2 \text{ m}^{-2} \text{ h}^{-1}$. This amount corresponds to 14% of the increase in total oxygen uptake (TOU) from the HY to the NO treatment. The oxygen consumption due to nitrification was $36 \mu\text{mol O}_2 \text{ m}^{-2} \text{ h}^{-1}$, and represented an additional 26% of the increase in TOU from the HY to the NO treatment. Overall, at least 40% of the increased oxygen uptake was not used for aerobic respiration, but in bacterial and chemical inorganic oxidation processes.

The additional doubling in the total oxygen uptake rates in the NOB treatment compared to the NO treatment was mostly due to respiration by the polychaetes (67%) and to additional oxidation in the burrow walls. We estimated the oxygen consumption in the *Marenzelleria* burrow walls by subtracting the oxygen consumption in the NO treatment and the *Marenzelleria* respiration in the Exetainers from the total oxygen consumption measured in the NOB treatment. The calculated respiration within the burrow walls was $73.3 \pm 22.9 \mu\text{mol m}^{-2} \text{ h}^{-1}$, corresponding to 17% of total respiration. The measured oxygen consumption rate by *Marenzelleria* ($20.7 \mu\text{mol O}_2 \text{ g}^{-1} \text{ DW h}^{-1}$) is considered a maximum respiration rate,

because O₂-saturated water was used in the incubations, which is not likely the case within the burrows where oxygen concentrations were expected to be substantially lower. Since chemoautotrophic and chemical oxidation processes such as nitrification and manganese, iron and sulfide oxidation were probably also stimulated by the greater exposure of sediment surface in the burrow walls, our estimate of the oxygen consumption rates in the burrow walls is likely underestimated.

Overall, however, the increased oxygen uptake measured in the sediments with *Marenzelleria* was insufficient to oxidize all reduced compounds, which were produced during anaerobic respiration deeper in the sediment. This is clearly seen by the increased efflux of Mn²⁺ and NH₄⁺ in the NOB treatment compared to the NO treatment. The increase in the flux of Mn²⁺ and NH₄⁺ was higher than that expected from the increase in surface area for solute exchange with the water column by the *Marenzelleria* burrows and suggests that *Marenzelleria* stimulated anaerobic bacterial processes. This is in agreement with the reported stimulation of bacterial sulfate reduction by *Marenzelleria* (Kristensen et al. 2011). The presence of free sulfide in the *Marenzelleria*-inhabited zone in our study supports this interpretation. High sulfide concentrations would also inhibit nitrification rates and stimulate DNRA (Burgin & Hamilton 2007). In addition, increased sulfide production can explain the low fluxes of Fe²⁺, because Fe²⁺ production would have been efficiently buffered by the formation of iron sulfides. Stimulation of anaerobic metabolism within the burrow walls could also explain the increased silica flux, because the ingestion of diatoms by *Marenzelleria* and the irrigation of the burrow walls due to the pumping activity of *Marenzelleria* (Quintana et al. 2011) would increase the release of dissolved silica to the water.

Our study confirms the scavenging effect of bioturbation on SRP (Rozan et al. 2002, Norkko et al. 2012), but this effect was relatively weak. This can be explained by the fact that the present experiments were carried out with sediments conditioned for >2 mo before the beginning of the measuring period. SRP release from the sediment following the establishment of anoxia or hypoxia is generally coupled to the reduction of iron oxyhydroxides and can be intense, but limited in time (Rozan et al. 2002). Once the reactive iron oxyhydroxide pool is consumed sorbed phosphorus is quickly released and the subsequent SRP efflux would only be supported by anaerobic carbon degradation rates and will slow down considerably. This would explain the low fluxes of

SRP in the hypoxic treatment. Under normoxic conditions, SRP release would be reduced or the flux direction would even be reversed as the re-oxidation of reduced metal pools would scavenge SRP from the water phase. This effect may be further enhanced by bioturbation (Rozan et al. 2002). It appears that a significant scavenging capacity in the NO and NOB treatments persisted even after the 2 mo long conditioning period.

Denitrification and DNRA rates under HY, NO and NOB conditions

The low rates of D_w and DNRA_w in all treatments were likely due to the low concentrations of NO₃⁻ in the overlying water (2.3 to 3.8 μM). This observation is common to many marine environments and many Baltic Sea sediments with low bottom water nitrate concentrations (Deutsch et al. 2010). In such systems, the removal of nitrogen as N₂ depends on the benthic oxygen uptake to support D_n (Eyre & Ferguson 2009). The 6-fold higher denitrification rates (from 1.5 to 9.5 μmol N m⁻² h⁻¹) and 10-fold lower rates of DNRA (from 3.8 to 0.4 μmol N m⁻² h⁻¹) in the NO treatment compared to the HY treatment indicate higher nitrogen loss and less efficient recycling of inorganic nitrogen in the NO treatment. Our results suggest that the increase of total denitrification was due to the formation of a thicker oxic surface layer that allowed nitrification to occur, which, in turn, generated nitrate to support the increased denitrification rates. This interpretation is also supported by the deeper oxygen penetration depth measured in the NO and NOB treatments compared to the HY treatment. Normoxic conditions would have resulted in increased N₂ loss and provided a negative feedback mechanism for nitrogen regeneration.

In the Baltic, colonization by macrofauna, particularly of the invasive genus *Marenzelleria*, is expected once normoxia is established (Schiedek 1997, Leppäkoski et al. 2002, Karlson et al. 2011). Our results indicate a 50% decrease in denitrification and a 3-fold increase of DNRA in sediment inhabited by *Marenzelleria* compared to normoxic non-bioturbated sediments, resulting in a net stimulation effect of denitrification by bottom water oxygenation of only a factor 3 compared to the HY treatment. This stimulation is only due to differences in D_n , since the contribution of D_w was not significantly different between the treatments.

This result is unusual, because it is commonly thought that denitrification is stimulated by the pre-

sence of macrofauna (Svensson 1998, Svensson et al. 2001). For example, Pelegri et al. (1994) measured a 3- and 5-fold increase of D_n and D_w , respectively, in the presence of a very large number of the amphipod *Corophium volutator* (19800 ind. m^{-2}). Tuominen et al. (1999) measured a 1.5-fold increase of D_n in sediments with 1500 ind. m^{-2} of the amphipod *Mono-poreia affinis*, and Nizzoli et al. (2007) measured a 3-fold increase of D_w in estuarine sediments with the polychaete *Nereis* spp. However, our results are in agreement with other published studies on *Marenzelleria*, which suggest that this organism does not stimulate denitrification (Karlson et al. 2005, Hietanen et al. 2007, Kristensen et al. 2011). Our findings indicate that *Marenzelleria* even reduced denitrification rates in comparison with oxidized non-bio-irrigated sediment and this may be due to the sulfidic conditions within the burrows (Kristensen et al. 2011).

The concentration series conducted in our ^{15}N -labeling experiments allowed us to assess the simultaneous occurrence of anammox and denitrification as potential N_2 -producing processes (Risgaard-Petersen et al. 2003, Trimmer et al. 2006, Racchetti et al. 2011). Since genuine $^{28}N_2$ production was constant and independent of the concentrations of added labeled nitrate, anammox did not appear to contribute significantly to N_2 production (Trimmer et al. 2006).

Stimulation of $^{30}N_2$ production in the bioturbated cores compared to normoxic non-bioturbated ones (Fig. 4) was possibly due to active transport of $^{15}NO_3^-$ from the water column in *Marenzelleria* burrows. Burrow walls were likely anoxic and denitrifiers would have primarily reduced $^{15}NO_3^-$ within burrows. The low $^{29}N_2$ production rates suggest that nitrification was suppressed. This is because nitrification would have produced new $^{14}NO_3^-$ from $^{14}NH_4^+$. The new $^{14}NO_3^-$ would have been used together with $^{15}NO_3^-$ and allowed the production of $^{29}N_2$. For the same reasons, $^{29}N_2$ production was insignificant under hypoxic conditions, but, in addition, the production of $^{30}N_2$ was lower compared to that in bioturbated sediments due to the absence of burrow-associated new sediment surfaces.

The observation that denitrification of water column nitrate was not significantly different in the 3 experimental treatments is somewhat surprising, as hypoxia should have reduced the diffusion length of nitrate to the denitrification zone. In addition, bioturbation by *Marenzelleria* should have increased the sediment surface area through which nitrate could diffuse and supported higher D_w , as suggested for

other burrowers (Nizzoli et al. 2007). In our system, however, dissolved sulfide increased close to the sediment–water interface in the hypoxic and bioturbated treatments, and sulfide toxicity to denitrifiers may have compensated the effects of a shorter diffusion distance (Burgin & Hamilton 2007, Aelion & Warttinger 2010).

Higher sulfate reduction rates in the presence of *Marenzelleria* could also explain the higher DNRA rates in the NOB treatment. Both hypoxia and the activity of *Marenzelleria* have been shown to increase sulfate reduction rates (Kristensen et al. 2011). High DNRA rates were also reported from sulfidic sediments that receive abundant labile organic carbon, e.g. sediment from affected, eutrophic systems and below mussel and fish farms (Brunet & Garcia-Gil 1996, Christensen et al. 2000, Nizzoli et al. 2006). Based on indirect evidence, Karlson et al. (2005) even suggested that DNRA was the main pathway of nitrate removal in Baltic Sea sediment. Our results suggest that DNRA only dominates over denitrification under hypoxic conditions and that ammonium regeneration via DNRA only represented a small fraction of the total recycled inorganic nitrogen (~10%), due to very low nitrate in the bottom water.

Summary and conclusions

This experimental study provides direct evidence of the anticipated recovery effects following recolonization of previously anoxic sediments by bioirrigating and bioturbating macrofauna in the Baltic Sea. Data from recent modeling studies emphasize the beneficial aspects of the invasive polychaete *Marenzelleria* spp. in counteracting eutrophication by increasing phosphorus retention and carbon mineralization (Norkko et al. 2012). The intense bioirrigation activity of this species may alter key sediment features, reduce the pool of labile organic carbon, and enhance phosphorus retention due to co-precipitation with iron oxyhydroxides. Modeling of a 10 yr long data set suggests that the colonization of Baltic Sea sediment by *Marenzelleria* would improve the quality of the benthic system through enhanced phosphorus retention in Baltic Sea sediment (Norkko et al. 2012). As a consequence, the frequency and risk of water column anoxia may be substantially reduced.

Our study provides data to test the predictions of the Norkko et al. model after the first months and presents new information on the behavior of nitrogen and silica and the associated changes in aerobic and

anaerobic respiration rates. Our findings suggest that the *Marenzelleria*-related scavenging effect of reactive phosphorus, while apparent, was weak. The change from hypoxic to normoxic macrofauna-free conditions led to better retention of silica and greater conversion of fixed nitrogen to N₂ gas. This would decrease the amount of recycled nutrients that would be available for primary production.

The stimulation of anaerobic metabolism, the enhanced release of total inorganic nitrogen and silica, and the reduced N₂ loss rates in the presence of *Marenzelleria* imply that the bioturbated treatment resulted in relatively higher recycling rates of fixed nitrogen compared to the normoxic treatment without infauna. Overall, these results suggest that, on the time scale of our experimental setup, *Marenzelleria* counteracted some beneficial effects that arose due to benthic oxygenation. It is apparent that the observed strong contrasts in biogeochemical flux characteristics and microbial rates have large implications for recovering aquatic ecosystems in the Baltic. Nevertheless, although we conducted our study over a substantially longer time period than previous studies, the experiment was still short compared to the long-term recovery expected for natural ecosystems of the Baltic, if effective nutrient-loading reduction measures are implemented (Meier et al. 2011). This emphasizes the need for extending the duration of these mesocosm experiments to substantially longer time periods.

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Appendix 1.

Table A1. Main chemical and physical parameters measured in the water flowing from the water tank reservoirs to the box core mesocosms. The water effluent was sampled 1 d before the first incubation was carried out

	Temperature (°C)	O ₂ (μM)	NH ₄ ⁺ (μM)	NO ₂ ⁻ (μM)	NO ₃ ⁻ (μM)	PO ₄ ³⁻ (μM)	H ₄ SiO ₄ (μM)
Normoxic reservoir	5.1	312.5	2.6	0.2	2.8	0.6	17.4
Hypoxic reservoir	5.1	48.4	3.4	0.6	1.0	0.5	19.4

Table A2. One-way parametric (D_w rates and PO₄³⁻ fluxes) and non-parametric (Kruskal-Wallis test, all remaining measurements) analysis of variance for the hypoxic (HY), normoxic (NO) and normoxic-bioturbated (NOB) treatments. Pairwise comparison was performed by means of Tukey test. Letters: significant differences between fluxes; same letter: no significant differences

	Analysis	p	HY	NO	NOB
O ₂ flux	$H_{2,33} = 29.13$	<0.001	a	b	c
CH ₄ flux	$H_{2,33} = 2.02$	0.365	a	a	a
Mn ²⁺ flux	$H_{2,33} = 26.02$	<0.001	a	a	b
Fe ²⁺ flux	$H_{2,33} = 5.30$	0.071	a	a	a
D_w rate	$F_{2,33} = 2.84$	0.073	a	a	a
D_n rate	$H_{2,33} = 31.14$	<0.001	a	b	c
D_{tot} rate	$H_{2,33} = 30.92$	<0.001	a	b	c
DNRA _n rate	$H_{2,15} = 5.43$	0.071	a	a	a
DNRA _w rate	$H_{2,15} = 9.87$	0.007	a	b	c
DNRA _{tot} rate	$H_{2,15} = 10.25$	0.006	a	b	c
NH ₄ ⁺ flux	$H_{2,33} = 27.88$	<0.001	a	b	c
NO ₃ ⁻ flux	$H_{2,33} = 22.11$	<0.001	a	b	b
PO ₄ ³⁻ flux	$F_{2,33} = 3.70$	0.036	a	a	b
H ₄ SiO ₄ flux	$H_{2,33} = 13.92$	<0.001	a	b	a

Table A3. Slope of the regression between $p^{29}\text{N}_2$ and $p^{30}\text{N}_2$ and the amount of the added labeled nitrate for the normoxic (NO), normoxic-bioturbated (NOB) and hypoxic (HY) treatments (\pm SE)

	NO		NOB		HY	
	Slope	p	Slope	p	Slope	p
$d(p^{29}\text{N}_2)/d(^{15}\text{NO}_3^-)$	0.101 \pm 0.021	0.041	0.044 \pm 0.008	0.035	-0.013 \pm 0.006	0.175
$d(p^{30}\text{N}_2)/d(^{15}\text{NO}_3^-)$	0.093 \pm 0.005	0.003	0.154 \pm 0.006	0.002	0.107 \pm 0.050	0.188

Table A4. Results of the analysis of covariance, testing the parallelism of $p^{29}\text{N}_2$ and $p^{30}\text{N}_2$ within the 3 treatments normoxic (NO), normoxic-bioturbated (NOB) and hypoxic (HY)

	NO			NOB			HY		
	df	F	p	df	F	p	df	F	p
Covariate (¹⁵ NO ₃ ⁻)	1	72.92	0.000	1	112.54	0.000	1	4.25	0.052
Dependent variable ($p^{29}\text{N}_2$ and $p^{30}\text{N}_2$)	1	25.92	0.000	1	4.93	0.038	1	23.91	0.000
Interaction between covariate and dependent variable	1	0.12	0.735	1	34.97	0.000	1	6.94	0.016
Residuals	20			20			20		