The Interplay of Macrofauna Functional Groups Shapes Nitrogen Cycling in Oligotrophic Estuarine Sediments

Mindaugas Zilius (mindaugas.zilius@jmtc.ku.lt)
Klaipedos universitetas https://orcid.org/0000-0002-2390-6636
Darius Daunys
Klaipedos universitetas
Marco Bartoli
University of Parma: Universita degli Studi di Parma
Ugo Marzocchi
Aarhus Universitet
Stefano Bonaglia
University of Gothenburg: Goteborgs Universitet
Ulisse Cardini
Stazione Zoologica Anton Dohrn Napoli
Giuseppe Castaldelli
University of Ferrara: Universita degli Studi di Ferrara

Research Article

Keywords: benthic-functioning, invertebrate-bacteria associations, nitrogen cycle, nitrate reduction, sediments, Baltic Sea

Posted Date: April 14th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-370095/v1

Abstract

The effects of single macrofauna species on benthic nitrogen (N) cycling has been extensively studied, whereas the effect of macrofauna communities on N-related processes remains poorly explored. In this study, we characterized benthic N-cycling in bioturbated sediments of an oligotrophic northern Baltic waters (Öre estuary). Solute fluxes and N transformations (N_2 fixation, denitrification and DNRA) were measured in sediments and in macrofauna-bacteria holobionts to partition the role of three dominant macrofauna taxa (*Limnecola balthica*, *Marenzelleria* sp. and *Monoporeia affinis*) in shaping N-cycling, and to disentangle the contribution of different functional groups within the community. In the studied area, benthic macrofauna comprised a low diversity community with extremely high local dominance of three macrofauna taxa, which are widespread and dominant in the Baltic. The biomass of these three taxa in the benthic community explained up to 30% of variation in measured biogeochemical processes, confirming their role in ecosystem functioning. The results also show that these taxa significantly contributed to the benthic metabolism and N-cycling (direct effect) as well as reworked sediments with positive feedback to dissimilative nitrate reduction (indirect effect). Taken together, these functions promoted a re-use of nutrient at the benthic level, limiting net losses (e.g. denitrification) and effluxes to bottom water. Finally, the detection of multiple N transformations in dominating macrofauna holobionts suggested a community-associated active and versatile microbiome, which alternatively contributes to the biogeochemical processes. The present study highlights hidden and interactive effects among microbes and macrofauna, which should be considered in analysing benthic functioning.

1. Introduction

The community of benthic invertebrates sustains ecosystem functioning in shallow coastal areas via functional group-specific mechanisms (Lohrer et al. 2004; Kristensen et al. 2014) and via common adaptations to the conditions within sediments (Karlson et al. 2005). Filter and deposit feeders generally dominate the benthic communities (e.g. Gogina et al. 2016). Filter feeders like bivalves may favour the transport of pelagic primary production to the sediment, promote water transparency and enhance the regeneration of nutrients to the water column, keeping primary producers growth elevated (Nakamura et al. 2000; Naldi et al. 2020). Surface or deep burrowing deposit feeders, like polychaetes, fertilise sediments with labile faeces and mix organic matter via sediment reworking, accelerating the rates of mineralization via priming effects (Kristensen 2000). By constructing and ventilating burrows, macrofauna alter redox conditions due to the transport of electron acceptors and metabolic end-products (Kristensen 2001; Hedman et al. 2011; Kristensen et al. 2014; Kauppi et al. 2018) and increase coupling between aerobic and anaerobic horizons (Nielsen et al. 2004; Pischedda et al. 2008; Kristensen et al. 2012). Therefore, macrofauna may stimulate an array of reactions and processes regulating benthic functioning. Macrofauna may also select and host unique microbiomes, which can contribute to biogeochemical cycling (Cardini et al. 2019; Zilius et al. 2020; Marzocchi et al. 2021).

Most studies on macrofauna-mediated benthic functioning were based on abundant species (Thrush et al. 2006; Smyth et al. 2018), or on reconstructed microcosms where different abundance levels of a

single species were manipulated (Karlson et al. 2005, 2007; Braeckman et al. 2010; Gilbertson et al. 2012; Bonaglia et al. 2014; Benelli et al. 2018; Kauppi et al. 2018; Samuiloviene et al. 2019). Alternatively, inferential multivariate statistical approaches were used to analyse the relationships between benthic communities and functional processes (Norkko et al. 2015; Villnas et al. 2018; Politi et al. 2019). However, emerging properties of natural communities, where different functional groups interact, still remain poorly explored and understood (Gamfeldt et al. 2014; Kaupi et al. 2018). Such emerging properties may either amplify or buffer the biogeochemical effects of a single species or functional groups (Clare et al. 2016a), since interactions may lead to non-additive effects.

The simultaneous analysis of whole benthic community functioning and metabolic activity of a single macrofauna species may help addressing the species role in the community, and to emphasize the possible interspecific interactions. The contribution of macrofauna to benthic functioning can occur in multiple ways: 1) directly, via their own metabolic rates (Welsh et al. 2015; Smyth et al. 2018), 2) indirectly via reworking of sediments, which stimulates microbial communities (Laverock et al. 2014; Bonaglia et al. 2013, 2014; Vasquez-Cardenas et al. 2016; Samuiloviene et al. 2019), and 3) via ecological interactions (predation, competition, facilitation) with other macrofauna (Clare et al. 2016a, b) or with bacteria (Zilius et al. 2020; Marzocchi et al. 2021). Increasing synergy between molecular tools and the use of stable isotope probing have allowed large improvements in our understanding of element cycling in holobionts and benthic macrofauna reworked sediments (Poulsen et al. 2014; Vasquez-Cardenas et al. 2016; Cardini et al. 2019; Zilius et al. 2020; Marzocchi et al. 2021; Politi et al. 2021).

In particular, benthic microbial nitrogen (N) cycling is largely affected by macrofauna activity. For example, dense bivalve populations can result in large ammonium (NH_4^+) effluxes to the bottom water, alleviating N limitation for microbial communities (Welsh et al. 2015; Smyth et al. 2018). Deep burrowing macrofauna, during ventilation, pump N- and oxygen (O_2)-rich bottom water downward (Nielsen et al. 2004; Mermillod-Blondin and Rosenberg 2006; Murphy and Reidenbach 2016). This stimulates microbial N-cycling pathways, including nitrification and denitrification (Tuominen et al. 1999; Bonaglia et al. 2013; Stief 2013; Bosch et al. 2015; Moares et al. 2018). Therefore, in macrofauna-reworked sediments, the abundance and the activity of microbial communities often exceed those of surrounding, non-bioturbated sediments (Laverock et al. 2014; Gilbertson et al. 2012; Yazdani Foshtomi et al. 2018; Samuiloviene et al. 2019). The exterior and/or interior of macrofauna offer additional habitats for microbial communities, which are actively involved in different N-cycling pathways such as nitrification, denitrification and dissimilatory nitrate reduction to ammonium (DNRA) or dinitrogen (N_2) fixation (Ray et al. 2019; Zilius et al. 2020; Marzocchi et al. 2021; Politi et al. 2021).

The present study shades light on how benthic biodiversity-ecosystem functioning affects N-cycling in a shallow coastal systems. The study was carried out in the Öre estuary (Northern Baltic Sea), which is characterized by low diversity macrofauna communities dominated by three taxa: the surface deposit feeder amphipod *Monoporeia affinis* (Lindström), the surface deposit and suspension feeding clam *Limnecola balthica* (L.) and the deep burrowing deposit feeding polychaete *Marenzelleria* sp. We hypothesized that the effects of macrofauna community on microbial processes largely depend on the

dominant macrofauna functional group or on the combination of co-dominant functional groups, each of them characterized by a particular bioturbation mode, feeding guild and potential to harbour active microbiota. Consequently, we expect the main biogeochemical processes in sediments to be predominantly the result of the collective effect of these different functional groups. Therefore, the aim of this study was to disentangle such collective effect on microbial N transformations. We used a methodologically integrated approach, which included benthic community (whole core) as well as holobiont (single individual) incubations, to disentangle and reconstruct N-cycling in non-manipulated benthic community, highlighting hidden and interactive effects among the abiotic environment, microbes and macrofauna.

2. Methods

2.1 Study site and sampling activities

The Öre estuary is a boreal estuarine system (~ 71 km^2) located on the Swedish coast of the Quark Strait, between the Bothnian Bay and Bothnian Sea (Northern Baltic Sea) (Fig. 1). The estuary is brackish (< 6), and oligotrophic due to limited nutrient inputs from the watershed (Hellemann et al. 2017; Voss et al. 2020), which mainly consists of coniferous forests and mires. This estuary is stratified depending on spatiotemporal gradients of salinity, changing upon seasonal river discharge, and summer temperature (Brydsten and Jansson 1989; Bartl et al. 2019). The estuarine sedimentary environment varies from silt to fine sandy deposits (Hellemann et al. 2017).

Sediments and animals for experimental activities were collected on 29 and 31 July 2019 in the area comprised between monitoring stations NB3, N8 and NB7 (Fig. 1). Sediments were collected on-board of R/V Botnica using a box corer (20 × 20 cm). After each deployment of the box corer, two large cores (i.d. 8 cm, length 30 cm) were subsampled for flux measurements and a small core (i.d. 4.2 cm, length 25 cm) was subsampled for sediment characterization. A total of 18 large cores and 6 small cores were collected. Bottom water (~ 30 m depth) was collected at the station NB8 with a Niskin-Type water sampler (30 L) for sediment core incubation and other experimental activities. In addition, macrofauna for holobiont incubations was collected using a Van Veen grab and carefully sieving sediments. Macrofauna was transferred to 10 L aquaria, containing continuously aerated bottom water and a nearly 2 cm thick layer of surface sediments from the sampling site. In the laboratory, open sediment cores were placed into an incubation tank, containing unfiltered aerated and well-stirred estuarine water in a temperature-controlled room (12°C). A stirring bar, driven by an external magnet at 40 rpm, was inserted in each core approximately 15 cm above the sediment interface to maintain the water phase mixed avoiding sediment resuspension. The cores were subsequently pre-incubated overnight.

2.2 Whole core measurements

After overnight preincubation, a gas-tight lid was placed on the top of each core and a dark incubation started. The experiment lasted from 7 to 9 h in order to keep final O_2 concentration within 20% of the

initial value. At the beginning and end of the incubation, 20 mL water aliquots were collected from each core, transferred into 12 mL exetainers (Labco Ltd) allowing overflow, and fixed with 200 µL of 7 M $ZnCl_2$ for O_2:Ar measurements. In addition, aliquots of 30 mL were filtered (Frisenette GF/F filters) into 12 mL plastic test tubes (for inorganic N and silica (DSi)) and in 10 mL glass tubes (for dissolved inorganic phosphorus – DIP), and they were immediately frozen at − 20 °C for later nutrient analysis (see Sect. 2.4 for details).

After the flux measurements, cores were opened, and left submerged in the incubation tank for 13 h. Afterwards, NO_3^- reduction processes were measured following the revised isotope pairing technique (r-IPT, Risgaard-Petersen et al. 2003). Briefly, all cores were spiked with $^{15}NO_3^-$ tracer (20 mM $Na^{15}NO_3$, 98 atom % ^{15}N, Sigma Aldrich) to a final concentration of 10 µM (n = 9) and 26 µM (n = 9). To calculate the isotopic enrichment, water samples for NO_3^- analysis were collected prior and after the tracer addition. The cores were then capped and incubated in the dark as described for flux measurements. At the end of incubations, the water and the whole sediment phases were gently mixed to a slurry. Thereafter, 20 mL aliquots of the slurry were transferred into 12 mL exetainers (Labco Ltd) allowing twice overflow, and fixed with 200 µL of 7 M $ZnCl_2$ for later $^{29}N_2$ and $^{30}N_2$ analyses. An additional 40 mL subsample was collected, transferred to 50 mL falcon vials and treated with 2 g of KCl for the determination of the exchangeable NH_4^+ pool and the $^{15}NH_4^+$ fraction (see Sect. 2.4 for details). After the incubations the sediments from all cores were carefully sieved (0.5 mm mesh size) to retrieve macrofauna for further taxonomic identification and determination of abundance and biomass.

2.3 Holobiont incubations

Individual incubations were employed to assess N-cycling pathways associated with the animal's holobionts. Incubations were carried out reproducing the experimental setup previously described in Marzocchi et al. (2021) and Politi et al. (2021). Briefly, animals were incubated in small bottom-capped Plexiglas cylindrical microcosms (total volume 227 ± 3 mL) partly filled with sterilized glass beads (Ø 1–1.3 mm; 4 mL – for *M. affinis*, 5 mL – for *L. balthica*, and 16 mL – for *Marenzelleria* sp. experiments), and the rest with filtered and aerated *in situ* water, amended with different isotopes (see Sect. 2.3.1 and 2.3.2 for details). The water was filtered (MCE filters, 142 mm diameter, pore size 0.22 µM, MF-Millipore™) to remove phytoplankton, suspended particles and bacteria, so that metabolic rates measured in the incubation could be solely attributed to the macrofauna and its associated microbiome. All microcosms were equipped with a stirring magnet for continuous water mixing (20 rpm) during incubation, and with gas tight lids on the top fitted with sampling ports for water subsampling and replacement.

2.3.1 Nitrate reduction

The r-IPT was used to assess NO_3^- reduction processes associated with animals, including denitrification, DNRA and anammox (Thamdrup and Dalsgaard 2002; Risgaard-Petersen et al. 2003). Three different macrofauna treatments, each with 5 replicates (containing 19–24 ind. of *M. affinis* or 2

ind. of *L. balthica* or 1–2 ind. of *Marenzelleria* sp.) and 1 control (only water), were applied. The first treatment had low $^{15}NO_3^-$ addition to a final concentration of 8.6 µM, the second treatment had high $^{15}NO_3^-$ addition to a final concentration of 25.3 µM, and the third treatment had $^{15}NH_4^+$ (15 mM $^{15}NH_4Cl$, 98 atom % ^{15}N, Sigma Aldrich) and $^{14}NO_3^-$ additions to final concentrations of 32.0 and 25.9 µM, respectively. The different $^{15}NO_3^-$ concentrations of treatments 1 and 2 were used to validate the IPT assumptions (Risgaard-Petersen et al. 2003). To calculate the degree of isotopic enrichment, water samples for NH_4^+ and NO_3^- analysis were collected prior and after the isotope addition. Before capping microcosms, they were preincubated for 1–3 h allowing the tracer diffusion into the animal's body. After this period, the microcosms were incubated in the dark for 20–39 h, depending on macrofauna species, at 12 ^oC. Following incubation, water aliquots were subsampled at three time points from each replicate, transferred into 12 mL exetainers (Labco Ltd) and poisoned with 200 µL of 7 M $ZnCl_2$ for gas measurements. In addition, dissolved O_2 was monitored with optodes (FireStingO2, PyroScience GmbH) to adjust the incubation time.

Slopes of the linear regression of $^{29}N_2$ and $^{30}N_2$ concentrations versus time were used to calculate rates of denitrification and anammox, using the equations from Thamdrup and Dalsgaard (2002). The slope of the linear regression of $^{15}NH_4^+$ concentration versus time was used to calculate rates of DNRA according to Bonaglia et al. (2016). All NO_3^- reduction rates were then calculated as a function of biomass dry weight. Finally, rates in the experimental chambers with animals were corrected with values detected in the controls.

2.3.2 Dinitrogen fixation

To determine rates of N_2 fixation, a stock solution of $^{30}N_2$-enriched 0.22 µm twice-filtered water was prepared using a modified version of the protocol described in Klawonn et al. (2015) as reported in Marzocchi et al. (2021). Before starting the incubation, the stock solution was gently transferred into five microcosms to minimize gas exchange with the atmosphere. After the macrofauna organisms were added (two individuals for each microcosm for *L. balthica* and *Marenzelleria* sp., and three individuals for each microcosm for *M. affinis*), the cores were closed and incubated in the dark for 6.3 to 6.5 h. Three additional microcosms were prepared and incubated as above but with unlabeled water to serve as a control for isotopic contamination. At the end of the incubation, the macrofauna was collected, weighted (wet weight), and stored at − 20°C for later ^{15}N incorporation analysis. Individuals of *L. balthica* were dissected to remove the soft tissues from the shell, and further to separate the foot from the rest of the body, before being weighted. In addition, ten non-incubated specimens of each incubated macrofauna species were weighted and stored as above for later determination of the natural abundance of N isotopes. Prior to the isotopic analysis, animals' tissues were freeze-dried for 48 h, ground to fine powder and weighed into tin capsules. Samples were analyzed for N elemental composition (%) and isotope ratios ($\delta^{15}N$). $^{15}N_2$ incorporation rates were calculated as in Cardini et al. (2019), for the entire animal.

$^{15}N_2$ incorporation was considered significant for those samples that showed an atom% excess that was more than two folds higher than the standard deviation of the atom% in the unlabelled samples.

2.3.3 Excretion and respiration

Macrofauna excretion and respiration were assessed to explain their contribution to net benthic fluxes. Briefly, six 22-mL glass microcosms with *L. balthica* (2 ind. per vial), *M. affinis* (6 ind. per vial), and *Marenzelleria* sp. (2 ind. per vial) with 0.22 µm twice-filtered water were set up and incubated in the dark, under continuous monitoring of O_2 with optodes (FireStingO2, PyroScience GmbH). In addition, two controls for each species incubation were added later to correct rates in vials with animals. Incubation was carried out in the dark at 12 ^oC and lasted from 2.5 to 5.5 h depending on macrofauna species. At the beginning and end of the incubation, a 5 mL aliquot was collected form each vial and filtered (Frisenette GF/F filters) into plastic test tubes for later NH_4^+ analyses. At the end of the experiment, animals were retrieved and weighed. Excretion and respiration rates were then normalized to biomass dry weight.

2.4 Analytical methods

Dissolved nutrient concentrations (NH_4^+, NO_2^-, NO_x^-, DSi, and DIP) were measured with a 5-channel continuous flow analyser (San^{++}, Skalar) using standard colorimetric methods (Grasshoff et al. 1983). NO_3^- concentration was calculated as the difference between NO_x^- and NO_2^-. Dissolved O_2 was quantified from O_2:Ar ratio measured by membrane inlet mass spectrometer (MIMS) at Ferrara University (Bay Instruments; Kana et al. 1994) and corrected for Ar concentration and solubility based on temperature and salinity (Colt 2012). ^{15}N-atom% in the dissolved N_2 pool in samples from N_2 fixation experiment was also estimated using MIMS. Isotopic samples for $^{29}N_2$ and $^{30}N_2$ production were analysed by gas chromatography-isotopic ratio mass spectrometry (GC-IRMS, Thermo Delta V Plus, Thermo Scientific) at the University of Southern Denmark following the protocol described by De Brabandere et al. (2015). Samples for $^{15}NH_4^+$ production were analysed by the same GC-IRMS after conversion of NH_4^+ to N_2 by the addition of alkaline hypobromite reagent (Warembourg 1993).

The elemental (C and N) and isotopic composition of N ($\delta^{15}N$) in sediments and animals were analysed by Isotope Ratio Mass spectrometry (IRMS, Delta plus V, Thermo Scientific) coupled with an elemental analyzer (Flash EA1112, Thermo Scientific) at Aarhus University. Measured $\delta^{15}N$ values were corrected using laboratory standards calibrated against international reference materials (USGS40 + 41).

2.5 Statistical analysis

The linear regression was employed to explain variability in net benthic fluxes and NO_3^- reduction processes using the dry biomass of benthic community (whole core incubations). The analysis of variance (one-way ANOVA) was used to test the significance of differences in NO_3^- reduction rates among species (holobiont incubations). The assumptions of normality and homogeneity of variance

were checked using Shapiro-Wilk test and Cochran's test, respectively. In the case of heteroscedasticity, data were square root or log(x) transformed. If assumptions were not met after transformation, non-parametric Mann-Whitney Rank Sum Test was used. For significant factors, post hoc pairwise comparisons were performed using the Student-Newman-Keuls (SNK) test. In addition, t-test was used to validate tracer effect on denitrification rates (holobiont incubation). Analyses were performed using SigmaPlot 14.0 software.

A distance-based linear model (distLM) was applied to explain the contribution of macrofauna taxa to variability of specific NO_3^- reduction process and benthic fluxes. The distLM was built on routinous stepwise selection, employing 9999 permutations at a significance level of $p < 0.05$. The resemblance matrices were built on Euclidean distance using normalized and logarithmically transformed fluxes and process rates (Clarke and Gorley 2006). Draftsman plots were used to examine distribution and potential multi-collinearity ($|r| > 0.6$) among predictor variables to be included in the model. The obtained results were visualized with distance-based redundancy analysis (db-RDA, Anderson et al. 2008), and vectors overlay for analysing predictor variables relationship with response vectors. The analysis was performed with the software PRIMER 6 (v.6, Primer-E Ltd.; Clarke and Gorley 2006).

3. Results

3.1 Macrofauna abundance and biomass

In total, 10 species or higher order taxa with an average abundance of 63 ± 3 ind. $core^{-1}$ were recovered after sieving sediments (Supplementary material, Table S1). The most abundant species *M. affinis* (33 ± 3 ind. $core^{-1}$) was followed by oligochaetes (17 ± 2 ind. $core^{-1}$), *Marenzelleria* sp. (11 ± 2 ind. $core^{-1}$) and *L. balthica* (≤ 4 ind. $core^{-1}$) (Table 1). Other taxa (*Pygospio elegans*, *Saduria entomon*, *Corophium volutator*, chironomids and hydrobiids) had much lower occurrence in cores (typically below 10% of total density) and extremely low numbers, altogether contributing by 6–38 individuals (16 ± 3 ind. $core^{-1}$ or 30% on average).

Table 1
Abundance and biomass of main macrofauna taxa and community in incubated cores (n = 18) from the Öre estuary. Results are represented by mean and standard error, and min – max values in brackets

Taxonomic unit	Characteristics			
	Abundance (ind. $core^{-1}$)		Biomass (mg_{dw} $core^{-1}$)	
	Average	Range	Average	Range
L. balthica	1.6 ± 0.4	(0–4)	38.3 ± 10.6	(0.0–160.5)
M. affinis	33.3 ± 2.7	(16–57)	17.2 ± 2.1	(0.4–31.4)
Marenzelleria sp.	9.4 ± 0.6	(5–14)	33.4 ± 6.6	(3.3–88.7)
Oligochaetes	16.8 ± 1.5	(9–32)	1.6 ± 0.3	(< 0.1–4.0)
Others	1.9 ± 0.4	(0–6)	0.8 ± 0.2	(0.0–2.6)
Total	63.2 ± 3.4	(42–92)	91.1 ± 10.1	(10.1–185.4)

The average biomass (91.1 ± 10.1 mg_{dw} $core^{-1}$) was typical for soft bottom coastal areas of the central and northern Baltic with a clear dominance of the polychaete *Marenzelleria* sp. (39%), the clam *L. balthica* (32%), and the amphipod *M. affinis* (26%) (Table 1).

3.2 Benthic fluxes and their relationship with macrofauna biomass

Measured total benthic O_2 uptake varied by a factor of nearly two, from − 939.9 to -1626.9 µmol O_2 m^{-2} h^{-1} (Fig. 2A). The sediment was always a source of DIN (11.9–104.7 µmol N m^{-2} h^{-1}) to the overlaying bottom water with equal contribution of NO_x^- and NH_4^+ (Fig. 2B). Net fluxes of NH_4^+ varied from 5.3 to 78.8 µmol N m^{-2} h^{-1}. The efflux of NO_3^- ranged from 2.1 to 45.9 µmol N m^{-2} h^{-1} whereas the efflux of NO_2^- ranged between 0.6 and 20.3 µmol N m^{-2} h^{-1}. The mean net flux of DIP was directed from the sediment to the near-bottom water, indicating sediments as source of P (Fig. 2C). Similar to DIP, DSi was also released (85.3–362.0 µmol Si m^{-2} h^{-1}) to bottom water (Fig. 2C).

The variation coefficients of the measured solute fluxes were large (0.17–1.32), suggesting pronounced small-scale variability of fluxes. Total macrofauna biomass explained 19–34% of the variability in O_2, NH_4^+, and NO_2^- fluxes with marginal significance level, but was not related to the transport of NO_3^-, DIP and DSi (Fig. 3).

3.3 Nitrate reduction in whole core incubations and its relationship with macrofauna biomass

Sedimentary NO_3^- reduction processes (denitrification and DNRA) were primarily (88–96%) fuelled by NO_3^- produced within sediments via nitrification (Fig. 4). The contribution of NO_3^- diffusion from the overlaying bottom water (D_w and $DNRA_w$) was of minor importance. D_n rates differed by a factor of two and ranged between 23.7 and 52.9 μmol N m^{-2} h^{-1}. D_w varied between 1.2 and 6.2 μmol N m^{-2} h^{-1}. DNRA accounted for 18–23% of the total measured NO_3^- reduction, and was in the range of 1.4–26.0 μmol N m^{-2} h^{-1} (Fig. 4B, C). Both $DNRA_w$ (0.1–3.9 μmol N m^{-2} h^{-1}) and $DNRA_n$ (1.2–2.4 μmol N m^{-2} h^{-1}) were variable among the cores, without any significant correlation with macrofauna biomass. Only the D_w was significantly related to the biomass of macrofaunal community (Fig. 5). Anammox rates were negligible in present study.

3.4 Partitioning of the macrofauna effects on N cycling processes and fluxes

Our distLM model explained 29.1% (sum of all canonical eigenvalues) of the total variation in NO_3^- reduction processes and fluxes using the biomass of the three dominant macrofauna taxa *L. balthica, M. affinis* and *Marenzelleria* sp. The rest of macrofauna, mainly represented by oligochaetes, was excluded from further model development due to its negligible contribution. Marginal test revealed that *M. affinis* and *Marenzelleria* sp. were significant (F = 2.2, p = 0.035 and F = 2.1, p = 0.044, respectively) in the distLM model, while *L. balthica* was marginally significant (F = 2.0, p = 0.055). Sequential tests showed that solely *M. affinis* had a significant effect (F = 2.2, p = 0.032) on variation of process rates and fluxes, contributing to the 12% of the total variability. *Marenzelleria* sp. (F = 0.8, p = 0.6) and *L. balthica* (F = 1.9, p = 0.08) explained numerically lower and marginally significant part of the total variation (6% and 11%, respectively) when individually included into the model. The main two axes, identified by the model, explained nearly 83% of the total variability.

The db-RDA depicted that net fluxes of DIP and NO_3^- reduction coupled to nitrification ($DNRA_n$ and D_n) were strongly associated to the biomass of *M. affinis* (Fig. 6). Nitrate reduction (D_w and $DNRA_w$) fuelled by NO_3^- from the overlaying water column and DSi fluxes were positively and strongly correlated to the biomass of *Marenzelleria* sp. The net O_2, NO_3^- and NH_4^+ fluxes were associated with *L. balthica*.

3.5 Respiration and N-cycling associated with animal holobionts

Measured animal respiration was in the range of 9.5–52.3 μmol O_2 g_{dw}^{-1} h^{-1} with significantly higher (SNK test, $p < 0.05$) rates observed for *M. affinis* as compared to the other two species (Fig. 7A). NH_4^+ production via animal excretion varied between 0.05 and 3.3 μmol N g_{dw}^{-1} h^{-1}. The results showed marginally significant (SNK test, p = 0.052) differences in NH_4^+ excretion rates among macrofauna species, peaking in *L. balthica*.

The highest denitrification rates (SNK test, $p < 0.05$) were measured in *M. affinis* followed by *L. balthica* and *Marenzelleria* sp. (Fig. 7B). Mean denitrification rates were not significantly different (t-test, $p > 0.05$) between low and high $^{15}NO_3^-$ levels in *M. affinis* and *Marenzelleria* sp., suggesting that the IPT assumptions were fulfilled. The same was not true for *L. balthica* incubations (Mann-Whitney Rank Sum Test, $p = 0.016$). For the latter taxa, we remark methodological difficulties when dealing with incubations of animals that due to the stress of manipulation or to the absence of sediment keep the shells closed, which limits tracer diffusion into their body. Similar to denitrification, significantly higher DNRA rates were observed in *M. affinis* (SNK test, $p < 0.05$), where they accounted for ~ 7% of the total measured NO_3^- reduction. Additions of combined $^{15}NH_4^+$ and $^{14}NO_3^-$ revealed measurable $^{29}N_2$ production, suggesting the presence of putative anammox in *M. affinis* and *L. balthica* holobionts. However, such process contributed by less than 0.5% to the total N_2 production (data not shown). N_2 fixation was observed in *L. balthica* and *Marenzelleria* sp. holobionts, with tendency towards higher rates in the former species (Fig. 7B), but the difference in the mean rates was not statistically significant (t-test, $p > 0.05$). Dissection of different clam body parts for $\delta^{15}N$ analysis indicated that N_2 fixation was likely occurring in the animal gut. N_2 fixation rates were below the detection limit of the method in *M. affinis*.

4. Discussion

4.1. The effect of benthic community on biogeochemical processes

We studied benthic respiration, net N fluxes and N-related microbial processes in sediments colonized by a relatively simple macrofaunal community, hosting three different functional groups. We aimed at understanding how the interplay among these functional groups affects specific-pathways of the benthic N-cycling. While the effects of individual functional groups are quite well understood (Mermillod-Blondin and Rosenberg 2006; Stief 2013, and references therein), relatively little is known on their aggregated effect in a whole community context. The three taxa investigated comprised 70% of the total macrofauna abundance and 98% of the total biomass in the low diversity benthic community of the Öre estuary, and explained nearly 30% of variation of the measured biogeochemical processes. Other environmental factors driven by hydrological or watershed attributes, such as sediment characteristics (C_{org}, redox state, and electron acceptor availability) as well as their spatial heterogeneity, are expected to account for the remaining variability (e.g. Gammal et al. 2018).

Single animal incubations allowed to quantify the direct contribution of macrofauna to O_2 respiration, which corresponded to nearly 22% of the total benthic O_2 uptake. *Marenzelleria* sp. alone accounted for nearly half of macrofaunal community respiration, indicating it as a keystone species in benthic metabolism. On the other hand, following multivariate analysis results, which address the community interaction with processes, the net O_2 flux was primarily correlated with higher density of *L. balthica*, likely due to molluscs-related indirect effects (i.e. stimulation of microbial community within sediments).

Although clams are semi-mobile subsurface dwelling organisms, and cannot produce comparable effects on solute exchange as polychaetes or amphipods (e.g. Michaud et al. 2005), their biodeposition of faeces may significantly stimulate the activity of microbial community in surface sediments, ultimately increasing the species role in benthic metabolism (Karlson et al. 2005).

It is difficult, however, to infer on the pathways that use O_2 in this benthic habitat, but oxidized surface sediment and the absence of dissolved free sulphides in pore water in the upper 10 cm (see also Lenstra et al. 2018) may indicate O_2 consumption via organic matter mineralisation, NH_4^+ oxidation to NO_3^- via nitrification or oxidation of metals (e.g. Mn^{2+} or Fe^{2+}). The vectors overlay in db-RDA (O_2, NH_4^+ and NO_3^- flux) support the idea that O_2 is consumed for NH_4^+ production and its subsequent oxidation. Considering NO_x^- efflux and NO_3^- reduction together, we can estimate how much O_2 was consumed for NH_4^+ oxidation, and mineralization within sediments (Fig. 8). As 2 moles of O_2 are used to oxidize 1 mole of NH_4^+ to NO_3^- then nitrification accounts for 11% of total O_2 uptake (70.2–206.3 μmol O_2 m^{-2} h^{-1}) in these sediments. Such O_2 demand for nitrification is among the highest in relative terms (as % of total O_2 consumption) among those reported in studies in the Baltic Sea using the IPT (< 6%; Karlson et al. 2005; Bonaglia et al. 2014; Bartoli et al. 2021), indicating favourable conditions for nitrifying bacteria. Besides the amount used to oxidize NH_4^+, a residual 67% of total O_2 uptake, corresponding to 949.0 ± 50.4 μmol O_2 m^{-2} h^{-1}, was likely used for organic N ammonification. Assuming 1 molar ratio between CO_2 and O_2 as the respiratory quotient and measured C/N molar ratio of 10.5 in upper sediments, then theoretical ammonification rates average 90.4 ± 1.4 μmol N m^{-2} h^{-1}. Alternatively, macrofaunal community directly via excretion supplied 28.3 ± 4.1 μmol N m^{-2} h^{-1}, which accounts for ~ 24% of total NH_4^+ production (excretion + ammonification). Macrofauna excretion, on the other hand, was equivalent to 90% of the measured NH_4^+ efflux at the sediment-water interface (Fig. 8). Among the different regressions between net fluxes and whole macrofaunal community biomass, those of net O_2 and NH_4^+ fluxes revealed a consistent trend. This was partly expected for the above mentioned reasons of direct macrofauna contribution to O_2 consumption and NH_4^+ production. Thus, we may consider that most of the NH_4^+ excreted by macrofauna was likely transferred to bottom waters, whereas another fraction, primarily regenerated via organic matter ammonification, was oxidized, assimilated or retained within sediments (Fig. 8). The results further show that clams alone contributed for up to 55% of measured excretion by the community. Our single macrofauna incubation suggests that bivalves like *L. balthica* excreted higher amounts of NH_4^+ per unit biomass when compared to other functional groups, as reported also in other studies (Vanni et al. 2017).

In the studied habitat, nearly 63% of all regenerated NH_4^+ was further oxidized to NO_x^- via nitrification (Fig. 8). Without continuous NH_4^+ supply via ammonification, excretion and/or upward transport from deeper layer such active nitrification would rapidly deplete the standing pool of NH_4^+ in the upper 2 cm sediment layer (473 μmol m^{-2}; Bartl et al. 2019). Multivariate analysis shows that *M. affinis* primarily

stimulated NO_3^- reduction pathways coupled to nitrification (D_n, $DNRA_n$), whereas *Marenzelleria* sp., due to NO_3^- downward transport from bottom water, stimulated the reduction of water column nitrate (D_w). Surface deposit dweller *M. affinis* actively mixes the uppermost sediment layer, and thereby increases solute exchange and sediment oxygenation (Viitasalo-Frösén et al. 2009). This is also supported by our db-RDA results on positive DIP relationship with *M. affinis*. In contrast, *Marenzelleria* sp., capable for construction and bioirrigation of relatively deep burrows, likely facilitated D_w and $DNRA_w$, in deeper layers. Similar effects by amphipods and polychaetes on NO_3^- reduction pathways were previously observed in experimental studies where individual functional groups were analyzed (Tuominen et al. 1999; Karlson et al. 2005; Bonaglia et al. 2013). Although macrofauna functional groups can produce different or contrasting effects on NO_3^- reduction pathways, these are largely regulated by NO_3^- availability (Karlson et al. 2005, 2007; Bonaglia et al. 2013; Nogaro and Burgin 2014; Murphy et al. 2016).

In a recent work by Hellemann et al. (2017) it was questioned whether the oligotrophic Öre estuary is functioning as a filter for inorganic N. They suggest that due to limited water column mixing and short water residence time, land generated NO_3^- is not readily available to denitrifiers in sediments. The phytoplankton assimilation and later sedimentation to the bottom is a likely possible link between surface and bottom layers (Bartl et al. 2019). Although particulate N associated to sedimented detritus is recycled to NH_4^+ and subsequently oxidized to NO_3^-, only a fraction of N is removed from the ecosystem through coupled nitrification-denitrification. Denitrification efficiency, which is the ratio between N_2 production and the sum of N_2 and DIN (NH_4^++NO_x^-) effluxes (Eyre and Ferguson 2009), was relatively low (40% on average), and suggested that ~ 60% of recycled organic particulate N to DIN was released back to bottom water where it could be assimilated or nitrified (Bartl et al. 2019). Only a minor fraction of inorganic N was later removed through NO_3^- diffusion into anoxic sediment layer (D_w and $DNRA_w$). Denitrification efficiency tended to decrease when plotted as a function of total macrofauna biomass (data not shown), suggesting that excretion and recycling had a relatively higher importance as compared to the stimulation of N losses. Overall, the stoichiometry of regenerated benthic inorganic nutrient (N (flux + D_n+$DNRA_n$) : Si (flux) : P (flux) = 28:52:1) suggested excess of N and Si, and P limitation. Such ecological nutrients stoichiometry is likely favoured by the activity of functionally diverse macrofaunal community. *M. affinis* and *Marenzelleria* sp. act as sediment re-workers in two distinct sediment layers, mixing fresh and old organic particles, and thus priming organic matter mineralization. This is evident by the active ammonification within sediments (Fig. 8). Whereas *L. balthica* regenerates part of the filtered/digested particulate N via its own excretion. Taken together all these community actions facilitate N accumulation in bottom water. While reworking sediments, the two burrowers maintain sediment oxidized, as suggested by limited reactive P release to the water column, despite active mineralization, and ultimately indicating the regeneration of geochemical buffers by macrofauna (e.g. oxidized metals).

4.2 Effects of macrofauna-microbes Holobionts to soft-bottom habitat functioning

Macrofauna, besides reworking sediments and ventilating burrows also hosts a complex microbiomes, which can be unique assemblage or inoculated from surrounding environment during feeding or burrowing activities (Poulsen et al. 2014; Ceullar-Gempeler and Leibold 2018; Hochstein et al. 2019; Samuiloviene et al. 2019; Marzocchi et al. 2021). Here, we show that bacteria associated with three key Baltic macrofauna species were metabolically diverse and capable of different N transformations such as denitrification, DNRA and/or N_2 fixation. Scaling up these process rates with the average macrofauna biomass at the study area, we were able to estimate the contribution of holobionts to multiple N-cycling pathways (Fig. 8). The holobionts of whole benthic community based on biomass estimations together contributed 1% of denitrification and 0.1% of DNRA measured in sediments, which was estimated to be 0.4 μmol N m^{-2} h^{-1}. The holobionts of clams and polychaetes were also capable for N_2 fixation (0.03 and 0.01 μmol N m^{-2} h^{-1}, respectively) (Fig. 8). Although the holobiont contribution to the N-cycling pathways in sediments is marginal in this study, this might be different in other seasons or habitats characterized by much higher macrofauna densities or larger specimens. As the abundance of this soft-bottom community can be higher in the other Baltic Sea areas (Gogina and Zettler 2010), we may also expect there higher holobionts contribution to the benthic N-cycling. However, it remains questionable to what extent our upscaling reflects *in situ* rates of N transformations in holobionts as we incubated adult macrofauna individuals while community includes different generation specimens (adults and juveniles), and for upscaling was used biomass data. Notably, the holobionts of *M. affinis* due to associated high denitrification rates and abundance can contributed up to ~ 8% of NO_3^- reduction (3.7 μmol N m^{-2} h^{-1}) if abundance data are used for calculations. To better understand the biomass- and abundance-specific effects on microbial processes in holobionts, measurements of the appropriate metabolic pathways should be carried out across different host age classes and biotic metrics in future studies.

Denitrification rates in animal holobionts from the Öre estuary were at least 66% higher than those measured at similar temperatures in chironomid larvae (*C. plumosus*; Politi et al. 2021). Added tracer concentrations markedly differed between these two experiments, with 2-fold higher concentrations in *C. plumosus*, which indicates much higher metabolic activity under lower NO_3^- concentration in *M. affinis* holobionts. The possible explanations for different rates might be attributed to variable number of denitrifying bacteria in hosts as well as their affinity for NO_3^- or quality of available carbon. Since these hosts occupy different niches in sediments and microbial communities display steep vertical zonation in sediments, a different bacteria with unique metabolic features originates in holobionts. Moreover, our finding of high denitrification rates associated with *M. affinis* was rather surprising given that transcription of denitrification marker genes, encoding NO_2^- reduction and its derivatives, typically occurs under low O_2 conditions (Härtig and Zumft 1999), similar to those in macrofaunal gut (Stief and Eller 2006; Bonaglia et al. 2017). We therefore expected higher activity of denitrifying bacteria in larger clam or polychaete gut. This allows to infer that animal body weight (or size) do not necessarily constrains bacterial activity. NO_3^- was also reduced via DNRA pathway, but this process was quantitatively less important. The re-occurrence of DNRA confirms that NH_4^+ production, associated with animals, is a complex process driven by both animal excretion and the activity of associated DNRA bacteria (Zilius et

al. 2020; Politi et al. 2021). However, bacterial NH_4^+ production in studied macrofauna remains quantitatively smaller as compared to excretion (Fig. 8).

Measured diazotrophic activity in animal holobionts from the oligotrophic Öre estuary was in the range of that observed in other animals from other eutrophic estuarine systems of the Baltic Sea (21.9–517.1 nmol g_{DW}^{-2} d^{-1}; Marzocchi et al. 2021; Politi et al. 2021). In line with these studies, N_2 fixation resulted markedly higher in filter-feeding macrofauna. However, such feeding mode does not necessarily favour N_2 fixation, as for example *L. balthica* can shift between suspension and deposit feeding (Riisgård and Kamermans 2001). Due to various feeding modes diazotrophic bacteria can inhabit different body parts including gills, foot, gut or body surface as has been seen from other N cycling pathways (Heisterkamp et al. 2013). Overall, the finding of diazotrophic activity in holobionts at different coastal sites of the Baltic Sea suggests that this process is widespread across diverse taxa of benthic macrofauna even when N concentrations are not limiting (Marzocchi et al. 2021; Politi et al. 2021). In future, more studies should address environmental or biological factors that regulate diazotrophic activity across different functional groups of macrofauna.

CONCLUSIVE REMARKS

In the studied area, the simplified macrofaunal community via its own metabolic activity, and to a lesser extend via associated microbiota, contributed to the benthic metabolism and N-cycling (direct effect), and reworked sediments with positive feedback to N mineralization, oxidation and reduction processes (indirect effect). Taken together, these functions promoted an excellent re-use of nutrient at the benthic level, a moderate loss and release to bottom water. The ecological stoichiometry of regenerated nutrient indicated N and Si excess, and P limitation, which is likely a consequence of the interplay among functional groups in the macrofaunal community, and maintain oligotrophic conditions this area. Such view of the benthic system, in the perspective of the whole system functioning, and of the stoichiometry of nutrient regeneration, can shed new light on macrofauna diversity and ecosystem functioning relationships along multiple environmental and human pressure gradients. Results of this study suggest that all macrofauna functional groups interacted with microbial communities as rates of microbial N-transformations were measured in single macrofauna incubations. The role of holobionts in the Öre estuary was of minor importance as compared to the activity of sediment-associated microbial communities, but this might be different along seasons or habitats. Dramatic changes in macrofauna populations, in particular of *M. affinis*, have already been observed in the studied area (Eriksson-Wiklund and Andersson 2014). Such drop of an even single functional group, may eventually alter biogeochemical *functions or services* (e.g. coupled ammonification-nitrification-denitrification) in surface sediments.

Declarations

ACKNOWLEDGEMENTS

We gratefully thank Umeå Marine Science Center (UMF) for hosting the INBALANCE Team, and for providing lab facilities and sampling boat. We kindly thank Jan Albertsson for precious suggestions while selecting sampling sites, Irma Vybernaitė-Lubienė for laboratory analysis and Tobia Politi for data handling; and Bo Thamdrup for making analytical instrumentation available at University of Southern Denmark.

FUNDING

The study was supported by the “Invertebrate–Bacterial Associations as Hotpots of Benthic Nitrogen Cycling in Estuarine Ecosystems (INBALANCE)” project, which has received funding from the European Social Fund (project No. 09.3.3-LMT-K-712-01-0069) under grant agreement with the Research Council of Lithuania (LMTLT).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AVAILABILITY OF DATA AND MATERIAL

Data can be accessed upon request to the corresponding author.

AUTHOR CONTRIBUTIONS

M.Z., M.B., U.C. and S.B. conceived the ideas and designed methodology; M.Z., M.B., D.D., U.M. and U.C. carried out sampling and the incubation experiments; S.B. G.C. and U.M. carried out the mass spectrometric and animal measurements; U.C., U.M. and S.B. led the data analysis; M.Z. and M.B. wrote the first draft of the manuscript. All authors contributed to the discussion and interpretation of data, and revised and approved the manuscript for submission.

References

1. Anderson M, Gorley R, Clarke K (2008) PERMANOVA + for PRIMER: guide to software and statistical methods. PRIMER-E, Plymouth, p 214
2. Bartl I, Hellemann D, Rabouille Ch, Schulz K, Tallberg P, Hietanen S, Voss M (2019) Particulate organic matter controls benthic microbial N retention and N removal in contrasting estuaries of the Baltic Sea. Biogeosciences 16:3543–3564. https://doi.org/10.5194/bg-16-3543-2019
3. Bartoli M, Nizzoli D, Zilius M, Bresciani M, Pusceddu A, Bianchelli S, Sundbäck K, Razinkovas-Baziukas A, Viaroli P (2021) Denitrification, nitrogen uptake, and organic matter quality undergo different seasonality in sandy and muddy sediments of a turbid estuary. Front Microbiol 11:612700. https://doi.org/10.3389/fmicb.2020.612700
4. Benelli S, Bartoli M, Zilius M, Vybernaite-Lubiene I, Ruginis T, Petkuviene J, Fano EA (2018) Microphytobenthos and chironomid larvae attenuate nutrient recycling in shallow-water sediments.

Freshw Biol 63:187–201. https://doi.org/10.1111/fwb.13052

5. Bonaglia S, Bartoli M, Gunnarsson JS, Rahm L, Raymond C, Svensson O, Shakeri Yekta S, Brüchert V (2013) Effect of reoxygenation and Marenzelleria spp. bioturbation on Baltic Sea sediment metabolism. Mar Ecol Prog Ser 482:43–55. https://doi.org/10.3354/meps10232
6. Bonaglia S, Deutsch B, Bartoli M, Marchant HK, Bruchert V (2014) Seasonal oxygen, nitrogen and phosphorus benthic cycling along an impacted Baltic Sea estuary: regulation and spatial patterns. Biogeochemistry 119:139–160. https://doi.org/10.1007/s10533-014-9953-6
7. Bonaglia S, Klawonn I, De Brabandere L, Deutsch B, Thamdrup B, Brüchert V (2016) Denitrification and DNRA at the Baltic Sea oxic–anoxic interface: Substrate spectrum and kinetics. Limnol Oceanogr 61(5):1900–1915. https://doi.org/10.1002/lno.10343
8. Bonaglia S, Brüchert V, Callac N, Vicenzi A, Fru EC, Nascimento FJA (2017) Methane fluxes from coastal sediments are enhanced by macrofauna. Sci Rep 7:13145. https://doi.org/10.1038/s41598-017-13263-w
9. Bosch JA, Cornwell JC, Kemp WM (2015) Short-term effects of nereid polychaete size and density on sediment inorganic nitrogen cycling under varying oxygen conditions. Mar Ecol Progr Ser 524:155–169. https://doi.org/10.3354/meps11185
10. Braeckman U, Provoost P, Gribsholt B, Van Gansbeke D, Middelburg JJ, Soetaert K, Vincx M, Vanaverbeke J (2010) Role of macrofauna functional traits and density in biogeochemical fluxes and bioturbation. Mar Ecol Progr Ser 399:173–186. https://doi.org/10.3354/meps08336
11. Brydsten L, Jansson M (1989) Studies of estuarine sediment dynamics using 137Cs from Tjernobyl accident as tracer. Estuar Coast Shelf Sci 28:249–259
12. Cardini U, Bartoli M, Lee R, Luecker S, Mooshammer M, Polzin J, Weber M, Petersen J (2019) Chemosymbiotic bivalves contribute to the nitrogen budget of seagrass ecosystems. ISME J 13:3131–3134. https://doi.org/10.1038/s41396-019-0486-9
13. Cuellar-Gempeler C, Leibold MA (2018) Multiple colonist pools shape fiddler crab-associated bacterial communities. ISME J 12(3):825–837. https://doi.org/10.1038/s41396-017-0014-8
14. Clare DS, Spencer M, Robinson LA, Frid CLJ (2016a) Species-specific effects on ecosystem functioning can be altered by interspecific interactions. PLoS ONE 11(11):e01657. https://doi.org/10.1371/journal.pone.0165739
15. Clare DS, Spencer M, Robinson LA, Frid CLJ (2016b) Species densities, biological interactions and benthic ecosystem functioning: an in situ experiment. Mar Ecol Prog Ser 547:149–161. https://doi.org/10.3354/meps11650
16. Clarke K, Gorley R (2006) PRIMER v6: User manual/tutorial. PRIMER-E, Plymouth, p 192
17. Colt J (2012) Dissolved gas concentration in water: Computation as functions of temperature, salinity and pressure, 2nd edn. Elsevier, London
18. De Brabandere L, Bonaglia S, Kononets MY, Viktorsson L, Stigebrandt A, Thamdrup B, Hall POJ (2015) Oxygenation of an anoxic fjord basin strongly stimulates benthic denitrification and DNRA. Biogeochemistry 126(1–2):131–152. https://doi.org/10.1007/s10533-015-0148-6

19. Eriksson Wiklund A-K, Andersson A (2014) Benthic competition and population dynamics of Monoporeia affinis and Marenzelleria sp. in the northern Baltic Sea. Estuar Coast Shelf Sci 144:46–53. https://doi.org/10.1016/j.ecss.2014.04.008
20. Eyre BD, Ferguson AJP (2009) Denitrification efficiency for defining critical loads of carbon in shallow coastal ecosystems. In: Andersen JH, Conley DJ (eds). Eutrophication in Coastal Ecosystems: Towards better understanding and management strategies Selected Papers from the Second International Symposium on Research and Management of Eutrophication in Coastal Ecosystems, 20–23 June 2006, Nyborg, Denmark. Springer Netherlands, Dordrecht, pp 137–146
21. Gamfeldt L, Lefcheck JS, Byrnes JEK, Cardinale BJ, Duffy JE, Griffin JN (2014) Marine biodiversity and ecosystem functioning: what's known and what's next? Oikos 124:252–265. https://doi.org/10.1111/oik.01549
22. Gammal J, Järnström M, Bernard G, Norrko J, Norkko A (2018) Environmental context mediates biodiversity–ecosystem functioning relationships in coastal soft-sediment habitats. Ecosystems 22:137–151. https://doi.org/10.1007/s10021-018-0258-9
23. Gilbertson WW, Solan M, Prosser JI (2012) Differential effects of microorganism–invertebrate interactions on benthic nitrogen cycling. FEMS Microbiol Ecol 82(1):11–22. https://doi.org/10.1111/j.1574-6941.2012.01400.x
24. Gogina M, Nygård H, Blomqvist M, Daunys D, Josefson AB, Kotta J, Maximov A, Warzocha J, Yermakov V. Gräwe U, Zettler ML (2016) The Baltic Sea scale inventory of benthic faunal communities. ICES J Mar Sci 73(4):1196–1213. https://doi.org/10.1093/icesjms/fsv265
25. Gogina M, Zettler ML (2010) Diversity and distribution of benthic macrofauna in the Baltic Sea Data inventory and its use for species distribution modelling and prediction. J Sea Res 64:313–332. https://doi.org/10.1016/j.seares.2010.04.005
26. Grassoff M, Ehrhardt M, Kremling K (1983) Methods of Seawater Analysis, 2ed. Verlag Chemie, Weinheimm
27. Härtig E, Zumft WG (1999) Kinetics of nirS expression (cytochrome cd1 nitrite reductase) in *Pseudomonas stutzeri* during the transition from aerobic respiration to denitrification: evidence for a denitrification-specific nitrate- and nitrite-responsive regulatory system. J Bacteriol Res 181(1):161–166. https://doi.org/10.1128/JB.181.1.161-166.1999
28. Hedman EJ, Gunnarsson JS, Samuelsson G, Gilbert F (2011) Particle reworking and solute transport by the sediment-living polychaetes *Marenzelleria neglecta* and *Hediste diversicolor*. J Exp Mar Biol Ecol 407:294–301. https://doi.org/10.1016/j.jembe.2011.06.026
29. Heisterkamp IM, Schramm A, Larsen LH, Svenningsen NB, Lavik G, de Beer D, Stief P (2013) Shell biofilm-associated nitrous oxide production inmarine molluscs: processes, precursors andrelative importance. Environ Microbiol 15(7):1943–1955. https://doi.org/10.1111/j.1462-2920.2012.02823.x
30. Hellemann D, Tallberg P, Bartl I, Voss M, Hietanen S (2017) Denitrification in an oligotrophic estuary: a delayed sink for riverine nitrate. Mar Ecol Progr Ser 583:63–80. https://doi.org/10.3354/meps12359

31. Hochstein R, Zhang Q, Sadowsky JM, Forbes VE (2019) The deposit feeder Capitella teleta has a unique and relatively complex microbiome likely supporting its ability to degrade pollutants. Sci Total Environ 670:547–554. https://doi.org/10.1016/j.scitotenv.2019.03.255
32. Kana TM, Darkangelo C, Hunt MD, Oldham JB, Bennett GE, Cornwell JC (1994) Membrane inlet mass spectrometer for rapid high-precision determination of N_2, O_2, and Ar in environmental water samples. Anal Chem 66(23):4166–4170. https://doi.org/10.1021/ac00095a009
33. Karlson K, Hulth S, Ringdahl K, Rosenberg R (2005) Experimental recolonisation of Baltic Sea reduced sediments: survival of benthic macrofauna and effects on nutrient cycling. Mar Ecol Progr Ser 294:35–49. https://doi.org/10.3354/meps294035
34. Karlson K, Hulth S, Rosenberg R (2007) Density of *Monoporeia affinis* and biogeochemistry in Baltic Sea sediments. J Exp Mar Biol Ecol 344(2):123–135. https://doi.org/10.1016/j.jembe.2006.11.016
35. Kauppi L, Bernard G, Bastrop R et al (2018) Increasing densities of an invasive polychaete enhance bioturbation with variable effects on solute fluxes. Sci Rep 8:7619. https://doi.org/10.1038/s41598-018-25989-2
36. Klawonn I, Lavik G, Böning P, Marchant H, Dekaezemacker J, Mohr W, Ploug H (2015) Simple approach for the preparation of $^{15-15}N_2$-enriched water for nitrogen fixation assessments: evaluation, application and recommendations. Front Microbiol 6:769. https://doi.org/10.3389/fmicb.2015.00769
37. Kristensen E (2000) Organic matter diagenesis at the oxic/anoxic interface in coastal marine sediments, with emphasis on the role of burrowing animals. Hydrobiologia 426:1–24. https://doi.org/10.1023/A:1003980226194
38. Kristensen E (2001) Impact of polychaetes (*Nereis* spp. and *Arenicola marina*) on carbon biogeochemistry in coastal marine sediments. Geochem Trans 2:92. https://doi.org/10.1186/1467-4866-2-92
39. Kristensen E et al (2012) What is bioturbation? The need for a precise definition for fauna in aquatic sciences. Mar Ecol Prog Ser 446:285–302. https://doi.org/10.3354/meps09506
40. Kristensen E, Delefosse M, Quintana CO, Flindt MR, Valdemarsen T (2014) Influence of benthic macrofauna community shifts on ecosystem functioning in shallow estuaries. Front Mar Sci 1:44. https://doi.org/10.3389/fmars.2014.00041
41. Laverock B, Tait K, Gilbert JA, Osborn AM, Widdicombe S (2014) Impacts of bioturbation on temporal variation in bacterial and archaeal nitrogen–cycling gene abundance in coastal sediments. Environ Microbiol Rep 6:113–121. https://doi.org/10.1111/1758-2229.12115
42. Lenstra WK, Egger M, van Helmond NAGM, Kritzberg E, Conley DJ, Slomp CP (2018) Large variations in iron input to an oligotrophic Baltic Sea estuary: impact on sedimentary phosphorus burial. Biogeosciences 15:6979–6996. https://doi.org/10.5194/bg-15-6979-2018
43. Lohrer AM, Thrush SF, Gibbs MM (2004) Bioturbators enhance ecosystem function through complex biogeochemical interactions. Nature 431:1092–1095. https://doi.org/10.1038/nature03042

44. Marzocchi U, Bonaglia S, Zaiko A, Quero GM, Vybernaite-Lubiene I, Politi T, Samuiloviene A, Zilius M, Bartoli M, Cardini U (2021) Zebra mussel holobionts fix and recycle nitrogen in lagoon sediments. Front Microbiol 11:610269. https://doi.org/10.3389/fmicb.2020.610269
45. Mermillod-Blondin F, Rosenberg R (2006) Ecosystem engineering: the impact of bioturbation on biogeochemical processes in marine and freshwater benthic habitats. Aquat Sci 68:434–442. https://doi.org/10.1007/s00027-006-0858-x
46. Moraes PC, Zilius M, Benelli S, Bartoli M (2018) Nitrification and denitrification in estuarine sediments with tube-dwelling benthic animals. Hydrobiologia 819:217–230. https://doi.org/10.1007/s10750-018-3639-3
47. Murphy EAK, Reidenbach MA (2016) Oxygen transport in periodically ventilated polychaete burrows. Mar Biol 163:208. https://doi.org/10.1007/s00227-016-2983-y
48. Nogaro G, Burgin AJ (2014) Influence of bioturbation on denitrification and dissimilatory nitrate reduction to ammonium (DNRA) in freshwater sediments. Biogeochemistry 120:279–294. https://doi.org/10.1007/s10533-014-9995-9
49. Nakamura Y, Kerciku F (2000) Effects of filter-feeding bivalves on the distribution of water quality and nutrient cycling in a eutrophic coastal lagoon. J Mar Syst 26(2):209–221. https://doi.org/10.1016/S0924-7963(00)00055-5
50. Naldi M, Nizzoli D, Bartoli M, Viaroli P, Viaroli P (2020) Effect of filter-feeding mollusks on growth of green macroalgae and nutrient cycling in a heavily exploited coastal lagoon. Estuar Coast Shelf Sci 239:106679. https://doi.org/10.1016/j.ecss.2020.106679
51. Nielsen OI, Gribsholt B, Kristensen E, Revsbech NP (2004) Microscale distribution of oxygen and nitrate in sediment inhabited by *Nereis diversicolor*: spatial patterns and estimated reaction rates. Aquat Microb Ecol 34:23–32. https://doi.org/10.3354/ame034023
52. Norkko J, Gammal J, Hewitt JE et al (2015) Seafloor Ecosystem Function Relationships: In Situ Patterns of Change Across Gradients of Increasing Hypoxic Stress. Ecosystems 18:1424–1439. https://doi.org/10.1007/s10021-015-9909-2
53. Pischedda L, Poggiale JC, Cuny P, Gilbert F (2008) imaging oxygen distribution in marine sediments. The importance of bioturbation and sediment heterogeneity. Acta Biotheor 56:123–135. https://doi.org/10.1007/s10441-008-9033-1
54. Politi T, Zilius M, Castaldelli G, Bartoli M, Daunys D (2019) Estuarine macrofauna affects benthic biogeochemistry in a hypertrophic lagoon. Water 11(6):1186. https://doi.org/10.3390/w11061186
55. Politi T, Barisevičiūte R, Bartoli M, Bonaglia S, Cardini C, Castaldelli G et al (2021) A bioturbator, a holobiont and a vector: the multifaceted role of *Chironomus plumosus* in shaping N-cycling. Freshw Biol (online). https://doi.org/10.1111/fwb.13696
56. Poulsen M, Kofoed MV, Larsen LH, Schramm A, Stief P (2014) *Chironomus plumosus* larvae increase fluxes of denitrification products and diversity of nitrate-reducing bacteria in freshwater sediment. Syst Appl Microbiol 37:51–59. https://doi.org/10.1016/j.syapm.2013.07.006

57. Ray NE, Henning MC, Fulweiler RW (2019) Nitrogen and phosphorus cycling in the digestive system and shell biofilm of the eastern oyster *Crassostrea virginica*. Mar Ecol Prog Ser 621:95–105. https://doi.org/10.3354/meps13007
58. Riisgård HU, Kamermans P (2001) Switching between deposit and suspension feeding in coastal zoobenthos. In: Reise K (ed) Ecological Comparisons of Sedimentary Shores. Ecological Studies (Analysis and Synthesis), vol 151. Springer, Berlin, pp 73–101. https://doi.org/10.1007/978-3-642-56557-1_5
59. Risgaard-Petersen N, Nielsen LP, Rysgaard S, Dalsgaard T, Meyer RL (2003) Application of the isotope pairing technique in sediments where anammox and denitrification coexist. Limnol Oceanogr-Meth 1:63–73. https://doi.org/0.4319/lom.2004.2.315
60. Samuiloviene A, Bartoli M, Bonaglia S, Cardini U, Vybernaite-Lubiene I, Marzocchi U, Petkuviene J, Politi T, Zaiko A, Zilius M (2019) The effect of chironomid larvae on nitrogen cycling and microbial communities in soft sediments. Water 11:1931. https://doi.org/10.3390/w1109193
61. Smyth AR, Murphy AE, Anderson IC, Song B (2018) Differential effects of bivalves on sediment nitrogen cycling in a shallow coastal bay. Estuaries Coast 41:1147–1163. https://doi.org/10.1007/s12237-017-0344-9
62. Stief P, Eller G (2006) The gut microenvironment of sediment-dwelling *Chironomus plumosus* larvae as characterised with O_2, pH, and redox microsensors. J Comp Physiol B 176:673–683. https://doi.org/10.1007/s00360-006-0090-y
63. Stief P (2013) Stimulation of microbial nitrogen cycling in aquatic ecosystems by benthic macrofauna: mechanisms and environmental implications. Biogeosciences 10:7829–7846. https://doi.org/10.5194/bg-10-7829-2013
64. Thamdrup B, Dalsgaard T (2002) Production of N_2 through anaerobic ammonium oxidation coupled to nitrate reduction in marine sediments. App Environ Microbiol 68(3):1312–1318. doi:10.1128/AEM.68.3.1312-1318.2002
65. Thrush SF, Hewitt JE, Gibbs M, Lundquist C, Norkko A (2006) Functional role of large organisms in intertidal communities: community effects and ecosystem function. Ecosystems 9:1029–1040. https://doi.org/10.1007/s10021-005-0068-8
66. Tuominen L, Mäkelä K, Lehtonen KK, Haahti H, Hietanen S, Kuparinen J (1999) Nutrient fluxes, porewater profiles and denitrification in sediment influenced by algal sedimentation and bioturbation by *Monoporeia affinis*. Estuar Coast Shelf Sci 49(1):83–97. https://doi.org/10.1006/ecss.1999.0492
67. Vanni MJ, McIntyre PB, Allen D et al (2017) A global database of nitrogen and phosphorus excretion rates of aquatic animals. Ecology 98:1475–1475. https://doi.org/10.1002/ecy.1792
68. Vasquez-Cardenas D, Quintana CO, Meysman FJR, Kristensen E, Boschker HTS (2016) Species-specific effects of two bioturbating polychaetes on sediment chemoautotrophic bacteria. Mar Ecol Prog Ser 549:55–68. https://doi.org/10.3354/meps11679
69. Viitasalo S, Laine A, Lehtiniemi M (2009) Habitat modification mediated by motile surface stirrers versus semi-motile burrowers: Potential for a positive feedback mechanism in a eutrophied

ecosystem. Mar Ecol Progr Ser 376:21–32. https://doi.org/10.3354/meps07788

70. Villnas A, Hewitt J, Snickars M, Westerbom M, Norkko A (2018) Template for using biological trait groupings when exploring large-scale variation in seafloor multifunctionality. Ecol Appl 28(1):78–94. https://doi.org/10.1002/eap.1630
71. Voss M, Asmala E, Bartl I et al (2020) Origin and fate of dissolved organic matter in four shallow Baltic Sea estuaries. Biogeochemistry. https://doi.org/10.1007/s10533-020-00703-5
72. Warembourg FR (1993) Nitrogen fixation in soil and plant systems. In: Knowles R, Blackburn TH (eds) Nitrogen isotope techniques. Academic Press, San Diego, pp 127–156
73. Welsh DT, Nizzoli D, Fano EA, Viaroli P (2015) Direct contribution of clams (*Ruditapes philippinarum*) to benthic fluxes, nitrification, denitrification and nitrous oxide emission in a farmed sediment. Estuar Coast Shelf Sci 154:84–93. https://doi.org/10.1016/j.ecss.2014.12.021
74. Yazdani Foshtomi M, Leliaert F, Derycke S, Willems A, Vincx M, Vanaverbeke J (2018) The effect of bio-irrigation by the polychaete *Lanice conchilega* on active denitrifiers: Distribution, diversity and composition of nosZ gene. PLoS ONE 13(2):e0192391. https://doi.org/10.1371/journal.pone.0192391
75. Zilius M, Bonaglia S, Broman E et al (2020) N_2 fixation dominates nitrogen cycling in a mangrove fiddler crab holobiont. Sci Rep 10:13966. https://doi.org/10.1038/s41598-020-70834-0

Figures

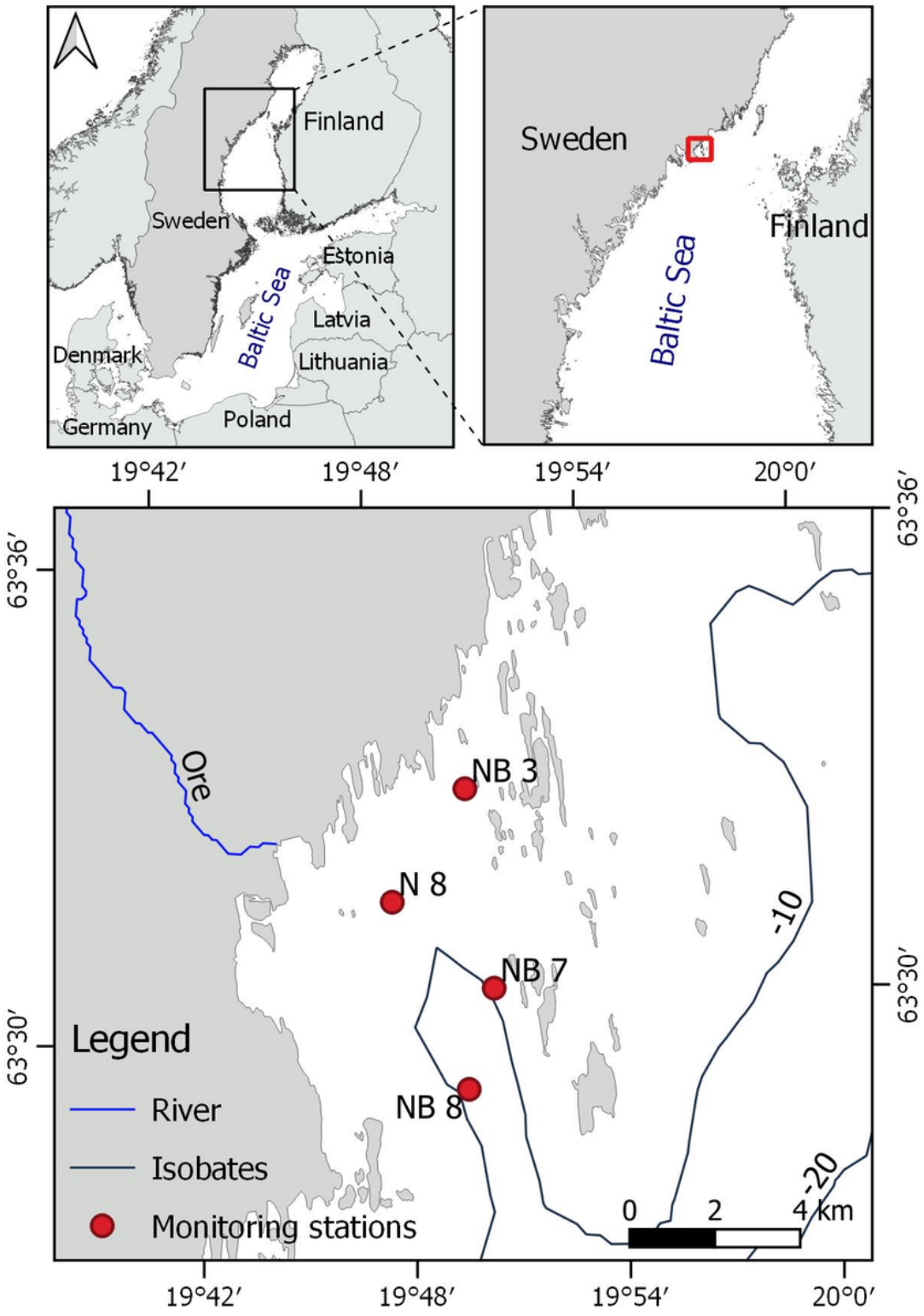

Figure 1

Map showing geographical location of Öre estuary with the sampling sites Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

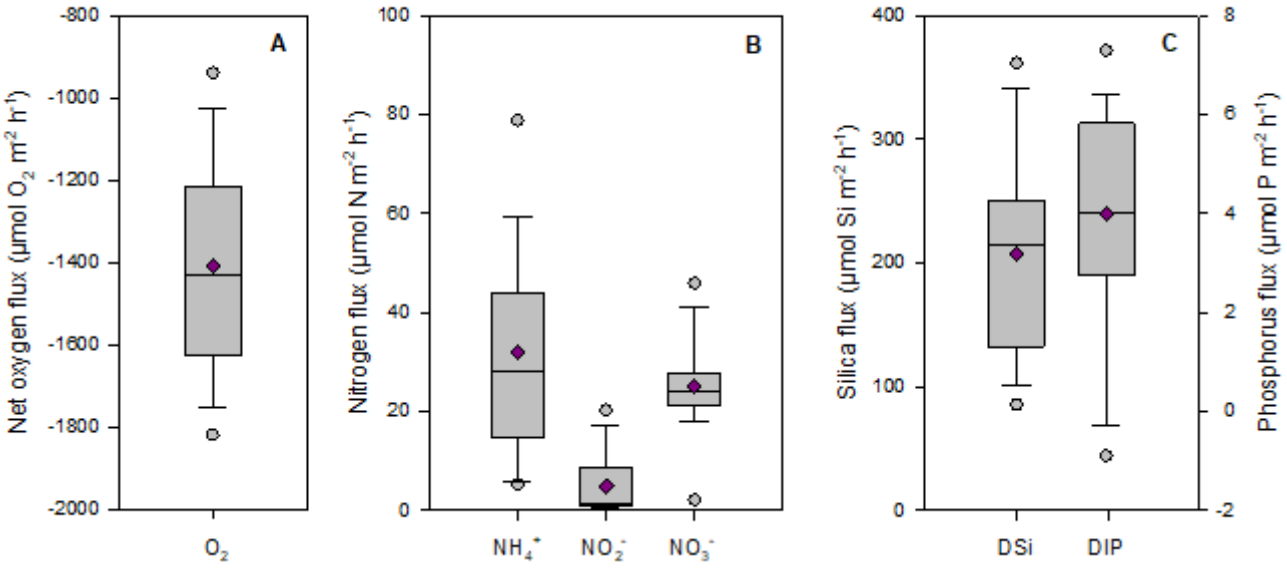

Figure 2

Net fluxes of dissolved oxygen (A), dissolved inorganic nitrogen (B), and silica and phosphorus (C) at the sediment–water interface. Data range (whiskers), upper and lower quartiles (edges), the median (horizontal line), and the mean (diamonds), and outliers (circles) are represented for 18 replicates

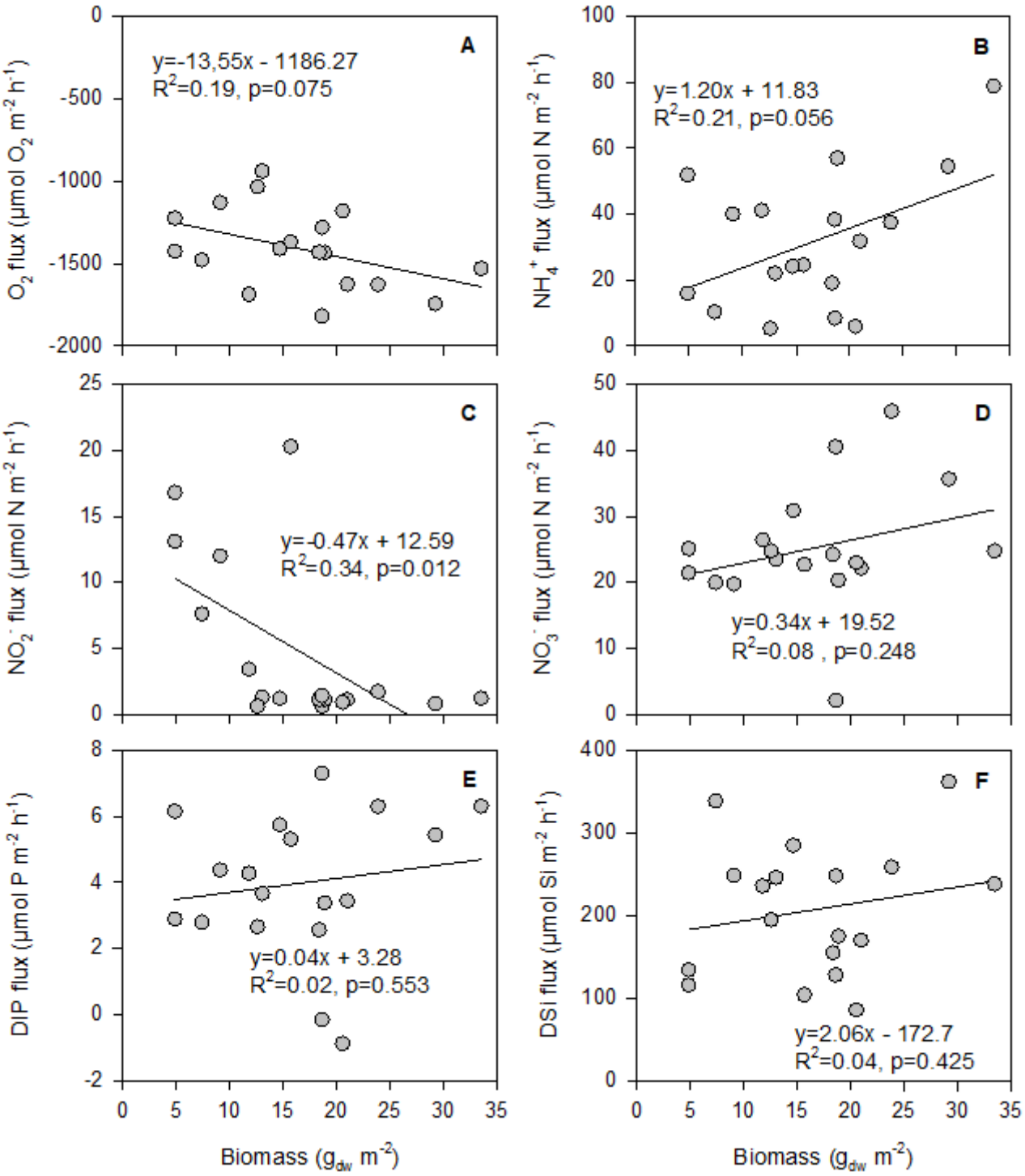

Figure 3

Linear regressions between macrofauna biomass and net fluxes of dissolved oxygen (A) ammonium (B), nitrite (C), nitrate (D), dissolved inorganic phosphorus (E) and silica (F) measured in whole core incubations (n=18)

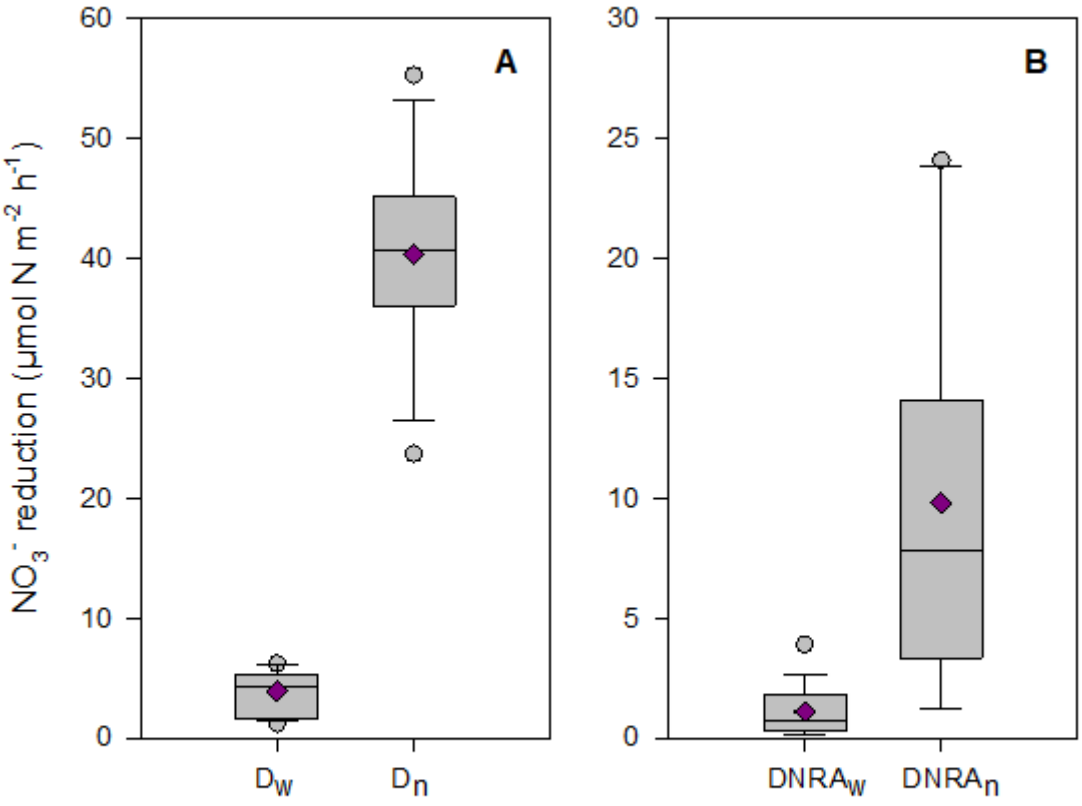

Figure 4

Denitrification of water column NO3− (Dw) and coupled nitrification-denitrification (Dn; A) and dissimilative nitrate reduction to ammonium of water column NO3− (DNRAw) and coupled nitrification-DNRA (DNRAn; B) measured in whole core incubations. Data range (whiskers), upper and lower quartiles (edges), the median (horizontal line), and the mean (dark pink diamond), and outliers (grey circle) are represented for n = 18 replicates

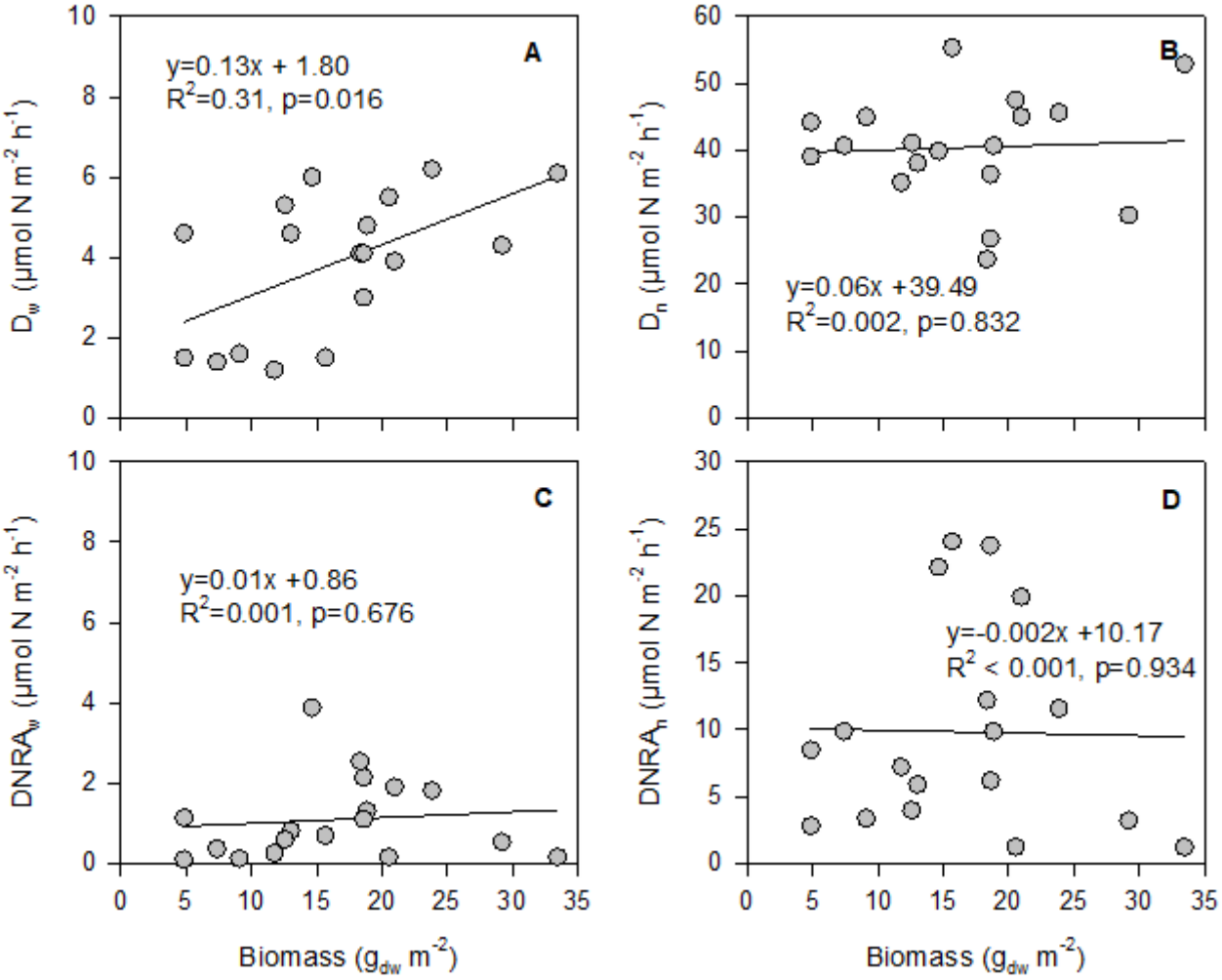

Figure 5

Linear regressions between total macrofauna biomass and NO3– reduction processes: denitrification of water column NO3– (A), coupled nitrification-denitrification (B), and dissimilative nitrate reduction to ammonium (DNRA) of water column NO3– (C) and coupled nitrification-DNRA (D) measured in whole core incubations (n = 18)

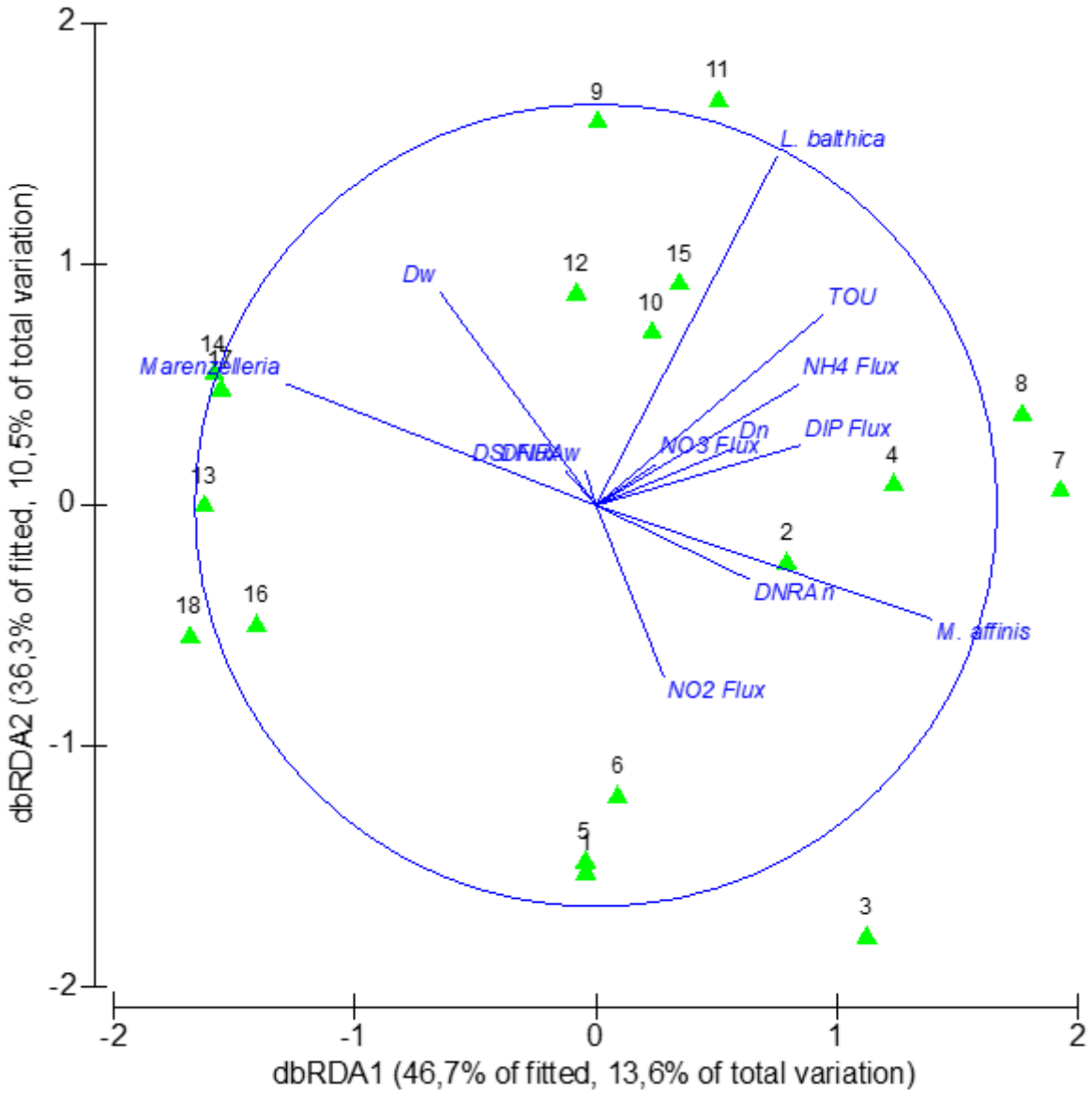

Figure 6

Distance based triplot of redundancy analysis (db-RDA) on fluxes (O2, NH4+, NO2−, NO3−, DIP, and DSi) and processes (denitrification – Dw, Dn, and dissimilative nitrate reduction to ammonium – DNRAn and DNRAw) in the Öre estuary, using the biomass of the dominant benthic macrofauna (L. balthica, M. affinis, Marenzelleria sp.) as explanatory variable. Numbers indicate single cores collected in the sampling area. The projection of any sample onto vectors approximates the measured value in that sample

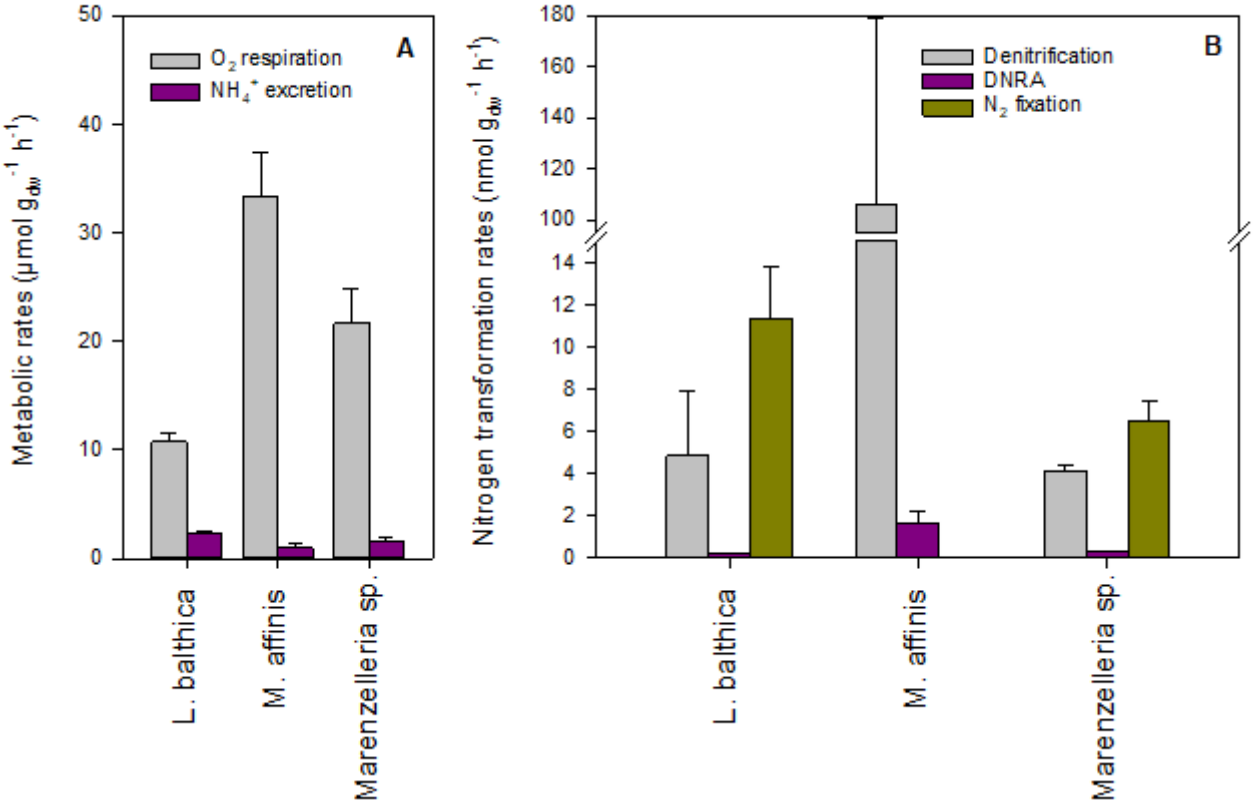

Figure 7

The rates of macrofauna metabolism (respiration and excretion) and associated microbial nitrogen transformations (denitrification, dissimilative nitrate reduction to ammonium (DNRA) and N2 fixation) are reported. Bars report means and standard errors based on replicates (n=3 to 10)

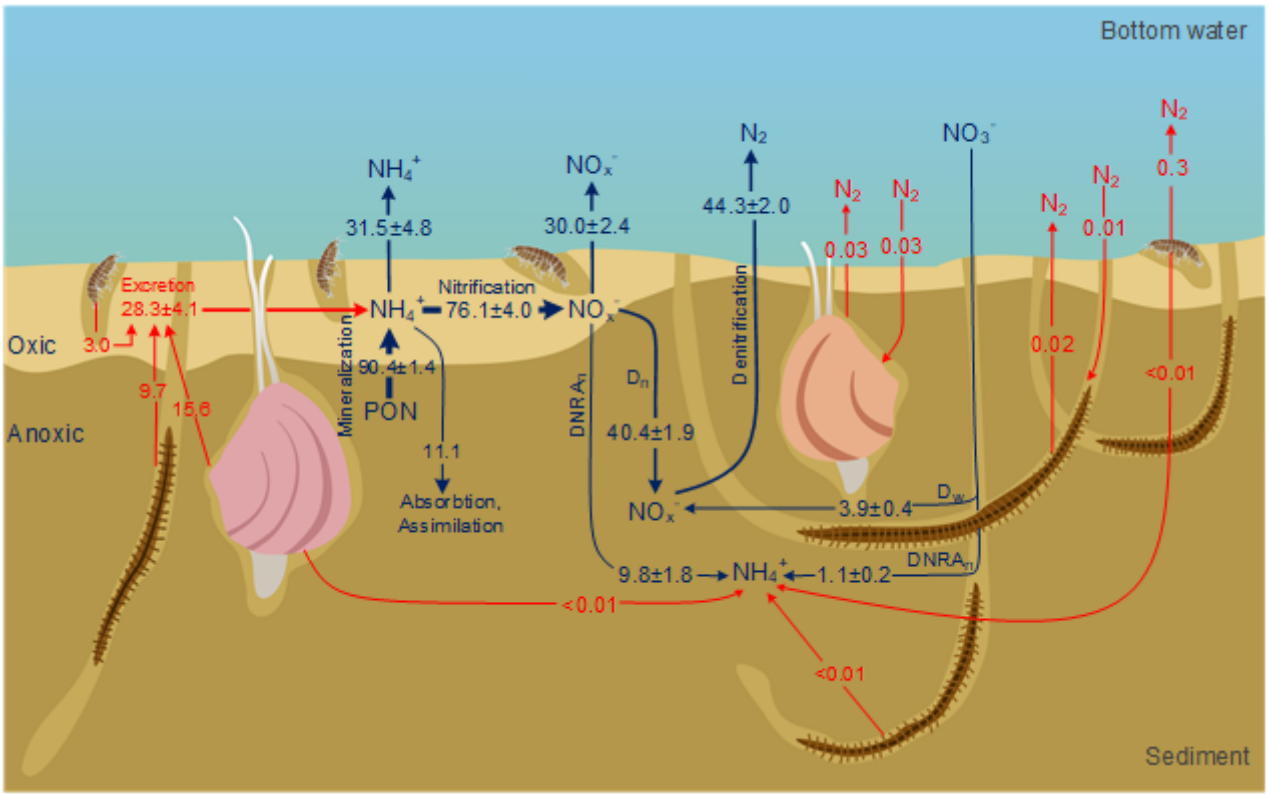

Figure 8

Flowchart of N-cycling in the Öre estuarine sediments hosting the dominant macrofauna species M. affinis, Marenzelleria sp., and L. balthica. Nitrogen transformations were calculated combining measured fluxes and processes in benthic community (unmanipulated whole core incubations = sediment + macrofauna) and macrofauna alone (individual incubations). The animal excretion rates and holobionts-mediated N-cycling were calculated based on mean macrofauna biomass in the study area. Equations used to calculate fluxes and process rates are provided in Table S2. Note that rates reported in figure are expressed as µmol N m−2 h−1. Results report means and standard errors based on experiment replicates (n=6 to 18, see the methods for details). Drawing by V. Gasiūnaitė

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SUPPLEMENTARYMATERIAL.docx