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Effects of copepods, diatoms and their interactions on denitrification in marine sediments: an experimental approach

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1. Introduction

Over the past century anthropogenic activities have dramatically increased the amount of reactive nitrogen on Earth. It has been estimated that nitrogen inputs have increased as much as ten-fold in coastal ecosystems (Paerl 2006; Pätsch et al. 2010). As a result, these, often nitrogen-limited areas (Howarth and Marino 2006) have experienced severe consequences of eutrophication, such as anoxia and changes in community structure (Koop-Jakobsen and Giblin 2010). Although the latter has often been observed (e.g. Dolbeth et al. 2003; Cardoso et al. 2002) it has proven difficult to predict and fully understand the effect of local eutrophication on the communities and their functioning.

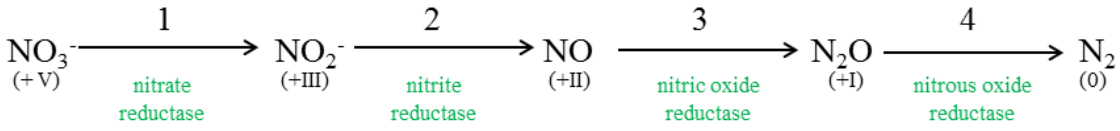


Figure 1: Denitrification pathway.

Roman numbers between brackets denote the oxidation state of the chemical N-species. Key enzymes are indicated in green. Based on Schreiber et al. 2012.

Denitrification is therefore a key process in the nitrogen cycle as it decreases the amount of reactive nitrogen available in these ecosystems (Fig. 1). It is a anaerobic process mediated by prokaryotes which can use nitrate (NO_3^-) or nitrite (NO_2^-) as a terminal electron acceptor in respiration. These substrates are converted to nitrous oxide (N_2O) or nitrogen gas (N_2). While nitrogen gas is a mostly harmless, omnipresent gas, nitrous oxide is an effective greenhouse gas and an important cause of stratospheric ozone destruction (Beaulieu et al. 2011). The fraction of denitrified N that escapes as N_2O rather than N_2 is influenced by a wide range of factors such as nitrate, organic carbon and oxygen availability (Herbert 1999).

Dissimilatory nitrate reduction to ammonia (DNRA) is a second major pathway of nitrate reduction. In this process, nitrate is converted to ammonium and no active nitrogen is removed from the system. During DNRA nitrous oxide can also be formed as an end-product (Seitzinger 1988). Contrary to denitrification, DNRA is not a strictly prokaryotic process as it has also been observed in diatoms (Kamp et al. 2011).

Another key pathway relevant to nitrate reduction is anaerobic ammonium oxidation (ANAMMOX, Burgin and Hamilton 2007). It is a microbial process in which ammonium is combined with nitrite, usually derived from nitrate reduction, and N_2 is produced (Burgin and Hamilton 2007). Contrary to the other two pathways which are in general mediated by a very

diverse assembly, ANAMMOX is apparently only conducted by members of the *Planctomycetes* group (Dong et al. 2009).

In estuaries, all three reduction pathways are mainly catalysed by benthic microbial assemblages. The relative importance of these assemblages is strongly influenced by the prevailing physico-chemical conditions (Herbert 1999). This has been attributed to the different competitive abilities of the microorganisms under changing environmental circumstances (Burgin and Hamilton 2007). Knowing how changes in and above the sediment affect the benthic bacterial community is therefore essential for predicting and understanding the impact of these changes on the nitrogen cycle (Magelhães et al. 2008). In the past decades, strong efforts have been made to realize this goal (e.g. Kaspar 1983; Koch et al. 1992; Teixeira et al. 2012). Key determinants of the removal pathways that have thus far been identified include abiotic factors such as relative availability of nitrate, labile carbon, and reduced sulfur (Burgin and Hamilton 2007), and biotic factors such as bioturbation (Binnerup et al. 1992) and benthic oxygen (O_2) production (Christensen et al. 1990).

The latter is mainly related to the presence of benthic microalgae such as diatoms. Through photosynthesis they can cause large diurnal fluctuations in O_2 (Bartoli et al. 2012). The oxygen produced by photosynthesis diffuses into the sediment where it not only inhibits anaerobic nitrate reduction, but also affects both the pH and the redox potential of the sediment (Risgaard-Petersen 2003).

Other indirect effects of diatoms on nitrate reducing bacteria include the excretion extracellular polymeric substances (EPS) and toxin production. EPS provides an additional carbon source for the heterotrophic bacteria (Risgaard-Petersen 2003). These photosynthetic products were found to stimulate bacterial growth (Mayor et al. 2012). They also form a matrix that stabilizes the sediment (biostabilization) and in which biofilm organisms, such as bacteria and the algae themselves, are embedded (Stal and de Brouwer 2003). Some species of diatoms can also produce cytotoxic compounds. These toxins are capable of affecting both grazers and bacteria (Ianora and Miralto 2010). Diatoms impact nitrogen fluxes directly through the assimilation of NO_3^- and other nutrients (Joye et al. 2003).

Next to microphytobenthos, also higher trophic levels can affect nitrogen fluxes in marine sediment (e.g. Kristensen 1988; Hunter 2012). The benthic fauna diversity in estuaries is high, both in size and functional diversity. The benthos spans up to 7 order of magnitude in length, from microfauna being just a few microns long to macrofauna reaching over a decimeter and in between is the meiofauna. Most of the research on the impact of benthos on N fluxes has been done on macrobenthos focusing mainly on the effects of bio-irrigation and bioturbation

(Binnerup et al. 1992; Braeckman et al. 2010). The effects of meiofauna (metazoans retained on a 38 μm sieve e.g. nematodes and harpacticoid copepods) on the N fluxes have almost completely been neglected, notwithstanding the fact that these abundant organisms (Gerlach 1970) interact in numerous ways with both bacteria and microphytobenthos .

Meiofauna are important grazers on both bacteria and algae, capable of eating their body weight equivalent in microorganisms each day (Montagne 1984). Doing so they can have a negative effect on the N fluxes by grazing upon the nitrate reducing bacteria, but also an indirect positive effect by reducing competitors of these bacteria (Parent and Morin 1999).

Furthermore, meiobenthos releases dissolved matter through excretion and respiration and particulate matter through production of faecal pellets, carcasses, moults, and dead eggs (Frangoulis et al. 2005). Excretion liberates C, N, and P compounds, that can directly be used by the lower tropic levels of the system. The quantity and composition of the outfluxes is dependent on a wide range of factors including the food concentration and quality and light conditions (Frangoulis et al. 2005).

Many of the meiobenthic species are capable of actively moving through the sediment. Doing so, they transport oxygen and organic matter deeper into the sediment, whilst at the same time transporting excretion products to the water column (Braeckman et al. 2010).

It is clear that the small-scale key players of marine sediments (bacteria, microphytobenthos and meiobenthos) interact with each other and with their environment in numerous ways and that these interactions are important for the N fluxes in the sediment. Notwithstanding the importance of denitrification as an ecosystem service, only a small body of research has been done on the importance of microphytobenthos on the N fluxes (Christensen et al. 1990; Risgaard-Petersen 2003; Risgaard-Petersen et al. 2004) and almost none on meiobenthos (but see Parent and Morin 1999).

This study was carried out to evaluate the influence and the importance of these small-scale biotic interactions on denitrification in marine sediments in order to provide a first evidence on the importance of meiobenthos on denitrification. To achieve this, microcosm experiments were performed with different combinations of players and under different light regimes. Sediment, harpacticoid copepods (Crustaceae, Copepoda) and diatoms were collected from an intertidal flat at Paulina Polder (Westerscheldt, The Netherlands). Nutrient fluxes and the denitrification potential were measured from each microcosm in order to unravel the role of each player on denitrification. In order to identify the denitrifying community from Paulina, denitrifying bacteria from the study site were isolated and identified.

The central hypothesis from this study stated that biological interactions between the three components (bacteria, diatoms, copepods) had an impact on denitrification. It was assumed that players and the interactions between them would indirectly affect the , most likely, very diverse denitrifying community and its activity.

2. Methodology

2.1. Field sampling

Silt (average median grain size: 53.63±0.75 µm) was collected from the intertidal mudflat Paulina (Westerscheldt estuary, The Netherlands; 51°20' N, 3°43' E) by scraping off the top layer (0-3cm) of the sediment during low tide. Seawater (salinity: 19.3; 1734±159 NO_3^- +NO_2^-N µg/L) was collected from the same site and was filtered over a 0.22 µm filter (Corning® 500mL Bottle Top Vacuum Filter) and stored in the dark at 4°C (filtered seawater: FSW).

2.2. Isolation of denitrifying bacteria

Four sediment samples (collected on 8/10/12) were serially diluted (from 10^{-1} to 10^{-7}) in Difco ™ Marine Broth (MB), enriched with 5 mM nitrate. The dilutions were flushed with helium to create anaerobic conditions. Ten percent acetylene was added to inhibit the N_2O reductase (cf. reaction 4, Fig. 1; Groffman et al. 2006). By blocking the final step in the denitrification pathway, N_2O becomes the terminal product of denitrification instead of N_2, The benefit of measuring N_2O instead of N_2 is that N_2O is not present in high atmospheric concentrations, reducing the risk of contamination of the gas sample with atmospheric input.

After incubation in the dark for 17 days at 15°C on a rotary shaker, nitrous oxide (N_2O) concentrations were determined by means of GC-TCD (Gas Chromatography- Thermal Conductivity Detector; MICRO E-0391, Interscience; LOD 13.55ppm N_2O). The two highest dilutions with detectable N_2O concentrations were selected for further plating. From each selected dilution, 75-100 µL MB was spread on Difco ™ Marine Agar (MA), enriched with 5 mM nitrate. The plates were incubated in an anaerobic chamber (80% N_2, 10% H_2, 10% CO_2; 27°C). Colonies were picked from the plates and restreaked until considered pure. The pure cultures were streaked both on MA enriched with 5 mM nitrate and MA without the nitrate addition and incubated once again in the anaerobic chamber. Only those that grew substantially better on MA with nitrate were streaked on MA and incubated aerobically. After three days, they were inoculated (100µL of bacterial suspension, with an optical density (600nm) of 0.5 in artificial seawater) in MB with 5 mM nitrate. The selected isolates were then treated as the dilution series to determine their denitrification activity, with the exception that they were incubated for 10 days at 28°C. They were identified through Sanger sequencing (Sanger et al. 1977) of the 16S rRNA gene. A partial 16S rDNA amplification was done using the primers 16R1093 and 16F0358 (Coenye et al. 1999). The PCR products were

then sequenced using an ABI3130xl sequencer (Applied Biosystems,USA). A BLAST search was performed to identify the isolates.

2.3. Experimental setup

Sediment (collected on 21/02/13) was defaunated by washing it over a 250 µm sieve. The sieved sediment (average median grain size: 56.89±0.25 µm) was divided in equal aliquots of 80 g and stored frozen (-18°C) in polyethylene containers (microcosms). A microcosm was defrosted two days before starting a treatment (Fig. 2). After adding 60 ml of filtered seawater (FSW), the thawed sediment was well mixed. Right before the start of a treatment, the FSW was removed by decantation.

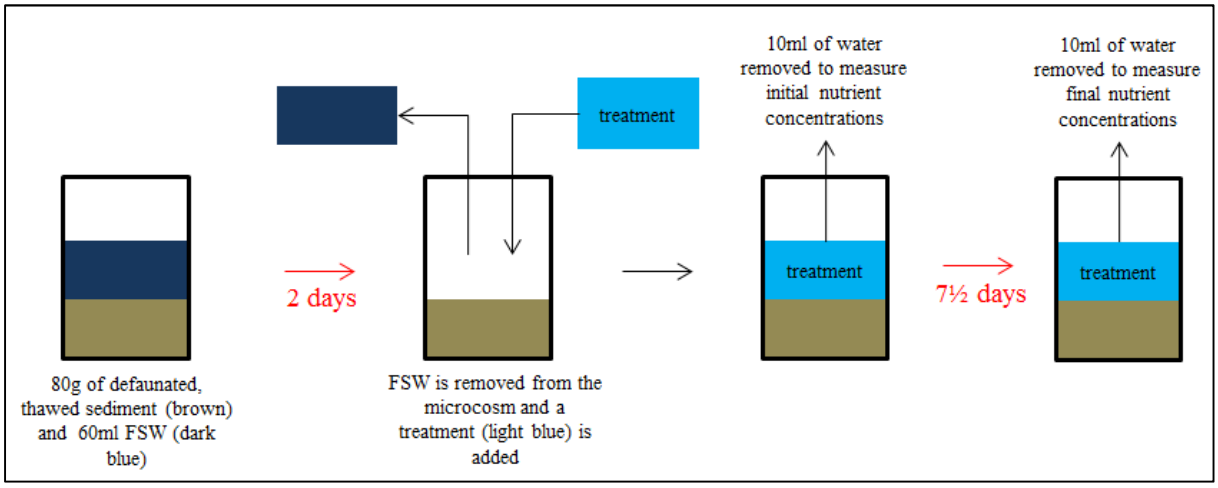


Figure 2: Scheme of the microcosm experiment. Microcosms are represented as rectangles. Incubation steps are indicated in red.

The experimental design included the following treatments:

1. Blank

   Microcosm containing 80g of previously defaunated and thawed sediment and 70ml FSW

2. Increased nitrate concentration (Increased NO_3 treatment)

   0.1 mmol KNO_3 in 70 ml FSW was added to the microcosm

3. Effect of copepods (Copepod treatment)

   200 phototactic copepods in 70 ml FSW collected from Paulina Polder. (Species composition of the study site cf. appendix: Cnudde & De Troch, unpublished)

4. Effect of diatoms (Diatom treatment)

400000 cells of *Navicula* sp. in 70 ml FSW were added to the microcosm.

5. Effect of diatoms and copepods (Copepod+diatom treatment)

   400000 cells of *Navicula* sp. and 200 copepods (as above) in 70 ml FSW were added

6. Spent medium (Spent medium treatment)

   70 ml of FSW, in which 200 copepods were fed 400 000 *Navicula* over a period of one week. Prior to the start of the treatment this spent medium was stored at -18°C after manually removing the copepods.

All six treatments were conducted both in the dark and under a diurnal (12h/12h) light regime. All treatments were replicated four times, for each light condition. Apart from the spent medium and blank, all treatments were first added to 70 ml of FSW, which was then supplemented to an aliquot of the previously defaunated and thawed sediment. After 1 hour, 10 ml of water was extracted for nutrient analysis (initial nutrient concentration; Fig. 2). All microcosms were incubated for seven and a half days, at 15°C. To avoid depletion of active nitrogen in the microcosms, half of the SW was replenished on day 5 and 6 of the experiment. At the end of the incubation period, 10 ml of SW was stored for nutrient analysis (final nutrient concentration; Fig. 2).

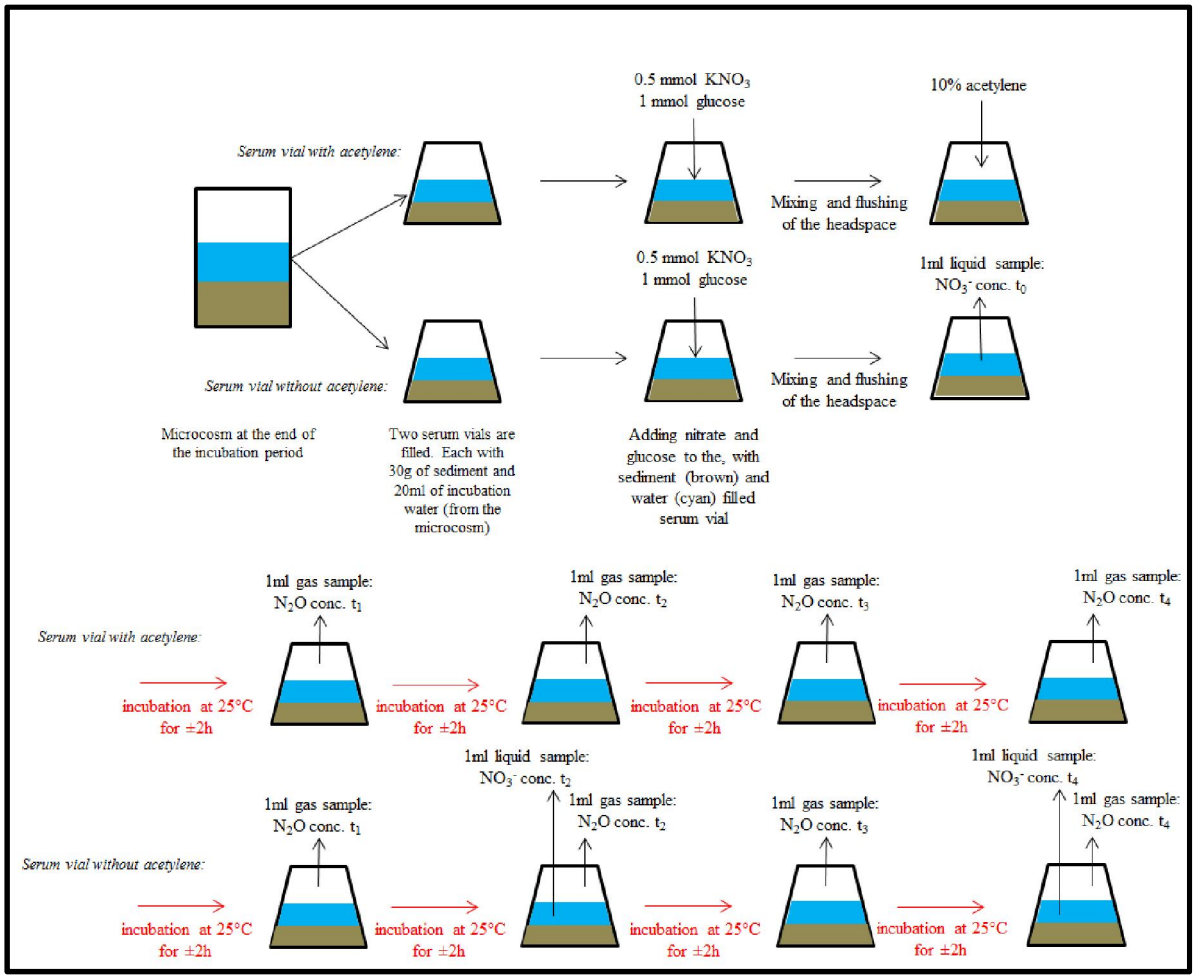


Figure 3: Experimental design of the denitrification potential experiment. Serum vials are represented as black trapezoids. Incubation steps are indicated in red.

The denitrification potential was measured using the acetylene inhibition technique according to Sørensen (1978; Fig 3). Briefly, a serum vial was filled with 30 g (wet weight) sediment and 20 ml incubation water (i.e. from the treatments). To prevent nitrogen and carbon limitation, the water was supplemented with 0.5 mmol KNO_3 and 1 mmol α-D-glucose. After rigorous shaking, 1 ml was extracted to determine the initial nitrite/nitrate concentration (t_0). The vials were hermetically sealed and flushed five times with helium to remove oxygen. After adding 10% acetylene (see above), the vials were incubated at 25°C and with a constant stirring rate of 90 rpm. The N_2O and CO_2 concentrations were measured every two hours by injecting 1 ml of headspace in a gas chromatograph (MICRO E-0391, Interscience). This was done four times (t_1-t_4) for each serum vial. At second and final sampling event, 800 µl of fluid was extracted for later nitrite/nitrate determination (indication for the expression rate of nitrate reductase, cf. reaction 1, Fig. 1). To determine the production rate of N_2O that was formed without acetylene inhibition (indication for the expression rate of nitrous oxide reductase, cf. reaction 4, Fig. 1), the process was repeated with a technical replicate where no acetylene was added to (Magalhães et al. 2011).

2.4. Nutrient analysis

Nutrient analysis of the samples from the incubation period (initial and final nutrient concentration) was done using an automatic chain (SAN^{plus} segmented flow analyser, SKALAR) according to Beyst et al. (2001).

Samples extracted from the serum vials for nitrite/nitrate determination (see before) were centrifuged (14000 rpm, 5 min) and the supernatants was stored frozen (-18°C) prior to analysis. Analysis of nitrate and nitrite was based on a colorimetric method as described by Smibert and Krieg (1994).

2.5 Data analysis

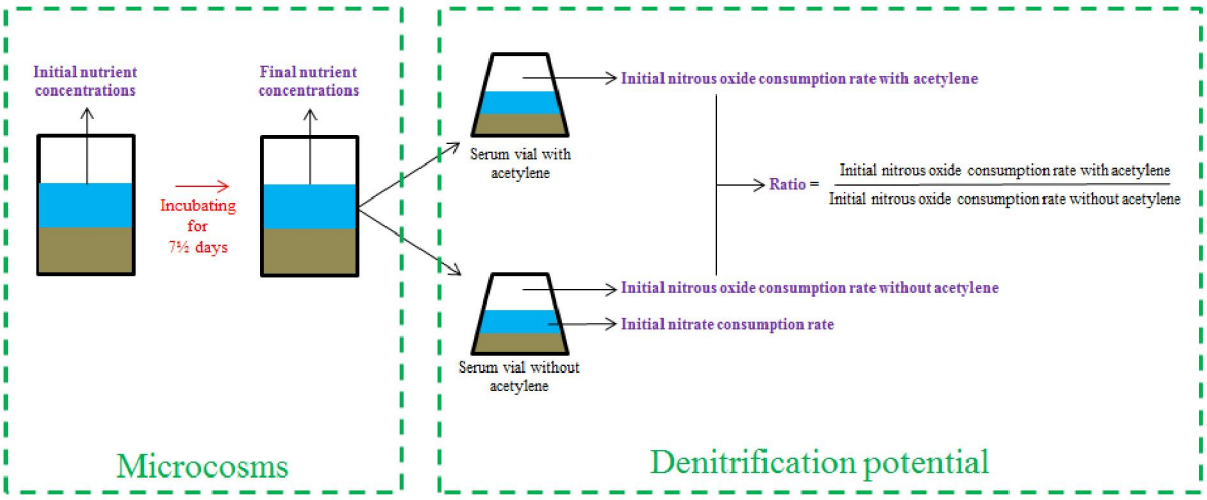


Figure 4: Scheme of the variables obtained from the microcosm experiment. The variables are printed in purple. Microcosms are represented as black rectangles and serum vials as black trapezoids Sediment is brown, incubation water is light blue.

Differences in initial and final nutrient concentrations (Fig. 4) between the treatments and light conditions were detected using a permutation-based two-way ANOVA (Wheeler 2010), performed in the software package R 2.15.0. Since the data did not meet the assumptions of a normal distribution based two-way ANOVA, only the permutation-based version of this test was appropriate. Pairwise differences were unravelled using Wilcoxon rank-sum post-hoc test using 95% confidence limits, with Bonferroni correction.

N_2O concentrations were corrected for headspace volume changes, pressure and dissolving of the gas into the liquid phase (Microsoft® Office Excel). The software package R 2.15.0. was used for further data analysis.

Gas production rates increased over time and an exponential regression was therefore fit to the data (Stenström et al. 1991): $p(t) = p_0 + \frac{qN_0}{\mu}(e^{\mu t} - 1)$, with p = the amount of gas at time

t, p_0 = the amount of product at t_0, qN_0 = the initial production rate and μ = the specific growth rate constant. Since the serum vials were flushed with helium, no N_2O was present at the start of the incubation and p_0 was set to zero. This function was augmented to fit the data for the nitrate consumption (in the serum vials) to $s(t) = s_0 - \frac{qN_0}{\mu}(e^{\mu t} - 1)$, with s = the amount of substrate (nitrate) at time t and s_0 = the amount of substrate at t_0.

For both equations, the specific growth rate constant (μ) was restricted to a range under which the deviation between the function and measurements were minimal ([0,01;0,5] and [0,001;0,05] for N_2O production and NO_3^- consumption, respectively).

The initial N_2O production rates obtained from the regression analysis are the production rates of N_2O at the start (t_0) of the denitrification potential experiment. The same is true for the initial NO_3^- consumption rate, which expresses the NO_3^-consumption rate at the start of the denitrification potential experiment. Thus the rates are corrected for microbial growth occurring after the nitrate and glucose addition.

Differences in the obtained qN_0 values for N_2O production (with and without the acetylene addition) and the qN_0 values for NO_3^- consumption (Fig. 4) between the different treatments and light conditions were analysed using a permutation-based two-way ANOVA (Wheeler 2010) since the data were not normally distributed. As with the nutrient concentrations, pairwise differences were unravelled using Wilcoxon rank-sum post hoc test using 95% confidence limits, with Bonferroni correction.

Differences in the ratio of the initial nitrous oxide production rates (with acetylene/without acetylene) between the different treatments and light conditions were analysed using a two-way ANOVA. Pairwise differences were detected using a Tukey's Honestly Significant Difference test.

3. Results

3.1. Isolation of denitrifying bacteria

Of the 52 isolates obtained, 14 grew substantially better on Marine Agar (MA) enriched with 5 mM nitrate, than on MA without the enrichment. Three out of these 14 selected isolates had a denitrification activity that was higher (0.006±0.002* mmol N_2O) than the control strain (*E.coli* LMG 5584; 0.001 mmol N_2O). Further, partial 16S rRNA gene sequencing revealed that these three isolates belong to one species (100% identical) from the genus *Marinobacter* (99.1% match).

3.2. Microcosm experiment

3.2.1. Nutrient levels

3.2.1.a Initial nutrient concentrations

Except for the spent medium and the increased nitrate treatment, the initial nutrient concentrations in the microcosms were the same for all treatments (Fig. 5). The results of the statistical tests are listed in Table 1. In the spent medium the nitrate concentration (34.33±10.63 x 10^{-3} mM) was three times lower than in the other treatments (129.51±2.34 x 10^{-3} mM). In the increased nitrate treatment, the nitrate concentration (1387.28±238.11 x 10^{-3} mM) was more than ten times higher than in the other treatments. As for nitrate, nitrite was significantly lower in the spent medium (1.42±0.32 x10^{-3} mM) compared to the other treatments (3.70±0.03 x10^{-3} mM). The phosphate concentration was eight times higher in the spent medium treatment (2.44±0.30 x10^{-3} mM) than in the other treatments (0.83±0.07 x 10^{-3} mM). The silica concentrations were slightly lower in the increased nitrate (57.43±4.56 x 10^{-3} mM) and spent medium (62.61±3.72 x 10^{-3} mM) treatment compared to the other treatments (70.51±0.03 x 10^{-3} mM). The initial concentration of ammonium (92.85±0.04 x 10^{-3} mM) did not differ between the treatments (Table 1).

* All averages are reported as average value ± standard error (SE).

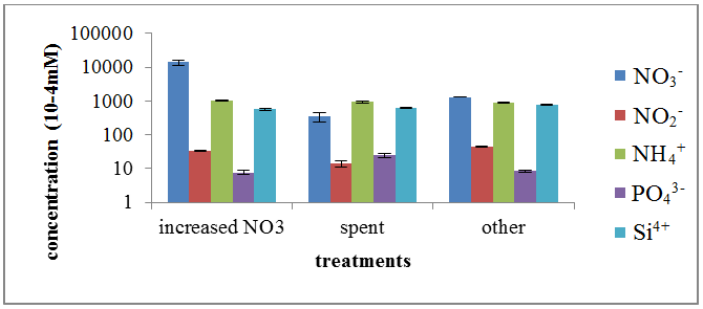


Figure 5: Initial nutrient concentrations for the different treatments (mean ± SE). Other treatments include copepod, diatom, copepod+diatom and blank treatments

	Df	NO_3^-	NO_2^-	NH_4^-	PO_4^{3-}	Si^{4+}
Treatments	5	**<2 x 10^{-16}**	**<2 x 10^{-16}**	0.09	**<2 x 10^{-16}**	**0.01**
Light conditions	1	0.8	0.8	0.7	1	0.2
Interaction	5	0.3	1	0.3	0.6	0.7

Table 1: p- values of permutation- based ANOVAs on the initial nutrient concentrations. Significant p-levels are printed in bold.

3.2.1.b Final nutrient concentrations

After seven and a half days of incubation, the nutrient concentrations did not differ significantly between light conditions (Table 2).

At the end of the incubation period, nitrate (Fig. 6a) was almost completely depleted (1.12± 0.03 x 10^{-3} mM). The final nitrite concentration (Fig. 6b) was low (0.30±0.01 x 10^{-3} mM) and differed significantly between treatments (Table 2). Post-hoc tests could however not reveal significant pairwise differences.

The ammonium concentration (Fig. 6c) increased about four times towards the end of the experiment (384.08±3.04 x 10^{-3} mM). The final ammonium concentration in the copepod treatment (462.23±9.70 x 10^{-3} mM) was significantly ($p = 0.001$) higher than the ammonium concentration in the diatom treatment (372.18±21.35 x 10^{-3} mM). The final ammonium concentration in the copepod+diatom treatment was the highest (485.69±34.52 x 10^{-3} mM) of all treatments, although it was not significantly different from the other treatments due to a larger variation between the replicates.

Phosphate concentrations (Fig. 6d) increased severely during the incubation period as the finale phosphate concentration (18.60±0.21 x 10^{-3} mM) was almost twenty times higher than the initial concentration. The final phosphate concentrations differed strongly between the different treatments. The highest phosphate concentrations were measured in the spent medium (26.32±2.37 x 10^{-3} mM), copepod (26.48±2.37 x 10^{-3} mM) and copepod+ diatom

(26.01±2.61 x 10^{-3} mM) treatment. The treatments with increased NO_3 (13.21±1.74 x 10^{-3} mM) and diatoms (13.80±1.83 x 10^{-3} mM) had the lowest phosphate concentration. Post-hoc comparisons revealed significant pairwise differences between spent medium and diatoms (p = 0.002), spent medium and increased nitrate (p = 0.0006) and copepods and increased nitrate (p = 0.001).

The final silica concentration was higher (83.95±0.84 x 10^{-3} mM) than the initial concentration and differed significantly among treatments, although no significant pairwise differences were detected.

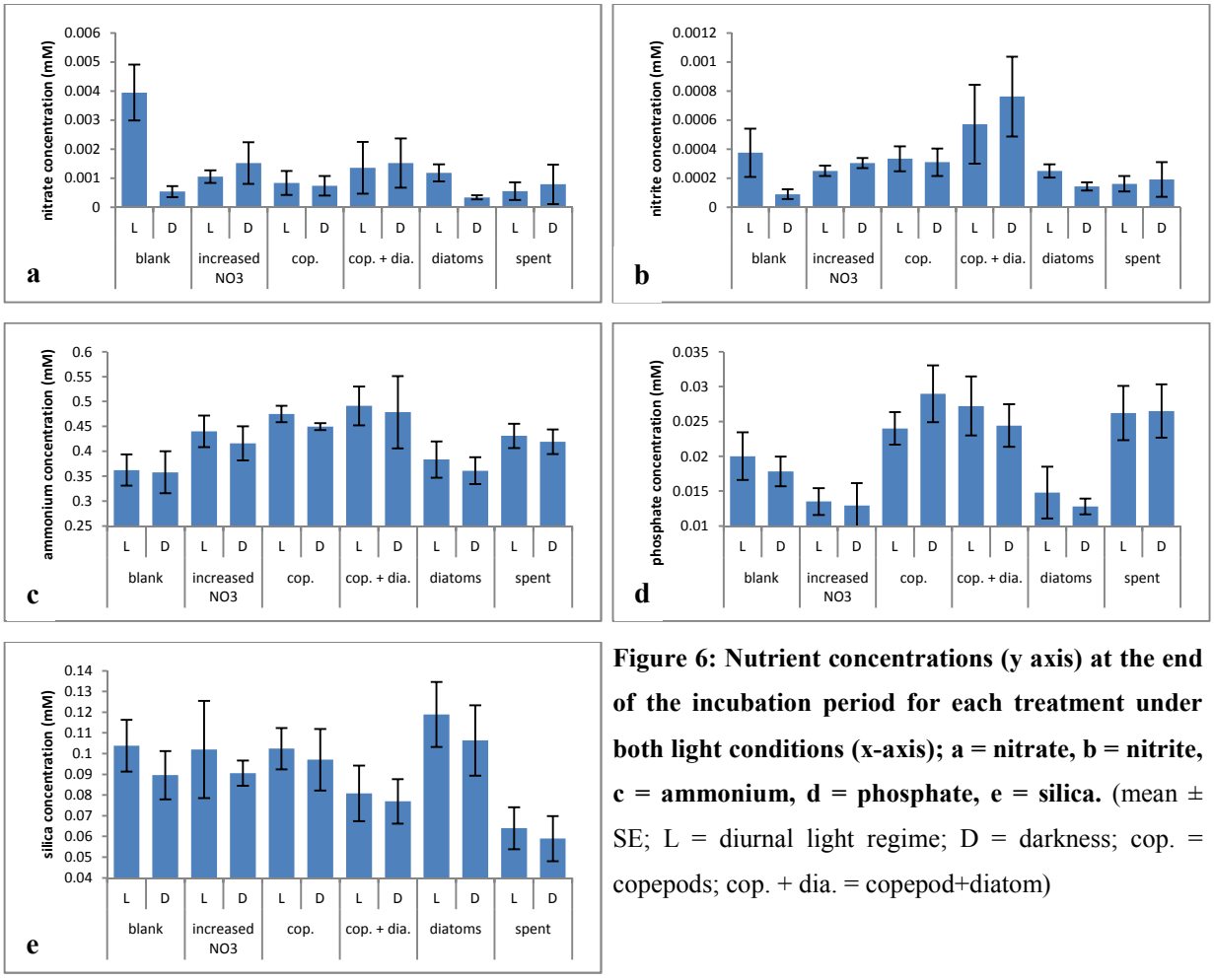


Figure 6: Nutrient concentrations (y axis) at the end of the incubation period for each treatment under both light conditions (x-axis); a = nitrate, b = nitrite, c = ammonium, d = phosphate, e = silica. (mean ± SE; L = diurnal light regime; D = darkness; cop. = copepods; cop. + dia. = copepod+diatom)

	Df	NO_3^-	NO_2^-	NH_4^-	PO_4^{3-}	Si^{4+}
Treatments	5	0.09	**0.006**	**0.007**	**<2 x 10^{-16}**	**0.02**
Light conditions	1	0.07	0.9	0.3	0.8	0.3
Interaction	5	**0.01**	0.5	1	1	1

Table 2: p- values of permutation- based ANOVAs on the final nutrient concentrations. Significant p-levels are printed in bold

3.2.2. Denitrification potential

During the measurement of the denitrification potential, nitrate was consumed, while nitrite and nitrous oxide were produced (Fig. 7). Consequently, as time passed, more nitrate disappeared in the serum vials and nitrite and nitrous oxide were formed (Fig. 4).

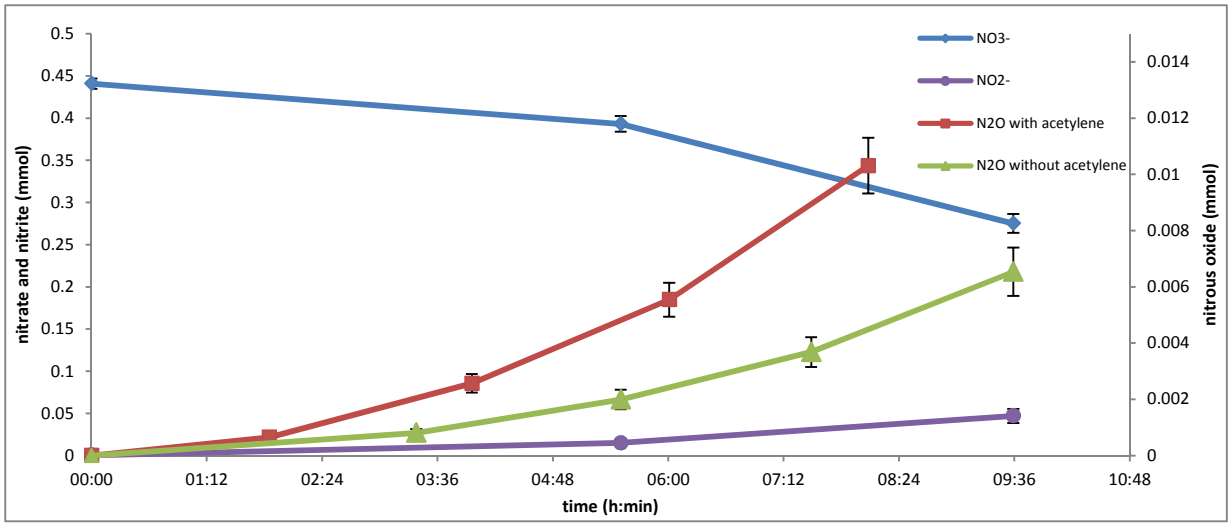
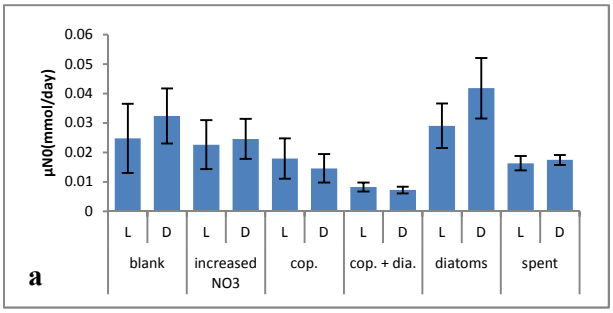


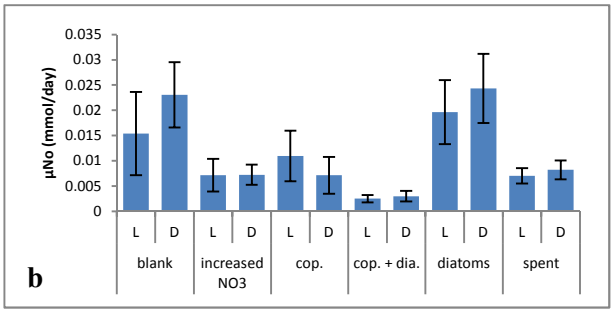
Figure 7:Nitrogen pools during the measurement of the denitrification potential. Amounts (mmol) are averaged over all samples, as is the time (h:min) ± SE. N_2O plotted on the right y axis NO_3^- and NO_2^- on the left y axis

3.2.2.a. Initial N_2O production and NO_3^- consumption rates

The initial nitrate consumption ranged between 0.301 and 0.889 mmol/day (average = 0.403±0.030 mmol/day; Fig. 8c). The initial nitrous oxide production rate with acetylene fluctuated between 0.005 and 0.060 mmol/day (average = 0.021±0.002 mmol/day; Fig. 8a) and without acetylene between 0.002 and 0.041 (average = 0.011±0.002 mmol/day; Fig. 8b).

The initial nitrate consumption and initial nitrous oxide production rates (with and without acetylene) did not differ between light conditions (Table 3). In contrast to the initial nitrate consumption rate, the nitrous oxide production rates did differ significantly between the treatments (Table 3). Both the nitrous oxide production rates were significantly lower for the copepod+diatom treatment compared to the blank ($p = 0.0006$ with acetylene; $p = 0.0006$ without acetylene), the diatoms ($p = 0.0003$ with acetylene; $p = 0.0006$ without acetylene) and spent medium ($p = 0.0006$ with acetylene; $p = 0.003$ without acetylene) treatment. The production rate for the copepod+diatom treatment with acetylene was also significantly lower ($p = 0.0006$) than the increased nitrate treatment.





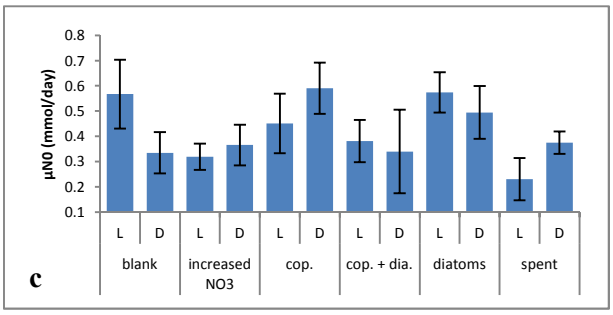


Figure 8: Initial consumption and production rates (y-axis) during the measurement of the denitrification potential for the different treatments and light conditions (x-axis); a = Initial nitrous oxide production rate (with acetylene), b = Initial nitrous oxide production rate (without acetylene), c = Initial nitrate consumption rate (mean ± SE; L = diurnal light regime; D = darkness; cop. = copepods; cop. + dia. = copepod+ diatom)

	Df	initial N_2O production rate with acetylene	initial N_2O production rate without acetylene	initial NO_3-consumption rates
Treatments	5	**0.009**	**0.002**	0.06
Light conditions	1	0.3	0.5	0.8
Interaction	5	0.9	0.9	0.3

Table 3: p- values of permutation- based ANOVAs on initial rates. Significant p- levels are printed in bold.

3.2.2.b. Ratio of the initial nitrous oxide production rates

The nitrous oxide production rates had a strong, positive linear correlation (Pearson's r = 0.93). The average ratio of production rate with acetylene over the rate in the measurement without acetylene was 0.46±0.03 and differed significantly between the treatments (Fig. 9, table 4). The ratios from the copepod+diatom treatment and the increased nitrate treatment were both significantly lower than the blank (p_{adj} = 0.002; 0.0004 resp.) and the diatom treatment (p_{adj} = 0.02; 0.003 resp.).

The correlation between the production rates and nitrate consumption was positive, but weak (Pearson's r = 0.30 with acetylene; Pearson's r = 0.27 without acetylene).

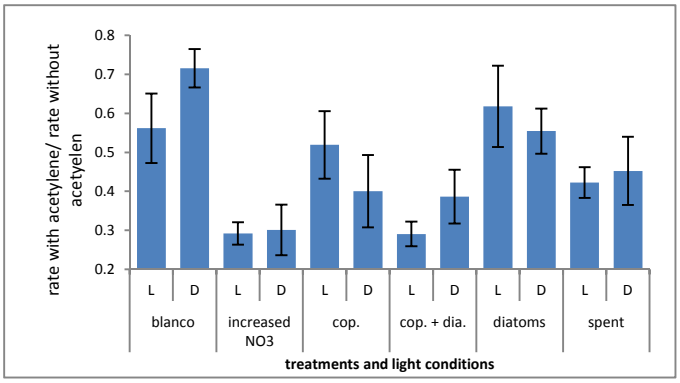


Figure 9: Ratio between nitrous oxide production rate with acetylene and without acetylene. (L = diurnal light regime; D = darkness ; cop. = copepods; cop. + dia. = copepod+ diatom)

Ratio (initial nitrous oxide production rates with acetylene /initial nitrous oxide production rate without acetylene)	
Treatments	**0.0001** (df = 5)
Light conditions	0.7(df = 1)
Interaction	0.4 (df = 5)

Table 4: p- values of the ANOVA on the ratio of the initial nitrous oxide production rates. Significant p-levels are printed in bold.

4.Discussion

4.1. Isolation of denitrifying bacteria

According to Gamble et al. (1977) about 10% of the anaerobically growing isolates are expected to be denitrifiers. Since 50 isolates were obtained, it could thus be expected that five of them would be denitrifiers. It turned out to be slightly less (three) and moreover, all of them belonged to the same species. Some denitrifiers may have been missed in our isolation setup. Possible reasons for this might be (1) a low denitrification activity, (2) the use of other electron acceptors than nitrate (Brettar and Höfle 2001) or (3) a false negative growth test. The growth test was done by comparing the growth on MA with and without the nitrate addition. However, the MA without the addition of nitrate may still contain small amounts of nitrate, as it was prepared with Difco^{TM} MA (1.6 mg NH_4NO_3/L).

The denitrifier that was isolated was a *Marinobacter* species. Denitrifying *Marinobacter* species have been isolated from marine sediments on many occasions (e.g. Gorshkova et al. 2003; Shivaji et al. 2005). They are wide-spread marine, gram-negative bacteria within the γ-subclass of the *Proteobacteria* (Nakano et al. 2010). Considering the ubiquity and metabolic capabilities of this genus, they probably play an important role in the biogeochemical cycling of nitrogen in marine environments (Handley and Lloyd 2013).

The *Marinobacter* sp. was isolated three times from the same isolation campaign. This either means that this species was omnipresent at the study site or that is was strongly favored by the isolation method used. A rich medium was used in this study on which the *Marinobacter* sp. could grow fast, outgrowing its more fastidious neighbours (Zengler et al. 2002).

The diversity of the denitrifying community in estuarine sediments was shown to be high (Braker et al. 2000; Smith et al. 2007) and it is therefore expected that more denitrifiers can be isolated from the study site through an increased isolation effort. Optimizing the media (Brettar and Höfle 2001; Heylen et al. 2006) and conditions (Ogilvie et al. 1997) used during the isolation campaign can further increase the yield. Ogilvie et al. (1997) for instance discovered that when working at 5 °C, most of the obtained isolates were denitrifiers, whereas fermenters dominated when the incubation temperature was set at 20°C. The use of selective media, with fewer electron acceptors other than nitrate itself, might also increase the proportion of denitrifiers obtained (Brettar and Höfle 2001). For this preliminary study on the denitrifying community of the study site such optimizations were not considered, but they should be considered in similar future isolation campaigns (see Van Hecke 2013).

4.2. Microcosm experiment

4.2.1. Nutrient levels

4.2.1.a. Initial nutrient concentrations

The spent medium that was added to the microcosm had a lower nitrate, nitrite and silica content than the filtered seawater which was used in the other treatments. It is likely that the diatoms, added to the setup as a food source for the copepods, were able to consume some nutrients before being consumed themselves by grazing copepods. Strikingly the ammonium concentration in the spent medium had not decreased, notwithstanding the preference of diatoms for ammonium uptake over nitrate (Waser et al. 1998). Since ammonia constitutes the lion's share of the total nitrogen excreted by the copepods (Frangoulis et al. 2005), it plausible that the ammonium consumption by diatoms was compensated by the excretion from the copepods. Since the phosphate concentration in the spent medium was even higher than in the filtered seawater, the phosphate, excreted by the copepods (Frangoulis et al. 2005) outweighed even the phosphate consumption.

4.2.1.b. Final nutrient concentrations

During the incubation period nitrate was almost completely depleted, while the ammonium concentrations increased about four times. Even if all the dissolved nitrate had been converted to ammonium through DNRA, the ammonium concentration would only have doubled. Furthermore, part of the nitrate will have been lost from the system through denitrification. Ammonium production from organic nitrogen decomposition must therefore have been important (Goeyens et al 1987). Since ammonium was able to accumulate, its consumption must have been limited. Nitrification might have been inhibited by a lack of oxygen (Bartoli et al. 2012) or high sulfide concentrations (An and Gardner 2002). The ammonium concentration was highest in the treatments with copepods. The extra ammonium might be a direct result from the copepod excretions, but also from an increased importance of DNRA compared to denitrification or a higher organic decomposition rate (see above). Reduced ammonium consumption due to sulfide inhibition of nitrification could also be a possibility (An and Gardner 2002). The spent medium had an intermediate final ammonium concentration. This indicates that whatever the reason for the higher ammonium in the treatments with copepods, it is at least partly caused by the excretion products from the copepods.

The lowest ammonium concentrations were found in the blank and the diatom treatment. It is unlikely that the deficit in ammonium was a result of the ammonium uptake by diatoms, since the concentrations in the blank, were almost the same as the diatom treatment, independent of the light conditions. It is possible that diatoms had survived the freezing of the sediment and were therefore still present in the blank (Souffreau et al. 2010), but they cannot have been numerous. Furthermore, in the dark, their growth, activity and nutrient uptake are expected to be negligible (Kamp et al. 2011).

The increased nitrate treatment had intermediate ammonium concentrations. The slight increase in ammonium might originate from the supplemented nitrate that was converted either directly into ammonium (DNRA) or indirectly through organic matter decomposition after it was first incorporated in the microbial biomass.

Phosphate concentrations had strongly increased during the incubation period, which proves, in accordance with the high ammonium concentrations, the importance of microbial organic matter decomposition in the microcosms (Suess 1979). There were striking differences in phosphate concentrations between treatments. Treatments with copepods or the waste products of copepods had a considerably higher phosphate content than the other treatments. The excretions of copepods therefore seem to be an important source of phosphate in the microcosms.

According to these findings, the presence and activity of copepods may be an important small-scale nitrogen and phosphorus source, either directly as excretion products or indirectly by changing the structure of activity of the bacterial community. Since primary production in temperate estuaries is typically nitrogen limited (Howarth and Marino 2006), the extra ammonium will directly benefit the microphytobenthos. In contrast, phosphorus is usually the limiting element for heterotrophic bacteria (Vadstein et al. 2012), which makes a phosphate addition from copepod excretions as important. In addition, a shift in the N:P ratio can cause a shift in dominance of primary producers (Howarth and Marino 2006).

4.2.2. Denitrification potential

During the measurement of the denitrification potential, more nitrate disappeared than nitrous oxide and nitrite were formed. The conversion to ammonium by DNRA is likely to be an important cause for this deficit (An and Gardner 2002). Since the acetylene inhibition of N_2O reductase is incomplete (Felber et al. 2012), unmeasured N_2 will still be produced. Lastly, part

of the unaccounted active nitrogen was probably present under the form of unmeasured intermediates, such as nitric oxide (Schreiber et al. 2012).

4.2.2.a. Initial N_2O production and NO_3^- consumption rates

In contrast to the initial N_2O production rate, the initial nitrate consumption did not differ significantly between the treatments. The initial nitrate consumption rate is a general indication for nitrate reduction processes (denitrification, DNRA and ANAMMOX) as it is an essential step in all three processes (cf. reaction 1, Fig. 1; Schreiber 2012). Initial nitrate consumption rates differed less strongly between treatments than the initial N_2O production rates did. In other words, differences between treatments in nitrate reduction rates were less pronounced than differences in denitrification activity. A reduced denitrification activity therefore seems to be compensated by alternative nitrate reduction pathways (DNRA and ANAMMOX).Under anaerobic conditions N_2O is mainly produced by denitrification, and only to a lesser extent by DNRA and ANAMMOX (Schreiber 2012). With the acetylene addition, the N_2O share derived from denitrification is even larger, as the acetylene blocks the final reduction of N_2O to N_2 (cf. reaction 4, Fig. 1). This is a reaction unique to denitrification (Schreiber 2012) and nitrous oxide production rates can therefore be seen as a measure for denitrification (Groffman et al. 2006).

The initial N_2O production rates did not differ between the blank and the diatom treatment. Consequently, diatoms had no or very little impact on denitrification in the microcosms. This is inconsistent with what is generally observed (Risgaard-Petersen 2003). That is, when ammonium is not limiting, the oxygen production by benthic microalgae enhances the rates of, and the coupling between, nitrification and denitrification (An and Joye 2001). However, it should be noted that the rate of denitrification can also be reduced by benthic oxygen production due to a longer diffusion path between water and the anoxic denitrification zone (Risgaard-Petersen et al. 1994). It is possible that positive and negative effects of the diatoms cancelled each other out, or that the overall effect was too small to be detected (Fig. 10a).

The copepods+diatoms treatment had the lowest initial N_2O production rates. The rates were also low for the copepod treatment. Consequently, copepods had a negative impact on denitrification. Not much is known about the impact of meiofauna on nitrogen fluxes, let alone how they can impact the N fluxes. However, the strongest effect of the copepods on denitrification was found when diatoms were added to the microcosm. Since the diatoms themselves had no effect on the N_2O production rates, it is unlikely that they were directly

responsible for the difference between the copepods+diatoms and the copepods treatments. The diatoms can however be indirectly responsible by influencing the composition of the copepod outfluxes, since the composition of the copepods excretion products depends on the food type (Frangoulis et al. 2005). The importance of the copepod excretions is also confirmed by the low initial N_2O production rates for the spent medium treatment (Fig. 10c). The outfluxes do not only provide nutrients, such as phosphate (see above), but also organic compounds to the microcosms. These contain high amounts of labile carbon (Frangoulis et al. 2005), which are known to stimulate DNRA over denitrification and ANAMMOX (Burgin and Hamilton 2007). Additionally, more organic matter results in a higher sulphate reduction rate (Berner et al. 1985). The main product of sulphate reduction is hydrogen sulphide, which inhibits denitrification and nitrification, but not DNRA (An and Gardner 2002). Bioturbation, caused by the actively moving copepods, enhances the sulphate reduction even further (Berner et al. 1985; Fig. 10d).

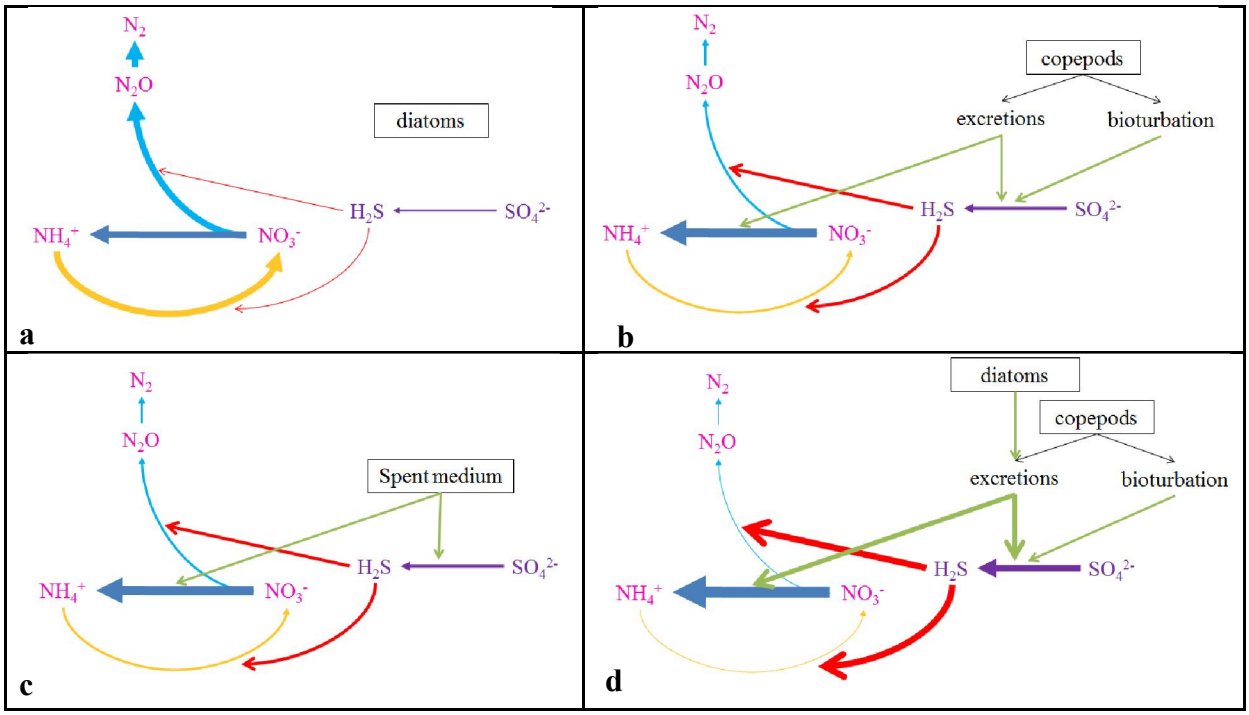


Figure 10: Summary of the assumed interactions to explain the observed differences in initial N_2O production rates. Treatments: a = diatom treatment; b = copepod treatment; c = spent medium treatment; d = copepod+diatom treatment. Inorganic nitrogen pools are indicated in pink; nitrate reducing pathways are indicated in blue: DNRA = dark blue and denitrification = light blue. Nitrification is indicated in orange. Treatments are placed in a black rectangle. Positive interactions are indicated as green arrows, negative ones as red arrows. The thickness of the arrows representsthe strength or rate of the interaction.

More evidence for the stimulation of DNRA by the copepod outfluxes is found in the final ammonium concentrations, which were higher in the treatments with copepods and the spent medium treatment, which contained their waste products.

If confirmed, these observations have important implications for the small-scale nutrient fluxes in estuarine sediment and the role of meiofauna herein. It was already known that meiofauna facilitates biomineralization of organic material and enhances nutrient regeneration (Coull 1999).They do so, not directly themselves, but by stimulating the bacterial community (Coull 1999). They can do this by producing excretion products and bioturbating the sediment . These same effects now also seem to be responsible for the enhancement of DNRA. Consequently, more active nitrogen will be preserved in the ecosystem with meiofauna present. By increasing the nutrient fluxes and reducing nutrient loss through denitrification, meiofauna is likely to actually enhance eutrophication.

The initial N_2O production rate with acetylene for the increased nitrate treatment was unexpectedly similar to the blank and the production rate without acetylene was even lower. In general, the denitrification rate is positively influenced by the nitrate load (e.g. Bartoli et al. 2012, Koch et al. 1992), but such a relation was not observed here. However, for a shorter, three and a half day long incubation period, the initial N_2O production rate with acetylene was almost twice as high for the increased nitrate treatment compared to the blank (data not shown). This indicates that the supplemented nitrate was depleted within the first few days and that the denitrifying community changed accordingly. This example clearly illustrates the importance of the temporal scale in this setup.

4.2.2.a. Ratio of the initial nitrous oxide production rates

The ratio between the initial N_2O production rate with acetylene (indication of the N_2 and N_2O produced) and the initial N_2O production rate without acetylene (indication of the N_2O reduced) was very low (0.46±0.03) compared to the net production rate between N_2O and N_2 generally observed in denitrification (0.0002- 0.06; Seitzinger 1998). This can, at least partly, be attributed to the limitations associated with the acetylene inhibition technique, such as the suppression of microbial respiration by acetylene and the incomplete inhibition of N_2O reductase (Felber et al. 2012). The N_2O will also have been produced by other processes, such as DNRA (Seitzinger 1988). The high nitrate concentrations added to the setup in order to measure the denitrification potential will also suppress N_2O reductase (Beaulieu et al. 2010). Likewise, hydrogen sulphide will block N_2O reductase (An and Joye 2001). Lastly, the shaking, done between the measurements of the gas production will have increased the chance of N_2O escaping before it was reduced to N_2.

The ratio differed significantly between treatments, suggesting a different influence of the treatments on the nitrous oxide reductase expression, compared to the nitric oxide reductase expression (cf. Fig. 1). The lowest ratio was obtained for the increased nitrate treatment. This might be attributed to a difference in reaction time of the enzyme expression to the fast changing nitrate concentration. The ratio was also low for the copepods+diatoms treatment. The lower ratio is a result of a low denitrification rate. Due to the low denitrification activity, not much more N_2O will be produced with acetylene, than without acetylene, hence, the ratio between them is small.

5. Conclusions

The denitrifying community at the study site could not fully be identified, since only one denitrifying species (*Marinobacter* sp.) was obtained. To obtain more isolates, the isolation effort should be increased, in combination with the use of optimized media and isolation conditions.

This study does however provide the first conclusive evidence for the impact of meiofauna on denitrification. Copepods were shown to decrease the denitrification potential through their activity and outfluxes. Diatoms themselves had no impact on denitrification, but when fed to copepods, they enhanced the negative impact of the meiofauna on the denitrification potential. The conditions created by the copepods favored DNRA over denitrification and therefore increased the amount of active nitrogen retained in the system. These results therefore show that the small-scale biological reactions between bacteria, copepods and diatoms have an important impact on denitrification and should therefore not be neglected.

6. Samenvatting

Onder invloed van de menselijke activiteit is gedurende de laatste eeuw de stikstofbelasting in aquatische systemen sterk toegenomen. In kustgebieden, zoals estuaria, is de stikstoftoevoer tot zelfs vertienvoudigd. Aangezien estuaria meestal stikstof gelimiteerd zijn, zijn ze bijzonder gevoelig voor een verhoogde N input. Eutrofiëring vormt tegenwoordig in veel estuaria dan ook een belangrijk probleem. Het is een complex proces, die de structuur van de estuariene gemeenschap sterk kan verstoren. Het beter begrijpen van de stikstofcyclus in estuaria onder sterk antropogene druk is daarom een belangrijke onderzoeksvraag.

Denitrificatie is een anaeroob proces binnen de stikstofcyclus, die grote hoeveelheden stikstof uit estuaria kan verwijderen. Prokaryoten zetten tijdens dit proces nitriet (NO_2^-) en nitraat (NO_3^-) om tot stikstofgas (N_2) en distikstofmonoxide (N_2O). Terwijl stikstof een overwegend inert gas is, is distikstofmonoxide een potentieel broeikasgas en mede verantwoordelijk voor de aantasting van de ozonlaag.

Dissimilatore nitraatreductie naar ammonium (DNRA) is een ander nitraat reducerende proces. Hierbij wordt nitraat omgezet naar ammonium (NH_4^+). Via dit anaeroob proces, wordt, in tegenstelling tot denitrificatie, de reactieve stikstof in het systeem behouden.

In estuaria worden DNRA en denitrificatie voornamelijk verwezenlijkt door de benthische microbiële gemeenschap. Het belang van beide reacties is afhankelijk van zowel de fysicochemische (omgeving) als biologische factoren. Deze laatste zijn vaak minder goed bestudeerd dan de fysicochemische factoren. Er zijn dan ook weinig gegevens over het belang van diatomeeën (kiezelwieren) en meiofauna (meercelligen die door een 1 mm zeef gaan maar achterblijven op een 38 µm zeef) op de stikstoffluxen in marien sediment, niettegenstaande de hoge abundantie en het belang van beide groepen in marien sediment.

Deze masterproef had als doel het belang van de kleinschalige biologische interacties tussen bacteriën, diatomeeën en meiofauna (Copepoda, Crustacea) op denitrificatie te onderzoeken.

Hiertoe werden labo experimenten ingericht waarin, verschillende combinaties gemaakt werden van de verschillende organismen. Sediment, copepoden en diatomeeën werden verzameld in de Paulinapolder (Westerschelde, Nederland). Om de denitrificerende gemeenschap van het studiegebied te identificeren werden denitrificerende bacteriën van het ingezamelde sediment geïsoleerd en geïdentificeerd.

Er werd verondersteld dat de kleinschalige biologische interacties, onderzocht in deze studie een belangrijke impact zouden hebben op denitrificatie. Het mechanisme hierachter zou

waarschijnlijk een, door de interacties, geïnduceerde verandering in de denitrificerende bacteriële gemeenschap zijn.

De isolatie campagne leverde drie denitrificerende isolaten op. Deze werden geïdentificeerd door sequenering van een fragment van het 16S gen. De drie isolaten bleken één en dezelfde soort te zijn, een *Marinobacter* species. Gezien er slechts één denitrificeerder geïsoleerd werd, kon de dentrificerende gemeenschap van het studiegebied niet geïdentificeerd worden. Meer denitrificerende isolaten van de Paulinapolder kunnen hoogst waarschijnlijk bekomen worden door een uitgebreidere isolatie campagne uit te voeren, met geoptimaliseerde media en isolatiecondities.

Om de biologische interacties tussen diatomeeën, copepoden en bacteriën te kunnen ontrafelen werden microcosmos experimenten ingericht waarin (1) diatomeeën, (2) copepoden, (3) copepoden en diatomeeën, (4) de afvalproducten van copepoden of (5) zeewater aangerijkt met nitraat werden toegevoegd aan het sediment. Er werd tevens een blanco behandeling gemaakt door enkel gefilterd, natuurlijk zeewater aan het sediment toe te voegen.

Het sediment werd zeven en een halve dag lang geïncubeerd onder twee lichtregimes (continu donker en 12u licht/ 12u donker). Bij het begin en op het einde van de incubatie werden de concentraties van de nutriënten (fosfaat, ammonium, nitraat, nitriet en silica) bepaald. Na de incubatieperiode werd het denitrificatiepotentieel van elke microcosmos bepaald. Hiervoor werd een overmaat aan nitraat en glucose toegevoegd aan het sediment, overgebracht in een fles, die vervolgens anaeroob geïncubeerd werd. De snelheid waarmee het nitraat verbruikt werd en distikstofoxide die gevormd werd in de fles werden bepaald. De nitraatconsumptie was een mate voor de nitraatreductie activiteit (zowel door DNRA als door denitrificatie). De N_2O productie was een mate voor denitrificatie. Gedurende de incubatieperiode (7.5 dagen) van de experimentele behandelingen verhoogden vooral de ammonium- en fosfaatconcentratie door de afbraak van organisch materiaal. Ze verschilden tevens tussen de verschillende behandelingen, maar niet tussen de verschillende lichtregimes. Er was meer ammonium en fosfaat aanwezig in de behandelingen met copepoden of deze waarbij excretieproducten van copepoden werden toegevoegd. Daaruit kon worden afgeleid dat de aanwezigheid van copepoden en hun afvalproducten leidt tot een belangrijke toename in de elementen fosfor en stikstof die zeer belangrijk en vaak limiterend zijn in estuaria.

Daarnaast bleken de behandelingen ook een belangrijk effect te hebben op het denitrificatiepotentieel met name de N_2O productie. De verschillen tussen de behandelingen waren veel minder uitgesproken voor de nitraatconsumptie . Hieruit kon geconcludeerd

worden dat een lagere denitrificatie deels gecompenseerd werd door een hogere DNRA activiteit. Het lichtregime had geen significant effect op het denitrificatie potentieel.

De N_2O productie was laag in de behandelingen met extra copepoden of met toegevoegde excretieproducten van copepoden. De copepoden hadden dus een negatieve invloed op denitrificatie. De laagste N_2O productie werd echter gevonden in het sediment waar zowel copepoden en diatomeeën werden aan toegevoegd. Diatomeeën op zich bleken echter geen invloed te hebben op de N_2O productie, maar konden het negatief effect van copepoden op de gasproductie wel versterken.

Door hun aanwezigheid en activiteit was meiofauna dus in staat om denitrificatie negatief te beïnvloeden. Deze resultaten tonen voor de eerste maal meiofauna een impact heeft op denitrificatie. De hypothese, waarin gesteld werd dat de kleinschalige biologische interacties een effect kunnen hebben op denitrificatie in marien sediment, werd hierdoor bevestigd.

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Appendix: Cnudde & De Troch, unpublished

Species composition of the copepod community from Paulina polder.

Family	Species
Leptastacidae	*Paraleptastacus spinicaudus*
Tachidiidae	*Microarthridion littorale*
	Tachidius discipes
Laophontidae	*Asellopsis intermedia*
	Platychelipus littoralis
	Paronychocamptus nanus
	Paronychocampus curticaudatus
Cletodidae	*Enhydrosoma gariene*
	Enhydrosoma sp 2
	Cletocamptus sp
Miraciidae	*Delavalia palustris*
	Robertsonia diademata
	Amphiascus sp 1
	Amphiascus sp 2
Nanopodidae	*Nannopus palustris*
Harpacticidae	*Harpacticus flexus*
	Harpacticus sp 2
Ectinosomatidae	*Ectinosoma sp*
	Pseudobradya minor
	Bradya sp