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Effect of high electron donor supply on dissimilatory nitrate reduction pathways in a bioreactor for nitrate removal

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HIGHLIGHTS

- High C/NO_3^- ratios and/or sulfide concentrations do not necessarily lead to a shift from DEN to DNRA.
- Microbial communities do not always use the energetically most favorable process.
- Reaction rates and biomass build-up are as important as the theoretical energy gain.

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ABSTRACT

The possible shift of a bioreactor for NO_3^- removal from predominantly denitrification (DEN) to dissimilatory nitrate reduction to ammonium (DNRA) by elevated electron donor supply was investigated. By increasing the C/NO_3^- ratio in one of two initially identical reactors, the production of high sulfide concentrations was induced. The response of the dissimilatory NO_3^- reduction processes to the increased availability of organic carbon and sulfide was monitored in a batch incubation system. The expected shift from a DEN- towards a DNRA-dominated bioreactor was not observed, also not under conditions where DNRA would be thermodynamically favorable. Remarkably, the microbial community exposed to a high C/NO_3^- ratio and sulfide concentration did not use the most energy-gaining process.

1. Introduction

The industrial and agricultural discharge of nitrogen compounds, especially nitrate (NO_3^-) and ammonium (NH_4^+), into groundwater, rivers and coastal areas has a substantial environmental impact (Sun and Nemati, 2012). Excess inorganic nitrogen in aquatic ecosystems causes eutrophication, resulting in increased occurrence of harmful algae blooms (Burgin and Hamilton, 2007) and the depletion of oxygen in bottom waters and sediments, leading to hypoxic zones (Diaz and Rosenberg, 2008). Therefore, the removal of NO_3^- from wastewaters and brines before entering rivers and the ocean is essential, and can be mediated by microbial processes in bioreactors.

Two microbially catalysed nitrogen removal processes used in wastewater treatment plants are denitrification (DEN) and anaerobic NH_4^+ oxidation (anammox). The end product of both processes, dinitrogen gas (N_2), is emitted from the wastewater treatment plants and has no harmful impact on the environment. However, NO_3^- can also be reduced to NH_4^+ via dissimilatory nitrate reduction to ammonium (DNRA) under anoxic/reduced conditions. Thereby, the nitrogen is recycled and remains as NH_4^+ within the ecosystem and thus DNRA does not alleviate eutrophication (Jäntti and Hietanen, 2012). The balance between these three NO_3^- converting processes is important as it defines whether fixed nitrogen is retained in or lost from wastewater bioreactors and in consequence from aquatic ecosystems.

Under NO_3^- limited conditions and high electron donor availability, DNRA is thought to be the favored NO_3^- reduction pathway (Christensen et al., 2000; Herbert, 1999; Tiedje, 1988), as per mole NO_3^- slightly more energy is gained by DNRA than by DEN with glucose as carbon source (Strohm et al., 2007). Under contrary conditions, i.e., high NO_3^- availability and electron donor limitation, DEN is the thermodynamically favorable pathway, as per mole electron donor more energy is gained.

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Beside nitrogen compounds, wastewaters are often contaminated with sulfide (S^{2-}, HS^- and H_2S). A high concentration of sulfide can inhibit DEN and anammox (Brunet and GarciaGil, 1996; Jin et al., 2013; Sørensen et al., 1980), and stimulate DNRA by serving as an additional electron source (Brunet and GarciaGil, 1996; Christensen et al., 2000).

In this study, the effect of high organic carbon and sulfide supply on NO_3^- reduction processes was investigated in two denitrifying upflow sludge-blanket bioreactors (USB) to test the hypothesis that high electron donor supply shifts microbial communities from DEN- to DNRA-dominated activity. The first reactor (R1) was an established denitrifying bioreactor; the second one (R2) was inoculated from R1 and by increasing the C/NO_3^- ratio sulfate reduction was stimulated. The long-term ($\geqslant$6 months) and short-term (120 min) influence of higher electron donor supply (organic carbon and sulfide) on anaerobic nitrogen cycling was evaluated in batch incubation experiments with granular sludge taken from R1 and R2. In these experiments, ^{15}N-labeled inorganic nitrogen compounds were used to trace the activities of DEN, DNRA, anammox, and NH_4^+ assimilation. Additionally, the microbial communities established in R1 and R2 were analyzed to determine potential differences by pyrosequencing, targeting functional genes involved in DEN and DNRA.

2. Methods

2.1. Upflow sludge-blanket (USB) bioreactors

Two wastewater reactors (R1 and R2) for the removal of NO_3^- from saline wastewaters were constructed and operated at the Faculty of Civil and Environmental Engineering at the Technion in Haifa, Israel. The two USB bioreactors consisted of a vertical tube with an inner diameter of 9 cm and had a working volume of around 2.8 L. Inside the reactors, the granular sludge and feeding solution were held at a constant temperature of 25 °C and mixed in time intervals with an internal stirrer. The reactor feeding solution (1% salinity) consisted of 128 mmol L^{-1} NaCl, 5.1 mmol L^{-1} $CaCl_2\times2H_2O$, 3.7 mmol L^{-1} $MgCl_2\times6H_2O$, 1.2 mmol L^{-1} $NaHCO_3$, 0.07 mmol L^{-1} KH_2PO_4, 0.35 mmol L^{-1} Na_2SO_4, and 1 mmol L^{-1} $NaNO_3$, was prepared with tap water and adjusted to a pH of 7.0. The reactor feeding solution was pumped upwards through the granular sludge and the effluent left the reactor through an outlet at the top.

The first reactor (R1) was an established denitrifying reactor and construction and handling was done according to Beliavski et al. (2010). As carbon source, 0.8 mmol L^{-1} acetic acid was added to the reactor feeding solution. The second reactor (R2) was prepared from granular sludge produced in R1, and the addition of 2.2 mmol L^{-1} ethanol as carbon source. By changing the C/NO_3^- ratio, the sulfide concentration increased inside R2 to approximately 1 mmol L^{-1} (determined according to Pachmayr, 1960) through stimulation of sulfate reduction. In R1, sulfide concentrations always remained below 3 μmol L^{-1}. The potential metabolic pathways and the calculated $\Delta G^{0'}$ values for both reactors are given in SupplInfo Table S1.

Granular sludge of these two reactors was sampled for batch incubation experiments to investigate the response of the NO_3^- reducing bacterial community to elevated electron donor supply in ^{15}N-labeling experiments.

2.2. Batch incubation experiments with granular sludge from R1 and R2

To quantify DEN and DNRA, $^{15}NO_3^-$ (99% ^{15}N at%, Cambridge Isotope Laboratories, Andover, MA, U.S.A.) was used as tracer in batch incubation experiments. The composition of the feeding solution was the same as for the main reactors but without sulfate. Accordingly, 0.8 mmol L^{-1} acetic acid was used as carbon source for the granular sludge taken from R1 and 2.2 mmol L^{-1} ethanol for the granular sludge taken from R2. The occurrence of anammox and N-assimilation was quantified in a separate batch experiment with $^{15}NH_4^+$ (98% ^{15}N at%, Cambridge Isotope Laboratories, Andover, MA, U.S.A.) as tracer. The experiments were performed on sludge from both reactors and can be summarized as follows:

Experiment 1: 1000 μmol L^{-1} $^{15}NO_3^-$, 250 μmol L^{-1} $^{14}NH_4^+$ and no sulfide added to the feeding solution.
Experiment 2: 1000 μmol L^{-1} $^{15}NO_3^-$, 250 μmol L^{-1} $^{14}NH_4^+$ and 1000 μmol L^{-1} Na_2S added to the feeding solution.
Experiment 3: 1000 μmol L^{-1} $^{14}NO_3^-$, 250 μmol L^{-1} $^{15}NH_4^+$ and no sulfide added to the feeding solution.

Before the start of the experiments and the addition of sulfide in experiment 2, the batch incubation feeding solution was adjusted to pH 7.0 and flushed with He for 20 min to establish anoxic conditions. Two bottles were run in parallel for each experiment and reactor, taking 2–3 mL fresh granular sludge from R1 and 1 mL from R2.

Feeding solution and fresh granular sludge were filled into a 100-mL glass bottle closed with a gastight stopper, avoiding inclusion of air bubbles. The granules were kept suspended within the bottle using a magnetic stirrer and a glass-coated stirring bar. Two needles were inserted into the stopper, one serving as the inlet connected to a reservoir of feeding solution, and the other one serving as a sampling port. During the experiment, all water samples were drawn through a filter, to keep the biomass inside the bottles.

Water samples (in total 12 mL) were taken every 20 min for analyses of NO_3^-, NH_{4tot}^+, N_2O, $^{15}NH_4^+$ (DNRA), $^{30}N_2$ (DEN) and $^{29}N_2$ (anammox). NO_3^- was analyzed by chemical conversion with VCl_3 to NO, which was quantified by the chemoluminescence detector of an NO_x-analyser (CLD 66, EcoPhysics, Germany) (Braman and Hendrix, 1989). NH_4^+ was analyzed according to the salicylate-hypochlorite method (Bower and Holm-Hansen, 1980). At every second sampling time point (40, 80, and 120 min), an additional sample (0.5 mL) was fixed in 2% ZnAc and analyzed for sulfide according to Pachmayr (1960). The sampled volume was replaced by fresh feeding solution and the resultant dilution was taken into account for rate calculations. All experiments were terminated after 120 min.

2.3. Rates of dissimilatory nitrate reduction processes (experiments 1 and 2)

All samples taken during the batch incubation experiments were analyzed to derive net turnover rates from concentration changes over time and the rates were normalized to the protein concentration of the granular sludge. Protein concentrations were determined in sub-samples of 1 mL freshly taken granules from each reactor. After extraction in 0.5 N NaOH at 80 °C for 20 min, proteins were quantified against bovine serum albumin standards according to Lowry et al. (1951).

For measuring the DEN activity (measured as $^{30}N_2$ production), 1 mL water sample was transferred into a 3-mL He-flushed, gastight exetainer (Exetainer; Labco, High Wycombe, UK), frozen at −20 °C and shipped to the laboratory in Bremen for further analysis. During thawing, 50 μL 50% $ZnCl_2$ was added to avoid further reduction of $^{15}NO_3^-$ to $^{30}N_2$. The exetainers were then left upside-down for 3 days at 21 °C to complete N_2 equilibration between medium and headspace. Subsequently, a headspace volume of 25–50 μL was analyzed for the isotope ratios of $^{28}N_2$, $^{29}N_2$, and $^{30}N_2$ by gas chromatography–isotopic ratio mass spectrometry (VG Optima, ISOTECH,