The influence of *Glyceria maxima* and nitrate input on the composition and nitrate metabolism of the dissimilatory nitrate-reducing bacterial community

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Abstract

The influence of nitrate addition and the presence of *Glyceria maxima* (reed sweetgrass) on the composition and nitrate metabolism of the dissimilatory nitrate-reducing bacterial community was investigated. Anoxic freshwater sediment was incubated in pots with or without *G. maxima* and with or without addition of nitrate. After incubation the sediments were sampled. Dilution series of these sediment samples were incubated in deep agar tubes and almost all colonies from the most diluted tubes were isolated and purified. When the nitrate concentration was low, 80% of the nitrate-reducing community in the rhizosphere of *G. maxima* consisted of NO_2^--accumulating or NH_4^+-producing *Bacillus* strains. In bulk sediment with low nitrate concentrations, denitrifying *Pseudomonas* and *Acinetobacter* strains were dominant. The difference in the composition of the nitrate-reducing community between sediment with or without plants disappeared when nitrate was added. Denitrifying pseudomonads then made up 78–86% of the nitrate-reducing community. This shows that when the nitrate concentration was low, the presence of *G. maxima* greatly influenced the nitrate-reducing community. However, when nitrate was present and available, the composition and nitrate metabolism of the dominant nitrate-reducing community was probably not affected by *G. maxima*.

Keywords: Denitrification; DNRA; Freshwater sediment; Nitrate; Root zone; Bacterial community

1. Introduction

There are two known dissimilatory nitrate-reducing pathways: denitrification and the dissimilatory nitrate reduction to ammonia (DNRA). Denitrification is the reduction of nitrate (NO_3^-) to nitric oxide (NO), nitrous oxide (N_2O) and, in most cases, dinitrogen (N_2). By this process mineral nitrogen concentrations in soils and sediments may be reduced. In contrast, the dissimilatory reduction of NO_3^- to ammonia (NH_4^+) leads to conservation of mineral nitrogen in soils and sediments. Denitrification is the dominant process in soils and in sediments [1–4], whereas DNRA can be considerable in marine and freshwater sediments and sometimes in soils

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[5–7,2]. Knowledge of the factors that determine the composition of the dissimilatory nitrate-reducing community would help to understand and predict temporal and spatial variation in denitrification and DNRA. The two processes are executed by different bacterial groups. The denitrifying population consists to a large extent of obligate oxidative organisms [4]. Most of the denitrifying bacteria found in soil, freshwater or estuarine sediments belong to the genera *Pseudomonas*, *Alcaligenes*, *Paracoccus* [4,1]. Bacteria dissimilating NO_3^- to NH_4^+ consists of facultative anaerobic, fermentative organisms, such as *Klebsiella*, *Enterobacter*, *Vibrio*, *Citrobacter*, *Achromobacter* and *Bacillus* [6,8,9,4]. When there is sufficient NO_3^-, the most important factor determining which of the two nitrate-reducing processes will be favoured is probably the ability to compete for organic substrates [4]. Another, important factor is the ratio of electron donors (i.e. organic substrates) and electron acceptors (i.e. nitrate and oxygen). Oxygen may also be an important environmental parameter in determining the composition of the nitrate-reducing community. In an anoxic environment without NO_3^- only the bacteria reducing NO_3^- to NH_4^+ will be able to grow, due to their fermentative abilities. A special environment in largely anoxic sediments is the root zone of aerenchymatous plant species, as these plants will release oxygen into the root zone [10–12]. The oxygen released may stimulate NO_3^- production and hence may lead to higher nitrate-reducing activities [13,14]. Little is known about the influence of wetland plant species, like *Glyceria maxima (Hartm.) Holmb.*, on the end-products of bacterial nitrate reduction, even though wetlands contribute substantially to global N_2O production [15,16].

The aim of the research presented was to examine the influence of *G. maxima* and the addition of nitrate on the composition and nitrate metabolism of the nitrate-reducing bacterial community.

2. Materials and methods

2.1. Sampling location and procedure

Bulk sediment (0–15 cm upper layer) was sampled in August 1990 at Junner Koeland, (52°30′N, 06°30′E) in a shallow, isolated meander of the river Vecht, in the Netherlands. *G. maxima* was the dominant aerenchymatous plant at this location. The sediment was transported to the laboratory in airtight buckets at 4°C, thoroughly mixed and the initial sediment parameters were determined. Six pots (500 ml) were filled with sediment, to study the effects of NO_3^- additions and the presence of *G. maxima*, on the species composition and nitrate metabolism of the nitrate-reducing community. In four of the six pots three non-sterile seedlings were planted. Pots with plants received either 0 or 531 µg NO_3^--N (g dry sediment)$^{-1}$, pots without plants received 0 or 235 µg NO_3^--N (g dry sediment)$^{-1}$. NO_3^- addition between sediments with and without plants differs because of differences in evapotranspiration and evaporation. The experiment was conducted in a growth chamber (Heraeus Vötsch, HPS-1500, Heraeus, Wijk bij Duurstede, Netherlands), air humidity was 80%, temperature 20°C–15°C with a light-dark regimen of

Table 1
Sediment parameters of Junner Koeland sediment before the experiment (initial) and after harvest

Added nitrate	Sediment type	NO_3^-	NH_4^+	pH		% Organic matter
				H_2O	KCl	
Initial	Bulk	2.2[b]*	23.1[c]	6.9[a]	6.3[a]	2.1
0	Rhizosphere	0.5[a]	5.8[ab]	6.9[a]	6.4[a]	1.8
	Bulk	1.7[ab]	10.0[b]	6.9[a]	6.4[a]	N.D.
531	Rhizosphere	1.3[ab]	4.1[a]	7.5[b]	6.7[b]	1.9
235	Bulk	1.1[ab]	15.7[c]	7.6[b]	6.8[b]	1.9

Concentrations are given in µg N (g dry sediment)$^{-1}$. *Significant differences are indicated with different letters ($P < 0.05$). N.D. = not determined.

16–8 h and a light intensity of 215 ± 10 µmol $(m^2 \cdot s)^{-1}$. After 9–12 weeks incubation, the roots had spread throughout the sediment and all sediment could be regarded as rhizosphere sediment. Rhizosphere sediment from the pots with plants and bulk sediment from the pots without plants were collected and the composition and nitrate metabolism of the nitrate-reducing community was determined.

2.2. Sediment analysis

The pH (H_2O) of the sediments was determined by shaking 5 g of moist sediment with 10 ml water for 2 h. The pH (KCl) and the mineral nitrogen concentrations were determined by shaking 5 g of moist sediment with 10 ml 1 M KCl also for 2 h. After shaking, samples were taken and centrifuged at $15\,000 \times g$ in a Biofuge A (Heraeus Christ, Dijkstra Verenigde, Almere, Netherlands) bench centrifuge for 10 min. The supernatants were analyzed for NO_3^-, NO_2^- and NH_4^+ using a Technicon Traacs 800 autoanalyzer (Technicon Instruments Corp., Tarrytown, NY, USA) with a detection level of 10 µM for all three compounds. The organic matter content was determined by analysis of weight losses after heating of about 5 g of dry sediment at 550°C for 4 h.

2.3. Composition of the dominant nitrate-reducing community

Three dilution series of sediment samples were made in deep agar tubes. The medium consisted of tryptic soy broth (TSB) (per litre: 19 g tryptone, 3 g soy peptone, 2.5 g dextrose, 5.0 g chloride and 2.5 g phosphate) with nitrate (4 mM) and 1% (w/v) agar,

pH 7.1–7.3 [4]. After incubation for 10 days at 20°C, as many as possible visually different colonies were picked from the most diluted tubes and subcultured aerobically until pure cultures were obtained. The isolated potentially nitrate-reducing bacteria were tentatively divided into different groups by four tests: the Gram test [17], the oxidase test with 1% (w/v) tetramethyl-*p*-phenylene-diamine-HCl, the catalase test with 10% (v/v) H_2O_2 and the oxidation-fermentation test with glucose as substrate [18]. The Gram-positive strains were examined microscopically for the possession of endospores. The Gram-negative strains were further identified with API-20NE and API-20E tests (Api System, S.A., Montalieu-Vercieu, France), to genus level.

2.4. Nitrate metabolism of the dominant nitrate-reducing bacteria

The cultures were tested on different media under different conditions. The first test was under anoxic conditions in deep agar tubes. The medium consisted of TSB with and without nitrate (10 mM). The tubes were incubated at 20°C. Based on this test the initially isolated strains were divided in three groups. The first group of isolates was non-culturable under the tested conditions. The second group of isolates was able to grow but did not consume NO_3^-: the non-nitrate reducing strains. The third group of isolates grew in the tubes and was also able to consume NO_3^-: the nitrate-reducing strains. The isolates producing gas in the presence of NO_3^- and without growth in the absence of NO_3^- were considered to be potentially denitrifying strains. When growth was observed in the tubes with and without NO_3^-, the strains were tentatively classified as bacteria able to

Table 2
The effect of different NO_3^- additions on the percentage non-culturable, non-nitrate-reducing and nitrate-reducing strains in the rhizosphere of *Glyceria maxima* and in bulk sediment

Sediment type	Rhizosphere $n = 57$	Bulk $n = 72$	Rhizosphere $n = 59$	Bulk $n = 38$
Added NO_3^-	0	0	530	235
% Non-culturable	32	8	20	58
% Non-nitrate reduction	42	14	34	24
% Nitrate reduction	26	78	46	18

Concentration added nitrate given in µg N (g dry sediment)$^{-1}$.

without nitrate addition

rhizosphere   bulk

N=15   N=56

with nitrate addition

rhizosphere   bulk

N=27   N=7

■ Pseudomonas   ▨ Acinetobacter

▩ Bacillus   □ Aeromonas

Fig. 1. Genus composition of the culturable nitrate-reducing bacterial community in the rhizosphere of *Glyceria maxima* and in bulk sediment, isolated from Junner Koeland sediment, amended with 0 and 235–531 μg NO_3^--N (g dry sediment)$^{-1}$.

series 2, Carlo-Erba Instruments, Milan, Italy) equipped with a hot wire detector and a Porapack Q column. N_2 was used as carrier gas (flow rate 30 ml per min). The column, injector and detector temperatures were 80°C, 120°C and 119°C, respectively. Peak area was computed by an integrator (model CR3A, Shimadzu, Interscience, Breda, Netherlands). The nitrate-reducing strains were divided into three groups based on the major end-products that were formed, group I: N_2O producers, reducing $> 80\%$ of the added NO_3^- to N_2O; group II: NO_2^- accumulators, reducing $> 50\%$ of the added NO_3^- to NO_2^- and N_2O; group III: NH_4^+ producers, reducing about 50% of the added NO_3^- to NH_4^+, and no NO_3^-, NO_2^- or N_2O were detectable.

2.5. Statistical analysis

Data of sediment parameters were analyzed by one-way ANOVA. Differences between means were tested for significance using the least significant difference (LSD) procedure.

3. Results and discussion

3.1. Sediment parameters

The sediment parameters of Junner Koeland are given in Table 1. After incubation, the NO_3^- concentration only decreased significantly, compared to the initial value, in the unamended rhizosphere sediment. In contrast the NH_4^+ concentrations, especially in the rhizosphere, decreased significantly during incubation compared to the initial NH_4^+ concentration. Only in bulk sediment to which 235 μg NO_3^--N (g dry sediment)$^{-1}$ had been added, the NH_4^+ concentration did not show significant decrease compared to the initial concentration. With the addition of NO_3^-, the $pH(H_2O)$ and the $pH(KCl)$ increased significantly.

3.2. Composition of the dominant nitrate-reducing bacteria

In Table 2, the effect of the different NO_3^- additions and the presence of *G. maxima* on the percentage non-culturable, non-nitrate-reducing and ni-

dissimilate NO_3^- to NH_4^+. To determine the metabolism of the nitrate-reducing strains more precisely these bacteria were again tested on TSB (100%) with and without NO_3^- in 100 ml serum bottles. The bottles were flushed with N_2 for 10 min and subsequently 10 kPa C_2H_2 was injected, to inhibit reduction of N_2O to N_2. The bottles were incubated for 2 weeks at 20°C. After the incubation period, the bottles were tested for N_2O production, NO_3^-, NO_2^- and NH_4^+ concentration. N_2O concentrations were measured using a gas chromatograph (6000 VEGA

without nitrate addition

rhizosphere
N=15

bulk
N=56

with nitrate addition

rhizosphere
N=27

bulk
N=7

■ N_2O-producer   ▨ NO_2^--accumulator

▧ NH_4^+-producer
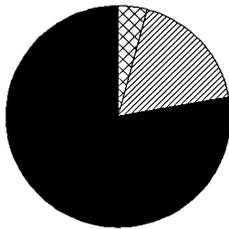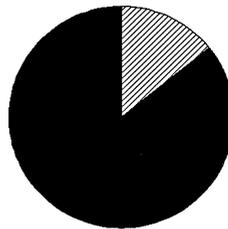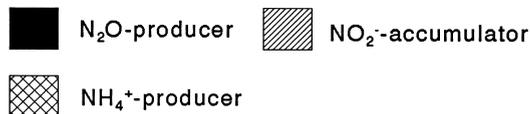
Fig. 2. Nitrate metabolism of the culturable nitrate-reducing bacterial community in the rhizosphere of *Glyceria maxima* and in bulk sediment, isolated from Junner Koeland sediment, amended with 0 and 235–531 µg NO_3^--N (g dry sediment)$^{-1}$.

trate-reducing strains are given. The percentage of nitrate-reducing strains in the rhizosphere increased when NO_3^- was added. In bulk sediment with NO_3^- addition the percentage of nitrate-reducing bacteria was low, due to the high proportion of non-culturable strains.

These percentages agreed well with the percentage non-culturable and non-nitrate-reducing strains initially isolated from various soils and sediments [19,1]. From the initial isolated bacteria 37% [19] to 41% [1] were non-culturable when transferred to liquid media

and 31% of the isolated strains were unable to reduce nitrate [19].

The culturable strains were identified to genus level regardless the ability to reduce NO_3^-. In this way the composition of the culturable strains could be compared to the composition of the culturable, nitrate-reducing strains. Although the percentage of strains unable to reduce NO_3^- was rather high, the genus composition was not affected by excluding the non-nitrate-reducing strains from the total number culturable, nitrate-reducing strains. Data presented in Fig. 1 show the genus composition of the culturable nitrate-reducing community present. The results show that different genera were present in the rhizosphere and bulk sediment when no NO_3^- was added. Since the tested strains were not consistently isolated from the most diluted tubes, to obtain as many as possible visually different strains ($n = 105$), the genus composition was also determined based on colonies isolated only from the most diluted tubes (10^{-8}, 10^{-9}, $n = 34$). The composition of the nitrate-reducing community based on strains isolated from these tubes was similar (data not shown) to the data presented in Fig. 1. So, the composition represents the dominant culturable nitrate-reducing genera (Fig. 1). From the deep agar tubes it was not possible, due to gas production, to determine the total number of potentially nitrate-reducing bacteria. Nonetheless, information concerning the number of gas-producing strains could be gained from these tubes. The number of gas-producing strains was the same in rhizosphere and bulk sediment without NO_3^- addition (data not shown). In the rhizosphere sediment without NO_3^- addition, the nitrate-reducing community consisted of 80% of Gram-positive strains. In the bulk sediment Gram-negative *Pseudomonas* and *Acinetobacter* strains were more abundant. When NO_3^- was added the difference between rhizosphere and bulk sediment almost disappeared. In both cases Gram-negative strains became dominant. The number of gas-producing strains increased by one (rhizosphere) or two (bulk) orders of magnitude, compared with no NO_3^- addition.

The genus composition in the rhizosphere and non-rhizosphere of *T. angustifolia* was different from that of *G. maxima*. In the rhizosphere of *T. angustifolia* many Enteriobacteriaceae and *Aeromonas/Vibrio* bacteria were present [20]. In soil the com-

position of the nitrate-reducing community was investigated by Smith and Zimmerman [21]. The nitrate-reducing community consisted of denitrifiers: *Pseudomonas*, *Flavobacterium* and *Alcaligenes* species and NO_2^- accumulators: *Bacillus*, *Enterobacter*, *Flavobacterium* and *Citrobacter* species. In an estuarine sediment a large percentage of the nitrate-reducing community belonged to the fermentative *Aeromonas/Vibrio* group [6,9]. Most strains isolated from a soil were Gram-positive bacteria, showing endospores, suggesting that they were *Bacillus* strains [19].

3.3. Nitrate metabolism of the dominant nitrate-reducing bacteria

Fig. 2 shows the nitrate metabolism of the dominant bacteria belonging to the nitrate-reducing community. In the rhizosphere without NO_3^- addition, the percentage of N_2O producers was only 7%, whereas the percentage of NO_2^- accumulators was 40% and NH_4^+ producers 53%. In the bulk sediment 71% of the isolated strains produced N_2O. When NO_3^- was added the nitrate-reducing community in the rhizosphere and bulk sediment consisted of 78% and 86% of N_2O producers, respectively. In the rhizosphere sediment only 4% of the *Bacillus* strains produced NH_4^+. Our results, concerning the nitrate metabolism of the nitrate-reducing community, did not differ much from the nitrate metabolism of the nitrate-reducing community isolated from the rhizosphere and non-rhizosphere of *Typha angustifolia* [20], taking the vague difference between NO_2^- accumulators and assumed NH_4^+ producers into account and excluding the unidentified bacteria [20].

3.4. Concluding remarks

Apparently the presence of the flooded aerenchymatous plant *G. maxima* was of great importance for the composition of the nitrate-reducing bacterial community under conditions of NO_3^- depletion. However, when NO_3^- was added, the presence of *G. maxima* had little influence on the composition and nitrate metabolism of the dominant nitrate-reducing community, provided nitrogen enters the environment as NO_3^-. The opposite may be true when only NH_4^+ is present. The presence of *G. maxima* is then probably important, due to the coupling between nitrification and denitrification in sediments [22], especially in the rhizosphere of aerenchymatous plants [13,12,4]. So, under NO_3^- limiting conditions, NO_3^- produced in the rhizosphere of *G. maxima* will subsequently be converted to NH_4^+ and the production of N_2O will be restricted. Only under conditions of sufficient NO_3^- supply denitrification and consequently N_2O production may occur but this is not affected by *G. maxima*. Thus *G. maxima* has no effect on the production of the greenhouse gas N_2O, either with or without the addition of NO_3^-.

The hypothesis is put forward that under NO_3^- limiting conditions the influence of the aerenchymatous plant *G. maxima* on the composition and nitrate metabolism of the nitrate-reducing community is large. When sufficient NO_3^- is available, the nitrate-reducing bacteria are less dependent on *G. maxima* and consequently the influence of *G. maxima* is minimal.

However, Brunel et al. [20] stated that the roots of *T. angustifolia* had no distinct effect on the dominance of denitrifiers or fermentative bacteria reducing NO_3^- to NH_4^+. The results of this study do differ from our results, also regarding the composition of the nitrate-reducing community. This could be due to the different aerenchymatous plant or to the fact that in the sediment of *T. angustifolia* NO_3^- was not limited.

In future, further research is necessary to verify the hypothesis, and to clarify if the sediment type, the aerenchymatous plant species or the nitrate concentration is an important parameter controlling the composition of the nitrate-reducing bacterial community. Also, further research is necessary to prove that NO_3^- availability quantitative leads to higher nitrate-reducing activity and dominance of denitrifying bacteria, regardless the presence of an aerenchymatous plant.

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