Effects of photoperiod on growth of and denitrification by *Pseudomonas chlororaphis* in the root zone of *Glyceria maxima*, studied in a gnotobiotic microcosm*

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Abstract

The emergent macrophyte *Glyceria maxima* was subjected to different photoperiods and grown with ammonium or nitrate as nitrogen source in presterilized microcosms with spatially separated root and non-root compartments. The microcosms were inoculated with the denitrifying bacterium *Pseudomonas chlororaphis*. The effect of the plant and the photoperiod on growth and denitrification by *P. chlororaphis* was assessed. The plant had a strong positive effect on the growth of the bacteria. The bacterial numbers in the root compartment of the planted microcosms were 19-32 times higher than found in the non-root sediment of the unplanted systems. Lengthening the photoperiod resulted in elevated bacterial numbers due to the higher carbon exudation of the plant. This effect was greater still in the nitrate-fed plants, where additional *P. chlororaphis* growth could proceed via denitrification, indicating nitrogen-limiting conditions in the microcosms. Higher porewater N₂O concentrations in the root compartments compared to the non-root compartments, which were highest for the long photoperiod, were also indicative of a plant-induced stimulation of denitrification. An effect of a diurnal oxygen release pattern of *G. maxima* on denitrification could not be detected. The gnotobiotic microcosm used in this study represents a potential system for the study of the behaviour and interactions of important bacterial groups, such as nitrifying and denitrifying bacteria where plant roots drive bacterial activity.

Introduction

The presence of aerenchymatous plants can have a major impact on the composition and functioning of microbial communities in flooded soils and sediments. Aerenchyma, which is one of the adaptations of a broad range of plant species rooting in anoxic environments (Blom and Voesenek, 1996; Justin and Armstrong, 1987), facilitates the diffusion of oxygen from atmosphere to the roots. Root-released oxygen may stimulate nitrogen cycling in the rhizosphere. The oxidation of ammonia into nitrite and nitrate by nitrifying bacteria, facilitated by the released oxygen, can subsequently stimulate denitrifying bacteria which reduce nitrate to N₂ or N₂O (Bodelier et al., 1996; Christensen and Sørensen, 1986; Reddy et al., 1989).

Studying the impact of oxygen-releasing plants on denitrifying bacteria, and their interactions with other nitrogen converting bacteria, is extremely difficult under in situ conditions. Information concerning regulating factors such as the quantity of oxygen and carbon released is far from complete, as is our knowledge regarding the soil volume that is influenced by the roots under natural conditions. Furthermore reliable in situ activity measurements are not available and population dynamics studies are hampered by gross underestimations as a large proportion of soil and sediment bacte-
ria resist laboratory culturing techniques (Ward et al., 1990). Moreover, the complexity of microbial activities and chemical processes in natural heterogeneous soil or sediment makes assessment of in situ microbial characteristics extremely difficult.

One of the purposes of this study was to focus upon specific microbial interactions in an attempt to gain an insight into the complex in situ situation. Therefore we have developed a closed gnotobiotic soil-plant system as a model approach for plant-microbe studies. With the aid of this system, we investigated the impact of oxygen-releasing plants on denitrification in physically separated root ("macro"-rhizosphere) and non-root sediment compartments. To obtain information on the potential behaviour of denitrifying bacteria in situations where oxygen-releasing plants regulate substrate availability, we chose to manipulate the photoperiod of the plant, which undergoes daily and seasonal fluctuations. By varying the hours of daylight of the plant, which is positively related to the carbon exudation (Whipps, 1992), we aimed to vary electron donor availability both with and without excess nitrate.

We determined the influence of the emergent macrophyte Glyceria maxima (Hartm.) Holmb. on the growth of the heterotrophic bacterium Pseudomonas chlororaphis. As the bacterial strain we used is unable to reduce nitrous oxide to dinitrogen, denitrifying activity could be determined by measuring accumulated nitrous oxide in a normal dinitrogen-rich atmosphere. Plants were subjected to a 14 or 20 hour photoperiod, and effects of carbon exudation and the diurnal oxygen release pattern of the plant on denitrification were studied by monitoring the nitrous oxide concentration in the pore water of the root- and non-root compartments.

Materials and methods

Description and operation of the model system

A schematic, vertical transection of the gnotobiotic model system, consisting of a stainless steel cylindrical sediment compartment covered by a glass cylinder, is shown in Figure 1. The sediment compartment and glass cover were connected by means of a clamping device which could be vertically positioned by a nut located in the supports of the system. In the centre of the sediment compartment a perforated stainless steel cylinder (Ø of holes: 1 mm), covered on the inside with nylon gauze (mesh size 30 μm, Merrem and La Porte, Zaltbommel, The Netherlands), served to separate the rooted sediment from the bulk sediment. To sample the pore water, rhizon soil solution samplers (Eijkelkamp, Giesbeek, The Netherlands) were vertically installed to a depth of 2 cm below the sediment surface. The samplers consisted of a hydrophillic porous polyethylene with a typical pore diameter of 0.1 μm. They were mounted on an injection needle which was connected to a second needle via silicone tubing and a luer-lock connection. By placing an evacuated blood-collecting tube (3 mL, Terumo Venoject, Leuven, Belgium) on the second needle, pore water could be withdrawn from the system. Three pore water samplers were evenly distributed in the non-root compartment at 2.0 cm from the root compartment and 0.5 cm from the outer wall of the system. One sampling device was located in the centre of the root compartment and was also used for nutrient addition, the nutrient solution being led through a hydrophillic filter (0.2 μm, Schleicher and Schuell, Dassel, Germany). The floodwater on top of the sediment (4 cm) was replenished by a silicone tube from one of the top connections, which was led through a hydrophillic filter. The gas atmosphere in the glass cylinder was refreshed by means of a membrane pump (Whisper 500, Whisper and Silaflax, USA). The air inlet and outlet were equipped with hydrophobic PTFE inline membrane filters (0.2 μm, Gelman Science, Ann Harbour, USA). The complete system was filled with sand before being autoclaved (121 °C). All materials used were selected and tested for resistance to several autoclaving cycles.

Organisms used and pre-cultivation

Plant

Glyceria maxima (Hartm.) Holmb., a common emergent macrophyte in the Netherlands, was used as a model plant as it is known to promote bacterial nitrification and denitrification in the soil surrounding its roots (Bodelier et al., 1996; Both et al., 1992). G. maxima is highly aerenchymatous (Smirnoff and Crawford, 1983) and the inability of its roots to survive anaerobic conditions necessitates continuous oxygenation of the root tissue resulting in oxygen leakage into the rhizosphere (Brändle and Crawford, 1987; Rees et al., 1987).

Seeds were collected from a G. maxima stand in a ditch near the institute during the 1992 growing season. They were surface sterilized by washing them for 20 minutes in 5% (v/v) NaHClO₃ with one subsequent rinse with 70% (v/v) ethanol and three washes...
with sterile demineralised water. The sterilized seeds were transferred to potato dextrose agar (Oxoid Unipath LTD, Basingstoke, UK) plates and incubated for 3 days at 28 °C to check for the absence of fungi. After 3 days, non-contaminated seeds were placed on water agar (1.5% w/v) with 10% (0.3 g L⁻¹) nutrient broth (Difco, Detroit, USA) and incubated in a cabinet for germination (day/night 10 h 25 °C/14 h 15 °C, photosynthetic photon flux density 8-11 μE m⁻² s⁻¹). Seeds which still showed signs of infection were cut out of the agar and removed. After 8 days 65% of the seeds had germinated. 17 days old seedlings were used for the experiment. All manipulations with seeds and seedlings were performed in sterile flow cabinets.

Bacterium

Pseudomonas chlororaphis ATCC 43928 is a relatively common soil heterotroph which is capable of denitrification and is able to grow in a defined medium. This species is present in the root zone of G. maxima and even tends to dominate it after amendment with nitrate (Nijburg et al., 1997). We used a strain only capable of reducing nitrate to nitrous oxide (Christensen and Tiedje, 1988) which is of methodological convenience. The culture had been stored at -80 °C. Prior to inoculation of the model system, P. chlororaphis was grown in nutrient broth cultures in 250 mL flasks at 25 °C with 100 rpm shaking. After subculturing three times, the cultures were centrifuged (10000xg, 15 minutes, 15 °C) and the pellet was resuspended in phosphate buffer saline (PBS) solution (containing in grams per litre: NaCl, 8.5; NaH₂PO₄·H₂O, 0.35; Na₂HPO₄·2H₂O, 1.34; pH 7.5) and centrifuged again. This washing procedure was repeated three times. The cell numbers of the washed suspension were determined microscopically using a Bürker-Türk counting chamber. Suspensions were diluted with PBS buffer to the desired inoculation density (see below).

Experimental design

24 model microcosms were each filled with 800 grams of dry calcareous river sand from the floodplain of the river Rhine (Bemmel, The Netherlands). Sediment characteristics were pH(H₂O) 8.3, 0.66% organic matter, and a water holding capacity of 24.51%. After flooding the sand with demineralized water, the systems were closed and autoclaved twice for 60 minutes (121 °C). In 16 of the systems 8 axenic G. maxima seedlings were planted in the rhizosphere com-
partment. The remaining 8 systems were used as unplanted controls. All manipulations with the systems were performed in a sterile flow cabinet. The systems were transferred to a Heraeus-Vötsch HPS-1500 growth chamber (day/night cycle 16 h, 22 °C/8 h, 15 °C; relative humidity 65%; photosynthetic photon flux density at plant level ±300 μE m⁻² s⁻¹). The air flow through the cylinders was 1.0 litre min⁻¹. The systems were positioned randomly in the cabinet and were rearranged regularly. After 14 days, the number of seedlings per system was reduced to 4. The randomly removed seedlings, roots included, were used to estimate the initial plant dry weights. Directly following thinning, 30 mL of a washed suspension of P. chlororaphis (see above), containing 2.7 × 10⁶ cells mL⁻¹, was inoculated per system to reach a final density of 1 × 10⁸ cells g⁻¹ dry sediment. Using a syringe with a 15 cm long needle, six 5 mL aliquots were injected randomly into the sediment, pulling up the needle while emptying. Most of the pore water had been removed before inoculation by means of the rhizon soil solution samplers. 10 glass beakers, with the same size and filled with the same amount of sediment as the microcosms, were inoculated with P. chlororaphis as described above. These beakers, receiving exactly the same autoclaving treatment as the microcosms, were used to stimulate the initial density of P. chlororaphis and the possible growth on residual carbon sources in the sand. P. chlororaphis numbers in these 10 beakers were determined after 3 days of incubation at 20 °C by plate counting, as described below. The moment of inoculation of the bacteria was regarded as the start of the experiment. Plant nutrients were added weekly by injection into the root compartment via the microporous sampling device in an exponentially increasing amount. The highest dose of the major elements (N, 3 mmol; P, 0.375 mmol; K, 1.125 mmol) was applied in weeks 7, 9. For the first 4 weeks all plants received NH₄⁺ as the sole nitrogen source. From week 5 half of the planted and unplanted systems received NO₃⁻ as the only nitrogen source to provide P. chlororaphis with sufficient electron acceptor and to study its denitrifying behaviour inside and outside the root zone of G. maxima. The remaining plants were kept on the NH₄⁺ regime. Also beginning in week 5, half of the planted systems were transferred to a growth cabinet with a photoperiod of 14 hours, and the other half was subjected to a 20 hour photoperiod. The day/night temperature was set at a constant value of 20 °C to prevent bacterial growth differences due to temperature differences. The selection of systems for the various treatments was at random with 4 replicates per treatment.

**Monitoring of the pore water**

In the weeks 1, 3 and 5-9, pore water samples from the root- and non-root compartments were collected prior to nutrient addition. To minimize bias of the dead volume in the sampler (±0.5 mL), we discarded the first 1 mL sample. The samples were immediately transferred to 4 °C and were analyzed for NH₄⁺, NO₃⁻, NO₂⁻ and pH the same day.

**Effects of G. maxima and photoperiod on denitrification**

To investigate the effects of the plant, the photoperiod and the diurnal oxygen release pattern on denitrification, porewater samples were collected from the planted and unplanted pots receiving only NO₃⁻. This was done 2 days after NO₃⁻ addition in week 5 and 6 at the end of the dark and light periods. Accumulation of nitrous oxide in the head space was monitored at the same time by withdrawing samples after the cylinders had been devoid of air flow for 4 hours. The gas-samples were analyzed for the presence of nitrous oxide the same day and for NO₃⁻ and NO₂⁻ the next day. The process of denitrification during one week was studied by sampling the pore water in week 1 daily from the moment of NO₃⁻ addition on.

**Harvesting and processing of plants and sediment**

After 9 weeks the systems were harvested. 2 duplicate microcosms of every treatment were processed per day. The floodwater was removed and stored at 4 °C for further analysis. Plant dry matter was determined by weighing after drying at 70 °C for 2 days. Moisture percentage of the thoroughly mixed sediment was determined gravimetrically after drying at 105 °C for 24 hours. Mineral nitrogen content was determined in 1 M KCl extracts (1:2.5, w/v). The numbers of P. chlororaphis were determined by plate counts. Two grams of sediment were transferred to 20 mL of PBS buffer in a 30 mL serum bottle (in triplicate) and placed on a rotary shaker for 4 hours (150 rpm, 20 °C). A 1 mL sample was then serially diluted (tenfold series dilutions) in PBS buffer solution. Out of every dilution series, 3 dilutions were subsampled (0.1 mL) and streaked on nutrient broth agar Petri dishes (in duplicate). The Petri dishes were incubated at 28 °C for


the colony-forming units (CFU’s) were counted after 1 week of incubation. For calculating the number of CFU’s per gram of dry sediment the dilution was used which showed 50-100 CFUs per plate.

Chemical analyses

NH$_4^+$, NO$_3^-$ and NO$_2^-$ in pore water samples, sediment extracts, nutrient solutions and floodwater were analyzed using a Technicon Traacs 800 autoanalyzer (Technicon Instr. Corp., Tarry Town, USA). Nitrous oxide in porewater samples was measured by injecting head space samples from venoject tubes into a gas chromatograph (Carlo Erba GC 6000) equipped with a Hayesep Q column (80 °C), an electron capture detector (ECD) (detector (nitrous oxide < 100 ppmv) and a hot wire detector (HWD) detector (nitrous oxide > 100 ppmv). The total amount of nitrous oxide in the gas and liquid phases of the venoject was calculated using the Jansen absorption coefficient for nitrous oxide (0.632, 20 °C, 1 atm; Tiedje, 1994). The chemical composition of the plant material was analyzed using dried, round (0.5 mm) plant material as described by Troelstra (1983) and Troelstra et al. (1995). The proportion of carboxylate and organic nitrogen were determined to provide an indication of the relative ammonium/nitrate uptake by plants (Troelstra, 1983).

Statistical analyses and calculations

Statistical analyses were performed using the WISTIX analytical software package (NH Analytical Software, St. Paul MN 55117, USA). Data were checked for normality by means of the Wilk-Shapiro test. Treatment effects of nitrogen form and photoperiod on plant biomass, plant chemical composition and numbers of P. chlororaphis were analyzed by a two-way ANOVA. Comparisons of means were performed using Tukey’s test. Pairwise comparisons of pore water NH$_4^+$, NO$_3^-$, and nitrous oxide concentrations at the light and dark period were analyzed using the sample t-test which accounts for inequality of variates as described by Snedecor and Cochran (1982). Porewater nitrate, nitrite and nitrous oxide concentrations during week 7 were analyzed per day of sampling using Tukey’s test. Data were either In or square transformed to obtain homogeneity of variances. Relative growth rates (RGR) per week were calculated using initial (W1) and final dry weights at harvest according to Equation 1.

\[ RGR = \frac{(\ln W2 - \ln W1)}{(t2 - t1)} \]

Results

General performance of the microcosm

The roots of the plant did not penetrate the nylon mesh and filled the root cylinder completely at harvest time. Visual evidence for release of oxygen by the roots was present in the form of Fe$_2$O$_3$ traces on the nylon mesh at the spots where the roots had been in contact with the mesh. All planted, nitrate-fed microcosms remained free of contamination during the complete experimental period and contained only P. chlororaphis. The remaining microcosms were contaminated with 3 different bacterial strains, with contamination reaching up to 45% of the total bacteria present. In all other microcosms, P. chlororaphis represented more than 75% of the bacteria present. The contaminating bacteria were characterized as an aerobic, Gram-negative, non-motile rod which formed transparent yellow colonies, an aerobic Gram-negative motile rod which formed non-transparent yellow colonies and a Gram-positive, coccolid-shaped bacterium capable of fermentation in Tryptic Soy Broth forming grey/white colonies. None of the contaminants could utilize nitrate as an electron acceptor and were also not present in the beakers used for the determination of the initial cell numbers.

The dynamics of the added ammonium and nitrate and pH of the pore water could be monitored during the entire experiment. Ammonium and nitrate concentrations in the unplanted systems increased approximately exponentially following nutrient addition (Figure 2A,B). Compared to the non-root compartment, ammonium concentrations in the pore water of the root compartment were always higher, whereas nitrate concentrations were equal. In the planted systems, ammonium and nitrate also began to accumulate from week 7. An increasing amount of the nitrate in the unplanted microcosms was reduced to nitrite, until week 7 after which nitrite remained constant (Figure 2C). Very little nitrite accumulation occurred in the planted systems. Pore water pH was in the range of 7.9-8.3 at the beginning of the experiment (data not shown). In the planted microcosms pore water pH of the root compartments decreased to ±7.0, whereas the pH in the non-root compartments and in the unplanted systems remained unchanged. Following the separation
level of pH 7.0 one week after starting the nitrate supply. From week 7 on there was a steep increase in pH in both compartments of the planted nitrate-fed microcosms to values of 7.7 and 8.1 in root- and non-root compartment, respectively. Photoperiod did not have any major influence on pore water pH. It is also noteworthy that the pH in the central “root” cylinder of the unplanted systems was often lower than in the corresponding non-root compartments.

Recovery of the added nitrogen was in the range of 51-69% and was the same for planted as well as unplanted systems (data not shown). Nitrate supply tended to lead to lower recoveries. Nitrate-fed plants acquired less nitrogen than ammonium-fed plants, 35-37% vs. 48-51% of the added nitrogen, respectively. Photoperiod seemed to have had no effect on overall nitrogen balances, except for the planted nitrate-fed microcosms where a longer photoperiod tended to result in higher N-loss.

Plant parameters

With ammonium as nitrogen source, a longer photoperiod led to higher biomass production, whereas with nitrate-fed plants biomass formation was not influenced by photoperiod (Table 1). Total dry weight of plants subjected to a 14 hour photoperiod did not differ for ammonium- or nitrate-fed plants. The ratio of shoot and root biomass was equal for all the treatments. The relative growth rate (RGR) of G. maxima was in the range of 0.83-0.89 week$^{-1}$ (Table 1). The growth rate of ammonium-fed plants with the longest photoperiod was significantly higher than the growth rate of the other treatments, which showed no significant difference with each other. Analysis of variance indicated that total biomass production was affected by both photoperiod and nitrogen source, RGR only by the photoperiod and shoot-root ratio by none of the treatments (Table 2).

Lengthening of the photoperiod resulted in lower K$^+$, Mg$^{2+}$, H$_2$PO$_4^-$, Cl$^-$, and organic nitrogen concentrations for the ammonium-fed plants (Table 3). There were no differences in the chemical composition of the nitrate-fed plants between the two photoperiods. When comparing ammonium- and nitrate-fed plants per photoperiod, it appeared that the carboxylate concentration (cations-inorganic anions) of the nitrate-fed plants was higher for both photoperiods. The carboxylate-organic nitrogen ratio was also higher for the nitrate-fed plants compared to the plants using ammonium as a nitrogen source (Table 3). This ratio was elevated only in ammonium and nitrate supply (week 5), the pH in the root compartments of the ammonium-fed plants continued to decrease to values of 6.3-6.4. The pH in the root compartment of the ammonium-fed plants was always lower than for the corresponding non-root sediment. In the planted columns with nitrate, pH in the root- and non-root compartment reached the same
Table 1. Total dry weight, shoot-root ratio (S/R) and relative growth rate (RGR) of Glyceria maxima, grown in gnotobiotic microcosms subjected to two different photoperiods (14 vs. 20 hours) and with ammonium or nitrate as nitrogen source. The sediment was inoculated with Pseudomonas chlororaphis (1 × 10⁶ cells g⁻¹ dry sediment). Values are means (±1 SE) of 4 replicate microcosms. Different letters per column indicate significant differences (Tukey’s test, p<0.05).

<table>
<thead>
<tr>
<th>Photoperiod/ N-source</th>
<th>Total dry weight (g)</th>
<th>S/R ratio</th>
<th>RGR (week⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14 h NH₄⁺</td>
<td>3.77±0.52a</td>
<td>1.78±0.30a</td>
<td>0.84±0.014a</td>
</tr>
<tr>
<td>14 h NO₃⁻</td>
<td>3.27±0.13a</td>
<td>1.14±0.02a</td>
<td>0.83±0.004a</td>
</tr>
<tr>
<td>20 h NH₄⁺</td>
<td>6.13±0.51b</td>
<td>1.36±0.17a</td>
<td>0.89±0.008b</td>
</tr>
<tr>
<td>20 h NO₃⁻</td>
<td>3.91±0.16a</td>
<td>1.25±0.07a</td>
<td>0.85±0.005a</td>
</tr>
</tbody>
</table>

Table 2. ANOVA results (degrees of freedom, F- and p-values) of total dry weight, shoot-root ratio (S/R) and relative growth rate (RGR) of Glyceria maxima grown in a gnotobiotic microcosms subjected to two photoperiods (14 vs. 20 hours) and with ammonium or nitrate as nitrogen source.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>df</th>
<th>Total dry weight</th>
<th>S/R ratio</th>
<th>RGR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light (L)</td>
<td>1</td>
<td>12.98</td>
<td>0.87</td>
<td>13.98</td>
</tr>
<tr>
<td>N-form (N)</td>
<td>1</td>
<td>15.78</td>
<td>4.61</td>
<td>4.68</td>
</tr>
<tr>
<td>L+N</td>
<td>1</td>
<td>5.14</td>
<td>2.28</td>
<td>1.07</td>
</tr>
</tbody>
</table>

Table 3. Chemical composition of Glyceria maxima (whole plant) grown in a waterlogged gnotobiotic microcosm subjected to two photoperiods (14 vs. 20 hours) and with ammonium or nitrate as nitrogen source. The sediment was inoculated with P. chlororaphis (1 × 10⁶ cells g⁻¹ dry sediment). Values are means for 4 replicate microcosms. Ion concentrations are expressed as meq kg⁻¹ dry matter, organic nitrogen as mmol N kg⁻¹ dry matter. Different letters indicate significant differences (Tukey’s test, p<0.05) between treatments.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Photoperiod:</th>
<th>14 h</th>
<th>20 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-form</td>
<td>NH₄⁺</td>
<td>NO₃⁻</td>
<td>NH₄⁺</td>
</tr>
<tr>
<td>K⁺</td>
<td>476 a</td>
<td>340 ab</td>
<td>237 c</td>
</tr>
<tr>
<td>Na⁺</td>
<td>252 a</td>
<td>314 a</td>
<td>211 a</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>245 a</td>
<td>238 a</td>
<td>232 a</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>79 a</td>
<td>77 a</td>
<td>52 b</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>12 a</td>
<td>6 ab</td>
<td>7 ab</td>
</tr>
<tr>
<td>H₂PO₄⁻</td>
<td>78 a</td>
<td>57 ab</td>
<td>34 b</td>
</tr>
<tr>
<td>NO₃⁻</td>
<td>1 b</td>
<td>91 a</td>
<td>0.25 b</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>462 a</td>
<td>299 b</td>
<td>269 b</td>
</tr>
<tr>
<td>SO₄²⁻</td>
<td>146 a</td>
<td>101 a</td>
<td>74 a</td>
</tr>
<tr>
<td>Carboxylate</td>
<td>395 bc</td>
<td>510 a</td>
<td>356 c</td>
</tr>
<tr>
<td>C/A/Norg</td>
<td>0.25 a</td>
<td>0.44 bc</td>
<td>0.41 c</td>
</tr>
</tbody>
</table>

Evidence of P. chlororaphis in the microcosms

Numbers of P. chlororaphis in the root compartments of the planted systems were all substantially higher in the non-root sediment (Figure 3). CFU's were in the range of 3.8 × 10⁶-1.1 × 10⁸ and 3.8 × 10⁶-1.07 × 10⁷ g⁻¹ dry sediment in the root- and non-root sediment, respectively. The planted/unplanted CFU ratios of the rooted sediment for ammonium- and nitrate-fed plants, respectively, were 12 and 15 for the short photoperiod and 19 and 32 for the long photoperiod. Elongation of the photoperiod resulted in significantly (p<0.05) higher growth of P. chlororaphis in the root- and non-root compartments of both ammonium- and nitrate-fed plants. From the ANOVA in Table 5 it is evident that growth of P. chlororaphis in the root zone of G. maxima was affected by the interaction of photoperiod and nitrogen source while growth in the non-root sediment was affected separately by photoperiod and nitrogen nutrition.

Effects of G. maxima and photoperiod on denitrification by P. chlororaphis

The pore water nitrous oxide concentrations were determined for planted and unplanted microcosms in week 5 and 6 at the end of the light and dark period 2 days after nitrate addition and the results are presented in Figure 4. There were no significant differences in nitrous oxide concentrations between the end of the light and dark period. This held true for both weeks 5 and 6 of the experiment. The presence of the plant resulted in an elevated nitrous oxide concentration,
Table 4. ANOVA results (degrees of freedom, F- and p values) of the main treatment effects (light, nitrogen form), and their interactions, on the chemical composition of Glyceria maxima, grown in a waterlogged gnotobiotic microcosm subjected to two different photoperiods (14 vs. 20 hours) and with ammonium or nitrate as nitrogen source. The sediment was inoculated with Pseudomonas chlororaphis (1 × 10^2 g^-1 dry sediment).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Treatment</th>
<th>df</th>
<th>F</th>
<th>p</th>
<th>F</th>
<th>p</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Light (L)</td>
<td>N-form (N)</td>
<td>L×N</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K^+</td>
<td>1</td>
<td>25.10</td>
<td>0.0003</td>
<td>0.010</td>
<td>0.7631</td>
<td>7.28</td>
<td>0.0194</td>
<td></td>
</tr>
<tr>
<td>Na^+</td>
<td>1</td>
<td>0.30</td>
<td>0.5968</td>
<td>6.39</td>
<td>0.0265</td>
<td>0.45</td>
<td>0.5144</td>
<td></td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>1</td>
<td>0.24</td>
<td>0.6365</td>
<td>0.00</td>
<td>0.9809</td>
<td>0.25</td>
<td>0.6246</td>
<td></td>
</tr>
<tr>
<td>Mg^{2+}</td>
<td>1</td>
<td>11.10</td>
<td>0.0060</td>
<td>2.27</td>
<td>0.1579</td>
<td>3.94</td>
<td>0.0706</td>
<td></td>
</tr>
<tr>
<td>NH_4^+</td>
<td>1</td>
<td>4.56</td>
<td>0.0540</td>
<td>5.94</td>
<td>0.0313</td>
<td>1.75</td>
<td>0.2106</td>
<td></td>
</tr>
<tr>
<td>H_2PO_4^-</td>
<td>1</td>
<td>16.60</td>
<td>0.0015</td>
<td>0.30</td>
<td>0.5950</td>
<td>6.54</td>
<td>0.0251</td>
<td></td>
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<tr>
<td>NO_3^-</td>
<td>1</td>
<td>5.95</td>
<td>0.0312</td>
<td>39.30</td>
<td>0.0000</td>
<td>5.48</td>
<td>0.0373</td>
<td></td>
</tr>
<tr>
<td>Cl^-</td>
<td>1</td>
<td>3.02</td>
<td>0.1080</td>
<td>7.84</td>
<td>0.0160</td>
<td>8.02</td>
<td>0.0151</td>
<td></td>
</tr>
<tr>
<td>SO_4^{2-}</td>
<td>1</td>
<td>1.70</td>
<td>0.2172</td>
<td>0.05</td>
<td>0.8243</td>
<td>7.47</td>
<td>0.0182</td>
<td></td>
</tr>
<tr>
<td>Carboxylate</td>
<td>1</td>
<td>2.17</td>
<td>0.1666</td>
<td>22.28</td>
<td>0.0005</td>
<td>0.01</td>
<td>0.9169</td>
<td></td>
</tr>
<tr>
<td>Organic N</td>
<td>1</td>
<td>20.08</td>
<td>0.0008</td>
<td>4.15</td>
<td>0.0644</td>
<td>5.77</td>
<td>0.0334</td>
<td></td>
</tr>
<tr>
<td>C-A/N_o rg</td>
<td>1</td>
<td>16.01</td>
<td>0.0018</td>
<td>27.18</td>
<td>0.0002</td>
<td>1.19</td>
<td>0.2966</td>
<td></td>
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</table>

The results show that the nitrous oxide concentrations amounted to 0.35-0.37 m^47-184%) between the replicates. The head space of the systems often failed to reach significance due to the high variation in nitrous oxide concentrations (CV 47-184%). In the unplanted microcosms, where nitrite concentrations were monitored daily, the nitrous oxide accumulation occurred in particular in the pore water of the root compartments. Despite the obvious trend toward higher nitrous oxide concentrations in the root compartments of the planted systems, differences were not significant between the replicates. The head space of the microcosms did not contain detectable amounts of nitrous oxide at any sampling event. A substantial amount of the added nitrate was reduced to nitrite, particularly in the unplanted microcosms where nitrite concentrations amounted to 0.35-0.37 mM in week 6. In the planted systems, the variation in nitrous oxide concentrations between replicates was substantial.

### Discussion

**General performance of the gnotobiotic microcosms**

To the best of our knowledge, this represents the first report describing the use of this type of gnotobiotic microcosm. Closed, gnotobiotic soil-plant systems have been used in plant-microbe interaction studies but have usually consisted of simple test tube set-up use for short incubation periods with very young seedlings (Bennett and Lynch, 1981; Turner and Newman, 1984). Neither of these studies provided information concerning sterility or contamination of the system. A larger experimental set-up was used by Trifonow et al. (1987), who studied rhizodeposition and ammonium depletion by means of a split root system with axenic oat roots. These authors regarded the system as being sterile when less than 50 colonies per mL were found in a 10-fold dilution of a soil suspension. Both (1990) constructed a gnotobiotic system for studying spatial distribution of nitrifying bacteria around roots of Plantago lanceolata. This elegant system was, however, contaminated with a Pseudomonas species. In our experimental design we succeeded in keeping a number of the microcosms free from contamination during the full 3-month experimental period, showing that the system can potentially be used for gnotobiotic studies. The systematic contamination of a number of the microcosms indicated that addition of the nutrient solutions was a common source of infection. Obviously, the 0.2 μm hydrophilic filter and the 0.1 μm porous polymer material of the sampling device were not always 100% effective in the exclusion of contaminants.

By regarding the completely rooted, central rhizosphere cylinder as a “macro-rhizosphere” from which carbon and oxygen are released into the surrounding non-root compartment, we could study microbial dynamics in the sediment layers adjacent to the root cylinder by subdividing the non-root sediment at the...
Figure 3. The number of colony-forming units of *Pseudomonas chlororaphis* in the root- and non-root compartment of gnotobiotic microcosms planted with or without *Glyceria maxima*. The plants were grown under two different photoperiods (14 vs. 20 hours) and with NH$_4^+$ or NO$_3^-$ as nitrogen source. T0 is the initial inoculum density determined 3 days after inoculation. Bars are means (±SE) of 4 replicate microcosms (except for unplanted + NH$_4^+$ where n = 1). Asterisks indicate significant differences between the 14- and 20 hour photoperiod (p<0.05, One way anova).

Table 5. ANOVA results (degrees of freedom, F- and p-values) of the number of colony-forming units of the bacterium *Pseudomonas chlororaphis* in the root zone of *Glyceria maxima* and in the non-root sediment. The plants were grown in waterlogged gnotobiotic microcosms subjected to two photoperiods (14 vs. 20 hours) and with ammonium or nitrate as nitrogen source.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>df</th>
<th>Variable</th>
<th>F</th>
<th>p</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light (L)</td>
<td>1</td>
<td>CFU root compartment</td>
<td>36.23</td>
<td>0.0001</td>
<td>11.39</td>
<td>0.0055</td>
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<tr>
<td>N-form (N)</td>
<td>1</td>
<td>CFU root compartment</td>
<td>21.13</td>
<td>0.0006</td>
<td>7.20</td>
<td>0.0199</td>
</tr>
<tr>
<td>LxN</td>
<td>1</td>
<td>CFU non-root compartment</td>
<td>7.05</td>
<td>0.0210</td>
<td>4.02</td>
<td>0.0680</td>
</tr>
</tbody>
</table>

of the experimental colony. This principle has eady been used in several studies in different context (ero and Thien, 1979; Helal and Sauerbeck, 1983; emdsson et al., 1987). Klemedtsson et al. were the authors looking at nitrification and dinitrification a non-gnotobiotic system. The “macro-rhizosphere” aciple overcomes the difficulties of defining the rhzosphere and facilitates the study of microbial process at defined distances from the roots. Other authors d an artificial root to overcome the traditional prob-bs (Martens, 1982; Odham, 1986). The problem of ficial root systems is that the amount and dynamics carbon, and oxygen release is set by the investi-ors, and it is questionable whether this reflects the “ural” dimensions of such highly variable process-
es. A close approximation of the natural situation was achieved by Høgberg and Sørensen (1993), who created a gel-stabilized system, in which microgradients of bacterial activity could be measured near single roots. The use of a gel matrix in combination with an intact root system is closer to the natural situation in comparison to artificial root systems. The mechanical resistance created by the gel matrix which can influence oxygen (Engelaar et al., 1993) and carbon (Barber and Gunn, 1974) release, will, however, differ.

The nitrogen added to our systems could not be completely recovered. In the ammonium-fed microcosms, 31-37% of the supplied nitrogen was not recovered. This can partially be explained by the method of nutrient supply, which occurred in small concentrated amounts. Apparently, a part of the nitrogen is retained in the dead volume of the hydrophillic filter + tubing of the rhizon soil solution sampling device. Although the sampling device was always rinsed with demineralized water after nutrient addition, we calculated that...
an amount of 12% of added nitrogen may not have reached the root compartment. Furthermore, ammonia volatilization may very well have occurred at the slightly alkaline pore water pH in the unplanted systems.

### Growth of G. maxima

Our model plant, *G. maxima*, grew normally in the closed cylinder. The relative growth rate (0.83-0.89 week⁻¹) was slightly lower than seedling RGR in natural situations (1.0-1.4 week⁻¹; Grime et al., 1988). This is probably due to the light-limited conditions created by the low photosynthetic photon flux density of 300 μE m⁻² s⁻¹ (≈65 W m⁻²), which is probably below the photosynthetic saturation. The chemical composition of the plants indicated that nutrient supply was sufficient. The plant content of the major elements (N; 1.70%, P; 0.17%, K; 1.41%, Ca; 0.24%, Mg; 0.08% in the dry matter) agreed very well with values for natural *G. maxima* stands at eutrophic freshwater locations (Dykyjova, 1978). The carboxylate-organic nitrogen ratio may give an indication of the relative amount of nitrate, as opposed to ammonium, taken up by the plant (Troelstra, 1983). In the case of ammonium uptake, this ratio will be lower as compared to nitrate uptake due to the lower carboxylate formation. The values in this study agree with this concept and may in principle be used as reference values for studies where the amount of nitrate acquired by the plant is determined by nitrification and denitrification. The differences between the ammonium- and nitrate-fed plants was not very large which is common in the Gramineae (Van Egmond, 1978). It is questionable, however, whether such a subtle difference will be reflected by the C-A/N org ratio as the nitrate production is relatively small and denitrification high. Furthermore, the elevated C-A/N org ratio in the ammonium-fed plants was related to the elongated photoperiod and not to a relative increase in nitrate nutrition, which may be problematic for future experimental interpretations. The photoperiod changed the organic N content of the ammonium-fed plants drastically, but did not affect the carboxylate content, which was influenced by the form of nitrogen as expected. In our experimental setting it might be more useful to use the carboxylate content as indicator of the relative

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**Figure 5.** Porewater $\text{N}_2\text{O}$ concentrations during week 7 in the root- and non-root compartment of gnotobiotic microcosms planted with *Glyceria maxima* and inoculated with *Pseudomonas chlororaphis*. The plants were grown under two different photoperiods (14 vs. 20 hours) and with NO$_3$ as nitrogen source. Bars indicate means (±SE) of 4 replicate microcosms. Different letters indicate significant differences between means of one sampling day ($p<0.05$, Tukey's test). Data were ln or square root transformed to obtain homogeneity of variances. The untransformed data are shown.
Effect of G. maxima and photoperiod on growth of
and denitrification by P. chlororaphis

The rhizosphere effect on the growth of _P. chlororaphis_ found in this study (R/S ratios 12-32, i.e. plant-
ed/unplanted) is in the range found fertile soils planted
with arable crops (Woldendorp, 1981). It was obvious
that the plant also stimulated the bacterial population
in the non-root sediment. The effect of the plant on
the non-root sediment was probably greatest directly
adjacent to the root compartment, and subdividing the
non-root sediment in layers of increasing distance from
the "macro-rhizosphere" should allow for the study of
microbial population dynamics across a gradient of
root-released substrates.

Although we were interested in the impact of plant-
carbon or oxygen release, as affected by photoperiod,
in the growth of and denitrification by _P. chlororaphis_
itis only possible to interpret the amount of oxygen and
carbon released by _G. maxima_ qualitatively. The total
number of _P. chlororaphis_ cells formed per microcosm
due to the presence of _G. maxima_ averaged 5.52×10^9
and 6.35×10^9 cells for the ammonium- and nitrate-
systems, respectively, of the 14 hour photoperiod
experiment. For the 20 hour photoperiod experiment
these values were 1.25×10^10 and 2.94×10^10. In the
nitrate-fed systems there is an excess of electron accept-
or which means that elevation of the photoperiod led
to an increase in the plant-derived carbon, which could
be converted bacterial biomass, with a factor of 4.6. His
value was only 2.3 in the ammonium-fed sys-
tems. The stimulation of carbon exudation and higher
cell numbers associated with the plants due to a
higher photoperiod was also found by Whippes (1984).
Apparently, there was a relative shortage of plant-
derived oxygen when taking the higher cell numbers
under account when nitrate was available. The total cell
numbers in the nitrate-fed microcosms were 1.2 and 2.4
times the numbers found for the ammonium-fed sys-
tems for the 14- and 20 hours photoperiod, respective-
Hence, the relative shortage of oxygen compared to
available amount of carbon increases with the hours
daylight. Extrapolation to the field situation pre-
tends better growth conditions for facultatively aerobic
rhizosphere bacteria in summer and autumn and less
suitable conditions in winter and spring. The ability
to regulate the degree of oxygen deprivation allows
one to mimic the field situation more closely provid-
ing the prospect of studying the competitive abilities
of nitrifying bacteria as they might occur in nature.
The plant-stimulated denitrification in the microcosms
is a phenomenon which has been described by many
studies (e.g. Prade and Trolldenier, 1990; Stefanson,
1972; Woldendorp, 1963). The impact of the plant
on denitrification in relation to its photosynthesis was
described by Scaglia et al. (1985) and Bakken (1988).
Since nitrate is not limiting, the effect of the phot-
operiod on denitrification in our study must be due to
the higher carbon availability. However, the nitrous
oxide production pattern in week 7 revealed an ini-
tial increase in denitrification followed by a decrease
which is consistent with the decreasing nitrate avail-
ability. The bacteria are probably outcompeted by the
plant for nitrate, but it is also possible that nitrous oxide
escapes from the sediment via the aerenchymatous tis-
tue of the plant as was demonstrated by Mosier et al.
(1990) and Reddy et al. (1989). However, we were
unable to detect any nitrous oxide in the head space of
the microcosms.

Providing _P. chlororaphis_ with nitrate in week 5 led
to immediate nitrite accumulation, which is an indica-
tion of excess electron acceptor, leading to an incom-
plete denitrification reaction (R A Kester, personal
communication). This appears even more likely if one
considers that nitrite accumulation in the unplanted
systems, which have a lower electron donor availabil-
ity, is substantially higher.

Effects of the diurnal photosynthetic cycle on den-
itrification could not be detected. The oxygen released
by the plants during the day might very well inhibit
it denitrification, as was found by Christensen and
Sørensen (1986) in the root zone of the macrophyte
_Litorolla uniflora_ (L.) Aschers. The high variation in
nitrous oxide porewater concentrations between the
replicate microcosms might also hamper detection of
possible differences between day and night measure-
ments.

The systems we describe in this study provide a
potential means of studying complex microbial inter-
actions in the vicinity of roots. The "macro" rhizo-
sphere principal, can be used as a simplification of the
highly complicated natural rhizosphere, enabling the
study of potential interactions of selected members of
microbial communities. Although this remains a model
approach, the dimensions and gradients of the plant-
derived microbial substrates (i.e. carbon, oxygen) are
still governed by the plant thus allowing for a clos-
er approximation of the natural situation. The system
does not claim to match in situ processes but can help to describe the specific behaviours and interactions of important functional bacterial groups, whose activities are dictated by the plant.

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References


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