

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

Paul L.E. Bodelier<sup>1</sup>, Albert G. Wijnhuizen<sup>1</sup>, Cees W.P.M. Blom<sup>2</sup> and Hendrikus J. Laanbroek<sup>3</sup>  
<sup>1</sup>Netherlands Institute of Ecology, Centre for Terrestrial Ecology, Department of Plant-Microorganism-Interactions, Department, P.O. Box 40, 6666 29, Heteren, The Netherlands\*\*, <sup>2</sup>Department of Ecology, University of Nijmegen, The Netherlands and <sup>3</sup> Netherlands Institute of Ecology, Centre for Limnology, Department of Microbial Ecology, Nieuwersluis, The Netherlands.

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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 with the nitrate-fed plants, where additional *P. chlororaphis* growth could proceed via denitrification, indicating oxygen-limiting conditions in the microcosms. Higher porewater N<sub>2</sub>O concentrations in the root compartments as 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 bacterial communities in flooded soils and sediments. The aerenchyma, which is one of the adaptations of a broad range of plant species rooting in anoxic environments (Blom and Voeselek, 1996; Justin and Armstrong, 1987), facilitates the diffusion of oxygen from the 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 N<sub>2</sub> or N<sub>2</sub>O (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-

\* Publication 2226, Netherlands Inst. of Ecol., Centre for Terrest. Ecol., Heteren, the Netherlands

\*\* FAX No: +31264723227. E-mail: bodelier@cto.nioo.knaw.nl

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 ( $\varnothing$  of holes: 1 mm), covered on the inside with nylon gauze (mesh size 30  $\mu\text{m}$ , 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 polymer with a typical pore diameter of 0.1  $\mu\text{m}$ . 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  $\mu\text{m}$ , 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 Silaflex, USA). The air inlet and outlet were equipped with hydrophobic PTFE inline membrane filters (0.2  $\mu\text{m}$ , 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 (Smirnov and Crawford, 1983) and the inability of its roots to survive anoxia 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)  $\text{NaHClO}_3$  with one subsequently rinse with 70% (v/v) ethanol and three washes

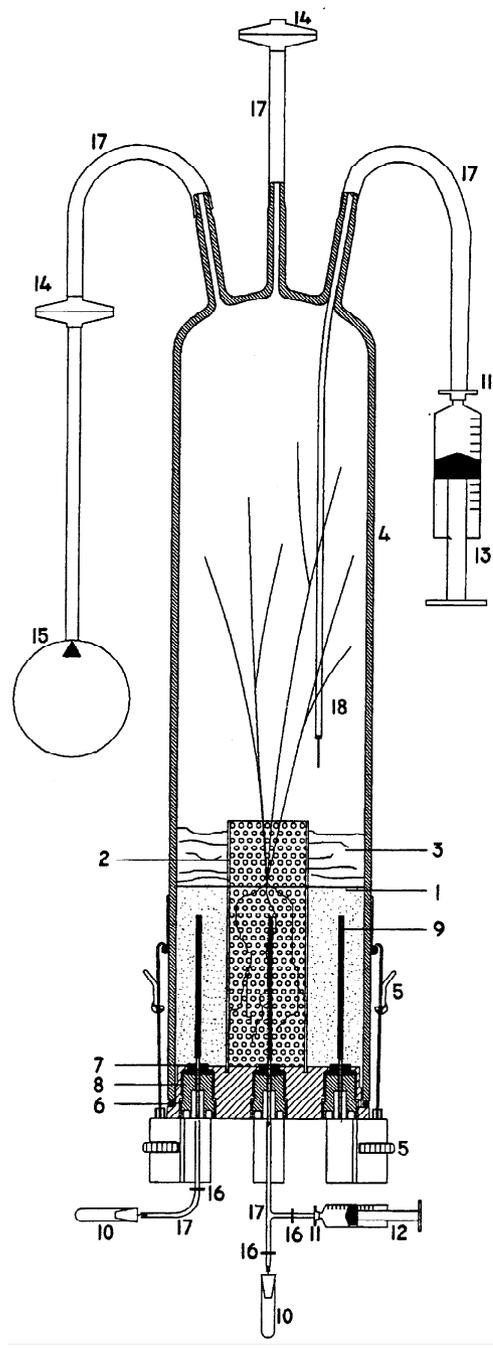


Figure 1. Graphical representation of a vertical transection of the gnotobiotic microcosm. 1: stainless steel cylindrical sediment compartment (12×9 cm; height×Ø), 2: stainless steel root cylinder (12×4 cm, h×Ø), 3: floodwater layer (4 cm), 4: glass cover (45×9.3 cm, h×Ø), 5: clamp device for pressing glass cover on soil compartment, 6: silicon greased airtight butylrubber gasket, 7: butylrubber septum, 8: bold housing harbouring the needle connecting the soil solution sampler, 9: rhizone soil solution sampler, 10: venoject sample collection tube, 11: hydrophillic filter (0.2 µm), 12: disposable syringe for nutrient addition, 13: syringe for floodwater addition, 14: hydrophobic PTFE in line membrane filter (0.2 µm), 15: air circulation pump, 16: tube clamps, 17: silicone tubing, 18: tube for floodwater addition.

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<sup>-1</sup>) 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<sup>-2</sup> s<sup>-1</sup>). 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 batch 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; NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, 0.35; Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O, 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(H<sub>2</sub>O) 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  $\pm 300 \mu\text{E m}^{-2} \text{s}^{-1}$ ). The air flow through the cylinders was  $1.0 \text{ litre min}^{-1}$ . 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  $2.7 \times 10^6$  cells  $\text{mL}^{-1}$ , was inoculated per system to reach a final density of  $1 \times 10^5$  cells  $\text{g}^{-1}$  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 rhizone 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 and 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, 8 and 9. For the first 4 weeks all plants received  $\text{NH}_4^+$  as the sole nitrogen source. From week 5 half of the planted and unplanted systems received  $\text{NO}_3^-$  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  $\text{NH}_4^+$  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 replicate microcosms 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 ( $\pm 0.5 \text{ mL}$ ), we discarded the first 3 mL sample. The samples were immediately transferred to 4 °C and were analyzed for  $\text{NH}_4^+$ ,  $\text{NO}_3^-$ ,  $\text{NO}_2^-$  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  $\text{NO}_3^-$ . This was done 2 days after  $\text{NO}_3^-$  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  $\text{NO}_3^-$  and  $\text{NO}_2^-$  the next day. The process of denitrification during one week was studied by sampling the pore water in week 7 daily from the moment of  $\text{NO}_3^-$  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 1.0 mL sample was then serially diluted (tenfold serial 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 and

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

$\text{NH}_4^+$ ,  $\text{NO}_3^-$  and  $\text{NO}_2^-$  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 Bunsen absorption coefficient for nitrous oxide (0.632, 20 °C, 1 atm; Tiedje, 1994). The chemical composition of the plant material was analyzed using dried, ground (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

All statistical analyses were performed using the SATISTIX 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 by Tukey's test. Pairwise comparisons of pore water  $\text{NO}_3^-$ ,  $\text{NO}_2^-$ , and nitrous oxide concentrations at the end of light and dark period were analyzed using the two-sample t-test which accounts for inequality of variances as described by Snedecor and Cochran (1982). Porewater nitrate, nitrite and nitrous oxide concentrations during week 7 were analyzed per day of sampling by Tukey's test. Data were either ln or square root transformed to obtain homogeneity of variances.

Relative growth rates (RGR) per week were calculated using initial (W1) and final dry weights at harvest (W2) according to Equation 1.

$$RGR = (\ln W_2 - \ln W_1) / (t_2 - t_1) \quad (1)$$

## 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  $\text{Fe}_2\text{O}_3$  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, coccoid-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  $\pm 7.0$ , whereas the pH in the non-root compartments and in the unplanted systems remained unchanged. Following the separation

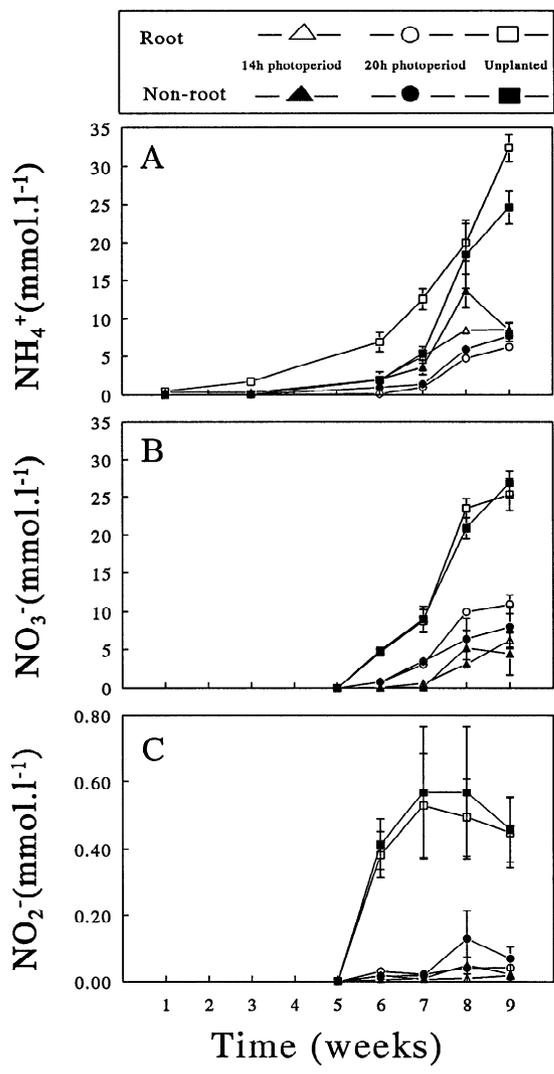


Figure 2. Porewater concentrations of  $\text{NH}_4^+$  (A),  $\text{NO}_3^-$  (B) and  $\text{NO}_2^-$  (C) in gnotobiotic microcosms planted with or without *Glycineria maxima* and inoculated with *Pseudomonas chlororaphis* and receiving only  $\text{NH}_4^+$  (A) or  $\text{NO}_3^-$  (B,C) as nitrogen source. The plants were grown under two different photoperiods (14 vs. 20 hours). Until week 5, all microcosms received ammonium; application of different nitrogen sources occurred from week 5 on. Values are means ( $\pm$ SE) of 4 replicate microcosms (except for unplanted microcosms +  $\text{NH}_4^+$  where  $n = 3$ ).

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

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<sup>-1</sup> (Table 1). The growth rate of ammonium-fed plants with the long 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  $\text{K}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{H}_2\text{PO}_4^-$ ,  $\text{Cl}^-$ , 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

**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* ( $1 \times 10^5$  cells  $g^{-1}$  dry sediment). Values are means ( $\pm 1$  SE) of 4 replicate microcosms. Different letters per column indicate significant differences (Tukey's test,  $p < 0.05$ )

Photoperiod/ N-source	Total dry weigh (g)	S/R ratio	RGR (week <sup>-1</sup> )
14 h/NH <sub>4</sub> <sup>+</sup>	3.77 $\pm$ 0.52a	1.78 $\pm$ 0.30a	0.84 $\pm$ 0.014a
14 h/NO <sub>3</sub> <sup>-</sup>	3.27 $\pm$ 0.13a	1.14 $\pm$ 0.02a	0.83 $\pm$ 0.004a
20 h/NH <sub>4</sub> <sup>+</sup>	6.13 $\pm$ 0.51b	1.36 $\pm$ 0.17a	0.89 $\pm$ 0.008b
20 h/NO <sub>3</sub> <sup>-</sup>	3.91 $\pm$ 0.16a	1.25 $\pm$ 0.07a	0.85 $\pm$ 0.005a

**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 microcosm subjected to two photoperiods (14 vs. 20 hours) and with ammonium or nitrate as nitrogen source

Treatment	df	Variable					
		Total dry weight		S/R ratio		RGR	
		F	$p$	F	$p$	F	$p$
Light (L)	1	12.98	0.0036	0.87	0.3691	13.98	0.0028
N-form (N)	1	15.78	0.0018	4.61	0.0528	4.68	0.0514
L $\times$ N	1	5.14	0.0426	2.28	0.1571	1.07	0.3224

the case of the ammonium-fed plants by the extension of the photoperiod. The analysis of variance revealed main effects of photoperiod on K<sup>+</sup>, Mg<sup>2+</sup>, H<sub>2</sub>PO<sub>4</sub><sup>-</sup>, NO<sub>3</sub><sup>-</sup> and organic nitrogen concentrations in the plant (Table 4). Nitrogen source had main effects on Na<sup>+</sup>, NH<sub>4</sub><sup>+</sup>, NO<sub>3</sub><sup>-</sup>, Cl<sup>-</sup> and carboxylate concentration. Interactions between photoperiod and form of N were found for the K<sup>+</sup>, H<sub>2</sub>PO<sub>4</sub>, Cl<sup>-</sup>, SO<sub>4</sub><sup>2-</sup>, NO<sub>3</sub><sup>-</sup> and organic N concentration. The (C-A)/N<sub>org</sub> ratio was affected by the photoperiod via the organic nitrogen and by the nitrogen nutrition via the carboxylate concentration. There was no interaction between light regime and nitrogen source in relation to the (C-A)/N<sub>org</sub> ratio.

#### Growth of *P. chlororaphis* in the microcosms

The numbers of *P. chlororaphis* in the root compartments of the planted systems were all substantially higher than in the non-root sediment (Figure 3). CFU's were in the range of  $3.8 \times 10^7$ - $1.1 \times 10^8$  and  $3.8 \times 10^6$ - $2.8 \times 10^7$   $g^{-1}$  dry sediment in the root- and non-root sediment, respectively. The planted/unplanted CFU

**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 in the microcosms was inoculated with *P. chlororaphis* ( $1 \times 10^5$   $g^{-1}$  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

Variable	Photoperiod:		14 h		20 h	
	N-form:		NH <sub>4</sub> <sup>+</sup>	NO <sub>3</sub> <sup>-</sup>	NH <sub>4</sub> <sup>+</sup>	NO <sub>3</sub> <sup>-</sup>
K <sup>+</sup>			476 a	402 ab	237 c	330 bc
Na <sup>+</sup>			252 a	314 a	211 a	318 a
Ca <sup>2+</sup>			245 a	238 a	232 a	239 a
Mg <sup>2+</sup>			79 a	77 a	52 b	70 ab
NH <sub>4</sub> <sup>+</sup>			12 a	6 ab	7 ab	5 b
H <sub>2</sub> PO <sub>4</sub> <sup>-</sup>			78 a	57 ab	34 b	47 b
NO <sub>3</sub> <sup>-</sup>			1 b	91 a	0.25 b	41a
Cl <sup>-</sup>			462 a	299 b	269 b	271 b
SO <sub>4</sub> <sup>2-</sup>			146 a	101 a	74 a	127 a
Carboxylate (C-A)			395 bc	510 a	356 c	476 ab
Organic N			1640 a	1158 b	892 b	932 b
C-A/N <sub>org</sub>			0.25 a	0.44 bc	0.41 c	0.53 b

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* ( $1 \times 10^5 \text{ g}^{-1}$  dry sediment)

Variable	df	Treatment					
		Light (L)		N-form (N)		L×N	
		F	<i>p</i>	F	<i>p</i>	F	<i>p</i>
K <sup>+</sup>	1	25.10	0.0003	0.010	0.7631	7.28	0.0194
Na <sup>+</sup>	1	0.30	0.5968	6.39	0.0265	0.45	0.5144
Ca <sup>2+</sup>	1	0.24	0.6365	0.00	0.9809	0.25	0.6246
Mg <sup>2+</sup>	1	11.10	0.0060	2.27	0.1579	3.94	0.0706
NH <sub>4</sub> <sup>+</sup>	1	4.56	0.0540	5.94	0.0313	1.75	0.2106
H <sub>2</sub> PO <sub>4</sub> <sup>-</sup>	1	16.60	0.0015	0.30	0.5950	6.54	0.0251
NO <sub>3</sub> <sup>-</sup>	1	5.95	0.0312	39.30	0.0000	5.48	0.0373
Cl <sup>-</sup>	1	3.02	0.1080	7.84	0.0160	8.02	0.0151
SO <sub>4</sub> <sup>2-</sup>	1	1.70	0.2172	0.05	0.8243	7.47	0.0182
Carboxylate	1	2.17	0.1666	22.28	0.0005	0.01	0.9169
Organic N	1	20.08	0.0008	4.15	0.0644	5.77	0.0334
C-A/N <sub>org</sub>	1	16.01	0.0018	27.18	0.0002	1.19	0.2966

compared to the unplanted systems. The plant-induced 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 often failed to reach significance due to the high variation in nitrous oxide concentrations (CV 47-184%) 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 (data not shown). Pore water nitrite concentrations in the planted systems were always lower than 0.10 mM and there was no photoperiod effect.

In week 7 of the experiment, porewater nitrous oxide concentrations were monitored daily (Figure 5), with the first sample being taken 5 hours after nitrate addition on the first day of the week. The nitrous oxide levels at day 1 of week 7 were in the same range as the values at day 2 of week 6. There was a distinct plant effect on nitrous oxide production during the week. Concentrations in the root compartment of the plants subjected to the long photoperiod, increased significantly to a value of 21 μM on day 2, and sub-

sequently decreased again to levels not significantly different from the non-root sediment and the unplanted systems. In the planted microcosms subjected to the short photoperiod, this elevation was much weaker and statistically not significant. In the unplanted systems nitrous oxide levels gradually increased averaging 4 μM on day 6. As in weeks 5 and 6, the variation in the nitrous oxide concentrations between replicate microcosms 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 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 Trofymow et al. (1987), who studied rhizodeposition and ammonium depletion by means of a split root system with axenic oat roots. These authors regarded their 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 any 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 hydrophillic 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

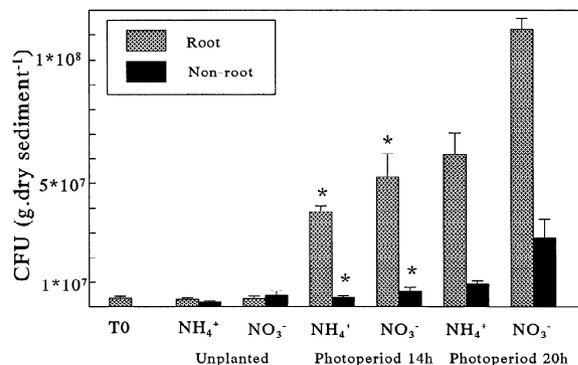


Figure 3. The number of colony-forming units of *Pseudomonas chlororaphis* in the root- and non-root compartment of gnotobiotic microcosms planted with or without *Glyceria maxima*. The plants were grown under two different photoperiods (14 vs. 20 hours) and with  $\text{NH}_4^+$  or  $\text{NO}_3^-$  as nitrogen source. T0 is the initial inoculum density determined 3 days after inoculation. Bars are means ( $\pm$ SE) of 4 replicate microcosms (except for unplanted +  $\text{NH}_4^+$  where  $n = 3$ ). Asterisks indicate significant differences between the 14- and 20 hour photoperiod ( $p < 0.5$ , 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

Treatment	df	Variable			
		CFU root compartment		CFU non-root compartment	
		F	$p$	F	$p$
Light (L)	1	36.23	0.0001	11.39	0.0055
N-form(N)	1	21.13	0.0006	7.20	0.0199
L $\times$ N	1	7.05	0.0210	4.02	0.0680

end of the experimental period. This principle has already been used in several studies in different context (Boero and Thien, 1979; Helal and Sauerbeck, 1983; Klemedtsson et al., 1987). Klemedtsson et al. were the only authors looking at nitrification and denitrification in a non-gnotobiotic system. The “macro-rhizosphere” principle overcomes the difficulties of defining the rhizosphere and facilitates the study of microbial processes at defined distances from the roots. Other authors used an artificial root to overcome the traditional problems (Martens, 1982; Odham, 1986). The problem of artificial root systems is that the amount and dynamics of carbon, and oxygen release is set by the investigators, and it is questionable whether this reflects the “natural” dimensions of such highly variable process-

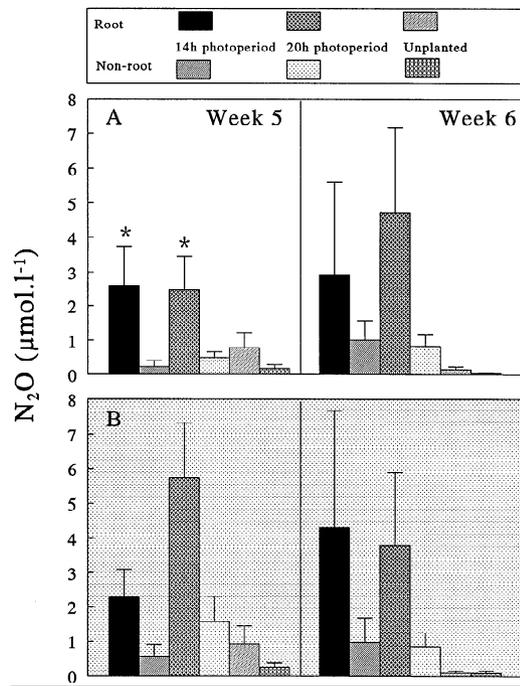


Figure 4. Porewater  $\text{N}_2\text{O}$  concentrations at the end of the light (A) and dark period (B), two days after  $\text{NO}_3^-$  addition in week 5 and 6, in gnotobiotic microcosms planted with or without *Glyceria maxima* and inoculated with *Pseudomonas chlororaphis*. The plants were grown under two different photoperiods (14 vs. 20 hours) and with  $\text{NO}_3^-$  as nitrogen source. Bars indicate means ( $\pm$ SE) of 4 replicate microcosms. Asterisks indicate significant differences between rhizosphere and bulk soil ( $p < 0.05$ , Rank-Sum test).

es. A close approximation of the natural situation was achieved by Højberg 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 hydrophilic 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

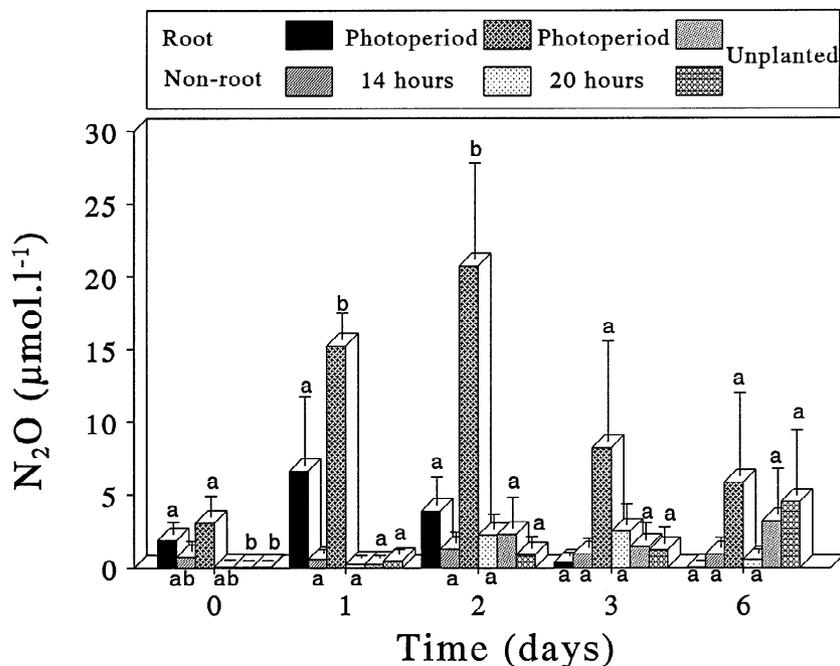


Figure 5. Porewater N<sub>2</sub>O concentrations during week 7 in the root- and non-root compartment of gnotobiotic microcosms planted with or without *Glyceria maxima* and inoculated with *Pseudomonas chlororaphis*. The plants were grown under two different photoperiods (14 vs. 20 hours) and with NO<sub>3</sub><sup>-</sup> 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.

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<sup>-1</sup>) was slightly lower than seedling RGR in natural situations (1.0-1.4 week<sup>-1</sup>; 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<sup>-2</sup> s<sup>-1</sup> (≈65 W m<sup>-2</sup>), 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<sub>org</sub> when the nitrate production is relatively small and denitrification high. Furthermore, the elevated C-A/N<sub>org</sub> 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

nitrate uptake instead of the C-A/N<sub>org</sub> ratio's. The values in the present study can be used as reference values in future experiments.

*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. planted/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, on the growth of and denitrification by *P. chlororaphis* it is 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  $5.52 \times 10^9$  and  $6.35 \times 10^9$  cells for the ammonium- and nitrate-fed systems, respectively, of the 14 hour photoperiod experiment. For the 20 hour photoperiod experiment these values were  $1.25 \times 10^{10}$  and  $2.94 \times 10^{10}$ . In the nitrate-fed systems there is an excess of electron acceptor 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. This value was only 2.3 in the ammonium-fed systems. The stimulation of carbon exudation and higher bacterial numbers associated with the plants due to a longer photoperiod was also found by Whipps (1984). Apparently, there was a relative shortage of plant-derived oxygen when taking the higher cell numbers into 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 systems for the 14- and 20 hours photoperiod, respectively. Hence, the relative shortage of oxygen compared to the available amount of carbon increases with the hours of daylight. Extrapolation to the field situation predicts better growth conditions for facultatively aerobic rhizosphere bacteria in summer and autumn and less favourable conditions in winter and spring. The ability to regulate the degree of oxygen deprivation allows

one to mimic the field situation more closely providing 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 photoperiod on denitrification in our study must be due to the higher carbon availability. However, the nitrous oxide production pattern in week 7 revealed an initial increase in denitrification followed by a decrease which is consistent with the decreasing nitrate availability. 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 tissue 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 indication of excess electron acceptor, leading to an incomplete 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 availability, is substantially higher.

Effects of the diurnal photosynthetic cycle on denitrification could not be detected. The oxygen released by the plants during the day might very well inhibit denitrification, as was found by Christensen and Sørensen (1986) in the root zone of the macrophyte *Litorea 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 measurements.

The systems we describe in this study provide a potential means of studying complex microbial interactions in the vicinity of roots. The "macro" rhizosphere 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 closer 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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