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FEMS Microbiology Ecology 25 (1998) 63–78

FEMS MICROBIOLOGY ECOLOGY

Interactions between nitrifying and denitrifying bacteria in gnotobiotic microcosms planted with the emergent macrophyte *Glyceria maxima*

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Received 30 January 1997; revised 5 August 1997; accepted 27 August 1997

Abstract

The population dynamics of the chemolithoautotrophic nitrifiers *Nitrosomonas europaea* and *Nitrobacter winogradskyi* were studied in gnotobiotic microcosms fed with ammonium in response to the presence or absence of the emergent macrophyte *Glyceria maxima* and the heterotrophic denitrifying bacterium *Pseudomonas chlororaphis*. By subjecting the plants to different day lengths, the effect of possibly limiting factors (i.e. oxygen and ammonium) on the interactions between the nitrifiers and denitrifying bacterium could be analysed. The presence of the plant had no effect on the growth of nitrifiers suggesting that, in addition to radial oxygen loss from the roots, other non-plant sources of oxygen (e.g. diffusion from the water layer) were important for nitrification. Potential nitrifying activities were suppressed by *G. maxima* due to ammonium uptake by the plants. Elongation of the day length in combination with the presence of *G. maxima* led to an increase in the number of *P. chlororaphis*. The presence of *P. chlororaphis* suppressed the growth of *N. winogradskyi*, but the growth of *N. europaea* and the potential nitrifying activities were not significantly affected. Potential denitrifying activities were stimulated by the plant, but showed no correlations with nitrifier activities or numbers. Apparently ammonium, and not oxygen, was the limiting factor for nitrification in the root zone of *G. maxima*. However, when the plant did not deplete the ammonium pool, *P. chlororaphis* could repress the nitrifiers indicating the latter's poor competitive status with respect to oxygen when the presence of root exudates allows for heterotrophic oxygen consumption.

Keywords: Nitrification; Denitrification; Microcosm; Oxygen; Ammonium; Root

1. Introduction

At anoxic/oxic interfaces in soils and sediments, ammonia can be oxidised to nitrate and subsequently reduced to N_2 or N_2O, leading to nitrogen loss from the ecosystem and emission of the greenhouse gas

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Publication no. 2286, Netherlands Institute of Ecology, Centre for Terrestrial Ecology.

0168-6496/98/$19.00
PII S0168-6496(97)00083-4

nitrous oxide [1]. In sediments, the upper few millimeters and the rhizosphere of oxygen-releasing, aerenchymatous plants are potential interfaces for coupled nitrification-denitrification. As denitrification depends on the presence of NO_3^-, nitrogen loss due to denitrification is dependent on the activity of the aerobic chemolithotrophic nitrifying bacteria. Ammonia-oxidising bacteria convert ammonia into nitrite which is oxidised to nitrate by nitrite-oxidising bacteria. Oxygen and ammonia availability are critical regulating factors of the nitrification process. All oxygen-consuming organisms and processes will compete for available oxygen in sediments, whereas ammonia availability will be determined mainly by plant uptake and bacterial consumption.

Nitrifying bacteria are assumed to be poor competitors for limiting amounts of oxygen [2–4] and compete poorly with plants [5,6] and bacteria [7] for limiting amounts of ammonia. Nevertheless, growth and activity of these organisms in the root zone of oxygen-releasing plants has been demonstrated to occur by means of cell counts, activity measurements and stimulated denitrification [8–11]. The survival mechanisms of aerobic nitrifiers in this environment, however, remain obscure. Longevity, especially under oxygen-poor conditions, seems to be a distinct trait of some nitrifiers [12–14]. Comparing nitrification in a freshwater lake sediment and in a permanently oxic dune soil, Bodelier et al. [11] found that ammonia oxidisers from sediment had higher affinities for oxygen than nitrifiers from well aerated terrestrial soils. Moreover, following a period of anoxia, these bacteria became fully active immediately after return to oxic conditions, whereas ammonia oxidisers from the dune soil exhibited a lag time prior to the onset of nitrifying activity.

Competitive success of nitrifiers for limiting amounts of oxygen with heterotrophic organisms has never been reported. Based on pure culture studies [15] and field data [11] it can be concluded that ammonia-oxidising cells can compete with heterotrophic cells on the basis of their oxygen uptake kinetics. Yield characteristics and uptake kinetics of the electron donor, however, are probably more important factors for the functioning of ammonia oxidisers in low oxygen habitats. Thus, in the rhizosphere of oxygen-releasing plants, the availability of substrates (i.e. organic substrates and ammonia) for nitrifying and heterotrophic bacteria will also influence the interactions of these organisms under oxygen-limiting conditions. Thus, in addition to releasing oxygen, the plant will also influence the interactions between nitrifying and denitrifying bacteria by releasing organic carbon and taking up ammonia.

The aim of this study was to test whether, and to what extent, nitrifiers are able to survive in the rhizosphere of oxygen-releasing plants and to investigate the possible influence of interactions with a denitrifying bacterium. Due to the elevated carbon release and subsequent stimulation of heterotrophs by *Glyceria maxima* [16], day length could be used as a 'natural' variable to study plant-induced effects on bacterial interactions. We studied the population dynamics of the nitrifiers *Nitrosomonas europaea* and *Nitrobacter winogradskyi* in the presence or absence of the denitrifier *Pseudomonas chlororaphis*, in gnotobiotic microcosms planted with *Glyceria maxima*. In this system, many of the uncertainties of studies of natural soils and sediments, such as underestimations of bacterial numbers due to limitations of culture methods, heterogeneity of soil or sediment, and definition of the rhizosphere, can be circumvented. The photoperiod imposed on the plant and the distance to the roots were used as variables to obtain information on the interactions between nitrifiers and denitrifiers in the plant-mediated oxic/anoxic zones.

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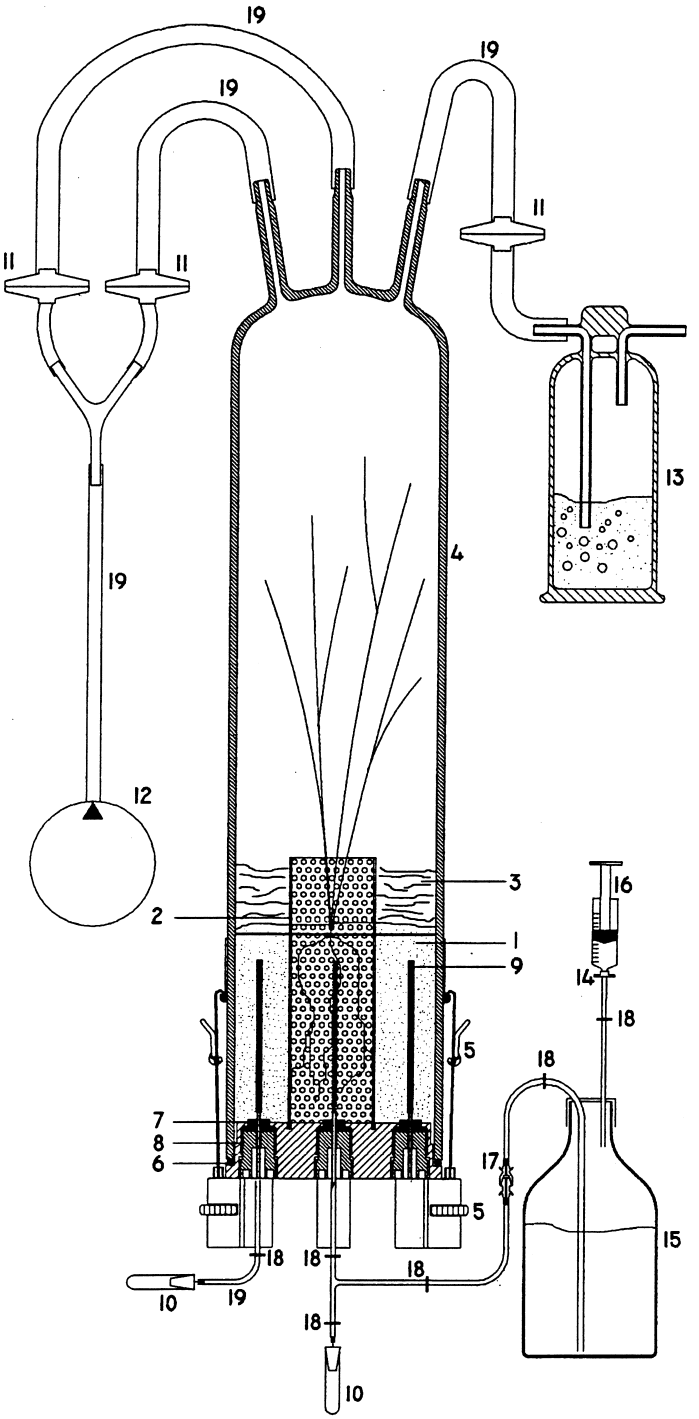
Fig. 1. A vertical cross-section of the gnotobiotic microcosm. 1: stainless steel cylindrical sediment compartment (12×9 cm, height×diameter), 2: stainless steel root cylinder (12×4 cm, h×diameter), 3: floodwater layer (4 cm), 4: glass cover (45×9.3 cm, h×diameter), 5: clamp device for securing glass cover on the soil compartment, 6: silicon greased airtight butylrubber gasket, 7: butylrubber septum, 8: housing containing the needle connecting the soil solution sampler, 9: rhizon soil solution sampler, 10: venoject sample collection tube, 11: hydrophobic PTFE in line membrane filter (0.2 μm), 12: air circulation pump, 13: NH_3 trap filled with H_3BO_3, 14: hydrophilic filter, 15: 500 ml flask for nutrient addition, 16: disposable syringe for pressurizing nutrient solution, 17: glass tube connection, 18: tube clamps, 19: silicone tubing.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2. Materials and methods

2.1. Description and operation of the model system

A schematic, vertical cross-section of the sterilisable microcosm used in this study is shown in Fig. 1. This system represents a modified version of that previously described [16]. The most important modification was the way in which nutrients and floodwater were added. Nutrient solution and floodwater were autoclaved in a flask which was connected to the rhizon soil solution sampling device in the root compartment. The oxygen-free nutrient solution was transferred from the flask to the microcosm by pressurising the flasks by means of a syringe filled with N_2 gas. Before addition of nutrients, the air present in the silicone tubing was withdrawn using a vacuum sample collection tube (Terumo, Belgium). Another modification was that the air leaving the system was led through a NH_3 trap which contained boric acid (H_3BO_3, 100 mM, pH < 4.8), to estimate ammonia volatilisation.

2.2. Organisms used and pre-cultivation

2.2.1. Plant

G. maxima (Hartm.) Holmb., a common emergent macrophyte in the Netherlands, was used as a model plant as it is known to produce stimulating effects on nitrification and denitrification [17,11]. *G. maxima* is highly aerenchymatous [18] and the inability of its roots to survive anoxia necessitates the continuous oxygenation of the root tissue resulting in oxygen leakage into the rhizosphere [19,20]. Sterile seedlings were obtained by surface sterilisation of the seeds with $NaHClO_3$ as described by Bodelier et al. [16].

2.2.2. Bacteria

N. europaea ATCC 19718 and *N. winogradskyi* ATCC 25391 were used as the nitrifying bacteria. The bacteria were cultivated separately in batch cultures in 500 ml flasks containing 200 ml medium of the following composition (mg l^{-1}): $(NH_4)_2SO_4$, 660 (*N. europaea*) or $NaNO_2$ (*N. winogradskyi*), 690; $MgSO_4 \cdot 7H_2O$, 40; $CaCl_2$, 0.02; NaCl, 500; KH_2PO_4, 100; and 1 ml trace element solution according to Verhagen and Laanbroek [7]. The pH of the media was adjusted to 7.7 with 1 M NaOH before autoclaving resulting in a final pH of 7.5. Bromothymol blue (5 ml, 0.04% w/v) solution was added to the *N. europaea* medium as pH indicator. During batch incubation the pH was adjusted with sterile Na_2CO_3 (1% w/v) to maintain pH 7.0–7.5. All cultures were incubated at 28°C. Prior to their use in the gnotobiotic microcosms, the batch cultures (200 ml) were centrifuged ($15\,000 \times g$, 20°C) in sterile, sealed centrifugation bottles (Nalgene, Rochester, MN, USA). Pellet were washed three times with an equal volume of batch medium without ammonium or nitrite. Cell numbers of the washed suspension were determined microscopically using a Bürker-Türk counting chamber. The suspensions were subsequently diluted to the desired inoculation density.

P. chlororaphis ATCC 43928 was used as a representative denitrifying bacterium. This species is a common soil heterotroph capable of denitrification isolated from agricultural soils [21] and was the dominant bacterial species isolated from dune soils (DeBoer, personal communication). *P. chlororaphis* also occurs in the root zone of *G. maxima*, where it tends to become dominant after amendment with nitrate [22]. We used a strain only capable of reducing nitrate to nitrous oxide [21] for methodological convenience. Growth conditions and handling prior to inoculation of the microcosms have been described elsewhere [16].

2.3. Experimental procedure

Calcareous river sand, pH(H_2O) 8.3, 0.66% organic matter, and 24.51% water holding capacity, originating from a floodplain from the river Rhine (Bemmel, The Netherlands), was used in these experiments. Separate experiments had indicated that this sand did not allow for any nitrifying activity and therefore cellulose (fibrous medium, Sigma Chemical Co., St. Louis, MO, USA) was added to the sand (2% w/w) to provide an adhering surface for the bacteria. Addition of cellulose led to a distinct stimulation of nitrification (data not shown). The mixture of sand and cellulose was sterilised by incubation at 200°C for 2×12 h. Autoclaved (121°C, 1 h) microcosms (24) were filled with 800 g of the heated sand-cellulose mixture in a sterile flow cabinet. After addition of sterile ultrapure milliQ water (Millipore BV, Etten-Leur, The Netherlands) the microcosms

were autoclaved again for 1 h (121°C). Prior to inoculation, the sand in the systems was percolated with 1 l of sterile ammonia oxidiser medium (see above), containing 1 mM NH_4^+, by pouring the buffer on top of the sand and subsequently removing it via the rhizon sampling device. This was done to supply the nitrifiers with substrate to establish themselves, to remove possible inhibiting compounds released during the sterilisation process, and to wash away any low molecular mass carbon compounds that might be used by *P. chlororaphis*. The sand was inoculated with 50 ml inoculum, containing washed cells of *N. europaea* and *N. winogradskyi*, leading to initial densities of 2.5×10^5 cells g^{-1} of dry sediment. The microcosms were incubated at 20°C at 70–80% water holding capacity, to allow 'establishment' of the nitrifiers. After 2 weeks the nitrate concentration in the pore water of the systems was 1–3 mM, indicating that nitrification had begun, and the pore water in the systems was removed to reduce the amount of nitrate present. At this point, half of the systems were inoculated with *P. chlororaphis* (1×10^4 g^{-1} dry sediment) and 16 microcosms, half with and half without *P. chlororaphis*, were planted with four sterile *G. maxima* seedlings as described by Bodelier et al. [16]. The sediment was flooded by the addition of sterile milliQ water. All microcosms were incubated in Heräus-Vötsch HPS 1500 growth cabinets (day/night cycle 16 h, 20°C/8 h, 20°C; relative humidity 70%; photosynthetic photon flux density at plant level ca. 300 μE m^{-2} s^{-1}). The air flow through the microcosms was 1.0 l min^{-1}. We also used beakers, receiving exactly the same treatment as the microcosms, to estimate the initial bacterial numbers as previously described [16]. Inoculation of *P. chlororaphis* and the planting of *G. maxima* was defined as week 1 of the experimental period. Plant nutrients were added weekly, by injection into the root compartment via the microsporous sampling device, in an exponentially increasing amount through week 5. From week 5 to 8 the highest doses of the major elements (N, 3.5 mmol; P, 0.448 mmol; K, 1.312 mmol) were applied. The unplanted systems did not receive any nutrients after week 5 to avoid toxic levels for the nitrifying bacteria. Pore water samples from the non-root and root compartments were collected prior to nutrient addition. The first 3 ml sample was discarded to avoid contamination with water from the dead space (approx. 0.5 ml) of the sampler. Samples were immediately transferred to 4°C and were analysed for NH_4^+, NO_3^-, NO_2^-, N_2O and pH the same day. At the beginning of week 5, half of the systems were subjected to a photoperiod of 12 h, whereas the other half were incubated with a 20 h photoperiod. Systems were randomly divided over the various treatments, with four replicate microcosms per treatment.

2.4. Harvesting and processing of plants and soil

The systems were harvested after 8 weeks. To avoid any possible bias, one microcosm of every treatment was processed per each of four harvest days. Floodwater was removed and stored at 4°C for further analysis. After cutting off the shoots, two stainless steel cylinders (5 and 6 cm in diameter) were inserted in the non-root compartment (N) creating three compartments of increasing distance (cm) from the root compartment (R): N1, 0–0.5; N2, 0.5–1.0; and N3, 1.0–3.5. The contents of each compartment, as well as the root compartment, were transferred to sterile glass containers for further analysis. Shoot and root dry matter was determined by weighing after drying at 70°C for 2 days. Moisture percentage, of thoroughly mixed sediment, was determined gravimetrically after drying at 105°C for 24 h. Mineral nitrogen content was determined in 1 M KCl extracts (1:2.5, w/v).

2.5. Microbial parameters

2.5.1. Enumeration of nitrifiers and denitrifiers

The numbers of *N. europaea* and *N. winogradskyi* were determined using a most probable number technique according to Bodelier et al. [11]. The technique was modified slightly in that the sand slurries were subjected to sonification (1 min, 47 kHz, Branson 2000 sonification bath) before serial dilution. Numbers of *P. chlororaphis* cells were determined by means of plate counting as described by Bodelier et al. [16].

2.5.2. Potential nitrifying activities

Potential nitrifying activities were taken as the slope of the nitrate+nitrite production vs time, dur-

ing a 6 h incubation period according to Verhagen et al. [23], except that slurries were incubated at 20°C instead of 25°C and nitrification stopped in the samples by centrifugation (15 000 × *g*, 15 min). This proved to be as efficient as addition of 2 M KCl (data not shown).

2.5.3. Denitrification activity

Denitrification activity assays were performed by the method of Tiedje [24] using the specific assay conditions described by Bodelier et al. [11]. The N_2O production was followed during an incubation period of 8 h in the presence of chloramphenicol to inhibit de novo enzyme synthesis. A 1 ml sample was withdrawn from the assay medium for analysis of NO_2^-, simultaneously with each N_2O sample.

2.5.4. Identification of bacterial contamination

Colonies which were different from *P. chlororaphis* were streaked out on fresh nutrient broth agar petri dishes to produce single colonies. Isolates were identified using a gas chromatograph-software system for the analysis of whole-cell fatty acid methyl ester (FAME, Microbial ID, Inc. [MIDI]) profiles as described by Janse et al. [25].

2.6. Chemical analyses

Concentrations of NH_4^+, NO_3^- and NO_2^- in pore water samples, sediment extracts, nutrient solutions, floodwater, NH_3 trap and in nitrification and denitrification assays were analysed using a Technicon Traacs 800 autoanalyser (Technicon Instr. Corp., Tarrytown, NY, USA). Nitrous oxide in pore water samples and denitrification assays was measured by injecting headspace samples from venoject tubes into a gas chromatograph (Carlo Erba GC 6000) equipped with a Hayesep Q column (80°C), a ECD detector (nitrous oxide < 100 ppmv) and a HWD detector (nitrous oxide > 100 ppmv). The total amount of nitrous oxide in gas and liquid phase of the venoject was calculated using the Bunsen absorption coefficient for nitrous oxide (0.632, 20°C, 1 atm) [24].

2.7. Statistical analyses

All statistical analyses were performed using the STATISTIX analytical software package (NH Analytical Software, St. Paul, MN, USA). Data were checked for normality by means of the Wilk-Shapiro test. Comparisons of means of plant parameters were performed with Tukey's test. Effects of the presence of *G. maxima* or *P. chlororaphis*, the photoperiod and the distance to the root were analysed by means of a Kruskall-Wallis one way ANOVA followed by a parametric ANOVA applied on the ranked variables as described by Potvin and Roff [26]. This procedure was performed to avoid a violation of the assumption of equal variances. Due to unequal sample sizes and inequality of variances it was not possible to test for interactions of treatment effects on the measured variables. Correlations between the measured variables were determined by means of the Spearman rank correlation test. Significance of the correlation coefficients was determined using the table presented by Zar [27].

3. Results

3.1. Plant parameters

Plant biomass production was significantly affected by the photoperiod but not by the presence of *P. chlororaphis* (Table 1). Plants subjected to a 20 h photoperiod produced 6.6–7.1 g of dry matter, while plants receiving only 12 h of daylight reached only 3.7–4.7 g (data not shown). Shoot/root ratio (4.3–5.8) and relative growth rates (RGR, 0.93–1.03 $week^{-1}$) did not differ between the treatments (data not shown). Analysis of RGR for main effects did, however, reveal a significant effect of the photoperiod (Table 1).

3.2. Microbiology

3.2.1. General aspects

All microcosms inoculated with *P. chlororaphis* remained free of contaminants during the complete experimental period of 3.5 months. Three of the 12 microcosms inoculated with only *N. europaea* and *N. winogradskyi* remained free of any contamination, one in each treatment. Of the remaining microcosms three were infected with *Acinetobacter lwoffi*, two with *Methylobacterium mesophilicum* and four con-

Table 1
Effects of the presence of *G. maxima*, the presence of *P. chlororaphis*, the photoperiod and the distance to the root compartment on the measured variables in gnotobiotic microcosms

Variable	Treatment			
	+/− plant	+/− *Pseudomonas*	Photoperiod	Distance to root compartment
Total plant dry matter	–	NS	(+)***	–
Relative growth rate	–	NS	(+)**	–
Potential nitrifying activity	(−)***	0.066	(−)**	NS
Potential denitrifying activity	(+)*	–	0.094	NS
Numbers of *P. chlororaphis*	(+)***	–	(+)*	NS
Numbers of *N. europaea*	NS	NS	0.091	NS
Numbers of *N. winogradskyi*	NS	(−)**	(−)**	NS
Ammonium content of the sediment	(−)***	NS	(−)**	(+)**
Nitrate content of the sediment	(−)***	NS	(−)***	0.081

The plants were subjected to different photoperiods (12 and 20 h) and were inoculated with the nitrifiers *N. europaea* and *N. winogradskyi* and either with or without the denitrifier *P. chlororaphis*.
***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, NS: not significant. When the 0.05 level was just not reached ($0.05 < P < 0.1$) the P value is given. (+) or (−) indicates a positive or negative treatment effect, respectively.

tained *P. chlororaphis*. *A. lwoffi* and *M. mesophilicum* infections did not apparently contribute to denitrification, based on their low cell numbers and the pore water nitrate concentrations in the unplanted systems, and only the microcosms containing *P. chlororaphis* infections were therefore discarded.

3.2.2. Nitrification

In Fig. 2 the most probable numbers results of *N. europaea* (A,C) and *N. winogradskyi* (B,D) are presented. Initial densities after inoculation were 1.41×10^4 and 8.31×10^4 cells g^{-1} dry sediment for *N. europaea* and *N. winogradskyi*, respectively. From the initial densities and Fig. 2 it is evident that substantial growth of both nitrifying strains occurred in all treatments. The increase in numbers was 2–3 orders of magnitude. The numbers of *N. europaea* were not significantly affected by the presence of *G. max-*

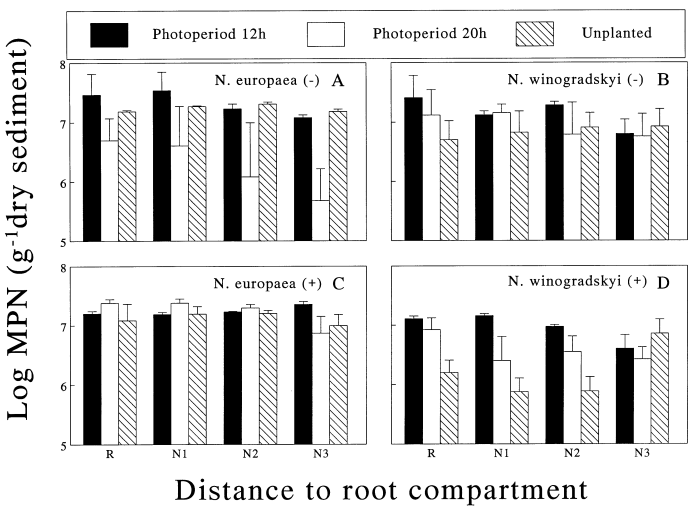

Fig. 2. Most probable numbers of *N. europaea* (A: (−) *P. chlororaphis*, C: (+) *P. chlororaphis*) and *N. winogradskyi* (B: (−) *P. chlororaphis*, D: (+) *P. chlororaphis*) in four sediment layers of gnotobiotic microcosms with or without *G. maxima*, which were subjected to two photoperiods (12 or 20 h). The sediment layers are defined as follows; R: root compartment and non-root layers N1, N2 and N3 at distances 0–5 cm, 0.5–1.0 cm and 1.0–3.5 cm from the root compartment, respectively.

ima or *P. chlororaphis* (Table 1). The effect of the photoperiod on numbers of *N. europaea* just failed to reach the 0.05 significance level (Table 1). There was also no effect of sediment layer, including the root compartment (Table 1). *P. chlororaphis* and the photoperiod had significant negative effects on the numbers of *N. winogradskyi* (Table 1).

The potential nitrifying activities (PNA) in the presence of *P. chlororaphis* (Fig. 3B) were generally lower than those without *P. chlororaphis* (Fig. 3A). This effect seemed to be more pronounced in the unplanted microcosms, but the effect of *P. chlororaphis* on PNA just failed to reach the significance level of 0.05 (Table 1). Elongation of the photoperiod led to an almost complete suppression of nitrifying activity in the root compartment. The PNA was significantly and negatively affected by the presence of the plant and the lengthening of the photoperiod (Table 1, Fig. 3). The distance to the root compartment had no significant effect on nitrifying activity.

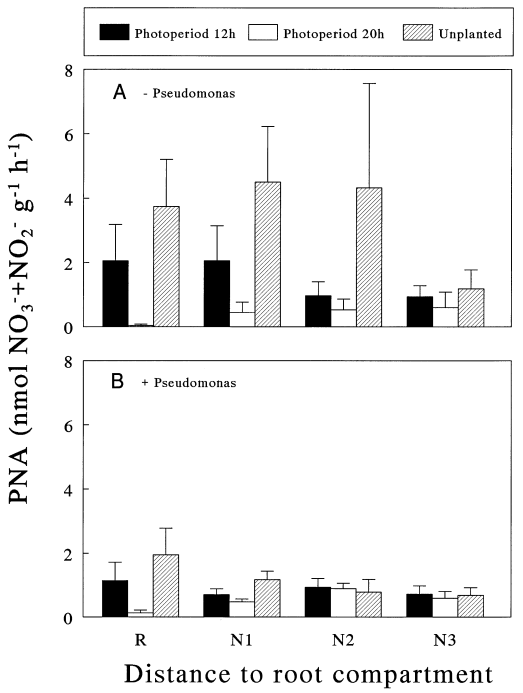

Fig. 3. Potential nitrifying activities in four sediment layers of gnotobiotic microcosms, planted with or without *G. maxima*, subjected to two photoperiods (12 or 20 h), and inoculated with the nitrifiers *N. europaea* and *N. winogradskyi* either without (A) or with (B) the denitrifier *P. chlororaphis*. The sediment layers are defined as in Fig. 2.

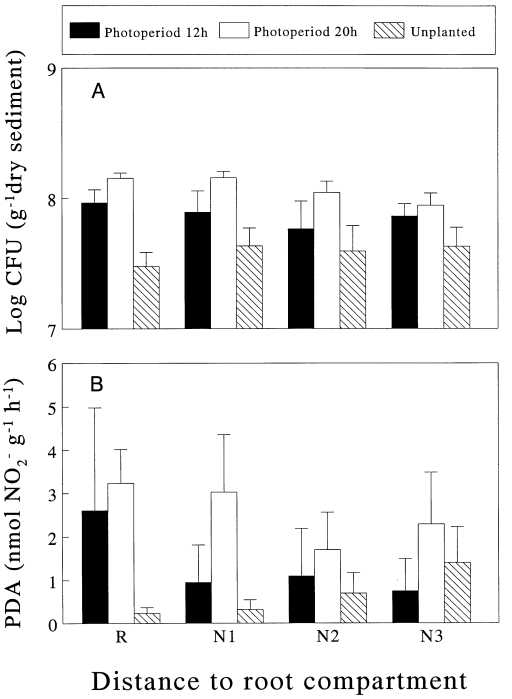

Fig. 4. Numbers of *P. chlororaphis* (A) and potential denitrifying activities (B) in gnotobiotic microcosms planted with or without *G. maxima*, which were subjected to two photoperiods (12 or 20 h), and inoculated with the nitrifiers *N. europaea* and *N. winogradskyi*. The sediment layers are defined as in Fig. 2.

3.2.3. Denitrification

The initial density of *P. chlororaphis*, determined after 3 days of incubation in a parallel series of glass beakers, was 1.17×10^7 CFU g^{-1} dry sediment. As we had inoculated 1×10^4 cells g^{-1} dry sediment, residual substrate present in the sediment apparently allowed for cell numbers to increase by 3 orders of magnitude. The final numbers were up to 5×10^7 CFU g^{-1} dry sediment in the unplanted microcosms, and 1.4×10^8 CFU g^{-1} dry sediment in the root compartment and the adjacent N1 layer of the 20 h photoperiod of the planted microcosms (Fig. 4A). The numbers of *P. chlororaphis* were stimulated by both the presence of *G. maxima* and lengthened photoperiod (Table 1). Again, there was no significant effect of the distance from the root compartment.

Table 2
Spearman rank correlations of measured variables of gnotobiotic microcosms planted with or without *G. maxima*, which was subjected to two photoperiods (12 and 20 h)

Variable	Plant dry matter[a]	Potential		Numbers of			Sediment	
		nitrifying activities	denitrifying activities	*P. chlororaphis*	*N. europaea*	*N. winogradskyi*	NH_4^+ content	NO_3^- content
Potential nitrifying activities	−0.78**	–	–	–	–	–	–	–
	($n=13$)							
Potential denitrifying activities	−0.03	−0.25	–	–	–	–	–	–
	($n=6$)	($n=40$)						
Numbers of *P. chlororaphis*	0.26	−0.22	−0.12	–	–	–	–	–
	($n=6$)	($n=46$)	(n=40)					
Numbers of *N. europaea*	−0.20	0.09	0.09	−0.46**	–	–	–	–
	($n=13$)	($n=72$)	(n=40)	($n=44$)				
Numbers of *N. winogradskyi*	0.12	−0.05	0.33*	−0.18	0.04	–	–	–
	($n=13$)	($n=78$)	(n=40)	($n=48$)	($n=72$)			
Sediment NH_4^+ content	−0.24	0.41**	−0.08	−0.54**	0.18	−0.26*	–	–
	($n=13$)	($n=78$)	($n=40$)	($n=48$)	($n=72$)	($n=80$)		
Sediment NO_3^- content	−0.24	0.54**	−0.13	0.60**	0.22	−0.16	0.82**	–
	($n=13$)	($n=78$)	($n=40$)	($n=48$)	($n=72$)	($n=80$)	($n=80$)	

The sediment was inoculated with the nitrifiers *N. europaea* and *N. winogradskyi* and either with or without the denitrifier *P. chlororaphis*. $^{**}P<0.01$, $^{*}P<0.05$. [a]Total plant dry weight related to measured variables in root compartment only.

The denitrifying activities are expressed as nmol NO_2^- derived from NO_3^- g^{-1} dry sediment h^{-1}. During the assays no N_2O could be detected, and the NO_2^- concentration in the assay increased linearly with time. The lowest value, 0.2 nmol NO_2^- g^{-1} dry sediment h^{-1}, was measured in the 'root' compartment of the unplanted systems, while the highest value (3.2 nmol NO_2^- g^{-1} h^{-1}, Fig. 4B) was found in the root compartment of the planted systems subjected to the 20 h photoperiod. Denitrifying activities were stimulated by the presence of *G. maxima* (Table 2). The photoperiod and the distance from the root compartment had no effect.

3.3. Sediment parameters

3.3.1. Final nitrogen content

Mineral N contents at the end of the experiment (Table 1) were influenced by the presence of *G. maxima*, the photoperiod and the distance to the root compartment (NH_4^+ only). The plants reduced the ammonium content compared to the unplanted microcosms (Fig. 5A,B) and was lowest in the microcosms subjected to the 20 h photoperiod, reaching levels of 8.5 mg NH_4^+ g^{-1} dry sediment in the root compartment of the microcosms with *P. chlororaphis*. The presence of *P. chlororaphis* had no significant effect (Table 1). In plant microcosms there was a gradient of increasing ammonium levels from the root compartment into the adjacent non-root layers. Nitrate concentrations were substantially lower than those for ammonium (Fig. 5C,D). Nitrate concentrations were also lowered by the presence of the plant and the longer photoperiod, whereas the presence of *P. chlororaphis* had no effect (Table 2). In contrast to the ammonium, the nitrate content was not significantly affected by the distance from the root compartment (Table 1). Nitrite was only detectable in the unplanted systems and reached values of 2.0–3.8 mg NO_2^- g^{-1} dry sediment (data not shown).

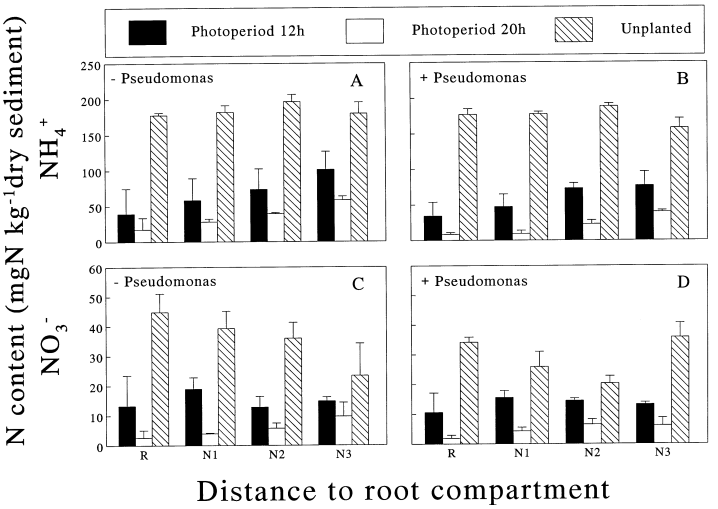

Fig. 5. Sediment ammonium (A,B) and nitrate (C,D) content in four sediment layers of gnotobiotic microcosms planted with or without *G. maxima*, which were subjected to two photoperiods (12 or 20 h), and inoculated with the nitrifiers *N. europaea* and *N. winogradskyi*, either with (B,D) or without (A,C) the denitrifier *P. chlororaphis*. The sediment layers are defined as in Fig. 2.

3.3.2. Pore water composition: NH_4^+, NO_3^- and N_2O

The concentrations of NH_4^+ and NO_3^- in the pore water of the root and non-root compartments are presented in Fig. 6. Ammonium concentrations in the absence (Fig. 6A) and in the presence of *P. chlororaphis* (Fig. 6B) were very similar. In the unplanted microcosms ammonium accumulated, as expected, from nutrient addition. In the planted systems ammonium started to accumulate from week 5 on, except for the root compartment of the microcosms subjected to the 20 h photoperiod. In all planted microcosms, ammonium concentrations of the pore

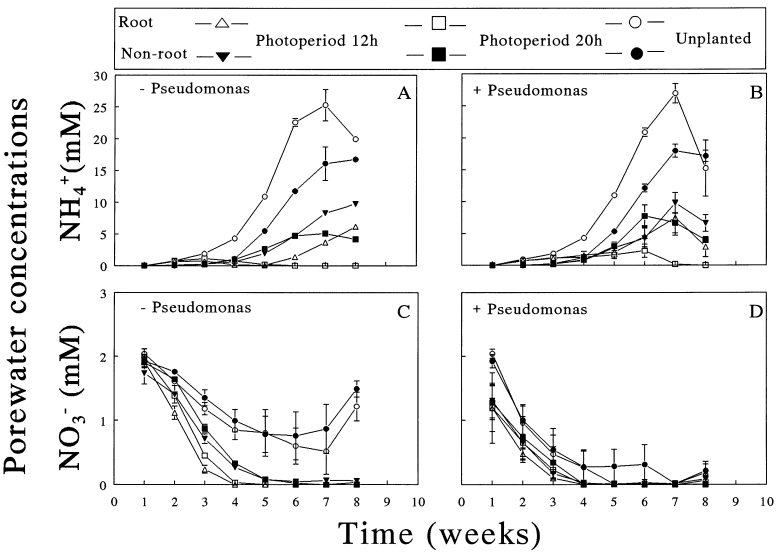

Fig. 6. Porewater ammonium (A,B) and nitrate (C,D) concentrations in the root and non-root compartments of gnotobiotic microcosms planted with or without *G. maxima* and inoculated with the nitrifiers *N. europaea* and *N. winogradskyi*, either with (B,D) or without (A,C) the denitrifier *P. chlororaphis*. Until week 5, all microcosms had been subjected to a photoperiod of 16 h. At the start of week 5, microcosms were subdivided into two series with different photoperiods of 12 and 20 h, respectively.

water were higher in the non-root compartment. The nitrate present, as the consequence of the preincubation period, decreased to almost undetectable levels in all microcosms, except for the unplanted systems without *P. chlororaphis* where the nitrate concentration remained in the range of 1–2 mM.

Nitrous oxide concentrations in the microcosms due to nitrifiers alone were in the range of 0.003–3.65 μmol N_2O l^{-1} pore water (Fig. 7A) and 1.04–25.28 μmol N_2O l^{-1} (Fig. 7B) in the presence of *P. chlororaphis*. In the absence of *P. chlororaphis*, N_2O production in the planted microcosms started earlier than in the unplanted systems, where appreciable amounts were only detected after week 3. Taking the substantial variation into account, it can be stated that the N_2O levels in the root and non-root compartments of the planted microcosms without *P. chlororaphis* stayed relatively constant through week 6. In weeks 7 and 8 the N_2O concentrations in the root compartment of the microcosms subjected to the 20 h photoperiod were significantly lower compared to those in the non-root compartment. This was not observed with the 12 h photoperiod. In the presence of *P. chlororaphis*, N_2O concentrations were 10–12 μmol l^{-1} at the first sampling event, which was 6 days after inoculation with *P. chlororaphis*, and remained at this level through week 4. After the separation to a long and short day length the patterns became more dynamic. In the unplanted systems, N_2O levels rose to approximately 25 μmol l^{-1} pore water (with *P. chlororaphis*). The amount of N_2O in the pore water started to decrease in the planted systems, especially in the root compartments. N_2O concentrations in the root compartment of the systems subjected to the long photoperiod were significantly lower in weeks 6–8 compared to the non-root compartment. With the short photoperiod this occurred only in week 8.

3.4. Correlation of measured variables

Spearman rank correlation coefficients of the measured variables are presented in Table 2. Potential nitrifying activities were negatively correlated with the total plant dry weight and were positively related with ammonium and nitrate concentrations in the sediment. The numbers of *N. europaea* were negatively correlated with the numbers of *P. chlororaphis*. The numbers of *N. winogradskyi* appeared to be positively linked to the denitrifying activity and were negatively correlated with the ammonium concentration. The numbers of *P. chlororaphis* were positively related with the nitrate concentration, but negatively with the ammonium content. Finally,

Table 3
Total number of cells formed in the experimental period of 8 weeks after the day of flooding

Photoperiod	(+/−) *P. chlororaphis*	*N. europaea* ($\times 10^{10}$)	*N. winogradskyi* ($\times 10^{10}$)	*P. chlororaphis* ($\times 10^{10}$)	Ratio *P. chlororaphis*/nitrifiers	
					N. europaea	*N. winogradskyi*
At day of flooding		0.44	0.29	0.94	2.14	3.24
Unplanted	(−)	0.59	0.39	–	–	–
Unplanted	(+)	0.70	0.40	2.67	3.79	6.65
12 h	(−)	1.49	1.17	–	–	–
12 h	(+)	0.71	0.68	5.53	7.79	8.13
20 h	(−)	−0.18	0.46	–	–	–
20 h	(+)	0.51	0.10	8.81	17.23	91.48

For *N. europaea* and *N. winogradskyi* the numbers formed were corrected for the initial densities[a] and the maximum number of cells formed in the non-flooded 'activation period'[b]. The numbers of *P. chlororaphis* were only corrected for the initial densities.

[a]Initial densities were determined 3 days after inoculation and were 1.41×10^4 g^{-1} dry sediment for *N. europaea*, 8.41×10^4 for *N. winogradskyi* and 1.17×10^7 for *P. chlororaphis*.

[b]For activation 0.8 mmol NH_4^+ per microcosm was added. The number of cells formed from this amount was calculated using the cell yields per mol N converted into nitrate at the end of the experiment in the unplanted systems without *P. chlororaphis*, being 1.88 mmol. The cell yields can be calculated for *N. europaea* and *N. winogradskyi* as 3.65×10^{12} and 5.50×10^{12} cells mol^{-1}, respectively. Hence in the non-flooded activation period 2.92×10^9 and 4.40×10^9 cells of *N. winogradskyi* and *N. europaea*, respectively, could have formed per microcosm. These numbers were subtracted from the total number of cells formed in the microcosms.

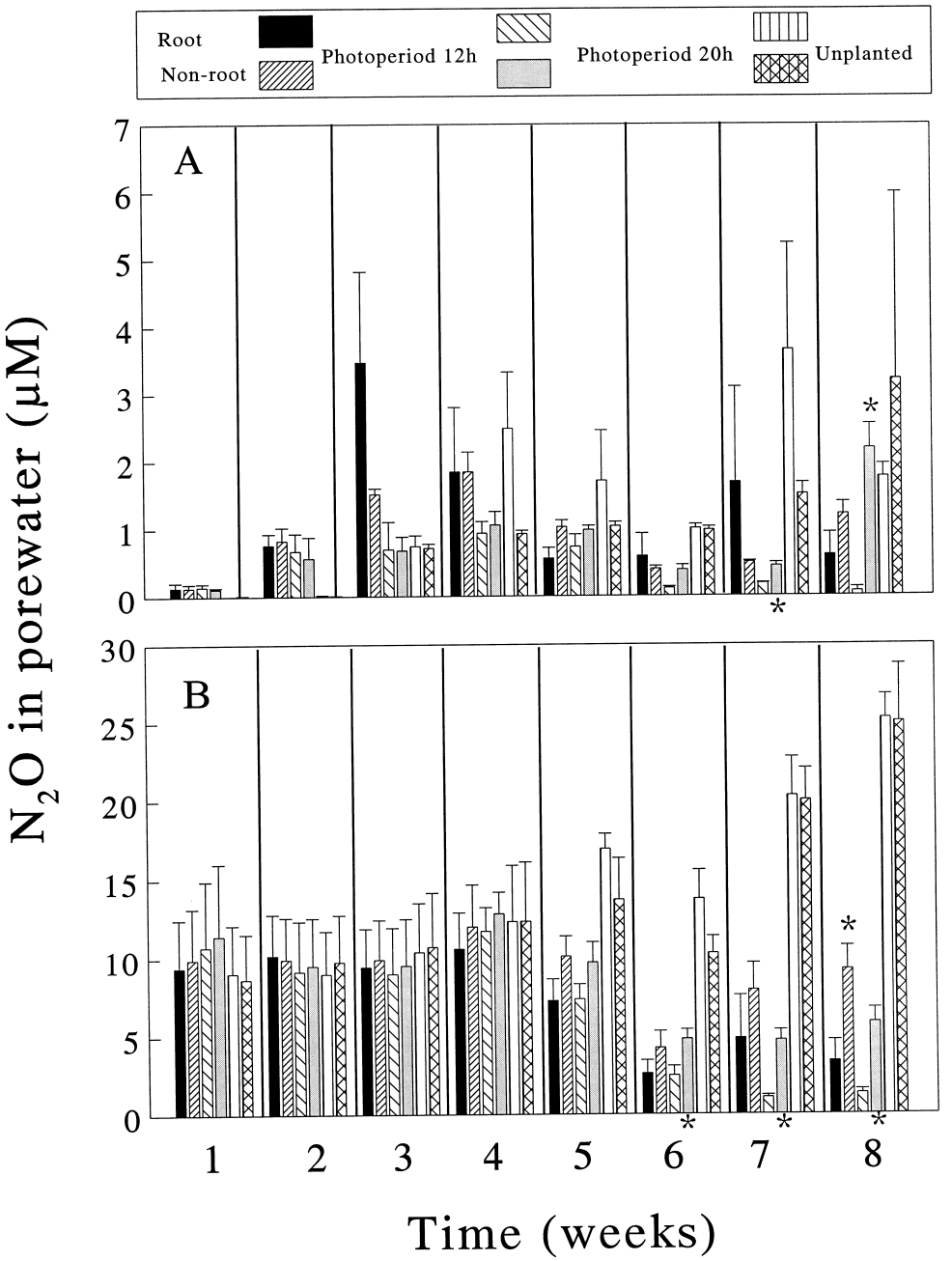

Fig. 7. Porewater nitrous oxide concentrations in the root and non-root compartments of gnotobiotic microcosms planted with or without *G. maxima* and inoculated with the nitrifiers *N. europaea* and *N. winogradskyi*, either with (B) or without (A) the denitrifier *P. chlororaphis*. Until week 5, all microcosms had been subjected to a photoperiod of 16 h. At the start of week 5, microcosms were subdivided into two series with different photoperiods of 12 and 20 h, respectively. Asterisks indicate significant differences between root and non-root compartments ($P < 0.05$, two sample *t*-test).

there appeared to be a strong positive relation between the ammonium and nitrate concentrations of the sediment.

4. Discussion

4.1. General

The modifications we applied to the gnotobiotic microcosms used in this study represented an improvement to the systems previously used, containing only *P. chlororaphis* [16], in which the sterilisation procedure and the method of nutrient addition was apparently not effective. The heat pretreatment of the sand and the addition of nutrients by means of a detachable flask led to a reduction in contamination. The microcosms inoculated with *P. chlororaphis* did not become infected. However, the systems inoculated with only nitrifiers were apparently more susceptible to contamination because of the available carbon and the slow growth of the nitrifiers.

4.2. Nitrification and denitrification

The major objectives of this study were to determine the impact of *G. maxima* on the interactions between nitrifying and denitrifying bacteria with the emphasis on the potential of nitrifiers to persist under limiting amounts of substrate (i.e. oxygen and ammonia). The increase in carbon availability, as reflected by the numbers of *P. chlororaphis*, due to the elongation of the day length imposes a higher competitive stress upon the nitrifiers. *G. maxima* and the longer photoperiod led to an increase in the numbers of *P. chlororaphis*, as has previously been reported [16], and the presence of *P. chlororaphis* significantly repressed growth of *N. winogradskyi*, but not of *N. europaea* (Table 1). This could be explained by the relatively poor oxygen kinetics of *N. winogradskyi* [28] or by competition for nitrite with *P. chlororaphis*. Although the growth of *N. europaea* was not affected, the potential activities tended to be lowered in the presence of *P. chlororaphis*. Activities were also lowered by the plant (Table 1). Apparently, the plant limits nitrification due to ammonium uptake, as was also found by Engelaar et al. [5] and Verhagen et al. [6]. This is most obvious for the 20 h photoperiod where the plants formed more biomass and thus required more ammonium. Although one would expect the treatment variables to interact, the data did not allow assessment by a two way ANOVA design. From Table 1 it can be deduced that the photoperiod affects every measured variable probably in interaction with the other treatments. The plant apparently imposes a more important stress on the nitrifiers than *P. chlororaphis*, especially for the longer photoperiod. However, for the 12 h photoperiod where ammonium is apparently not limiting, nitrification tended to be higher in the absence of *P. chlororaphis*, indicating that part of the oxygen was consumed by the heterotroph. Negative effects of *P. chlororaphis* on nitrifiers due to reasons other than uptake of oxygen (e.g. production of antibiotics) cannot be excluded.

In an attempt to elucidate competition for oxygen, we calculated the total number of cells formed after flooding. From Table 3 it can be seen that the ratio between *P. chlororaphis* and *N. europaea* in the unplanted systems hardly changed compared to day 0. The numbers of *N. winogradskyi* decreased relative to the numbers of *P. chlororaphis* in the unplanted systems. These ratios increased substantially when the photoperiod was elongated. This may be due to growth inhibition of the nitrifiers due to plant N uptake. However, for the 12 h photoperiod, where sufficient ammonium is available, the numbers of *P. chlororaphis* increased while the nitrifiers remained at the level of the unplanted systems, indicating oxygen limitation. For the 12 h photoperiod when *P. chlororaphis* was not present, the nitrifiers had no competitors for oxygen and continued to multiply. Hence, plant-induced growth of the nitrifiers appeared only when there was sufficient ammonium and no *P. chlororaphis* present. However, the stimulation of nitrification by oxygen released by the plant was not very substantial, as the growth in the unplanted systems, using oxygen diffused from the water layer, was nearly as high. To form the amount of nitrate present in the unplanted systems at the end of the experiment, 3.85 mmol O_2 would be needed corresponding to a flux of 8.76 mmol O_2 m^{-2} day^{-1} (period 56 days, surface area 7.85×10^{-3} m^2). Jensen et al. [14] measured an oxygen flux due to nitrification in the top 1.5 cm of a sediment core flooded with air saturated water of 37.6 mmol O_2 m^{-2}

day^{-1}, demonstrating that the growth in our study might very well have been the consequence of the diffusive flux from the water layer. Hence, in studies addressing the effect of oxygen-releasing plants on nitrification, it is essential that the upper sediment layer receiving oxygen from the water layer should be omitted from activity measurements and bacterial counts. The idea that oxygen from the water layer greatly influences the nitrification profile is supported by the fact that the radial distance from the root compartment had no effect on the distribution and activity of nitrifiers. It remains a possibility that the large volumes (50–100 ml) used for nutrient addition might have played a role in the distribution of plant-derived carbon and oxygen through the microcosms.

Denitrification activity was enhanced by the presence of *G. maxima* (Table 1). However, in the short-term assays we could never detect any N_2O, while nitrite accumulated linearly. An explanation might be a shortage of electron donor which can lead to incomplete denitrification (R.A. Kester, unpublished results). Although a substantial part was of nitrifier origin, the pore water composition also indicated N_2O production by denitrification (Fig. 7). At the moment of inoculation of *P. chlororaphis* nitrate was present from the activation period of the nitrifiers. This led to an immediate nitrite accumulation in the microcosms with *P. chlororaphis* of up to 0.9 mM in the pore water (data not shown), supporting the carbon shortage theory. Inhibition of the nitrite reductase by oxygen in the microcosms certainly also needs to be considered due to the lack of N_2O production in denitrification assays.

Nitrous oxide accumulated to a level of 10 μM, and remained unchanged through week 4 in the microcosms with *Pseudomonas*. The subsequent decrease of N_2O in the planted systems may have been caused by N_2O uptake by or escape via the plant, as previously reported [9,29], or by nitrate-limiting conditions due to plant nitrate uptake. The presence of nitrate in the sediment at the end of the experiment makes the latter unlikely, although pore water nitrate concentrations were low. This discrepancy between nitrate concentrations in the pore water and KCl extracts indicates a spatial variability of nitrate availability in the microcosms. Addition of chloramphenicol to the denitrification assay medium should allow one to relate the denitrification activity to nitrate production. However, no relation could be found between the potential nitrifying activity or nitrate content of the sediment and denitrification activity (Table 2). Apparently factors other than nitrate production influenced the detected enzyme levels. The availability of carbon may be the key factor as seen by Ambus [30], where addition of glucose stimulated denitrification 2.5-fold in streamside soil while addition of nitrate had no effect. Dendooven and Anderson [31] have also demonstrated that even under aerobic conditions a high nitrate reductase potential was maintained enabling denitrifiers to react to the presence of carbon and anoxicity.

4.3. Synthesis

To our knowledge there are no reports available which have addressed the issues of the present study by means of gnotobiotic systems. Experiments with pure cultures of nitrifiers in pre-sterilised sand or soil in the presence of a plant have only been performed under drained conditions. The numbers of nitrifiers observed in the present study were comparable to those found in gnotobiotic sand columns planted with *Pisum sativum* L. and continuously fed with ammonium [32], where ammonium and oxygen are not limiting. Even under flooded conditions, it appears that the availability of ammonium, and not oxygen, may be more important, as indicated by the positive correlation between mineral ammonium content and potential nitrifying activities (Table 2). The suppression of nitrification due to ammonium uptake by the plant with 20 h of daylight might be comparable to in situ conditions in summer. The maximal vegetation may be responsible for the observed repression of nitrification in the root zone of *G. maxima* [11]. The 12 h photoperiod might reflect the situation in early spring, where the initially lower ammonium demand of the plants allows nitrifiers to become active. But even under these reduced photosynthetic periods nitrifiers have to compete for oxygen with heterotrophic bacteria such as *P. chlororaphis* in our experiment.

In addition to the decisive role of ammonium, this study reveals that the survival of nitrifiers in the root zone of aerenchymatous plants does not depend on their competitive abilities. The presence of *P. chlororaphis* already tends to lower nitrification in both

the unplanted systems and the planted columns at the 12 h photoperiod. It is likely that increasing the available carbon would lead to an even more severe suppression of nitrification as was demonstrated for biofilms [33,34] and sludge reactors [35]. Nevertheless, when ammonium is available, nitrifiers are able to persist and to function at low oxygen conditions. Unravelling the mechanisms underlying the persistence of nitrifiers in adverse habitats, without the possession of competitive abilities, is worthwhile since these mechanisms in soil or sediments may be ecologically of greater importance than the rapid and efficient growth displayed by 'laboratory' bacteria.

Acknowledgments

This study was supported by the Foundation of Life Sciences of the Netherlands Organisation of Pure Research. We thank J.W. Woldendorp, S.R. Troelstra, W. de Boer and G.A. Kowalchuk for valuable discussion and critical reading of the manuscript. The authors would like to thank Dr. Søren Christensen for providing the *Pseudomonas chlororaphis* strain, G. Wijlhuizen for the drawings of the microcosm.

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