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Competition for nitrate and glucose between *Pseudomonas fluorescens* and *Bacillus licheniformis* under continuous or fluctuating anoxic conditions

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Abstract

The dissimilatory nitrate-reducing bacterial community in the rhizosphere of aerenchymatous plant species such as *Glyceria maxima*, consists of oxidative, denitrifying and fermentative nitrate-ammonifying bacteria. To study the respective ecological niches of both types of nitrate-reducing bacteria, competition for nitrate or glucose between the representative denitrifier *Pseudomonas fluorescens* and the representative fermentative nitrate-ammonifying *Bacillus licheniformis* under continuous or fluctuating anoxic conditions were performed in continuous culture. Competition started by mixing the separate, steady-state mono-cultures of the two species at different ratios. All the experiments were performed at a dilution rate of 0.05 h^{-1}. The competition was followed by measuring concentrations of nitrogen, glucose and fatty acids and by determining the cell numbers of *P. fluorescens* and *B. licheniformis*. Under continuous anoxic nitrate-limited conditions and under certain fluctuating anoxic conditions (8 h 10% and 16 h 0% air saturation), *B. licheniformis* was able to maintain itself in the chemostat at a low percentage of 4–7%. Under continuous anoxic glucose-limited conditions and under specific fluctuating anoxic (16 h 10% and 8 h 0% air saturation) conditions, *B. licheniformis* washed out. The outcome of the competition was explained by a higher affinity of *P. fluorescens* for nitrate and glucose compared to *B. licheniformis*. *B. licheniformis* was able to maintain itself in the chemostat under continuous anoxic nitrate-limited conditions and under certain fluctuating anoxic conditions (8 h 10% and 16 h 0% air saturation) due to the fermentation of the remaining glucose.

Keywords: Competition; Nitrate reduction; Chemostat; *Pseudomonas fluorescens*; *Bacillus licheniformis*

1. Introduction

Denitrification and dissimilatory nitrate reduction to ammonium, for short nitrate ammonification, are the two known dissimilatory nitrate-reducing pathways. Denitrification by oxidative bacteria such as *Pseudomonas* spp. , leads to the formation of gaseous products such as nitric oxide (NO), nitrous oxide (N_2O) and molecular nitrogen (N_2) [1]. Fermentative

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strains, such as *Bacillus* spp., reduce nitrate (NO_3^-) to nitrite (NO_2^-) and subsequently to ammonium (NH_4^+) [2]. The distribution and activity of denitrifying and nitrate-ammonifying bacteria is mainly determined by the relative availability of carbon, nitrate and oxygen [2–4]. In the rhizosphere of flooded aerenchymatous plants oxygen is released, therefore affecting the electron donor/electron acceptor ratio [5,6]. Nitrification can occur in the rhizosphere of aerenchymatous plants due to this oxygen release [7]. For example, in the rhizosphere of *Glyceria maxima* (Hartman) Holm. (reed sweet grass) nitrification is stimulated [8,9]. Diffusion of this nitrate to anoxic sites enables nitrate reduction to take place. The nitrate concentration will be lowered not only by nitrate reduction but also by nitrate uptake by the plant [10,11]. Organic substrates are also released into the rhizosphere of aerenchymatous plants [12,13], thereby increasing the concentration of available organic substrates.

It has been shown that in the rhizosphere of *G. maxima* fermentative bacteria potentially dissimilating NO_3^- to NH_4^+ were dominant when NO_3^- was limiting or absent [11,14]. Under all other conditions, potentially denitrifying bacteria become dominant. *G. maxima* only exerts a major influence on the nitrate-reducing bacterial community under conditions of NO_3^- limitation, probably due to the excretion of organic compounds. Based on these results, we postulated that when NO_3^- is not present in an anoxic sediment, bacteria capable of dissimilating NO_3^- to NH_4^+ become dominant due to their fermentative metabolism, since they are able to generate energy in the absence of external electron acceptors. However, when oxygen is released into the rhizosphere nitrification can take place. At that moment, the NO_3^- uptake rate by the fermentative community, able to reduce nitrate, is greater than that of the relatively few denitrifying cells, and hence most nitrate will be reduced to ammonium. If the maximum growth rate (μ_{max}) of the denitrifying bacteria, however, is greater than that of the fermentative nitrate-reducing bacteria, the denitrifying bacteria will outgrow the others and the proportion of nitrate reduced to dinitrogen will increase, especially when NO_3^- is available over a longer time period.

In order to understand and predict the behaviour of a potentially nitrate-reducing community in relation to NO_3^- availability, competition experiments were performed in a chemostat with the denitrifying species *Pseudomonas fluorescens* and the fermentative nitrate-reducing species *Bacillus licheniformis*, both isolated from the rhizosphere of *G. maxima* [11].

The aim of these experiments was to determine which factor(s) (carbon, nitrate or oxygen) controls the competition between *P. fluorescens* and *B. licheniformis* under continuous or fluctuating anoxic conditions.

2. Materials and methods

2.1. Bacterial strains

The experiments were performed with a denitrifying strain of *Pseudomonas fluorescens* (Biovar III or IV) and a nitrate-ammonifying strain of *Bacillus licheniformis*, able to dissimilate NO_3^- to NH_4^+, both isolated as dominant representatives of the nitrate-reducing community from the rhizosphere of *G. maxima* [11]. Using these two strains experiments were performed to determine optimal growth conditions before the competition experiments were started. The results showed (data not shown) that with D-glucose as the carbon and electron donor, the growth characteristics of the two species were most optimal for the chemostat experiments. Glucose is also known to be excreted by the roots [15]. *P. fluorescens* denitrifies NO_3^- entirely to dinitrogen without accumulation of N_2O. *B. licheniformis* reduces NO_3^- completely to NH_4^+, without flocculation of the cells or accumulation of NO_2^-, when ammonium was available in the medium. The reduction of nitrate beyond the level of nitrite by *B. licheniformis* was dependent on the presence of ammonium [16].

Before starting the chemostat experiments, the maximum specific growth rates (μ_{max}) of *P. fluorescens* and *B. licheniformis* on glucose or acetate as electron donor in the presence or absence of nitrate as electron acceptor were determined in triplicate in 250-ml flasks flushed with dinitrogen. The pH of the medium at the start of the incubations was 7.5, temperature fluctuated between 22°C and 25°C. During 24–30 h samples were taken to determine the optical density (O.D.), the total number of bacteria and the glucose, acetate and mineral nitrogen concentrations.

The μ_{max} was determined during the exponential growth phase of the strains. The μ_{max} values were also determined for both species in a pH-stat (pH 7.5), temperature 25°C, with glucose as electron donor and O_2 as electron acceptor (10% and 80% air saturation). The μ_{max} values of *P. fluorescens* and *B. licheniformis* are given in Table 1.

2.2. Medium and growth conditions

The mineral medium for the chemostat experiments (Biostat M fermenters, B. Braun, Melsungen, Germany; 1.2 l culture volume) consisted of (per litre): 0.2 g $MgSO_4 \cdot 7H_2O$, 0.584 g NaCl, 0.0294 g $CaCl_2 \cdot 2H_2O$, 1200 mg K_2HPO_4, 920 mg (NH_4^+) H_2PO_4 (8.0 mmol l^{-1}) and 10 ml trace-element solution. Trace-element solution contained (per litre): $FeCl_2 \cdot 4H_2O$ 2 g, Na_2-EDTA (Titriplex III) 4.3 g, H_3BO_3 62 mg, $CuCl_2 \cdot 2H_2O$ 17 mg, $NiCl_2 \cdot 6H_2O$ 24 mg, $CoCl_2 \cdot 6H_2O$ 24 mg, $MnCl_2 \cdot 4H_2O$ 0.1 g, $NaMoO_4 \cdot 2H_2O$ 24 mg and $ZnCl_2$ 68 mg. Glucose and nitrate were used in different ratios as electron donor and acceptor, respectively. The stock solutions of D-glucose and the phosphate buffers were autoclaved separately and added to the mineral medium after sterilization. All solutions were kept under oxygen-free nitrogen gas (5.0 Hoek Loos, Schiedam, The Netherlands). *P. fluorescens* and *B. licheniformis* were cultured separately in two chemostats until steady-state conditions were obtained [4]. The experiments were performed at pH 7.5 (automatic titration with 0.5 mol l^{-1} HCl and 0.5 mol l^{-1} NaOH) and at a temperature of 25°C. The cultures were stirred at 150 r.p.m. at a dilution rate (D) of 0.05 h^{-1}. Purity was checked on solid medium with Tryptic Soy Broth (TSB) and 1% agar (w/v) enriched with 10 mmol l^{-1} KNO_3. Steady states were reached after 5–8 volume changes when the optical density (O.D.), the cell numbers of *P. fluorescens* and *B. licheniformis*, the electron donor and electron acceptor concentrations remained constant.

2.3. Competition experiments in the chemostat

The competition experiments started when the two steady-state chemostat cultures of *P. fluorescens* and *B. licheniformis* were mixed. Therefore 250-ml cultures were anoxically taken from the steady-state cultures and added to the other chemostat. The cell numbers of *P. fluorescens* and *B. licheniformis* at steady-state conditions determined the mixing ratios of the competition experiments.

The experiments were conducted under (i) electron donor-limited conditions: 2.5 mmol l^{-1} D-glucose+20 mmol l^{-1} nitrate (C/N = 0.75), (ii) electron acceptor-limited conditions: 20 mmol l^{-1} D-glucose+2.5 mmol l^{-1} nitrate (C/N = 48.0) [4]. The C/N ratios were based on the stoichiometric equations, assuming that *P. fluorescens* reduced nitrate entirely to dinitrogen and glucose was oxidised to CO_2. For *B. licheniformis* the assumption was made that glucose was totally oxidised to acetate and CO_2 and nitrate reduced to ammonium.

To our knowledge nothing is known about the actual oxygen concentrations in the rhizosphere of *G. maxima*. However, there is a positive correlation between the photosynthetic activity and the amount of oxygen released in the rhizosphere [12]. Therefore, to determine the effect of different periods of photosynthesis (summer 16 h, winter 8 h) and hence oxygen concentration, competition between the two species was also tested at two different daily cycles of 10% air saturation with the same glucose and nitrate concentration: 10 mmol l^{-1} D-glucose+10 mmol l^{-1} nitrate (C/N = 6.0) [4]. With a C/N ratio of 6.0, nitrate is limited only for the denitrifying strain. The two anoxic steady-state cultures were flushed with air directly after mixing. One chemostat had a regime of 8 h 10% and 16 h 0% air saturation, whereas the other chemostat had a regime of 16 h 10% and 8 h 0% air saturation as measured in the culture. The oxygen concentration was measured with an Ingold oxygen electrode (Ingold, Frankfurt, Germany). Every day samples were taken 4–7 h after the continuous cultures had shifted to 10% air saturation, to determine the O.D. (660 nm), the cell numbers of *P. fluorescens* and *B. licheniformis* and the concentrations of NO_3^-, NO_2^-, NH_4^+, glucose and fatty acids. The competition experiments were continued until stable coexistence was obtained or until one of the strains had washed out.

2.4. Determination of the numbers of P. fluorescens and B. licheniformis

During the competition experiments the numbers

of *P. fluorescens* and *B. licheniformis* were determined daily by Bürker-Türk counting chamber and by plating chemostat samples onto agar (1%, w/v) plates containing TSB medium enriched with 10 mmol l^{-1} KNO_3. The plates were incubated at 20°C and 37°C. At 37°C only *B. licheniformis* was able to grow. The recovery of *B. licheniformis* cells at this temperature was the same as at 20°C. The recovery of *P. fluorescens* and *B. licheniformis* cells determined by plate counts was in the same order of magnitude as the total cell count by Bürker-Türk.

2.5. Analytical methods

The O.D. was measured with a Vitatron MCP (660 nm) (Meyvis Instruments, Bergen op Zoom, The Netherlands). During the competition experiments concentrations of NO_3^-, NO_2^- and NH_4^+ were determined with a Technicon Traacs 800 autoanalyser (Technicon Instruments Corp., Tarrytown, NY, USA). The detection limit for the three compounds was 0.010 mmol l^{-1}. Glucose was determined using a test combination for glucose (Boehringer Mannheim Diagnostica, Mannheim, Germany) based on the GOD-POD method. The detection level was 0.050 mmol l^{-1}. The fatty acids were analysed on a high performance liquid chromatograph (HPLC) (Pharmica Biotech., Roosendaal, The Netherlands). Separation proceeded on an OAHY (Merck) organic acids column, at an eluent flow-rate of 0.6 ml min^{-1} (10 mmol l^{-1} H_2SO_4, 10 μl sample loop-injected, column temperature 60°C). Sample components were identified with the use of internal standards and quantified by simultaneous UV absorption (206 nm) and refraction index (RI) measurements. The detection level for the fatty acids was 0.010 mmol l^{-1}.

3. Results

3.1. Growth characteristics of P. fluorescens and B. licheniformis

The μ_{max} values of *P. fluorescens* and *B. licheniformis* are given in Table 1. The pH of the batch cultures decreased only slightly during the incubation, no more than 0.1–0.2 pH units (data not shown). The results in Table 1 show that under all conditions tested, the μ_{max} of *P. fluorescens* was higher than that of *B. licheniformis*. There was no difference in μ_{max} between 10% and 80% air saturation, indicating excess electron acceptor even at 10% air saturation. *P. fluorescens* was able to utilise acetate with nitrate as electron donor, whereas *B. licheniformis* was able to ferment glucose under anoxic conditions.

The steady-state concentrations of mineral nitrogen, glucose and volatile fatty acids in glucose- or nitrate-limited chemostat mono-cultures of *P. fluorescens* and *B. licheniformis* under continuous anoxic conditions are given in Table 2.

Under glucose-limited conditions (C/N = 0.75) *P. fluorescens* reduced 8.3 mmol l^{-1} nitrate, without the accumulation of nitrite, and a small amount of acetate was produced. Under nitrate-limited conditions (C/N = 6.0), 6.50 mmol l^{-1} glucose remained in the chemostat and there was no nitrate, nitrite or acetate detectable (Table 2). These concentrations agree well with the stoichiometric equation regarding the reduction of nitrate with glucose as carbon source, leaving the endogenous metabolism of *P. fluorescens* out of consideration. The glucose concentration at steady state also under nitrate-limited conditions but with excess glucose (C/N = 48.0) was much lower than expected regarding the above mentioned equation (Table 2). This indicates that glucose

Table 1
Maximum growth rates (μ_{max}) of *Pseudomonas fluorescens* and *Bacillus licheniformis* under different incubation conditions

	μ_{max} (h^{-1})	μ_{max} (h^{-1})	μ_{max} (h^{-1})	μ_{max} (h^{-1})
Electron donor	D-Glucose	D-Glucose	D-Glucose	Acetate
Electron acceptor	O_2	NO_3^-	None	NO_3^-
P. fluorescens	0.36 ± 0.14	0.15 ± 0.01	no growth	0.08 ± 0.01
B. licheniformis	0.24 ± 0.03	0.11 ± 0.01	0.10 ± 0.02	No growth

With nitrate or none electron acceptor the μ_{max} values were determined in batch cultures: initial pH 7.5, temperature 22–25°C. With oxygen as electron acceptor, 10% and 80% air saturation, the μ_{max} values were determined in a pH-stat: pH 7.5, temperature 25°C.

was not completely oxidised to CO_2 and another oxidised organic carbon product must be present. This was indeed the case: with excess glucose *P. fluorescens* produced an unidentified organic carbon product. This product was detectable on the HPLC with RI measurement, whereas with UV absorption the product was not detectable, indicating that the unknown product is not an organic acid. Under these conditions no pyruvate, succinate or formate was detected.

The results showed that *B. licheniformis* reduced more than half of the available NO_3^- only to NO_2^- without the formation of NH_4^+, under glucose-limited conditions (C/N = 0.75) (Table 2). Under this condition the nitrogen balance was almost 100%. With 10 mmol l^{-1} glucose and nitrate (C/N = 6.0), the NO_2^- concentration was low and a slight increase in NH_4^+ was detectable. Under nitrate-limited conditions (C/N = 48.0) the NH_4^+ concentration decreased to 4.65 mmol l^{-1}. Under this condition the culture of *B. licheniformis* contained different organic carbon products: lactate, formate and acetate. The nitrogen balances with C/N ratio of 6.0 and 48.0 were 61% and 44%, respectively (Table 2).

The unknown organic carbon product, present in the *P. fluorescens* culture under nitrate-limited conditions (C/N = 48.0), as well as succinate and pyruvate, were not detectable in the cultures of *B. licheniformis* under any of the conditions applied. Ethanol was present at low concentrations in the *P. fluorescens* and in the *B. licheniformis* cultures, but could not be measured accurately.

3.2. Competition under continuous anoxic glucose-limited conditions

Competition between *P. fluorescens* and *B. licheniformis* under glucose-limited conditions (C/N = 0.75), is presented in Fig. 1. Competition started when 250 ml of *B. licheniformis* culture (2.78×10^8 ml^{-1}) was added to the steady-state culture of *P. fluorescens*, resulting in a mixing ratio between *P. fluorescens* and *B. licheniformis* of 5.41. The cell percentage of *P. fluorescens* decreased sharply after mixing, concomitantly with the accumulation of nitrite in the culture (Fig. 1A,C). After 4 volume changes of the competition, the cell percentage of *P. fluorescens* increased steadily and reached 97–98% of the total cell numbers determined on the TSB plates at the end of the experiment. At the same time, nitrite disappeared again from the culture. There were no organic carbon products detectable; only at the end of the competition experiment 0.75 mmol l^{-1} acetate was measured.

In the chemostat where 250 ml *P. fluorescens* culture (5.34×10^8 ml^{-1}) was added to the steady-state culture of *B. licheniformis*, the competition started at a ratio between *P. fluorescens* and *B. licheniformis* of 0.78 (Fig. 1D–F). When *P. fluorescens* was added the NO_2^- concentration rapidly decreased to zero (Fig. 1D). NO_2^- was probably reduced by *P. fluorescens* with acetate as the carbon source. Acetate decreased from 2.19 mmol l^{-1} to zero in less than 1 volume change and the cell numbers of *P. fluorescens* rapidly increased. When NO_2^- could not be detected any

Table 2
Steady-state concentrations of mineral nitrogen, glucose and volatile fatty acids in glucose-or-nitrate limited cultures of *Pseudomonas fluorescens* and *Bacillus licheniformis* under continuous anoxic conditions (the concentrations are given in mmol l^{-1})

	Incubation conditions			NO_3^-	NO_2^-	NH_4^+	D-Glucose	Acetate	Lactate	Formate
	Glucose	Nitrate	C/N ratio							
Pseudomonas fluorescens	2.5	20	0.75	11.68	0.0	6.26	< 0.05	0.35		
	10	10	6.0	< 0.01	0.0	5.10	6.50	0.0		
	20	2.5	48.0	< 0.01	< 0.01	6.50	7.86	0.0		
Bacillus licheniformis	2.5	20	0.75	9.52	10.64	7.04	< 0.05	2.19	0.00	0.00
	10	10	6.0	0.02	1.06	9.90	< 0.05	6.77	0.00	0.00
	20	2.5	48.0	< 0.01	< 0.01	4.65	< 0.05	7.15	5.28	5.58

The chemostat conditions were: Dilution rate 0.05 h^{-1}, temperature 25°C and pH 7.5. The initial NH_4^+ concentration in the medium was 8.0 mmol l^{-1}.

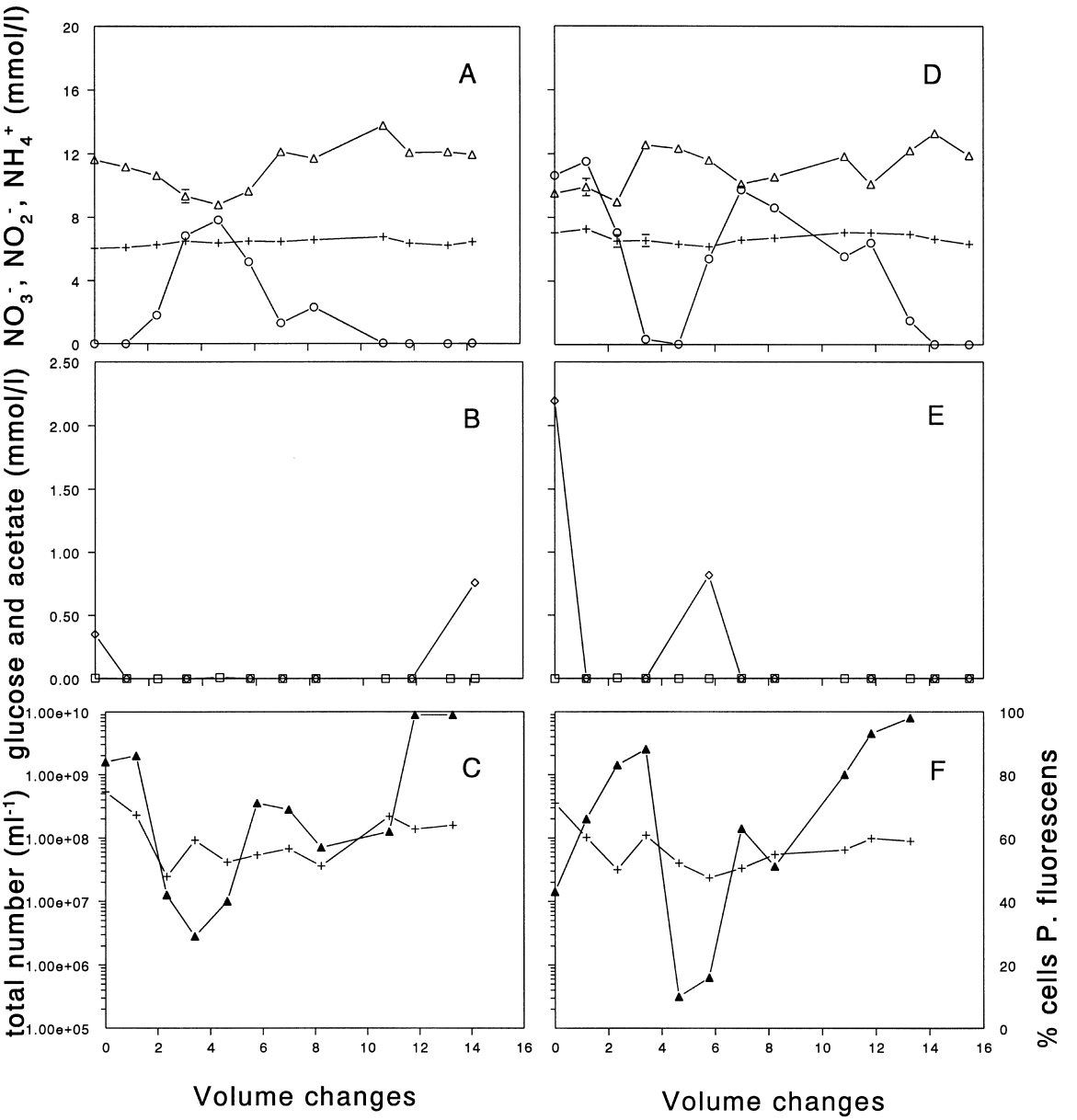

Fig. 1. Competition for glucose between *Pseudomonas fluorescens* and *Bacillus licheniformis* in a glucose-limited chemostat under continuous anoxic conditions. Dilution rate (D) was 0.05 h^{-1}. The medium contained 2.5 mmol l^{-1} D-glucose and 20 mmol l^{-1} nitrate (C/N = 0.75). At the start of the experiment steady-state mono-cultures of both strains were mixed. The data in A–C are the result of adding 250 ml of the *B. licheniformis* culture to the culture of *P. fluorescens*. The competition started with a ratio between *P. fluorescens* and *B. licheniformis* of 5.41. The data in D–F resulted from 250 ml of the *P. fluorescens* culture being added to the culture of *B. licheniformis*. Initial ratio between *P. fluorescens* and *B. licheniformis* cells at the competition was 0.78. Panels A and D present concentrations of nitrate (open triangles), nitrite (open circles), ammonium (crosses); panels B and E present concentrations of glucose (open squares) and acetate (open diamonds); and panels C and F present total numbers of *Pseudomonas fluorescens* plus *Bacillus licheniformis* determined by plate counts (crosses) and the percentage cell counts of *Pseudomonas fluorescens* compared to the total plate counts (closed triangles).

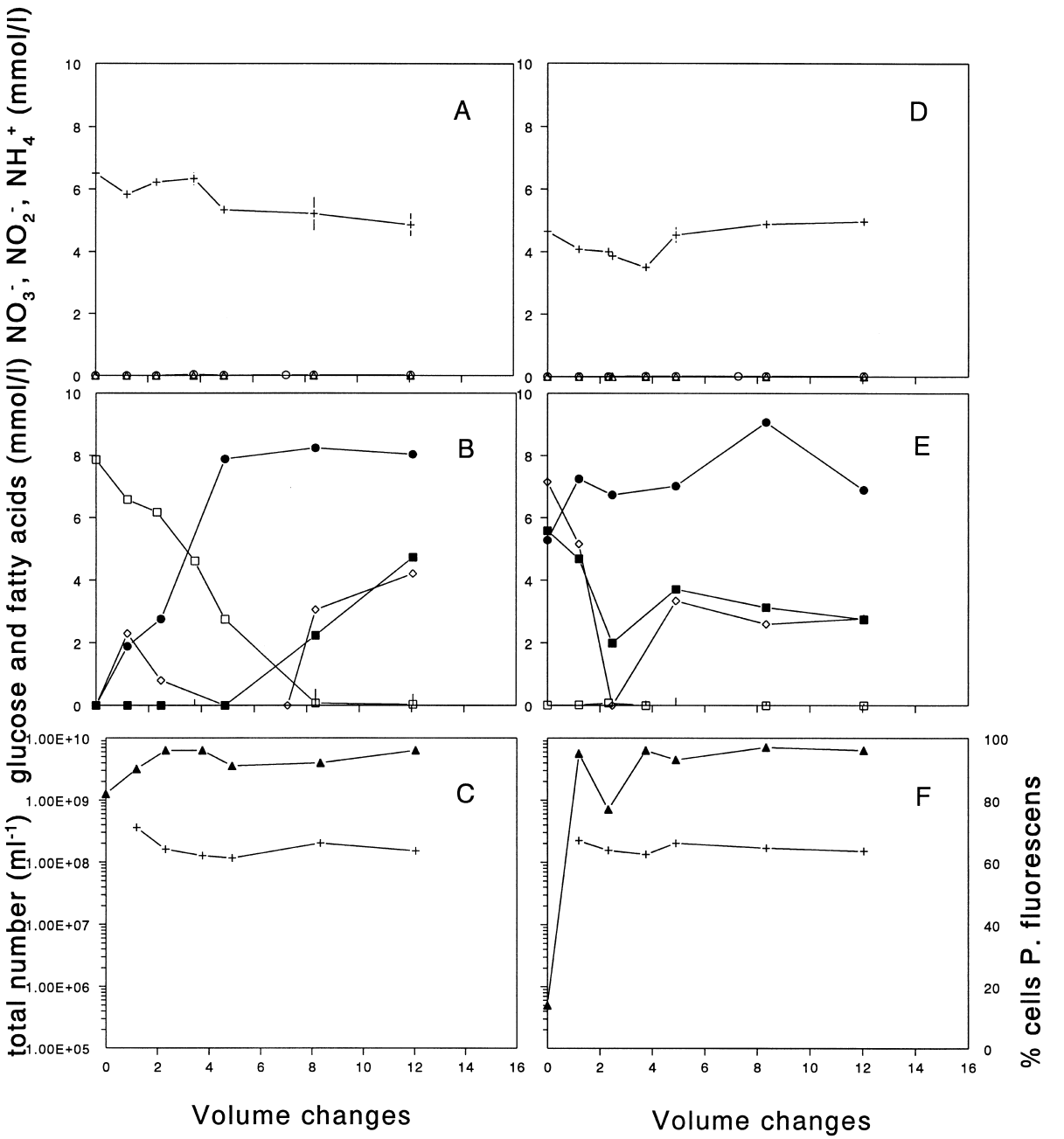

Fig. 2. Competition for nitrate between *Pseudomonas fluorescens* and *Bacillus licheniformis* in a nitrate-limited chemostat under continuous anoxic conditions. Dilution rate (D) was 0.05 h^{-1}. The medium contained 20 mmol l^{-1} D-glucose and 2.5 mmol l^{-1} nitrate (C/N = 48.0). The data in A–C are the result of adding 250 ml of the *B. licheniformis* culture to the culture of *P. fluorescens*. The competition started with a ratio between *P. fluorescens* and *B. licheniformis* of 4.67. The data in D–F resulted from 250 ml of the *P. fluorescens* culture being added to the culture of *B. licheniformis*. Initial ratio between *P. fluorescens* and *B. licheniformis* cells at the competition was 0.16. Panels A and D present concentrations of nitrate (open triangles), nitrite (open circles) and ammonium (crosses); panels B and E present glucose (open squares), acetate (open diamonds), formate (closed squares) and lactate (closed circles); and panels C and E present total numbers of *Pseudomonas fluorescens* plus *Bacillus licheniformis* determined by plate counts (crosses) and the percentage cell counts of *Pseudomonas fluorescens* compared to the total plate counts (closed triangles).

longer, the cell percentage of *P. fluorescens* decreased while the acetate and NO_2^- concentrations increased again. After 6 volume changes, the number of *P. fluorescens* started gradually to increase, acetate decreased again rapidly and the NO_2^- concentration slowly decreased until it was not detectable after 14 volume changes. In addition under this mixing regime *P. fluorescens* out-competed *B. licheniformis*, comprising 98–99% of the total number of colonies determined on the plates at the end of the experiment.

3.3. Competition under continuous anoxic nitrate-limited conditions

The competition experiment with glucose (20 mmol l^{-1}) and nitrate (2.5 mmol l^{-1}), with nitrate being the limited substrate for both strains (C/N = 48.0), is presented in Fig. 2.

Competition started when *B. licheniformis* was added (7.46×10^8 ml^{-1}) to the steady-state culture of *P. fluorescens*, resulting in a mixing ratio between *P. fluorescens* and *B. licheniformis* of 4.67 (Fig. 2A–C). During competition the glucose concentration decreased to zero over a time period of 8.2 volume changes, whereas the concentration of lactate gradually increased to 8 mmol l^{-1}. The formate and acetate concentrations were 4.5 mmol l^{-1} at the end of the experiment. There were no strong fluctuations in cell numbers of *P. fluorescens* or *B. licheniformis*. The cell percentage of *P. fluorescens* reached a constant level of 96% of the total plate counts. *B. licheniformis* remained constant at around 4%. The competition resulted in coexistence, although the number of the fermentative *B. licheniformis* strain was rather low, i.e. 1×10^7 cells ml^{-1}. Apparently, the remaining glucose was fermented by *B. licheniformis* to lactate, formate and acetate. The unknown organic carbon product present during steady-state in the pure culture of *P. fluorescens* was not detectable any more after 2 volume changes; this product was apparently fermented by *B. licheniformis* (data not shown).

In the other competition experiment, a 250-ml culture of *P. fluorescens* (5.74×10^8 ml^{-1}) was added to the *B. licheniformis* culture, resulting in a initial competition ratio between *P. fluorescens* and *B. licheniformis* of 0.16 (Fig. 2D–F). The concentrations of acetate and formate decreased and the number of *P. fluorescens* rapidly increased during the first 2.5 volume changes. The lactate concentrations slightly fluctuated but overall remained the same. The formate and acetate concentration stabilised around 2.7 mmol l^{-1}. Also, in this experiment the percentage of the denitrifying *P. fluorescens* reached a constant level of 96%. *B. licheniformis* remained in the chemostat by fermenting the remaining glucose.

3.4. Competition under fluctuating oxic-anoxic conditions

The competition experiment with glucose (10 mmol l^{-1}) and nitrate (10 mmol l^{-1}) (C/N = 6.0) under fluctuating oxic conditions is presented in Fig. 3.

B. licheniformis was added to the *P. fluorescens* culture, resulting in a cell ratio of *P. fluorescens*/*B. licheniformis* of 2.24 with an oxygen regime of 8 h 10% and 16 h 0% air saturation (Fig. 3A–C). In the chemostat where *P. fluorescens* was added to the *B. licheniformis* culture the initial cell ratio of *P. fluorescens*/*B. licheniformis* was 0.20, with an oxygen regime of 16 h 10% air saturation and 8 h 0% (Fig. 3D–F). The samples were all taken in a period 4–7 h after the chemostats shifted to 10% air saturation, since the time of sampling during these competition experiments was critical. The competition with 8 h 10% air saturation resulted in coexistence, with *P. fluorescens* comprising 96% of the total population. Under this competition condition, nitrate was limited for *P. fluorescens* and despite the regime of 8 h 10% air saturation, a part of the glucose must have been available for *B. licheniformis*.

In the experiment with 16 h 10% air saturation (Fig. 3D–F), *P. fluorescens* oxidised all the available acetate prior to 2 volume changes. The number of *P. fluorescens* increased and no glucose remained for *B. licheniformis*. This competition experiment resulted in a wash-out of *B. licheniformis*.

4. Discussion

The fermentation products of *B. licheniformis* with a dilution rate of 0.05 h^{-1} were acetate, lactate, formate, ethanol and CO_2. The fermentation balance of

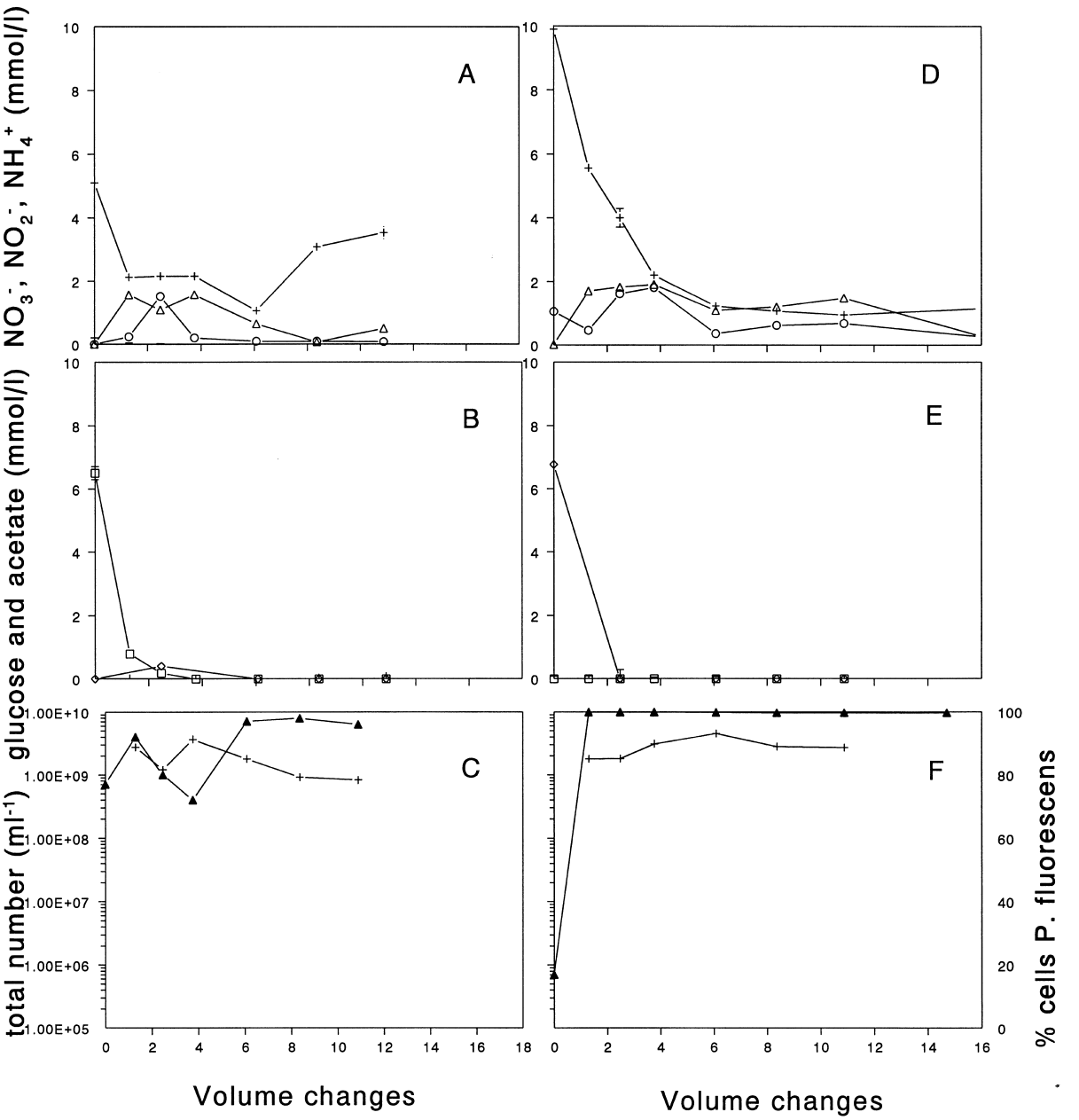

Fig. 3. Competition between *Pseudomonas fluorescens* and *Bacillus licheniformis* in a chemostat under fluctuating oxic-anoxic conditions. Dilution rate (D) was 0.05 h^{-1}. The medium contained 10 mmol l^{-1} D-glucose and 10 mmol l^{-1} nitrate (C/N = 6.0). The data in A–C are the result of adding 250 ml of the *B. licheniformis* culture to the culture of *P. fluorescens*. The competition started with a ratio between *P. fluorescens* and *B. licheniformis* of 2.24. The data in D–F resulted from 250 ml of the *P. fluorescens* culture being added to the culture of *B. licheniformis*. Initial ratio between *P. fluorescens* and *B. licheniformis* cells at the competition was 0.13. The competition experiments were performed with an oxygen regime of 8 h 10% and 16 h 0% air saturation (A–C) and with a regime of 16 h 10% and 8 h 0% air saturation (D–F). Panels A and D present concentrations of nitrate (open triangles), nitrite (open circles), ammonium (crosses); panels B and E present concentrations of glucose (open squares) and acetate (open diamonds); and panels C and F present total numbers of *Pseudomonas fluorescens* plus *Bacillus licheniformis* determined by plate counts (crosses) and the percentage cell counts of *Pseudomonas fluorescens* compared to the total plate counts (closed triangles).

B. licheniformis with glucose as carbon source is dependent on the growth rate [17].

The competition experiment under glucose-limited conditions (C/N = 0.75) resulted in a wash-out of *B. licheniformis* (Fig. 1C,F). There was no NH_4^+ production, and at least at the end of the competition no NO_2^- accumulation. The high nitrite concentration at the beginning of the competition experiment with a ratio of *P. fluorescens*/*B. licheniformis* of 0.78 (Fig. 1D) had been caused by *B. licheniformis*, reducing nitrate only to nitrite without accumulation of ammonium. The addition of *P. fluorescens* led to a linear decrease of the formed nitrite from the beginning of the competition experiment.

During these competition experiments there was in both chemostats an unexpected temporary NO_2^- accumulation (Fig. 1A,D) and, at the same time, a sharp decrease in cell numbers of *P. fluorescens* (Fig. 1C,F). The different rates of nitrate and nitrite reduction by *P. fluorescens* or the competition between nitrate and nitrite reduction pathways for electrons [18,19] may account for a part of the NO_2^- accumulation. However, the explanation for the NO_2^- accumulation remains incomplete.

The competition experiments under nitrate-limited conditions (C/N = 48.0) (Fig. 2) resulted in coexistence of both species, although the cell numbers of *B. licheniformis* were very low: 4% of the total numbers of cells determined by plate counts. *B. licheniformis* was apparently dependent on the fermentation of glucose. The reason for the low number of *B. licheniformis* in these competition experiments, despite the high concentration of glucose available, is not known.

The following conclusions can be drawn regarding the competition experiments that were performed under continuous anoxic conditions with glucose or nitrate limitation. Under glucose-limited conditions (C/N = 0.75) the competition resulted in the wash-out of *B. licheniformis*, indicating that the affinity for glucose is higher for *P. fluorescens* than for *B. licheniformis*. Under nitrate-limited conditions (C/N = 48.0) *P. fluorescens* also wins the competition for nitrate, but *B. licheniformis* is able to maintain itself in the chemostat due to the possibility to ferment the remaining glucose. Under the tested conditions *P. fluorescens* has also a higher affinity for nitrate than *B. licheniformis*. These conclusions are in agreement with other experiments. Enrichments in the chemostat under anoxic conditions with a dilution rate of 0.05 h^{-1} with glycerol as carbon source, in the presence of limiting amounts of nitrate, resulted in coexistence of fermentative enterobacteria and bacteria with an oxidative metabolism [20]. Fermentative bacteria were only able to maintain high numbers when the supply of oxygen or nitrate was limited. The conclusion was drawn that oxidative bacteria such as pseudomonads and acinetobacteria were better competitors for limiting amounts of electron acceptors.

The results of the competition under fluctuating anoxic conditions, 8 h 10% and 16 h 0% air saturation (Fig. 3A–C) to 16 h 10% and 8 h 0% air saturation (Fig. 3D–F), showed that the numbers of *P. fluorescens* cells contributed for 96% and 100% of the total number of bacteria determined by plate counts, respectively. This indicates that with longer oxic periods, *P. fluorescens* utilised all the available glucose with nitrate or oxygen as electron acceptor. With shorter oxic periods *B. licheniformis* was able to maintain itself, probably due to fermentation of the remaining glucose during anoxic periods as the nitrate concentration was limiting under these conditions for *P. fluorescens*.

The results showed that *P. fluorescens* was able to utilise nitrate and oxygen simultaneously, since the samples taken during the periods of 10% air saturation contained low to not detectable nitrate concentrations. However, anoxic niches could have been formed in the chemostat during the 10% air saturation periods, since stirring activity might have been too low [20].

Other studies have shown that the synthesis and activity of the reductases involved by the reduction of nitrate may occur under different oxygen concentrations. The mechanism of oxygen inhibition or repression of denitrification appears to differ for each reductase and denitrifying species [21–25]. There are two types of nitrite reductase found in denitrifiers, an oxygen tolerant cytochrome cd_1 nitrite reductase and an oxygen sensitive Cu-containing nitrite reductase [26]. It is the general opinion that the Cu-type nitrite reductase cannot be active in intact cells in the presence of oxygen [21]. It is likely that *P. fluorescens* contains a cytochrome cd_1 nitrite reductase [27].

Based on our results we can attempt to describe the dissimilatory processes related to nitrate in the rhizosphere of aerenchymatous plants and to discuss this in relation to the hypothesis formulated. The results of this study showed that *P. fluorescens* had a higher affinity for both glucose and nitrate than *B. licheniformis*. *B. licheniformis* was only able to maintain itself in the chemostat under glucose excess by fermenting the remaining glucose. Assuming that carbon is in excess and the electron-acceptor concentrations are limiting, as is often the case in the rhizosphere of waterlogged plants, fermentative bacteria are able to grow and thereby increasing their biomass. *B. licheniformis* produces nitrate reductase in the presence and absence of nitrate [28]. By oxygen release in the rhizosphere of aerenchymatous plants, nitrification might take place [9] and the nitrate produced might be reduced to either ammonium or dinitrogen. Initially, the nitrate reduction pathway to be taken depends largely on the biomass of the two dissimilatory nitrate-reducing subcommunities present. After a relative long period of anoxic conditions, most available nitrate will be reduced to NH_4^+ due to the larger active biomass of the fermentative bacteria [11,14]. When the period of nitrate availability increases, the oxidative denitrifying strains will dominate, since the denitrifying strains apparently have a higher affinity for nitrate and a higher growth rate than fermentative bacteria. The results of our experiments support the above mentioned processes, however, under glucose-limited conditions (C/N = 0.75) (Table 2 and Fig. 1D) *B. licheniformis* reduced nitrate only to nitrite instead of ammonium. Under these circumstances there was no reason to use nitrite as electron sink as the reduction of nitrate was the energy providing step [2,18].

The rhizosphere of aerenchymatous plants is a very heterogeneous environment due to the release of oxygen and different organic carbon compounds. Around the roots there may be a zone shifting between aerobic processes during the day and anaerobic processes during the night [5]. In nature the competition between denitrifying and nitrate-ammonifying bacteria for nitrate and organic carbon can also be controlled by other factors than affinities and growth rates. Therefore, in future more research is necessary to examine the competition between denitrifying and fermentative nitrate-reducing bacteria in the rhizosphere of aerenchymatous plants.

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