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Mixed Culture Hydrogenotrophic Nitrate Reduction in Drinking Water

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Abstract. Isolation and identification of the bacteria from a hydrogenotrophic reactor for the denitrification of drinking water revealed that several microorganisms are involved. *Acinetobacter* sp., *Aeromonas* sp., *Pseudomonas* sp. and *Shewanella putrefaciens* were repeatedly isolated from the hydrogenotrophic sludge and postulated to be of primary importance in the process. Nitrate reduction to nitrite appears to be a property of a diverse group of organisms. Nitrite reduction was found to be stimulated by the presence of organic growth factors. Thus, in a mixed culture, hydrogenotrophic denitrification reactor, NO_2^- formed by NO_3^--reducers can be converted by true denitrifiers thriving on organic growth factors either present in the raw water, or excreted by the microbial community. Mixotrophic growth also contributes to NO_2^- reduction. Finally, chemolithotrophic bacteria participate in the nitrite to nitrogen gas conversion.

Introduction

Hydrogen metabolism is widely distributed among various well-described physiological groups of bacteria, such as the methanogenic and acetogenic bacteria [49], the phototrophic bacteria [38, 48], the sulfate-reducers [69, 70], the organotrophic fermentative bacteria [15, 28], or the N_2-fixing bacteria [50]. Hydrogen is also reported to be a driving mechanism for the microbial degradation of micropollutants, such as atrazine, in soils and drinking water [67].

The aerobic hydrogen bacteria belong to different taxonomic groups. They are characterized by the ability to obtain energy by oxidation of gaseous hydrogen via an electron transport chain and concomitant synthesis of cell material by the reductive assimilation of CO_2 via the Calvin cycle, i.e. to grow chemolithoautotrophically [2]. They are all facultative autotrophs with one exception, *Hydrogenobacter thermophilus*, which is an obligate autotroph [10]. The hydrogen bac-

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teria all show a versatile metabolism; many of them, especially those regarded as *Pseudomonas* and *Alcaligenes*, are characterized by the ability to use a wide spectrum of carbon substrates [10].

Anaerobic growth on hydrogen is associated with dissimilatory nitrate reduction and confined to only a few species [10]. Two different pathways of dissimilatory nitrate reduction can be distinguished. In the first, nitrate is converted to gaseous products such as N_2 or N_2O, a process called denitrification. In the second, nitrate is converted to NH_4^+ [14, 59]. So far, only *Paracoccus denitrificans* and closely related strains have been found to be able to denitrify nitrate, nitrite, or nitrous oxide under hydrogenotrophic conditions [2, 4, 34]. The hydrogen oxidizers *Alcaligenes eutrophus* and *Hydrogenophaga* (*Pseudomonas*) *pseudoflava* are able to grow heterotrophically as denitrifiers with nitrate as an electron acceptor. Growth under autotrophic anaerobic conditions with nitrate, however, is minimal or absent in these species [2]. Hydrogen is also a potential electron donor for dissimilatory nitrate reduction to ammonium, a process first observed in the highly reducing environment of the rumen [22, 31, 37] and later in representatives of the genera *Clostridium* [65], *Desulfovibrio* [6], *Wolinella* [11] and *Campylobacter* [60].

Paracoccus denitrificans is one of the most intensively studied denitrifying microorganisms due to its nutritional versatility [59, 65]. Its well-known capability to reduce nitrogenous compounds with hydrogen is at the basis of several new approaches for denitrification of drinking water [20, 26, 35, 39, 58]. The concentration of nitrate in ground and surface water shows an increasing trend. The use of hydrogen as a reductant for denitrification is currently under investigation in a 1.0 $m^3\ h^{-1}$ pilot plant on a water production center fed from a storage reservoir with nitrate-rich surface water [39]. The hydrogenotrophic treatment concept offers a number of important benefits, such as food-grade quality of the reductant, process reliability, low excess sludge production and no need for intensive monitoring or biological post-treatment to control and remove residual reductant [20, 26, 39].

In-depth insight about the hydrogenotrophic reduction of oxidized nitrogen compounds relates to only a few organisms [59]. To our knowledge, this is the first report about the ecology, stability, and dynamics of a mixed culture, hydrogen consuming denitrification system. To study the bacterial populations of the pilot-reactor, microorganisms were enriched and purified from the in-situ biomass and the reactor effluent under different conditions. Isolates have been tentatively identified and the importance of hydrogen as a microbial reductant and energy source has been assessed.

Methods

Sampling Procedure

A simplified scheme of the hydrogenotrophic denitrification system is shown in Fig. 1. The process–technological aspects have been described elsewhere [39]. A mixture of H_2 and CO_2 (97.5 : 2.5) was recirculated and diffused from the bottom of the left column, in counter-current with the nitrate-rich surface water. The main denitrifying activity occurred in this part of the plant. The water subsequently entered an upflow column where, in the bottom part, the residual dissolved H_2 was

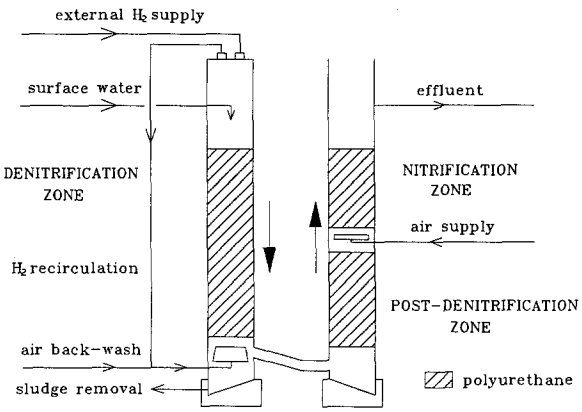


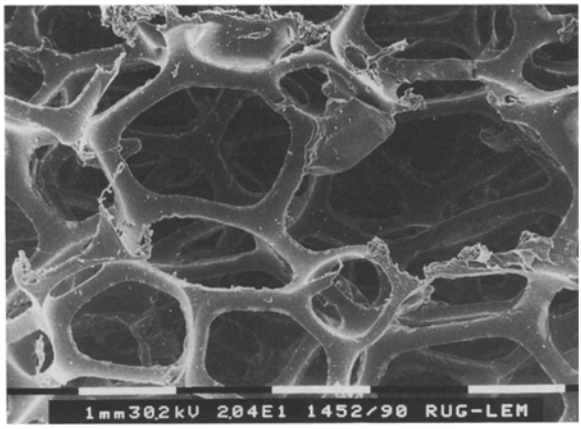
Fig. 1. Schematic diagram of the hydrogenotrophic denitrification plant. (The columns had a diameter of 0.30 m and a height of 4.05 m.)

removed. In the upper zone, the water was reaerated and potential traces of intermediate nitrogenous compounds, such as nitrite, were reoxidized to nitrate. Lamellar reticulated polyurethane with a mean porosity of 20 pores per inch (Fig. 2a) was used as carrier in all parts of the plant. During steady-state operation, samples were taken from the colonized matrix (Fig. 2b, c). In addition, homogenized samples were taken from the final effluent and from the sedimented hydrogenotrophic sludge periodically removed by back-washing of the system.

Microbial Enumeration Techniques

Water samples were taken weekly from the influent and the effluent of the plant and, after dilution, spread onto solid culture media. The best suitable medium for enumeration of total heterotrophic colony-forming units was determined on the basis of a separate study [40]. Lab-Lemco (Oxoid; 1 g liter^{-1}) agar (15 g liter^{-1}) amended with KNO_3 (1 g liter^{-1}) and incubated at 28°C during 3 days gave the highest recovery. The denitrifying population was enumerated after incubation under anaerobic conditions. For the enumeration of total hydrogenotrophic NO_3^- reducing organisms, the following mineral medium was used (in g liter^{-1}) : $FeSO_4 \cdot 7H_2O$, 0.1; Na^{2-} EDTA, 0.14; $MgSO_4 \cdot 7H_2O$, 0.5; $CaCl_2 \cdot 2H_2O$, 0.01; NH_4Cl, 1.0; KNO_3, 1.0; $Na_2HPO_4 \cdot 2H_20$, 4.5; KH_2PO_4, 1.5; $NaHCO_3$, 0.5; agar, 15; trace elements, 1 ml stock solution. The latter contained (in mg 100 ml^{-1}) : Na^{2-} EDTA, 500; $NiSO_4 \cdot 6H_2O$, 450; $MnCl_2 \cdot 4H_2O$, 50; $ZnSO_4 \cdot 7H_2O$, 10; $CuSO_4 \cdot 5H_2O$, 3; $Na_2MoO_4 \cdot 2H_2O$, 5; $CoCl_2 \cdot 6H_2O$, 5; H_3BO_4, 10; $Na_2SiO_3 \cdot 9H_2O$, 2; $AlCl_3 \cdot 6H_2O$, 5; $Na_2SeO_3 \cdot 6H_2O$, 2. The pH of the medium was adjusted to 7.0 with dilute HCl. The inoculated plates were transferred into anaerobic jars with H_2 : CO_2 (80:20) and incubated at 28°C for 10 days. All counts were performed in duplicate.

The means of influent and effluent bacterial counts were tested statistically with a t-test.



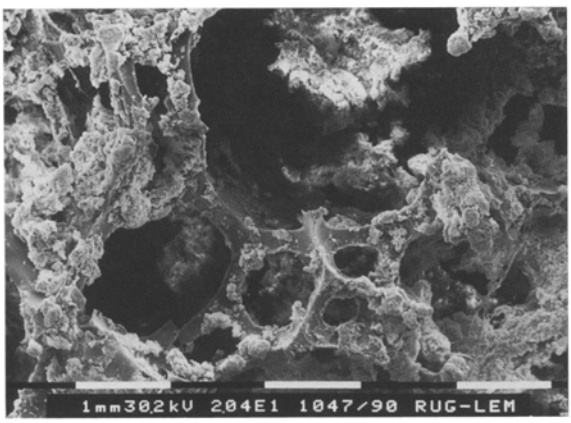


Fig. 2. Scanning electron micrographs of uncolonized (a) and colonized (b, c) polyurethane carrier. (a, b) bar = 1 mm; (c) bar = 10 µm.

Strain Isolation and Identification

Samples were taken from the reactor biofilm under mesotrophic (±20°C) and psychrotrophic (<10°C) operating conditions to evaluate the effect of temperature on the composition of the microbial community. The samples were diluted and treated as water samples for further enrichment or direct plating. Mineral agar medium was inoculated with 100 µl of sample and incubated under a $H_2 : CO_2$ (80:20) atmosphere. Bacterial colonies from these plates were repeatedly transferred and incubated under analogous conditions. Enrichment cultures were obtained in a continuously stirred and controlled fermentation vessel (300 ml). The basic medium consisted of (in g liter^{-1}) : KH_2PO_4, 0.6; K_2HPO_4, 1.6; $MgSO_4 \cdot 7H_2O$, 0.8; trace element solution [61], 4 ml and H_2SO_4, 1.25 ml. The medium was amended with 0.2% KNO_3 and 0.5% acetate (heterotrophic) or carbonate (autotrophic) and continuously gassed with H_2/CO_2 (90/10) under controlled pH conditions (7.0). The heterotrophic and autotrophic enrichment cultures were both plated on mineral agar medium and enumerated after 5 and 10 days, respectively (autotrophic growth was slower). Based on morphology and cell shape, representatives of each colony type were picked and repeatedly transferred to solid and to liquid mineral medium. All cultures were incubated anaerobically at their respective isolation temperature (mesotrophic or psychrotrophic).

Some of the purified strains have been identified based on API 20E and 20NE tests (API, La Balme-les-Grottes, France), gas chromatographic fatty acid analysis [44, 56], polyacrylamide gel electrophoresis (SDS-PAGE) of whole cell proteins [32, 33], DNA-rRNA hybridizations [16, 18], and % guanine-cytosine determination of their DNA [17].

On other occasions samples were taken from the reactor effluent and processed under mesotrophic conditions as described above, except that the isolated strains were not further identified. These strains were tentatively designated with strain numbers.

Nitrate-Nitrite Reduction Assay

The capacity of the purified strains to reduce NO_3^- and/or NO_2^- was tested under autotrophic, heterotrophic, and mixotrophic conditions in 10-ml liquid Durham broth tubes. Heterotrophic and hydrogenotrophic NO_3^- reduction were tested by transferring pure cultures from the initial solid mineral medium into Nitrate broth (Difco) and liquid mineral medium, respectively. Mixotrophic NO_3^- reduction was examined in liquid mineral medium amended with 50 mg liter^{-1} yeast extract or 62.5 mg liter^{-1} glucose. Hydrogenotrophic NO_2^- reduction was tested in liquid mineral medium without KNO_3, but amended with 0.50 or 0.15 g liter^{-1} KNO_2. All were incubated at 28°C. Psychrotrophic isolates were tested at 10°C. Nitrate broth was incubated microaerophilically while all autotrophic and mixotrophic tests were incubated on a shaker under a H_2/CO_2 atmosphere. All experiments were repeated twice using different subcultures. The broth was tested for NO_3^-, NO_2^-, and NH_4^+. Gas-producing strains were presumed to be denitrifiers and confirmed for NO_3^- reduction in a gas-tight vessel. After incubation the gases were analyzed by gas chromatography. Reference tests were performed with *Paracoccus denitrif-*

icans LMG 4218 and *Alcaligenes eutrophus* LMG 1196. Nitrate, nitrite, and ammonium were determined qualitatively and, in some cases, also quantitatively using a Technicon Autoanalyzer equipped with a cadmium reduction column.

Hydrophobicity Test

Hydrophobicity was assessed by the affinity to adhere to octane as previously described [54]. After growth in liquid medium, cells were harvested by centrifugation at 10,000 *g* for 10 min. The cell pellet was washed twice in phosphate buffered saline (in g liter^{-1}: NaCl, 8; KH_2PO_4, 0.34; K_2HPO_4, 1.21) and centrifuged again. Finally, the cells were resuspended in the same buffer to an optical density of 0.60 ± 0.02 at 600 nm using a double beam spectrophotometer (Shimadzu UV 190). To 5-ml aliquots of the cell suspensions, 0.5 ml octane was added. The mixture was vortexed for 1 min and left for 10 min to allow complete phase separation. The aqueous phase was transferred to 4-ml cuvettes and the optical density of treated and untreated (without octane) cell suspensions was measured at 600 nm. Hydrophobicity was calculated as the relative percentage of bacteria removed from the aqueous phase. Tests were performed in duplicate.

Assimilable Organic Carbon Determination

Assimilable organic carbon (AOC) is that portion of the total organic carbon (TOC) which can readily be converted to cell mass. It is expressed as a carbon concentration by means of a conversion factor or calibration [29]. In a first approach, the AOC was determined by the method of Van der Kooij [62, 64, 63]. The maximum level of growth, expressed as the maximum colony count Nmax, was measured after inoculation of a representative water sample with *Pseudomonas fluorescens* strain P17 and *Spirillum* species strain Nox. The original method of Van der Kooij was slightly modified. Previous research has shown that prior to inoculation, pasteurization was ineffective to kill the surface water biota. Therefore, the indigenous bacteria were eliminated from the sample by membrane-filtration of 250 ml with pre-washed 0.22 μm Durapore (Millipore) membrane filters. This implies that only the dissolved fraction of the AOC was determined. Static incubation was performed at 28°C instead of the prescribed 15°C. From the Nmax values, AOC concentrations were calculated with the yield coefficient of the test organisms for acetate. An alternative method applied was the modified method of Werner [27, 66], where the membrane-filtered water sample (250 ml) was inoculated with a protozoa-free suspension of indigenous organisms. During incubation at 28°C on a shaker, the growth curve of the mixed culture was determined. Total heterotrophic plate counts were performed on Lab-Lemco (Oxoid; 8 g liter^{-1}) agar (15 g liter^{-1}) plates amended with KNO_3 (1 g liter^{-1}) and incubated during 4 days at 28°C. The results were plotted as mean log colony-forming units per ml versus incubation time. The slope (μ) is an indication of the substrate biodegradability while the growth factor $f = \log (y/y_o)$ indicates the substrate quantity with y and y_o as the maximum and initial cell density. All AOC measurements were performed in duplicate. Bacterial counts were done in triplicate. Further details of the experimental procedure are described by Liessens et al. [40].

Results

Colonization Phenomena

The polyurethane matrix was colonized with a dense biofilm in a time period of about one month (Fig. 2b, c).

The importance of NO_2^- as a key intermediate in the hydrogenotrophic denitrification process was apparent during the first start-up period of the plant (Fig. 3A). Nitrate was rapidly reduced but nearly quantitatively converted to NO_2^-. Continuous operation gradually decreased the NO_2^- concentration until normal operational conditions were reached (day 20). This phenomenon was also observed in laboratory reactors (data not shown). After one year of operation, the reactor was emptied and the carrier material was washed and dried until no viable biomass remained on the polyurethane. After finishing the cleaning procedure, a thin layer of dried biomass remained on the matrix. The carrier was mounted into the columns and the reactor was restarted. During this period, however, no NO_2^- accumulation was observed (Fig. 3B). The latter results suggest that the residual dead biomass served as supplementary carbon and/or energy source for the nitrite-reducing hydrogenotrophs.

The possible relation between NO_2^- reduction and organic carbon was evaluated by determination of the assimilable organic carbon (AOC) in the influent and effluent of the main hydrogenotrophic denitrification column. Parallel research has shown that for a heterotrophic denitrification system, soluble microbial products excreted by the dense microbial population cause a significant increase in dissolved AOC in the effluent [40]. From the results in Table 1 and Fig. 4, there is no indication that the dissolved AOC concentration increases throughout hydrogenotrophic treatment, which may indicate a consumption of the in-reactor excreted microbial products.

Hydrogen as a Microbial Reductant

The importance of H_2 as reductant in the process is shown in Fig. 5. Interruption of the H_2 supply gradually increased the effluent nitrate to a level of 79% of the influent value. The NO_2^- concentration did not increase throughout this experiment. The remaining observed activity indicates that heterotrophic microorganisms are also involved.

The importance of H_2 is also reflected by the relative amount of potential hydrogenotrophic NO_3^- reducers on the total heterotrophic population (Fig. 6). The data show that hydrogenotrophic microorganisms are already present in the incoming water and comprise an important part of the autochtonous NO_3^- reducers. In the reactor effluent, for a series of 12 measurements, there was an average change in heterotrophs, heterotrophic NO_3^- reducers, and hydrogenotrophic NO_3^- reducers of -0.1, $+0.5$ and $+0.8$ log CFU ml^{-1}, respectively. For the total heterotrophs and the total heterotrophic NO_3^--reducing population, only six (sample numbers 2, 4, 7, 8, 9, 12) and four (sample numbers 1, 2, 6, 7), respectively, effluent values were significantly different from the influent value at a level of 0.05. On the other hand, all hydrogenotrophic counts showed significant differences between influent and effluent. These data suggest a slight growth of the latter group in the reactor system.

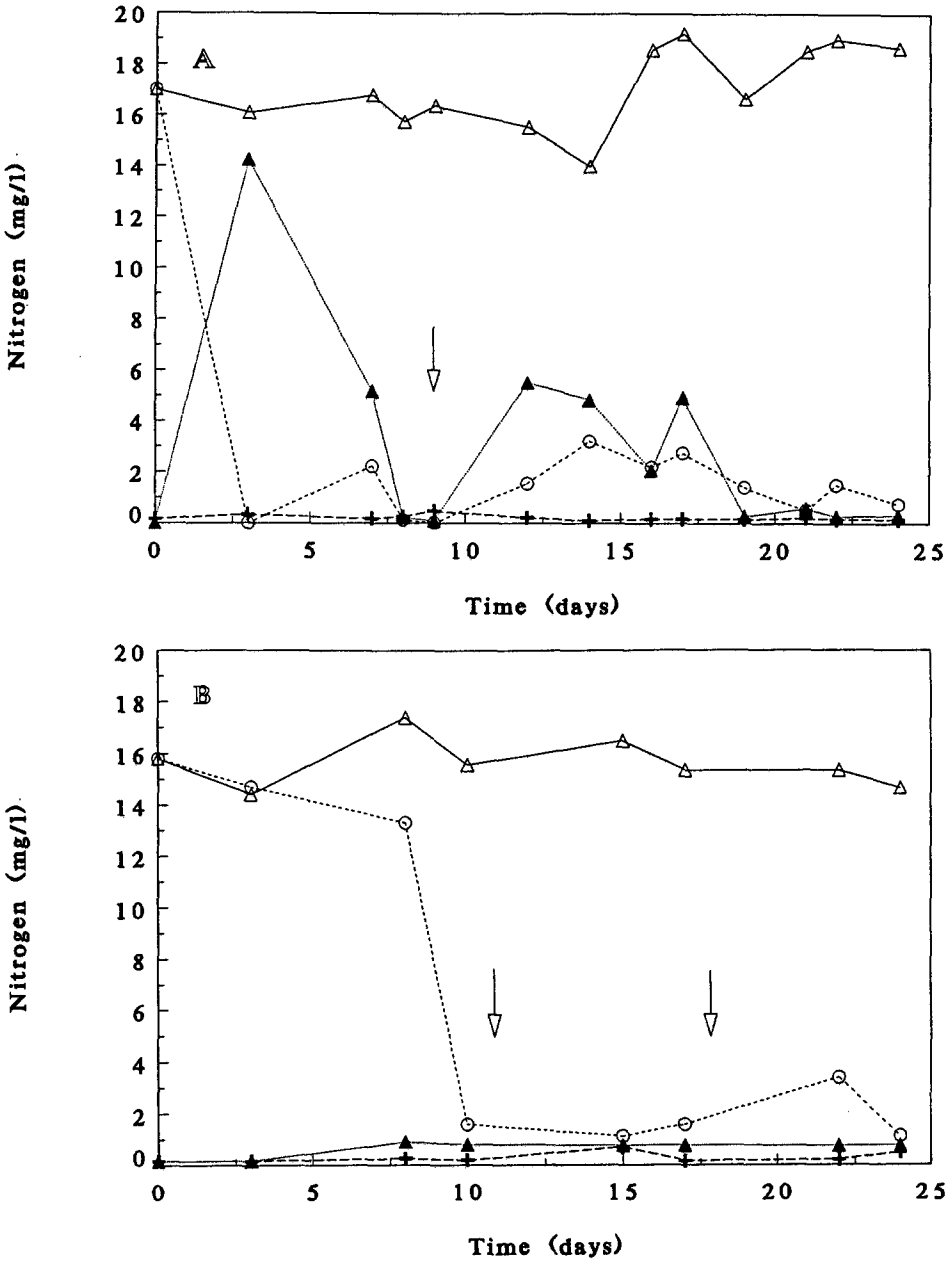


Fig. 3. Effluent characteristics of the reactor during first (A) and second (B) start-up. The symbols are as follows: —△—, NO_3^--N influent; ····⊙···, NO_3^--N effluent; – – + – –, NO_2^--N influent; —▲—, NO_2^--N effluent.

Table 1. Dissolved AOC[a] in the treatment plant influent and effluent according to the modified Van der Kooij method (in μg acetate-C eq liter^{-1})

Date of analysis	Influent	Effluent
March 19, 1991	283 ± 36	279 ± 16
March 25, 1991	11 ± 4	7.5 ± 0.5

[a] Arithmetic means of duplicate analyses with indication of the lowest and highest value

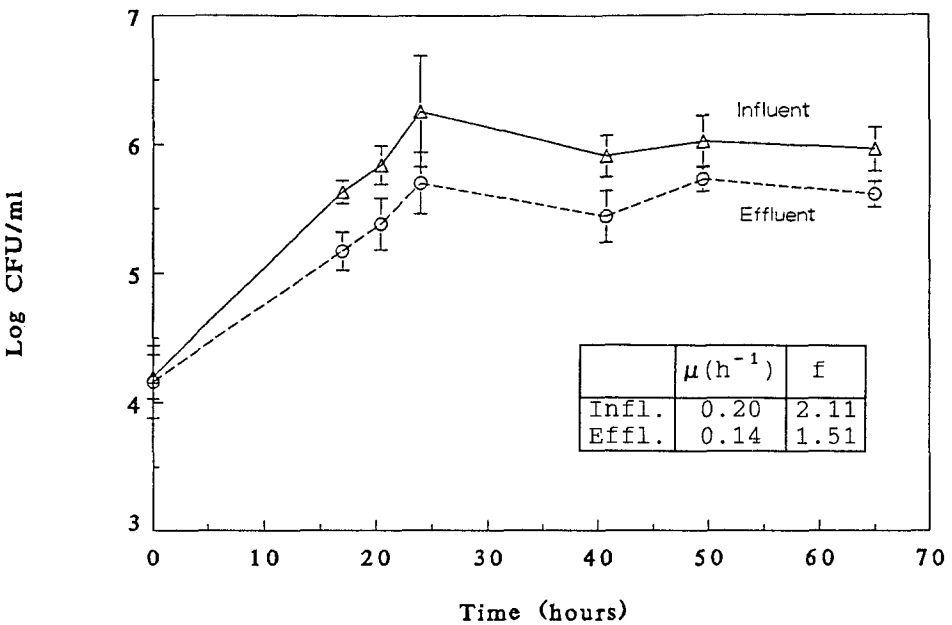


Fig. 4. Growth curve of a natural inoculum in the influent and effluent of the treatment plant according to the modified Werner method. Data are geometric means ± SD of triplicate bacterial counts. Indication is given of the growth rate μ and the growth factor f.

Isolation and Identification

The isolation procedure, starting from reactor biofilms, yielded 136 pure cultures. Based on whole-cell PAGE protein patterns, 31 mesotrophic (summer) and 54 psychrotrophic (winter) different primarily Gram-negative strains were obtained. Table 2 gives an overview of their identification. More information on the identified microorganisms in relation to the isolation procedure and identification methods applied, is given by Vanbrabant et al. [61].

It appears that *Acinetobacter* sp., *Pseudomonas* sp., *Aeromonas* sp. and *Shewanella putrefaciens* play an important role in the denitrification process, as representatives of these taxa were repeatedly isolated during both procedures. It is also striking that, in addition, *Acinetobacter* sp. and *Shewanella putrefaciens* were both isolated from laboratory reactors which were started and operated indepen-

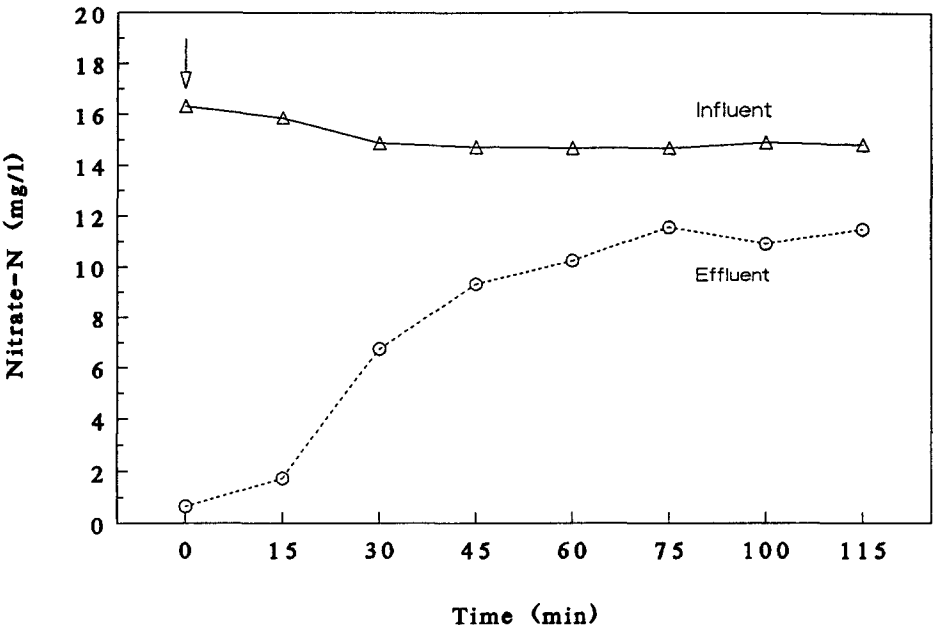


Fig. 5. Effect of interrupting H_2 supply on denitrification performance. The experiment was initiated at t = 0 (arrow).

dently from the pilot-reactor (data not shown). The former were seeded with soil extract and canal water during start-up [20].

The nitrate–nitrite reduction capacity of the different strains isolated from reactor biofilms was tested. *Acinetobacter* sp., *Aureobacterium* sp., *Aureobacterium saperdae*, *Bacillus* sp., and two unidentified isolates did not reduce NO_3^- at all. For all other mesotrophic isolates, no reduction beyond NO_2^- occurred under hydrogenotrophic conditions. When grown heterotrophically, *Pseudomonas* (*syringae*), *Pseudomonas* (*stutzeri*) and one unidentified strain reduced NO_3^- to N_2; *Serratia odorifera* reduced NO_3^- to NH_4^+; *Aeromonas hydrophila*, *Comamonas acidovorans*, *Pseudomonas* (*mendocina*), *Shewanella putrefaciens*, and one unidentified strain reduced NO_3^- to NO_2^-. Under psychrotrophic incubation, none of the isolates showed NO_3^- reduction beyond NO_2^-, both in heterotrophic and autotrophic growth conditions. In general, nearly two-thirds of the isolates were capable of oxidizing H_2, with NO_3^- as terminal electron acceptor. However, under these conditions, none of the identified strains from this series of isolates showed complete denitrification.

The isolation procedure of mesotrophic hydrogenotrophs was repeated for the reactor effluent. Based on colony morphology and cell shape, 21 different axenic cultures were obtained and designated with strain numbers (1 to 18, iC5, eC6 and eC7). Colonies from the initial axenic plates were in turn inoculated into heterotrophic and autotrophic Durham broth tubes. After incubation, the liquid culture was tested for intermediate nitrogenous compounds (Table 3). One group of the isolates showed minimal NO_3^- reduction (group A). Another group did not go beyond NO_2^- (group B). As for the previous series of isolates, a third group of

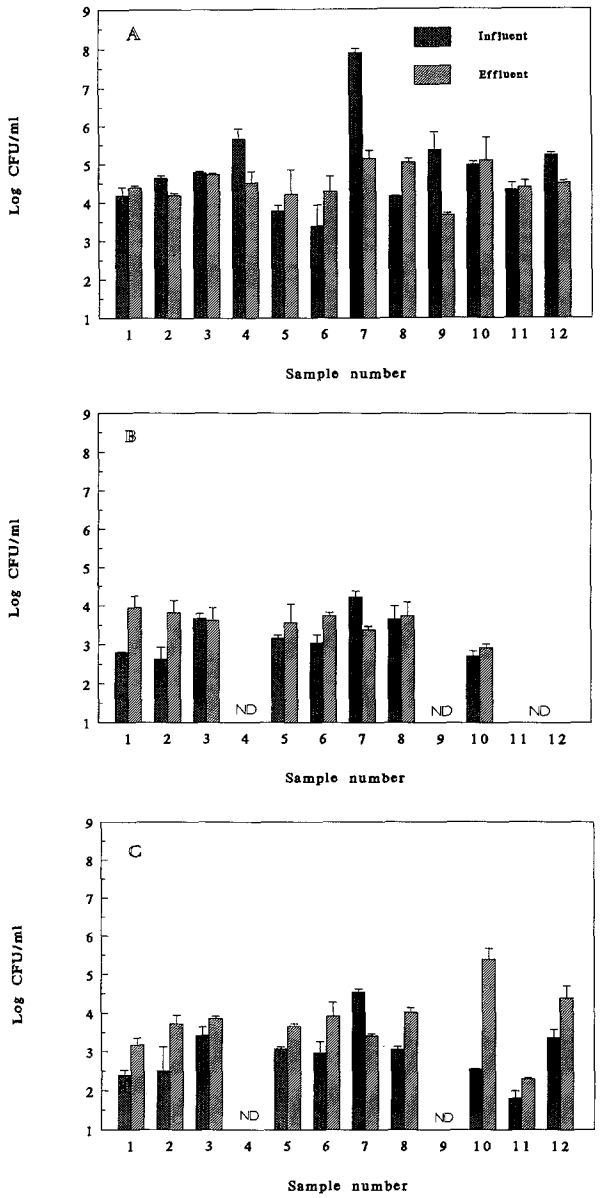


Fig. 6. Bacterial enumerations in the influent and the effluent of the reactor operating under steady state conditions. (A) Heterotrophic population, (B) heterotrophic NO_3^--reducing population, and (C) hydrogenotrophic NO_3^--reducing population. Data are geometric means ± SD. The sample numbers refer to samples taken during a time course of 3 months with an interval of ca. 1 week. ND = not determined.

strains (group C) could reduce NO_3^- to NO_2^- with H_2, but required heterotrophic growth conditions to further reduce NO_2^- to N_2 or NH_4^+. To exclude the possibility of NO_2^--toxicity, the strains were transferred from the initial solid plate into liquid mineral medium without NO_3^-, but amended with 0.15 and 0.50 g liter^{-1} KNO_2, respectively. None of these strains showed any growth after prolonged hydrogenotrophic incubation. This confirmed the inability of these bacteria to develop in a medium with molecular H_2 and NO_2^- as the sole electron donor and acceptor,

Table 2. Isolation and identification of microorganisms from hydrogenotrophic denitrifying biofilms; mesotrophic versus psychrotrophic isolation

Code number	Mesotrophic	Frequency[a]	Code number	Psychrotrophic	Frequency[a]
α1	*Acinetobacter* sp.	3		*Acinetobacter* sp.	25
α2	*Aeromonas hydrophila*	5		*Aeromonas hydrophila*	1
α3	*Aureobacterium* sp. (*A. saperdae*)[b]	1	β1	*Aeromonas sobria*	1
α4	*Aureobacterium* sp.	2			
α5	*Bacillus* sp.	3		*Bacillus* sp.	1
α6	*Comamonas acidovorans*	2	β2	*Micrococcus lylae*	1
α7	*Serratia odorifera*	2			
α8	*Pseudomonas* sp. (*P. syringae*)	3	β3	*Pseudomonas* sp. (*P. aureofaciens*)	7
α9	*Pseudomonas* sp. (*P. mendocina*)	1			
α10	*Pseudomonas* sp. (*P. stutzeri*)	4			
α11	*Shewanella putrefaciens*	1		Shewanella putrefaciens	11
	Unidentified strains	4		Unidentified strains	7

[a] Frequency of occurrence in a total of 31 mesotrophic or 54 psychrotrophic isolates
[b] Names between parentheses need further confirmation

Table 3. Characterization of nitrate–nitrite reduction capacity of strains isolated from reactor effluent, under heterotrophic and hydrogenotrophic conditions

Strain designation	Incubation conditions[a]	Ultimate NO_3^- reduction product	Group designation
3, 4, 5, 11, 13, 15, 18	HE		
		No or weak	A
	HY	NO_3^- reduction	
1, 2, 10, 12, 14, 17	HE	NO_2^-	B
	HY	NO_2^-	
6, 7, 8, 16, iC5, eC6, eC7	HE	N_2	C
	HY	NO_2^-	
9	HE	NH_4^+	
	HY	NO_2^-	
VB 26, 28, 33, 34, 35, 53, 66, 72	HE	N_2	D
	HY	N_2	

[a] HE = heterotrophic; HY = hydrogenotrophic

respectively. Prolonged attempts using an isolation procedure for autohydrogenotrophic denitrifiers [2] finally yielded eight isolates (VB26, 28, 33, 34, 35, 53, 66 and 72) capable of reducing NO_3^- all the way to N_2 both heterotrophically and hydrogenotrophically (group D). The identification of these isolates remains uncer-

Table 4. Nitrate-reducing isolates and the reduction product under different nutritional conditions

Strain designation	Heterotrophic	Hydrogenotrophic	Mixotrophic	Group designation
5, 15, 18	–[a]	–	NH_4^+	A
iC5, eC5	N_2	NO_2^-	N_2/NH_4^+	C
9	NH_4^+	NO_2^-	NH_4^+	
Paracoccus denitrificans LMG 4218	N_2	NO_2^-	N_2	C
Alcaligenes eutrophus LMG 1196	N_2	NO_2^-	N_2	

[a] –, No or very weak NO_3^- reduction observed

tain. Based on whole cell PAGE protein patterns, they can be divided into three different groups. The protein patterns, however, show no resemblance to *Paracoccus denitrificans*, *Alcaligenes eutrophus*, or *Hydrogenophaga pseudoflava*. Further research is being carried out.

A representative selection of the strains was examined for mixotrophic growth characteristics. A trace amount of yeast extract (50 mg liter^{-1}) was added to hydrogenotrophically incubated test tubes after NO_3^- (1 g liter^{-1} KNO_3) was quantitatively converted to NO_2^-. Upon reincubation under a H_2/CO_2 atmosphere, six strains were found to further reduce the available NO_2^-. The characteristics of these strains are summarized in Table 4. In addition to our isolates, *Paracoccus denitrificans* and *Alcaligenes eutrophus* were also tested. Yeast extract was beneficial for their growth and NO_2^- reduction. Yeast extract, a mixture of B-vitamins, amino acids, and ribonucleic acids from yeast, can provide essential nutrients for which de novo synthesis under chemolithotrophic conditions is impossible (auxotrophy). Also, the organic compounds of yeast extract can be used for energy and/or cell material generation (mixotrophy). To further elucidate these aspects, the isolates (5, 15, 18, iC5, eC7, and 9) were transferred from the initial hydrogenotrophic agar plates and grown in the appropriate liquid mineral test medium amended with 62.5 mg liter^{-1} glucose. Complete NO_2^- reduction was obtained, which indicates that these strains cannot reduce NO_2^- under strict auxotrophic conditions.

Hydrogenotrophically grown cells were generally characterized by their ability to densely stick to the glass tube wall, a phenomenon which was not observed during heterotrophic growth. The fact that H_2 can induce hydrophobic growth was confirmed by hydrophobicity measurements of three well-grown isolates (6, 7, and 9). Under heterotrophic conditions, hydrophobicity amounted to 11.6%, 0%, and 7.2%, while under hydrogenotrophic conditions, hydrophobicity increased up to 82%, 90%, and 85% respectively.

Table 5. Different functional groups participating in the nitrate-reduction process

Group designation		Use of reductant organics	H_2	NO_3^- to NO_2^-	NO_2^- to N_2	Examples of isolates[a]
No or weak NO_3^- reduction	A_1	+	−	t[b]	−	α1, α3 to α5 3, 4, 11, 13
	A_2	+	−	t	−	
		t ———	+[c]	+	[+][d]	5, 15, 18
Nitrate reduction only	B	+	+	+	−	α2, α6, α9, α11 1, 2, 10, 12, 14, 17
Complete NO_3^- reduction provided organics	C_1	+		+	+	α8, α10
		+	+	+	−	6, 7, 8, 16, eC6
	C_2	+		+	[+]	α7
			+	+	−	iC5, eC7, 9
		t ———	+	+	[+]	
Auto- and heterotrophic complete NO_3^- reduction	D	+	+	+	+	VB 26, 28, 33, 34, 35, 53, 66, 72

[a] Reference is made to the identified and unidentified mesotrophic isolates from Tables 2 and 3
[b] t = traces
[c] t ——— + both together
[d] [ ] NH_4^+ can be formed

Discussion

Niches in Hydrogenotrophic NO_3^- Reduction

Different groups of microorganisms appear to be involved in hydrogenotrophic NO_3^- reduction (Table 5). Several strains isolated have no direct use of nitrate or hydrogen (subgroup A_1); they must thrive on organics entering with the water or on metabolites produced by the dominant denitrifiers. The subgroup A_2 brings about complete removal of oxidized nitrogenous species by using H_2, provided it can acquire a small amount of organics. It is not clear if the latter are derived from the water as such, or from the other associated strains. Group B can use organics or H_2 and NO_3^-, but does not go beyond NO_2^-. These organisms are probably responsible for an important part of the nitrate to nitrite conversion. The subgroup C_1 can bring about complete denitrification provided a large supply of organic reductant is available, but in practice probably contributes only to the hydrogenotrophic conversion of nitrate to nitrite. The subgroup C_2 is interesting because, provided it can acquire some organics besides H_2, it brings about the conversion of NO_3^- to NO_2^- and subsequently to N_2 or NH_4^+. In the total context of drinking water production, the latter conversion is not desirable. Normally, formation of substantial amounts (> 0.1 mg liter^{-1}) of ammonium were not observed, suggesting that the mixotrophic reducers of NO_3^- to NH_4^+ are not a dominant group. The most important group is D: these organisms are capable of autotrophically reducing NO_3^- via NO_2^- all the way to N_2.

Microbial Diversity

On the basis of the available literature, one would expect a strong biological selection under conditions with hydrogen as electron donor and nitrate as acceptor. From our study however, it can be concluded that the approach of "single" culture hydrogenotrophic nitrate removal, inspired by the well-described *Paracoccus denitrificans*, is far from evident under natural process conditions. The rapid colonization of the carrier material in the reactor results from the abundant presence of potential hydrogenotrophic nitrate reducing microorganisms in the storage reservoir. A large diversity of organisms was shown to be involved; *Acinetobacter* sp., *Aeromonas* sp., *Pseudomonas* sp., and *Shewanella putrefaciens* were repeatedly isolated. In a hydrogenotrophic denitrification reactor at Rasseln (Germany), Selenka and Dressler [58] classified the causal chemolithotrophic strains as representatives of the genera *Pseudomonas*, *Alcaligenes*, and *Achromobacter*. *Aeromonas hydrophila*, *Pseudomonas* sp., and *Acinetobacter* sp. are well-known to colonize support media in water treatment facilities [3, 9, 36] and were frequently isolated from the distribution network fed from a river water treatment plant [8, 47]. *Aeromonas* is one of the most prevalent nitrate-respiring organisms in estuarine environments [7, 43]. The opportunistic presence of *Acinetobacter* sp. not capable of reducing NO_3^- remains unclear. The latter organism can be involved in creating anoxic conditions.

Few bacterial species are able to both denitrify NO_3^- or NO_2^- to N_2 and dissimilate it to NH_4^+. A clear exception appears to be the strain of *Shewanella putrefaciens* studied by Samuelsson [55]. Representatives of this taxon were repeatedly isolated from the reactor. This phenomenon can account for the simultaneous NH_4^+ formation and gas production in some of the test tube assays.

As shown in Table 5, NO_2^- is the key intermediate in hydrogenotrophic NO_3^- reduction. Enteric bacteria were the first reported to use molecular H_2 in bacterial NO_3^- reduction with NO_2^- as final reduction product, thereby relying on organic carbon as sole carbon source [2, 65]. Under carbon-limited conditions, NO_2^- is accumulated and exported from the cells [60]. Under strict hydrogenotrophic conditions, most of the H_2-oxidizing bacteria reviewed by Aragno and Schlegel [2] reduce NO_3^- only to NO_2^-, which accounts for all representatives of our groups B and C. Nitrite is further reduced by organisms of the subgroups A_2 and C_2 only in the presence of trace amounts of organic carbon. Group A_2 represents strict mixotrophs, as growth and NO_3^- reduction are scanty under either strict auto- or heterotrophic conditions. It is likely that in the mixed culture, the number of potential NO_3^--reducers, including the NO_2^--accumulators, exceeds the number of true denitrifiers. The same observation was made in soils [23] and wastewater treatment plants [68]. Therefore, nitrate reduction to nitrite is not the rate-limiting step.

Organic growth factors are generally not required for the H_2-oxidizing species [2]. The requirement as noticed for several strains (subgroup C_2) in order to further reduce NO_2^- under hydrogenotrophic conditions is peculiar, because growth factors are not necessary for the metabolism of H_2 and the fixation of CO_2 under strict lithotrophic conditions with NO_3^-. Nor can growth factors be required for the NO_2^- reducing system as such, since yeast extract is not a necessary supplement to the medium for growth with NO_3^- and glucose.

From our survey, it appears there is evidence that bacteria exist which are able to proliferate in media in which the NO_2^-–H_2 system acts as sole energy source. Hydrogenotrophic nitrite reduction under putative autotrophic conditions was brought about by the isolates VB 26, 28, 33, 34, 35, 53, 66, and 72.

For the subgroups A_2 and C_2, the accelerated growth in the presence of yeast extract indicates that the bacteria are not strict autotrophs. This phenomenon was first described as a case of chemo-metatrophy sensu Lwoff [42, 65] or auto-heterotrophy [24], in which the energy is derived from an inorganic conversion, while organic growth substances are nevertheless indispensable for the proliferation of the organism. Later, the term mixotrophy was introduced [4, 5, 51] to describe the concomitant functioning of heterotrophic and autotrophic biosynthetic processes within a cell. Kluyver and Verhoeven [34] had shown in their adaptation experiment that cells of *Paracoccus denitrificans* grown autotrophically, at the expense of H_2 and NO_3^-, are able to oxidize organic compounds simultaneously and independently. Mixotrophy has also been demonstrated for *Alcaligenes eutrophus* [10, 53] and *Thiobacillus intermedius* [41], where 90% or more of the cell carbon may be derived from the organic substance and 10% or less from bicarbonate. The functional status and selective advantage of the mixotrophic way of life have been described previously [25, 52]. The mixotrophic mechanisms may allow these cells, when competing under predominantly H_2-determined autotrophic conditions, to compete with hydrogenotrophic denitrifiers.

The organic carbon used for NO_2^- reduction can be partly provided by the raw water, decaying cells, or by overflow metabolism of the densely established biofilm. The internal supply of organic carbon in the biofilm is capable of maintaining a considerable denitrification rate, as also reported by Nielsen et al. [46]. Another possible source of organic carbon which cannot be excluded is the chemosynthetic conversion of CO_2 to low molecular carbon compounds such as acetate, a well-defined feature of homoacetogenic bacteria [1, 19]. Overproduction and excretion of soluble microbial products is well-described for aerobic [57] and anaerobic [30] environments, oligotrophic biofilms [9, 45], and for aerobic autotrophs assimilating CO_2 via the Calvin cycle [12, 13].

The absence of an increased concentration of dissolved AOC in the effluent of the reactor is in contrast with the findings for a methanol-fed system [40] and biological processes in general [9]. This may also indicate that internally produced organic compounds are continuously drained through biosynthesis of a mixotrophic group of microorganisms. More evidence is given by the temporary nitrite accumulation during the initial start-up of the reactor with "virgin" polyurethane. Once a biofilm is established on the carrier, the internal carbon generation is started and NO_2^- reduction proceeds. During the second start-up period, residual organic carbon was still present on the carrier; hence, no NO_2^- accumulation was observed. Nitrite production is also reported only in the early phase of biomass development for a 50 $m^3\ h^{-1}$ hydrogenotrophic denitrification plant in Germany [58]. The formation of hydrophobic clumps under hydrogenotrophic conditions may also result from the beneficial effect it has for the cells, with respect to energy and/or carbon conservation. Aragno and Schlegel [2] also reported that heterotrophically grown cells of some H_2 oxidizing strains will not grow on solid media under autotrophic conditions unless arranged in the form of clumps.

Our results corroborate literature data that indicate hydrogenotrophic NO_2^- reduction may be strongly influenced by the availability of reducing power. The low energy efficiency of nitrite reduction [21] and the dependence of the hydrogen oxidizers on reverse electron transport during their anabolism [10] are probably at the basis of the growth factor requirement. The organic carbon supplied may unburden the anabolic reactions. Reducing equivalents used for NO_2^- reduction can thus come from the pool of electrons that otherwise would be necessary for CO_2-fixing reactions. The nature of the organics involved and the levels at which regulation occurs need further elucidation.

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