Proceedings of the 4th German-Israeli Status Seminar. B. Moav, V. Hilge, and H. Rosenthal (Eds). EAS Special Publication No.17, Oostende, Belgium.
39-54, 1992

Nitrite Accumulation by Denitrifiers Isolated from Fluidized Bed Reactors Operated in an Aquaculture Unit

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Abstract

In fluidized bed reactors, applied for nitrate removal in an aquaculture unit, denitrification of nitrate was found to result in an accumulation of nitrite. Isolation of denitrifying bacteria from these reactors revealed that most of these isolates produced nitrite from nitrate under controlled laboratory conditions. Two of the isolates, one with accumulated nitrite while respiring on nitrate, and the other not, were subject to further studies. It was found that nitrite accumulation in one of these isolates could be adequately explained by differences in the relative rates of nitrate and nitrite reduction. Inhibiting concentrations of nitrite were lower for the isolate which did not accumulate nitrite. Incubation of the nitrite-accumulating isolate with different carbon sources resulted in differences in the relative nitrite accumulation (as compared with the amount of nitrate reduced).

KEYWORDS: denitrifying bacteria, fluidized bed reactors

Introduction

One of the characteristics of modern aquaculture is the trend to develop recirculated or semi-recirculated fish culture systems, in which fish can be grown at densities higher than so far known in more conventional fish culture systems, such as still water ponds. Although the investment and overhead costs of these intensive fish culture systems are usually high, certain local conditions might favor the use of such systems over the use of more conventional culture methods (Muir, 1981).

The high fish densities at which these systems are operated and the corresponding large feed inputs would cause a rapid deterioration of the water quality were it not that methods assuring a proper water treatment are an intrinsic part of the overall system design.

Water purification by means of so called biofilters is often employed in intensive fish culture systems. Most of the biofilters used are aimed at reducing the levels of ammonia in the culture system by facilitating growth of nitrifying bacteria which oxidize ammonia via nitrite to nitrate. Nitrate, the end product of this process, is allowed to accumulate in the system as it is far less toxic to fish than the more reduced inorganic nitrogen forms. However, a build-up of nitrate to high concentrations in the culture system should be prevented for mainly two reasons. Firstly, high concentrations of nitrate might be toxic to some fish species while, secondly, limitations exist in many countries as to the maximal allowed levels of nitrate in the effluent waters.

A pilot-scale biofiltration system aimed at reducing the levels of inorganic nitrogen from an intensive fish culture unit has been operative for two years at the experimental fish culture station near Ginosar (Israel). The biofiltration system consisted of an aerobic trickling filter and two anaerobic fluidized bed columns which facilitated the growth of nitrifying and denitrifying organisms, respectively. Under the operational conditions tested, nitrite accumulation was found to take place in both treatment steps (van Rijn and Rivera, 1990). Denitrifying bacteria were isolated from the fluidized bed columns and two of these isolates, one of which accumulates nitrite during the process of

denitrification while the other does not, were subjected to further examination. Kinetics of nitrate and nitrite removal by these bacteria were examined at various ambient concentrations of these substrates. Furthermore, the effect of different carbon sources on the accumulation of nitrite was examined.

Materials and Methods

1. ENRICHMENT OF DENITRIFYING BACTERIA

To a crude sample from one of the fluidized bed columns, nitrate (KNO_3: 50 mg liter^{-1}) was added and the sample was incubated at a temperature of 27°C for 24 h under anaerobic conditions. Anaerobic conditions were established by nitrogen flushing of sealed culture vessels. After this incubation period a subsample was diluted (1:100) with minimal medium for isolation of denitrifying bacteria with yeast extract (0.4%) as a carbon source (Jeter and Ingraham, 1981) and again incubated under anaerobic conditions. This step was repeated until a sample was obtained with a low concentration of organic matter and a high reduction of nitrate.

2. ISOLATION

A bacterial suspension from the enrichment cultures was streaked on solid agar with addition of minerals similar to that of the medium used for enrichment. Individual colonies from the solid medium were transferred to liquid medium, and were once more transferred to solid medium after growth in the liquid medium was observed. This step was related until axenic cultures were obtained. The axenic isolates obtained were tested for denitrifying properties by incubation in deep agar tubes. The formation of gas pockets in these tubes pointed to an isolate with denitrifying properties.

3. TAXONOMIC TEST

Identification of the bacterial isolates was performed by means of API-20NE.

4. NITRATE AND NITRATE REDUCTION

Isolates were harvested in the stationary growth phase and distributed in the medium as described previously: a concentration of around 1 mg liter^{-1} protein. Nitrate and nitrite concentrations varied among the different runs (0-1200 mg liter^{-1} $NO_{3(2)}$-N). The changes in ammonia, nitrite, nitrate and protein, during anaerobic incubation in sealed culture vessels, were followed over a period in which a linear disappearance of either nitrate or nitrite took place (up to 24 h). Reduction rates of nitrate and nitrite were calculated by using linear regression analysis. The maximum standard error was 10%. Rates were expressed as nitrate-N or nitrite-N reduction per h per mg protein, whereby the initial protein concentration of the sample was used for calculation. Protein concentrations during different runs did not change by more than 5%. During the period of linear reduction of nitrate and nitrite, ammonia depletion did not take place.

Results and Discussion

Changes in ammonia, nitrite and nitrate concentrations (influent - effluent) were determined in pilot-scale fluidized bed reactors operated at three different flow rates. It was found (Table I) that at the lowest flow rate examined (0.17 m^{-3} m^{-2} min^{-1}) nitrate was removed by the fluidized bed, while ammonia and nitrite concentrations were higher in the effluent water. The same picture emerged at a flow rate of 0.34 m^{-3} m^{-2} min^{-1} and at the highest flow rate (0.68 m^{-3} m^{-2} min^{-1}) both nitrate and ammonia were removed by the columns while nitrite was shown to accumulate. Examination of even higher flow rates (not shown) resulted in large fluctuations in nitrate removal and even led to accumulation. It can be concluded that, although it was possible to operate the fluidized bed columns at a flow rate (0.64 m^{-3} m^{-2} min^{-1}) at which ammonia and nitrate removal from the treated water occurred, nitrite removal did not occur and accumulation at all flow rates was examined. Nitrite accumulation by biological denitrification systems has been described by several authors (Wilderer et al., 1987; Nilson et al., 1980; Richard and Leprince, 1982; Kurt et al., 1984). It is considered to be a serious problem in the biological treatment of drinking water, as the admissible concentration of nitrite in some countries is as low as 0.1 mg

Table I. Removal/accumulation rates of ammonia, nitrite and nitrate operated at three different flow rates[a].

Flow rate ($m\ min^{-1}$)	NH_4-N	NO_2-N	NO_3-N	n
		($mg\ N\ m^{-3}\ min^{-1}$)		
0.17	+11.8 (10.4)	+35.4 (23.0)	-145.5 (34.2)	10
0.34	+12.5 (21.4)	+38.7 (10.1)	-186.2 (61.2)	16
0.68	-28.9 (18.3)	+27.5 (16.8)	-198.1 (99.1)	42

[a] Positive values indicate accumulation rates; negative values indicate removal rates. Numbers in parentheses indicate standard deviation (From van Rijn and Rivera, 1990).

NO_2 $liter^{-1}$ (European Community, 1980). Also in fish culture systems too high nitrite concentrations pose serious problems, as nitrite is toxic to fish at relatively low concentrations (Colt and Tchobanoglous, 1976; Russo and Thurston, 1977).

Subsamples of the fluidized bed columns obtained on two different dates were incubated in the laboratory under anaerobic conditions in the presence of nitrate, nitrite and ammonia. After 24 h of incubation under these conditions it was found (Table II) that the samples obtained on August 20, 1989 showed an almost complete nitrate removal over the 24 h period while 67% of the nitrate-nitrogen which was removed was found to accumulate as nitrite-nitrogen. In the sample obtained from the fluidized bed reactor in October 1989 nitrate reduction was also apparent, while only 23% of the nitrate-nitrogen was accumulated as nitrite-nitrogen.

The nitrite accumulation observed during the 24 h incubation of the field samples can be attributed to the action of nitrate respiring bacteria as the runs were conducted in the presence of ammonia (repressing the nitrate assimilatory pathway) and under

Table II. Changes of inorganic nitrogen in samples obtained from fluidized bed columns after 24 h of incubation under anaerobic conditions[a].

Date of sampling		NH_4-N	NO_2-N (mg N liter^{-1})	NO_3-N
20.8.1989	initial	41.4	39.8	35.8
	after 24 h	38.9	63.1	1.2
15.10.1989	initial	67.2	2.2	42.5
	after 24 h	62.5	6.9	22.4

[a] Equal portions of samples obtained from the fluidized bed reactors (COD: 200 mg liter^{-1} O_2) were incubated at 27°C with addition of a mineral mix as described in Materials and Methods. Incubation was conducted with duplicates and average values are given (Adapted from: van Rijn and Rivera, 1990).

strictly anaerobic conditions (excluding the production of nitrite nitrification). Under these conditions various factors might underlie the observed accumulation of nitrite. Betlach and Tiedje (1981) found that nitrite accumulation in some strain of denitrifying organisms could be explained by the consistently lower activity of nitrite reductase than nitrate reductase. Differences in induction periods for the nitrate and nitrite reductase enzymes (Williams et al., 1978), differences in oxygen tolerance of the various reductases (Krul and Veeningen, 1977; Hochstein et al., 1984), and the quantity and nature of electron donors (Knowles, 1982) are additional factors which have been shown to cause accumulation of nitrite in denitrifiers. Studies on fluidized bed reactors (van der Hoek and Klapwijk, 1987) revealed that by increasing the buffering capacity of the system, nitrite accumulation could be prevented. It was postulated that the high pH levels generated in the active denitrifying biofilms caused inhibition of nitrite reductase and, therefore, nitrite accumulation.

Six strains of denitrifiers were isolated from samples obtained from the fluidized bed reactors. The results of a comparison of some taxonomic parameters using the API-20NE identification system is shown in Table III. Based on these characteristics, strain E could be identified as *Pseudomonas acidovorans*, strains D, E and F could be grouped in the genus *Pseudomonas*, with strains A and B being in the *Flavobacterium* genus. It was not possible to identify strain C to genus level by means of this test.

Anaerobic incubation of these strains with a follow-up of the changes in ammonia, nitrite and nitrate revealed that five out of the six strains accumulated nitrite upon reduction of nitrate anaerobic conditions (Figure 1). Only in the case of strain A (*Flavobacterium* sp.) no nitrite was found to accumulate in the medium during nitrate reduction. The finding, that after 10 h of incubation no further changes in nitrate and nitrite levels were observed, probably indicates that at this sampling time the carbon source had become exhausted.

Two of the isolates, D and A, were subjected to further studies, the first one showing the accumulation of nitrite during denitrification, while the other one denitrified nitrate without accumulating nitrite.

The rate of nitrate reduction of strain D (*Pseudomonas* sp.) at various ambient concentrations of nitrate is depicted in Figure 2A. Nitrate reduction rates followed Michaelis-Menten kinetics and a maximum nitrate removal rate of 4,17 mg NO_3-N/mg protein/h was reached at an ambient nitrate concentration of 300 mg $liter^{-1}$. The nitrite reduction at the various nitrate concentrations (as calculated from the disappearance of nitrate and the accumulation of nitrite) followed the same trend as nitrate reductase activity (Figure 2B), but was considerably lower. The accumulation of nitrite at the various ambient nitrate concentrations (Figure 2C) increased with growing concentrations of nitrate. Finally, the nitrite reduction rates against various concentrations of ambient nitrite were examined (Figure 2D). It was found that inhibition of nitrite reduction occurred at ambient nitrite concentrations between 45-55 mg $liter^{-1}$ NO_2-N.

It becomes clear from these results that nitrite accumulation in the medium is due to the fact that nitrate reductase activity

Table III. Different reactions of six bacterial isolates when screened with API-20NE.

Strain	NO_3	TRP	GLU	ADH	URE	ESC	GEL	PNPG	GLUa	ARAa	MNEa
A	+	-	-	-	+	+	-	-	+	-	+
B	+	-	-	-	-	-	+	-	-	-	-
C	+	-	-	-	-	+	+	+	+	-	+
D	+	-	-	+	+	-	-	-	-	-	-
E	+	-	-	-	-	-	-	-	-	-	-
F	+	-	-	-	+	-	-	-	-	-	-

Strain	MANa	NAGa	MALa	GNTa	CAPa	ADIa	MLTa	CITa	PACa	OX
A	-	+	+	-	-	-	-	-	-	-
B	-	-	+	-	-	-	-	-	+	-
C	+	+	+	+	-	-	-	-	-	-
D	+	-	-	+	+	+	+	+	+	+
E	+	-	-	+	+	+	+	-	+	+
F	+	-	-	+	+	+	+	-	+	+

Abbreviations: NO_3-reduction of nitrate; TRP-tryptophan, indole production; GLU-glucose. acidification; ADH-arginine, dihydrolase; URE-urea, urease; ESC-esculin, hydrolysis; GEL-gelatine, hydrolysis; PNPG-p-nitro-phenyl- D-galactopyranoside, -galactosidase; GLUa-glucose assimilation; ARAa-arabinose assimilation; MNEa- mannose assimilation; MANa- mannitol assimilation; NAGa-N-acetyl-glucosamine assimilation; MALa- maltose assimilation; GNTa- gluconate assimilation; CAPa- caprate assimilation; ADIa- adipate assimilation; MLTa- malate assimilation; CITa- citrate assimilation; PACa- phenyl-acetate assimilation; OX-cytochrome oxidase.

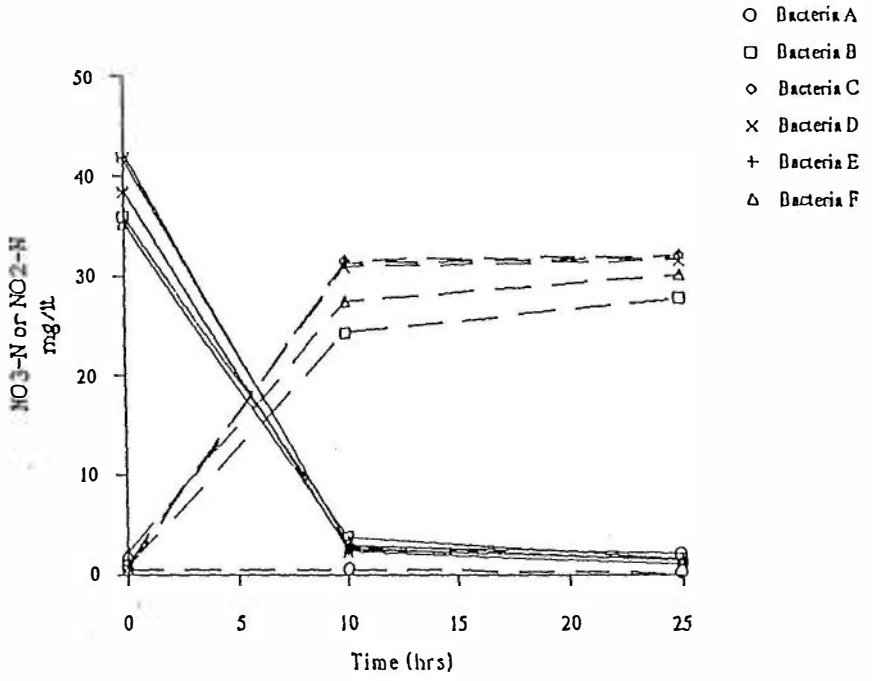

Figure 1: Anaerobic incubation of denitrifying isolates with nitrate as an electron acceptor and yeast extract (o.4%) as an election donor. Cells were grown in medium described in Materials and methods. Ammonia was added in excess and was not depleted during the incubation period. Nitrate (-), Nitrite (--).

exceeds nitrite reductase activity in this bacterium. The sudden drop in both nitrate and nitrite reduction at high ambient nitrate concentrations was probably due to the fact that at these concentrations, inhibition of nitrite occurred, since during these runs the concentrations of nitrite (Figure 2C) were in the range found to be inhibiting (Figure 2D).

Nitrite inhibition at concentrations as low as 28-42 mg liter^{-1} NO_2-N were found to be toxic to some other strains of denitrifying bacteria (Tiedje, 1990).

A similar experiment was conducted with strain A (*Flavobacterium*

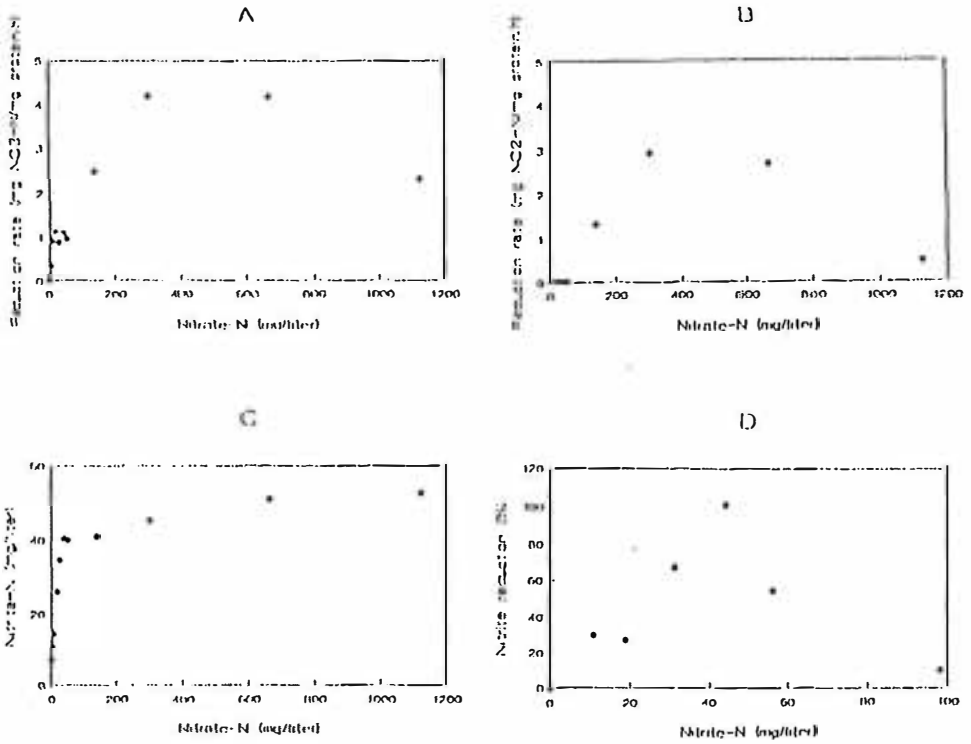

Figure 2: Isolate D (*Pseudomonas* sp.). Rates of nitrate (A) and nitrite (B) reduction at various ambient nitrate concentrations, accumulation of nitrite reduction rates at various ambient nitrite concentrations (D). The initial protein concentration was around 1 mg liter^{-1} in each of the runs. The incubation period was up to 24 h. Ammonia was added to the medium (Materials and Methods) in excess and was not depleted during any of the runs. Yeast extract was used as a carbon source.

sp.) and results are presented in Figure 3. Nitrate reduction rates over the range of ambient nitrate concentrations reached a maximum value of 7.1 mg NO_3-N/mg protein/h at the highest ambient nitrate concentration examined (Figure 3A). Nitrite accumulation did not take place in any of the runs. The nitrite reduction rates at various ambient nitrite concentrations of 21.6 mg liter^{-1} while beyond this concentration the nitrite reduction rates decreased due to the possible toxic effect of nitrite on the cells. The maximum removal rate of nitrate by this bacterium was considerably higher than that of bacterium D.

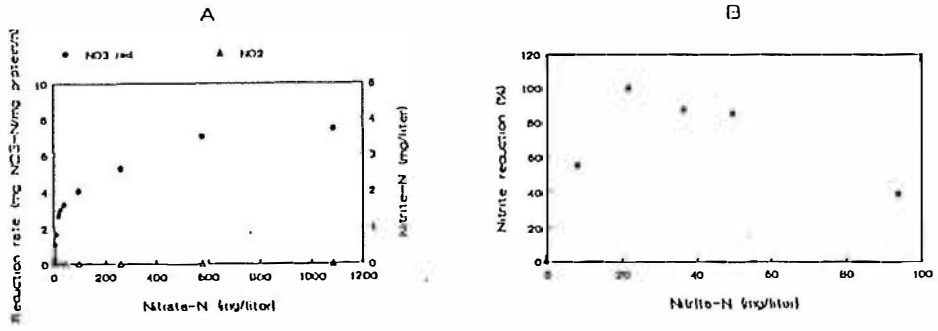

Figure 3: Isolate A (*Flavobacterium* sp.). Rates of nitrate reduction and absolute nitrite accumulation at various ambient nitrite concentrations (A) and relative nitrite reduction rates at various ambient nitrite concentrations (B). Experimental conditions as in Figure 2.

The level at which nitrite causes inhibition in this strain was lower than for strain D. The higher sensitivity of strain A to nitrite might be explained by the fact that an accumulation of nitrate to high levels is prevented due to its relatively active nitrite reductase. This contrasts with bacterium D which exhibits a higher tolerance to nitrite, due probably to the fact that the organism itself causes an increase in nitrite in its environment.

It remains to be examined if the results obtained in these experiments are generally true for denitrifying bacteria that do and do not accumulate nitrite. If so, one might assume that in an environment where both types of bacteria are present (e.g. the fluidized bed columns), the concentration of nitrite might be an important factor controlling the dominance of one type over the other.

Both bacteria were grown on a wide array of organic substrates under anaerobic conditions and the denitrification rate was followed up over a period of 24 h (Figure 4). Both isolates were capable of growth on all organic carbon sources tested. As would be expected, the different carbon sources used (each with its specific energy yield) caused different rates of nitrate reduction. Based on the results obtained in this experiment, it is impossible to discern whether the amount of nitrate reduced is

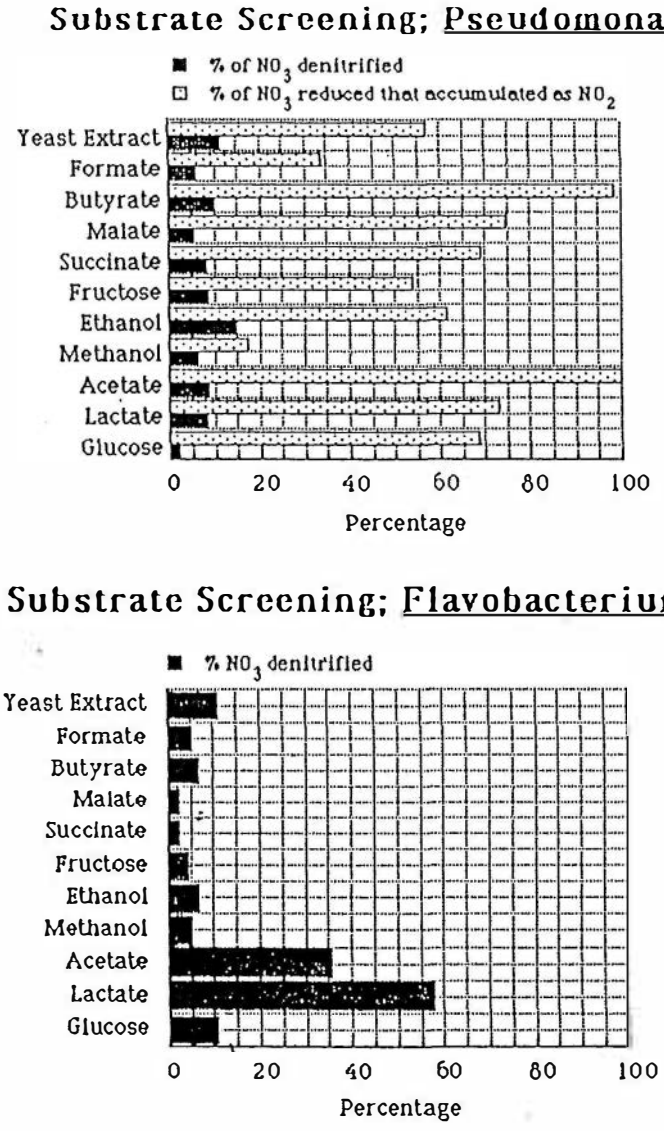

Figure 4: Nitrate reduction (as a percentage of the initial concentration) and nitrite accumulation (as a percentage of the nitrate reduced) in isolates D (*Pseudomonas* sp.) and A (*Flavobacterium* sp.) as a function of different carbon sources. The concentration of yeast extract was 0.4%, while all other carbon sources were added in equimolar amounts (10mM). The experiment was conducted for 24 h with medium as described in Materials and Methods. No ammonia depletion occurred during the various runs. The initial nitrate concentration was 500 mg liter^{-1} NO_3-N.

actually the maximum amount of nitrate which could be reduced by these isolates over 24 h, as carbon depletion might have occurred during the incubation period. Nitrite accumulation in strain A was not apparent, while its accumulation (as a percent of nitrate reduced) in isolate D varied among the different carbon sources. The differences in nitrite accumulation of these cells when grown with different carbon sources might indicate the kinetic parameters of denitrification change according to the carbon source supplied. This finding is supported by earlier studies in which it was found that the availability of electron donor (Nommik, 1956; Wijler and Delwiche, 1954), as well as the type of electron donor (Bremmer, 1977), influence the composition of products from denitrification. It can be concluded, therefore, that the choice of carbon source might to some extent prevent nitrite accumulation in the nitrite accumulating denitrifier examined in this study. It remains to be examined, however, if other carbon sources will cause a reduction of nitrate to nitrogen without the accumulation of nitrite.

Conclusions

In this study a comparison of two denitrifying bacterial strains revealed that the nitrite accumulation observed in one of these strains was due to differences in activity of the nitrate and nitrite reductase enzymes. Nitrite was found to inhibit the activity of the strain that did not accumulate nitrite at lower concentrations, than did the strain that did accumulate nitrite. Furthermore, growth rates (not shown), and denitrification rates of the strain that did not accumulate nitrite, were found to be higher than those of the nitrite-accumulating strain. Based on the experimental evidence for those strains only, it could be possible to selectively enrich the non-nitrite-accumulating strain. In addition, it was found that different carbon sources affected the rate of nitrite accumulation in the nitrite-accumulating strain.

To what extent these findings at this stage can be applied to improve the nitrite removal of fluidized bed reactors remains to be examined. It might be assumed, however, that a more thorough understanding of the microbial processes taking place in these fluidized bed reactors will eventually lead to such an improvement.

Acknowledgments

The authors would like to thank Ing. Guillermo Rivera for technical assistance. The study was supported by the German Ministry for Research and Technology (BMFT) in the framework of the joint German-Israeli programme on aquaculture (NCRD project AQ 6/533).

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