J. Chem. Technol. Biotechnol. 1998, **72**, 213–220

Nitrate and Nitrite Reduction of a Sulphide-Rich Environment

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(Received 27 August 1997; revised version received 2 March 1998; accepted 20 March 1998)

Abstract: A sulphide-rich anaerobic sludge acclimated with a molasses wastewater was used to carry out studies on nitrate and nitrite reductions in continuously stirred batch reactors. It was shown that a COD/N-NO_x ratio as high as 65·6 mg mg^{-1} did not promote dissimilatory reduction of nitrogen oxides to ammonia. Denitrification was characterized by a probable accumulation of gaseous intermediates, nitric oxide (NO) and nitrous oxide (N_2O), by sulphide consumption with concomitant elemental sulphur production and by an increase of the redox potential. In addition, sulphate reducers were completely inhibited by nitrogenous oxides. Cultures performed without any carbon source proved that denitrifiers were able to use sulphides as electron donors. Furthermore, while a lag phase preceded nitrate denitrification, nitrite was consumed immediately. Chemical reduction of nitrite by ferrous iron (Fe^{2+}) was considered to be responsible for this difference. Evidence of such a chemodenitrification has been presented by using a sterilized sludge which kept its ability to reduce nitrite while it lost its capacity to use nitrate. Moreover, this chemical activity was favoured by Fe^{2+} addition. Finally, it has been suggested that during the cultures performed with non-sterilized sludge, a biological reduction of the ferric ions (Fe^{3+}) would be coupled to nitrite chemodenitrification and would allow a regeneration of Fe^{2+}. © 1998 SCI

J. Chem. Technol. Biotechnol. **72**, 213–220 (1998)

Key words: denitrification; sulphide; nitrate; nitrite; redox potential; chemodenitrification

NOTATION

COD	Chemical oxygen demand (g O_2 dm^{-3})
EDTA	Ethylene-diaminetetraacetic acid
NAR	Nitrate reductase
NIR	Nitrite reductase
N-gas	N-N_2O + N-N_2
N-NO_x	N-NO_2 + N-NO_3
TKN	Total Kjeldahl nitrogen
TOC	Total organic carbon
TSS	Total suspended solids
VFA	Volatile fatty acids
VSS	Volatile suspended solids

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Contract/grant sponsor: Agence de l'Environement et de la Maitrise de l'Energie (ADEME), Paris, France.

1 INTRODUCTION

The anaerobic digestion of effluents from food and beverage industries produces methane, carbon dioxide, ammonia and biomass. Owing to the presence of a high sulphate content, large amounts of sulphide are also generated. Its free form (H_2S) is very corrosive, in addition to its high degree of toxicity. H_2S denatures proteins inside the cytoplasm.[1] It may combine with iron of cytochromes and other iron-containing compounds in

 J. Chem. Technol. Biotechnol. 0268-2575/98/$17.50. Printed in Great Britain

the cell[2] or could be responsible for an indirect toxicity by rendering the iron insoluble as FeS and, thus, not available for ferredoxin and cytochrome *c* synthesis.[3] The hydrogen sulphide may also interfere with the various coenzymes A and M sulphide linkages.[4] Therefore, it causes inhibition of various bacteria such as methanogens, sulphate-reducing organisms and denitrifiers. Despite its toxicity, sulphide can be used as an electron donor by denitrifying bacteria such as *Thiobacillus denitrificans*.[5–8]

Ammonia, which results from protein digestion, has to be removed since its discharge in receiving waters causes ecological and public health problems. Biological nitrification and denitrification are the most widely used treatment for nitrogen elimination from digested wastewaters. Therefore, the traditional overall carbon and nitrogen removals are performed in a three-unit process which includes an anaerobic digester, an anoxic denitrifying step and a nitrifying reactor (Fig. 1(a)). Nitrogenous oxides produced from ammonia by aerobic autotrophic bacteria are recycled to the denitrification reactor where they are reduced to molecular nitrogen (N_2), mainly by heterotrophic bacteria which use the remaining organic matter from the digested wastewater as electron donors. In such a process, three reactors are needed. Moreover, in the case of a nitrogen-rich wastewater, the carbon originating from the digested effluent could be insufficient for a total nitrogen oxides removal. Therefore, it is often necessary to add an external carbon source to achieve a complete denitrification.

To solve these problems, it would be very attractive to combine denitrification and anaerobic digestion in an integrated process (Fig. 1(b)). Its feasibility depends on the ability to denitrify in a methanogenic environment. It has been shown that denitrification could take place in marine sediments[9] or in digested sludge.[10–15] Denitrifying organisms would survive in such nitrate-free environments by carrying out a low level of fermentation.[14] This anaerobic metabolism is the only explanation for the survival of these typically aerobic bacteria under conditions of extreme electron acceptor starvation. Moreover, this hypothesis is supported by recent genetic studies showing that *Pseudomonas* has an anaerobic operator sequence, *anr*, controlling expression of the operon that carries the ability to ferment arginine.[14]

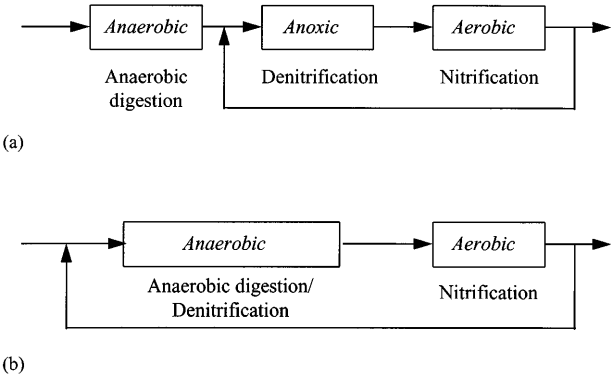

Fig. 1. Biological carbon and nitrogen removal processes: (a) classical configuration, (b) configuration proposed.

However, dissimilatory nitrate reduction to ammonia has been found to be the main nitrate reduction pathway in methanogenic environments.[9,15–17] It seems that a low electron donor/electron acceptor ratio[10] and/or a high initial nitrate load[12] promote denitrification activity in anaerobic sludge. Furthermore, it has been suggested that the nature of the carbon source could influence the reduction pathways of the nitrogen oxides.[11,18–20] For instance, denitrification would prevail against N-oxides ammonification in the case of a volatile fatty acids (VFA)-containing effluent whatever the COD/N-NO_x ratio. Conversely, ammonia production would be predominant in glycerol and glucose media (fermentable substrates).[21]

In the case of wastewaters containing fermentable carbon, such as food processing effluents, a determination of the conditions for a complete reduction to N_2 (if they exist) is needed.

In addition to the problem of the dissimilatory N-oxide reduction to ammonia, it has been shown that nitrates strongly inhibit methanogenesis.[12,16,22–24] This inhibition, which would not be directly related to a redox change,[21,23,25] still remains unclear. Clarens *et al.* showed that *Methanosarcina mazei* was much more inhibited by the intermediate compounds of denitrification than by nitrate itself.[26] This inhibition also depends on the methanogenic bacteria involved since Belay *et al.* showed that some methanogenic strains could grow at very high nitrate concentrations (2800 mg N-NO_3 dm^{-3}).[27]

The effect of nitrate on the anaerobic digestion of a molasses wastewater has been recently investigated using discontinuous cultures.[28] A lag phase preceded nitrate reduction. It appeared that during this phase, methanogenic activity was not affected by nitrate. Methane production failed only when denitrification began after about 40 h. It has been suggested that the rise in redox potential and the transient nitrite accumulation which occured during nitrate denitrification could be responsible for the inhibition of methanogens, instead of nitrate itself. In addition, sulphide consumption was observed during nitrate reduction. Finally, it has been proved that a high COD/N-NO_3 ratio did not promote nitrate dissimilation to NH_4^+ using a molasses wastewater as carbon substrate.

To compare nitrate and nitrite reductions in a sulphide-rich environment, new discontinuous cultures using nitrite or nitrate and a molasses effluent were proposed. First, the influence of the COD/N-NO_2 ratio on the nitrogen reduction pathway was evaluated. Then, the nitrite reduction was studied, the redox potential was monitored and the sulphide content was determined. Finally, denitrification was investigated in batch cultures performed without carbon substrate.

2 MATERIALS AND METHODS

2.1 Biomass and culture conditions

A methanogenic consortium originating from a 20 dm^3 laboratory scale digester fed with a diluted molasses wastewater was anaerobically transferred to sealed batch reactors (1 dm^3) deoxygenated through argon bubbling (for few minutes) before inoculation. The were agitated with a magntic stirrer (stirrer speed at 400 rpm) and placed in a temperature-controlled room ($34 \pm 1°C$). Gas production was periodically determined using a 100 cm^3 air-tight syringe; pressure in the flasks was periodically decreased to atmospheric pressure. pH was monitored with an Ingold pH-meter (2301) in each sample. Concentrated molasses wastewater (24 or 10 g, d (density) = 1·3) was introduced in each reactor. This substrate contained a high COD content (total COD = 460 g dm^{-3} and soluble COD = 430 g dm^{-3}). Its total organic carbon (TOC), total nitrogen (TKN) and sulphate concentrations were respectively 260, 36 and 63 g dm^{-3}. The pH was adjusted to 7·3 through addition of a few drops of 37% (w/v) HCl solution. The redox potential (E_0) was continuously monitored using an Ingold pH transmitter (2400) and a combination redox electrode (Ag/AgCI reference system, KCl 3 M, $Eh_{ref}^{35°C} = 199·8$ mV). Runs were replicated three-fold and their initial conditions are reported in Table 1.

Nitrite and nitrate chemodenitrification was studied using glass flasks. Before sterilization (autoclave, 50 min, 130°C), concentrated molasses wastewater (2·5 g) was fed to the sludge (100 cm^3) and the pH was adjusted to 6·8 by addition of HCl (37%, w/v). To promote the reduction of nitrogen oxides by Fe^{2+}, $FeCl_2$ was supplied to some flasks (final concentration in the broth: 70 mmol dm^{-3}). Then the flasks were deoxygenated through argon bubbling and sealed. After sterilization, nitrogen oxides were added using 0·2 μm filters and the pH was controlled (the sterilizing treatment increases the pH). A pH value of 7·3 was necessary to perform the experiments with the same initial conditions as previous ones. The flasks were agitated in a thermostatically controlled orbital shaker (35°C, 250 rpm).

The study of the autotrophic denitrification was performed in 1 dm^3 batch reactors, as previously described.[28] To exhaust all the carbon residuals remaining, the sludge was left in starvation for a few days. Then, nitrogenous oxides were added to the broth.

TABLE 1
Initial Culture Conditions and Nitrogen Balance for the Three Different Batch Culture Experiments Performed in this Study

	Culture 1	*Culture 2*	*Culture 3*
Culture conditions			
TOC (mg dm^{-3})	3910	3800	5790
^{soluble}COD (mg dm^{-3})	8170	8020	12 145
[N − NO_3] (mg dm^{-3})	210	0	0
[N − NO_2] (mg dm^{-3})	0	197	185
pH	7·4	7·3	7·3
Volume (cm^3)	1000	1000	1000
VSS (mg dm^{-3})	2200	2200	2200
COD/N − NO_x	38·9	40·7	65·6
Nitrogen balance			
Reduced N − NO_x (mg)[a]	212·4	190	174·7
N-gas produced (mg)	206·7	187·8	173·3
Initial TKN (mg dm^{-3})	1700	1960	1974
Final TKN (mg dm^{-3})	1708	1870	1974
% denitrification	97·3	98·8	99·2

[a] Reduced N − NO_x was evaluated considering the amount of nitrogen oxides removed from the broth at each sampling and thus not available for reduction.

2.2 Analyses

Samples were collected from each culture. After centrifugation (10 min, 8000g), the TOC content was determined through UV oxidation using a Dohrman DC-80 and the COD measurements were done by the potassium dichromate ferrous ammonium sulphate method.[29] Suspended solids (SS) and volatile suspended solids (VSS) analyses were performed according to standard methods.[29] Sulphate, nitrate and nitrite were measured by ion chromatography (Dionex 100). Separation and elution were carried out on an IonPac. AS4A analytical column using carbonate/bicarbonate eluent (1·8 mmol dm^{-3} Na_2CO_3/1·7 mmol dm^{-3} $NaHCO_3$) and a sulphuric regeneration (H_2SO_4, 25 mmol dm^{-3}). Regenerant and eluent flow rates were respectively 5 cm^3 min^{-1} and 2 cm^3 min^{-1}. Dissolved sulphides were assayed through a method developed by Percheron *et al.*[30] Its principle is based on the complete oxidation of an unstable compound (sulphide) into its stable form (sulphate) using a strong oxidant: hydrogen peroxide. The sulphate content of the sample was determined before and after this treatment. The difference between both results gave the total dissolved sulphide. To avoid H_2S exhaust, this oxidation has to be performed immediately after sampling, without cell separation. A titrimetric method after distillation using a Büchi apparatus was used to measure ammonia and total Kjeldahl nitrogen (TKN). Gas composition (CO_2, CH_4, O_2, H_2, N_2O and N_2) was evaluated by gas chromatography (Shimadzu GC8A) using an Hayesep 80–100 mesh column, a molecular sieve column and a katharometer detector (argon carrier). NO was detected using a molecular sieve column and helium as gas vector and a Shimadzu GC14A instrument.

2.3 Calculations

To evaluate the recovery of reduced nitrate and to quantify the denitrification/dissimilatory reduction to

ammonia ratio a nitrogen balance was done for each culture. For that purpose, the total $N - N_2$ produced ($P^n_{N-N_2}$) was evaluated according to eqn (1).

$$P^n_{N-N_2} = \sum_{i=1}^{n} [N - N_2]^i \cdot V^i_g + (V^n_{GH} \cdot [N - N_2]^n - V^0_{GH} \cdot [N - N_2]^0) \quad (1)$$

$P^2_{N-N_2}$: Total N-N_2 production at $t = n$ (mg); V^i_g: Biogas produced between $t = i - 1$ and $t = i$ (dm^3); V^n_{GH}: Volume of the gas headspace of each reactor at $t = n$ (dm^3); $[N\text{-}N_2]^i$: Dinitrogen content in the produced biogas at $t = i$ (mg dm^{-3}).

The accumulation of nitrogen gas in the headspace of reactors has to be considered in this calculation. Moreover, the nitrogenous oxides ($N - NO_x$) sampled and thus not available for reduction were also taken into consideration to determine the true amount of $N - NO_x$ reduced.

The denitrification rate was calculated according to eqn (2).

$$\text{Denitrification rate (\%)} = \left(1 - \frac{(N_{reduced} - N_{produced})}{N_{reduced}}\right) \times 100 \quad (2)$$

with

$$N_{reduced} = (N - NO_x)_i - (N - NO_x)_s - (N - NO_x)_f \text{ (mg)}$$

$$N_{produced} = P^n_{N-N_2O} + P_{N-N_2} \text{ (mg)}$$

$(N - NO_x)_i$ = initial nitrate or nitrite content (mg N); $(N - NO_x)_s$ = nitrate and/or nitrite taken in samples (mg N); $(N - NO_x)_f$ = final nitrate and/or nitrite content (mg N) = 0 in our experiments.

3 RESULTS

3.1 Influence of the COD/N $- NO_x$ ratio on denitrification

It has been recently shown that a COD/$N - NO_3$ ratio has not any effect on the shift between denitrification and nitrate dissimilation to NH_4^+ using a molasses wastewater.[28] At the highest ratio tested during this work (29·2 mg mg^{-1}) no ammonification was detected but complete denitrification was observed. To complete these results, three new batch reactors were run at higher COD/$N - NO_x$ ratios using not only nitrate but also nitrite. Table 1 shows that despite a ratio as high as 65·6 nitrogen oxides were completely reduced to N_2. In addition, the TKN content did not increase during the experiment which confirms that nitrate and nitrite had not been reduced to ammonia.

3.2 Characteristics of nitrate denitrification

A lag phase was usually observed before nitrate reduction (Fig. 2). It has been previously shown that sulphide is responsible for a transient inhibition of denitrification since the higher the initial sulphide content, the longer the lag phase.[27]

Nitrate denitrification was also characterized by a transient nitrite accumulation. Furthermore, from Fig. 2 it appears that molecular nitrogen production started after the complete reduction of nitrogen oxides which suggests an accumulation of intermediates such as nitric oxide (NO) and nitrous oxide (N_2O). A few hours before the nitrogen release, NO and N_2O were effectively detected in the gas headspace of the reactors (respectively 6 and 10% (w/v) at $t = 70$ h). However, it is difficult to quantify accurately NO by gas chromatography, because of its instability.

Finally, during the denitrification of nitrate, sulphide consumption and an increase in the redox potential occurred while sulphate reduction was totally inhibited (Fig. 3). In control cultures performed without nitrogen oxide, the redox potential fell to −500 mV, sulphate reduction began at $t = 40$ h and produced sulphide (results not shown). Concurrently, the colour of the medium in the denitrifying reactors turned from black to brown and yellow particles appeared in the broth, suggesting the formation of elemental sulphur.

It might be thought that the redox potential and the sulphide content are directly related to each other. However Fig. 3 shows that this may not be so obvious since the redox potential increased about 20 h before the sulphide decreased.

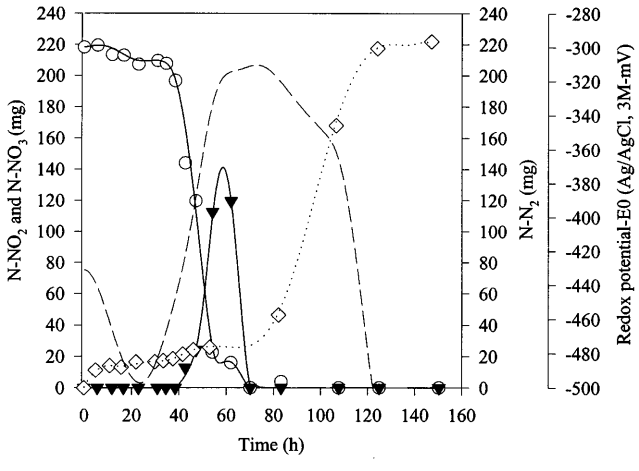

Fig. 2. Nitrate reduction in batch culture using a molasses wastewater as an organic carbon source at an initial nitrate concentration of 210 mg N dm^{-1}. ○, $N - NO_3$ (mg); ▼ $N - NO_2$ (mg); ◇, total produced $N - N_2 - P^n_{N-N_2}$ (mg) (culture 1, Table 1).

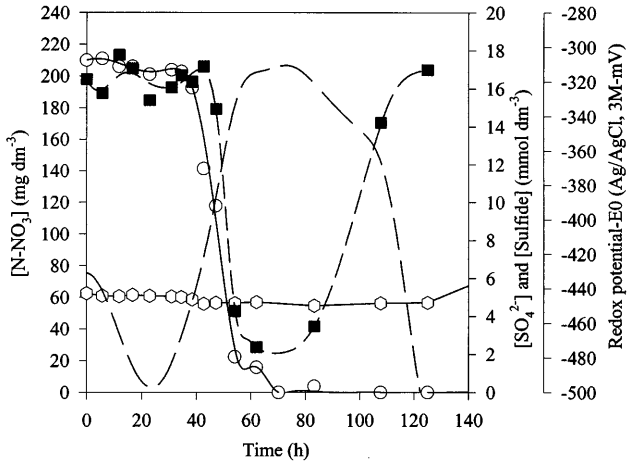

Fig. 3. Nitrate reduction in batch culture using a molasses wastewater as an organic carbon source at an initial nitrate concentration of 210 mg N dm^{-3}. ○, $N - NO_3$ (mg); ■ dissolved sulphide (mmol dm^{-3}); ⬡ SO_4^{2-} (mmol dm^{-3}); — —, redox potential (E_0—mV) (culture 1, Table 1).

3.3 Nitrite denitrification

Unlike nitrate reduction, nitrite denitrification began immediately without any significant lag phase (Fig. 4). In addition, we did not observe any methane production. Of course, it cannot be concluded if nitrite or its reduction products were responsible for this failure. Figure 4 shows that nitrite was truly denitrified since the $N - N_2$ produced is equal to the $N - NO_2$ initially supplied. Moreover, the evolution of the nitrite content suggests an accumulation of NO and N_2O, as in the case of nitrate. Indeed, during the first 30 operating hours, 50% of the initial nitrite as reduced without any gas release. Furthermore, a gradual increase of the N_2O content in the headspace of the reactors till about 5% (v/v) at $t = 35$ h has been observed. Ten hours later, N_2O was not detected in the gas. However the nitrogen

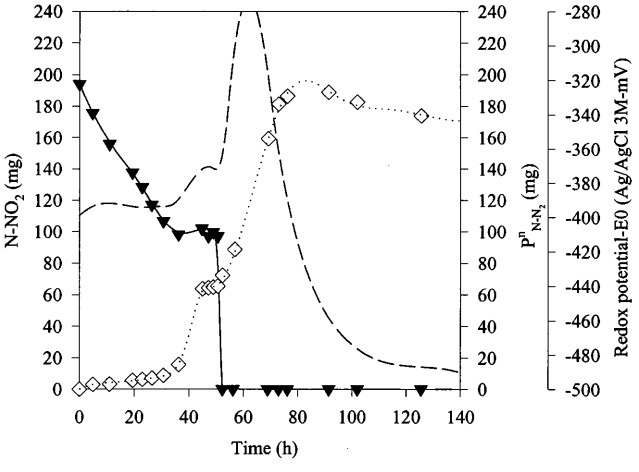

Fig. 4. Nitrite reduction in batch culture using a molasses wastewater as an organic carbon source at an initial nitrite concentration of 197 mg N dm^{-3}. ▼, $N - NO_2$ (mg); ◇, total produced $N - N_2 - P^n_{N-N_2}$ (mg); — —, redox potential (E_0—mV) (culture 2, Table 1).

balance shows that at $t = 45$ h, 25 mg of the initial $N - NO_2$ was not recovered. Therefore, it is thought that the missing nitrogen was possibly NO in a free form or bound to metalloproteins since the N_2O disappeared at $t = 45$ h. Finally, the reason for sudden acceleration of the nitrite reduction near $t = 50$ h remains unclear. It was coupled to a large increase of the redox potential.

Again, sulphate reduction was inhibited during the experiment (Fig. 5). Like nitrate denitrification, nitrite reduction used sulphides, probably as electron donors. One can remark that the sulphide consumption did not cause a significant redox potential increase which occurred only after the sulphide concentration decreased. In addition, the colour in the reactors turned brown, and yellow particles appeared in the broth as previously noticed during the nitrate reduction, suggesting the formation of elemental sulphur.

3.4 Chemical denitrification of nitrite

Chemodenitrification could be the process for NO and N_2O production and could also explain the linear nitrite reduction systematically observed at the beginning of the experiments. Several mechanisms have been proposed, such as a decomposition of NH_2OH or a reaction of HNO_2 with phenolic groups of organic matter.[31–33] However the major mechanism is a reduction of nitrite by metals such as iron in the reduced state (eqn (3)).

$$Fe^{2+} + NO_2^- + 2H^+ \rightarrow Fe^{3+} + NO + H_2O \quad (3)$$

This endergonic reaction depends strongly on pH. It is favoured at acidic pH and very slow at pH 7.

From Fig. 6, it appears that after sterilization, the sludge used during this study could not reduce nitrate

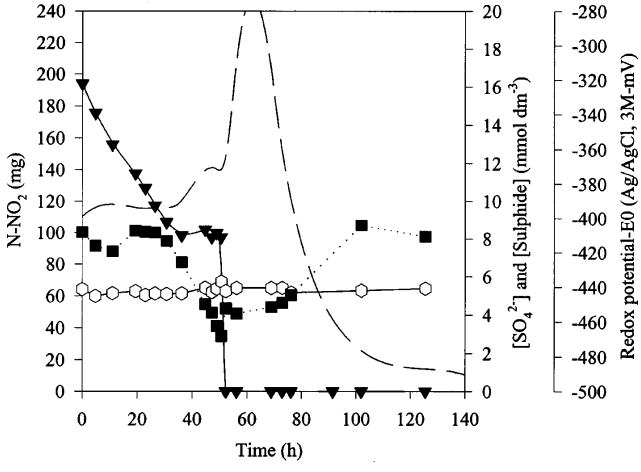

Fig. 5. Nitrite reduction in batch culture using a molasses wastewater as an organic carbon source at an initial nitrite concentration of 197 mg N dm^{-3}. ▼, $N - NO_2$ (mg dm^{-3}); ■, dissolved sulphide (mmol dm^{-3}); ⬡, SO_4^{2-} (mmol dm^{-3}); — —, redox potential (E_0—mV) (culture 2, Table 1).

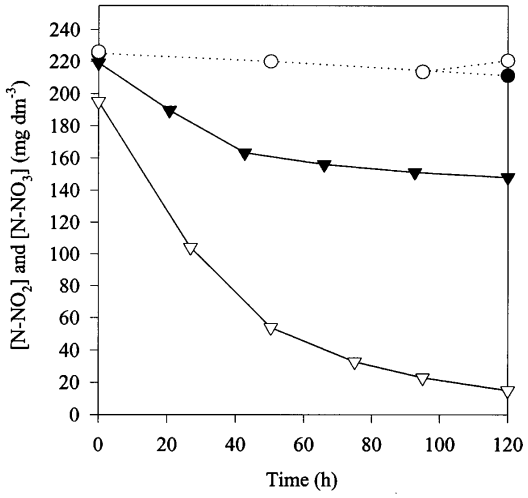

Fig. 6. Evidence of chemical nitrite reduction by Fe^{2+} in sterilized sludges compared with nitrate experiments as control flasks, with and without $FeCl_2$ addition. ●, $N-NO_3$ (mg dm^{-3}); ○, $N-NO_3$ (mg dm^{-3}) + Fe^{2+} as $FeCl_2$ (70 mmol dm^{-3}); ▼, $N-NO_2$ (mg dm^{-3}); ▽, $N-NO_2$ (mg dm^{-3}) + Fe^{2+} as $FeCl_2$ (70 mmol dm^{-3}).

even when iron was supplied. Conversely, nitrite reduction still occurred but stopped after a few hours probably because ferrous iron became limiting. Indeed, in cultures enriched with iron, the nitrite reduction proceeded much more quickly and it was complete. This experiment suggests that an abiotic mechanism can be considered to explain the slow and linear nitrite reduction which occurred from the onset in the cultures described previously. However, during this linear phase, nitrite was reduced by 50% while after sterilization, the nitrogenous oxide content never decreased below 70% of its initial value (in standard conditions, i.e. without iron addition).

3.5 Evidence of autotrophic denitrification using a sludge acclimated to a molasses wastewater

It has been previously shown that the reduction of nitrogenous oxides was associated with sulphide consumption and concurrent elemental sulphur production. It has been proposed that denitrifiers can use sulphides as electron donors. The existence of such an autotrophic phenomenon during our cultures had to be proved even though it had been shown by Mulder *et al.*[34] in a similar process. For that purpose, two new cultures were performed without any organic carbon source supply. Before the addition of nitrate or nitrite, the sludge was left in starvation for a few days conditions to ascertain that all the organic carbon residuals remaining had been degraded.

From Fig. 7 it can be seen that the TOC content did not decrease during the study. It was probably organic matter which was difficult to biodegrade. Nitrate and nitrite were completely reduced without any organic carbon consumption. According to the stoichiometry of

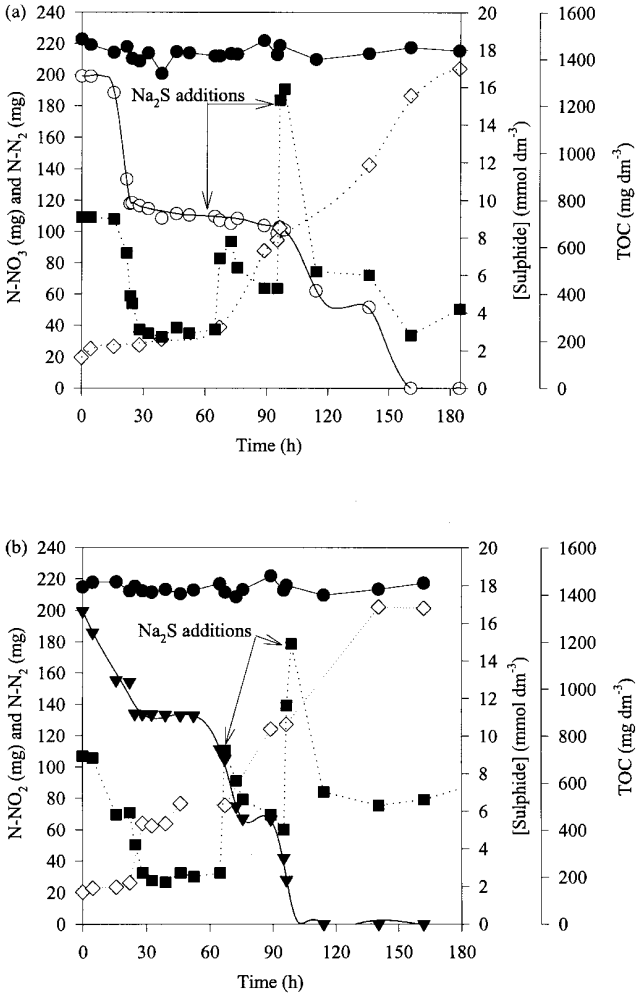

Fig. 7. Evidence of autotrophic denitrification using a sludge acclimated to a molasses wastewater after a period of starvation to remove the non-biodegradable organic matter. Nitrate (a) or nitrite (b) at an initial concentration of 200 mg dm^{-3} were used as electron acceptors. ○, $N-NO_3$ (mg); ▼, $N-NO_2$ (mg); ◇, total produced $N-N_2-P^n_{N-N_2}$ (mg); ■, dissolved sulphide (mmol dm^{-3}); ●, TOC (mg dm^{-3}).

heterotrophic denitrification, the complete reductions of 200 mg $N-NO_3$ or $N-NO_2$ need respectively 570 and 342 mg COD. Considering a COD/TOC ratio of 3·0 in the sludge (result not shown), that means that a minimum decrease of the TOC contents of 190 and 114 mg for, respectively, nitrate and nitrate reductions should have been observed if heterotrophic denitrification had occurred. Moreover, the consumption of sulphide was directly related to the nitrogen oxide consumption at the beginning of the culture. Denitrification kept its previously-described characteristics. The change in nitrate and nitrite content in particular the existence of plateaus, was probably due to NO and N_2O accumulations rather than to a sulphide limitation or to a sulphide-use threshold. Indeed, Na_2S additions did not change the kinetics of the nitrite and nitrate reductions. Moreover, N_2 production occurs without nitrate or nitrite reduction respectively at $t = 70$ h and

$t = 25$ h (Fig. 7) which suggests the use of NO and/or N_2O.

4 DISCUSSION

Production of NO and N_2O was suggested during the nitrate reduction experiment (Fig. 2). It is known that NO has a high affinity for metal ions located in the active sites of enzymes. This may have affected the activity of reductases which contain Fe—S groups, haems and copper ions. It has been suggested that bacteria could be protected from this toxic compound by metalloproteins which exhibit reversible NO-binding and release NO on interaction with the reductases.[31] This phenomenon, which makes uncertain the dissolved NO quantification, would not be efficient enough in these experiments and the NO and N_2O accumulations, in addition to nitrite build-up, could be responsible for the transient inhibition of nitrate reductases (NAR) which appears between 50 and 70 h (Fig. 2).

The aforementioned observations on the nitrite reduction experiments suggest that, when nitrite reduction failed at $t = 35$ h (Fig. 3), the N_2O content in the headspace was maximal and the amount of free NO was probably very high. Therefore, the inhibition of nitrite reductases (NIR) could have been due to their accumulation. It can be noticed that the N_2O reduction which appeared at $t = 35$ h slowed down considerably when the N_2O content in the headspace became limiting. It seems that the quick N_2 production and probably the inhibition of the NIR and NO reductase (NOR) caused the exhaustion of N_2O. Such as inhibition of the NOR and the NIR by nitrate and NO respectively has been reported by others.[32]

The difference observed in the nitrite reduction with or without sterilization could be explained by a combined process with chemodenitrification and biological reactions. In that way, Zumft[31] and Von Schulthess *et al.*[32] described that cultures of *Escherichia coli* were capable of reducing Fe^{3+} to Fe^{2+}. Then, ferrous iron chemically reacts with nitrite to produce ferric iron and NO. Fe^{3+} can be reused for a new cycle. During our experiments, the N_2 production which started near $t = 35$ h (Fig. 4) and which corresponded to biological activity caused the failure of the chemodenitrification. It is possible that this biological denitrification induced a modification of the culture conditions, for instance a slight increase of the redox potential, and/or stopped the biological Fe^{2+} production from Fe^{3+}. Indeed, Achtnich *et al.*[35] showed that the activities of ferric iron reducers were affected by denitrifiers. They suggested a competition for electron donors between both flora.

This chemical theory makes easier the understanding of the redox potential evolution which did not change during the possibly abiotic phase and then increased greatly when a biological nitrite reduction would have appeared. Finally, chemodenitrification would explain why a lag phase preceded nitrate reduction while it did not exist during nitrite reduction.

However, even if chemodenitrification could be an important phenomenon in this study, a biological explanation for the linear and immediate nitrite reduction must not be turned down. For instance, NAR activity could have been affected by an inhibitor originating from the molasses wastewater, while NIR would be insensitive to such a compound. Its degradation during acetogenesis would have allowed the development of NAR. Furthermore NIR could be constitutive while NAR synthesis induced by nitrate could be inhibited, for instance by H_2S.

5 CONCLUSIONS

It has been shown that a $COD/(N - NO_x)$ ratio up to 65 does not promote nitrogenous oxides ammonification. In addition, while nitrate denitrification began after a lag phase, nitrite reduction was immediate.

Evidence is presently accumulating that chemodenitrification of nitrite could explain this difference. Indeed, sterilized sludges were able to reduce the nitrogenous oxide. The phenomenon was increased when ferrous iron was supplied to the broth. Biological reduction of ferric iron would also be coupled to this chemical reaction and restore free Fe^{2+} which would be available for a new cycle. However, the difference between nitrate and nitrite reductions might be the result of a biological phenomenon such as possible inhibition of the NAR activity by a toxic compound, the NIR remaining insensitive. To conclude, further experiments are needed. For instance, it would be very interesting to chelate iron using EDTA to know if denitrification still occurs immediately after addition of nitrite under these new conditions. If so, the chemical theory will have to be re-examined.

Using a molasses wastewater, nitrate and nitrite reductions were also characterized by sulphide consumption, elemental sulphur production and an increase in the redox potential. It has been proved that denitrifiers used sulphide as an electron donor.

ACKNOWLEDGEMENTS

This work was supported by a research grant from the 'Agence de l'Environement et de la Maitrise de l'Energie', (ADEME), Paris, France.

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