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NITRATE AND NITRITE REDUCTIONS WITH ANAEROBIC SLUDGE USING VARIOUS CARBON SOURCES: GLUCOSE, GLYCEROL, ACETIC ACID, LACTIC ACID AND METHANOL

J. C. AKUNNA, C. BIZEAU and R. MOLETTA*

Institut National de la Recherche Agronomique, Laboratoire de Biotechnologie de l'Environnement des IAA, Boulevard Général de Gaulle, 11100 Narbonne, France

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Abstract—Batch-tests were used to determine the potentials of digested sludge to reduce nitrate and nitrite in the presence of five different carbon sources: glucose, glycerol, acetic acid, lactic acid and methanol. Ammonium accumulation was found in glucose and glycerol media. Dissimilatory reduction to ammonium accounted for up to 50% of reduced nitrate and nitrite. The rest were denitrified. In the media containing these carbon substrates volatile fatty acids, particularly acetic acid, were produced and ammonification was higher than denitrification activities only when glucose and glycerol were still present in the media. Ammonium production was higher in nitrite cultures than in nitrate cultures. In the culture media with acetic and lactic acids and methanol, ammonium was not detected. Nitrate/nitrite reduction in acetic and lactic acids media was essentially denitrification activity. Up to 100% of reduced nitrate and nitrite in the culture media with these acids were denitrified at average rates between 27 and 23 mg $N\text{-}NO_x$/g MLVSSh, nitrite reduction rate being about 14% lower than total nitrate reduction rate. COD requirements for nitrate and nitrite reductions were generally lower in cultures with acetic and lactic acids than in glucose and glycerol cultures. Methanol culture media showed a very small reduction rate for the $N\text{-}NO_x$ indicating the absence (or presence in very small quantity) of the bacteria capable of denitrifying with this substrate.

Key words—anaerobic sludge, denitrification, ammonium production (ammonification), nitrate, nitrite, glucose, glycerol, acetic acid, lactic acid, methanol

INTRODUCTION

A single integrated denitrification/methane production system has been proposed (Hanaki and Polprasert, 1989; Kuroda *et al.*, 1988; Akunna *et al.*, 1992). When treating sewage wastewaters with an anaerobic baffled reactor equipped with a separate nitrification unit, Garuti *et al.* (1991) obtained more than 80% COD, suspended solids and nitrogen removal efficiency and produced 50% less sludge than the traditional nitrification/denitrification process. But according to some workers (Garcia, 1982; Tiedje, 1981, 1988), dissimilatory nitrate reduction to ammonium is the major nitrate reduction pathway in anaerobic digesters, because of the abundance of facultative and obligate anaerobes (ammonium formers). It is also believed that nitrate–ammonium reduction is favoured in carbon-rich (or nitrate-poor) environments (Tiedje, 1981, 1988). This was confirmed in our recent studies with glucose as carbon source (Akunna *et al.*, 1992). In sediment slurry, the same behaviour was also observed (King and Nedwell, 1985).

The ammonium formed is used for the synthesis of organic nitrogen compounds and the excess is excreted into the culture. In experiments with pure cultures of *Escherichia coli* (a facultative anaerobe) with glucose and glycerol, it was found that up to 75% of added nitrite can accumulate as ammonium (Cole, 1978). Anaerobic sludge has been found to exhibit higher ammonification (60–70%) than denitrification (30–40%) potentials (Kaspar *et al.*, 1981).

Knowing that ammonium-formers are abundant in anaerobic conditions, nitrate reduction in such systems is not advisable since it would result in nitrogen conservation instead of nitrogen losses via gaseous products of denitrification.

In this work, therefore, we have tried to find out the effects of the nature of carbon substrates in the nitrate–ammonium and nitrate–nitrogen gas reduction pathways, using an anaerobic methane-producing sludge that was not previously acclimatized to nitrate or nitrite. To really appreciate the activities of ammonium formers (presumed abundant in digested sludge) and true denitrifiers (assumed absent or present in small amounts in this type of sludge), these experiments were carried out in a

*Author to whom all correspondence should be addressed.

non-growth medium in order to evaluate the potentialities of the bacterial flora already abundant in the digester.

Two series of experiments were carried out using nitrate and nitrite respectively in order to compare and evaluate their individual performances.

MATERIALS AND METHODS

Preparation of batch cultures

Anaerobic sludge was obtained from a 2-year-old laboratory pilot anaerobic digester enriched with glucose, peptone and yeast extracts as carbon and nitrogen sources. Before the sludge inoculum was taken the digester was left running without feeding for about 1 week in order to exhaust all the carbon residuals remaining to avoid interferences with the carbon sources to be studied. At the time of sludge sampling, biogas from the digester was made up of about 65% methane and 33% carbon dioxide.

A synthetic non-growth medium containing monopotassium and dipotassium phosphate (3000 mg/l) (served also as buffer agents); $NaHCO_3$, 400 mg/l; $MgSO_4 \cdot 7H_2O$, 5 mg/l and some trace elements ($FeCl_2$, 0.5 mg/l; $CaCl_2$, 0.5 mg/l; KCl_2, 0.5 mg/l and $CoCl_2$, 0.1 mg/l) were put in five 500 ml-capacity incubation bottles. Each bottle contained 360 ml of synthetic medium and 40 ml of sludge inoculum which summed up to a total volume of 400 ml. Into each bottle, 578 mg KNO_3 (corresponding to 200 mg $N\text{-}NO_3$/l in 400 ml solution) was added. Glucose (1125 mg), glycerol (986 mg), acetic acid (1000 mg), lactic acid (1125 mg) and methanol (800 mg) were, respectively, added to the bottles. The amount of each carbon substrate in 400 ml solution corresponded to approx. 3000 mg COD/l, except the acetic acid medium which was slightly less (Table 1).

The same operation was repeated using nitrite. 394 mg $NaNO_2$ (or 200 mg $N\text{-}NO_2$/l in 400 ml solution), was added to each bottle followed by the addition of the carbon substrates as done in the tests with nitrate. Before incubation at 30°C, the pH of each bottle was adjusted to about 7.5 using 10 N NaOH. Anaerobic condition was established in each bottle by flushing with pure argon for 5–10 min. Table 1 summarizes the composition of each culture media.

Analytical methods

Eight samples were collected from each test bottle during the 4-day experimental period. MLSS and MLVSS analysis were performed as described in *Standard Methods* (APHA, 1985) for the samples collected at the beginning and end of the experiment. Filtrate COD was measured by the potassium dichromate–ferrous ammonium sulphate method. Nitrate and nitrite nitrogen concentrations were determined on samples filtered through 0.2 μm nylon membrane syringe filters (Nalgene) by ion chromatography system using conductivity detection (Dionex DX 100), and ammonium nitrogen by distillation method (Rodier, 1975). Concentrations of glucose, glycerol and volatile fatty acids were determined by HPLC with a HPX 87 H column (Bio-Rad) and a refractive index detector (Waters).

RESULTS AND DISCUSSION

MLVSS at the end of the experiments was about 0.3 g/l in the glucose and glycerol media and about 0.2 g/l in the acetic and lactic acid media (an increase of 0.1 and 0.2 g/l, respectively). The increase in MLVSS accounted for 10 and 5% of the nitrogen removed, respectively, for these media. The growth observed was significant knowing that the medium was initially considered as a non-growth medium with only nitrate and nitrite as nitrogen sources. However, the growth was small for the amount of COD utilized. Thus, nitrate and nitrite assimilated for biomass synthesis were not taken into account in nitrogen balance calculations. There was no significant change in the MLVSS content of the methanol culture medium.

For nitrate reduction, $N\text{-}NO_x = N\text{-}NO_3 + N\text{-}NO_2$. All references to nitrogen eliminated, do not refer to $N\text{-}NO_3$ which has only been converted to $N\text{-}NO_2$.

Glucose (nitrate culture medium)

Figure 1 shows that with glucose nitrate reduction started earlier than with most of the carbon substrates used. The conversion of nitrate was rapid (Fig. 2). During this period (between 6 and 18 h) ammonium and a large amount of nitrite accumulated in the medium (Figs 3 and 4) and the pH value fell from 7.5 to 6.7 (Fig. 5). Glucose concentration fell from 2900 to 600 mg/l at 18 h (Fig. 9). Volatile fatty acids, principally acetic acid and glycerol, were also produced in the medium.

At 18 h, there was no more nitrate (Fig. 2) and the nitrite accumulated was maximum (140 mg $N\text{-}NO_2$/l). Nitrite reduction beyond 18 h was slow

Table 1. Characteristics of each culture medium

	Glucose medium	Glycerol medium	Acetic acid medium	Lactic acid medium	Methanol medium
Total vol. (ml)	400	400	400	400	400
MLVSS (mg/l)	100	100	100	100	100
$N\text{-}NH_4$ (mg/l)	0	0	0	0	0
	First series: nitrate reduction				
COD soluble (mg/l)	2937	2966	2569	2679	2946
$N\text{-}NO_3$ (mg/l)	200	200	200	200	200
$COD/N\text{-}NO_3$	14.7	14.8	12.9	13.4	14.7
C/N	5.4	4.8	4.8	5.0	3.7
pH initial	7.5	7.5	7.4	7.4	7.5
	Second series: nitrite reduction				
COD soluble (mg/l)	2990	2900	2600	3100	3010
$N\text{-}NO_2$ (mg/l)	200	200	200	200	200
$COD/N\text{-}NO_2$	15.0	14.5	13.0	15.5	15.0
C/N	5.5	4.7	4.8	5.7	3.8
pH initial	7.5	7.5	7.5	7.5	7.5

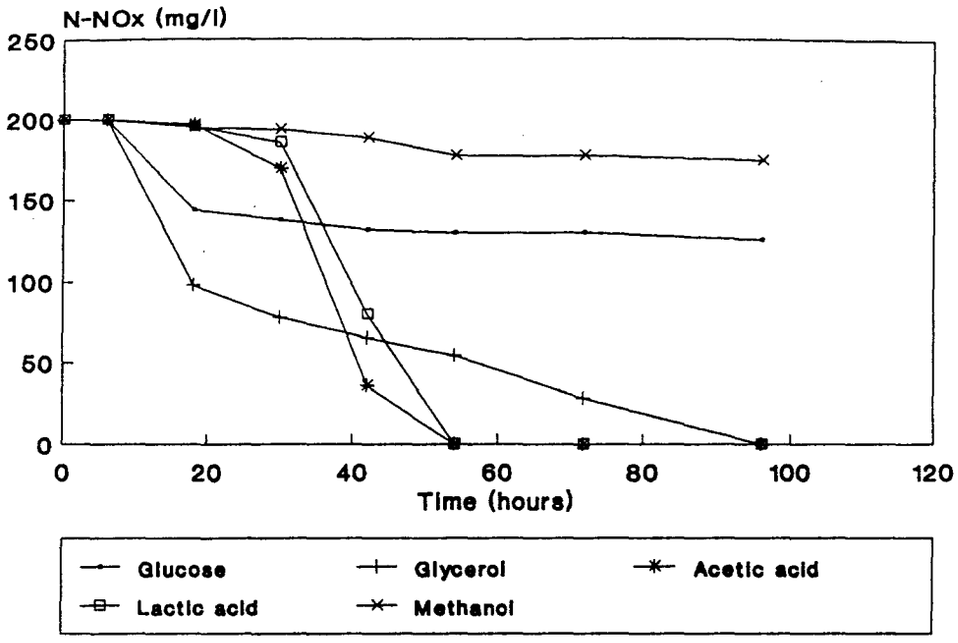

Fig. 1. Time–course of total nitrogen reduction (Figs 2 and 3).

probably because of the toxic effects due to its high concentration coupled with very low pH value (6.7). Acetic acid and glycerol content of the culture did not change appreciably during this period thus confirming the inhibition effects. Ammonium accumulation rate also decreased considerably. Its concentration of about 35 mg $N\text{-}NH_4$/l at 42 h did not significantly change afterwards. At the end of the experiment about 126 mg/l of nitrite nitrogen was also found remaining in the media. Denitrification activity counted for about 50% of the total nitrate lost.

These results suggest that with glucose, ammonification is greater during the acidification process. The initial pH fall means that denitrification activity

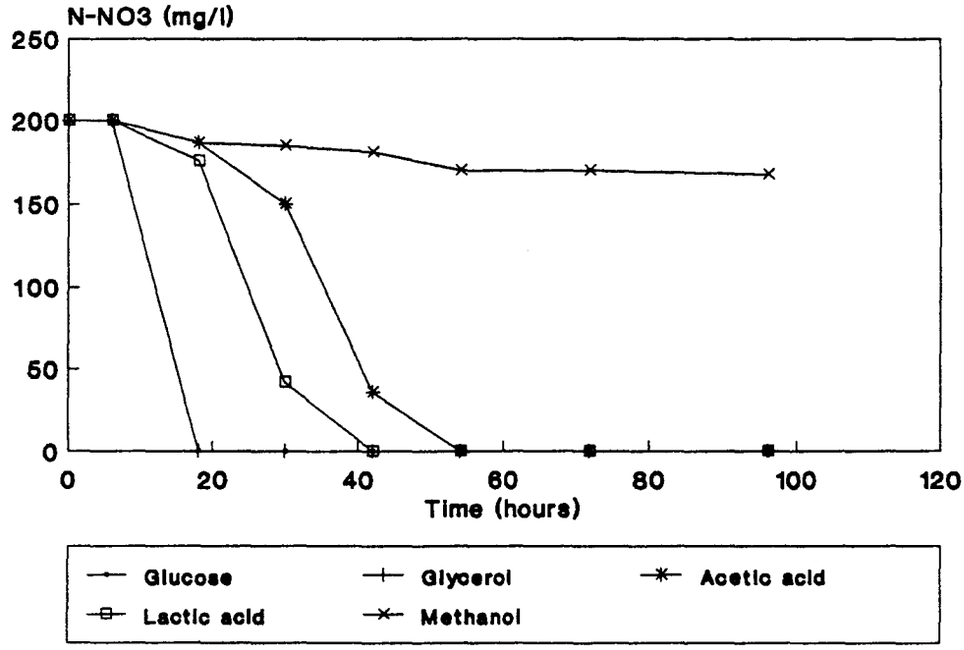

Fig. 2. Nitrate consumption in nitrate culture vs time.

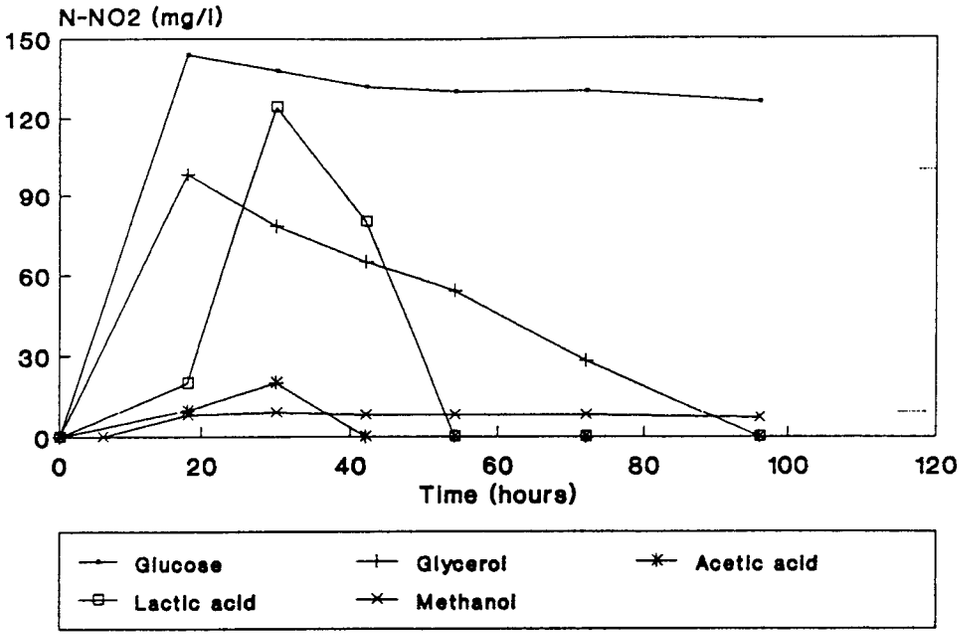

Fig. 3. Nitrite accumulation and reduction in nitrate cultures.

was not high enough to cancel the effects of acid production.

Glucose (nitrite culture medium)

The same behaviour as in the nitrate culture was observed in the nitrite culture except the absence of obvious inhibition (Figs 6–8). As a result, acidification (Figs 8 and 9), ammonification (Fig. 7) and denitrification processes were higher in this medium. The lowest pH value, 6.5, was observed at 70 mg $N\text{-}NO_2$/l compared with almost the same pH value at 140 mg $N\text{-}NO_2$/l noted in the nitrate culture.

All the glucose disappeared after 30 h (Fig. 9) with the production of acetic acid. Unlike in the nitrate medium, there was no accumulation of glycerol which is an intermediary by-product of glucose transformation. Beyond 18 h the acetic acid production rate decreased. The marked difference between the acid production in the nitrate and nitrite

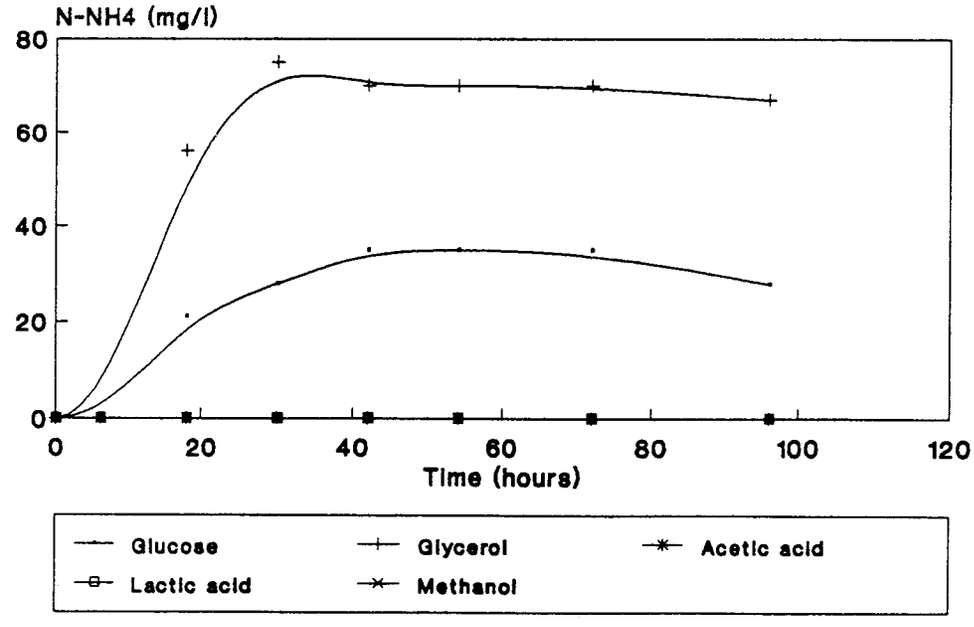

Fig. 4. Ammonium accumulation in nitrate cultures.

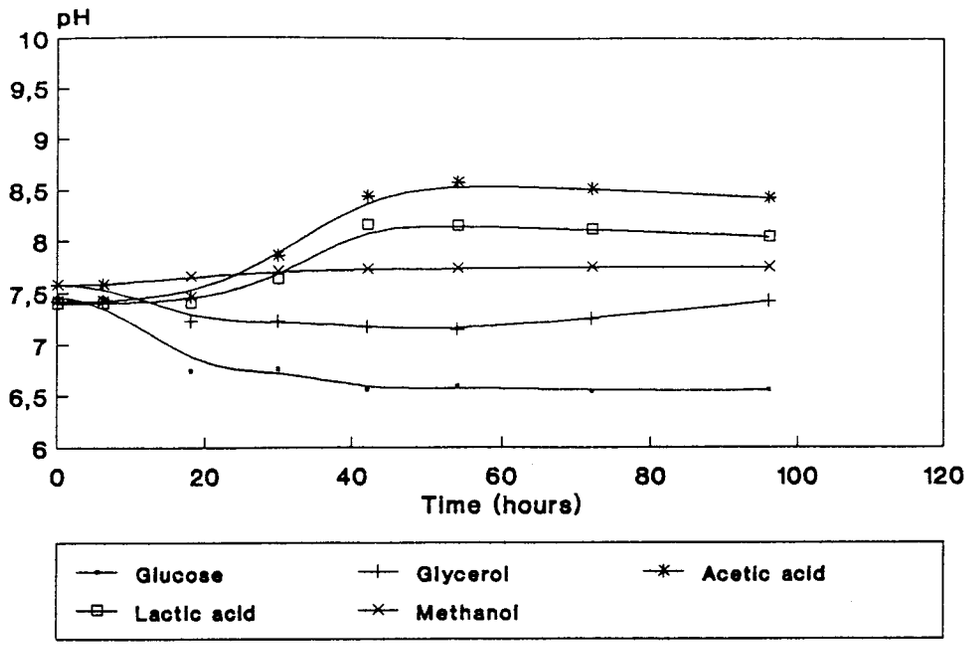

Fig. 5. pH variations in nitrate curves vs time.

media was due to lower activities observed in the former.

It is interesting to note that nitrite reduction after 30 h did not result in significant ammonium production (Fig. 7), suggesting a relatively higher denitrification activity. Since in Fig. 9 it can be seen that there was no more glucose in the medium after 30 h, it indicates that the ammonification process was greater when glucose was in the medium (before its total conversion to simpler products such as acetic acid).

Glycerol (nitrate culture medium)

High initial nitrate conversion rate was also noticed in the glycerol culture medium between 6 and 18 h during which the medium pH value fell from 7.5 to 7.2 (Fig. 5). Nitrite and ammonium were the principal by-products of the reaction just as in the

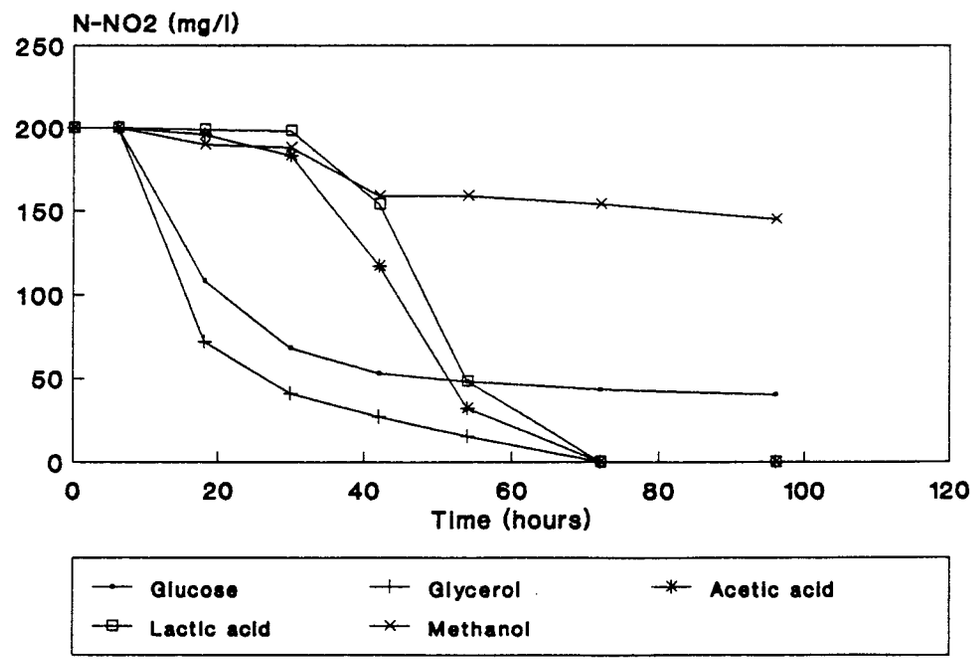

Fig. 6. Time-course nitrate reduction in nitrate cultures.

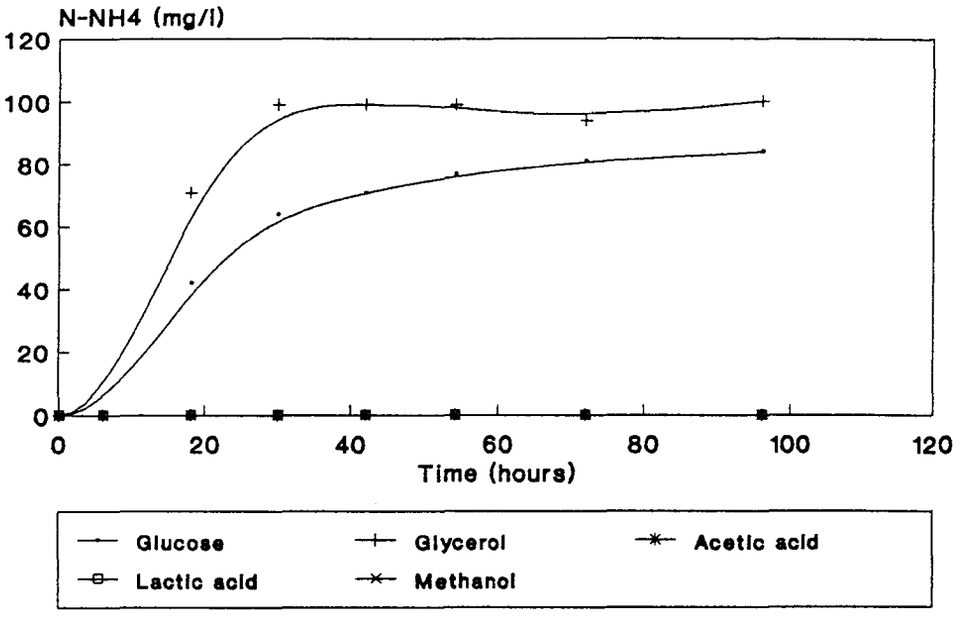

Fig. 7. Ammonium accumulation in nitrite cultures.

glucose medium (Figs 3 and 4). Maximum nitrite concentration of 100 mg $N\text{-}NO_2/l$ was observed at 18 h. The ammonium concentration in the medium was high, increasing from 0 at 6 h to 56 mg $N\text{-}NH_4/l$ at 18 h. A nitrogen balance shows that about 20% of the added nitrate nitrogen was lost by denitrification during this period. All the nitrate disappeared at this time just as in the glucose medium (Fig. 2). During the same period all the glycerol was used up with the production of volatile fatty acids composed principally of acetic acid (Fig. 10).

From 18 h, reduction of accumulated nitrite continued but at a lower rate than the initial reduction rate. The pH values started rising which was a good indication of denitrification activity. Ammonium accumulation rate decreased drastically and its concentration became relatively constant after 30 h at about 77 mg $N\text{-}NH_4/l$. Nitrite reduction continued after 30 h but no significant increase in the ammonium content of the medium was noted. At 96 h, all the nitrites in the medium were eliminated resulting in more than 60% denitrification activity.

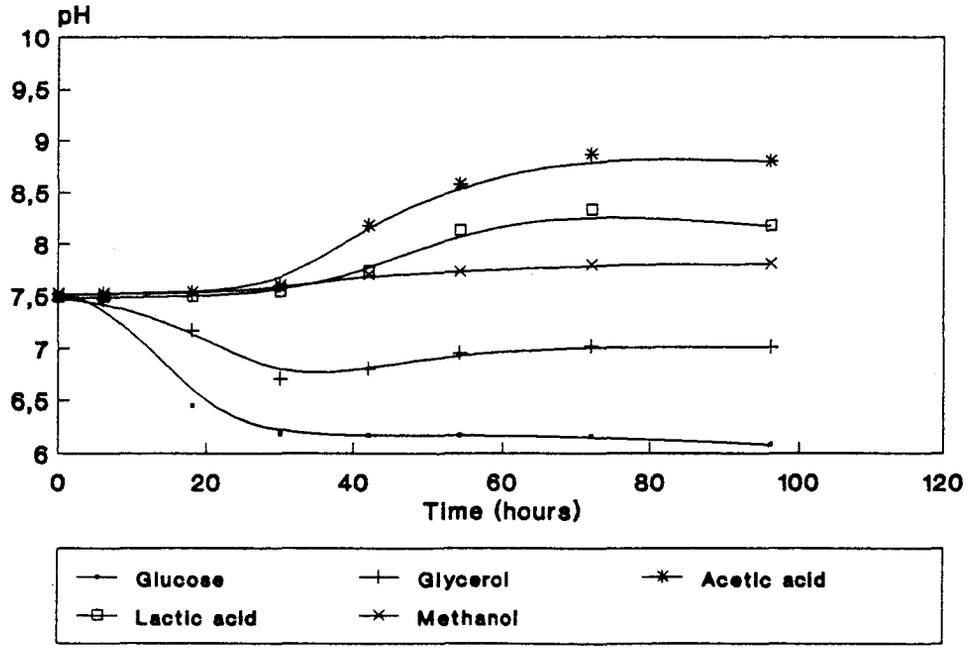

Fig. 8. pH variations in nitrite cultures vs time.

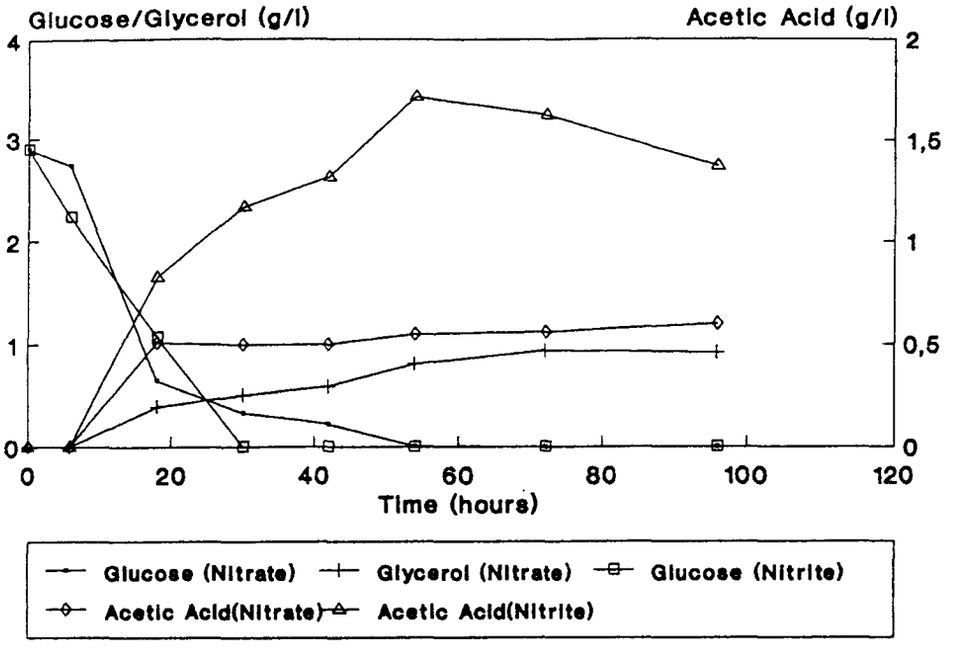

Fig. 9. Glucose consumption and glycerol and acetic acid production and consumption in nitrate and nitrite media.

From Fig. 10 it will be seen that the high denitrification activity observed after 30 h occurred when there was little or no glycerol in the medium. Just as in the glucose culture medium, ammonification was believed to be favoured when all the glycerol had not been converted to simpler molecules.

Glycerol (nitrite culture medium)

Similar results in the glycerol–nitrate culture medium were obtained in glycerol–nitrite culture medium except that at the end of the experiment, 50% of reduced nitrite was found as ammonium compared with 38% observed in the glycerol–nitrate culture medium.

Acetic and lactic acids (nitrate and nitrite culture media)

The reduction of nitrate in the presence of these acids did not start as rapidly as in the cases of glucose and glycerol. An 18-h passive period elapsed before significant nitrate reduction was observed (Figs 1–3). During this period, ammonium accumulation was not observed and the pH values were constant (Figs 4 and 5). From 18 h, nitrate reduction started and the rate was maximum between 30 and 42 h in the two media. Nitrite accumulation was noticed in the lactic acid medium, up to 120 mg $N\text{-}NO_2$/l at 30 h when the pH value was 7.7. The accumulated nitrite did not pose problems as evidenced by its continued reduction, probably due to favourable pH value. Figures 4 and 5 show clearly that nitrate reduction in these media does not result in ammonium accumulation, but results in hydroxide ion production which raises the alkalinity of the media, evidence of denitrification activity. All the added nitrate in these two media was denitrified.

The same observations were made in nitrite culture. A longer passive period (30 h) was the major difference noted (Figs 6–8).

The absence of ammonium in these culture media suggests that ammonium formers in the digested sludge used do not consume fatty acids to carry out dissimilatory nitrate or nitrite reduction to ammonium. It could also imply that the nitrate/nitrite–ammonium reduction pathway takes place principally during acidification (or fermentation) of carbon substrates, suggesting that ammonium formers and acidifiers (fermenters) use the same kind of carbon substrates.

Ammonium might have been formed in these media (acetic and lactic acid), but only at the rate and amounts required for the synthesis of organic nitrogen compounds. This is true for assimilatory nitrate reduction. The assimilatory process is believed to be regulated by the ammonium and organic nitrogen in the medium (Tiedje, 1988). Nitrate being the only nitrogen source in these experiments, the multiplication of denitrifying populations must have been slow since assimilatory nitrate reduction is known to be a slow process. This might explain the 18 h passive period observed in these media.

Methanol (nitrate and nitrite culture media)

Figures 1–8 show that negligible reactions occurred in the methanol culture medium. Nitrate and nitrite reductions were very low. There was no ammonium

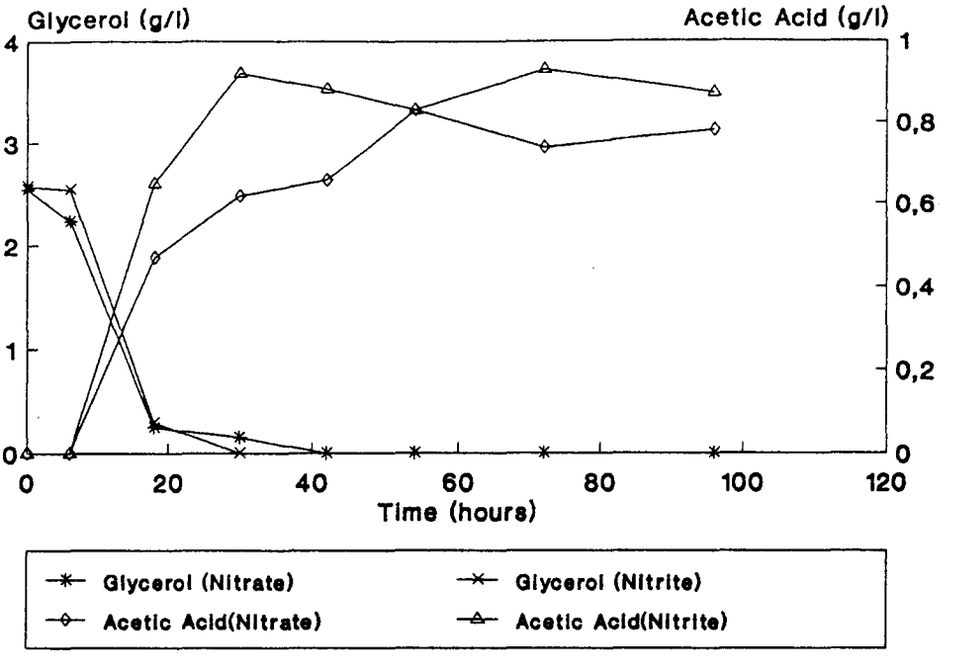

Fig. 10. Glycerol consumption and acetic acid production and consumption in nitrate and nitrite media.

accumulation and the pH values were more or less constant throughout the incubation period. COD measurements showed that methanol was not significantly consumed.

Since ammonium accumulation was not detected in the methanol culture media, it suggests that ammonium formers in digested sludge do not consume alcohols for nitrate–ammonium dissimilatory reduction. On the other hand, denitrification activity was also very low. Methanol has been used successfully as carbon substrate for denitrification (McCarty *et al.*, 1969; Sperl and Hoare, 1971; Nurse, 1980; Blaszczyk *et al.*, 1981). The very low or even negligible reduction rate observed may be due to the absence of the bacteria type capable of consuming it for nitrate reduction. Or that these bacteria were present but they needed a longer adaptation period for their multiplication. It has been reported that denitrification with methanol results in a selective enrichment for bacteria belonging to the genus *Hyphomicrobium* (Sperl and Hoare, 1971; Nurse, 1980).

Comparison of the performances of nitrate- and nitrite-containing culture media

Ammonium accumulation. In both nitrate and nitrite cultures, significant ammonium accumulation was observed only in glucose and glycerol media. At the end of the experiment, up to 50 and 38% of lost nitrate nitrogen were, respectively, found as ammonium nitrogen in the glucose and glycerol media. But in the nitrite culture, 52 and 50% of reduced nitrite–nitrogen were accumulated as ammonium–nitrogen in glucose media, respectively. There seems to be a greater tendency to produce ammonium in nitrite culture.

Reduction rates. Table 2 shows the average nitrogen reduction rates for each of the carbon sources according to the form of nitrogen present in the medium. These rates were calculated from Figs 1 and 6 for nitrate and nitrite culture media, respectively. For nitrate media they represent the reduction rate of $N\text{-}NO_x$ between 6 and 96 h in the glucose and glycerol cultures and between 18 and 54 h in the

Table 2. Average $N\text{-}NO_x$ reduction rates for each of the carbon substrates

Carbon substrate	Form of $N\text{-}NO_x$	Average $N\text{-}NO_x$ reduction rates (mg N/g MLVSSh)	Major reduction pathways
Glucose	Nitrate	2.7	High ammonification and low denitrification
	Nitrite	5.9	High ammonification and low denitrification
Glycerol	Nitrate	7.4	Ammonification and denitrification
	Nitrite	10.1	Ammonification and denitrification
Acetic acid	Nitrate	27.8	Denitrification
	Nitrite	23.8	Denitrification
Lactic acid	Nitrate	27.8	Denitrification
	Nitrite	23.8	Denitrification
Methanol	Nitrate	—	Reduction very low
	Nitrite	—	Reduction very low

Table 3. Ratio between the COD consumed and the N-NO_x eliminated for the different carbon substrates

Form of carbon	Ratio $\frac{\text{mg COD}}{\text{mg N-NO}_x}$		Principal COD-consuming reaction(s)
	Nitrate	Nitrite	
Glucose	5.6	4.9	Ammonification, denitrification and acidification
Glycerol	5.6	4.1	Ammonification, denitrification and acidification
Acetic acid	3.7	2.0	Denitrification
Lactic acid	4.1	2.8	Denitrification

acetic and lactic acid cultures. For the nitrite media, they were calculated between 6 and 96 h for glucose culture, between 6 and 72 h for glycerol and between 30 and 72 h for acetic and lactic acid cultures. The reduction rate of each culture medium was then divided by their MLVSS content at the end of the experiment.

For the glucose and glycerol media, the average nitrite–nitrogen reduction rate was significantly greater than the average nitrate–nitrogen reduction rate. This was because of the ammonification process which was greater in the nitrite culture. Toxicity of high nitrite concentration (140 mg $N\text{-}NO_2$/l) at low pH value (6.7) in the glucose culture during nitrate reduction might have contributed to the relatively lower reduction rate observed. Denitrification rates were 14% greater with nitrate than with nitrite in acetic and lactic acid medium. These two media showed identical performances.

COD consumption. At the end of the experiments, the COD consumed was compared to the nitrate–nitrogen and nitrite–nitrogen completely eliminated for each of the carbon media. The ratios obtained are shown in Table 3. As expected, this table shows that the COD requirements for nitrite reduction were lower than for nitrate reduction. The greater COD consumption in glucose and glycerol media was expected since some of the consumed COD could have been used by the acid formers for the acidification (or fermentation) of these substrates.

For the acetic and lactic acids, the major source of COD consumption was denitrification activity. Assimilation of these acids for cell synthesis might have been responsible for the difference between theoretical and experimental ratios (theoretical ratios not counting yield are 2.86 for nitrate and 1.71 for nitrite).

CONCLUSIONS

This study has shown that digested sludges not initially acclimated to nitrate or nitrite have the potential of reducing N-NO_x. And the by-products of the reduction depend on the nature of the carbon substrate present in the medium.

In the presence of glucose and glycerol, the production of volatile fatty acids dominated by acetic acid was observed and ammonification was greater than denitrification only when these carbon substrates were still present in the media. Denitrification and ammonification activities were greater in the glycerol than in the glucose experiment. With either glycerol or glucose, up to 50% of reduced nitrate or nitrite were found as ammonium. The rest were assumed lost through denitrification. The ammonium production affinity was slightly higher in cultures with nitrite.

Nitrate and nitrite reductions in acetic and lactic acid media did not result in ammonium accumulation. Up to 100% of reduced nitrate or nitrite were denitrified with these carbon substrates at average rates of 27 and 23 mg N-NO_x/g MLVSSh with nitrate and nitrite substrates, respectively. It can thus be concluded that ammonium formers in the anaerobic sludge used in this study do not easily use fatty acids for dissimilatory nitrate–/nitrite–ammonium reactions.

Bacteria capable of reducing nitrate or nitrite in methanol culture media did not seem to be in the digested sludge used. Or, they might have existed but in a very small amount that needed a very long adaptation period. The absence of accumulated ammonium indicated that the fermentative bacteria abundant in the digested sludge were not capable of using methanol for dissimilatory nitrate– or nitrite–ammonium reduction.

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