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EFFECT OF NITRATE ON METHANOGENESIS AT LOW REDOX POTENTIAL

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ABSTRACT

Batch tests were used to study the contribution of redox potential to the inhibition of methanogenesis by nitrates. Using cultures containing cysteine solution which lowered the redox potential to about -300 mV, it was found that methane production started only after the total reduction of all oxides of nitrogen. However, it was observed that cysteine enhanced methanogenesis for cultures not containing nitrate. These cultures produced more methane than those without cysteine. Furthermore, cysteine was also found to enhance denitrification. Denitrification rates were 7 and 3.5 mg NO_3-N $gVSS^{-1}$ h^{-1} for cysteine and non cysteine-containing cultures respectively. This study shows that to have simultaneous denitrification and methanogenesis processes in a continuous single reactor would require a reactor system with zones of different nitrate concentrations, so that denitrifying and methanogenic populations could coexist and flourish.

Keywords : Anaerobic sludge, denitrification, methane, nitrate, redox potential..

INTRODUCTION

In the past twenty years anaerobic digestion has proven to be the best alternative to aerobic processes for the treatment of high strength wastes. The main reasons usually cited for this development are the lower sludge production and lower energy consumption [1].

However, anaerobic digestion alone cannot ensure high quality effluent as required for discharge into most inland watercourses. Additional treatment processes, including nitrogen removal, may be needed. Biological nitrogen removal involves nitrification, which is an aerobic process and denitrification, which takes place in anaerobic conditions. Denitrification processes require organic carbon as electron donor for the reaction. Consequently a complete treatment of a high strength wastewater which requires both nitrogen and organic carbon removal will involve two or more reactors, one aerobic for nitrification and other (s) anaerobic for both denitrification and anaerobic organic carbon removal. Possible combinations of aerobic and anaerobic processes have been reported [2-4].

The study of combined denitrification and methanogenesis has been attempted by some researchers [2, 3, 5-7]. It has been observed that nitrate inhibits methanogenesis and consequently under completely mixed conditions the two processes do not proceed simultaneously [2, 3, 8]. Methanogenesis was found to commence after the total reduction of nitrates (or nitrogen oxides). Thus, the inhibition of methanogenesis by nitrate is reversible. These authors ruled out the competition for organic carbon between the denitrifiers and the methanogens as the possible cause of the inhibition because their studies were carried out with organic carbon concentrations well in excess of denitrification requirements. Nitrate and its denitrification products have been shown recently to inhibit methanogenesis by pure methanogenic strains [9, 10].

The success reported by some researchers (and in our earlier works) in carrying out simultaneous denitrification and methanogenesis in a single reactor [2, 5-7] could therefore be attributed to their utilisation of biofilm reactors. In biofilm reactors, the methanogens in micro-zones devoid of nitrates within the reactor can continue to produce methane while denitrification occurs in other zones, thereby creating the impression that no inhibition exists. Another way to succeed is to use batch reactors in which the two processes are separated in time [3].

Since methanogenic bacteria generally require very low redox potential (E_h) environments [11], it is suspected that the relatively high redox potential associated with the presence of nitrates might be one cause of the inhibition of

methanogenesis observed in the presence of these compounds. This assumption appears logical since the nitrate inhibition is reversible.

The aim of this study, therefore, was to investigate the contribution of redox potential in the nitrate inhibition of methanogenesis.

MATERIALS AND METHOD

Two series of experiments were carried out, firstly to confirm the inhibition of methanogenesis by nitrate and secondly to investigate the effect of lowering the redox potential. Both studies were carried out in the presence of surplus organic carbon to eliminate the effect of competition which might occur in carbon-limited conditions. Glucose was used as the carbon source for the first experiment (Batch A) while the second experiment (Batch B) was carried out mainly with anaerobic sludge treating glucose media but which contained organic carbon in the form of volatile fatty acids (mainly acetic and propionic acids) due to acidogenesis of the glucose that occurred prior to the test. The purpose of using sludge containing acetic acid in the second experiment was to ensure that the most preferred carbon source for both the methanogens [11] and the denitrifying bacteria [12] was present at the beginning of the experiment, in order to avoid possible delays of methanogenesis which might occur due to acidogenesis and acetogenesis processes if glucose was the only source of organic carbon present.

Batch A

The first experiment was to determine the effect of nitrate on methane production in the presence of excess organic carbon. Two 500 ml incubation bottles (and their duplicates), A1 and A2, each received 250 ml of culture medium consisting of the following substances: glucose 2.2 g l^{-1}; yeast extract 50 mg l^{-1}; peptone 120 mg l^{-1}; monopotassium and dipotassium phosphate (buffer agents) 3 g l^{-1}; sodium bicarbonate 400 mg l^{-1}; magnesium sulphate 5 mg l^{-1}; and trace elements as described in [12]. Each bottle was then innoculated with 50 ml of anaerobic sludge from a 2-year old laboratory anaerobic digester treating glucose enriched synthetic wastewater. Potassium nitrate was added to one set of the bottles (A2) to give a final concentration of 156 mg NO_3-N l^{-1}. The pH of each medium was adjusted to 7.5 with a few drops of 10N sodium hydroxide. Anaerobic conditions were established by flushing each bottle with pure argon gas for 5-10 minutes. The bottles were then placed on an agitating table in a room maintained at 35°C. Samples were collected at the beginning of the incubation time and periodically afterwards using syringes. Table 1 summarises the test conditions at the beginning of the experiment. Acetic acid concentration at t = 0 was about 100 mg l^{-1}. It was contained in the seed sludge.

Batch B

A solution of cysteine was prepared using the method proposed by Homann *et al.* [13] by dissolving 6 g of sodium hydroxide in 500 ml of distilled water, followed by boiling and cooling, and the addition of 6.25 g of L-cysteine and 6.25 g of sodium sulphide. The solution was then purged with nitrogen gas and autoclaved. The prepared solution was then allowed to cool to room temperature.

In parallel to the preparation of the cysteine solution, 395 ml of anaerobic sludge obtained from the same source as in Batch A was put into four different 500 ml-capacity incubation bottles (and their duplicates) B1, B2, B3 and B4. The seed sludge contained organic substrates only in the form of volatile fatty acids (following the acidogenesis of glucose). The bottles were covered and anaerobic conditions established by flushing each bottle with pure argon gas for 5-10 minutes. The bottles were then placed on an agitating table in a room maintained at 35°C. Gas samples were collected periodically with a syringe for analysis. Methanogenesis was established a couple of hours later but the cultures were left for 24 hours before adding the following solutions with a syringe:

B1: 5 ml of distilled water

B2: 5 ml of distilled water and 6 drops of the cysteine solution

B3: 290 mg of potassium nitrate in 5 ml of distilled water

B4: 290 mg of potassium nitrate in 5 ml of distilled water and 6 drops of the cysteine solution.

Table 1. Characteristics of Batch A at time, t = 0.

Bottle Ref.	pH	E_h (mV)	VSS (mg l^{-1})	COD (mg l^{-1})	NO_3-N (mg l^{-1})
A1	7.5	-250	100	2400	0
A2	7.5	-200	100	2400	156

After a second flushing with argon gas, the bottles were returned to the agitating table in the incubation room. Samples were collected at the beginning of the incubation time and periodically afterwards with syringes.

The characteristics of the test bottles at the beginning of the experiment, and after the addition of the solutions are summarised in Table 2. The volatile suspended solids (VSS) concentration at the beginning of the experiment was about the same in each bottle, 600 mg l^{-1}.

It was initially planned to exhaust accumulated gas in all the bottles at the same time, but the idea was discarded because gas production rates were different in the bottles. In the end no gas exhaustion was carried out in cultures with added nitrate simply because the gas production rates in these bottles were not high enough develop to excessive pressure build-up.

Analytical methods

Methane, nitrogen and carbon dioxide were measured by gas chromatography (Schimazu GC-8A). Nitrate and nitrite were analysed by ion chromatography (Dionex 100). Ammonium nitrogen was analysed by the distillation and titration method. Volatile fatty acids analysis was done using a gas chromatography fitted with a flame ionisation detector (Chrompac CP 1000). A full description of the analytical procedure can be found elsewhere [14]. pH and redox potential were measured with a pH meter (Bayern 537), equipped with a combination pH electrode (Ingold U402-S7) and a redox platinum electrode (Ingold PT 4805-S7). Volatile suspended solids (VSS) and chemical oxygen demand (COD) were determined as described in Standard Methods [16].

RESULTS AND DISCUSSION

For nitrate reduction, NO_x-N refers to the sum of nitrate and nitrite. All references to nitrate removal do not refer to nitrate converted only to nitrite. The tests were carried out in duplicates and the results obtained from two identical test bottles were found to be similar. The results reported in this section were not average figures but those obtained from one set of the experiments.

Effect of nitrates on methane production (Batch A)

Figure 1 shows the results from the Batch A test. At the start of the experiment E_h values were -200 and -250 mV in the bottles with and without nitrate respectively. Methane production was observed in the bottle without nitrate 24 hours after the start of the experiment and continued all through the experimental period.

Table 2. Characteristics of Batch B at time, t = 0.

Bottle Ref	pH	E_h (mV)	Acetic acid (mg l^{-1})	Propionic acid (mg l^{-1})	NO_3-N (mg l^{-1})	Cysteine addition
B1	7.1	-280	670	270	0	No
B2	7.3	-300	670	300	0	Yes
B3	7.1	-215	740	200	100	No
B4	7.3	-291	700	300	100	Yes

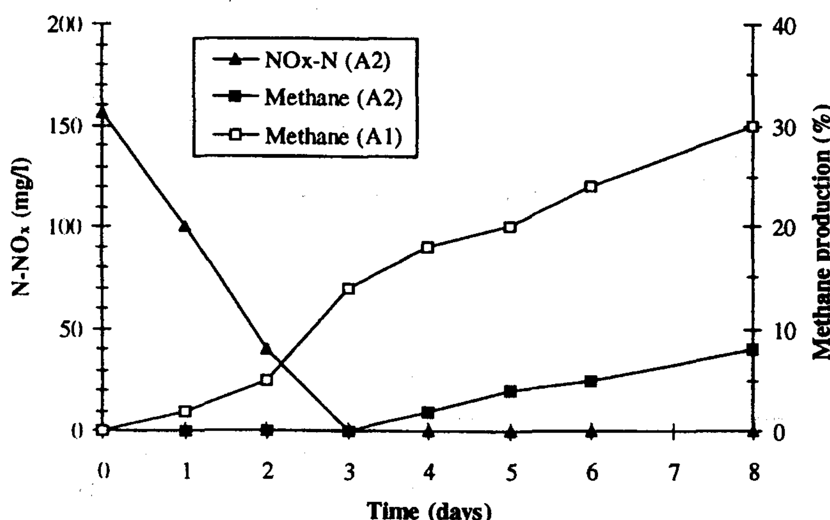

Figure 1. Methane production and nitrate reduction in the Batch A test.

For the bottle containing nitrate, it took three days for nitrate to disappear completely. Mass balance showed that about 13% of the added nitrate was converted to ammonium nitrogen. The rest was converted to nitrogen gas. The figures also show that while nitrate was still in the solution, methane was not detected in the bottle. Methane production started only after all the nitrate had been removed from the solution and increased gradually all through the duration of the experiment.

This experiment clearly showed that the presence of nitrates has a reversible inhibition effect on methane-producing bacteria. The fact that acetic acid was present in both cultures (100 mg l^{-1}) at the beginning of the experiment suggested that this inhibition affected both the acetoclastic methanogens (which convert acetic acid to methane) and the hydrogenophilic methanogens that convert carbon dioxide and hydrogen to methane. The presence of surplus organic carbon reduced the likelihood of competition between the nitrate-reducing bacteria and the methane-producing bacteria. The higher redox potential of the culture containing nitrate was suspected to be the cause of the inhibition. The validity of this hypothesis was tested by the Batch B experiments.

Effect of nitrate and cysteine on methane production (Batch B)

The analysis of the gas produced from each of the test bottles before and after the addition of water (B1), water and cysteine solution (B2), nitrate solution (B3) and nitrate solution with cysteine (B4) respectively produced the results shown in the Figure 2. All the test bottles were producing methane gas before the addition of the respective solutions. After the additions the cultures without nitrate continued methane production but those with nitrate stopped methane production. Figure 3 shows the variation of nitrate in both cultures with time.

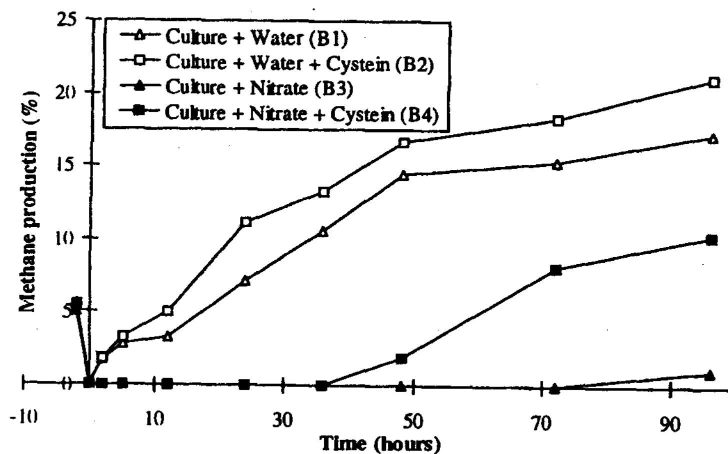

Figure 2. Effect of cysteine and nitrate on methane production.

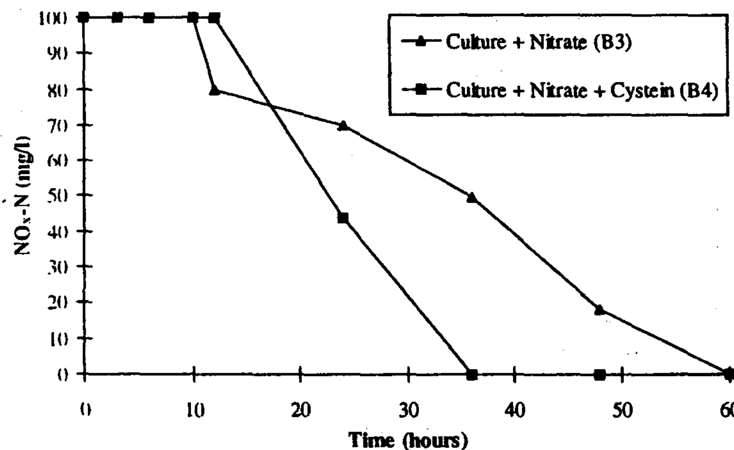

Figure 3. Effect of cysteine on nitrate reduction.

Superimposing Figure 3 on Figure 2 it will be seen that methane production in both bottles to which nitrate was added started after the total removal of nitrate from both solutions. The effect of the cysteine was to accelerate the rate of nitrate removal (since nitrate removal was achieved faster in the culture with cysteine) so that methanogenesis could be re-established faster, after 36 hours (Figure 3).

It is important to note that in this test, the organic carbon was in the form of volatile fatty acids (mainly acetic and propionic acids) unlike in the Batch A experiments where glucose was used. The results therefore suggest that the form of the carbon source had no relevance to the delayed methane production in the presence of nitrate. However, it is evident that the form of carbon source had an influence on the end-products of nitrate reduction. In this experiment (Batch B), all the nitrates were converted to nitrogen gas (denitrification), and no ammonium nitrogen accumulation was observed. These findings confirmed the conclusions of our previous work [12].

These results suggest that the higher redox potential in cultures containing nitrate was not the only cause of inhibition of the methane producing bacteria. Therefore, simultaneous denitrification and methanogenesis in a continuous system appears virtually impossible if nitrogen oxides are always to be found present in the entire system.

Effect of cysteine on methane production and the rate of nitrate reduction

Figures 2 and 3 show that for cultures without nitrate, methane gas production in the culture containing the cysteine solution was higher than that in the culture without the cysteine solution. Moreover, the presence of cysteine enhanced nitrate reduction in cultures with added nitrate. For the same quantity of initial nitrate content (100 NO_3-N mg l^{-1}), the culture medium added cysteine reduced all the nitrates up to 24 hours faster. Nitrate reduction rates were estimated to be 7 and 3.5 mg NO_3-N $gVSS^{-1}$ h^{-1} for cysteine- and non cysteine-containing cultures respectively. Thus, the nitrate reduction rate was doubled by using the cysteine solution to lower the cultures redox potential. Cysteine could have been partially used by denitrifying bacteria as a growth factor, which would explain this increase of the nitrate reduction rate.

CONCLUSION

In this study it was found that in the presence of nitrate and surplus organic substrates the methanogens were rendered inactive but their activities re-established after the total reduction of all nitrogen oxides in the culture medium.

The cause of nitrate inhibition of methanogenesis could not be attributed entirely to the higher redox potential associated with the presence of oxides of nitrogen. This study showed that even with cysteine solution which lowered the redox potential of medium with added nitrate to -300 mV, the inhibition of methanogenesis persisted and stopped after the total reduction of all nitrogen oxides. Therefore, simultaneous denitrification and methanogenesis processes in a continuous single reactor would require a reactor system with zones of different nitrate concentrations, so that denitrifying and methanogenic populations could coexist and flourish.

It was also observed that lower redox potential enhanced both methanogenic and denitrification activities. Denitrification rate was doubled in the cultures containing nitrates with the cysteine solution. However, the possibility of cysteine serving as electron donor for denitrification might explain this high nitrate reduction rate.

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