Inhibitory effects of nitrate reduction on methanogenesis in the presence of different electron donors

A. E. Tugtas and S. G. Pavlostathis

ABSTRACT

The preferential utilization of different electron donors and their effects on the nitrate reduction and methanogenesis in a mixed, mesophilic (35°C) methanogenic culture were investigated. Batch methanogenic cultures were fed with dextrin/peptone (D/P), propionate, acetate, and H_2/CO_2 at an initial COD of 500 mg/L and an initial nitrate concentration of 50 mg N/L. Immediate cessation of methane production was observed in all nitrate-amended cultures. Methane production completely recovered in the D/P- and acetate-fed cultures, and partially recovered or did not recover in the propionate- and H_2/CO_2-fed, nitrate-amended cultures, respectively. Accumulation of denitrification intermediates was observed in both the propionate- and H_2/CO_2-fed cultures, which resulted in inhibition of fermentation and/or methanogenesis. The fastest and the slowest nitrate reduction were observed in the acetate- and propionate-fed cultures, respectively.

Key words | competition, electron donor, inhibition, methanogenesis, nitrate reduction

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INTRODUCTION

Combined anaerobic treatment technologies, which include both anaerobic digestion and nitrate reduction processes, have been widely applied for the treatment of high strength organic C and N-bearing wastes. However, it has been reported that accumulation of denitrification intermediates (e.g., nitrite NO_2^-, nitric oxide NO, nitrous oxide N_2O) during nitrate reduction, lead to suppression of methanogenesis (e.g., Klüber & Conrad 1998; Tugtas & Pavlostathis 2007*a*). Nitric oxide exerted the most and nitrate exerted the least inhibitory effect on the fermentative/methanogenic consortia among all other denitrification intermediates (Tugtas & Pavlostathis 2007*a*).

Nitrate reduction occurs in two distinct pathways: dissimilatory nitrate reduction to nitrogen gas (denitrification) and dissimilatory nitrate reduction to ammonia (DNRA). Denitrification proceeds in a stepwise manner in which nitrate (NO_3^-) is reduced to nitrite (NO_2^-), nitric oxide (NO), nitrous oxide (N_2O) and nitrogen gas (N_2) (Rittmann & McCarty 2001). The DNRA process involves nitrate reduction to nitrite and then to ammonia. DNRA occurs either at very low redox potential values, in the presence of sulfide, or at high COD/N values (Myers 1972; Knowles 1982; Akunna *et al.* 1992; Tugtas & Pavlostathis 2007*a*, *b*). Nitrate reducers are very versatile in terms of energy source utilization. In fact, the energy sources of nitrate reducers include all three substrate classes known to be used by microorganisms: organic (organotrophs), inorganic (lithotrophs), and light (phototrophs) (Tiedje 1988). Nitrate reducers utilize a variety of organic substrates, such as glucose, glutamic acid (Marazioti *et al.* 2003) and VFAs (Elefsiniotis *et al.* 2004), as well as hydrogen gas (Scheid *et al.* 2003), which are also utilized by fermenters/methanogens. Nitrate reducers and fermenters/methanogens can coexist in natural and engineered systems. This coexistence may cause competition for the same substrate(s) due to the wide variety of electron donors utilized by nitrate reducers (Roy & Conrad 1999). The preferential use of carbon/electron donor sources by nitrate reducers may be different in pure cultures compared to that of mixed cultures. In addition to the potential competition,

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the intermediates of denitrification are known to inhibit various bacterial species (Klüber & Conrad 1998). Therefore, the accumulation of denitrification intermediates may result in the build up of these compounds and, thus, may cause inhibition of nitrate reduction and/or fermentation and methanogenesis in mixed, overall methanogenic systems.

The objective of the research reported here was to investigate the utilization of different electron donors by nitrate reducers and their effect on the overall fermentative/methanogenic system in a mixed methanogenic culture.

MATERIALS AND METHODS

Mixed methanogenic culture

A mixed, sulfide-acclimated methanogenic culture developed with an inoculum obtained from a mesophilic (35°C), municipal anaerobic digester, fed with a concentrated dextrin/peptone (D/P) solution (4 g/L dextrin, 2 g/L peptone in the feed solution) and nutrient media was used in this study (Tugtas & Pavlostathis 2007*a*). The culture was maintained at 35°C and was fed twice a week with a hydraulic (and solids) retention time of 35 days and was continuously mixed using a magnetic stirrer. The culture was maintained under the above-stated conditions for three years before the initiation of this study. The steady-state pH, gas-phase CH_4 and CO_2 and the total solids (TS) and volatile solids (VS) concentrations of the culture were 7.1 ± 0.1, 60.7 ± 0.8%, 39.2 ± 0.4%, 6,900 ± 300 mg/L, and 2,200 ± 100 mg/L (mean ± standard deviation), respectively.

Batch nitrate reduction assay

A batch assay was performed to test the effect of different electron donors on the mixed methanogenic culture in the presence of nitrate using 160 mL serum bottles (110 mL liquid volume) sealed with rubber stoppers and aluminum crimps and pre-flushed with helium gas. An aliquot of 70 mL of the sulfide-acclimated methanogenic culture and 20 mL culture media were anaerobically transferred to each serum bottle. Four different substrates were used in this assay: a mixture of dextrin and peptone (D/P), propionate, acetate, and H_2/CO_2. Each electron donor was added to the serum bottles resulting in an initial chemical oxygen demand (COD) of 500 mg/L. In order to provide a total of 500 mg COD/L with a H_2/CO_2 mixture (80% to 20% v/v, respectively), 332 mL of the H_2/CO_2 mixture at 35°C was added to the bottle headspace. For each substrate, one of the cultures was nitrate-free and the other one was amended with 50 mg nitrate-N/L using a $NaNO_3$ stock solution. The COD/N value of 10 was selected in order to prevent the prevalence of DNRA in this experiment (Akunna *et al.* 1992). The initial biomass concentration in all culture series was 1,468 ± 11 mg VS/L. Incubation of all cultures was carried out in the dark at 35°C with continuous mixing using a tumbler at 4 rpm.

Analytical methods

TS, VS, and pH measurements were conducted according to procedures described in *Standard Methods* (APHA 2005). Nitrate and nitrite were measured by ion chromatography (conductivity detection). Methane, nitrous oxide, nitrogen gas, and volatile fatty acids (VFAs) were measured by gas chromatography (thermal conductivity and flame ionization detection). Details on these methods have been previously reported (Tugtas & Pavlostathis 2007*a*).

RESULTS AND DISCUSSION

The assay testing the preferential utilization of the four electron donors lasted for 8 days. The initial pH value was 7.1 ± 0.02 (mean ± standard deviation) in all four culture series. The final pH values for the nitrate-free/nitrate-amended cultures were 6.9/7.2, 7.2/7.4, 7.3/7.4, and 7.0/7.2 for the D/P-, propionate-, acetate-, and H_2/CO_2-fed cultures, respectively. Methane recovery was monitored and the initial volumetric methane production rates were calculated using linear regression starting at the recovery time (Table 1). The initial methane production rate in the nitrate-free cultures was as follows in decreasing order: D/P > H_2/CO_2 > acetate > propionate (Table 1). The observed highest methane production in the D/P-fed culture was probably due to the prior acclimation of the culture to this substrate. Acetate and H_2/CO_2 are used by methanogens, which contributed to the observed fast initial methane production.

Table 1 | Initial methane production rate and COD utilization in nitrate-free (control) and nitrate-amended mixed methanogenic cultures amended with different electron donors

	Methane production			COD Processed (%)			
Culture series/Electron donor	**Initial rate (mL/L·d)***	r^2	**Normalized rate (%)†**	**CH_4**	**VFAs**	**Nitrate reduction‡**	**Total§**
Nitrate-free cultures							
D/P	21.4 ± 4.7	0.835	100	100	ND	NA	100
Propionate	8.4 ± 0.6	0.985	100	100	ND	NA	100
Acetate	15.9 ± 2.0	0.968	100	100	ND	NA	100
H_2/CO_2	19.7 ± 2.3	0.972	100	100	ND	NA	100
Nitrate-amended cultures¶							
D/P	10.1 ± 1.7	0.973	47	85	ND	31	116
Propionate	2.1 ± 0.1	0.998	25	34	22	31	87
Acetate	9.7 ± 0.1	0.999	61	79	ND	31	110
H_2/CO_2	ND	ND	ND	ND	ND	31	31

*Results of linear regression (mean ± standard deviation; $n \geq 3$) of single culture data starting at the recovery time.
†Normalized to the initial methane production rate of the control culture observed at each culture series.
‡Fraction of COD utilized for the complete reduction of nitrate to N2 neglecting microbial growth (calculated).
§Normalized to the total COD utilized for methane production in each control culture.
¶Initial nitrate concentration 50 mg N/L.
NA, not applicable.
ND, not detected.

Propionate has to go through fermentation and the culture was not exposed to high propionate concentrations before, therefore, the observed slow methane production may be the result of slow utilization of propionate.

Addition of nitrate resulted in an immediate suppression of methanogenesis in all four cultures (Figure 1B). Upon the complete removal of all denitrification intermediates, methane production fully recovered in the D/P- and acetate-fed cultures, partially recovered in the propionate-fed culture, and did not recover in the H_2/CO_2-fed culture (Figures 1B and 1C). The initial rate of methane production in the nitrate-amended cultures fed with different electron donors were as follows in decreasing order: D/P > acetate > propionate (Table 1). Stimulation of methanogenesis in the presence of acetate compared to propionate and H_2/CO_2 was reported in nitrate-amended soil microcosms (Roy & Conrad 1999). Because the initial methane production rate was different for each substrate in the nitrate-free cultures, in order to determine the effect of nitrate addition on methane production, the rates of the nitrate-amended cultures were normalized to the methane production rate in the corresponding nitrate-free

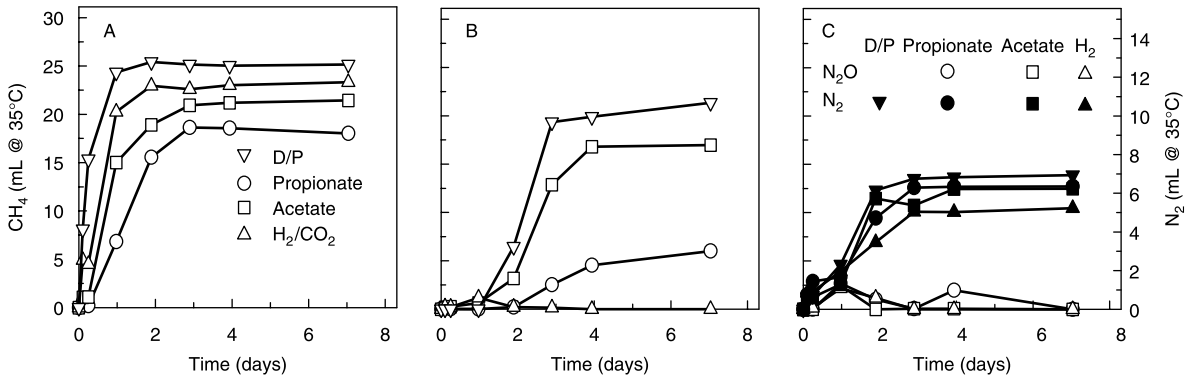

Figure 1 | Methane production profiles in the nitrate-free (A), nitrate-amended (B) cultures, and production and consumption of N_2O and production of N_2 in the nitrate-amended cultures (C).

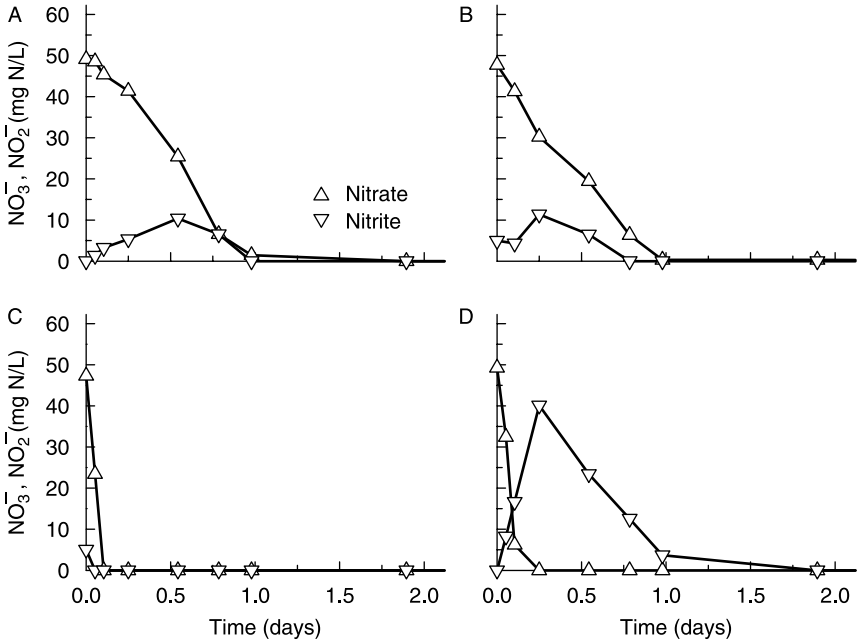

Figure 2 | Nitrate and nitrite profiles in D/P- (A), propionate- (B), acetate- (C), and H_2/CO_2-fed (D) cultures.

culture (Table 1). Methanogenesis was affected mostly in the H_2/CO_2-fed culture followed in decreasing order by the propionate-, D/P-, and acetate-fed cultures (Table 1).

Accumulation of N_2O was observed in the propionate-, acetate-, and H_2/CO_2-fed cultures for 4, 2, and 3 days, respectively (Figure 1C). The fastest nitrate reduction rates were observed in the acetate- and H_2/CO_2-fed cultures, followed by D/P- and propionate-fed cultures (Figures 2C and 2D; Table 2). However, significant nitrite accumulation was observed in the H_2/CO_2-fed culture. In addition, accumulation of nitric oxide (NO) was observed qualitatively in chromatograms during gas analysis of both the propionate- and H_2/CO_2-fed cultures. The initial rate of N_2 production was as follows in decreasing order: D/P > acetate > propionate > H_2/CO_2 (Table 2). Roy & Conrad (1999) also reported stimulation of denitrification mostly by acetate followed by propionate and H_2 in rice field soil microcosms. Slower reduction of the denitrification intermediates associated with the type of the electron donor, could be the reason for slower N_2 production in the cultures in which the fastest nitrate reduction was observed (Table 2).

Acetate and propionate production was observed in the D/P-fed culture (Figure 3A). Slightly higher acetic acid production was observed in the nitrate-amended, D/P-fed culture as opposed to the nitrate-free control culture. The COD required for the complete nitrate reduction was calculated based on 2.857 mg COD/mg NO_3^--N (Table 1). The total processed COD in the D/P-fed, nitrate-amended culture was similar to that of the nitrate-free control culture, suggesting that all of the initial COD was utilized by nitrate reducers, fermenters, and methanogens (Table 1). Although the propionate utilization profiles were the same in both the nitrate-free and nitrate-amended, propionate-fed cultures at the beginning of the incubation, the propionate utilization rate became slower concomitant to complete cessation of nitrate reduction. Approximately

Table 2 | Nitrate reduction and N_2 production rates in methanogenic cultures fed with different types of electron donors

	Nitrate reduction		N_2 production	
Electron donor	Rate (mg NO_3^--N/L·d)*	r^2	Initial rate (mL/L·d)*	r^2
D/P	55.6 ± 3.5	0.987	3.1 ± 0.3	0.962
Propionate	51.3 ± 3.4	0.992	2.0 ± 0.2	0.892
Acetate	456.2 ± 0.1	0.999	3.2 ± 1.1	0.986
H_2/CO_2	282.0 ± 0.2	0.882	1.6 ± 0.1	0.973

*Mean ± standard error ($n \geq 3$).

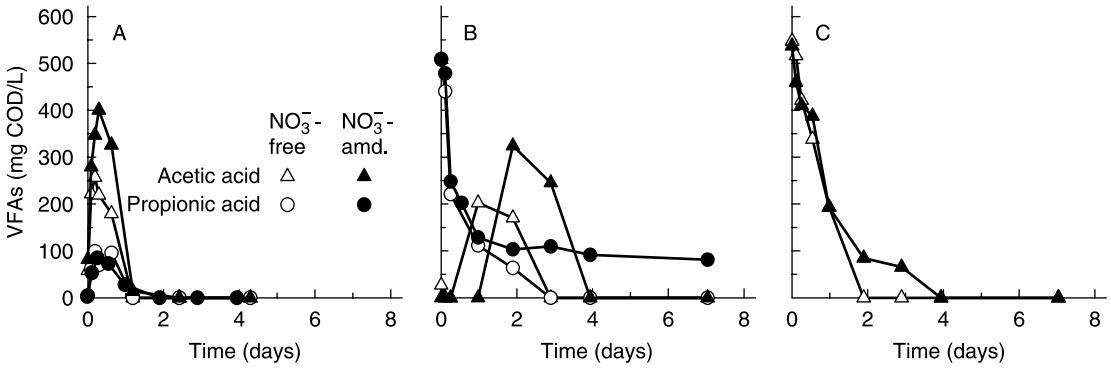

Figure 3 | VFA production and consumption profiles in the cultures fed with D/P (A), propionate (B), and acetate (C). VFAs were not measured in the H_2/CO_2-fed cultures.

22% of the initially added COD remained as propionate at the end of the incubation (Figure 3B; Table 1). As mentioned above, the methane production recovered only partially in the nitrate-amended, propionate-fed culture accounting for only 34% of the initial COD and 13% of the initial COD remained unutilized (Table 1). In addition, acetate production was observed as a result of cessation of nitrate reduction suggesting that propionate was directly utilized by nitrate reducers. The acetate utilization profiles were similar in both the nitrate-free and nitrate-amended, acetate-fed cultures (Figure 3C). At the time when complete nitrate reduction occurred, acetate utilization slowed down, which is attributed to the cessation of acetate utilization by denitrifiers (Figures 2C and 3C). The total processed COD was close to that of the acetate-fed, nitrate-free culture (Table 1). In the H_2/CO_2-fed culture, 31% of the initially added COD was utilized for nitrate reduction and the remainder was not consumed due to complete inhibition of methanogenesis as a result of accumulation of denitrification intermediates (Table 1).

CONCLUSIONS

The fastest nitrate reduction was observed in the acetate-fed culture and methane production fully recovered at the end of the incubation. However, the slowest nitrate reduction was observed in the propionate-fed culture and methane production only partially recovered. Although fast nitrate reduction was observed in the H_2/CO_2-fed culture, methane production did not recover due to accumulation of denitrification intermediates. The utilization of different electron donors by nitrate reducers within a methanogenic system resulted in accumulation of different levels of denitrification intermediates, which in turn had a different impact on methanogenesis (i.e., varying from complete inhibition to full recovery).

The results of this study are useful in understanding the fate of carbon- and nitrogen-bearing wastes as well as microbial process interactions in anoxic/anaerobic systems. Such information can then be used to manage and operate treatment systems with the goal of maximizing their treatment capacity and efficiency.

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