A membrane biofilm reactor achieves aerobic methane oxidation coupled to denitrification (AME-D) with high efficiency

O. Modin, K. Fukushi, F. Nakajima and K. Yamamoto

ABSTRACT

Methane would potentially be an inexpensive, widely available electron donor for denitrification of wastewaters poor in organics. Currently, no methanotrophic microbe is known to denitrify. However, aerobic methane oxidation coupled to denitrification (AME-D) has been observed in several laboratory studies. In the AME-D process, aerobic methanotrophs oxidise methane and release organic metabolites and lysis products, which are used by coexisting denitrifiers as electron donors for denitrification. Due to the presence of oxygen, the denitrification efficiency in terms of methane-to-nitrate consumption is usually low. To improve this efficiency the use of a membrane biofilm reactor was investigated. The denitrification efficiency of an AME-D culture in (1) a suspended growth reactor, and (2) a membrane biofilm reactor was studied. The methane-to-nitrate consumption ratio for the suspended culture was 8.7. For the membrane-attached culture the ratio was 2.2. The results clearly indicated that the membrane-attached biofilm was superior to the suspended culture in terms of denitrification efficiency. This study showed that for practical application of the AME-D process, focus should be placed on development of a biofilm reactor.

Key words | biofilm, denitrification, membrane, methanotrophs

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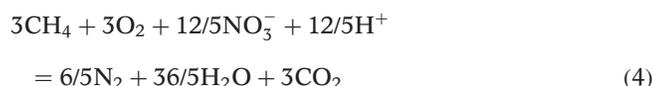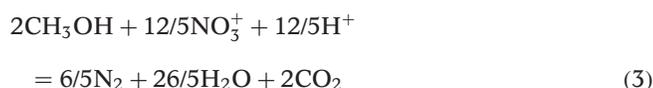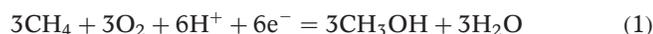The University of Tokyo,
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INTRODUCTION

Methane could potentially be used as an electron donor for denitrification of wastewater poor in organics. This process has been investigated in several laboratory studies (reviewed in Modin *et al.* 2007*a*). Currently, no single microorganism is known to couple methane oxidation and denitrification. Instead, most studies have focused on aerobic methane oxidation coupled to denitrification (AME-D). In the AME-D process, aerobic methanotrophs oxidise methane and release soluble organic metabolites and lysis products, which are used by coexisting denitrifiers as electron donors for denitrification (Rhee & Fuhs 1978; Meschner & Hamer 1985; Costa *et al.* 2000; Eisentraeger *et al.* 2001). Recently, anoxic methane oxidation coupled to denitrification (ANME-D) has also been observed. The process was accomplished by a slow-growing association of

archaea and bacteria (Raghoebarsing *et al.* 2006). Currently, however, the aerobic process appears more attractive for engineering applications due to the ease at which an AME-D culture can be enriched (Modin *et al.* 2007*b*).

The denitrification efficiency of the AME-D process, in other words the ratio of methane-to-nitrate consumed, is usually low. Theoretically, under the most favourable circumstances for AME-D, the methanotrophs convert methane to methanol, which is then used by denitrifiers for denitrification (Equations (1)–(3)). This would result in the most optimal molar methane-to-nitrate ratio being 1.25 (Equation (4)). In reality, however, this ratio is unlikely to be achieved. The presence of oxygen (which is required in an AME-D reactor) typically inhibits denitrification. This means a large fraction of the organics made available by

the methanotrophs may be oxidised aerobically by coexisting heterotrophs. Denitrification may be confined to anoxic regions in flocs or be carried out by aerobic denitrifiers if such bacteria are present in the culture.

$$3CH_4 + 3O_2 + 6H^+ + 6e^- = 3CH_3OH + 3H_2O \quad (1)$$

$$CH_3OH + H_2O = CO_2 + 6H^+ + 6e^- \quad (2)$$

$$2CH_3OH + 12/5NO_3^+ + 12/5H^+$$
$$= 6/5N_2 + 26/5H_2O + 2CO_2 \quad (3)$$

$$3CH_4 + 3O_2 + 12/5NO_3^- + 12/5H^+$$
$$= 6/5N_2 + 36/5H_2O + 3CO_2 \quad (4)$$

In this study, we compare the denitrification efficiency of an AME-D culture grown in suspension and on a membrane. We hypothesise that a membrane biofilm reactor, in which methane and oxygen are supplied through the lumen of a hollow-fibre membrane to a biofilm growing on the membrane surface, would be a promising reactor configuration for optimising the efficiency of the AME-D process. In this setup, methanotrophs should thrive near the membrane surface, where high concentrations of both methane and oxygen are available. Further out in the biofilm, at a distance from the membrane surface, the oxygen has been consumed and anoxic conditions prevail. In this space, denitrifiers are likely to grow utilising organics diffusing out from the inner portions of the biofilm and nitrate diffusing in from the bulk liquid (Figure 1).

MATERIALS AND METHODS

Culture enrichment and growth medium

The AME-D culture was originally enriched from an activated sludge sample (Modin *et al.* 2007*b*). Experiments were carried out in a nitrate minerals salts medium (NMS) of the following composition (mg L^{-1}): MgSO$_4$·7H$_2$O 1000; CaCl$_2$·2H$_2$O 270; FeSO$_4$·7H$_2$O 9.1; and KNO$_3$ 1444. The medium also contained 2 mL L^{-1} of phosphate buffer and 1 mL L^{-1} of trace element solution. The phosphate buffer contained (g L^{-1}): KH$_2$PO$_4$ 24.4; and Na$_2$HPO$_4$ 10.2.
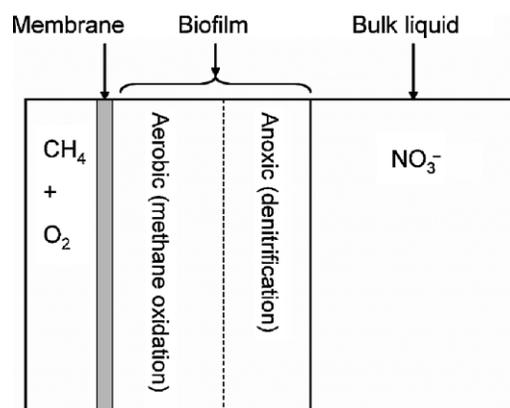

Figure 1 | Theoretical biofilm development in a membrane biofilm reactor where methane and oxygen are supplied through the lumen of a hollow-fibre membrane to a biofilm growing on the membrane surface. The inner portion of the biofilm should be aerobic promoting methane oxidation while the outer portion should be anoxic promoting denitrification.

The trace element solution contained (mg L^{-1}): FeSO$_4$·7H$_2$O 2486; MnCl$_2$·4H$_2$O 500; ZnCl$_2$ 50; NiSO$_4$·6H$_2$O 101; CoCl$_2$·6H$_2$O 50; Na$_2$MoO$_4$·2H$_2$O 26; H$_3$BO$_3$ 50; CuSO$_4$·5H$_2$O 310; and 5 mL 35% HCl. The final pH of the medium was adjusted to 7.0.

Suspended culture experiment

The batch experiment with suspended culture was carried out in a 1.1 L bottle with 250 mL liquid. The headspace was pressurised with methane (44 kPa), oxygen (45 kPa) and helium (101 kPa). The change in gas phase composition as well as liquid phase nitrate and nitrite concentrations were measured over time. The gas pressure was measured at each sampling occasion. The amount of each gas in mol units was calculated using the ideal gas law. The dissolved portion of the gas was calculated using solubility constants for gases in water (CH$_4$: 7.08×10^4 kPa M^{-1}, O$_2$: 7.89×10^4 kPa M^{-1}, N$_2$O: 4.11×10^4 kPa M^{-1} (Kaye & Laby Online 2007)). The bottle was placed on a magnetic stirrer, inoculated with 10 mL enrichment culture and incubated at room temperature (approximately 25°C).

Membrane biofilm experiments

The membrane biofilm reactor was constructed using a 30 cm long silicone tube with an outside diameter of 3 mm and a wall thickness of 0.5 mm. The silicone tube, which

acted as a gas-permeable membrane, was placed centrally in a clear acrylic pipe with an inside diameter of 2.5 cm. NMS medium was recirculated through the pipe from a 1.1 L reservoir with a helium-filled headspace to achieve anoxic conditions in the bulk liquid. The total liquid volume in the system was 800 mL. The reactor was inoculated with the previously described AME-D enrichment culture. Nitrate, nitrite and dissolved organic carbon (DOC) concentrations in the bulk liquid was measured over time. The silicone tube was connected to a 600 mL gas reservoir where methane, oxygen and helium were mixed at elevated pressure. The amount of methane penetrating into the biofilm could be calculated by measuring the change in methane content of the gas reservoir. The biofilm thickness was measured non-destructively using a method developed by Freitas dos Santos & Livingston (1995). The clear pipe containing the silicone tubing with attached biofilm was placed between a light source and a lens. An image of the biofilm-covered silicone tube was projected on a screen a distance away from the lens. The biofilm thickness could be calculated based on the diameter measured on the screen. The inaccuracy of this method was approximately 20 μm.

Analytical methods

Gaseous methane, oxygen, nitrogen and nitrous oxide were measured by gas chromatography (Shimadzu 8A) with a thermal conductivity detector. The gases were separated on either a Molecular Sieve 5A or an activated carbon column. Helium was used as the carrier gas. Nitrate and nitrite were analysed using ion chromatography (IC) on a Metrohm 761 Compact IC. Dissolved organic carbon was measured by filtering the sample through 0.45 μm PTFE filter and analysis with a Shimadzu TOC-5000A total organic carbon analyser.

RESULTS AND DISCUSSION

Suspended culture experiment

The batch experiment was run for 8 days. The amounts of methane and oxygen and concentration of nitrate-nitrogen in the batch are shown in Figure 2. The mol ratio between oxygen and methane consumption by the culture was 1.39.
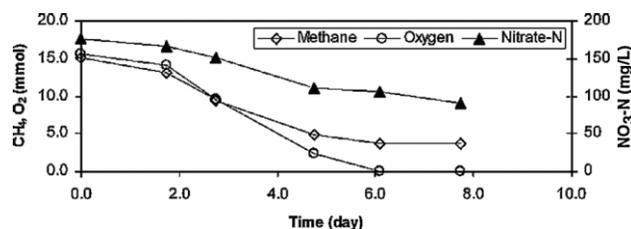

Figure 2 | Amount of methane and oxygen, and concentration of nitrate-nitrogen in the suspended batch culture over time.

This meant there was an excess of methane present, which led to oxygen being depleted first. Nitrate consumption largely followed methane and oxygen consumption, although some nitrate removal was observed even after oxygen had been completely consumed. This nitrate removal was most likely due to denitrification on endogenous metabolism. Nitrite was only detected on one occasion. This occurred after 1.8 days when the nitrite concentration was 1.2 mg $NO_2-N L^{-1}$. At the following sampling occasions the nitrite concentration was zero. Small amounts of nitrogen and nitrous oxide were also detected in the headspace. The nitrogen content could not be accurately quantified due to potential air contamination of the needle during the sampling procedure, the nitrous oxide content, however, was 0.18 mmol at the end of the run. The cumulative methane and nitrate consumptions in mmol are shown in Figure 3. At the end of the run, the methane-to-nitrate consumption ratio was 8.7.

Membrane biofilm experiment

The membrane biofilm reactor was inoculated with the AME-D enrichment culture. The gas reservoir was pressurised with methane, oxygen and helium with the following approximate partial pressures: $CH_4 \sim 30$ kPa, $O_2 \sim 30$ kPa and He ~ 80 kPa. The gas reservoir was refilled every 2–3 days. After 5 days of operation, a biofilm
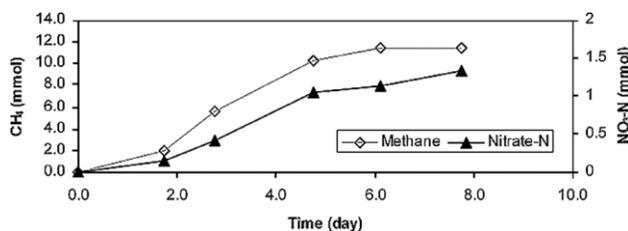

Figure 3 | Cumulative consumption of methane and nitrate by the suspended batch culture over time.
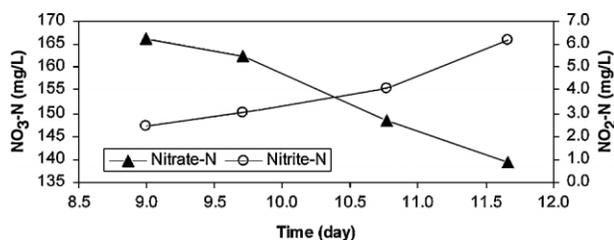
Figure 4 | Nitrate-nitrogen and nitrite-nitrogen concentrations in the bulk liquid of the membrane biofilm reactor.

was clearly visible on the membrane surface. At this time, all the liquid was exchanged for fresh NMS medium. After 7 days, the average biofilm thickness was 570 μm. Biofilm thickness was measured at three points along the tube: 10 cm from the bottom end, the middle and from the top end. The variation between the different measurement points was less than the inaccuracy of the method (~20 μm). From the beginning of the 10th day until the end of the 12th day of operation, the methane penetration and nitrate and nitrite concentrations were measured daily. As shown in Figure 4, a significant amount of nitrite was produced by the biofilm, ranging from 2.5 $mgNO_2-N\,L^{-1}$ at the start of the measurements to 6.2 $mgNO_2-N\,l^{-1}$ at the end. The nitrate concentration dropped from 166 to 139 mg $NO_3-N\,L^{-1}$ during this time. The removal of total inorganic nitrogen (TIN) (nitrate + nitrite) was compared to the disappearance of methane from the gas reservoir (Figure 5). The cumulative methane-to-nitrate consumption ratio at the end of the run was 2.2. It should also be noted that small amounts of methane could also be found in the headspace of the liquid reservoir indicating that not all the methane that penetrated the silicone membrane was utilised inside the biofilm. Thus, the actual consumption ratio may have been even smaller.

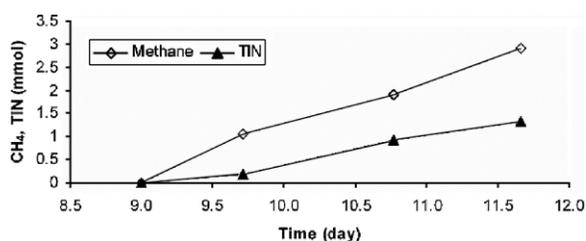The biofilm thickness was measured at the beginning and the end of this 3-day period and was found to be

Figure 5 | Cumulative removal of total inorganic nitrogen and methane in the membrane biofilm reactor.

590 μm on both occasions, thus there did not appear to be any significant increase in biofilm biomass during this period. Neither was there any clear increase in the DOC concentration, which fluctuated between 3.0 and 5.1 mg L^{-1}.

Comparison of the reactor configurations

Up until now, most studies on AME-D have used suspended culture reactors. The reported efficiencies range from 4.0 to 12.7 mol CH_4 mol^{-1} NO_3^- consumed (Houbron *et al.* 1999; Modin *et al.* 2007a). The ratio achieved with the suspended culture in this study (8.7) falls within this range. Although some studies using attached-growth have been carried out (Werner & Kayser 1991; Rajapakse & Scutt 1999), no methane-to-nitrate ratios are available from those studies. Our experiments indicate that a membrane biofilm reactor is significantly more efficient for AME-D than a suspended culture reactor. Due to the dense heterogeneous structure of biofilms, anoxic regions, in which denitrification is the favoured respiration process, are more likely to develop. Moreover, in a biofilm the microbes are in close contact with each other, which in this case might enhance carbon transfer from the methanotrophs to the denitrifiers.

A disadvantage of the membrane biofilm reactor configuration used in this study is that methane and oxygen are mixed in the membrane lumen, which might result in a flammable gas mixture and pose a safety hazard for this type of reactor. Thus, other biofilm reactor configurations in which methane and oxygen are kept separate should be investigated for AME-D. Such studies are currently underway in our laboratory. Furthermore, in this study, some methane penetrated the biofilm and could be found in the bulk liquid. Since methane is a greenhouse gas it is desirable that no methane leaves the reactor. This can potentially be achieved by manipulating the intramembrane pressures of oxygen and methane. Reportedly, complete methane utilisation was observed in a methanotrophic membrane biofilm reactor for trichloroethylene removal (Clapp *et al.* 1999).

CONCLUSIONS

The efficiency of the AME-D process in terms of methane-to-nitrate consumption was investigated both with

suspended and membrane-attached culture. In the suspended culture reactor, the overall ratio was 8.7 mol CH_4 mol^{-1} NO_3^-. In the membrane biofilm reactor, the overall ratio was 2.2 mol CH_4 mol^{-1} NO_3^-. The results clearly indicated that the membrane-attached biofilm was superior to the suspended-culture for AME-D. The low ratio obtained with the biofilm setup shows that the AME-D process is a promising alternative for denitrification of wastewaters poor in organics. It also shows that future research should focus on the development of a biofilm reactor for this process.

ACKNOWLEDGEMENTS

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