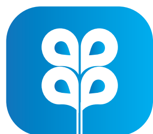

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Membrane Diffuser Coupled Bioreactor for Methanotrophic Denitrification under Non-aerated Condition: Suggestion as a Post-denitrification Option

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Abstract

Methanotrophic denitrification under a non-aerated condition (without external supply of oxygen or air) was investigated in a bioreactor coupled with a membrane diffuser. Batch experiment demonstrated that both methane consumption and nitrogen production rates were not high in the absence of oxygen, but most of the nitrate was reduced into N_2 with 88% recovery efficiency. The methane utilized for nitrate reduction was determined at 1.63 mmol CH_4/mmol NO_3^--N, which was 2.6 times higher than the theoretical value. In spite of no oxygen supply, methanotrophic denitrification was well performed in the bioreactor, due to enhanced mass transfer of the methane by the membrane diffuser and utilization of oxygen remaining in the influent. The denitrification efficiency and specific denitrification rate were 47% and 1.69 mg NO_3^--N/g VSS·hr, respectively, which were slightly lower than for methanotrophic denitrification under an aerobic condition. The average concentration of total organic carbon in the effluent was as low as 2.45 mg/L, which indicates that it can be applicable as a post-denitrification method for the reclamation of secondary wastewater effluent. The dominant fatty acid methyl ester of mixed culture in the bioreactor was $C_{16:1\omega7c}$ and $C_{18:1\omega7c}$, which was predominantly found in type I and II methanotrophs, respectively. This study presents the potential of methanotrophic denitrification without externally excess oxygen supply as a post-denitrification option for various water treatment or reclamation.

Keywords: Bioreactor, Membrane diffuser, Methanotrophic denitrification, Secondary wastewater effluent, Specific denitrification rate

1. Introduction

Post biological denitrification is applied to a variety of environmental areas, such as groundwater remediation, water treatment, and wastewater reclamation [1, 2]. Technologies related to biological denitrification could be divided into heterotrophic and autotrophic denitrification in terms of the microorganisms involved and carbon source utilized. Heterotrophic denitrification requiring external carbon source is the most common and proven due to its high kinetic performance and stability. However, the addition of an external carbon source for post-denitrification may lead to an increase in operation cost and cause problems, such as excess biological growth and residual organic carbon in the treated effluent. Autotrophic denitrification using elemental sulphur or hydrogen gas can be an option for heterotrophic denitrification with several advantages: less sludge production and no external carbon source required [3-5]. Nonetheless, intermediate sulphate occurrences and high operating cost for the use of hydrogen gas can be addressed as major defects of autotrophic denitrification.

Denitrification using methane as a sole carbon source is already known [6] and application of this to water treatment has recently been attempted [7]. Since methanotrophic reaction converting methane to methanol is most likely to occur in the presence of oxygen, methanotrophic denitrification has so far been known as aerobic oxidation of methane with denitrification (AeOM-D). The methanotrophs utilize methane monooxygenase (MMO) to oxidize methane to methanol (or other organic carbon) and these groups are currently classified into three specific types depending on the metabolic pathway: type I (ribulose monophosphate [RuMP] pathway), type II (serine pathway), and type X (simultaneous RuMP and serine pathway) [8]. The stoichiometry for AeOM-D is shown in Eqs. (1) and (2).

$$5CH_4 + 2.5O_2 \rightarrow 5CH_3OH \quad (1)$$

$$5CH_3OH + 6NO_3^- \rightarrow 5CO_2 + 3N_2 + 6OH^- + 7H_2O \quad (2)$$

Meanwhile, more recent studies have confirmed that meth-

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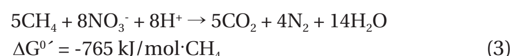
ane can be directly used as a carbon source for denitrification in the absence of oxygen, that is, anaerobic oxidation of methane with denitrification (AOM-D) [8, 9]. The stoichiometry for AOM-D is shown in Eq. (3) and the reaction is thermodynamically favoured [10].



$$5CH_4 + 8NO_3^- + 8H^+ \rightarrow 5CO_2 + 4N_2 + 14H_2O \quad \Delta G^{0'} = -765\ kJ/mol \cdot CH_4 \tag{3}$$

AOM-D has several advantages over AeOM-D, when applied for water treatment: 1) it does not require oxygen (or air) supply; 2) it theoretically needs 25% less CH_4 for denitrification; and 3) it does not generate by-products, such as methanol, which may cause deterioration of effluent. However, few studies have been successfully done due to the limited rate for denitrification and slow growth of the related methanotrophs [11, 12]. Pure cultures have not yet been isolated; however, several studies presented that anaerobic methane oxidation can be conducted by anaerobic methanotrophic archaea [13, 14].

Since AOM-D has such drawbacks in denitrification rate and methanotrophic growth, it is of importance in enhancing kinetics for the mass transfer of methane. Mass transfer of methane to water is very low due to its high dimensionless Henry's constant (28.4 at 20°C). Therefore, exposure of methanotrophs to abundant methane will be a key factor to enhance the AOM-D performance. Islas-Lima et al. [15] confirmed the capability of AOM-D in anaerobic batch assays, through varying methane partial pressures in the headspace. The nitrate removal efficiency reached 99.3% regardless of the methane partial pressure; but the denitrification rate was dependent on the methane partial pressure, indicating that the dissolved concentration of methane is the key kinetic parameter for AOM-D. In order to improve the dissolution of hydrogen, microporous membranes have often been successfully used as a gas diffuser [16, 17]. Similarly, the mass transfer of methane will be one of the key factors to attain denitrification using methane as a sole carbon source.

The overarching goal of this study was to demonstrate the possibility of denitrification using methane as a sole carbon source without an external supply of oxygen, and evaluate its applicability as a post-denitrification technology. Specifically, we investigated 1) the capability of a mixed culture consortium, consisting of anaerobic digested sludge and activated sludge for methanotrophic denitrification under anaerobic condition; 2) kinetic improvement of the microporous membrane especially for the mass transfer of methane; and 3) the performance of bioreactor coupled with membrane diffuser for methanotrophic denitrification under a non-aerated condition. We also investigated the microbial community structure of the mixed culture in the bioreactor, using fatty acid methyl ester (FAME) methods.

2. Materials and Methods

2.1. Mixed Culture Consortium and Medium

The mixed culture used as a seed for all experiments consisted of activated sludge and anaerobic digested sludge, taken from the 'J' wastewater treatment plant (Seoul, Korea) in a 1:1 (w/w) mixing ratio. The growth medium was prepared with the following composition: $MgSO_4 \cdot 7H_2O$, 1,000 mg/L; $CaCl_2 \cdot 2H_2O$, 270 mg/L; $FeSO_4 \cdot 7H_2O$, 9.1 mg/L; 2 mL of phosphate buffer; and 1 mL of trace mineral solution. The composition of buffer and mineral solution in the medium were in the following concentrations: KH_2PO_4, 24,400 mg/L; Na_2HPO_4, 10,200 mg/L; $FeSO_4 \cdot 7H_2O$, 2,486 mg/L; $MnCl_2 \cdot 4H_2O$, 500 mg/L; $ZnCl_2$, 50 mg/L; $NiSO_4 \cdot 6H_2O$, 101 mg/L; $CoCl_2 \cdot 6H_2O$, 50 mg/L; $Na_2MoO_4 \cdot 2H_2O$, 26 mg/L; H_3BO_3, 50 mg/L; $CuSO_4 \cdot 5H_2O$, 310 mg/L; and 35% HCl 5 mL [18]. Nitrate was added to the medium solution at 30 and 20 mg NO_3^--N/L for anaerobic batch test and bioreactor operation, respectively.

2.2. Batch Test

A 500-mL glass bottle with a rubber screw cap was prepared for the batch test. The working volume was set at 300 mL after adding medium and seed. The initial concentration of the seed was 1,000 mg VSS/L. The batch experiment was carried out under anaerobic condition. In order to remove dissolved oxygen (DO) in the medium, the glass bottle was purged with helium gas for 1 min, and then 20 mL of 99.99% CH_4 was added to 200 mL of headspace. The vials were horizontally placed on a rotary shaker and continuously mixed at 400 rpm. The batch experiment was conducted at room temperature (21°C ± 1°C). CH_4 and N_2 concentrations in the headspace were periodically measured during the experimental period. Liquid sample was taken at the end of experiment in order to measure the NO_3^--N concentration.

2.3. CH_4 Dissolution Test: Kinetic Assessment

A cylindrical acrylic reactor (10 cm I.D. × 60 cm long, 2.5 L working volume) filled with deionized water was prepared to estimate kinetics for the CH_4 dissolution of a microporous membrane diffuser. The dissolution test was carried out by supplying CH_4 at various flow rates from 5 to 40 mL/min. The microporous membrane module was designed with four hollow-fiber membranes, which were made of polytetrafluoroethylene (PTFE) with mean pore size of 0.25 µm. The overall surface area of the membrane was 105.73 cm². A commercial tubular diffuser (Alita industries, Baldwin Park, CA, USA) was simultaneously tested as a control. Compared to the microporous membrane, the polyethylene (PE) tubular diffuser had a larger mean pore size and smaller surface area, which were 20 µm and 51.24 cm², respectively. Liquid sample was collected every 30 min and the concentration of methane was analysed using the headspace method. The volumetric mass transfer coefficient (k_La) of methane was calculated by the following equation:

$$\frac{dC}{dt} = k_La\left(C^* - C\right) \tag{4}$$

where k_La is the volumetric mass transfer coefficient (hr^{-1}), C^* is the saturated concentration of methane (mg/L), and C is the concentration of methane at each sampling time (t) (mg/L).

2.4. Continuous Operation of Bioreactor Coupled with Membrane Diffuser

The same reactor (2.5 L) used for kinetic experiment was prepared as a sequencing batch reactor type bioreactor for methanotrophic denitrification under a non-aerated condition (Fig. 1). The bioreactor was operated with a 24-hr fill-and-draw cycle that consisted of a 0.5-hr feeding, 23-hr reaction, and 0.5-hr settling and supernatant decanting period.

A PTFE hollow-fiber membrane diffuser was installed for diffusing methane into the bioreactor. The membrane diffuser

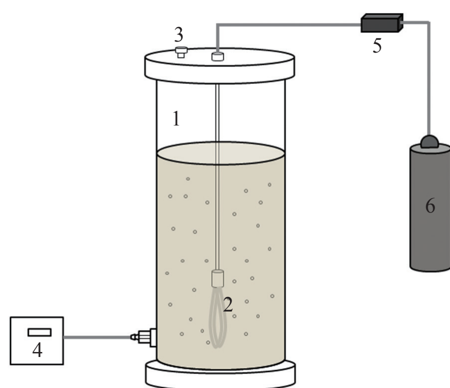


Fig. 1. Schematic of membrane coupled bioreactor for methanotrophic denitrification: 1, bioreactor; 2, membrane module; 3, off gas port; 4, fill and draw pump; 5, gas flow meter; and 6, methane.

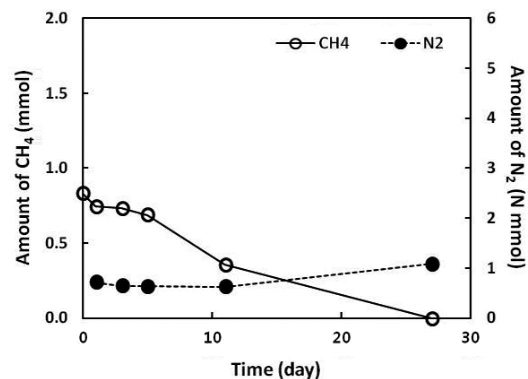


Fig. 2. Variation of amount of CH_4 and N_2 in headspace of anaerobic batch test.

was the same one previously tested in the kinetic experiment. Methane was continuously sparged through the membrane at 10 mL/min and the flow rate was controlled by an electric gas flow meter (TSC-210; New Flow System, Seoul, Korea). The reactor was operated under room temperature condition and it was continuously stirred at 400 rpm by magnetic stirrer during the reaction period. The mixed culture used for the batch experiment was used as a seed at 1,000 mg VSS/L at the start of operation. NO_3^--N, NO_2^--N, and total organic carbon (TOC) in the effluent were periodically measured.

2.5. Analytical Methods

The concentration of CH_4 and N_2 was analysed using gas chromatography (DS6200; Donam Instruments Inc., Seongnam, Korea) equipped with a thermal conductivity detector using an 8-ft HayeSep Q column (Alltech, USA). The temperatures of oven and detector were 40°C and 120°C, respectively. Helium was used as a carrier gas. NO_3^--N, NO_2^--N were measured using ion chromatography (ICS-900; Dionex, Sunnyvale, CA, USA) and TOC in the effluent was measured using a TOC analyzer (TOC-V CPH; Shimadzu, Kyoto, Japan).

2.6. Fatty Acid Methyl Esters of Mixed Culture

FAMEs technology was used for analysis of microbial composition in the mixed culture during operation of the bioreactor. The mixed culture sample was collected from the reactor during the reaction period, and then centrifuged at 2,000 rpm for 7 min. Pelleted materials after centrifugation were transferred in 30 mL of glass tube. The 15 mL of 0.2 M methanolic potassium hydroxide was added and pellet was resuspended using a vortex mixer. The glass tube was placed in a 37°C water bath for 1 hr and remixed every 10 min during this period. Approximately 3 mL of acetic acid was added for neutralization. Thereafter, 10 mL of hexane was added to the solution, which was in turn mixed by vortex mixer, and centrifuged at 2,000 rpm for 3 min. The 5 mL of the overlying hexane layer was transferred to a test tube and evaporated to dryness under flowing N_2. Finally, the remaining material in the test tube was re-dissolved by 0.5 mL of 1:1 of hexane and methyl tertburyl ether and transferred to a gas chromatography vial. FAMEs were analyzed by gas chromatography (Agilent/HP 6890 series II; Agilent, Santa Clara, CA, USA) equipped with Hewlett-Packard methyl phenyl silicone fused silica capillary column (25 m × 0.22 mm × 0.33 m). The initial oven temperature was programmed from 170°C to 270°C at 5°C/min. Hydrogen gas was used as a carrier gas. The FAME peaks were identified by Sherlock MIS software (MIDI Inc., Newark, DE, USA).

3. Results and Discussion

3.1. Batch Experiment

Batch experiments under anaerobic condition were carried out for 27 days to check the capability of the mixed culture seed for AOM-D, utilizing CH_4 as the sole carbon source for denitrification. Fig. 2 shows the variation of CH_4 and N_2 amount in the headspace during incubation. The CH_4 in the headspace was unchanged in 5 days, and then began to decrease. A substantial amount of CH_4 appeared to decrease between 5 and 11 days, which accounted for a 57% decrease in CH_4 amount. Consequently, CH_4 completely disappeared from the headspace at the end of experiment. The maximum CH_4 consumption rate was calculated at 0.09 mmol/day. The change in N_2 content in the headspace was also identified as shown in Fig. 2. The amount of N_2 in anaerobic batch started to increase after 11 days of incuba-

Table 1. CH_4 utilized for denitrification in batch test

Component	Initial (mmol)	Final (mmol)	Overall rate (mmol/day)	Theoretical CH_4 requirement for denitrification (CH_4/NO_3^-)	CH_4 utilized for dentrification (CH_4/ NO_3^-)
CH_4 (gas phase)	0.83	0	0.03	-	-
N_2 (gas phase)	0.63	1.08	0.02	-	-
NO_3^--N[a]	0.74	0.23	0.02	0.63	1.63

[a]Mass in liquid sample.

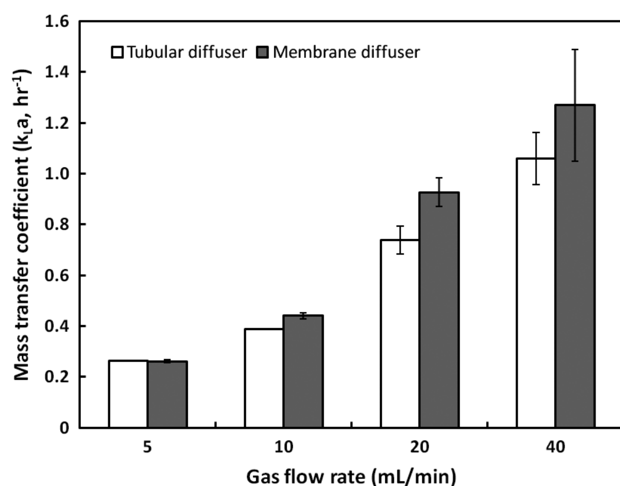


Fig. 3. Mass transfer coefficient (k_La) of methane at various flow rates.

tion and reached 1.08 mmol at the end of experiment.

Concentrations of NO_3^--N in the liquor were measured for the samples taken at the end of the batch experiment. The amount of NO_3^--N remaining was 0.23 mmol, which corresponded to 69% of NO_3^--N removal efficiency. The experimentally estimated CH_4 utilized for NO_3^--N reduction was 1.63, which was about 2.6 times that of the theoretical value (Table 1). The mass balance for nitrogen was calculated by comparing the mass production (0.45 mmol as N) of N_2 with the mass depletion of NO_3^--N (0.51 mmol). The mass recovery of N_2 was 88% indicating that most NO_3^--N removed was converted to N_2 gas through denitrification. These overall results indicated that the mixed culture used in this study could utilize CH_4 as a sole carbon source for denitrification under anaerobic condition, even though the CH_4 consumption rate was most likely lower than that of AeOM-D [19].

3.2. Kinetics for CH_4 Dissolution of Microporous Membrane Diffuser

The volumetric mass transfer coefficient (k_La) of methane, depending on flow rates, is shown in Fig. 3. The k_La value describes the rate of dissolution of methane into water and could be affected by the gas component and its concentration, characteristics of the diffuser, and various environmental conditions [20]. As the gas flow rate increased, the k_La steadily increased regardless of the diffuser type and the dissolved methane concentration consequently reached 20.4 ± 0.16 mg/L, which is close to the saturation value (23 mg/L at 20°C). The membrane diffuser showed a higher k_La than the tubular diffuser in all ranges of gas flow rate tested. This was probably attributed to the higher surface area and small pore size of the membrane diffuser than of the tubular diffuser. The smaller pore size could generate finer bubbles, which were most likely well dissolved in the water [21, 22]. The maximum k_La for the membrane and tubular diffuser was 1.27 and 1.06 hr^{-1} at 40 mL/min, respectively.

3.3. Operation of Non-aerated Bioreactor

Fig. 4 shows the denitrification efficiency and specific denitrification rate (SDR) obtained from operation of the non-aerated bioreactor for 50 days. The bioreactor was operated without external oxygen (or air) supply, but DO concentration was maintained in the range between 0.3 and 0.5 mg/L due to oxygen remaining in the medium solution. In spite of continuous purging N_2 into the medium storage tank, the average DO concentration in the medium tank was 1.0 mg/L.

The denitrification efficiency increased and reached a maximum of 69% after 30 days of operation. In order to check the capability of AOM-D under strict anaerobic condition, sodium dithionite ($Na_2O_4S_2$) was added at 2 mmol/day after 35 days, and then the DO concentration was rapidly decreased to 0.05 mg/L. Anaerobic condition in the reactor was double-checked by adding resazurin to the effluent as an indicator. The denitrification efficiency drastically dropped as soon as $Na_2O_4S_2$ was added. The above results indicate that the presence of DO (at least 0.5 mg/L) is necessary to perform denitrification using methane as a sole carbon source, since the direct utilization of methane for AOM-D under strict anaerobic condition is too slow to occur in 23 hr of reaction time. It is generally accepted that low DO is a favourable condition for denitrification, but not for methanotrophic denitrification. Methanotrophic denitrification requires DO for methanotrophs to assimilate carbon via the RuMP or serine pathway [8]. Even though the potential of AOM-D was already confirmed in the anaerobic batch experiment, the rate of methanotrophic reaction was very slow without oxygen [23, 24]. Based on this study and a previous study [25], DO concentration in the bioreactor for methanotrophic denitrification is preferably at a narrow range between 0.5 and 0.8 mg/L, which is achievable in a non-aerated manner. Thus, secondary wastewater effluent would be a good candidate for non-aerated methanotrophic denitrification being applied with inherent DO in the range between 0.9 and 3.7 mg/L [26].

Table 2 shows a comparison of the SDR obtained from the bioreactor with other previous studies. The SDR was very variable among studies, and the maximum SDR of 1.69 mg NO_3^--N/g VSS·hr obtained from this study was slightly lower than from the previous studies. This was attributable to the following reasons:

Table 2. Specific denitrification rate (SDR) of bioreactor under non-aerated condition, and comparison with other studies

Condition	Inoculum	Reactor	SDR (mg NO_3^--N/g VSS·hr)	Reference
Aerobic (21°C–23°C)	Activated sludge	SBR, continuous gas flow	2.36	[25]
Anoxic (30°C)	Anaerobic sludge	Batch	10.42	[15]
Anoxic (30°C)	Denitrificant/methanotrophic biomass	SBR	5.42	[8]
Anaerobic (35°C)	Bacteria related to *M. oxyfera*	Batch	0.33	[27]
Non-areated (20°C)	Activated & anaerobic digester sludge	SBR, continuous gas flow	1.69	This study
Anaerobic (20°C)	Activated & anaerobic digester sludge	Batch	0.09	This study

SBR: sequencing batch reactor.

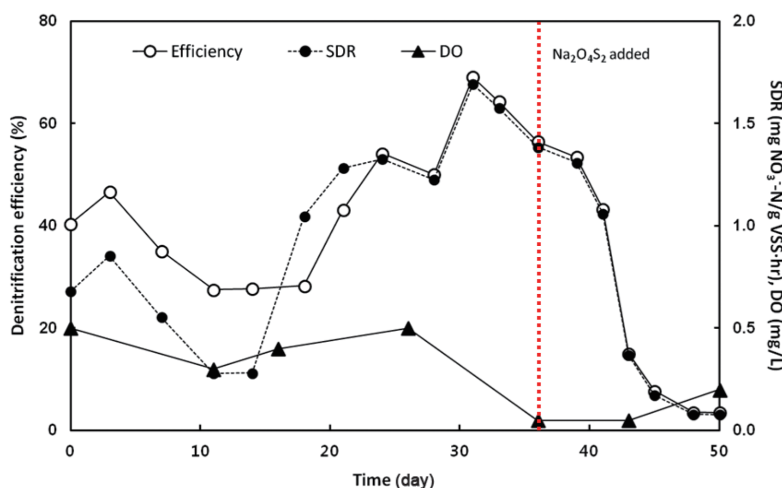


Fig. 4. Denitrification performances of bioreactor under non-aerated condition. SDR: specific denitrification rate, DO: dissolved oxygen, VSS: volatile suspended solids.

the high biomass concentration of the mixed culture not pure culture used in this study. However, the obtained SDR showed a similar level to that of another continuous process under aerobic condition [25].

Fig. 5 shows the variation of TOC concentrations in the effluent. One of the most prevalent concerns in the operation of post denitrification is the deterioration of effluent quality due to excessive presence of unutilized organic substances. In methanotrophic denitrification, the remaining organic substances could mainly be dissolved CH_4 or methanol. In this study, the TOC concentration averaged 2.45 mg/L during the overall period of operation. In general, the TOC concentration of secondary effluent from most wastewater treatment plants is around 11 mg/L [28]. This result means that concern about residual organic matter after denitrification was not significant in methanotrophic denitrification, since methane or unutilized methanol was not predominant.

This study suggests that methanotrophic denitrification under non-aerated condition can be a promising option for water reclamation of secondary effluent or groundwater remediation with a relatively high denitrification performance and less residual organic concentration. Although the capability of AOM-D under anaerobic condition was not available due to the slow rate, methanotrophic denitrification could be achieved in a bioreactor without excess supply of oxygen by utilizing the inherent DO in water.

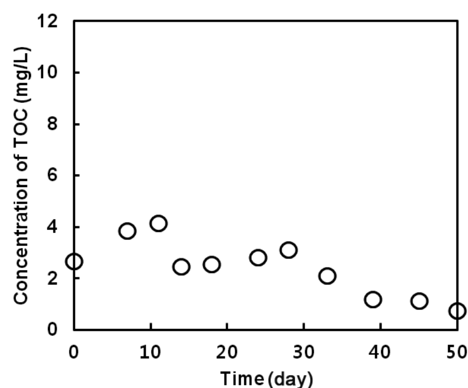


Fig. 5. Concentration of total organic carbon (TOC) in effluent of bioreactor.

3.4. FAMEs Analysis for Methanotrophs in Bioreactor

Table 3 shows the proportion of major FAMEs extracted from the mixed culture in the bioreactor. Of four major FAMEs identified, the most predominant FAME was $C_{16:1\omega7c}$, which is known to

Table 3. Proportion of FAMEs from mixed culture of bioreactor and comparison with FAMEs for methanotrophs obtained in the other studies

FAMEs	Proportion (%)	Dominant FAMEs in literature		
		FAMEs	Species of methanotrophs	Reference
$C_{14:0}$	10.8	-	-	-
$C_{16:0}$	16.6	C_{16} sp.	*Methylomonas* sp.	[31]
$C_{16:1\omega7c}$	45.6	C_{16} sp.	*Methylococcus capsulatus*	[31]
$C_{18:1\omega7c}$	27.0	C_{18} sp.	*Methylosinus trichosporium*	[31]
		C_{18} sp.	*Methylobacterium organophilum*	[31]
		$C_{18:1\omega8c}$	*Methylosinus* and *Methylocystis*	[32]
		$C_{18:1\omega7c}$	*Methylocella* and *Methylocapsa*	[32]

FAME: fatty acid methyl ester.

be a typical FAME for type I methanotrophs, such as *Methylococcus luteus* (UQM3304), *Methylococcus whittenburyi* (UQM3310), and *Methylomonas gracilis* [29]. Secondly, the dominant FAME in the mixed culture was $C_{18:1\omega7c}$, which is normally found in an anaerobic digester in high proportion [30]. Also, in several studies, type II methanotrophs have C_{18} FAME including $C_{18:1\omega7c}$ as their major component [29, 31, 32]. Type I methanotrophs are known to be a major microbial group found in most AeOM-D processes; whereas, type II methanotrophs are dominantly found under anoxic or anaerobic condition, with lower DO concentration, compared to methane concentration [33, 34]. This study revealed that both methanotrophs of types I and II were copresent in the bioreactor due to the seed selected (mixed culture of activated sludge and anaerobic digested sludge) and environment (low DO concentration). However, those FAME profiles are also found in other microorganisms as well as methanotrophic bacteria. Hence, in order to more accurately identify methanotrophs in mixed culture, some other methods, based on genomic technology, may be necessary in the future.

4. Conclusions

This study presents the applicability of methanotrophic denitrification under non-aerated condition for the reclamation of secondary wastewater effluent. In batch experiment, methane can be utilized as a sole carbon source for denitrification under anaerobic condition, but rates for methane consumption and nitrate reduction were slow. A microporous membrane diffuser could enhance the mass transfer of methane, so that it was incorporated into the bioreactor. A relatively good denitrification performance was achieved in the bioreactor under non-aerated condition. In spite of no external supply of oxygen, oxidation of methane by methanotrophs occurred by utilizing the residual DO in the influent. The residual TOC concentration in effluents from the bioreactor was much lower than in common secondary effluent. The copresence of two different types of methanotrophs of types I and II in the bioreactor well represented the characteristics of the non-aerated bioreactor providing a favourable circumstance for both types of methanotrophs. The DO concentration in the secondary effluent might be inherently in the range to support methanotrophic denitrification, so that methanotrophic denitrification will be attainable without external oxygen supply. This study suggests that methanotrophic denitrification under non-aerated condition will be a promising option to current post denitrification for the reclamation of secondary wastewater effluent or remediation of groundwater.

Acknowledgments

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