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Upflow anaerobic-microaerobic fixed biofilm reactor integrating methanogenesis with partial nitrification

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Abstract

An anaerobic-microaerobic fixed biofilm (AMFB) reactor, that integrates methanogenesis with partial nitrification within a single unit was investigated to achieve carbon removal simultaneously with ammonium oxidation in dilute wastewater. Membrane aeration was used for a controlled and efficient oxygen supply for partial nitrification and to prevent oxygen related inhibition of methanogens in the AMFB reactor. Removal of chemical oxygen demand (COD) and ammonium oxidation was first tested on synthetic wastewater, followed by domestic wastewater. The COD removal efficiency ranged between 92-99% on synthetic wastewater at hydraulic retention time (HRT) of 8-24 h. Nearly complete removal of biochemical oxygen demand (BOD₅) was obtained for domestic wastewater. Influent COD was mainly removed by fermentation and methanogenesis, resulting in high methane yields of up to 0.33 L CH₄ g COD⁻¹ anaerobic. Ammonium oxidation efficiency of 69-86% was obtained. Microbial community analysis showed proliferation of fermenters and methanogens exclusively in the anaerobic section of the reactor, while aerobic heterotrophs and nitrifiers were mainly identified in the membrane aerated section. This study first proves that the single-stage AMFB reactor can treat municipal wastewater economically to meet the wastewater standards, although further research for improving water quality (e.g., denitrification) would be required.

Keywords: wastewater treatment, anaerobic COD removal, partial nitrification, microaerobic, methane.
1. INTRODUCTION

The development of compact wastewater treatment systems has garnered much attention in the past decade. This interest can be attributed to many reasons including: expansion/retrofitting of wastewater treatment plants (WWTPs) in urban and peri-urban areas with limited land availability; cold region plants placed inside heated buildings and small-scale decentralised WWTPs for rural and remote communities. Compact treatment systems currently in the marketplace typically utilize the activated sludge process and modifications thereof. Since this biological treatment depends on aerobic microbial metabolism, intensive oxygen supply is essential which features high energy consumption and operating costs. Methane-producing anaerobic biotechnologies such as anaerobic digestion (AD) provide an energy-efficient alternative to aerobic technologies. Contrary to aerobic counterparts, AD does not require oxygen for removal of organics and produces less sludge. Rapid improvements in bioreactor technology (e.g., immobilization, granulation, membranes, etc.) has resulted in advent of compact high-rate anaerobic bioreactors with treatment efficiency and process stability close to aerobic technologies. Multiple studies have reported organic removal efficiencies of 90-95% at a short hydraulic retention time (HRT) of 9-12 h in fixed-bed/film anaerobic digesters [1, 2]. The immobilisation of the microbial community on inert media allows excellent biomass retention leading to enhanced degradation rate, reduced digestion time and a small footprint. Moderate effluent quality can also be achieved without the need for solid-separation. Despite the advantages, a major bottleneck in the widespread adoption of such compact AD systems for domestic wastewater treatment are limitations in removing nutrients, especially nitrogen.
Wastewaters typically contain considerable levels of nitrogen-based compounds such as ammonium that needs to be removed before discharge to water body for ensuring water quality and protecting aquatic ecosystems (e.g., algal bloom and toxicity to aquatic organisms). European (EN 12-566) and American standards (NS Standard 40) for small scale wastewater treatments, a niche market for anaerobic compacted systems, mandate effluent $NH_4^+$-N concentrations of below 10 mg L$^{-1}$ [3]. Anaerobic systems transform a portion of nitrogen to soluble ammonia rather than removing it by nitrification. The requirement of oxygen for nitrification coupled with oxygen sensitivity of methanogens limits removal of nitrogenous pollutants in anaerobic reactors. Thus, additional removal steps are required after anaerobic digesters to achieve effluent quality suitable for discharge, which makes the entire process complex and expensive. A tangible solution to this challenge is a single compact reactor that combines AD with nitrification. This may be accomplished by exploiting the ability of methanogens to withstand low levels of dissolved oxygen (DO) combined with relatively low DO requirements for partial nitrification (i.e., nitritation), the oxidation of ammonia to nitrite. Transformation of nitrite to N$_2$ can be achieved in post-denitrification with less dose of electron donor, another potential benefit of partial nitrification.

Sustained methanogenic activity in mixed cultures under microaerobic conditions of 0.1 – 0.5 mg L$^{-1}$ of DO has been widely reported [4-6]. This tolerance in mixed cultures is attributed to oxygen consumption by facultative microorganisms which creates localised anaerobic environments where methanogens are protected. Similarly, low DO concentrations (< 1 mg L$^{-1}$) are common in bioreactors catalysing partial nitrification [7-9]. Therefore, methanogenesis and partial nitrification could be combined in a single reactor provided low DO concentrations can be maintained by controllable and efficient oxygen supply; to maximize oxygen consumption by ammonia oxidizing bacteria (AOB), while minimizing oxygen related inhibition of methanogens. Advancement in
membrane-based aeration allows for oxygen delivery at high rates and transfer efficiencies. Membrane systems have been applied where a conventional aeration system is unable to meet the oxygen requirements of a high rate system. With bubble-free aeration using membranes, oxygen transfer efficacy close to 100% is achievable with consequent energy savings [10, 11]. In addition, the oxygen supply rate can be efficiently controlled by the intramembrane oxygen partial pressure and membrane surface area. Modulation of membrane into different geometries (e.g., tubular or flat membrane, etc.) allows for membrane integration into different reactor designs, increased membrane surface to volume ratio and high volumetric mass transfer [11, 12].

This study presents the development and operation of an upflow bioreactor that can simultaneously accomplish carbon and ammonium removal in wastewater under microaerobic conditions. Organic carbon was removed by methanogenic biodegradation (anaerobic process), while ammonium removal was achieved by partial nitrification (aerobic process). To simplify workflow and attain a small land footprint, the aerobic and anaerobic processes were integrated in a single reactor by staging former on top of the latter without any physical separation. In both treatment steps, the active biomass was immobilised on a porous carbon felt media (fixed-biofilm). Micro-porous gas membrane was used for efficient oxygen delivery for partial nitrification, allowing simultaneous methanogenesis retained at the bottom of the reactor.

2. MATERIAL AND METHODS

2.1 Bioreactor Design
The schematic of the anaerobic-microaerobic fixed biofilm (AMFB) reactor developed in this study is depicted in Figure 1. The reactor was fabricated using a plexiglass column with an inner diameter of 13 cm and a height of 105 cm. The empty bed volume of the reactor was ~ 14 L. The reactor was divided into two main sections: a lower anaerobic section occupying 8 L of the volume and an upper microaerobic section with a volume of 4 L. The headspace occupied ~ 0.5 L of the reactor volume. The anaerobic zone was built for degradation of organic matter, while the microaerobic zone was designed primarily for partial nitrification (nitrite accumulation) using a gas membrane. The anaerobic and microaerobic zones were fluidically bridged without any physical barrier. A middle zone of 1.5 L in liquid volume bridged the two sections. Two separate recirculation loops were used for mixing the liquids in the anaerobic and microaerobic zones. A circulation rate of 8 L h\(^{-1}\) was applied for both zones using peristaltic pumps (Masterflex L/S 7523-80, Cole-Parmer, Canada).

In both zones, the active biomass was immobilised on carbon-felt media (Speer Canada, Kitchener, ON, Canada). Carbon felt was selected due to low cost ($0.85/kg), and high porosity (91%) and surface area (0.5 m\(^2\)/g) which minimize mass transfer limitations and biofilm shearing, a major bottleneck in fixed-biofilm reactors [1, 13]. The anaerobic zone was packed with three carbon felt modules: CF1, CF2 and CF3. The modules were prepared by packing cylindrical pieces of carbon felt on a stainless-steel mesh support with individual dimensions of 12.8 cm (diameter) X 15 cm (height). Similarly, the microaerobic zone consisted of three carbon felt modules: CF4, CF5 and CF6. These hollow cylindrical modules had an inner and external diameter of 4 cm and 12.8 cm, respectively, with a height of 5.5 cm.
A woven carbon cloth membrane with hydrophobic micro-porous layer (Model# W1S1009, Fuel Cell store, TX, USA) was used for bubble-less aeration, creating the microaerobic zone. The custom-made membrane module consisted of a hollow steel tube with wide channels milled down its length and capped at the bottom end with a rubber stopper. The membrane was wrapped around the steel tube and sealed using a specialized adhesive (Fuel Cell store, TX, USA). Stainless-steel fittings connected the module to the removable reactor top (Figure 1). Based on the assumption that most of the organics in wastewater would be stabilised to methane and carbon dioxide in the anaerobic zone, the air supply through the gas membrane was intended principally for partial nitrification in this study. The membrane had a thickness of 410 µm, an effective surface area of ~ 0.1 m² and occupied ~1.5 L of the microaerobic zone. To enhance gas transfer efficiency, the membrane module was operated in a dead-end mode. The membrane was maintained at the desired air-pressure by using a mass flow meter (FMA-1609A, Omega, Canada) to control the air flow rate to the membrane module. The membrane pressure was monitored using a manometer (Omega Instruments, USA) connected to the membrane module through a stainless-steel fitting. The membrane was inserted through the hollowed center of the media modules (CF4-CF6) in the microaerobic zone (Figure 1).

### 2.2 Bioreactor Start-up and Operation

The AMFB bioreactor start-up took 8 weeks and entailed two activities: 1) enrichment of methanogens in the anaerobic zone, and 2) enrichment of nitrifying microorganisms in the microaerobic zone. For enrichment of methanogens, the AMFB reactor was packed with media modules CF1 to CF3 and inoculated with 3 L of anaerobic digester sludge (MLVSS 10,800 ± 750 mg L⁻¹) sampled from
Galt wastewater treatment plant (Cambridge, ON, Canada). The reactor was fed with an acetate medium for 4 weeks at an HRT of 72 h to facilitate the formation of methanogenic biofilms on the media. In tandem, a separate procedure was followed for enrichment of nitrifiers. Media modules CF4 to CF6 were incubated with recycle activated sludge in an ammonium medium (31 mg L⁻¹ of NH₄⁺-N) in a separate 3L container and was continuously sparged with air for 3 weeks to grow nitrifying biofilms. Subsequently, the media modules enriched with nitrifying biofilm were stacked in the AMFB reactor along with the gas membrane module. From this point onwards, the reactor was operated as a staged anaerobic-microaerobic system for 4 weeks with the acetate medium at an HRT of 72 h. After achieving a pseudo-steady state for COD removal, the bioreactor was operated in phases described in Table 1 (overall 8 phases). In phases 1, 3, 5 and 6, COD and ammonium removal was tested at HRTs of 24, 12 and 8 h on the acetate medium, amounting to COD loading rates from 0.35 to 2.1 kgCOD d⁻¹m⁻³ (based on reactor volume, m⁻³) and NH₄⁺-N loading rates from 0.03 to 0.08 kgNH₄⁻-N d⁻¹m⁻³. To distinguish between the contribution of anaerobic and aerobic oxidation to overall COD reduction, anaerobic control tests were conducted in phases 2 and 4 by ceasing air supply to the membrane module. Similar procedure was also followed to ascertain COD removal characteristics on domestic wastewater in phases 7 and 8. Before changing each phase, steady state condition was confirmed by observing less than 10% variation in effluent COD and ammonium concentrations.

Acetate medium or domestic wastewater was fed at the base of the anaerobic zone using a peristaltic pump (Masterflex L/S 7523-80, Cole-Parmer, Canada) and circulated through the carbon felt media, CF1-CF3. The feed was transported upwards to the microaerobic zone, passed through media CF4-CF6, and exited the reactor at the upper section of the microaerobic zone. The COD, nitrogen, and DO concentrations were monitored through the sampling ports (Port 1, Port 2 and Port3) installed along the reactor. The
removable cover of the reactor had lines for biogas sampling and monitoring. The reactor was operated at a room temperature of 22±1 °C at all phases and a pH of 7.6 was maintained in the anaerobic zone with a pH controller (Cole Parmer, QC, Canada) using 1.5 M NaOH as the alkaline solution.

2.3 Membrane Performance

The aeration system for partial nitrification needs to adequately match the oxygen demand for oxidation of ammonia to nitrite. The ability of the membrane module to meet this criterion was assessed by evaluating the oxygen supply rate (OSR) prior to reactor installation. The OSR (gO₂ d⁻¹ or gO₂ m⁻² d⁻¹) was evaluated based on the oxygen transfer rate from the gas membrane to the bulk liquid under abiotic conditions with distilled water [14]; the time course of DO concentration in the bulk liquid under different air pressures of 14, 28 and 41 kPa. Detailed procedure for estimation of OSR is provided in supporting information (SI). A stoichiometric oxygen demand for partial nitrification was computed at 1.4-3.8 gO₂ d⁻¹ for NH₄⁺-N load in phases 1 to 6 (3.40 mg of O₂ required per mg of NH₄⁺-N), given that most of the organic COD would be stabilized anaerobically. Experimental OSR of 1.08 gO₂ d⁻¹, 2.1 gO₂ d⁻¹ and 3.3 gO₂ d⁻¹ were obtained by adjusting intramembrane air pressure to 14, 28 and 41 kPa, respectively. This matching OSR (1.08 - 3.3 gO₂ d⁻¹) to the stoichiometric requirement (1.5-3.8 gO₂ d⁻¹) clearly demonstrated the ability of the membrane module to sufficiently meet the oxygen demand for partial nitrification in the AMFB reactor. The O₂ mass transfer with clean deionized water can be higher than that with wastewater in bioreactors having immobilized biomass. To mitigate O₂ transfer limitations, highly porous
(~ 91%) carbon felt media was used to ensure efficient O₂ transfer even to the local microbial microenvironments in the AMFB reactor. The liquid in the microaerobic zone was also recirculated at a rate of 2 L L⁻¹ microaerobic zone.

2.4 Reactor Performance

The performance of the AMFB reactor was evaluated in terms of COD and ammonium removal efficiency (%) and was calculated as:

\[ E(\%) = \left( \frac{C_{in} - C_{out}}{C_{in}} \right) \cdot 100 \]  

Where: \( E \) is the COD or ammonium removal efficiency, \( C_{in} \) and \( C_{out} \) are the corresponding influent and effluent concentrations (g L⁻¹) of COD or ammonium.

The oxygen uptake rate for nitrification (OURₕ) was based on requirement of 3.40 mg and 4.54 mg of O₂ for conversion of 1 mg of ammonium nitrogen to nitrite and nitrate, respectively. The OURₕ (g/d) was calculated as [15]:

\[ \text{OURₕ} = F \left[ 3.40 (\text{NO}_2-\text{N}_{eff}) + 4.54 (\text{NO}_3-\text{N}_{eff}) \right]/V \]  

Where: \( \text{NO}_2-\text{N}_{eff} \) is the effluent nitrite concentration (mg L⁻¹), \( \text{NO}_3-\text{N}_{eff} \) is the effluent nitrate concentration (mg L⁻¹), \( F \) is the effluent flow rate (L d⁻¹) and \( V \) is the reactor volume (L).
The oxygen uptake efficiency (OUE$_N$) is a measure of the amount of oxygen supplied to the system utilised for nitrification. The OUE$_N$ (%) was calculated as:

\[
\text{OUE}_N = \frac{\text{OUR}_N}{\text{OSR}} \times 100
\]

Where: OSR is the oxygen supply rate

2.5 DNA extraction, PCR amplification of 16S rRNA and Illumina Sequencing

After completion of all the phases, the reactor was dismantled and media modules were removed from the anaerobic and aerobic zone under a sterile fume hood. Pieces of carbon felt media (1 cm X 1 cm X 5 cm) were excised from modules in the anaerobic (CF1 and CF3) and aerobic zone (CF4 and CF6) using a sterile surgical scissor and stored at -20°C until used for analysis. Genomic DNA (gDNA) was extracted using the PowerSoil™ DNA isolation kit (MOBIOL Laboratories, Carlsbad, USA) as outlined in the instructions provided by the manufacturer. The extracted DNA was then used as a template to amplify the 16S rRNA genes by PCR. Universal prokaryotic primer 515F-Y (GTGYCAGCMGCCGCGGTAA) and 926R (CCGYCAATTYMTTTRAGTTT) were used to amplify the genes in both bacteria and archaea. Each primer contained a six-base index sequence for sample multiplexing as well as Illumina flow cell binding and sequencing sites. PCR mix (25 μl total volume) contained 1X ThermoPol Buffer buffer, 0.2 μM forward primer, 0.2 μM reverse primer, 200 μM dNTPs, 15 μg BSA, 0.625 U Taq DNA polymerase, 1 μl of template (1 to 20 ng). The PCR was
performed as follows: 95°C for 3 min, 35 cycles of 95°C for 30 sec, 55°C for 30 sec, 68°C for 1 min, and a final extension of 68°C for 7 min.

Indexed PCR amplicons were quantified in a 1.5% agarose gel containing GelRed and equal quantities of each amplicon were pooled. The pooled 16S rRNA amplicons were excised from an agarose gel and purified using Wizard SV Gel and PCR Clean-Up System (Promega, cat. no. A9282). The Illumina library was denatured and diluted following Illumina guidelines (Document no. 15039740 v01). A 2.5 pM library containing 10% PhiX was sequenced on a MiSeq instrument (Illumina Inc., San Diego, USA) using a 2 x 250 cycle MiSeq Reagent Kit v2 (Illumina Canada Inc, cat. no. MS-102-2003). Paired-end reads were assembled using PANDAseq [16]. Merged reads were clustered with UPARSE [17] which collapsed the data into groups of highly similar sequences. The data are compressed into unique taxa that are within 97% similarity to one another, which reflects a species cut-off for bacteria and archaea. The most abundant sequence within each 97% cluster is selected as the “representative sequence” for each operational taxonomic unit (OTU). All representative sequences were classified by RDP [18] with a stringent confidence threshold (0.8).

2.6 Medium Composition and Analytical Methods

The acetate medium contained (per litre): 0.640-1.510 g CH₃COONa, 0.100-0.125 g NH₄Cl, 74 g KCl, 32 g K₂HPO₄, 20 g KH₂PO₄, 0.8 g yeast extract, 0.128 g of KH₂PO₄, 0.434 g of Na₂HPO₄, 0.685 g of NaNO₃, 0.4804 of Na₂S·9H₂O, 0.001 g of CaCl₂·2H₂O, 0.001 g of FeSO₄·7H₂O, 0.1647 g of MgCl₂·6H₂O and 1 mL of trace solution. The composition of trace solution consisted of (per liter): 0.1 g ZnSO₄·7H₂O, 0.03 g MnCl₂·4H₂O, 0.3 g H₃BO₃, 0.2 g CoCl₂·6H₂O, 0.01 g CuCl₂·2H₂O, 0.01 mg
NiCl₂·6H₂O, and 0.03 g Na₂SeO₃. The medium was autoclaved, and then the pH was adjusted to 7.6 ± 0.2 using 0.1 N H₂SO₄. The concentration of sodium acetate was adjusted to increase COD loading rate (phase 1 to 6 in Table 1). The ammonium medium (31 mg L⁻¹ of NH₄⁺-N) for enrichment of nitrifiers during start-up was same in composition to the acetate medium but devoid of any acetate. During phases 7 and 8, the reactor was fed with wastewater collected at a pilot wastewater treatment facility (University of Waterloo, ON, Canada). To avoid carbon felt blockage, solids were removed using a 1 mm sieve prior to reactor feeding.

The concentrations of chemical oxygen demand (COD), biochemical oxygen demand (BOD₅) and mixed liquor volatile suspended solids (MLVSS) were determined with the Standard Methods [19]. Ammonium concentration was measured with an Auto Analyzer 3 (Bran-Luebbe, Germany). Nitrate and nitrite were quantified using Dionex ICS-3000 HPLC equipped with an ionic conductivity detector (Dionex Corporation, Sunnyvale, California, USA). The off-gas flow rate was measured using a MilliGascounter™ (Ritter Apparatus, Bochum, Germany). The off-gas composition was quantified using a gas chromatograph equipped with a thermal conductivity detector (GC-TCD, SRI GC 310C, USA). The GC-TCD equipped with a packed column (PorapakQ, 6 ft x 1/8 inches, 80/100 mesh, Agilent Tech., USA) used helium (99.999 %, PraxAir, Canada) gas as the carrier gas at a flow rate of 10 mL min⁻¹ and a pressure of 21 psi. The temperatures of the column oven and detector were 41°C and 200°C, respectively. A benchtop DO meter (Model 01972, Cole Parmer, IL, USA) was used for DO measurements.
3. RESULTS AND DISCUSSION

3.1 Removal of organic carbon in AMFB reactor

High COD removal was consistently observed in the AMFB reactor fed with the acetate medium, as shown in Figure 2A. Almost complete COD removal efficiency of ~99% (effluent COD 6-7 mg L\(^{-1}\), Figure 3A) was obtained at HRTs of 24 h (phase 1, 0.35 kgCOD\(\cdot\)d\(^{-1}\)\(\cdot\)m\(^{-3}\)) and 12 h (phase 3, 0.68 kgCOD\(\cdot\)d\(^{-1}\)\(\cdot\)m\(^{-3}\)) under anaerobic-microaerobic conditions. High COD removal of ~96% was also achieved when the COD loading rate was doubled to 1.56 kgCOD\(\cdot\)d\(^{-1}\)\(\cdot\)m\(^{-3}\) (phase 5). Further reduction of HRT to 8 h (phase 6, 2.1 kgCOD\(\cdot\)d\(^{-1}\)\(\cdot\)m\(^{-3}\)) marginally reduced the removal efficiency to 92% (effluent COD ~ 85 mg L\(^{-1}\)). Anaerobic tests at HRT of 24 h (phase 2) and 12 h (phase 4) resulted in COD removal efficiency of 97% and 93%, respectively (Figure 2A). This was only 2-6% lower to the COD removal obtained at same loading rates and HRT under anaerobic-microaerobic conditions (phases 1 and 3), implying that fermentation and methanogenesis was primarily responsible for organic removal in the bioreactor.

Importantly, almost complete BOD\(_5\) removal (98%) was obtained at phases 7 and 8 when domestic wastewater was fed to the AMFB reactor. The average BOD\(_5\) concentration in the final effluent was only 6±5 mg L\(^{-1}\) (Table 2) with an influent BOD\(_5\) of 178±22 mg L\(^{-1}\). This result clearly demonstrated that the AMFB reactor can treat domestic wastewater to meet the federal wastewater effluent standard in Canada and many other countries (e.g., < BOD\(_5\) 25 mg L\(^{-1}\)). The BOD\(_5\) was mainly removed under anaerobic conditions as indicated by similar effluent BOD\(_5\) concentration under anaerobic-microaerobic (BOD\(_5\) 4±5 mg L\(^{-1}\), phase 7) and complete anaerobic conditions (BOD\(_5\) 6±3 mg L\(^{-1}\), phase 8). This result is consistent to COD removal in the bioreactor fed with acetate medium (see Figure 2), proving that AMFB reactor can adequately treat domestic wastewater without intensive aeration. Interestingly, there was
large difference between effluent BOD$_5$ (4±5 mg L$^{-1}$) and COD concentration (121±7 mg L$^{-1}$), which indicates accumulation of non-biodegradable or slowly biodegradable organics in AMFB effluent. Future research is required for characterizing organics accumulated in anaerobic and micro-aerobic conditions. The suspended solids (SS) were not measured during the experiments due to clean effluent from the AMFB reactor; for instance, effluent COD concentration was 6-16 mg L$^{-1}$ in Phases 1-3 and effluent BOD concentration was 4-6 mg L$^{-1}$ in Phases 7 and 8. Microbial reactions (e.g., hydrolysis) and filtration through carbon felt media would keep effluent SS low. The AMFB reactor was fed with wastewater filtered with 1mm sieve to mitigate media clogging, which would account for low COD and BOD in the effluent. Particulates in the effluent would increase or media blockage would occur in the AMFB reactor that receives wastewater directly. Sieving wastewater as pretreatment would be essential for treating domestic wastewater with the AMFB reactor, and hence future research is required to more investigate sieving processes (e.g., different sieving sizes) in parallel with reactor performance.

Overall, the organic removal in the AMFB reactor was comparable to those reported for anaerobic-aerobic systems with physical separation of each zone. Li et al. [20] recently reported COD removal efficiencies of over 98 % in a continuous aerobic-anaerobic coupled (CAAC) system comprising of a moving bed bioreactor (MBBR) connected to an anoxic, anaerobic and aerobic zone in series and fed with artificial wastewater at OLR of 1.5 -1.7 kg m$^{-3}$ d$^{-1}$. Similarly, COD removal efficiencies of 92-99% were obtained for sequential or baffled anaerobic-aerobic bioreactors treating poultry slaughterhouse and potato starch wastewater at OLR of 2.5 -4.5 Kg m$^{-3}$ d$^{-1}$ [21, 22]. These results highlight that the integration of anaerobic and aerobic zones without physical separation, as in the AMFB reactor, can achieve equally high degradation efficiencies with a potentially smaller footprint.
3.2 Characterization of COD removal in anaerobic and aerobic zones

In order to optimise the design and operating protocol of the AMFB reactor, it is essential to ascertain not only the overall removal of organics, but also the fraction of organic matter that is removed by anaerobic and microaerobic processes. The COD removal in each zone was estimated based on the COD concentration of samples collected from port 2 and 3 representing anaerobic and microaerobic zone, respectively (Figure 1). Figure 2B shows that about 81-95% of the COD in the acetate feed was anaerobically removed at all HRTs; aerobic oxidation only contributed between 5-10% to overall COD removal. The COD removal in phases 1, 3 and 5 were comparable to those obtained in phases 2 and 4 (completely anaerobic conditions), validating that most of COD was removed in the anaerobic zone. These results also support that membrane aeration was not utilised for COD removal, but mainly for ammonium oxidation, which was the intended objective for creating the microaerobic condition using the gas membrane. For domestic wastewater (phase 7), the anaerobic COD removal was 50±8 % (Figure 2B), which was over three quarters of the overall COD removal (68%), while the aerobic zone accounted for less than one quarter of the total COD removal (16%).

3.3 Ammonium removal in the AMFB reactor

Ammonium oxidation efficiency exceeded 68 % at all HRTs on acetate medium (contained 0.125-0.150 g L⁻¹ of NH₄Cl), as shown in Figure 2A. A maximum oxidation efficiency of 86% was obtained at HRT of 24 h (phase 1, 0.03 kgNH₄-N d⁻¹ m⁻³). A
comparable ammonium oxidation of 74-78% was also achieved at HRT of 12 h (phase 3 and 5, $0.05-0.06 \, K \, g_{NH_4-N} \, d^{-1} m_v^{-3}$). Reducing HRT further to 8 h (phase 6, $0.08 \, K \, g_{NH_4-N} \, d^{-1} m_v^{-3}$) lowered ammonium oxidation efficiency to 69%. Anaerobic tests at HRT of 24 h (phase 2) and 12 h (phase 4) indicated a marginal ammonium removal of 6-8% probably via cell synthesis. This difference in ammonium removal under anaerobic-microaerobic (74-86%) and completely anaerobic conditions confirmed ammonium oxidation in the microaerobic zone, as designed. An ammonium oxidation efficiency of 67% (effluent $NH_4^+$-N of 12 mg L$^{-1}$, Table 2) was achieved on domestic wastewater with active membrane aeration (phase 7, $0.05 \, K \, g_{NH_4-N} \, d^{-1} m_v^{-3}$).

Nitrite was the primary metabolite of ammonium oxidation at all phases. The $NO_2^-$-N concentrations in the effluent were considerably higher than $NO_3^-$-N concentrations (Table 2). Furthermore, nitrogen balance indicated that effluent $NO_2^-$-N concentrations accounted for 60-72% of the $NH_4^+$ - N removed in each phase. Higher $NO_2^-$-N concentrations were indicative of ammonium removal by partial nitrification, the intended purpose of creating the microaerobic zone. Such nitrite accumulation due to partial nitrification is a common phenomenon under oxygen limited conditions [23-25]. Indeed, DO concentrations in the microaerobic zone ranged from 0.2 to 0.9 mg L$^{-1}$ during the experiments and matched the low DO requirements for partial nitrification (DO $< 1$ mg L$^{-1}$).

The low DO concentrations in the microaerobic zone also affirmed the efficacy of the carbon-based membrane system to adequately match the stoichiometric oxygen requirement for nitrification in different phases. Matching oxygen supply to the stoichiometric oxygen requirement also resulted in high OUE$_N$ (%), which is a measure of the amount of oxygen used for nitrification.
to the amount of oxygen transferred through membrane aeration. OUE\textsubscript{N} of 62-75\% was obtained at different phases (Table 2), much higher than the OUE\textsubscript{N} of 15-30\% reported in nitrifying bioreactors with air diffusers [26].

The discharge of nitrite to water body can cause toxic or inhibitory effects on aquatic life and would require post-denitrification step. Methane that is produced in the AMFB reactor (discussed below) could be used as the electron donor for denitrification [27], with significant energy and cost benefits. Full nitrification to nitrate may also achieved in AMFB without system change simply by increasing membrane surface area or air pressure.

3.4 Methane production

Methane percentage at different phases is illustrated in Figure 4, while the corresponding methane yields are indicated in Table 2. Methane gas yields, normalised to the mass of COD removed anaerobically (L gCOD\textsuperscript{-1}\textsubscript{anaerobic}) ranged between 0.28 – 0.33 L gCOD\textsuperscript{-1}\textsubscript{anaerobic} on acetate feed (phase 1 to 5) and were comparable to the methane yields reported for anaerobic digesters having immobilized biomass and treating synthetic wastewater. Methane yields in the range of 0.22 to 0.26 LgCOD\textsuperscript{-1}\textsubscript{removed} was obtained for an anaerobic immobilized bio-plates reactor treating dilute (500 mgL\textsuperscript{-1}) synthetic wastewater with acetate at OLR of 1-2 kgCODd\textsuperscript{-1}m\textsuperscript{-3} [28]. Similarly, a methane yield of 0.29-0.32 LgCOD\textsuperscript{-1}\textsubscript{removed} was reported for an anaerobic biofilm reactor treating low strength wastewater [29]. The methane yield was 0.22-23 L gCOD\textsuperscript{-1}\textsubscript{anaerobic} on domestic wastewater (phase 7 and 8). These results confirmed that methanogenic degradation was responsible for COD removal in the anaerobic zone and methanogenesis was not
inhibited at the low DO of 0.1-0.3 mg L\(^{-1}\) in the anaerobic zone (Table 2). This work first proves that organic wastewater can be anaerobically treated simultaneously with nitritation in a single bioreactor (AMFB). The upflow AMFB reactor equipped with porous media and gas membrane achieved BOD removal of 98\%, ammonium oxidation of 65\% and methane yield of 0.12 L\(_{\text{CH}_4}\) gCOD\(^{-1}\) reactor for domestic wastewater at HRT of only 12 h. This HRT is close to that used for activated sludge processes including secondary clarifier (aeration tank 8 h and secondary clarifier 2-3 h), indicating the footprint of the AMFB reactor to be similar to existing wastewater treatment facilities.

Aeration diluted methane composition in the off-gas and it ranged between 8-25\%; in comparison, a higher methane gas composition of 40-55\% was obtained in anaerobic tests (Figure 4). Nitrogen gas was consistently present in the off-gas even in the anaerobic phases (Phases 2, 4, and 8), though the percentage composition was lower to that measured in phases with active aeration. Daily biogas production rate of 1.6-3 L d\(^{-1}\) under the anaerobic conditions can theoretically flush the entire volume of the headspace \(~0.5\ L\); the AMFB reactor was operated for at least 7 days before sampling biogas. Probably due to lack of mixing in the headspace, the biogas in the headspace was diluted instead of complete replacement with CH\(_4\) and CO\(_2\). The literature also reported N\(_2\) gas accumulation in the headspace of anaerobic bioreactors treating dilute wastewater [28, 30]. Yoo et al [30] reported N\(_2\) percentage of 59\% in the headspace of an anaerobic fluidized membrane bioreactor treating domestic wastewater. Similarly, Wu et al. [28] observed N\(_2\) content of 14-22\% in an anaerobic digester treating low-strength wastewater.

Conceivably, utilization of the biogas as renewable bioenergy would be challenging due to biogas dilution by nitrogen, so flaring the off-gas seems realistic as many municipal wastewater treatment plants routinely burn the biogas produced from anaerobic
digesters [31]. Actually, the AMFB reactor was designed for small scale and decentralized municipal wastewater treatment facilities where installation and operation of complex systems for biogas energy could be challenging. If denitrification is essential to improve effluent quality and protect receiving water body from eutrophication, methane can be reused as the electron donor for nitrite reduction, as previously discussed. Instead of conventional denitrification in continuous stirred tank reactors, membrane biofilm reactors equipped with gas-permeable membranes for methane delivery will be ideal for methane-utilizing denitrification [32]. The lack of aeration and small sludge production in the AMFB reactor could provide significant benefits for rural WWTPs over activated sludge, but large-scale AMFB reactor tests are essential to confirm these advantages.

Table 3 summarizes COD and nitrogen balances in the AMFB reactor fed with domestic wastewater. Nitrogen balance had good closure in Phase 7. In comparison, unaccounted electron sinks were considerable at 22-48% of the influent COD. The most significant electron sinks of the untracked electron sinks include dissolved methane, biomass growth, and other exogenous electron acceptors present in wastewater. Dissolved methane would be one of main untracked electron sinks, since aqueous methane can account for 14-35% of influent COD in methanogenesis-based dilute wastewater treatment [33]. Biomass growth is another electron sink, which can range from 7 to 11% of influent COD [33]. Interestingly, unaccounted electron sinks fluctuated from 22 to 48% in Phases 7 and 8, which implies that the concentration of other electron acceptors present in domestic wastewater may change, such as sulfate. H₂S may be produced in the AMFB reactor, adversely affecting performance of the AMFB reactor, although effluent quality and methane yield were steady in this work. For success of AMFB reactor application to rural domestic wastewater treatment, future study of tracking sulfate and H₂S would be required.
3.5 Microbial Community

Figure 5 shows that archaeal and bacterial composition clearly differed between the microaerobic and anaerobic zone. The archaeal composition in the anaerobic zone stood at 6%, while they constituted only 0.03% of the identified prokaryotes in the aerobic zone. This relatively higher abundance of archaea in the anaerobic zone along with the known affiliation of methanogens with the archaea domain affirmed COD removal in the anaerobic zone by methanogenic degradation. This is also consistent to substantial COD removal in the anaerobic zone and methane production in the bioreactor. Methanogens such as *Methanothrix*, *Methanobacterium*, *Methanomassiliicoccus*, *Methanospirillum*, *Methanobrevibacter*, *Methanolinea* and *Methanosphaera* [34-39] comprised over 50% of the archaea in the anaerobic zone (Figure 5a). The abundance of methanogens in the anaerobic zone was also considerably higher to that in the aerobic zone (0.2%). The archaeal composition in the AMFB reactor was comparable to that reported for AD sludge (4.7-5.6%) treating wastewater and biosolids from activated sludge system [40, 41].

The bacterial composition stood at 94% for the anaerobic zone and 99.97% for the aerobic zone (Figure 5b). Of these, however, the bacterial populations were obviously different according to the functions in each zone. In the anaerobic zone dominant genera were related to anaerobic fermentation such as *Mucinivorans* (20%) *Bacteroidales* (17%) and *Bacteroidetes* (6%) as carbohydrate and sugar fermenters [42], *Candidatus Cloacamonas* (11%) as H₂-producing bacteria [40], and *Smithella* (10%) as propionate-oxidizing bacteria [43]. This result is consistent to substantial COD removal by fermentation and methanogenesis in the anaerobic zone. In the aerobic zone, bacterial diversity increased since many oxygen-favorable heterotrophic bacteria such as
Litorilinea, Aridibacter, Aquicella and Dokdonella were detected [44-47], and could be related to COD removal in the aerobic zone. Nitrifiers such as Nitrosomonas and Nitrospira were identified exclusively in the microaerobic zone and constituted 4 and 2 % of the bacterial composition, respectively (Figure 5b). Considering that ammonium removal was primarily catalyzed by partial oxidation as indicated by high $\text{NO}_2^-$-N concentration in the effluent along with low DO concentrations in the aerobic zone (Table 2), higher abundance of AOB was expected. Recognised AOB may not be main ammonia oxidizing organisms in the aerobic zone. Enrichment of alternative ammonia-oxidizing organisms has been previously proposed in low DO nitrifying bioreactors [9, 48]. For example, members of Gammaproteobacteria that constituted 4 % of the bacterial composition in the aerobic zone have been suggested to be involved in nitrification under low DO conditions [9]. In addition, Nitrosophaera, a known ammonia-oxidizing archaeon [49] was also identified in the microaerobic zone suggesting this genus might contribute to ammonia oxidation in the AMFB reactor, even though its relative abundance was small (0.04% of total prokaryote). Some uncertainties were identified in molecular biology data as reported in mixed-culture bioreactors [50, 51] but the microbial community supports COD removal by fermentation and methanogenesis in the anaerobic zone and nitritation and partial COD oxidation in the aerobic zone.

4. CONCLUSION

The AMFB reactor is a compact, simple technology suitable for small scale and decentralised wastewater treatment. By integrating methanogenesis with partial nitrification (using gas membrane) in a single reactor, the AMFB design features simultaneous carbon and ammonium removal. Membrane aeration allows for a controlled and balanced oxygen supply for partial
nitrification, while preventing oxygen related inhibition of methanogens. High COD removal of 92-99% was obtained on acetate medium (HRT 8-24 h, COD 0.35-2.1 kgCODd⁻¹m⁻³). A BOD₅ removal efficiency of > 97% was achieved on domestic wastewater (HRT 12 h, BOD₅ 0.25 kgBODd⁻¹m⁻³) with the effluent BOD₅ concentration of 4±5 mg L⁻¹ meeting the regulatory wastewater effluent standard (e.g., < BOD₅ 25 mg L⁻¹). A majority of the COD was removed anaerobically, as designed, and anaerobic COD removal resulted in high methane yields of 0.22-0.33 L gCOD⁻¹ anaerobic. Ammonium oxidation in AMFB reactor ranged between 69 to 86% (0.03 – 0.08 KgNH₄⁻N d⁻¹m⁻³) and nitrite was accumulated at 15.6-24.2 mg N L⁻¹ in the effluent, along with low nitrate (0.5-5.5 mg N L⁻¹). Microbial community analysis showed a clear distinction between microbial populations in the anaerobic and membrane aerated section. Known methanogens and nitrifiers were identified exclusively in the anaerobic and membrane aerated section, respectively, first demonstrating simultaneous methanogenesis and nitritation in the single stage AMFB reactor.

ACKNOWLEDGEMENTS
This work was financially supported by Natural Sciences and Engineering Research Council of Canada, Discovery Grant. The authors wish to thank Mr. Bum Kyu Kim for technical support.

REFERENCES


Table 1. Phases of reactor operation

<table>
<thead>
<tr>
<th>Phase</th>
<th>Substrate</th>
<th>COD (mg L⁻¹)</th>
<th>BOD₅ (mg L⁻¹)</th>
<th>NH₄⁺ – N (mg L⁻¹)</th>
<th>HRT (h)</th>
<th>Aeration</th>
<th>MP (kPa)</th>
<th>*COD loading rate (Kg d⁻¹ m⁻³)</th>
<th>*NH₄⁺– N loading rate (Kg d⁻¹ m⁻³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Acetate, Ammonium</td>
<td>492</td>
<td>41</td>
<td>24</td>
<td>Yes</td>
<td>14</td>
<td>0.35 (0.61)**</td>
<td>0.03 (0.10)***</td>
<td></td>
</tr>
<tr>
<td>2</td>
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<td>492</td>
<td>41</td>
<td>24</td>
<td>No</td>
<td>28</td>
<td>0.35 (0.61)**</td>
<td>0.03 (0.10)***</td>
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</tr>
<tr>
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<td>Yes</td>
<td>28</td>
<td>0.68 (1.18)**</td>
<td>0.05 (0.2)***</td>
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<tr>
<td>4</td>
<td>Acetate, Ammonium</td>
<td>1095</td>
<td>43</td>
<td>12</td>
<td>No</td>
<td>28</td>
<td>1.56 (2.73)**</td>
<td>0.06 (0.21)***</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Acetate, Ammonium</td>
<td>1095</td>
<td>43</td>
<td>12</td>
<td>Yes</td>
<td>28</td>
<td>1.56 (2.73)**</td>
<td>0.06 (0.21)***</td>
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</tr>
<tr>
<td>6</td>
<td>Acetate, Ammonium</td>
<td>980</td>
<td>37</td>
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<td>Yes</td>
<td>41</td>
<td>2.1 (3.67)**</td>
<td>0.08 (0.28)***</td>
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<tr>
<td>7</td>
<td>Real Wastewater</td>
<td>381</td>
<td>178</td>
<td>34</td>
<td>Yes</td>
<td>28</td>
<td>0.54 (0.95)**</td>
<td>0.05 (0.17)***</td>
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<tr>
<td>8</td>
<td>Real Wastewater</td>
<td>298</td>
<td>130</td>
<td>30</td>
<td>No</td>
<td></td>
<td>0.43 (0.74)**</td>
<td>0.04 (0.15)***</td>
<td></td>
</tr>
</tbody>
</table>

*Based on reactor volume  
**Based on anaerobic zone volume (Fig. 1)  
***Based on microaerobic zone volume (Fig. 1)
Table 2. Reactor performance in different phases of operation

<table>
<thead>
<tr>
<th>Phase</th>
<th>Effluent COD/BOD₅ (mg L⁻¹)</th>
<th>Effluent NH₄⁺-N (mg L⁻¹)</th>
<th>Effluent NO₂⁻-N (mg L⁻¹)</th>
<th>Effluent NO₃⁻-N (mg L⁻¹)</th>
<th>OUE₅ (%)</th>
<th>CH₄ Yield (L gCOD⁻¹ anaerobic)</th>
<th>DO (Ae) ** (mg L⁻¹)</th>
<th>DO (An.) *** (mg L⁻¹)</th>
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<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>6</td>
<td>17</td>
<td>2.1</td>
<td>63</td>
<td>0.28 (0.27)§</td>
<td>0.5</td>
<td>0.2</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
<td>38</td>
<td>19.3</td>
<td>2.5</td>
<td>74</td>
<td>0.33 (0.33)§</td>
<td>0.7</td>
<td>0.1</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>10</td>
<td>19.3</td>
<td>2.5</td>
<td>74</td>
<td>0.23 (0.22)§</td>
<td>0.7</td>
<td>0.2</td>
</tr>
<tr>
<td>4</td>
<td>81</td>
<td>40</td>
<td>24.2</td>
<td>0.5</td>
<td>76</td>
<td>0.31 (0.30)§</td>
<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td>5</td>
<td>42</td>
<td>11</td>
<td>24.2</td>
<td>0.5</td>
<td>76</td>
<td>0.31 (0.28)§</td>
<td>0.3</td>
<td>0.2</td>
</tr>
<tr>
<td>6</td>
<td>87</td>
<td>12</td>
<td>19.1</td>
<td>1.6</td>
<td>66</td>
<td>0.28 (0.25)§</td>
<td>0.4</td>
<td>0.3</td>
</tr>
<tr>
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<td>121/4#</td>
<td>12</td>
<td>19.1</td>
<td>1.6</td>
<td>66</td>
<td>0.23 (0.12)§</td>
<td>0.9</td>
<td>0.5</td>
</tr>
<tr>
<td>8</td>
<td>144/6#</td>
<td>25</td>
<td>15.6</td>
<td>5.5</td>
<td>74</td>
<td>0.22 (0.22)§</td>
<td>0.2</td>
<td>0.3</td>
</tr>
</tbody>
</table>

# Average effluent BOD₅ concentration
OUE₅ is the oxygen uptake efficiency calculated as per Eq. 3
* Dissolved methane concentration measured in the anaerobic zone of the reactor (Fig. 1)
** Dissolved oxygen (DO) concentration in the microaerobic zone of the reactor (Fig. 1)
*** Dissolved oxygen (DO) concentration in the anaerobic zone of the reactor (Fig. 1)
§ Methane yield normalised to total COD removed (L/gCOD removed)
Table 3. COD and nitrogen balance in the AMFB reactor fed with domestic wastewater

<table>
<thead>
<tr>
<th>Phase</th>
<th>Influent COD (g d⁻¹)</th>
<th>Effluent COD (g d⁻¹)</th>
<th>Methane gas (g d⁻¹)</th>
<th>Unaccounted e⁻ sink (g d⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>7.62</td>
<td>2.42</td>
<td>1.56</td>
<td>3.64 (48%)</td>
</tr>
<tr>
<td>8</td>
<td>5.96</td>
<td>2.88</td>
<td>1.76</td>
<td>1.32 (22%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Nitrogen balance</th>
<th>Influent NH₄⁺-N (g d⁻¹)</th>
<th>Effluent NH₄⁺-N (g d⁻¹)</th>
<th>Effluent N₂O₅⁻ - N + N₂O₅⁻ - N (g d⁻¹)</th>
<th>Unaccounted e⁻ sink (g d⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>0.680</td>
<td>0.240</td>
<td>0.422</td>
<td>0.018 (2.6%)</td>
</tr>
</tbody>
</table>

*percentage of the influent COD or nitrogen
List of figures:

Figure 1. General schematics of the reactor (not drawn to scale).

Figure 2. Removal efficiency of (A). COD and $NH_4^+$ in the reactor (B). COD in the anaerobic and microaerobic zone.

Figure 3. Influent and effluent concentration of (A). COD (B). $NH_4^+$ at different phases.

Figure 4. Off-gas and methane composition at different phases of AMFB operation.

Figure 5. Relative abundance of (A) archaeal and (B) bacterial communities in anaerobic and aerobic zone. “Others” indicate relative abundance of archaeal genera below 0.01% or bacterial genera below 1%.
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Highlights

- Methanogenesis and partial nitrification were combined in a single bioreactor
- High COD removal efficiency of 92-99% was achieved
- Majority of the influent BOD$_5$ (80-91%) was anaerobically oxidized
- Ammonium oxidation ranged between 69-86%, with nitrite accumulation of 15-24 mg/L
- Known methanogens and nitrifiers were identified
Graphical Abstract