Effects of C/N ratio on nitrous oxide production from nitrification in a laboratory-scale biological aerated filter reactor
Qiang He, Yinying Zhu, Leilei Fan, Hainan Ai, Xiaoliu Huangfu and Mei Chen

ABSTRACT
Emission of nitrous oxide (N₂O) during biological wastewater treatment is of growing concern. This paper reports findings of the effects of carbon/nitrogen (C/N) ratio on N₂O production rates in a laboratory-scale biological aerated filter (BAF) reactor, focusing on the biofilm during nitrification. Polymerase chain reaction–denaturing gradient gel electrophoresis (PCR-DGGE) and microelectrode technology were utilized to evaluate the mechanisms associated with N₂O production during wastewater treatment using BAF. Results indicated that the ability of N₂O emission in biofilm at C/N ratio of 2 was much stronger than at C/N ratios of 5 and 8. PCR-DGGE analysis showed that the microbial community structures differed completely after the acclimatization at tested C/N ratios (i.e., 2, 5, and 8). Measurements of critical parameters including dissolved oxygen, oxidation reduction potential, NH₄⁺-N, NO₃⁻/N, and NO₂⁻/N also demonstrated that the internal micro-environment of the biofilm benefited N₂O production. DNA analysis showed that Proteobacteria comprised the majority of the bacteria, which might mainly result in N₂O emission. Based on these results, C/N ratio is one of the parameters that play an important role in the N₂O emission from the BAF reactors during nitrification.

Key words | biological aerated filter, carbon/nitrogen ratio, microelectrode technology, nitrification, nitrous oxide

INTRODUCTION
Nitrous oxide (N₂O) is a strong greenhouse gas that has a bad effect on stratospheric ozone and has a more than 300-fold stronger greenhouse effect than carbon dioxide (CO₂) (Czepiel et al. 1996; Ravishankara et al. 2009). N₂O accounts for 10% of the total global warming effect of all greenhouse gas emissions, even though N₂O emissions are approximately 0.03% of total greenhouse gas emissions (Heidberg & Redlich 1996). Studies found that N₂O could be produced from biological nitrogen removal processes in wastewater treatment systems (Tallec et al. 2006; Foley et al. 2010; Okabe et al. 2011a).

It is also confirmed that both nitrification and denitrification processes are responsible for N₂O emission during wastewater treatment (Kampschreur et al. 2009a, 2009b; Ahn et al. 2010; Desloover et al. 2012). Nitrification is a two-step process where ammonia-oxidizing bacteria (AOB) firstly oxidize ammonium (NH₄⁺) to nitrite (NO₂⁻), and then nitrite-oxidizing bacteria (NOB) oxidize nitrite to nitrate (NO₃⁻). Especially during nitrification, N₂O could be mainly produced by AOB through two pathways: (1) the reduction of nitrite to N₂O as the final product of AOB denitrification, and (2) the by-product of incomplete oxidation of NH₂OH (Colliver & Stephenson 2000; Law et al. 2012; Wunderlin et al. 2012; Pan et al. 2013).

The biological aerated filter (BAF), one of the most popular wastewater treatment processes based on the biofilm, has been widely applied to treat wastewater in medium and small scale plant recently (Fatihah & Donnelly 2009; Ray et al. 2012). Previous researches mainly pay great attention to the N₂O emissions from the activated sludge systems (Okabe et al. 2011b; Kong et al. 2013; Rathnayake et al. 2015). For example, in a laboratory-scale process, a microelectrode was applied to detect N₂O emissions from reactors, indicating that the microbial community structure impacts on the
N₂O emissions (Okabe et al. 2011b). However, there has been little study on the emissions of N₂O during nitrification in BAF reactors.

Previous researches reported that N₂O emissions during nitrification were affected by many water qualities, including dissolved oxygen (DO), pH, carbon to nitrogen (C/N) ratio, NH₄⁺-N and NO₂⁻-N (Zheng et al. 1994; Schulthess et al. 1995; Schulthess & Gujer 1996; Chung & Chung 2000; Burgess et al. 2002). Some studies have indicated that lower DO could lead to higher N₂O emissions (Kampschreur et al. 2008; Tallec et al. 2008). Water pH is considered as a critical factor impacting N₂O emissions. Hynes and coworkers had pointed out that the maximum emission was observed at pH 8.5, and minimum emission of N₂O was observed at pH 6.0 (Hynes & Knowles 1984). Moreover, C/N ratio also plays an important role in N₂O emissions. In an intermittently aerated bioreactor, up to 20–30% of the nitrogen load transformed to N₂O when the C/N ratio was below 3.5 (Ito-kawa et al. 2001). Similar observations were gained in other cases; e.g., the N₂O emission reduced from 4.5% to 0.2% of the nitrogen load with the increase of the C/N ratio (Park et al. 2000). Further increasing C/N (>10) resulted in the increase of N₂O emissions (Vanniel et al. 1993). However, few data have been obtained to illustrate how C/N ratio affects N₂O emissions during nitrification from biofilm systems, especially from BAF reactors.

The aim of the present study is to find the release mechanism of N₂O and transformation of nitrogen in BAF under different C/N ratios. Nitrification process in a laboratory-scale BAF was employed in three separate reactors with three selected C/N ratios (i.e., 2, 5, and 8). N₂O emissions from the biofilm of three reactors were measured by a micro-electrode to analyze the cause of N₂O emission. Moreover, polymerase chain reaction–denaturing gradient gel electrophoresis (PCR-DGGE) was applied to investigate the species diversity on the biofilm. Based on these findings, an effective range of C/N ratios for controlling N₂O emissions can be proposed, which may provide a breakthrough for biological nitrogen removal processes in wastewater treatment plants.

MATERIALS AND METHODS

Materials

Tris, proteinase K, sodium dodecyl sulfonate (SDS), hexadecyl-trimethyl-ammonium bromide (CTAB), acrylamide, deionized formamide, methylene bis acrylamide, urea, ammonium persulfate, agar powder, tetramethylethylenediamine (TEMED), and agarose were obtained from Sigma-Aldrich Company. Ribozyme, Taq DNA polymerase, 10× PCR buffer, dNTPs, primer, DNA maker (DL500), 6× loading buffer, PMD-19T connection kit, DH5α competent cell, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal), and isopropyl β-D-thiogalactoside were purchased from TaKaRa, Japan. Ethylenediaminetetraacetic acid (EDTA) disodium salt, trypthone, beef extract, and ampicillin were obtained from Beijing Dingguo Changsheng Biotechnology Co. Ltd. KH₂PO₄, HCl, Na₂HPO₄, isopropanol, chloroform, isoamyl alcohol, acetic acid, and ethanol were obtained from Sinopharm Chemical Reagent Co., Ltd.

Characteristics of test wastewater

A synthetic wastewater was used in BAF as influent, which mainly consisted of starch, glucose, NH₄Cl, KH₂PO₄, MgSO₄·7H₂O, CaCl₂, FeSO₄·7H₂O, Na₂MoO₄·2H₂O, ZnSO₄·7H₂O, CoCl₂, and MnSO₄. Its main characteristics are shown in Table 1.

Reactor operation

Three laboratory-scale BAF reactors were used in nitrogen removal processes at the same time. Three reactors were operated almost under the same condition, except for different C/N ratios. The main body of the reactor consisted of a tube made of hyaline organic glass (Figure 1). The reactor contained a total working volume of 44 L (height of 140 cm and diameter of 20 cm) and a headspace volume of 16 L, giving a total reactor volume of 60 L. Ceramsite with grain size of 3–5 cm, total weight 42 kg, was used as support material for biofilms. Wastewater entered into the reactor from the bottom of the tube, and then flowed upward through the entire tube. Finally, water was expelled from the outfall at the top of the reactor after the treatment under different hydraulic retention time (HRT) during nitrification. The true and dry densities of the ceramsite were 1,634 kg/m³ and 961 kg/m³, respectively. The mixed liquor

<table>
<thead>
<tr>
<th>No.</th>
<th>C/N</th>
<th>COD</th>
<th>TN</th>
<th>NH₄-N</th>
<th>NO₂⁻-N</th>
<th>NO₃⁻-N</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2:1</td>
<td>70–90</td>
<td>31–35</td>
<td>28–30</td>
<td>0–1.3</td>
<td>0–0.32</td>
</tr>
<tr>
<td>2</td>
<td>5:1</td>
<td>150–188</td>
<td>31–36</td>
<td>25–30</td>
<td>0–1.0</td>
<td>0–0.09</td>
</tr>
<tr>
<td>3</td>
<td>8:1</td>
<td>195–248</td>
<td>30–34</td>
<td>24–29</td>
<td>0–1.2</td>
<td>0–0.10</td>
</tr>
</tbody>
</table>
temperature in the reactor tube was controlled at 20 ± 3 °C using a water jacket to mimic common temperature condition.

The seed sludge (total suspended solids = 5,240 mg/L and volatile suspended solids (VSS) = 3,675 mg/L) was collected from the Jiguanshi sewage treatment plant in Chongqing, China. The sludge was then cultivated in the reactor for 2 days and operated in a batch mode for degassing and biomass attachment. After this, the reactor was fed with synthetic municipal wastewater at an HRT of 6 h. After 10 days, most of the particles in the reactor were coated with biomass. The final effluent VSS was the only excess sludge from the system. The BAF system was run in a steady-state for more than 100 days. Then, measurement of microelectrodes and PCR-DGGE were conducted to investigate the N₂O emission mechanism from the BAF system’s biofilm.

In order to find the effects of C/N ratios on N₂O emissions, three tests were conducted at various C/N ratios (2, 5, and 8) in BAF reactors with DO concentration of 4 mg/L and HRT of 6 h. An air pump pumped air into the bottom of the BAF system (WT-ZWS24Z, China).

Analytical procedure

The grab influent and effluent samples of nitrification were collected at regular time interval (every 3 days) in airtight bottles at 4 °C for monitoring the conventional indexes of BAF reactors. For getting more precise data, some regular parameters, i.e., chemical oxygen demand (COD), NH₄⁺-N, NO₂⁻-N, NO₃⁻-N, and total nitrogen (TN), in the influent and effluent were measured three times by using standard methods, respectively (APHA 2005). A dissolved oxygen meter (JBP-607, Weiye, Shanghai, China), an ORP meter (320P-83, Orion, USA), and a pH meter (PHSJ-4F, Leici, Shanghai, China) were used to measure DO, oxidation reduction potential (ORP), and pH.

N₂O measurement

The off-gas grab samples were collected from the zone reactor with a 1 L auto-sealed gas sample bag, which was connected to the gas collecting hole. The water outlet port was sealed by water to grab gas with a volume of 1 L. The N₂O concentration in the off-gas was measured with a gas chromatograph (Agilent, USA) equipped with a Porpak Q chromatographic column and electron capture detector. Temperature of the injector, column, and detector were 100, 50, and 300 °C, respectively. Nitrogen gas was used as carrier gas, whose flow rate was 40 mL/min. The method of determining the N₂O concentration in the biofilm is described in the ‘Microelectrode system’ section.

DNA extraction and PCR-DGGE

PCR-DGGE technology was utilized to identify the bacterial species present in the biofilm. Extraction of biofilm DNA and PCR-DGGE procedures were adopted from previous studies published by Li et al. (2014). Briefly, approximately 5 g ceramsite sample was collected from the middle of the biofilm reactors after biofilm culturing and an additional operation of 115 days. This ceramic biofilm sample was ground fully and transferred to a 2 mL centrifuge tube. Then, 1.35 mL DNA extract and 30 μL proteinase K (10 g/L) were added to the tube simultaneously. The tube was immediately shaken at 37 °C for 30 min using a temperature control table; we then added the mixture to 150 μL of 20% SDS. The sample was then placed in a water bath at 65 °C for 2 h; every 15-20 minutes, the sample was mixed by rotating up and down. After the bath, the sample was then placed in a refrigerator to freeze again at −20 °C, and then was placed in 65 °C water to melt. The freezing and thawing procedure was carried out three times. The sample was then centrifuged at 5,000 rev/min for 10 min at room temperature (∼20 °C); the supernatant was then transferred to a new 2 mL centrifuge tube containing the same volume of mixture solution (chloroform:isoamyl alcohol (v/v) = 24:1). The sample was again centrifuged at 5,000 rev/min for 10 min at room temperature (∼20 °C) and the supernatant was transferred to a new 2 mL centrifuge tube.
Isoamyl alcohol (isoamyl alcohol:mixture (v:v) = 0.6) was then added to the mixture after 1 h, the precipitate was obtained using high speed centrifugation (12,000 rev/min for 15 min) and then it was washed with 75% ethanol and under room temperature. Finally, the DNA was dissolved in sterile water containing RNA enzyme, and stored under −20°C.

Molecular fingerprinting of bacterial communities during BAF was conducted through PCR-DGGE by using the primers F338 (with GC-clamp) and R518 (5’-CCCCATACGGGAGGCAGCAG-3’) and 518 R (5’-ATTACC GCGGCGCTGG-3’) (Li et al. 2014). The touchdown PCR was performed in a thermocycler (Bio-Rad, USA) using the following procedure: an initial denaturing step of 5 min at 94°C, followed by 30 cycles with denaturing (94°C for 45 s), annealing (60°C for 45 s), and elongation (72°C for 90 s), followed by a final extension at 72°C for 10 min.

Then, DGGE was conducted for the PCR products on a gene mutation detection system (The Dcode™ Universal Mutation Detection System, Bio-Rad, USA) by using gradient gel electrophoresis under the following procedure: a voltage of 200 V and 60°C for 10 min, followed by a voltage of 80 V and 60°C for 900 min. After electrophoresis, the gel was removed and was placed in a 5 g/mL ethidium bromide solution for 25 min.

Cloning, sequencing and phylogenetic analysis of the 16S rRNA

The steady-state concentration profiles of N₂O, DO, NO₃, NO₂, and NH₄⁺ as a function of biofilm depth were obtained using a microelectrode system (Rathnayake et al. 2015). Additionally, pH and ORP as a function of the film thickness were also determined by using the microelectrode system with the corresponding sensors (Rathnayake et al. 2015). N₂O, DO, pH, and ORP electrodes were obtained from Unisence, Denmark, while NO₃, NO₂, and NH₄⁺ electrodes were manufactured according the procedures recommended by Gieseke & Beer (2004). The granules were acclimated in the synthetic medium located in the middle of the column for the batch experiments for at least 1 h before microsensor measurements to ensure that steady-state profiles were obtained. Physicochemical parameters of the medium were almost unchanged during the measurements.

The microelectrode system has three parts (Unisence, Denmark). The main part controls the microelectrode detection process. The micro-propulsion system included a motor control system (precision 1 μm), a manual dual head push device (precision 10 μm), and the test stand. These were used to measure the microelectrode in the detection process. Finally, the detection component, made of a universal microscope, was used to observe the electrode and the sample interface.

Microelectrode system

The steady-state concentration profiles of N₂O, DO, NO₃, NO₂ and NH₄⁺ as a function of biofilm depth were obtained using a microelectrode system (Rathnayake et al. 2015). Additionally, pH and ORP as a function of the film thickness were also determined by using the microelectrode system with the corresponding sensors (Rathnayake et al. 2015). N₂O, DO, pH, and ORP electrodes were obtained from Unisence, Denmark, while NO₃, NO₂ and NH₄⁺ electrodes were manufactured according the procedures recommended by Gieseke & Beer (2004). The granules were acclimated in the synthetic medium located in the middle of the column for the batch experiments for at least 1 h before microsensor measurements to ensure that steady-state profiles were obtained. Physicochemical parameters of the medium were almost unchanged during the measurements.

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RESULT AND DISCUSSION

The performance of the BAF reactors and the effects at different C/N ratios

In order to understand the pollutant removal efficiency of BAF reactors, conventional effluent quality indices were tested at various C/N ratios, during stable operation of the reactors. The COD residual of the effluent is shown in Figure 2.

BAF reactors showed highly effective reduction in COD for various C/N ratios tested, indicating effective organic pollutant removal (Figure 2). The COD of the influent had only a negligible influence on the resulting effluent COD,
although it is of note that the COD levels were relatively low in all effluents tested in the present study (C/N ratios were 2, 5 and 8). Low COD levels (∼20 mg/L) resulted in difficulty for further COD removal and might be responsible for the similar COD concentration for the tested C/N in inlets.

The effect of BAF reactors on the concentration of TN in influent and effluent was also analyzed. The results for all effluents with varying C/N ratios are shown in Figure 3, where the average effluent TN concentrations were 28.37, 21.20, and 20.68 mg/L for effluents with a C/N ratio of 2, 5, and 8 respectively. This indicates that the removal rate of TN increased with an increase in C/N ratio, which may be due to low COD levels, resulting in less electron donor availability, as seen with effluents 2 and 5.

The level of NH$_4^+$-N removal in effluents with varying C/N ratios is summarized in Figure 4. The corresponding average NH$_4^+$-N concentrations in effluents were 6.84, 2.15, and 2.83 mg/L for C/N ratios of 2, 5, and 8, respectively.
showing more effective N removal at higher C/N ratios, with the maximum achieved removal rate at a C/N ratio of 5 and the minimum achieved rate at a C/N ratio of 2.

As shown in Figure 5, most of the NO$_3^-$-N in the influent was consumed, resulting in effluent concentrations of 16.41, 17.00, and 15.74 mg/L NO$_3^-$-N respectively for effluents with C/N ratios of 2, 5, and 8. Relatively constant NO$_3^-$-N concentrations were seen for effluents with C/N ratios of 2 and 5, with a reduction in NO$_3^-$-N concentrations observed with the further increased C/N ratio of 8.

The changes in the NO$_2^-$-N concentrations of influent and effluent are shown in Figure 6 for all varying C/N ratios. NO$_2^-$-N production was relatively low in all three influents, with concentrations of 0.15, 0.03, and 0.07 mg/L, for C/N ratio of 2, 5, and 8 respectively, and there appears to be the highest NO$_2^-$-N concentrations with the lowest C/N ratio of 2.

**Effects of C/N ratio on the production rate of gaseous N$_2$O in nitrification**

Gaseous N$_2$O production was measured using gas chromatography, for all C/N ratio effluents, as shown in Figure 7.

The N$_2$O production rates were 0.2390, 0.1257, and 0.2137 mg/h respectively for effluents with a C/N ratio of 2, 5, and 8. The highest levels of NO$_2$ production were observed in effluent with a C/N ratio of 5, 1.9-fold higher than in effluent with a C/N ratio of 2, and 1.7-fold higher than when C/N ratio increased to 8. N$_2$O emission factors based on the TN removal have also been calculated and results showed a similar trend in that N$_2$O emission factors were $1.15 \pm 0.91\%$, $0.14 \pm 0.04\%$, and $0.24 \pm 0.06\%$ for C/N ratio of 2, 5, and 8, respectively. C/N may be a critical factor in the production of N$_2$O and studies by Itokawa et al. found that during steady-state operation of an intermittently aerated bioreactor treating high-strength wastewater, 20–30% of the nitrogen load was emitted as N$_2$O when the C/N ratio was below 3.5 (Itokawa et al. 2001). While more research is required to establish the optimal functional range, these data suggest it may be effective to control C/N ratios to 4–5 when treating water using either activated sludge or biofilm processes, in order to minimize the production of N$_2$O.
Microbial community analysis of BAF at different C/N ratios

The bacteria were isolated from the biofilm environment and, for all samples, 16S rDNA V3 regions were amplified using DGGE electrophoresis and an ethidium bromide gel imaging system with Quantity One software (final concentration 0.5 g/mL), with imaging performed using DGGE gel staining. Following long-term acclimatization and stabilization at different C/N ratios, the microbial community structures differed significantly between the seed sludge and the BAF biofilm, in response to the varying conditions in each reactor (Figure 8).

Fourteen predominant bands were identified by DGGE analysis and were sequenced to identify the bacterial strains present within the microbial community. Comparing the 16S rRNA sequences, the majority of the bacteria were members of Proteobacteria (Table 2, bands 1, 2, 4, 6, 9, 10, 11, 13 and 14), with other groups present, such as Ignavibacteria (Table 2, band 3), Alveolata (Table 2, band 7), Firmicutes (Table 2, band 5) and Bacteroidetes (Table 2, band 8).

According to their rRNA sequence, Proteobacteria can be divided into five classes, (α, β, γ, δ, and ε), where β-proteobacteria and γ-proteobacteria mainly use organic matter as an electron source to respire aerobically and ferment under oxygen-free conditions, both of which are important steps in organic matter degradation in waste-water treatment systems. Studies have confirmed that β-proteobacteria are usually aerobic or facultative aerobic bacteria, which are capable of aerobic ammonia oxidation. Therefore, it has been suggested that oxygen is a sufficient electron source when β-proteobacteria dominate the water environment (Ikenaga et al. 2003). The δ-proteobacteria are reported to play a significant role in the depletion of COD, and some common bacteria found in biological filters, such as AOB, NOB and other denitrifying bacteria species, belong to Proteobacteria classes α-, β- and γ- respectively (Kumar et al. 2010). Some studies have found that α-proteobacteria can also exist as anoxic or anaerobic denitrifying bacteria (Shapleigh 2011); therefore Proteobacteria are one of the most important microbial strains in the process of nitrogen removal and COD depletion.

Proteobacteria were found to exist in abundance in reactors under all different conditions, especially, Nitrosomonas oligotropha (Table 2, band 4), which was the dominant microbial group at C/N ratio of 5 h and is likely to be a dominant group in the transformation of NH₄⁺-N to NO₂⁻-N. In addition, Nitrosomonas oligotropha had a negative impact on the production of hydroxylamine, which resulted in a decrease in N₂O emissions.

Research about the biofilm micro-environment of BAF at different C/N ratios

The depth profile of the biofilm micro-environment was studied using microelectrode measurements during the nitrification phase as shown in Figure 9.

Biofilm depth increased significantly with an increase in C/N ratios, where ratios of 2, 5 and 8 resulted in a corresponding thickness of 210, 300, and 375 μm, respectively, as shown in Figure 9(a), highlighting a relationship between biofilm thickness and the C/N ratio of influent.

There was a trend of gradual reduction in DO concentration seen with increasing biofilm depth. With a
maximum C/N ratio of 8, a thicker biofilm resulted in a larger range of reduction in DO concentrations. Effluents with larger C/N ratios and therefore high COD contents resulted in the intermediate propagation of heterotrophic microorganisms, which might be responsible for producing a thicker depth of biofilm.

Figure 9(b) shows the changes in ORP signal values, according to the depth of the biofilm at different C/N ratios. The ORP signal value appears to decrease slightly with depth of biofilm and both surface and inner layers of the biofilm have a strong oxidative capacity. The biofilm was relatively thin and this small depth may have resulted in the ORP signal value being dominated by DO concentrations of the influent, as ORP signal value also showed a reduction in relation to increased biofilm depth, consistent with the trend seen in DO concentrations (Figure 9(a)).

$\text{N}_2\text{O}$ concentrations decreased in accordance with increased depth of biofilm, by between 0.112 mg/L and 0.096 mg/L at C/N ratio of 2, and between 0.021 mg/L and 0.008 mg/L at a C/N ratio of 8, while at a C/N ratio of 5, the $\text{N}_2\text{O}$ concentrations changed less in accordance with depth of biofilm, remaining between 0.002 and 0.004 mg/L (Figure 9(c)). Through horizontal comparison of producing ability under varying conditions, biofilm $\text{N}_2\text{O}$ production at a C/N ratio of 2 was higher than at higher C/N ratios of 5 or 8. A larger concentration of $\text{N}_2\text{O}$ in the biofilm resulted in a larger amount of $\text{N}_2\text{O}$ being released into the water or gas phase. Thus, the phenomenon of $\text{N}_2\text{O}$ production in biofilm micro-environments can explain the gas phase $\text{N}_2\text{O}$ production observed. When the C/N ratio was 5, $\text{N}_2\text{O}$ production in the biofilm was negligible, suggesting that control of the influent C/N ratio to 5 would reduce production and emissions of $\text{N}_2\text{O}$.

When the C/N ratio was at its lowest in the present study (i.e., 2), the concentrations of $\text{NH}_4^+$-N in the biofilm were relatively high compared to other C/N ratio effluents and decreased from 8.110 mg/L to 7.855 mg/L with depth of biofilm. However, the concentrations of $\text{NH}_4^+$-N changed only negligibly according to biofilm depth, for effluents with C/N ratios of 5 and 8, staying at 3.8 mg/L and 3.0 mg/L respectively (Figure 9(d)).

It could be seen in Figure 9(e) that at a C/N ratio of 2, the $\text{NO}_3^-$-N concentrations ranged from 20.292 mg/L to 21.989 mg/L according to depth of biofilm, with the depth of 100 μm being the critical point for shift in $\text{NO}_3^-$-N concentrations. The $\text{NO}_3^-$-N concentrations in the effluent with a C/N ratio of 5 followed a similar pattern, ranging from 19.894 mg/L to 21.485 mg/L, while in effluent with a C/N ratio of 8, the $\text{NO}_3^-$-N concentrations followed a different trend and decreased from 17.609 mg/L to 16.969 mg/L with depth of biofilm. Although it is of note that there were very small variations in $\text{NO}_3^-$-N and DO concentrations, there was a consistent overall response. The $\text{NO}_3^-$-N concentrations had a negative relationship with C/N ratios, possibly as a larger amount of heterotrophic bacteria are produced with higher C/N ratios, which results in higher levels of DO consumption (Rathnayake et al. 2015).

All $\text{NO}_3^-$-N concentrations increased in accordance with depth of biofilm, with C/N ratio 2 effluent increasing from 0.141 mg/L to 0.156 mg/L, C/N ratio 5 effluent having

<table>
<thead>
<tr>
<th>Band number</th>
<th>Most similar strain</th>
<th>Accession number</th>
<th>Semblance</th>
<th>Most similar groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Band 1</td>
<td>Ferrovum myxofaciens</td>
<td>NR_117782</td>
<td>96%</td>
<td>Proteobacteria</td>
</tr>
<tr>
<td>Band 2</td>
<td>Lacibacterium aquatile</td>
<td>NR_125556</td>
<td>98%</td>
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<tr>
<td>Band 3</td>
<td>Melioribacter roseus</td>
<td>NR_074796</td>
<td>86%</td>
<td>Ignavibacteriae</td>
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<td>Band 4</td>
<td>Nitrosononas oligotropha</td>
<td>NR_114770</td>
<td>95%</td>
<td>Proteobacteria</td>
</tr>
<tr>
<td>Band 5</td>
<td>Lactococcus chungangensis</td>
<td>NR_044357</td>
<td>99%</td>
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</tr>
<tr>
<td>Band 6</td>
<td>Nitrosononas oligotropha</td>
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<td>Proteobacteria</td>
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<tr>
<td>Band 7</td>
<td>Epistylis riograndensis</td>
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<tr>
<td>Band 8</td>
<td>Chitinophaga jiangningensis</td>
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<td>Band 9</td>
<td>Rhodanobacter lindaniclasticus</td>
<td>NR_024878</td>
<td>99%</td>
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<td>Band 10</td>
<td>Zooshikella ganghwensis</td>
<td>NR_025668</td>
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<td>Band 11</td>
<td>Rhodanobacter lindaniclasticus</td>
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</tr>
<tr>
<td>Band 13</td>
<td>Lysobacter brunescens</td>
<td>NR_041004</td>
<td>100%</td>
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<tr>
<td>Band 14</td>
<td>Reyranella graminifolii</td>
<td>NR_126180</td>
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<td>Proteobacteria</td>
</tr>
</tbody>
</table>
the maximum increase from 0.099 mg/L to 0.178 mg/L, and C/N ratio 8 effluent increasing from 0.115 mg/L to 0.142 mg/L. Generally, the NO$_2$-N concentrations were relatively low in all conditions, indicating that there were low numbers of NO$_2$-N producing bacteria in the biofilm and reactor environment.
CONCLUSIONS

A laboratory-scale nitrification BAF process was developed in three separate reactors to identify the release mechanism of N₂O and the migration and transformation of nitrogen under different C/N ratios during nitrification.

- The reduction in COD and NH₄⁺-N and the production of NO₃⁻-N occurred in the BAF reactor. The COD levels in the effluent were altered by the C/N ratios but the effect was negligible. With an increase in the C/N ratio, the TN removal rate increased and the production of both NO₃⁻-N and NO₂⁻-N decreased. It was observed that the removal rate of NH₄⁺-N was at its lowest with a low C/N ratio of 2 and at its maximum with a higher C/N ratio of 5.
- The C/N ratio is one of the key factors in the production of N₂O and the average production rates in the aerobic zone were 0.2390, 0.1257 and 0.214 mg/L, when C/N ratios were at 2, 5 and 8, respectively.
- The microbial community structures differed significantly between the seed sludge and the BAF biofilm following long-term acclimatization and stabilization at different C/N ratios, although in all reactors it was found that Proteobacteria were the dominant bacteria, which may have a major impact on N₂O emission.

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