In situ probing the effect of potentials on the microenvironment of heterotrophic denitrification biofilm with microelectrodes

Yong Xiao a, Song Wu a, b, Zhao-Hui Yang b, Ze-Jie Wang a, Chang-Zhou Yan a, Feng Zhao a,c

a Key Laboratory of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen 361021, China
b College of Environmental Science and Engineering, Hunan University, Changsha 410082, China

HIGHLIGHTS
- Heterotrophic biofilm can use electrode as electron donor for denitrification.
- High pH suppressed the activities of reductases involving in denitrification.
- Low potential reduced the electron supply for denitrification processes.

ABSTRACT
Bio-electrochemical reactor provides a promising technology to remove nitrogen contaminants in water. In this study, a heterotrophic biofilm for denitrification process was developed, and stable total nitrogen removal efficiencies (>80%) were achieved. Fluorescence in situ hybridization showed that genes norB mainly transcribed in inner biofilm while genes nosZ showed similar transcription activities in the entire biofilm. The microelectrodes of pH and nitrous oxide (N₂O) were applied to profile the microenvironment of denitrification biofilm. In all measurements, the microenvironment pH decreased as a function of biofilm depth. The highest N₂O concentration of 90 µM in biofilm was detected when the bulk solution pH was 7.3, and an applied potential of −0.2 V did not decrease the yield of N₂O in biofilm at pH 7.3. Nevertheless, pH 9.5 or an applied potential of −0.4 V seemed not to suppress the yield of N₂O in biofilm.

1. Introduction
Nowadays, fast movement of human society towards urbanization, industrialization and agricultural activities has introduced various nitrogen-containing contaminants such as nitrate and ammonia into environment. Nitrogen contamination can introduce severe environmental problems, e.g., eutrophication of water, deterioration of water quality and potential hazard to human or animal health. Therefore, different denitrification technologies, especially biological denitrification, have been developed to remove nitrate contaminants from water environment.

In some wastewater, organic substance is limited and biological denitrification efficiency is reduced since organic substances are demanded as electron donors for most bacteria to reduce nitrate/nitrite. Therefore, a biological denitrification technique with bio-electrochemical reactor (BER) using electrode or electrolysis-sourced hydrogen as electron donor for denitrification was developed (Sakakibara and Kuroda, 1993; Islam and Suidan, 1998; Prosnansky et al., 2002; Zhao et al., 2011).
microbial fuel cells have been greatly investigated for wastewater treatment (Liu et al., 2004; Min et al., 2005) and the combination of microbial fuel cells and denitrification process could also be classified as one type of BER (Clauwaert et al., 2007; Virdis et al., 2008; Virdis et al., 2010; Yu et al., 2011; Puig et al., 2012). Dissolved oxygen (DO) and pH are usually considered as the most important factors impacting denitrifying performance of BERs (Watanabe et al., 2001; Prosnansky et al., 2002; Clauwaert et al., 2009). Current density, which might change the activities of bacteria and sequentially affect the denitrification process, has also been evaluated (Sakakibara and Kuroda, 1993; Islam and Suidan, 1998; Prosnansky et al., 2002; Zhao et al., 2011). Though potential is another operation parameter influencing the performance of BERs, little attention has been paid to the effect of potential on biofilm. Furthermore, most studies report the performance of entire reactor, and few studies have investigated the effect of potential on the microenvironment of biofilm.

The object of this research was to quantify microenvironment variation of denitrification biofilm on electrode surface at different potentials and pH. Microelectrode has been considered as a powerful tool for profiling microenvironment of bio-aggregates/biofilm (Okabe et al., 1999; Li and Bishop, 2004), hence, the microelectrodes of pH and nitrous oxide (N₂O) were used for the measurement of pH and N₂O concentration in biofilm on electrode surface in the current work, respectively. In addition, to interpret the microenvironment profile of N₂O, fluorescence in situ hybridization (FISH) targeting mRNA was used to infer the transcription activity of N₂O related reductases in denitrifying bacteria.

2. Materials and methods

2.1. Biofilm culture

In the present study, a lab-scale reactor with an efficient volume of 1 L was used for denitrification biofilm culture, and, to gain a denitrification biofilm, four pieces of carbon felt (4 cm in length and 4 cm in width, Gansu Haoshi Carbon Fiber, China) were used as electrodes (Fig. SM-1 in Supplementary Material (SM)). 200 mL of activated sludge collected from the sludge settling tank of Jimei Domestic Wastewater Treatment Plant was added into the reactor, and then the reactor was filled with 800 mL medium of Jimei Domestic Wastewater Treatment Plant was added into the reactor. Commercial pH and N₂O microelectrodes with a diameter of 50 μm (Unisense A/S, Denmark) were used to penetrate the biofilm and measure the pH and N₂O in the microenvironment, respectively. A MicroProfiling System (Unisense) was used to control the penetration and acquire data. Different constant potentials of the biofilm (−0.2 and −0.4 V vs. Ag/AgCl, except when noted) were held by a CHI660D electrochemical workstation (CH Instruments, China), and the measurement without an applied potential, i.e. an open circuit, was defined as "control". The three-electrode measurement system was established as shown in Fig. 1. The carbon felt with denitrification biofilm, a blank carbon felt (8 cm²) and an Ag/AgCl electrode served as the working electrode, counter electrode and reference electrode (RE1 in Fig. 1), respectively.

Before each measurement, the polarization lasted at least 100 min to make the biofilm achieve a steady current (Fig. SM-2). Following the manufacturer's instructions, microelectrodes were calibrated before each application. The calibration of pH microelectrode was performed under the same potential to offset the effect of electric field on the microelectrode. The step size of

![Fig. 1. Schematic diagram of the microelectrode measurements. RE1, WE and CE are Ag/AgCl reference electrode, working electrode and counter electrode for three electrodes system, respectively. ME is the microelectrode for measurement, Motor is used for controlling the depth of penetration, RE2 is a Ag/AgCl reference electrode for pH microelectrode measurement only.](image-url)
When a biofilm was grown on electrode, the steady 
\( \frac{\text{O}_2}{\text{C}_0} \) concentration in effluent were shown in Figs. SM-3 and SM-4, respec-
tive. The image of the cross section of the surface layer (depth 0–800 \( \mu m \)) and the inner layer (depth 800–1700 \( \mu m \)) of biofilm were excised from the carbon felt on microscopy for FISH analysis, respectively. Two oligonucleotide probes, fluorescein isothiocyanate (FITC) labeled nos1 (5’-CAAGT-TYTCCAAAGACCCGT-3’) for mRNA of nosZ and 5-tetrachloro-fluoro-
rescein (TET) labeled nor1 (5’-TBTGTCAGCTGCTGGTNGA-3’) for mRNA of norB, were designed using Primer Premier 5 (PREMIER Biosoft International, USA) based on the cDNA sequences of norB or nosZ.

FISH hybridization buffer with 30% of formamide and standard wash buffer were prepared freshly according to Pernthaler’s report (Pernthaler et al., 2001), and the final concentration of nos1 and nor1 was 5 \( \mu m \) each. FISH was conducted according to a fixation-
free protocol (Yilmaz et al., 2010). The slides were imaged by a DP72 CCD in an IX71 inverted microscope (Olympus, Japan). Three replications of hybridization were conducted to test the reproducibility.

3. Results and discussion
3.1. Denitrification performance of biofilm

After being cultured for 2 wk, the heterotrophic denitrification biofilm achieved stable TN removal efficiencies (>80%), and the DO in bulk solution of reactor was 0.3–0.5 mg L\(^{-1}\). The TN in influ-
ent and effluent before the microelectrode measurement was shown in Fig. 2. The COD in influent and effluent and the NO\(_3\)-N and NO\(_2\)-N in effluent were shown in Figs. SM-3 and SM-4, respec-
tively. The results indicate that the biofilm performed the function of denitrification well and was suitable for subsequent analysis. Three pieces of carbon felt with biofilm were cultured at different pH values and applied potentials to investigate the effects of pH and potentials on nitrogen removal efficiency. Results showed that an increase in pH decreased the nitrogen removal efficiency (\( p < 0.05 \)) (Table SM-1). The results also indicate that the biofilm can remove nitrogen with electrode as electron donor since there was no organic substance in bulk solution when a negative potential was applied. Moreover, a low potential (e.g. at \(-0.4 \text{ V}\)) decreased the nitrogen removal efficiency (\( p < 0.01 \)), compared with that with control or at \(-0.2 \text{ V}\). The image of the cross section of the biofilm taken by a microscopy was shown in Fig. SM-5, and microelectrode measurement was 50 \( \mu m \), which was controlled by a motor (Fig. 1). For each measuring point, the microelectrode waited for 10 s and measured for 10 s and then moved to the next point. Three sites on each biofilm were randomly selected and measured to examine the reproducibility. The temperature was controlled at 25 \( \pm 0.5 \) \(^{\circ}\)C during the measurement.

Although a neutral pH was usually suggested as the optimum pH for denitrifying bacteria (Wang et al., 1995), nitrous oxide reductase NosZ showed a high activity at pH 9.2 (Kucera et al., 1986). Therefore, a neutral pH of 7.3 and an alkaline pH of 9.5 of bulk solution were selected to profile the biofilm microenvironment, i.e. pH and \( \text{N}_2\text{O} \) concentration. After the measurement at 7.3, the biofilm was cultured at bulk solution of pH 9.5 for 3 d and then conducted the same measurement. Each biofilm was measured at all the three potential and the two pH. During the microelectrode measure-
ment, the bulk solution contained SynM1 medium.

2.4. FISH

RNA-targeted FISH was used to detect the transcription activity of two genes of \( \text{norB} \) and \( \text{nosZ} \) that associated with the generation and reduction of \( \text{N}_2\text{O} \), respectively. After the thickness of biofilm was determined by measuring the cross section with a microscopy, the surface layer (depth 0–800 \( \mu m \)) and the inner layer (depth 800–1700 \( \mu m \)) of biofilm were excised from the carbon felt on microscopy for FISH analysis, respectively. Two oligonucleotide probes, fluorescein isothiocyanate (FITC) labeled nos1 (5’-CAAGT-TYTCCAAAGACCCGT-3’) for mRNA of nosZ and 5-tetrachloro-fluo-
rescein (TET) labeled nor1 (5’-TBTGTCAGCTGCTGGTNGA-3’) for mRNA of norB, were designed using Primer Premier 5 (PREMIER Biosoft International, USA) based on the cDNA sequences of norB or nosZ.

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free protocol (Yilmaz et al., 2010). The slides were imaged by a DP72 CCD in an IX71 inverted microscope (Olympus, Japan). Three replications of hybridization were conducted to test the reproducibility.

3.3. pH profile in biofilm

The effect of potentials on pH was examined by a pH microelec-

The electrochemical results of the biofilm are presented in Fig. 3, which shows the reduction peaks at potentials of \(-0.2 \text{ V}\) for pH 7.3 and \(-0.17 \text{ V}\) for pH 9.5. The reduction peak presents an electron transfer between the biofilm and the electrode by comparing the current recorded from the bacteria-free carbon felt. It indicates that the biofilm may use the electrons from electrode to reduce \( \text{NO}_3^- \). When a biofilm was grown on electrode, the steady state current was higher than the blank electrode, especially when the pH was 7.3 (Fig. SM-2). These results indicate that there are electron transfer processes between the biofilm and electrode. It also suggests that nitrate may be removed by the heterotrophic biofilm using electrode as electron donor in BERs. Since the reduction of nitrate associates with alkali production (Eq. (1)), the potential of reduction peak shifted from \(-0.2 \text{ to } -0.17 \text{ V}\) while the pH of bulk solution was adjusted from 7.3 to 9.5. This shift of reduction peak is in accord with the theoretical calculation results from Nernst equation.

\[
\text{NO}_3^- + 5[\text{H}] \rightarrow 1/2\text{N}_2 + 2\text{H}_2\text{O} + \text{OH}^- \quad (1)
\]

3.2. pH profile in biofilm

The effect of potentials on pH was examined by a pH microelec-
trode penetrating into the biofilm. Fig. 4 shows the pH variation at different depth of biofilm. The pH values decreased from 7.1 of the
biofilm surface to 6.8 at depth of 1700 lm when the pH in bulk solution was 7.3. When the pH in bulk solution was 9.5, the microenvironment pH of biofilm decreased from 9.3 to 7.2 and the variation range was six times larger than that at pH 7.3. Moreover, the biofilm showed different effects on the microenvironment pH at different potentials. At a same depth of biofilm, the pH in biofilm at /C0.2 V was higher than that of control; however, the potential of /C0.4 V showed little effect on the biofilm pH when the pH of bulk solution was 9.5.

Using glucose as electron donor, the stoichiometric equation is as follow (Akunna et al., 1992):

\[ \text{NO}_3^- + 5/2\text{C}_6\text{H}_12\text{O}_6 \rightarrow 1/2\text{N}_2 + 5/4\text{CO}_2 + 3/4\text{H}_2\text{O} + \text{OH}^- \]  

According to Eq. (2), 1 mol of OH\(^-\) and 5/4 mol of CO\(_2\) will be produced while 1 mol of NO\(_3^-\) is reduced. According to Eqs. (3) and (4), the 5/4 mol of produced CO\(_2\) can consume as much as 5/2 mol of OH\(^-\) and therefore may acidify the microenvironment in biofilm.

\[ \text{CO}_2 + \text{OH}^- \rightarrow \text{HCO}_3^- \]  

\[ \text{HCO}_3^- + \text{OH}^- \rightarrow \text{CO}_3^{2-} \]  

When pH of bulk solution was about 7.3, most of produced CO\(_2\) may exist as hydrate since the concentration of OH\(^-\) is very low. Therefore, the lowest microenvironment pH in biofilm was still higher than 6.8, though microenvironment pH in biofilm deeper than 300 lm have decreased to about 7.0 (Fig. 4a). When pH in the bulk solution was 9.5, the concentration of OH\(^-\) was one hundred times higher than that at pH 7.3 and the CO\(_2\) could react with OH\(^-\) as shown in Eqs. (3) and (4). Therefore, produced CO\(_2\) may significantly decrease the pH from 9.5 in surface biofilm to 7.2 in inner biofilm (Fig. 4b). Besides, the 1700 µm-thick biofilm played an important role in maintaining the low pH in inner biofilm by blocking the fast diffusion of CO\(_2\) from inner biofilm to bulk solution.

According to the electrochemical data of biofilm (Fig. 3), electron transfer may occur between biofilm and electrode when the potential was below –0.2 V at pH 7.3 or below –0.17 V at pH 9.5. The electrochemical reactions between the biofilm and the electrode reduced the amount production of CO\(_2\) or acidic substances from respiring glucose and sequentially raised the pH in biofilm. At same depth of biofilm, the microenvironment pH in biofilm being held at –0.2 V was higher than that in the control. Previous research reported that applying a low potential to biofilm will lower the oxidation–reduction potential in biofilm (Babauta et al., 2011). Low oxidation–reduction potential may change the metabolic pathway of glucose to yield organic acid. Therefore, the pH in biofilm at –0.4 V was lower than that at –0.2 V.

3.3. N\(_2\)O profile in biofilm

The effect of potentials on N\(_2\)O concentration was examined by a N\(_2\)O microelectrode penetrating into biofilm, and the profile of N\(_2\)O concentration in different depth of biofilm was shown in Fig. 5. When the pH of bulk solution was 7.3, N\(_2\)O concentration in biofilm increased from 2 to 90 µM as the depth changing from 600 to 1100 µm and then decreased to 75 µM at depth of 1600 µm. It indicates that, as the depth increasing from 600 to 1100 µm, the activity of NO reductases is increasing but the activity of N\(_2\)O reductases is decreasing, respectively. When the pH in bulk solution was adjusted to 9.5, N\(_2\)O concentration in biofilm increased from 10 to 21 µM as the depth increased from 0 to 500 µm.
After that, it decreased to 2 μM and stabilized at the biofilm between 1400 and 1700 μm.

Biological denitrification is a complex biochemical reaction involving a variety of enzymes and intermediates. Four reductases of Nar, Nir, Nor and Nos are the primary enzymes reducing nitrate to nitrogen gas (Fig. SM-6) (Zumft, 1997). Among these reductases, the reductase Nor involves in reducing nitric oxide to N₂O while the reductase Nos reduces N₂O to nitrogen gas. NosZ protein is the main component of Nos, which is encoded by gene nosZ. NorB is the cytochrome b subunits of NO reductase, and norB is the coding gene of NorB.

The transcription activities of genes can indirectly reflect the activities of corresponding enzymes. Since many denitrification bacteria encode more than one reductase that involves in the denitrification (Zumft, 1997), we designed two specific probes (nor1 targeting the mRNA of norB; nos1 targeting the mRNA of nosZ rather than species specific 16s rRNA gene probes) to detect the transcription activities of the two genes in the surface layer and the inner layer of biofilm using FISH technology. The FISH images are shown in Fig. 6 when the pH of bulk solution was 7.3. The green points in the images represent the bacteria transcribed nosZ genes while the red points represent the bacteria transcribed norB genes. There were 112 ± 46 red cells and 161 ± 67 green cells for the biofilm surface, and 287 ± 108 red cells and 162 ± 65 green cells for the inner biofilm. The results showed that much more red cells were found in inner biofilm than that in biofilm surface (p < 0.01), i.e. more bacteria transcribed norB genes in inner biofilm and consequently the N₂O might be mainly produced in inner biofilm. Unlike the norB genes, the transcription activity of nosZ genes in surface biofilm was similar to that in inner biofilm since the numbers of green cells were similar in both images. Therefore, the N₂O was mainly produced in the inner biofilm while the reduction of N₂O occurred in the entire biofilm at pH 7.3.

The pH is the main factor affecting the N₂O concentration in biofilm when there is no potential/current effect, e.g. the N₂O concentration in biofilm decreases as the microenvironment pH increases from 6.8 to 7.1. Above N₂O of 70 μM was detected in biofilm where the microenvironment pH was lower than 6.8 and the depth was deeper than 900 μm (Fig. 4a and 5a). The FISH analysis shows that much more norB genes were transcribed in the layer of biofilm than the nosZ genes (Fig. 6). These results suggested that the activities of N₂O reductases (such as NosZ) were higher than the activities of NO reductases (such as NorB) in the pH range of 6.8–7.1, while the activities of NO reductases were higher than the activities of N₂O reductases at pH lower than 6.8.

It was also concluded from Fig. 4b and 5b that the activities of NO reductases may be higher than that of N₂O reductases in the pH range of 7.5–8.0. Though N₂O reductase of NosZ from Paracoccus denitrificans showed high activity at pH 9.2 (Kučera et al., 1986), most previous research recommended neutral pH as the optimum condition for most environmental strains of denitrifying bacteria (Wang et al., 1995; Godini et al., 2011). Considering the microenvironment profiles of pH and N₂O, we summarized that the activities of N₂O reductases were higher than that of NO reductases in the pH range (i.e. between 6.8 and 7.5; or >8.0), the activities of NO reductases were higher than that of N₂O reductases in the pH range (i.e. between 7.5 and 8.0; or <6.8) (Fig. SM-7).

While a potential of −0.2 V was applied to the biofilm at pH 7.3, the N₂O concentration in bulk solution was as low as the control, and the N₂O concentration in biofilm increased from 5 to 70 μM as the depth increasing from 150 to 900 μm. Then, the N₂O concentration stabilized at 70 μM in the biofilm depth between 900 and 1700 μm. Considering the pH change curve at different potentials, −0.2 V might enhance the activities of NO reductases and N₂O reductases in biofilm. When −0.4 V was applied to the biofilm, the N₂O concentration was very low in the entire biofilm. Low potential might change the metabolic pathway of glucose to yield organic acid, which can reduce the electron supply for denitrification. Therefore, the applied potential of −0.4 V might mainly suppress the production of N₂O because of the reduced electron supply since the pH in this case was similar to that in biofilm at −0.2 V.

The N₂O concentration was below 2 μM in the entire biofilm when the potential of −0.2 or −0.4 V were applied to the biofilm at pH 9.5. The low N₂O concentration at −0.2 V might be the result of the low output rate N₂O induced by high pH in biofilm (Fig. 4b). Low N₂O concentration in biofilm at −0.4 V might be mainly induced by the reduction of electron supply. Although pH is an important factor affecting denitrification process, the change of potential seems to have double effects on pH and electron supply and sequentially affect the transformation of N₂O in biofilm in BERs.

4. Conclusions

Effect of potential on the pH and N₂O concentration in microenvironment of denitrification biofilm was demonstrated using microelectrodes. Electrons can be transferred between electrode and biofilm, and nitrate may be removed by heterotrophic biofilm using electrode as electron donor. Potentials and pH are two important factors to achieve high nitrogen removal efficiency in
BERs. Raising the pH of bulk solution from 7.3 to 9.5 or holding a potential of −0.4 V suppressed the yield of N₂O in biofilm. High pH mainly suppressed the activities of reductases involving in denitrification, and low potential mainly reduced the electron supply for denitrification.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2013.06.065.

References


