Nitrate Removal and Dynamics of Microbial Community of A Hydrogen-Based Membrane Biofilm Reactor at Diverse Nitrate Loadings and Distances from Hydrogen Supply End

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Abstract: The back-diffusion of inactive gases severely inhibits the hydrogen (H2) delivery rate of the close-end operated hydrogen-based membrane biofilm reactor (H2-based MBfR). Nevertheless, less is known about the response of microbial communities in H2-based MBfR to the impact of the gases’ back-diffusion. In this research, the denitrification performance and microbial dynamics were studied in a H2-based MBfR operated at close-end mode with a fixed H2 pressure of 0.04 MPa and fed with nitrate (NO3−) containing influent. Results of single-factor and microsensor measurement experiments indicate that the H2 availability was the decisive factor that limits NO3− removal at the influent NO3− concentration of 30 mg N/L. High-throughput sequencing results revealed that (1) the increase of NO3− loading from 10 to 20–30 mg N/L resulted in the shift of dominant functional bacteria from Dechloromonas to Hydrogenophaga in the biofilm; (2) excessive NO3− loading led to the declined relative abundance of Hydrogenophaga and basic metabolic pathways as well as counts of most denitrifying enzyme genes; and (3) in most cases, the decreased quantity of N metabolism-related functional bacteria and genes with increasing distance from the H2 supply end corroborates that the microbial community structure in H2-based MBfR was significantly impacted by the gases’ back-diffusion.

Keywords: back-diffusion; biofilm; denitrification; microbial community; nitrate loading

1. Introduction

Nitrate (NO3−) contamination of surface and groundwater has become a significant challenge due to the uncontrolled discharge of wastewater and the intensive use of fertilizers [1,2]. Given the organic-deficient nature of surface and groundwater (in which, the content of dissolved organic matter is commonly below 18 mg/L) [3,4], the autotrophic denitrification technique is therefore a more reasonable option than its heterotrophic counterpart, despite the higher biodegradation kinetics rate of the latter. As an emerging autotrophic denitrification approach, H2-based membrane biofilm reactor (H2-based MBfR), has gained widespread popularity in recent years for the purification of NO3−-contaminated surface and groundwater, mainly attributed to the unique advantages, namely, allowing efficient and cost-effective NO3− elimination with minimal bio-sludge yield and without
need for external carbon source [5–7]. In H2-based MBfR, H2 gas, the exogenous electron donor, driven by the H2-concentration gradient across the walls of the hollow-fiber membranes (HFMs), diffuses passively from the intramembrane to the HFMs-attached biofilm. In the biofilm, H2 is oxidized by the denitrifying bacteria (DNB) to reduce the NO3− that diffuses from the bulk liquid [8]. Given the abundant presence of inorganic carbon (mainly bicarbonate) in most surface and groundwater [9–11], the denitrification performance of the H2-based MBfR system is commonly largely dependent on the availability of H2 and NO3− within the counter-diffusional biofilm.

The gas-based MBfR is usually operated with either open-end or close-end HFMs. In the case of open-end operation, due to the markedly higher gas velocity of advective transport in the intramembrane than the diffusive transfer across the walls of HFMs, the intramembrane gas was uniformly distributed with an elevated concentration level, which enables the high microbial activity in the biofilm along the HFMs [12]. Nonetheless, in addition to the massive loss of gas, the open-end operation is inapplicable to H2-based MBfR as it creates an explosive atmosphere. Alternatively, the H2-based MBfR is extensively equipped with close-end HFMs, on account of electron donor (i.e., H2) saving and operational safety [13–15]. Unfortunately, close-end HFMs always suffer from the back-diffusion of inactive gases such as nitrogenous and water vapor gases, especially the N2 from bulk liquid and hydrogenotrophic denitrification process, which may severely reduce the overall HFM efficiency in terms of H2 delivery [8,12,16]. According to a previous model-predicted result, the gas transfer rate of HFMs operated at the open-end mode was obviously greater than that of the close-end operation, leading to an approximately 116% increase in the contaminant removal flux of the system [12].

As displayed in Figure 1, in principle, as a result of the continuous H2 consumption by the biofilm along the HFMs, accompanied by the back-diffusion of inactive gases (mainly N2) from the biofilm and bulk liquid into the intramembrane as well as the flow of gases toward the distal end of HFMs, the partial pressure of H2 and N2 gradually increases and decreases with the increase in distance from the H2 supply end, respectively; the diffusion of N2 from the intramembrane into the biofilm is due to its concentration at the end of the HFMs [5]. Until now, to the best of our knowledge, only one single research exists that covers the effects of gas back-diffusion on the gas profiles of biofilm, the gas transfer rate of membrane, and the pollutant removal efficiency of the gas-based membrane biofilm reactor system [12]. No study has been published to give insights into the impacts of gas back-diffusion on the dynamics of a microbial community in H2-based MBfR. Research is needed to address this knowledge gap. The objectives of this study are as follows: (1) to evaluate how the denitrification performance of H2-based MBfR responds to influent NO3− concentration variation, and (2) to reveal the evolution of the microbial community structure in the biofilm with changing NO3− loadings and distances from the H2 supply end.

![Figure 1](image-url)
2. Materials and Methods

2.1. Reactor Configuration

A schematic of the lab-scale H$_2$-based MBfR used in this study is shown in Figure 2. The reactor contained 20 polyvinyl chloride made HFM modules (effective length = 450 mm, inner diameter = 1.0 mm, outer diameter = 1.5 mm, pore size = 0.01 µm, membrane surface area = 0.042 m$^2$) assembled in a vertical plexiglass cylindrical shell (inner diameter = 45 mm, height = 500 mm, effective volume = 0.6 L). An ultrapure H$_2$ tank was connected to the lower end of the HFM module for pressurized H$_2$ supplementation. The upper end of the module was sealed using waterproof epoxy glue. A gas regulator was linked to the H$_2$ tank for H$_2$ supplying pressure adjustment. Synthetic medium (see Section 2.2 for details) was pumped from the bottom of the reactor via a peristaltic pump (BT101L-DG-1, Lead Fluid, Baoding, China). To guarantee the complete mixing of bulk liquid, a recirculation pump (BT101L-YZ15/25, Lead Fluid, Baoding, China) was operated at a high flowrate of 100 mL/min. Effluent was collected from the outlet at the top of the reactor.

![Figure 2. Schematic of the H$_2$-based membrane biofilm reactor (MBfR) setup.](image-url)

2.2. Synthetic Influent

The synthetic influent was prepared using tap water amended with NaNO$_3$, NaHCO$_3$, and mineral trace elements. NO$_3$~$^-$/ND$^-$ was added according to the demand, and 252 mg/L NaHCO$_3$ was added as the sole carbon source to maintain the autotrophic bacteria growth [13]. The composition of mineral trace elements was identical to our previous study (in µg/L) [17]: FeSO$_4$·7H$_2$O; 1000, CaCl$_2$·2H$_2$O; 1000, FeSO$_4$·7H$_2$O; 13, ZnSO$_4$·7H$_2$O; 4, MnCl$_2$·4H$_2$O; 38, H$_3$BO$_3$; 25, CoCl$_2$·6H$_2$O; 1, CuCl$_2$·2H$_2$O; 1, NiCl$_2$·6H$_2$O; 4, Na$_2$MoO$_4$·2H$_2$O; and 4, Na$_2$SeO$_3$. The bulk liquid pH was adjusted to around 7.5 with phosphate buffer (216 mg/L Na$_2$HPO$_4$·12H$_2$O + 236 mg/L KH$_2$PO$_4$) to eradicate the impact of pH drop on the denitrification performance. The synthetic medium was purged with pure N$_2$ for 30 min to maintain the anaerobic condition of the influent prior to entering the reactor [18].

2.3. Experimental Operation

The inoculated biomass was obtained from a long-term operated denitrifying H$_2$-based MBfR in our lab [19]. A H$_2$ pressure of 0.04 MPa was used throughout the whole experiment period, which is a quintessential empirical parameter extensively implemented in preceding H$_2$-based MBfRs [9,13,20].
The start-up processes of the reactor were as follows: a relatively low influent NO$_3^-$ concentration of 10 mg N/L was initially used to facilitate the start-up of the reactor, and the influent flowrate was set at 1 mL/min, resulting in a hydraulic retention time (HRT) of 10 h; once the complete removal of NO$_3^-$ was achieved, the influent flowrate was increased to 2 mL/min, corresponding to an HRT of 5 h. Following experiments, in which the influent NO$_3^-$ concentration was sequentially maintained at 10, 20, and 30 mg N/L in phases I, II and III, respectively, were carried out to investigate the effects of NO$_3^-$ availability on the denitrification performance of the system and the dynamics of microbial communities in biofilm. In each phase, 40 days operation was performed to enable the system performance and microbial community structure to reach stabilization. Specifically, a NO$_3^-$ influent concentration of 10–30 mg N/L was adopted since this range is close to the typical NO$_3^-$ concentration in real contaminated groundwater [21–23], and is the representative concentration range extensively investigated in H$_2$-based MBfRs for NO$_3^-$ removal [14,20,24–26].

2.4. Analytical Methods of Aquatic Samples

Denitrification performance of the H$_2$-based MBfR was ascertained by measuring the influent and effluent concentrations of NO$_3^-$ and NO$_2^-$. The collected influent and effluent samples were filtered immediately through a 0.22 µm polyvinylidene fluoride (PVDF) syringe filter (Sangon Biotech, Shanghai, China), and then stored at 4 °C until analyzed. Aquatic NO$_3^-$ and NO$_2^-$ concentrations were analyzed by ion chromatography (ICS-1000, Dionex, Sunnyvale, CA, USA) equipped with a Dionex AS-19 column (4 × 250 mm, 4 µm) using 55 mM sodium hydroxide as the eluent. The NO$_3^-$ concentrations inside the biofilm were determined by a microsensor measuring unit, and the detailed analytical procedure can be found in our previous research [27].

NO$_3^-$ removal flux of the system (J in g/(m$^2$d)) was calculated by Equation (1) [15,28].

$$J = \frac{Q}{A}(S_{\text{inf}}-S_{\text{eff}})$$

where $Q$ is the influent flow rate (m$^3$/d); $A$ is the membrane surface area (m$^2$); and $S_{\text{inf}}$ and $S_{\text{eff}}$ are the influent and effluent NO$_3^-$ concentrations (g/m$^3$), respectively.

2.5. Biofilm Sampling and Analysis

In order to evaluate the microbial community structure variation as a function of changing influent NO$_3^-$ concentration, the biofilm sample was obtained by stripping off the entire biofilm from one of the 20 HFMs at the end of phases I, II, and III, named bio-sample N1, N2, and N3, respectively. Additionally, to figure out the microbial community structure at diverse locations of the biofilm fed with a specific NO$_3^-$ loading, as shown in Figure 2, we sampled the biomass on a single HFM from the sampling ports 1, 2, and 3 (with a distance of 5, 20, and 35 cm from the H$_2$ supply end, respectively) at the end of phase III, which was named bio-sample D, M, and U, respectively. It should be noted that after the biofilm sampling at the end of phases I and II, new biofilms could be reconstructed on the single HFM within 14–21 days; meanwhile, stable NO$_3^-$ removal was achieved at the remaining 19–26 days. All the bio-samples were stored at −80 °C until analyzed.

The collected bio-samples were delivered to Novogene Co., Ltd. (Suzhou, China) for high-throughput pyrosequencing analysis to investigate the structure and dynamics of the microbial community. Extraction of genomic DNA was conducted using the cetyltrimethylammonium bromide (CTAB)/sodium dodecyl sulfate (SDS) method [29], and bacterial 16S rRNA genes of V4-V5 regions were amplified using primers 515F (5′-GTGCCAGCMGCCGCGG-3′) and 907R (5′-CCGTCAATTCMTTTRAGTTT-3′) [30]. After purification with the Qiagen Gel Extraction Kit (Qiagen, Germany), the amplicon library was generated using the TruSeq DNA PCR-Free Kit (Illumina, San Diego, CA, USA). Paired-end reads were merged by using FLASH (V1.2.7), and high quality tags were screened on the basis of QIIME (V1.9.1) [31], then they were assigned into operational taxonomic units with a similarity threshold of 97% by Uparse V7.0.1001 [32]. The Silva Database [33] was
employed on the basis of the Mothur algorithm for taxonomic annotation, and sequence alignment was processed by the MUSCLE (V 3.8.31) [34]. PICRUSt, a classical and powerful platform for predicting functional genes [35,36], was applied based on the whole qualified sequencing results using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database [37], and more details are provided in Langille et al. [38]. In this research, we especially focused on the KEGG orthology of nitrogen metabolism related pathways.

3. Results and Discussion

3.1. Denitrification Performance

After 60 days running of the H2-based MBfR at conditions where HRT and NO3− loading equaled 10 h and 10 mg N/L, respectively, biofilms with a relatively uniform thickness of nearly 600 µm were naturally formed on the surface of the HFMs, and complete NO3− removal was achieved, implying the successful start-up of the system. Following the stabilization of the system after the HRT was shifted from 10 to 5 h, experiments were conducted to evaluate the effects of electron acceptor availability on the denitrification performance. As delineated in Figure 3, the effluent NO3− concentration in phases I, II, and III was 0.12, 0.77, and 7.86 mg N/L, respectively, and NO2− was only detected in the effluent in phase III with a concentration of 3.2 mg N/L. According to the existing reference [14], excessively high NO3− loading could lead to NO2− accumulation in the effluent of H2-based MBfR. Noticeably, the average NO3− removal flux (calculated via Equation (1)) of the system was markedly enhanced from 0.67 g/(m2·d) to 1.31 g/(m2·d), as the influent NO3− concentration was increased from 10 mg N/L in phase I to 20 mg N/L in phase II, but the further increase of NO3− loading to 30 mg N/L in phase III merely resulted in a slightly higher NO3− removal efficiency (1.50 g/(m2·d)). In combination with the NO3− removal and NO2− accumulation results, it can be surmised that the relatively higher NO3− removal flux at the influent NO3− concentration of 30 mg N/L was because the quantity of the electron donor (H2) was insufficient to completely reduce NO2− to N2.

![Figure 3](image_url)

**Figure 3.** The effect of influent NO3− concentration on the denitrification performance of H2-based MBfR.

The microsensor measurement results, as displayed in Figure 4, indicate that NO3− was always abundant at varying depths of the biofilm when 30 mg N/L NO3− was added to the influent, while an influent NO3− concentration of 20 mg N/L resulted in the formation of an inefficient denitrifying zone in the biofilm interior, where the DNB activity was inhibited because of the lower local NO3− concentration than the half-maximum-rate concentrations of NO3− for DNB (KDNB = 0.2 mg N/L) [39]. The above findings led us to reasonably extrapolate that the availability of electron donor (i.e., H2) was the dominant limiting factor for NO3− removal in phase III. As illustrated earlier, the H2 availability for biofilm utilization is directly subject to the back-diffusion of N2 from the biofilm interior and bulk liquid. However, no single study exists that adequately covers the influence of the gases’ back-diffusion on the dynamics of microbial communities in the counter-diffusional biofilm of a H2-based MBfR.
Therefore, we concentrated in this study on the clarification of the microbial community structure characteristics of the counter-diffusional biofilm colonized on the close-end HFMs, which is conductive to furthering our comprehension of biofilm-related contaminant removal behavior.

3.2. Microbial Community Analysis

To delve into the dynamics of microbial communities, the relatively high abundance of bacteria (top 8) at the genus level were investigated at different phases (bio-samples N1, N2, and N3) as well as distances from the H2 supply end (bio-samples D, M, and U). The qualified sequence reads of bio-samples N1, N2, N3, D, M, and U were 59,290, 61,121, 65,304, 61,093, 62,610, and 47,935, respectively. As shown in Figure 5, in phase I, the most abundant genera pertaining to autotrophic denitrification were found to be Hydrogenophaga and Dechloromonas with a relative abundance of 13.3% and 44.8%, respectively. Hydrogenophaga, known as an unculturable H2-oxidizing bacteria [40], played a dominant role in leading to NO3− removal in a number of H2-based MBfRs [41–43]. The genus of Dechloromonas was deemed to be capable of eliminating NO3− and ClO4− using dissimilatory nitrate reductases and/or specialized perchlorate reductases [42,44–46]. The further increase in influent NO3− concentration from 10 mg N/L in phase I to 20 mg N/L in phase II gave rise to the overwhelming percentage (relative abundance equals 49%) of Hydrogenophaga as well as the strikingly decreased population (<1.0%) of Dechloromonas in the biofilm. This is in accordance with the results of preceding studies that Hydrogenophaga outperformed Dechloromonas in terms of H2 utilization and NO3− reduction in the case of an elevated NO3− loading [24,42,47]. Intriguingly, the relative abundance of Hydrogenophaga dropped to 17.2% at the influent NO3− concentration of 30 mg N/L in phase III. The possible explanations are the following: (1) the shortage of electron donors, which is supported by the observation that a mass of NO3− accumulation, given the susceptibility of Hydrogenophaga to toxicity of NO2− [1,13]; and (3) the enrichment of unidentified bacterial strains, which were affiliated to the order of Sphingobacterales, and might be involved in the conversion process of NO3− to NO [48].

Concerning the microbial communities at diverse locations of the biofilm fed with influent containing 30 mg N/L NO3−, Hydrogenophaga, was always the primary genus regardless of the distance from the H2 supply end, where its relative abundance was dramatically increased from 13.1% at the distance of 5 cm to 21.7% at the distance of 35 cm. This is unsurprising in light of the fact that attributed to the impact of back-diffusion, the quantity of H2 that could penetrate the HFMs and be available for the utilization of DNB proliferated at different positions of biofilm was negatively correlated with the distance from the H2 supply end. As a frequently discovered autotrophic DNB in H2-based MBfRs [19,47], Methyloversatilis was found to be the second most abundant functional bacteria for NO3− removal, possessing a relatively stable relative abundance ranging from 4.0–5.2% at varying locations of the biofilm. The unidentified_Sphingobacterales, a possible NO3− consumer as mentioned earlier, with its content variation tendency with changing positions was similar to that of Hydrogenophaga, but merely
occupied a quite lower population (0.3–1.4%). The unidentified *Nitrospiraceae*, the well-known autotrophic nitrite-oxidizing bacteria (NOB) [43], preferred to enrich at the upper side instead of the lower side of the biofilm, presumably due to the fact that the low H₂ pressure at the upper side of the intramembrane facilitated the entrance into the biofilm and the subsequent consumption of trace of dissolved oxygen arisen from the influent by the NOB.

Throughout the whole experiment, other DNBs such as *Azomonas* [42,49] were detected in the bio-samples but with an exceedingly low relative abundance. Since SO₄²⁻ was included in the influent, some genera involved in sulfur reduction (*Desulfovibrio, Sediminibacterium*) [50,51] and sulfide oxidation (*Flavobacterium* and *Sulfuritalea*) [18,52] were also discovered, thus a sulfur-relating microcirculation could occur in the counter-diffusional biofilm.

### 3.3. Predictive Functional Genes

PICRUSt was applied to predict the functional genes in the bio-samples on the basis of the high-throughput sequencing results of the 16S rRNA gene, and the odds ratios of the predictive functional genes are summarized in Figure 6. The variation tendencies in predicted relative abundance of a few basic metabolic pathways including xenobiotics biodegradation and metabolism, membrane transport, and energy metabolism are in full agreement with those of the detected dominant DNB (i.e., *Hydrogenophaga*) at conditions of varying NO₃⁻ loadings and distances from the H₂ supply end. Xenobiotics biodegradation and metabolism is closely related to the resistance of microorganisms to the toxicity of the exogenous contaminant [53]. The predicted highest abundance (5.87%) of the genes relating to xenobiotics biodegradation and metabolism appeared in phase II, probably due to the largest population of *Hydrogenophaga* and poor accumulation of noxious intermediates at this stage. With a similar trend, membrane transport, known to play a fundamental role in the substantial and ATP transportation process [54], occupied a conspicuously greater relative abundance of 17.69% in phase II than those (9.11–14.47%) in phases I and III. The evolution of denitrification behavior of functional bacteria is correlated to the changes of the nitrogen metabolism-related genes, belonging to energy metabolism-related genes. The nitrogen metabolism-related genes were most abundant with a proportion of 0.95% in phase II. It is noteworthy that the gene abundance involved in nitrogen metabolism was increased from 0.83% in the upside to 0.88% in the downside of the biofilm; this mirrored the variation tendency in the abundance of the genes involved in membrane transport with changing distance from the H₂ supply end. The foregoing function prediction results can support that the microbial metabolism at different locations of the biofilm in the H₂-based MBfR was significantly affected by the back-diffusion of inactive gases.
According to a preceding reference [27], the biofilm depth (from the bulk liquid side) that the electron donor (H₂) could reach was negatively correlated with NO₃⁻ loading; thus the activity of the nitrite reductase in DNB that grew in the vicinity of the HFM side might suffer from the shortage of H₂. Regarding the nitrous oxide reductase genes, the counts in phase I were found to be strikingly greater than those in phases II and III. The limited expression of nitrous oxide reductase in phases II and III is presumably associated with the insufficient supply of electron donors in the case of high electron acceptor (NO₃⁻) loading.

To figure out the nitrogen reductase involved in the nitrogen metabolism pathway, copy numbers of functional enzymes relating to the autotrophic denitrification process including nitrate, nitrite, nitric oxide, and nitrous oxide reductases were examined, as exhibited in Figure 7. The results regarding the counts of predictive functional genes encoding for nitrate reductase consisting of alpha subunit, beta subunit, gamma subunit, cytochrome, and electron transfer subunit are shown in Table 1. As to the influent NO₃⁻ concentration series, the largest counts of nitrate reductase genes of 57,554 and nitric oxide reductase genes of 19,475 were found in phase II, probably due to the greatest population of *Hydrogenophaga* genera in the biofilm, as shown in Figure 5. It is worth noting that despite the proportion of *Hydrogenophaga* and counts of nitrate enzyme genes in phase II were obviously greater than those in phase III (Figures 5 and 7), however, the NO₃⁻ removal flux in phase II was slightly lower than that in phase III (Figure 3). This can be ascribed to the decreased availability of NO₃⁻ for DNB in the biofilm interior in phase II (Figure 4), which gave rise to the declined overall activity of these functional bacteria. Although the nitrite reductase genes were more enriched in phase III rather than phase II, a considerable amount of NO₂⁻ was accumulated in the effluent in phase III (Figure 3). According to a preceding reference [27], the biofilm depth (from the bulk liquid side) that the electron donor (H₂) could reach was negatively correlated with NO₃⁻ loading; thus the activity of the nitrite reductase in DNB that grew in the vicinity of the HFM side might suffer from the shortage of H₂. Regarding the nitrous oxide reductase genes, the counts in phase I were found to be strikingly greater than those in phases II and III. The limited expression of nitrous oxide reductase in phases II and III is presumably associated with the insufficient supply of electron donors in the case of high electron acceptor (NO₃⁻) loading.

**Figure 6.** Odds ratios of predictive functional genes in the biofilm samples based on PICRUSt analysis. Genes with unknown functions and low relative abundance (<1%) are eliminated.
were investigated in a close-end operated H2-based MBfR. Based on the analysis of the concentration variations of NO3\(^-\) and/or its intermediate product (i.e., NO2\(^-\)) in the effluent and biofilm as a function changing NO3\(^-\) loading, the H2 availability for biofilm utilization was found to be the main limiting factor for NO3\(^-\) removal at an influent NO3\(^-\) concentration of 30 mg N/L. Microbial community analysis results suggest that at NO3\(^-\) loadings of 20–30 mg N/L, Hydrogenophaga was always recognized as the dominant functional bacteria in the collected bio-samples, regardless of the distance from the H2 supply end; an influent NO3\(^-\) concentration of 20 mg N/L was found to facilitate the enrichment of Hydrogenophaga; the relative abundance of Hydrogenophaga was negatively correlated with the distance from the H2 supply end. Functional genes analysis results corroborate that the variation trends of...

### Table 1. Counts of predictive functional genes and descriptions involved in the nitrogen metabolism pathway.

| Reductase                  | KO_Hierarchy   | KEGG_Description             | N1  | N2  | N3  | D   | M   | U   |
|----------------------------|----------------|------------------------------|-----|-----|-----|-----|-----|-----|
| Nitrate reductase          | K00370         | nitrate reductase alpha subunit | 12,718 | 18,940 | 16,095 | 15,010 | 14,465 | 14,245 |
|                            | K00371         | nitrate reductase beta subunit | 12,783 | 18,984 | 16,111 | 15,421 | 14,485 | 14,296 |
|                            | K00374         | nitrate reductase gamma subunit | 8040 | 18,639 | 15,925 | 12,799 | 14,172 | 13,816 |
|                            | K02567         | nitrate reductase (cytochrome), electron transfer subunit | 5236 | 501 | 437 | 694 | 372 | 408 |
| Nitrite reductase          | K00366         | nitrite reductase (NO-forming) | 3676 | 1837 | 3077 | 7378 | 6180 | 6000 |
| Nitric oxide reductase     | K04561         | nitric oxide reductase subunit B | 14,325 | 19,475 | 16,173 | 13,828 | 14,392 | 14,122 |
| Nitrous oxide reductase    | K00376         | nitrous-oxide reductase       | 10,580 | 1231 | 890 | 1666 | 1152 | 1040 |

![Figure 7. Counts of predictive functional genes involved in the nitrogen metabolism pathway.](image-url)

As the distance from the H2 supply end was increased, the counts of nitrate, nitrite, and nitrous oxide reductase genes gradually decreased, with this variation trend identical to those in relative abundances of Hydrogenophaga and predicted genes involved in N metabolism. For instance, the counts of nitrate, nitrite, and nitrous oxide reductase genes in the biomass at the upside of HFMs was decreased by 1428, 1378, and 626, respectively, in comparison to those at the downside. This result offers evidence that the expression of most denitrifying enzymes of DNB in the biofilm was significantly hampered by the back-diffusion of inactive gases, especially those that grew at the locations far from the H2 supply end. In particular, in most cases of diverse NO3\(^-\) loading and distance from the H2 supply end, the counts of genes encoding for nitrous oxide reductase are the fewest among denitrifying enzymes, likely as a consequence of the prioritized consumption of electron donor by other N metabolism-related enzymes in the case of electron donor deficiency. The relatively less expression of nitrous oxide reductase implies the possible accumulation of denitrifying intermediates (i.e., N2O). A previous mechanism study results suggested that biofilm systems could result in obviously greater amount of N2O emissions than suspended-growth systems, attributed to their nature in terms of microbial stratification and substrate gradients [55].

### 4. Conclusions

In this study, the NO3\(^-\) removal performance and characteristics of microbial community structure were investigated in a close-end operated H2-based MBfR. Based on the analysis of the concentration variations of NO3\(^-\) and/or its intermediate product (i.e., NO2\(^-\)) in the effluent and biofilm as a function changing NO3\(^-\) loading, the H2 availability for biofilm utilization was found to be the main limiting factor for NO3\(^-\) removal at an influent NO3\(^-\) concentration of 30 mg N/L. Microbial community analysis results suggest that at NO3\(^-\) loadings of 20–30 mg N/L, Hydrogenophaga was always recognized as the dominant functional bacteria in the collected bio-samples, regardless of the distance from the H2 supply end; an influent NO3\(^-\) concentration of 20 mg N/L was found to facilitate the enrichment of Hydrogenophaga; the relative abundance of Hydrogenophaga was negatively correlated with the distance from the H2 supply end. Functional genes analysis results corroborate that the variation trends of
relative abundance of basic metabolic pathways and counts of functional enzyme genes with varying \text{NO}_3^-\text{ loading and distance from the H}_2\text{ supply end are, in most cases, in good agreement with the population evolution of } Hydrogenophaga; due to the impact of the gases’ back-diffusion, a majority of functional genes pertaining to the microbial metabolism as well as the denitrification process gradually decreased from the downside to upside of HFMs.

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