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Effect of self-alkalization on nitrite accumulation in a high-rate denitrification system: Performance, microflora and enzymatic activities

Wei Li [a], Xiao-Yu Shan [a], Zhi-Yao Wang [a], Xiao-Yu Lin [a], Chen-Xu Li [a], Chao-Yang Cai [a], Ghulam Abbas [a], Meng Zhang [a], Li-Dong Shen [b], Zhi-Qiang Hu [c], He-Ping Zhao [a, d, *], Ping Zheng [a, **]

[a] Department of Environmental Engineering, College of Environmental and Resource Science, Zhejiang University, Hangzhou, China
[b] Department of Agricultural Resource and Environment, College of Applied Meteorology, Nanjing University of Information Science and Technology, Nanjing, China
[c] Department of Civil and Environmental Engineering, University of Missouri, Columbia, USA
[d] MOE Key Lab of Environmental Remediation and Ecosystem Health, College of Environmental and Resource Science, Zhejiang University, Hangzhou, China

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ABSTRACT

The self-alkalization of denitrifying automatic circulation (DAC) reactor resulted in a large increase of pH up to 9.20 and caused a tremendous accumulation of nitrite up to 451.1 ± 49.0 mgN L^{-1} at nitrate loading rate (NLR) from 35 kgN m^{-3} d^{-1} to 55 kgN m^{-3} d^{-1}. The nitrite accumulation was greatly relieved even at the same NLR once the pH was maintained at 7.6 ± 0.2 in the system. Enzymatic assays indicated that the long-term bacterial exposure to high pH significantly inhibited the activity of copper type nitrite reductase (NirK) rather than the cytochrome cd_1 type nitrite reductase (NirS). The terminal restriction fragment length polymorphism (T-RFLP) analysis revealed that the dominant denitrifying bacteria shifted from the NirS-containing *Thauear* sp. 27 to the NirK-containing *Hyphomicrobium nitrativorans* strain NL23 during the self-alkalization. The significant nitrite accumulation in the high-rate denitrification system could be therefore, due to the inhibition of Cu-containing NirK by high pH from the self-alkalization. The results suggest that the NirK-containing *H. nitrativorans* strain NL23 could be an ideal functional bacterium for the conversion of nitrate to nitrite, i.e. denitritation, which could be combined with anaerobic ammonium oxidation (Anammox) to develop a new process for nitrogen removal from wastewater.

1. Introduction

The discharge of high strength nitrate wastewater may cause an alarming increase in nitrate level in water bodies. The permissible level of nitrate in drinking water is 10 mg/L NO_3^-–N in USA and 12 mg/L in Europe (USEPA, 1987). Since nitrate concentration could be higher than 1000 mg/L in the wastewater from fertilizer, explosive, metal finishing, and nuclear industries, the removal of nitrate nitrogen becomes necessary. Biological denitrification is widely applied in nitrogen removal from wastewater (Lu et al., 2014; Grady et al., 1999) because of its high efficiency and low costs (Isaka et al., 2012). In recent years, some high-rate denitrifying systems have been developed (Li et al., 2015; Rabah and Dahab, 2004).

As it is well-known, the denitrification is an alkalization process (Lee and Rittmann, 2003). Using methanol as an electron donor, for example, 1 g NO_3^-–N removal produces 3.57 g $CaCO_3$ alkalinity (mainly in the form of HCO_3^- and CO_3^{2-}) according to Eq. (1) (McCarty et al., 1969). The self-alkalization of denitrification often leads to a pH higher than 9.0 in the continuous or sequencing batch denitrifying reactors (Li et al., 2014; Gong et al., 2013). As a result, nitrite accumulation is frequently reported in the denitrifying reactors with high pH (Baeseman et al., 2006; Lee and Rittmann, 2003; Glass and Silverstein, 1998). Normally the nitrite

* Corresponding author.
** Corresponding author.
E-mail addresses: zhaohp@zju.edu.cn (H.-P. Zhao), pzheng@zju.edu.cn (P. Zheng).

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accumulation should be avoided since nitrite released to water bodies is more toxic than nitrate to aquatic life. On the contrary, the nitrite accumulation should be promoted in some anammox-based processes because nitrite is a necessary substrate of anammox (Strous et al., 1999). It was reported that a pH of 9.0 caused significant accumulation of nitrite in the denitrification at a high NO_3^-–N concentration of 2700 mg N L^{-1} (Glass and Silverstein, 1998). It was also reported that a pH below 6.0 even caused more serious accumulation of nitrite than a pH above 8.5 (Cao et al., 2013; Thomsen et al., 1994). So far, most of the information about the effect of pH on nitrite accumulation comes from the studies of denitrifying system at low nitrate loading rates (NLRs) (<10 kgN m^{-3} d^{-1}). Little information is available about the nitrite accumulation and the effect of self-alkalization on nitrite accumulation at high NLRs.

$$NO_3^- + 1.08CH_3OH \rightarrow 0.064C_5H_7NO_2 + 0.47N_2 + 0.52HCO_3^- + 0.24CO_3^{2-} + 1.68H_2O \quad (1)$$

Because methanol is often applied in the denitrification, so a lot of methylotrophs are involved in the process (Mustakhimov et al., 2013). No matter full-scale plants or lab-scale reactors fed with methanol (NLR < 10 kgN m^{-3} d^{-1}), methylotrophic denitrifiers such as *Hyphomicrobium* spp. are frequently detected as the dominant denitrifying bacteria (Lu et al., 2014; Isaka et al., 2012; Lew et al., 2012). Up till now, however, the relevance of nitrite accumulation to methylotrophs is not clear.

Four enzymes, i.e. nitrate reductase (NAR and/or NAP), nitrite reductase (NirK or NirS), nitric oxide reductase (cNOR or qNOR) and nitrous oxide reductase (N_2OR), are involved in denitrification (Zumft, 1997). From the enzymology point of view, nitrite accumulation means an imbalance of enzymatic reactions. What caused the imbalance of enzymatic reactions? Is the imbalance of enzymatic reactions relevant to self-alkalization? These two questions need to be answered.

In our lab, a novel denitrifying automatic circulation (DAC) reactor has been developed with NLR up to 55 kg N m^{-3} d^{-1} (Li et al., 2015), and a significant nitrite accumulation was observed at a high pH of 9.2 from the self-alkalization (Li et al., 2015). The objective of this study was to evaluate the impact of self-alkalization on: (1) denitrification process, including the conversion of intermediates such as nitrite and nitrous oxide; (2) succession of denitrifying population in the microbial community through polymerase chain reaction-terminal restriction fragment length polymorphism (PCR-T-RFLP) analysis; (3) activity of key denitrifying enzymes such as nitrate reductase and nitrite reductase.

2. Materials and methods

2.1. DAC bioreactor operation

The experiments were carried out in a plexiglass DAC reactor (Fig. S1) with an effective volume of 1.25 L (Li et al., 2014a). The DAC reactor was inoculated with the denitrifying granular sludge obtained from a high-load lab-scale reactor, which had been operated for more than 100 days (Li et al., 2013). The initial NLR of DAC reactor was set at 25 kg N m^{-3} d^{-1}. The COD/NO_3^-–N ratio was fixed at 5.0, and the influent NO_3^-–N concentration was increased stepwise from 1000 to 2200 mg N L^{-1}. The hydraulic retention time (HRT) was 0.96 h, and effluent recycle ratio (recycle flow to inflow ratio) was 2.0. The influent and the recycle flow were mixed in the manifold before entering the reactor at the bottom. A high up-flow velocity was applied to get a suitable shear force for sludge granulation. The volatile suspended solids (VSS) was kept at 30 ± 3 g/L by removing sludge regularly. The VSS/SS was about 50%–55%. At NLR of 55 kg N m^{-3} d^{-1}, an automatic pH controller (Fig. S1) was used to maintain a constant pH of 7.4 by adding 2.5 M HCl (pH-control phase). The experimental temperature was set at 30 ± 1 °C. The pH of synthetic wastewater was 7.0–7.2. The composition of synthetic wastewater is available in the Support Information (SI).

2.2. Chemical analysis

The influent and effluent samples were taken using a syringe and filtered through a disposable Millipore filter unit (0.22 μm pore size) immediately. The pH, alkalinity, nitrate, nitrite, COD, suspended solids (SS) and VSS were all determined according to the standard methods (APHA, 2005). Details about the determination of N_2O, N_2, CO_2 and qPCR assays are presented in the SI. All the tests were performed in triplicate and the results were expressed as mean ± standard deviation. An analysis of variance (ANOVA) was performed by using IBM SPSS Statistics® Desktop version 19.0 (IBM, USA) and $p < 0.05$ was considered to be statistically significant.

2.3. Determination of NAR and NIR activities

The denitrifying sludge was withdrawn and washed 3 times with 0.01 M phosphate buffer (pH = 7.4) first (McGrath and Quinn, 2000), and then sonicated at 20 kHz and 4 °C for 5 min to break the denitrifying cells. The debris was then centrifuged at 12,000 g and 4 °C for 10 min and the crude extracts in supernatant were obtained for the determination of enzymatic activities. According to the literature, the enzymatic activities were based on protein content with bovine serum albumin as the standard (Lowry et al., 1951).

The NAR and NIR activities were determined using methylviologen as the electron donor (Kristjansson and Hollocher, 1980). 300 μL of crude extracts was added to a cuvette containing 1 mM $NaNO_3$ or 1 mM $NaNO_2$, 1 mM methylviologen and 5 mM sodium hyposulfite ($Na_2S_2O_4$) with total volume of 2 mL. The pH was adjusted as needed (0.1 M carbonate buffer and 0.1 M HCl was used for pH of 9.2 ± 0.1 and 7.2 ± 0.1, respectively). The incubation was conducted at 30 °C for 30 min, followed by determination of nitrite produced in NAR activity assay or consumed in NIR activity assay. The specific activities of NAR or NIR were expressed as μg produced nitrite • h^{-1} g^{-1} protein or μg consumed nitrite • h^{-1} g^{-1} protein. The diethyl dithio carbamate (DDC 10 mM) was used to identify the type of nitrite reductase with the samples harvested at NLR of 55 kgN m^{-3} d^{-1} with and without pH control.

2.4. DNA extraction and PCR amplification

The DNA was extracted from 0.25 to 0.30 g fresh denitrifying sludge by using a Power Soil DNA kit (Mo Bio Laboratories, CA, U.S.A) for the samples at NLR of 35 kgN m^{-3} d^{-1} (at day 38), 45 kgN m^{-3} d^{-1} (at day 52), 55 kgN m^{-3} d^{-1} (at day 68) and the samples at NLR of 55 kgN m^{-3} d^{-1} with pH control (at day 100), respectively. DNA concentration was determined using the NanoDrop spectrophotometer (ND-1000, Isogen Life Science, Netherlands).

The primers 11F (5′-GTTTGATCCTGGCTCAG-3′), and 1387R (5′-GGG CGG WGT GTA CAA GGC-3′) were applied to target the 16S rRNA gene (Green et al., 2004; Marchesi et al., 1998). PCR amplification was carried out in a total volume of 25 μL, containing 2 μL dNTPs mixtures (2.5 mM) (Takara, Japan), 2.5 μL 10 × PCR buffering solution (containing 15 mM magnesium ions) (Takara,