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Nitrogen removal characteristics of indigenous aerobic denitrifiers and changes in the microbial community of a reservoir enclosure system via *in situ* oxygen enhancement using water lifting and aeration technology

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HIGHLIGHTS

- The indigenous aerobic denitrifiers were enhanced *in situ*.
- The enhanced system performed very well in terms of nitrogen removal and the inhibition of Fe, Mn and P pollutants.
- The N-functional bacteria were obviously increased via *in situ* oxygen enhanced.
- Nitrogen source, Fe, Mn, DO were the most important factors affecting the bacterial community function and composition.

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ABSTRACT

Indigenous aerobic denitrifiers of a reservoir system were enhanced *in situ* by water lifting and aeration technology. Nitrogen removal characteristics and changes in the bacterial community were investigated. Results from a 30-day experiment showed that the TN in the enhanced water system decreased from 1.08–2.02 to 0.75–0.91 mg/L and that TN removal rates varied between 21.74% and 52.54% without nitrite accumulation, and TN removal rate of surface sediments reached 41.37 ± 1.55%. The densities of aerobic denitrifiers in the enhanced system increased. Furthermore, the enhanced system showed a clear inhibition of Fe, Mn, and P performances. Community analysis using Miseq showed that diversity was higher in the *in situ* oxygen enhanced system than in the control system. In addition, the microbial composition was significantly different between systems. It can be concluded that *in situ* enhancement of indigenous aerobic denitrifiers is very effective in removing nitrogen from water reservoir systems.

1. Introduction

Excessive nitrogen concentration, often in the form of nitrate and ammonia, leads to poor water quality and has recently become a growing concern. Increasing N export has been related to water eutrophication in coasts, lakes, and especially in drinking water reservoirs. There is still some debate over whether N alone is the main driver of these problems, but there is no doubt that an increase in N loading causes water quality degradation. In the past few years, bioremediation has attracted growing attention because it has lower maintenance costs and is more efficient at removing pollutants than other methods (Zhu et al., 2008). Traditional biotreatment processes for nitrogen removal involve autotrophic nitrification and heterotrophic denitrification. Nitrification is achieved under aerobic conditions, while denitrification requires anaerobic and anoxic conditions through a sequence of intermediates (nitrate, nitrite, nitric oxide, and nitrous oxide), resulting in nitrogen gas. Because of their different oxygen requirements, these two steps are separated spatially and temporally. Since oxygen inhibits the reaction steps, traditional processes are impractical in natural waters, especially in reservoirs. The discovery of the first aerobic denitrifying bacteria, *Thiosphaera pantotropha* (Robertson and Kuenen, 1983), led to a novel method for removing nitrogen which is not limited by oxygen. Moreover, aerobic denitrification occurs in natural systems. Gao et al. (2010) demonstrated that aerobic denitrification exists in permeable sea sediments (Gao et al. (2010), and Coban et al. (2015) quantified the rates of aerobic denitrification (Coban et al., 2015a).

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Microbiologists have defined aerobic denitrification as the co-respiration or co-metabolism of oxygen and nitrate. Aerobic denitrification has attractive advantages: nitrification and denitrification can occur in the same system, and denitrification can cause sufficient alkalinity to partly balance the acidity of nitrification. Consequently, more researchers in recent years have focused on nitrogen removal using aerobic denitrifiers. Some full-scale bioaugmentation experiments with aerobic denitrifying bacteria have been conducted successfully. For example, Robertson et al. (1989) applied bioaugmentation to biologically remove nitrogen from wastewater (Robertson et al., 1989). These authors introduced and maintained aerobic denitrifiers in a complex nitrifying community, allowing nitrification and denitrification to occur concurrently in the same aerobic unit. Patureau et al. (1997) successfully combined an aerobic denitrifier, *Microvirgula aerodenitrificans* (Patureau et al., 1997), with a nitrifying consortium, despite the fact that the denitrifying activity of the aerobic bioreactor declined over time. Cattaneo et al. (2003) studied the performance of *Pseudomonas denitrificans* in a fluidized bed and in a stirred tank reactor, and found that bacteria successfully removed nitrogen in the fluidized bed reactor (Cattaneo et al., 2003). Recently, Chen et al. (2015) found that PCN bacteria capable of nitrogen removal could be used to treat municipal wastewater in a pilot scale SBR (Chen et al., 2015). This approach was able to meet the strict requirements of the National Municipal Wastewater Discharge Standards of China (chemical oxygen demand (COD) < 50 mg/L, total nitrogen (TN) < 15 mg/L, total phosphorus (TP) < 0.5 mg/L). It is well known that the successful application of bioaugmentation technology depends on the adaptation of microbial strains to indigenous microorganisms, which means the introduced microbial strains should survive and remain active in the receiving systems. Although bioaugmentation seems simple at first, many attempts to use bioaugmentation have failed owing to the poor *in situ* survival or low activity of bioaugmentation strains (Thompson et al., 2005). Problems concerning the adaptation of inoculated microorganisms, insufficient substrate, competition between introduced species and indigenous biomass, and grazing by protozoa have been suggested as possible reasons for experimental failure. Moreover, when adding a considerable amount of "inoculated bacteria strains" to natural water, especially in drinking water reservoirs, environmental safety cannot be ignored (Wu et al., 2014).

Research on bacterial inoculation or *in situ* enhancement of indigenous aerobic denitrifying bacteria for nitrogen pollution removal or bioremediation of reservoir systems is scarce when compared to research on bioaugmentation in soils, municipal wastewater, and groundwater systems. It is known that the quality of water reservoirs is affected by endogenous pollutants released into the overlying water under anoxic conditions (Gantzer et al., 2009). Many studies demonstrated that anoxia could re-introduce N, P, Fe, and Mn from sediments into overlying water layers (Gantzer et al., 2009; Chai et al., 2011). To effectively control pollutants released from sediments, aerobic conditions in reservoirs must be kept using hypolimnetic oxygenation (Beutel and Horne, 1999). During the past few decades, WLA (water lifting and aeration) technology has been developed and used effectively to increase dissolved oxygen concentration and improve water quality of micro-polluted drinking water reservoirs (Cong et al., 2006, 2009; Bryant et al., 2011; Gerling et al., 2014). Meanwhile, aerobic denitrifying species with nitrogen removal characteristics have also been isolated from reservoirs (Wei et al., 2010; Guo et al., 2013; Huang et al., 2015a,b). This suggests that aerobic denitrification is an effective way to decrease endogenous nitrogen pollution in aquatic ecosystems. However, the underlying mechanisms are not well understood (Gao et al., 2010; Coban et al., 2015b).

In the present study, indigenous aerobic denitrifiers from the enclosure system of Zhoucun drinking water reservoir were enhanced *in situ* using WLA technology. WLA is used to mix and oxygenate water, facilitating the growth of aerobic denitrifiers and enhancing the water denitrification by aerobic microorganisms (Huang et al., 2012). The objectives of this study were: (i) to investigate the feasibility and efficiency of nitrogen removal by indigenous aerobic denitrifiers and the inhibition of Fe, Mn, and P pollutants via *in situ* oxygenation; (ii) to examine the bacterial diversity and abundance, and to find out which genera of bacterioplankton are present at different times in enhanced and control systems; and (iii) to investigate the relationship between the bacterioplankton community structure and environmental driving factors, focusing especially on the bacteria involved in nitrogen cycling across the whole experimental period.

2. Methods

2.1. Experimental system

Enhanced system: An enhanced system was used to simulate the WLA (water lifting and aeration) technology system. Compressed air was released in the bottom of the enhanced experimental system in the form of small bubbles, which increased dissolved oxygen concentration through direct mixing and oxygenation (Supplementary Fig. S1). **Control system:** An experimental system without aeration, placed at the bottom of the reservoir, was used as control group (Supplementary Fig. S2).

2.2. Nitrogen removal in the enhanced system

The enhanced experiment was conducted in three periods with three different oxygen concentration levels (high oxygen concentration, medium oxygen concentration, and low oxygen concentration). In order to study the nitrogen removal performance, nitrate, nitrite, ammonia, TN (total nitrogen), and TDN (total dissolved nitrogen) concentrations were measured in each period. All parameters were measured in triplicate ($n = 3$). In order to access the inhibition of TN in sediments, the TN of surface sediments was also measured at specific times in both enhanced and control systems.

2.3. Changes of oligotrophic aerobic denitrifiers

In order to investigate whether the ability of indigenous bacteria to remove nitrogen could be improved in the enhanced system, the numbers of oligotrophic aerobic denitrifiers were measured in enclosure systems. The density of oligotrophic aerobic denitrification bacteria in the experimental systems were measured by plate counts (Huang et al., 2012, 2015c). The numbers of aerobic denitrification bacteria of water samples (0.5 m, 5.0 m, 7.5 m, 10.0 m, and 13.0 m) were tested via gradient dilution. The gradient dilutions were as follows: 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}, respectively. Then 0.2 mL diluents were streaked onto a solid screening medium (included (g/L): CH_3COONa, 0.10; $NaNO_3$, 0.02; $K_2HPO_4{\cdot}3H_2O$, 0.02; $CaCl_2$, 0.01; $MgCl_2{\cdot}6H_2O$, 0.01; and agar, 20; pH 7.2.) in triplicate and incubated at 30 °C for 5 days. Prominent single colonies were harvested and calculated.

2.4. Quantification of nirS and nirK genes abundance

Quantitative PCR was used to estimate the numbers of nirS and nirK copies in water and sediment systems collected at 20 sites during the experimental period of Oct. 16–Nov. 14. DNA was extracted from an approximately 2 L water sample (every water sample) and ~50 mL of surface sediment (0–2 cm) for water and