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New nitrogen removal pathways in a full-scale hybrid constructed wetland proposed from high-throughput sequencing and isotopic tracing results

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ABSTRACT

Constructed Wetlands (CWs), which usually feature limited supply of electron acceptors, possess complex microbial nitrogen removal pathways. Based on Illumina high-throughput sequencer, ^{15}N isotopic tracer, and three years of physicochemical analysis, in this study, we report on a complex microbial consortium responsible for the nitrogen removal in a full-scale hybrid CW, consisted of vertical-baffled flow wetland (VBFW, first stage) and horizontal subsurface flow wetland (HSFW, second stage), receiving municipal sewage. Two organotrophic anaerobic ammonium oxidation (anammox) bacteria, *Candidatus* 'Anammoxoglobus propionicus' and 'Brocadia fulgida', chemolitho-autotrophic denitrifiers mainly *Thiobacillus denitrificans*, heterotrophic denitrifiers, ammonia oxidizers, and nitrite oxidizers were found in the HSFW, which achieved a mean total nitrogen removal load of 3.7 g N m^{-2} d^{-1} in the summer season. A phylogenetic analysis and isotopic incubation experiment revealed that simultaneous autotrophic denitrification, heterotrophic denitrification and anammox processes contributed to the nitrogen loss from the system, which composed of a complex nitrogen removal scheme in a full-scale CW for low-strength municipal sewage treatment. Sulphur-oxidizing *Thiobacillus denitrificans*, the most abundant bacterial species in HSFW sediments, can convert nitrate to nitrite and support anammox bacteria with the necessary substrate to complete nitrogen removal. The present study confirmed the presence of anammox process in a full-scale CW and indicated that the nitrogen removal pathways in CWs are much more complex than we previous thought.

1. Introduction

Wetlands (natural/constructed) generally possess a complex hydrology and nutrient cycling that influences the biogeochemistry in the system (Gutknecht et al., 2006). Constructed wetlands (CWs) have been used to treat different types of wastewater, which are primarily synthesized by versatile microbial activity and produce different intermediates and harmless end products (Faulwetter et al., 2009). The exchange of substrates is a suitable survival strategy for microbes especially in anoxic conditions, such as marine sediments (Orphan et al., 2001), freshwater ecosystems (Zhou et al., 2014), or even in CWs, where the scarcity of electron acceptors is common.

Previous studies have demonstrated microbial co-metabolisms significantly influence the C, N and S cycle in the marine ecosystem due to the limited supply of electron acceptors (Thompson et al., 2012). In such environments, bacterial associations between methanotrophic archaea and sulfate-reducing bacteria (Boetius et al., 2000), *Thioploca* and anaerobic ammonium oxidation (anammox) (Prokopenko et al., 2013) bacterial symbiosis have been confirmed. The latter symbiotic consortium was found in the Soledad basin where anammox bacteria obtain both ammonium

Abbreviations: Anammox, anaerobic ammonium oxidation; COD, chemical oxygen demand; CWP, clean water pond; CWs, constructed wetlands; DO, Dissolved Oxygen; FSWs, free water surface wetlands; GC–MS, gas chromatography–mass spectroscopy; HSFW, horizontal subsurface flow wetland; NAD, natural aeration ditch; NO_2^--N, nitrite-nitrogen; NO_3^--N, nitrate-nitrogen; ORP, oxidation-reduction potential; SPs, sedimentation ponds; SS, suspended solid; ST, septic tank; TN, total nitrogen; VBFW, vertical-baffled flow wetland.

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and nitrite for its metabolism from the filamentous *Thioploca*, and *Thioploca* driven anammox activity was estimated to contribute to more than half of the total benthic nitrogen production (Prokopenko et al., 2013). Anammox bacteria has also been found to occur as a syntrophic relationship with *Candidatus* 'Methanoperedens nitroreducens' in an anaerobic lab-scale bioreactor, where anammox bacteria gained nitrite from nitrate reduction and methane oxidation (Haroon et al., 2013). In freshwater ecosystems particularly in lake environments, microbial associations were observed during metabolite exchange between photoautotrophs and methanogenic archaea in the oxic water column (Grossart et al., 2011), while bacteria and a methanotrophic archaeal consortium was found in anoxic sediments (Raghoebarsing et al., 2006). Moreover, anammox activity was found positively correlated with the denitrifiaction process in inland river sediments (Zhou et al., 2014). These findings suggest microbial symbiosis and co-metabolism commonly exist in natural ecosystems.

Thiobacillus denitrificans is a common species of *Thiobacillus* genus, found in various environments including sewage treatment facilities (Shao et al., 2010). This species can act as an autotrophic denitrifier in carbon-limited environments. *T. denitrificans* converts nitrate to nitrite, nitrous oxide and even nitrogen as autotrophic denitrification products (Robertson and Kuenen, 2006; Shao et al., 2010). Subsequently, anammox bacteria can use substrate (NO_2^-) from *T. denitrificans* and ammonium from the surrounding environment to produce nitrogen in an anoxic environment. The reactions can be described by the following equations (1 and 2). This nitrogen removal pathway based on a combination of anammox and autotrophic denitrification using sulfide as an electron donor has been artificially developed as a new nitrogen removal process called denitrifying ammonium oxidation (DEAMOX) (Kalyuzhnyi et al., 2006; Liu et al., 2015). Therefore, *T. denitrificans* can play a similar role like *Thioploca*, to supply nitrite for the anammox bacteria in carbon limited environment, as reported by Prokopenko et al. (2013).

$$NO_3^- + HS^- + H^+ \xrightarrow{Thiobacillus} NO_2^- + S^\circ + H_2O \quad (1)$$

$$NH_4^+ + NO_2^- \xrightarrow{Anammox} N_2 + 2H_2O \quad (2)$$

A new type of hybrid CW, consisted of the vertical-baffled flow wetland (VBFW) and horizontal subsurface flow wetland (HSFW) in series, has been developed and successfully applied in South China in more than 10 full-scale cases (Zhai et al., 2011). Based on three-year continual monitoring data, we found a simultaneous removal of total nitrogen (TN) and ammonium nitrogen (NH_4^+-N) along the HSFW, second stage of the hybrid CW, but the limited decline in chemical oxygen demand (COD) in high-temperature seasons. It was hypothesized that anammox process occurs in summer in this hybrid CW when treating low-strength municipal sewage. An Illumina high-throughput sequencer, ^{15}N isotopic tracer and detailed physiochemical analysis were applied to confirm the presence of anammox activity. The microbial diversity and associations concerning the nitrogen removal in CWs were also discussed.

2. Materials and methods

2.1. Study site

The study was conducted in the Qinghe Agro-park Wastewater Treatment Plant, a hybrid CW system receiving municipal sewage and located in Chongqing City in south-west China. The plant has been in operation since January 2011, treating 500–600 m^3/d of sewage collected from a community of approximately 6000 people. The hybrid CW plant consists of a septic tank (ST, 450 m^3), two sedimentation ponds (SPs, 200 m^3 each) in parallel, a lagoon (640 m^3), five VBFWs (708 m^2 total) in parallel, five HSFWs (2740 m^2 total) in parallel, and a clean water pond (CWP, 340 m^3). The overall layout of the plant is presented in Fig. 1. The average hydraulic retention times for each treatment stage are approximately 19, 8, 28, 18, 18 and 15 h for the ST, SP, lagoon, VBFW, HSFW and CWP, respectively, and nearly 106 h for the total treatment plant. Gravel with a size of approximately 10 mm was used as the medium in the vegetated beds. The porosities of the medium in both VBFWs and HSFWs are 48%. *Cyperus alternifolius*, an ornamental emergent fast-growing perennial plant, was applied as a macrophyte in the VBFWs and HSFWs, whereas *Eichhornia crassipes*, a floating plant, was used for the SP and CWP. The hybrid CW is composed of a "U" pattern of continuous baffled flow VBFWs and an "S" pattern of shallow plug flow HSFWs in a series. Both VBFWs and HSFWs are loaded continuously with hydraulic loading rates (HLRs) varied from 0.71–0.85 m/d and 0.18–0.2 m/d, respectively.

For the second stage, an HSFW normally consists of three to five sections of the HSFW beds with a water depth of 0.4 to 0.6 m. Between every two sections of HSFW beds, a shallow cascaded natural aeration ditch (NAD) was constructed. More detailed information about this hybrid CW can be found in previous research papers (Zhai et al., 2012, 2011).

2.2. Sampling and physiochemical analysis

Twenty-eight sampling points were installed along the flow route of the sewage treatment plant (Fig. 1) to investigate the concentration of the water physiochemical parameters. From point 5 to 26 in the HSFW bed (consist 3 sections: HF1 point 6–12, HF2 point 13–19 and HF3 point 20–26), water samples were drawn through pre-installed perforated pipes located 25 cm from the top of the gravel beds. For other sampling points where free water surface was available, water samples were collected directly with a glassy sampling device. Triplicate samples were collected from each sampling point, three times a month from Jan. 2011 to Dec. 2013 with the exception of the rainy periods and holiday times. The present study only focused on summer season (June to October) nitrogen removal performance. A total of 24 lots of water quality sampling results were presented in this study. The samples for temperature, oxidation-reduction potential (ORP), dissolved oxygen (DO), turbidity and pH were analysed in the field with portable sensors as described by Zhai et al. (2012). The COD, suspended solid (SS), TN, NH_4^+-N, nitrate-nitrogen (NO_3^--N) and nitrite-nitrogen (NO_2^--N) samples were pretreated with 40 mg/L of $HgCl_2$ onsite to prevent conversion of NO_2^--N and NO_3^--N after sampling and stored in a cooler, transported to the laboratory at Chongqing University within 3 h and analysed according to the Chinese National Standard methods (Bureau, 2002).

For the isotopic tracing and microbial analysis, substrate samples, mixtures of gravel and sediment, were collected from 6 selected sites along the monitored HSFW beds (Fig. 1) by opening the vegetated bed and excavating with a shovel at a depth of approximately 30 mm from the top of the bed. The substrate samples were sealed on-site in glass bottles and stored in a cooler at 4 °C and then transported to the laboratory for microbial study and anammox rate measurements. The sampling campaign was conducted once a month from June to September in 2014.

2.3. Measuring anammox rate with ^{15}N isotopic tracing technology

Anammox rates of the substrate samples were measured using a combination of an isotopic tracing technology ($^{14}NH_4Cl$ and ^{15}N-labled $Na^{15}NO_2$) for the confirmation of anammox activity and its ratio to denitrification (Thamdrup and Dalsgaard, 2002). The mano-