Water Research 102 (2016) 321–329

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres

Pathways regulating the removal of nitrogen in planted and unplanted subsurface flow constructed wetlands

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ARTICLE INFO

Article history:
Received 17 March 2016
Received in revised form
22 June 2016
Accepted 23 June 2016
Available online 25 June 2016

Keywords:
Functional genes
Wastewater treatment
Anammox
Denitrification

ABSTRACT

Single-stage constructed wetlands (CWs) are characterized by a low potential for N removal. Understanding the pathways regulating N cycling as well as their dependence on environmental variables might improve the potential of CWs for N removal and results in more accurate simulation tools. In this study we employed qPCR targeting marker functional genes (*amoA*, *nirK*, *nirS*, clade I and II *nosZ*) or microorganisms (anammox) regulating key pathways of N cycling to unravel their relative importance. Furthermore, the influence of plant species on treatment performance was studied. Our findings indicated nitrification-denitrification as the principal route of N removal in CWs, while anammox did not have a strong contribution. Evidence was also arisen that ammonia oxidizing archaea (AOA) contributed on NH_3 oxidation. Overall, plant species had a weak effect on the abundance of N functional genes (*amoA* of AOA), but it strongly affected the performance of CWs in terms of N removal in the following order: unplanted < *Phragmites communis* < *Typha latifolia*. These findings suggest that plant species stimulate N removal by upregulating the rates that the responsible biochemical pathways operate, probably by increasing O_2 supply. In addition, our study revealed differences in indicators linked to N_2O emissions. The abundance of clade II *nosZ* genes remained low across the season scaling down a strong contribution in the reduction of the emitted N_2O. The increasing ratios of *nosZ*/Σ*nir* and *nirS*/*nirK* with the progress of season indicate a shift in the composition of denitrifiers towards strains with a lower genetic potential for N_2O release. Similar trends were observed among the treatments but the mechanisms differed. The planted treatments stimulated an increase in the Σ*nosZ*/Σ*nir* ratio, while the unplanted an increase in the *nirS*/*nirK* ratio.

1. Introduction

Constructed wetlands (CWs) have been extensively used around the world to treat municipal (Crites et al., 2014) and other types of wastewater (Vymazal, 2014) due to advantages associated with the lower construction and operation costs compared to conventional wastewater treatment plants (Tsagarakis et al., 2003; Mburu et al., 2013; Wu et al., 2015). However, the effectiveness of CWs in terms of N removal has been characterized as poor and constrains their performance. For instance, Kadlec and Knight (1996) reported average removal of roughly 44% for total nitrogen (TN) in CWs in the North America. Vymazal (2007), analyzing published data from CWs operating at a wide range of inflow loadings, found that removal efficiencies of N ranged from 40 to 55% with the lowest removal efficiencies to be mainly observed in single-stage CWs. These findings indicate that CWs commonly fail to achieve the increasingly strict standards imposed by environmental agencies for effluent discharge to water bodies or for recycling purposes (Paranychianakis et al., 2015).

The poor performance of single-stage CWs might partly arise from our limited understanding on the processes that regulate the biogeochemical cycle of N, their relative contribution on N cycling, and the influence of environmental factors, and hence, on the adoption of appropriate design and operation conditions. In fact, our perception on N cycling in CWs remains still rooted on the classical view of nitrification and denitrification processes. Recent studies have revealed that anammox and heterotrophic nitrification-aerobic denitrification processes may also have important roles that however, depends on CWs configuration, operation and the environmental factors (Zhu et al., 2011; Coban et al., 2015; Zhi et al., 2015). Data on functional genes abundance

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http://dx.doi.org/10.1016/j.watres.2016.06.048
0043-1354/

have been widely used to gain insights for the relative importance of the pathways involved in N cycling in terrestrial and aquatic ecosystems (Bru et al., 2011; Tsiknia et al., 2015), and only recently pertinent studies have started to be published for CWs (Chon et al., 2011; Ji et al., 2012; Coban et al., 2015). In particular, Ji et al. (2012) reported stratified distribution of N functional genes with the depth and high rates of NO_3 reduction to NH_4 in a pilot-scale CW. Furthermore, strong interactions between the biochemical cycles of C and N with a remarkable influence on the composition and the activity of functional microorganisms have also been reported. In a tidal-flow CW, NH_3 oxidation was hinted as the dominant pathway of NH_4 removal in effluents with a C/N ratio less than six based on functional genes abundance, but when the C/N ratio increased the contribution of anammox process was enhanced (Zhi and Ji, 2014).

It has been well documented that nitrification, a central pathway of N cycling, constrains the potential of CWs to remove N due the low availability of O_2 that is preferentially used by the heterotrophs inhibiting the growth and activity of ammonia oxidizers (Crites et al., 2014). Plant species affect the rates that O_2 is released to the rhizosphere of subsurface flow (SSF) CWs, exerting a strong control on the N pathways operating and on their rates (Nivala et al., 2013; Crites et al., 2014). Differences among plant species in terms of O_2 release in the rhizosphere have been positively correlated (35–76%) with the removal of TN (Mei et al., 2014). Plant species also affected the composition of nitrifying and denitrifying bacteria in the rhizosphere compared to the bulk sediment in constructed (Ruiz-Rueda et al., 2009) and natural wetlands (Bañeras et al., 2012; Trias et al., 2012) implying potential impacts on the performance of CWs. However, links between treatment performance of CWs and the abundance and/or the composition of functional microbial communities remain scarce or they are limited to static observations.

Herein, we investigate i) the pathways regulating the cycling of N in SSF-CWs, ii) the influence of plant species on the pathways operating and the treatment performance of CWs in terms of N removal, and iii) the seasonal trends of the N operating pathways of CWs and their treatment performance. We hypothesized that the absence of vegetation or the plant species by itself would impose direct and indirect controls on the treatment performance through its effects on environmental variables (e.g. redox potential, O_2 release) and/or on the abundance and composition of N functional genes, providing thus a proxy to evaluate the pathways operating and their importance on N cycling. To achieve these targets, we have employed quantitative PCR (qPCR) targeting marker functional genes of certain biochemical pathways of N biogeochemical cycle including the *amoA* genes of archaeal (AOA) and bacterial (AOB) ammonia oxidizers, the denitrifying genes (*nirK*, *nirS*, *nosZ* clade I and II), and the 16S rRNA gene of anammox bacteria. These data are linked to N removal efficiencies estimated from N mass balances to determine the relative importance of these pathways.

2. Materials and methods

2.1. Pilot constructed wetlands and experimental set up

The experiment included six pilot CWs that were operated under field conditions. The climatic conditions prevailed during the experimental period and the sampling dates are shown in Fig. S1. Polyethylene tanks, which were painted in white to avoid their overheating during summertime, were used as CWs basins. Their dimensions were 95 (length) x 45 (width) x 48 (height) cm and were filled in with gravel with a mean diameter of 7 mm. CWs were planted in pairs with the plant species *Phragmites australis* or *Typha latifolia* in June 2013, or they were left unplanted. The wetland basins were fed with a low-strength nutrient solution (TN: 5 mg/L) until the end of October 2013 to allow for the successful establishment of the vegetation and the aquatic microbial communities. The feeding of CWs with wastewater started on November 7, 2013 by using a modified OECD synthetic wastewater, that contained 200 mg/L glucose, 100 mg/L urea, 20 mg/L NaH_2PO_4, 5 mg/L $CaCl_2$, 2.5 mg/L $MgSO_4$, 1.5 mg/L KH_2PO_4, and micronutrients. CWs were supplied with wastewater from a central tank (1000 L) by peristaltic pumps operating continuously for 11 h and resting for 1 h, so that overheating problems of the pumps are avoided. Hence, the pumps operated for 22 h per day at a flow rate of 1.2 L/h. The influent entered the CW from the one side and was collected from the other to ensure conditions of horizontal flow, while the water surface was maintained 2 cm below the gravel surface. The synthetic wastewater was prepared every three days from October to April and every two days from May to September to minimize the effect of elevated temperatures on the mineralization of organic matter in the tank. The net volume of CWs was estimated to be 52.4 ± 1.8 L at the beginning of the experiment that corresponded to a theoretical hydraulic residence time (HRT) of two days. The theoretical HRT was re-calculated at the end of the operation period and showed no significant change (51.6 ± 2.2 L). Hydrologic balances were also performed at certain dates by collecting the effluent volume to estimate the effect of plant species on water losses from CWs.

2.2. Sample collection and chemical analyses

Water samples were collected early in the morning from the inlet and outlet of CWs and they were immediately analyzed for NH_4^+-N, NO_3–-N, urea, and total kjeldahl N (TKN). NH_4^+-N and NO_3–-N concentrations were measured colorimetrically in a Perkin-Elmer spectrophotometer (Lambda 25) with the Nessler reagent and the Cd-reduction method, respectively. TKN was determined by a semi-automated kjeldahl device and urea colorimetrically with the Greenan et al. (1995) protocol. pH and redox potential (Eh) were measured with a Thermo Scientific Orion 5-Star Portable multimeter.

2.3. DNA extraction and quantitative PCR (qPCR) assays

Microbial genomic DNA was extracted in seven dates (November 07 (day 0), November 27, December 16, January 13, April 10, June 10, and July 28) throughout the study period. Since no standard protocol has been developed so far to extract DNA from the gravel of CWs, we tested two protocols for their efficiency and the consistency of the obtained results. The first protocol tested was that described by Moore et al. (2004) and the second one was the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA). Comparisons in terms of the extracted quantity and quality DNA and consistency of the results obtained by qPCR showed a superiority of the latter, which was eventually selected for the performed DNA extractions. In brief, the protocol included two steps. Firstly, 20 g of gravel were sampled from 5 to 15 cm depth and placed in sterilized 50-ml falcon tubes. Twenty milliliters of sterilized phosphate bovine buffer solution was added in each tube and shaken for 1.5 h to detach the biofilm from gravel's surface. Then, the gravel was removed and the liquid was centrifuged at 10,000 rpm for 15 min and the supernatant was discarded. In the second step, the pellet was extracted with the PowerSoil® DNA Isolation Kit. In each sampling date two gravel samples were taken from each CW for DNA extraction. Then, the two replicates of DNA from each basin were pooled and its quality was checked in 1% agarose gel. The extracted DNA was quantified with a Pearl Nano-Photometer® (Implen) before it stored at −80 °C.

The abundance of ammonia monooxygenase (*amoA*) genes of