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

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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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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

AOB and AOA, nitrite reductase genes (*nirS*, *nirK*) and N_2O-reductase genes (*nosZ* clade I and II) was monitored by qPCR using the primer pairs amoA-1F/amoA-2R (Rotthauwe et al., 1997), Arch-amoAF/Arch-amoAR (Francis et al., 2005), nirSCd3aF/nirSR3cd (Throbäck et al., 2004), nirK876/nirK1040 (Henry et al., 2004), nosZ2F/nosZ2R (Henry et al., 2006), and nosZ-II-F/nosZ-II-R (Jones et al., 2013), respectively. The abundance of anammox 16S rRNA genes was quantified with the A438f/A684r primer pair (Humbert et al., 2012). qPCR runs were carried out in a StepOnePlus™ Real-Time PCR System (Applied Biosystems) in reactions of 20 μl using the KAPA SYBR Fast Master Mix (2×) qPCR Kit (KAPA Biosystems) and 2 μl of genomic DNA. All reactions were completed with a melting curve starting at 60 °C, with an increase of 0.5 °C, up to 95 °C to verify amplicon specificity. Standard curves were constructed using serial dilutions, 10^3–10^7 (*amoA* of AOA and AOB, *nirS*, *nirK*, *nosZ* clade I) and 10^2–10^6 (*nosZ* clade II and anammox 16S rRNA) of linearized plasmids (pGEM-T, Promega) containing cloned fragments from each gene. Controls without template resulted in undetectable products for all samples, while inhibitory effects were not detected at dilutions greater than 1/10. The amplification efficiencies varied between 72% and 96% and the R^2 values of the standard curves ranged from 0.995 to 0.997. The detailed protocols of qPCR as well as the amplification efficiency are summarized in Table S1.

2.4. Hydrological and N mass balance of CWs

With the progress of time, water losses between treatments were differentiated significantly due to different evapotranspiration (ET) rates. In order to proceed in a more accurate estimation of the N removal potential of various CWs, N mass balance calculations were performed at selected dates. Water losses through ET were estimated by collecting the whole volume of effluent leaving the CWs basins. Then, the N mass was calculated by measuring the nitrogenous compounds (NH_4^+-N, NO_3−-N, TKN) concentration in the effluent and multiplying their sum with the effluent volume estimated in the previous step.

2.5. Statistical analysis

All statistical analyses were performed with the R statistical platform (R Development Core Team, 2013). The plots were generated with the *ggplot2* package. Aligned-rank transformation and non-parametric analysis of variance (ANOVA) on mixed-effect models was performed with the *ARTool* package to evaluate the effect of the treatments and time. The aligned rank transformation is a powerful alternative to parametric F-tests for cases that the criterion of normality is not satisfied. Thus, untransformed data of the N functional genes were modeled considering the treatments and the time as independent variables in order to examine if these factors or their interaction had any significant effect on the explanation of the variance in the data. The art () function from the *ARTool* package, that performs the aligned-rank transformation, was applied in a mixed-effect model of the form:

Y ~ Time + Treatment + Time:Treatment + (1|Random effect)

Subsequently, the *anova* () function was used to evaluate the significance of these effects (Wobbrock et al., 2011).

Pairwise relationships among the N functional genes and their significance were tested with the Pearson correlation coefficient. The variables were tested for normality before further analysis and these (functional genes) that did not follow the normal distribution were subjected to Box-Cox transformation.

3. Results

3.1. Nitrogen forms and physico-chemical conditions

Both treatments and season significantly affected the concentration of nitrogenous compounds in CWs. In the early period of operation (November 07 – December 12) the performance of CWs in terms of NH_4^+-N removal was not affected by the presence of vegetation or the plant species (Fig. 1a). During that period the concentration of NH_4^+-N in the effluent followed a decreasing trend. This trend was continued for CWs planted with *T. latifolia* by January 22. By contrast, NH_4^+-N concentration followed an increasing trend in CWs planted with *P. australis*, and especially in the unplanted CWs, reaching greater concentrations compared to CWs planted with *T. latifolia* in December 19 and January 6, respectively. Thereafter, and until April 2 NH_4^+-N concentration remained more or less constant between treatments with the greatest concentrations measured in the unplanted CWs followed by *P. australis*, while the lowest concentrations were observed in CWs planted with *T. latifolia*. From this date until the end of the study, the NH_4^+-N concentration showed a sharp increase in the planted CWs only, which in the case of *P. australis* CWs reached the concentration of the unplanted CWs (Fig. 1a). The concentration of TKN (Fig. 1b) followed strictly the pattern of NH_4^+-N concentration and it accounted for the majority of TKN suggesting that organic-N consisted of only a minor fraction of the TN. Urea was not detected in the effluent from CWs in any of the samplings or treatments implying its rapid hydrolysis to NH_4^+.

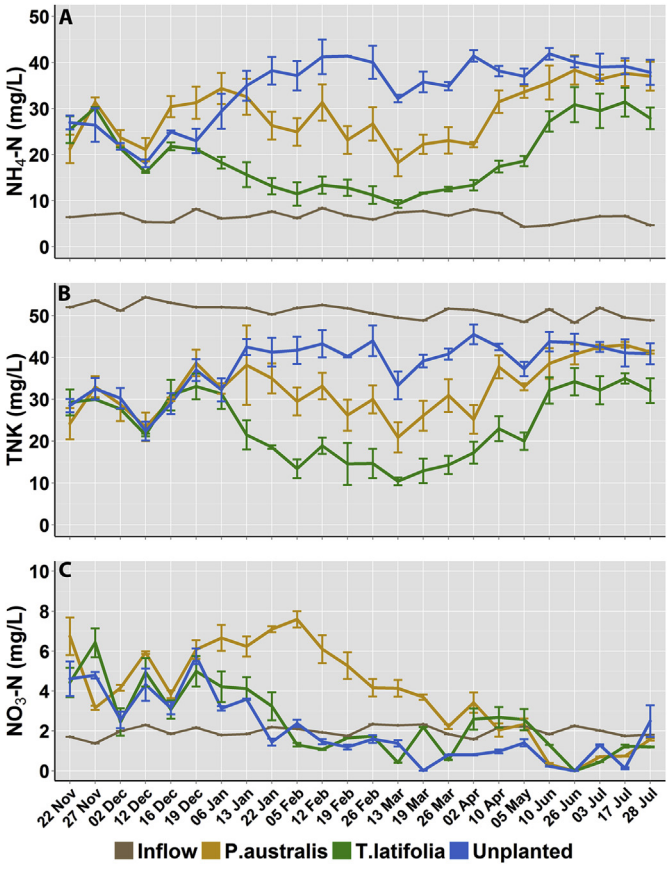

Fig. 1. Concentration of N-forms in constructed wetlands unplanted or planted with *T. latifolia* and *P. australis*. (a) Ammonium concentration; (b) Nitrates concentration; and (c) Total kjendal N concentration.

There was no significant effect of the treatments on the concentration of NO_3−-N, until December 19 (Fig. 1c). Then, and until February 05 the concentration of NO_3−-N increased in CWs planted with *P. australis*, while the opposite trend was observed for unplanted CWs and CWs planted with *T. latifolia*. Thereafter, the concentration of NO_3−-N in CWs planted with *P. australis* decreased to reach eventually that of unplanted or planted with *T. latifolia* CWs in May 05 (Fig. 1c).

With regard to the physicochemical conditions, the Eh was not affected by the presence of vegetation or the plant species itself (Fig. S2). In addition, no significant seasonal variations were observed. By contrast, planted CWs showed slightly lower values of pH compared to the unplanted ones (Fig. S3). Seasonal fluctuations were also observed. pH slightly declined during the winter period but recovered to the initial or slightly greater values in the summer, particularly in the planted CWs probably due to the high ET rates.

3.2. Nitrogen mass balance

Plant species had a strong influence on the hydrology of CWs by regulating the ET rate, and particularly from May 05 onwards, when the differences in terms of ET between the treatments were exacerbated (Fig. S4). Thus, a nitrogen mass balance was applied to account for the effect of variable ET on N removal. The estimates revealed even greater differences in the potential of CWs to remove N (Fig. 2). More specifically, CWs planted with *T. latifolia* showed the greatest N removal followed by those planted with *P. australis*. Uptake by vegetation was not taken into account in N mass balance estimations since i) uptake was accounted for a small only fraction of the applied N (7.4–8.4%; Table S2), and ii) plant biomass had been well developed when the application of wastewater started (see "Materials and Methods" section), thus the uptake of N was not expected to have impacted the estimations derived.

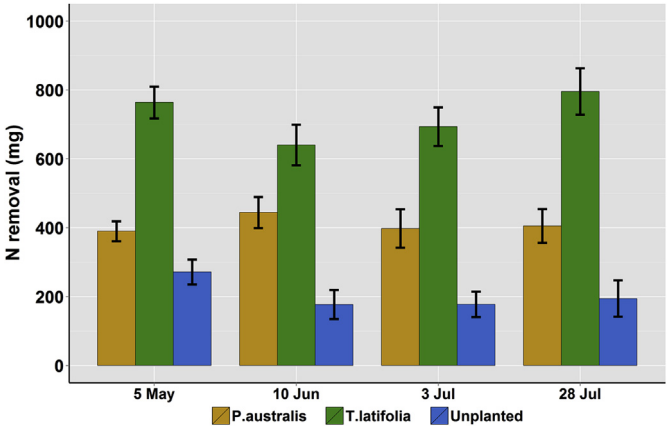

Fig. 2. Mass balance of N in constructed wetlands unplanted or planted with *T. latifolia* and *P. australis*. The mass balance was performed by taking into account the evapotranspiration losses and the concentration of N-forms presented in Fig. 1.

3.3. Abundance of functional genes regulating the cycling of nitrogen

The abundance and the ratios of the genes or microorganisms involved in the cycling of N were significantly affected by the treatment and the sampling time (Table 1). In addition, significant interactions were observed for the *amoA* genes of AOA, *nirK* genes and the ratio of *nirK*/*nirS* genes (Table 1). Relatively low abundances (10^3–10^4/g gravel) of *amoA* genes of AOB were measured early in the operation period (November 07 and November 27) of the CWs (Fig. 3a.). Thereafter, the abundance of *amoA* genes increased by approximately two orders of magnitude and remained relatively constant by the end of the study (Fig. 3a). No significant differences were observed between treatments during that period. With regard to *amoA* genes of AOA, they showed a lower abundance compared to that of AOB (Fig. 3b). In the unplanted CWs the abundance of *amoA* genes of AOA remained constant during the whole experimental period, while in the planted CWs their abundance followed a pattern similar to that of AOB (Fig. 3b). Greater abundance of *amoA* genes of AOA was observed in the unplanted CWs early in the operation period (November 07 and November 27) but this effect changed in December 16 and January 13 with species planted with *T. latifolia* to show the greatest abundance (Fig. 3b).

Relatively high numbers of *nirK* genes were measured at the beginning of the operation period that further increased in the latter samplings (December 16 and January 13) reaching a peak in April 10 ($\approx 10^8$). Then, an abrupt decrease occurred at levels similar to those measured early in the operation period (Fig. 4a). The abundance of *nirS* genes was lower compared to that of the *nirK* genes (Fig. 4b). They followed quite a similar pattern to that of *nirK*, but with a milder decrease at the end of the experimental period. The abundance of clade I *nosZ* genes also followed a seasonal pattern that resembled those of *nirK* and *nirS* genes (Fig. 4c). With regard to the abundance of clade II *nosZ* genes, it was not affected either by the season or the treatments imposed and remained at very low numbers (Fig. 4d) compared to the other denitrifying genes. The ratio of *nirS*/*nirK* genes increased with time and was also significantly affected by the treatment with the unplanted CWs to show greater values compared to the planted CWs (Fig. 5a). The *nosZ* clade I/Σ*nir* ratio also increased with the progress of season but in that case the greatest values were observed in the planted CWs (Fig. 5b). Shifts were observed for *nosZ* clade I/*nirK* (Fig. 5c) ratios, which also followed an increasing trend. Regarding the abundance of 16S rRNA gene of the anammox bacteria, it remained at very low numbers ($\approx 2 \times 10^3$) throughout the study period (Fig. 6).

Significant correlations were established between some of the functional genes investigated in this work (Table 2). The abundance of *nirS* genes was strongly and positively correlated with the abundance of *nirK*, *amoA* genes of AOA and clade I *nosZ* genes and negatively with that of *nosZ* clade II. *nirK* genes were also positively correlated with *nosZ* clade I and *amoA* genes of AOA, while a negative correlation was obtained with *amoA* genes of AOB. Clade I *nosZ* genes showed a negative relationship with the clade II *nosZ* genes. Anammox 16S rRNA genes were only correlated with *amoA* genes of AOB.

Table 1
Mixed-effects analysis of variance of nitrogen functional genes in response to the treatment (plant species) and sampling time.

	amoA (AOA)	*amoA* (AOB)	*nirS*	*nirK*	*nosZ* clade I	*nosZ* clade II	Anammox (16S rRNA)	AOA/AOB	*nirS*/*nirK*	∑nos/∑nir
Treatment	***	ns	ns	ns	***	ns	ns	***	**	***
Sampl. time	ns	*	***	***	**	ns	ns	ns	***	ns
Treatment × Sampl. time	ns	ns	ns	*	ns	ns	ns	ns	*	**

ns: not significant; *p < 0.05; **p < 0.01; ***p < 0.001.

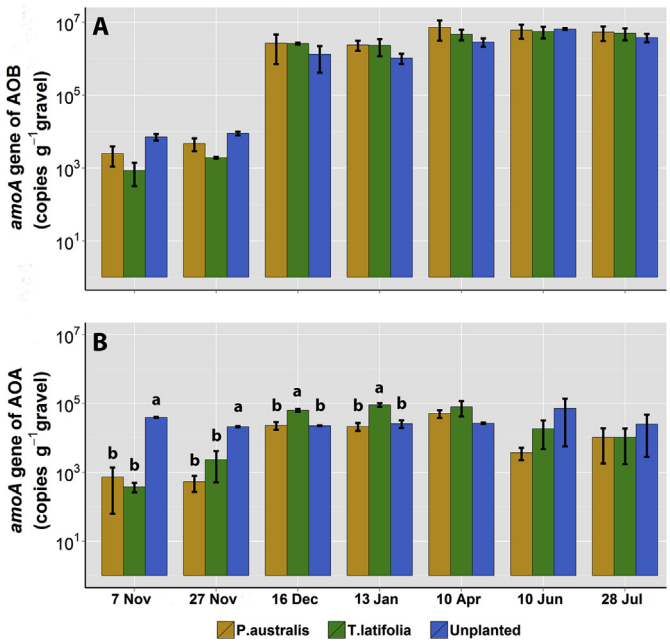

Fig. 3. Abundance of *amoA* gene copies in constructed wetlands unplanted or planted with *T. latifolia* and *P. australis*. (a) *amoA* gene copies of AOA; and (b) *amoA* gene copies of AOB.

4. Discussion

The prevailing physico-chemical conditions have a strong effect on the processes regulating the biogeochemical cycling of N (Thamdrup, 2012). In this work pH ranged at levels that do not impose limitations to the processes regulating the cycling of N, even though the differences that were observed between the vegetated and non-vegetated CWs. The slightly lower pH values in the planted CWs have probably been arisen from the higher oxidation rates of organic matter and NH_4^+ oxidation (Fig. 1), but also from the release of organic acids by the roots in the planted CWs (Bais et al., 2006). The values of Eh were indicative of anoxic/reducing conditions in accordance to studies with similarly short HRTs (Pedescoll et al., 2013; Corbella and Puigagut, 2015). The lack of differences between the treatments and strong seasonal variations contrasts previous findings (Stein et al., 2007), but it can be explained by the O_2 demands that exceeded the potential of plant species to release O_2 in the rootzone eliminating thus the differences between CWs. Similarly, Rodriguez and Brisson (2016) did not report differences in the 1st of a series of CWs planted with *P australis* and *Phalaris arundinacea* although in the 2nd series such differences became clear.

The mineralization of organic-N occurred at high rates as indicated by the low concentrations of organic-N and the non-detectable levels of urea in the effluent of all the treatments. This observation suggests that organic-N mineralization does not constitute a bottleneck on the performance of CWs, at least at the loadings applied in this work. By contrast, NH_4^+ concentration differed between the treatments suggesting that ammonia oxidation represented the limiting step in the potential of CWs for N removal. The response of *amoA* genes of AOB to the transition from the oligotrophic to nutrient rich conditions (Fig. 3) supports the argument that nitrification represents an important pathway of N cycling in CWs. Despite the variable performance of CWs in terms of NH_4^+ removal, the abundance of *amoA* genes of AOB did not differ between the unplanted and the planted CWs implying that

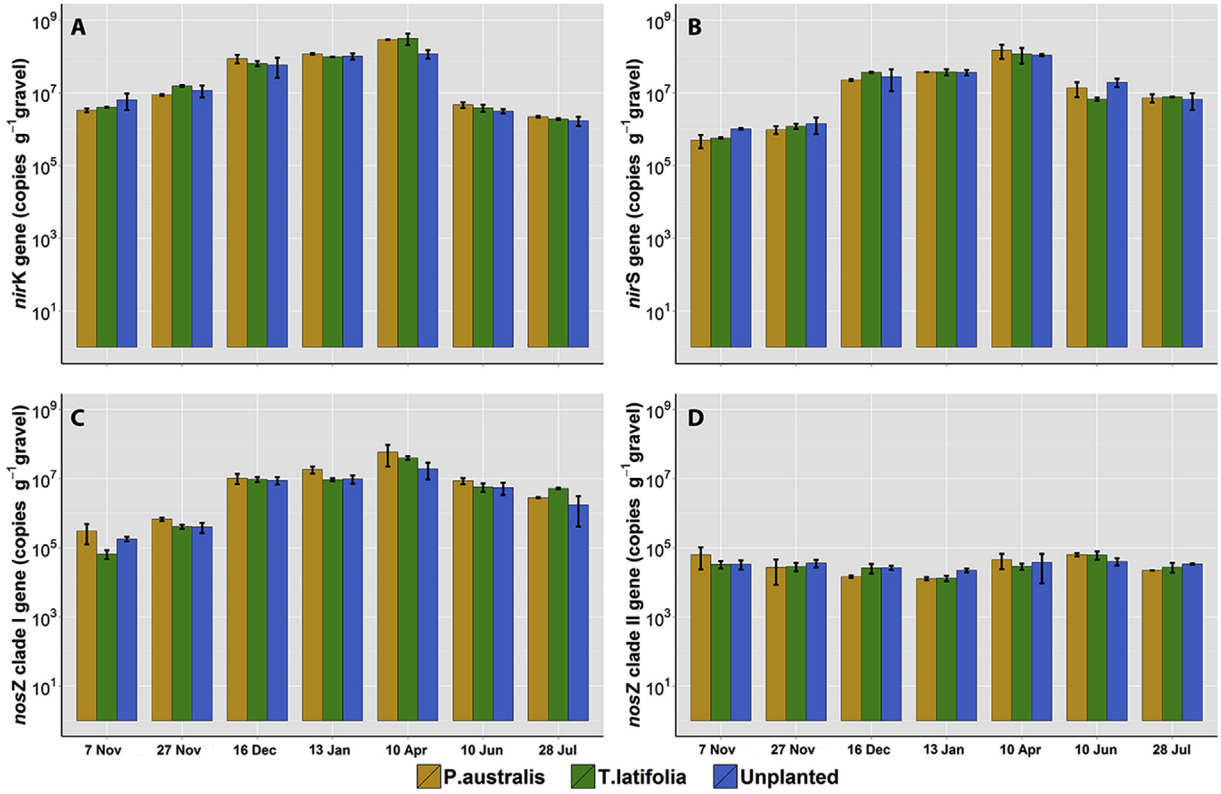

Fig. 4. Abundance of denitrifying gene copies in constructed wetlands unplanted or planted with *T. latifolia* and *P. australis*. (a) *nirK* gene copies; (b) *nirS* gene copies; (c) clade I *nosZ* gene copies; and (d) clade II *nosZ* gene copies.

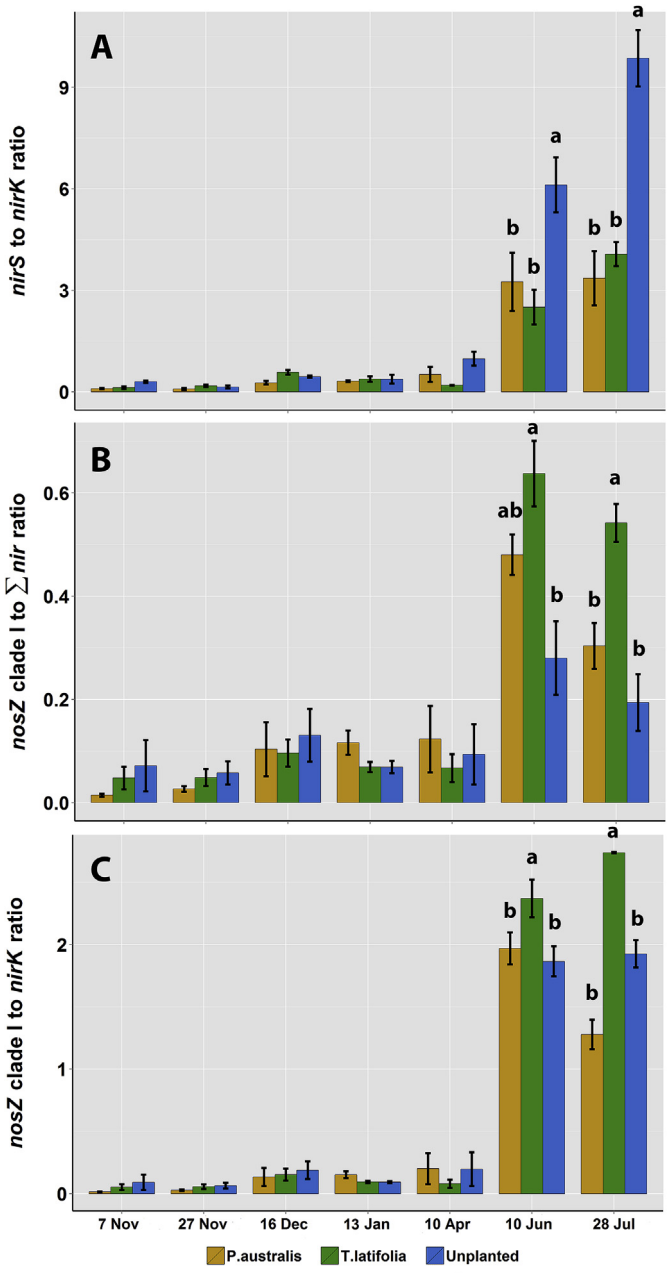

Fig. 5. Evolution of the ratio of denitrifying genes in constructed wetlands unplanted or planted with *P. australis* and *T. latifolia*. (a) ratio of *nirS*/*nirK* genes, (b) ratio of *nosZ* clade I/Σnir genes, and (c) ratio of *nosZ* clade I/*nirK* genes.

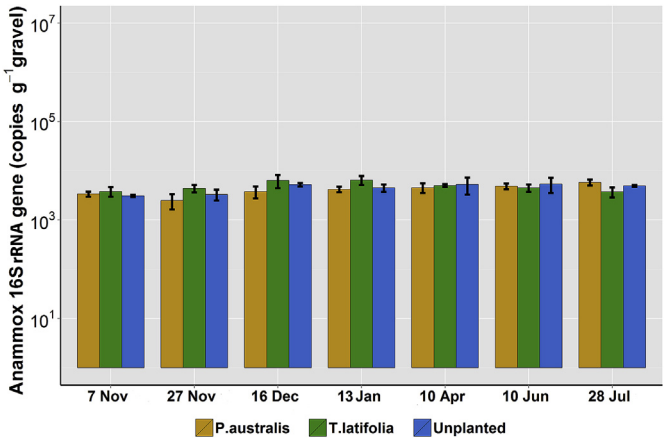

Fig. 6. Abundance of 16S rRNA gene copies of anammox bacteria in constructed wetlands unplanted or planted with *T. latifolia* and *P. australis*.

nitrification was rather constrained by the activity of ammonia oxidizers. This constrain might have arisen from O_2 availability. Vegetation has a prominent role in supplying SSF-CWs with O_2 (Crites et al., 2014) and the release rates are strongly mediated by the plant species (Mei et al., 2014). The denser canopy of CWs planted with *T. latifolia* compared to *P. australis* (Table S2), which probably implies a greater rooting density as well, supported greater rates of O_2 release stimulating NH_4^+ oxidation. In agreement, Maltais-Landry et al. (2009) found that applying artificial aeration in unplanted and planted CWs stimulated the oxidation of NH_4^+ particularly in the unplanted ones.

The *amoA* genes of AOA showed also a similar response to effluent application in the planted CWs. This pattern is somewhat contrasting taking into account the oligotrophic lifestyle of AOA and the commonly reported decrease in the ratio of AOA/AOB with the increasing NH_4^+ concentration (Prosser and Nicol, 2012; Zhang et al., 2015). This pattern might have arisen from the heterogeneity in NH_4^+ concentration at microscales and/or the adaptation of AOA to micro-oxygenic conditions (Berg et al., 2015; Sollai et al., 2015) particularly close to the roots. This finding provides evidence that AOA have also an important role in the regulation of NH_4^+/NH_3 oxidation in CWs. That influence appears to be dependent on NH_4^+ concentration and the plant species. The increase of NH_4^+ concentration in the planted CWs during the summertime (May 05-June 26) was not accompanied by changes in the abundance of *amoA* genes of AOA and AOB and probably did not imply any changes in NH_3 oxidation potential. It was rather a seasonal effect arising from the greater ET rates in the planted CWs (Fig. S4), in accordance to earlier studies (Headley et al., 2012; Milani and Toscano, 2013; Pedescoll et al., 2013) and accounted for a great proportion (>90%) of this increase as it was indicated by N mass balance calculations (Fig. 2). Moreover, release of NH_4^+ due to the mineralization of the organic matter derived from plant litter might have also contributed.

Overall, the activity of denitrifying organisms is not constrained in CWs operating under anoxic conditions (Fig. S2) and excess of organic-C (Crites et al., 2014). Thus, depleted concentrations of NO_3- should be expected in the effluent of all CWs, independently of the plant species and its effects on NH_4^+ oxidation. Indeed, such a pattern of NO_3- was observed that was consistent with the increase in the abundance of denitrifying genes following effluent application. The favored conditions for denitrification in CWs prevented the occurrence of seasonal pattern similar to that observed for NH_4^+ concentration during the summer period. Since there were no differences in the abundance of denitrifying genes between the treatments (Fig. 4), the slightly higher concentration of NO_3- in CWs planted with *P. australis* early in the operation period (January 06 to March 26) could be attributed to a plant species effect on denitrifiers composition. Indeed, rhizosphere effects on the composition of denitrifying community and the denitrification potential of CWs have been reported (Ruiz-Rueda et al., 2009; Hallin et al., 2015). Thereafter, this effect declined and eventually disappeared suggesting that effluent composition eventually compensated for the influence of plant species on the composition of denitrifiers.

nirS genes showed a greater response to effluent application compared to *nirK* genes (Fig. 4) implying a more important role in the regulation of denitrification. A few studies have revealed distinct patterns of *nirS* and *nirK* denitrifiers between the

Table 2
Pearson's correlation coefficients (r) between nitrogen functional and 16S rRNA genes.

	AOA *amoA*	AOB *amoA*	*nirS*	*nirK*	*nosZ* clade I	*nosZ* clade II	Anammox 16S
AOA *amoA*	1						
AOB *amoA*	ns	1					
nirS	0.43 **	ns	1				
nirK	0.51 ***	−0.35 *	0.75 ***	1			
nosZ clade I	ns	0.34 *	0.78 ***	0.59 ***	1		
nosZ clade II	ns	ns	−0.32 *	ns	−0.35 *	1	
Anammox 16S	ns	0.54 ***	ns	ns	ns	ns	1

Significance: ns: not significant; *p < 0.05; **p < 0.01; ***p < 0.001.

environments, although, the evolutionary drivers have not yet elucidated (Yuan et al., 2012; Baker et al., 2015; Tsiknia et al., 2015). Both genes followed seasonal patterns with the *nirK* genes to show a steeper decline late in the operation period compared to *nirS* genes. The reasons that have led to this response remain obscure. They might be related to the higher temperatures that prevailed during that period (Fig. S1) which in turn may have induced shifts in the composition of denitrifiers and/or to the natural evolution of denitrifying communities in CWs with the progress of time. Independently of the driving factor(s), this shift is documented by the increase in the ratios of *nosZ* clade I/Σ*nir* genes and *nirS*/*nirK* genes with the progress of season and indicates the prevalence of denitrifying communities enriched in *nosZ* genes (Fig. 5). This shift is explained by the dominance of *nirS* denitrifiers (Fig. 5a) during the summer which more commonly encode for *nosZ* genes in the gene inventory of denitrification (Graf et al., 2014) and *nirK* strains enriched with the *nosZ* gene (Fig. 5c).

At the same time alternative routes, like the anammox pathway, did not seem to have a strong contribution on N cycling as it could be inferred by the low abundance of 16S rRNA genes which is probably and overestimated since 16S rRNA primers target also other taxa of Planctomycetes (Humbert et al., 2010; Coban et al., 2015). Abundances of hydrazine synthase (*hzsA*) gene that exceeded 10^5 copies/g did not result in detected activity of anammox process in a SSF-CW treating polluted groundwater (Coban et al., 2015) providing further support for the low contribution of anammox in N cycling at the abundances observed in the present study. These findings are in accordance to the suggestion of Saeed and Sun (2012) that the classical nitrification–denitrification route is the major pathway of N removal in the SSF-CWs.

The observed seasonal shift in the composition of denitrifiers with the progress of time could be linked with corresponding changes in their genetic potential to emit N_2O. Increases in the ratio of *nirS*/*nirK* and *nosZ*/Σ*nir* genes with the progress of time, as those observed in the present study, have been linked with lower rates of N_2O emission (Jones et al., 2014). The abundance of clade II *nosZ* genes has a strong influence on the regulation of N_2O emissions, at least in the terrestrial ecosystems (Jones et al., 2013). In this study, however, their abundance was not affected by the treatment, despite the differences observed between them in terms of N removal and hence on the rates of the released N_2O. This pattern suggests that *nosZ* clade II denitrifiers do not have an important role in the reduction of N_2O emissions in CWs. In accordance, clade II *nosZ* genes were occasionally detected in the rhizosphere of aquatic plants in CWs operating with mine drainage (Hallin et al., 2015). The indicators of the genetic potential of CWs to emit N_2O were strongly differentiated among the treatments (Fig. 5) implying differences in the importance of the operating mechanisms. Although the rating of these mechanisms is not possible from a theoretical point of view, earlier work including unplanted CWs and planted with *P. australis* and *Typha angustifolia* reported lower rates of N_2O emissions from the latter (Maltais-Landry et al., 2009) providing support to the argument that plant species affect the mechanisms regulating N_2O emissions and/or their rates. These findings outline the possibility to control N_2O emissions by selecting suitable plant species and operation conditions and possibly point out and to other ecosystems.

The strong relationships (Table 2) between the functional genes of N might indicate preferential cooperation or a proxy of the contribution of the functional genes on the corresponding process (AOB and AOA; *nirK* and *nirS*). The relationships between ammonia oxidizing (*amoA* of AOA and AOB) and denitrifying genes have been probably arisen from their interdependence on the substrates (NH_4^+, NO_3-) rather from artifacts resulting from *nirK* genes, which commonly co-occur in these microbial groups (Long et al., 2015; Tsiknia et al., 2015). Studies in other ecosystems have shown that nitrifying and denitrifying genes occupy overlapping niches or that their relationships indicate physiological coupling that follows seasonal patterns (Abell et al., 2010; Smith et al., 2015). In the present study, however, the relationships between the N functional genes did not differ between seasons probably due to the mild seasonal shifts that observed in the abundance of N functional genes during the early (November 07-November 27) and the late (June 10-July 28) samplings.

With regard to the contribution of plant uptake, our findings confirmed those of previous studies (Vymazal, 2007; Crites et al., 2014; Zheng et al., 2015) that uptake and accumulation of N in hypergeous biomass does not constitute an important mechanism of removal (Table S2).

5. Conclusions

Our findings indicate nitrification-denitrification as the principal route of N removal in SSF-CWs. Ammonia oxidation was identified as the process that constrained the potential of CWs to remove N. The presence of vegetation, and the plant species by themselves, determined the rate that the whole route proceeded, probably by affecting O_2 release to the rhizosphere. The data provided evidence for an active involvement of AOA in the oxidation of NH_3 to NO_2^- and that effect was dependent on the plant species. The composition of denitrifying communities shifted towards to a community with a lower genetic potential for N_2O emission, but this finding has to be further elucidated. Moreover, the indicators of the genetic potential of CWs to emit N_2O differed among the treatments implying differences in the type and the importance of the underlying mechanisms, but also the possibility to control N_2O emissions through the selection of appropriate plant species. In addition, qPCR data on the abundance of *nosZ* clade II denitrifiers does not support a strong contribution on the assimilation of N_2O. Finally, the anammox process does not have a strong contribution on N cycling despite the anaerobic conditions that prevailed in CWs throughout the study. The high ET rates that prevailed during the summertime in the planted CWs masked the positive effects of vegetation on N removal when effluent concentrations were only

considered. Mass balance analysis, however, revealed their potential for N removal was not constrained.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2016.06.048.

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