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BACTERIAL ESTABLISHMENT DURING THE FIRST YEAR OF OPERATION OF AN UNSATURATED AND A PARTIALLY SATURATED VERTICAL FLOW CONSTRUCTED WETLANDS

Catiane Pelissari [1]
Cristina Ávila Martin [2]
Alcione A. Alves [3]
* Pablo H. Sezerino [1]

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Abstract

Based on seven sampling campaigns during the first year of operation of an unsaturated (UVF) and a partially saturated vertical (SVF) subsurface flow constructed wetland the dynamics of nitrogen transforming bacteria were unravelled. Ammonia oxidizing populations (*Nitrosomonas* and *Nitrosospira*) showed large variations throughout the study, especially in the top layer of both wetlands, whereas in the bottom layers lower abundance and no temporal variation was found. Nitrite oxidizing bacteria (*Nitrobacter* and *Nitrospira*) displayed little fluctuations and were identified in higher abundance in the bottom of the UVF wetland, and the top of the SVF wetland. Denitrifying bacteria exhibited no significant changes over time in both wetlands. In addition, the saturation of the bottom part of the SVF caused denitrification to occur over time in greater magnitude in the SVF wetland than in the UVF wetland, coinciding with the greater abundance of denitrifiers in this wetland configuration.

Keywords: constructed wetlands, bacterial temporal dynamics, start-up period, nitrifying-denitrifying biofilm development.

[1] GESAD-Decentralized Sanitation Research Group, Department of Sanitary and Environmental Engineering, Federal University of Santa Catarina, Trindade, Florianópolis, Santa Catarina, 88040-900, Brazil.
[2] AIMEN Technology Center, Porriño, Pontevedra, Spain.
[3] Federal University of Fronteira Sul, Cerro Largo, Rio Grande do Sul, 97900-000, Brazil.
**Corresponding author*: GESAD-Decentralized Sanitation Research Group, Department of Sanitary and Environmental Engineering, Federal University of Santa Catarina, Trindade, Florianópolis, Santa Catarina, 88040-900, Brazil. Email: pablo.sezerino@ufsc.br

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Introduction

Constructed wetlands (CW) are systems widely used in the world for treatment of various wastewater types, under the most varied technological arrangements and configurations. Vertical subsurface flow (VF) is a CW modality that is employed primarily for carbonaceous organic matter removal and nitrification. The greater performance of this wetland type over the horizontal subsurface (HF) and free water surface flow wetlands occurs due to its unsaturated characteristics, provided by the intermittent feeding and drainage through the bottom, which originates predominantly aerobic environments along the filter media depth (Kadlec and Wallace, 2009). In this way, VF wetlands can be employed either as sole treatment technologies or combined with others CW modalities, especially when total nitrogen removal is aimed, constituting hybrid systems (Álvarez *et al.,* 2017; Torrijos *et al.,* 2016).

Over time, many improvements have been conducted especially to maximize the nitrogen removal in VF wetlands, including the use of hybrid systems (Vymazal, 2013), systems with fill and drain cycles known as tidal flow (Austin *et al.,* 2006; Hu *et al.,* 2014), the recirculation of final effluent (Al-Zreiqat *et al.,* 2018; Foladori *et al.,* 2013), the use of aeration in the VF wetland with waste gas purification from biological treatment (Zhang *et al.,* 2018), as well as the intermittent aeration (Jia *et al.,* 2018), the application of a filter media that promotes greater microbial activity such as biochar (de Rozari *et al.*, 2018), or even the use of filter media that have nutrient adsorption capacity (Saeed and Sun, 2011; Yakar *et al.,* 2018). Recently it was evidenced that operating a classical VF wetland with a bottom layer saturation, known as partial saturated vertical flow (SVF) wetland, increased total nitrogen (TN) removal (Carvalho Jr. *et al.,* 2018; Dong and Sun, 2007; Martínez, *et al.,* 2018; Pelissari *et al.,* 2017a; 2018), being nitrification-denitrification (NDN) process the main nitrogen removal pathway in this unit. Pelissari *et al.* (2018) showed that a SVF wetland removed twice as total nitrogen (TN) load (56 %) compared to an unsaturated VF wetland (36 %). Moreover, low oxidized nitrogen in the SVF wetland effluent was identified (1 mg L^{-1} vs. 26 mg L^{-1} in SVF and VF wetland effluents, respectively). Dong and Sun (2007) showed a TN removal efficiency of 25 % for a VF wetland and 37 % for a SVF wetland.

Despite CW have been long considered a black box, it is already clear that nitrogen transformations in this system are linked to the microbial community developed in the filter media (Button *et al.,* 2015; Langergraber, 2007; Meng *et al.,* 2014). In this context, recent studies conducted in VF and SVF wetlands have shown new perspectives related to the microbial dynamics linked to nitrogen transformation pathways. Firstly, it was discovered that the first step of nitrification in VF and SVF wetland is performed by bacteria and archaea, being ammonia oxidizing archaea (AOA) more metabolically active than ammonia oxidizing that bacteria (AOB) (Pelissari *et. al.,* 2017b; 2018). Hereafter, in SVF and VF wetlands 'nitrification aggregates' were identified in the biofilm of the filter beds, where AOA and nitrite oxidizing bacteria (NOB) showed an association in the nitrification process. Moreover, active denitrifying populations increased in the bottom layer of a SVF wetland (Pelissari *et al.,* 2018). Recently, Lai *et al.* (2020) showed that the

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increase of the COD/N ratio promoted the absolute abundances of the denitrifying functional genes in a VF wetland. In another study, heterotrophic nitrification and aerobic denitrification bacteria were identified (Tan *et al.*, 2020).

In this way, microbial populations can serve as the most sensitive and rapid bioindicator in response to various pollutants, including nitrogen transformations, due to their rapid growth rates and quick response to changes (Urakawa and Bernhard, 2017). However, many studies reporting microbial dynamics are based on punctual sampling campaigns. This sampling strategy reports the situation of microbial structure at the time the sample was collected and not a real behavior based on temporal and operational variations of CW system. The current study evaluated the interactions of nitrifying and denitrifying populations in a typical unsaturated vertical (UVF) wetland and a SVF wetland operated under the same conditions treating urban wastewater. A periodical assessment of the nitrogen transforming bacterial communities was carried out over a year of operation by microbiological analyses.

Materials and methods

Description of the wastewater treatment plant

The wastewater treatment system was implemented in south Brazil (27º 35' 48'' latitude 48° 32' 57'' longitude), under subtropical climate. This was comprised by a septic tank (3 m^3) as primary treatment, followed by an UVF and a SVF wetland operated in parallel (Fig. 1). The wetlands were commissioned in June 2015 and were designed to treat urban wastewater for a demand of a residential family composed by 5 people equivalent.

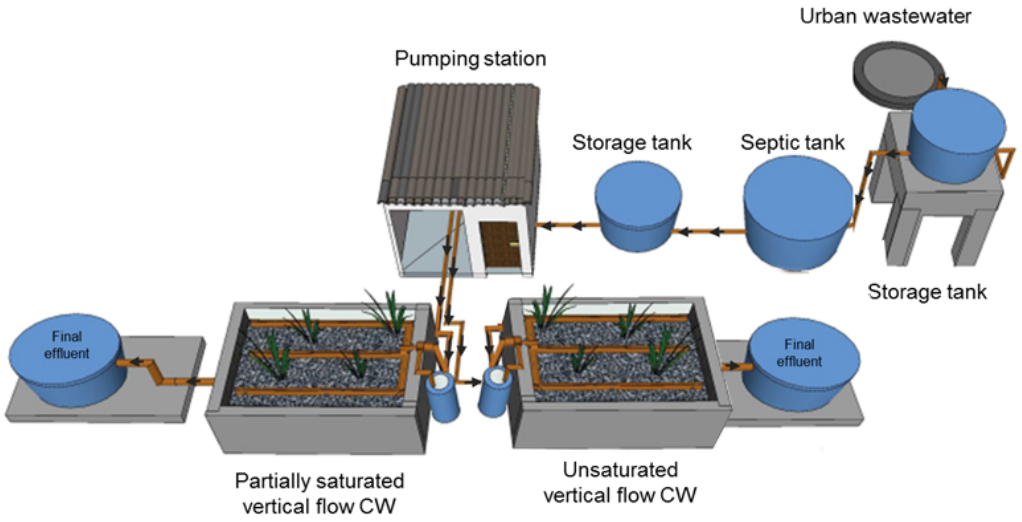

Figure 1. Diagram of the wastewater treatment plant indicating the treatment line.

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Each wetland had a surface area of 7.5 m² (2.3 m × 3.3 m) and was planted with *Typha domingensis*. The UVF wetland was operated typically unsaturated (0.70 m free drainage), with sand (d_{10}= 0.21 mm and Cu= 5.1) as filter media. The SVF wetland had the bottom part (0.40 m) saturated (57 % of total depth) by setting the outlet pipe at that height and the bed media was sand (d_{10}= 0.29 mm and Cu= 4.05). Each wetland received an average flow of 470 L d^{-1}, resulting in organic (OLR) and hydraulic loading rates (HLR) of 35 g COD m^{-2} d^{-1} and 63 mm d^{-1}, respectively. Both wetlands operated with cycles of feed and rest of 3.5 days. Feeding was done by intermittent pumping, totaling around 3 (Mo and Thu) and 4 (Tu and Wed) pulses d^{-1} (157 and 117 L per $pulse^{-1}$, respectively).

Influent and effluent samples from each CW were grabbed fortnightly during the study period (June 2015 to July 2016). These samples were collected after of a drainage period of approximately 15 min, referent the first feeding pulse of the day (around 9 am). Samples were taken to the adjacent laboratory for analysis of the following water quality parameters: pH, alkalinity, total suspended solids (TSS), chemical oxygen demand (COD), biochemical oxygen demand (BOD_5), total nitrogen (TN), ammonium nitrogen (NH_4-N) and oxidized nitrogen species (NO_x-N). The determination of these parameters was done by following standard methods (APHA, 2005). NH_4-N analysis was conducted following Vogel (1988).

Sampling and assessment of bacterial dynamics

Quantitative essay of functional genes from total eubacteria (16S rRNA), nitrifying (*amoA*) and denitrifying bacteria (*norB* and *nosZ*), as well as diversity of bacterial community were determined through quantitative polymerase chain reaction (qPCR) and next generation sequencing (NGS) analyses, respectively.

During the first year of CW operation (July 2015 to July 2016), filter media samples from top (0 - 15 cm depth) and bottom (60 - 70 cm depth) layers of UVF and SVF wetlands were collected at two points in each layer. Samples were collected every two months totalizing seven sampling campaigns throughout the study (first, third, fifth, seventh, ninth, eleventh and twelfth month of operation). All samples were collected with the aid of a properly sterilized soil sampler. Moreover, the filter media collection and storange of the samples followed recommendations of Pelissari *et al.* (2017; 2018). qPCR analysis was performed at all sampling campaigns. Meanwhile, NGS assays were conducted in samples collected at the end of each season of the year (winter and spring 2015, and summer, fall and winter 2016).

DNA extraction from of approximately 0.25 g of filter media was carried out in triplicate at each sampling campaign using protocol of Power Microbiome™ DNA Isolation kit (MOBIO Laboratories, Inc., Carls- bad, CA). DNA extracts were kept frozen at −80 °C until further analysis. qPCR analysis of eubacteria was conducted on the V3 hypervariable region of 16S rRNA following Prenafeta-Boldú *et al.* (2012). Nitrifying population was quantified by ammonia monooxygenase α-subunit encoding gene (*amoA*) as

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recommended by Rotthauwe *et al.* (1997). The denitrifying population was accessed by means of two genes expressed during the denitrification process. *norB* catalytic subunit of nitric oxide reductase and typical denitryifer *nosZ* (clade I) that catalytic subunit of nitrous oxide reductase, were quantified (Braker and Tiedje, 2003; Calder *et al.,* 2014). All samples were analyzed in triplicate by means of three independent samples. The standard curve of each target gene was designed by using FunGene data base (http://fungene.cme.msu.edu/) five gBlocks® Gene Fragments (IDT, Integrated DNA Technologies). Serial dilutions (10^{10} to 10^2 gene copies μl^{-1}) from synthetic genes were subjected to qPCR assays in duplicate. qPCR reactions fitted quality standards efficiencies between 95 and 110 % and R^2 above 0.995. qPCR reaction was analyzed using the 7500 real-time PCR system (Applied Biosystems, The Netherlands).

A 16S rRNA assessment was performed through MiSeq platform to study the diversity of bacterial populations. 16S rRNA libraries targeting V3–V4 regions from eubacterial populations were sequenced by utilizing MiSeq Illumina sequencing platformat at Neoprospecta microbiome technologies following manufacturer's instructions. Primer set U341F (5'-CCTACGGGRSGCAGCAG–3') and 806R (5'-GGACTACHVGGGTWTCTAAT–3') were used. Downstream MiSeq data analysis was carried out by using QIIME software version 1.8.0. The obtained DNA reads were compiled in FASTq files for bioinformatics processing. Quality filtering of the reads was performed at Q25, prior to the grouping into operational taxonomic units (OTUs) at 97 % sequence homology cutoff. The following steps were performed using QIIME: Denoising using Denoiser (Reeder and Knight, 2010), reference sequences for each OTU were obtained via the first method of UCLUST algorithm (Edgar, 2010), for sequence alignment and chimera detection the algorithms PyNAST and ChimeraSlayer (Caporaso *et al.,* 2010; Haas *et al.,* 2011) were used. OTUs were then taxonomically classified using BLASTn against GreenGenes and RDP (Bayesian Classifier) database and compiled into each taxonomic level (DeSantis *et al.,* 2006).

Statistical data analyses

The Shapiro-Wilk test was performed on conventional wastewater quality parameters to determine whether data were normally distributed. Given that data followed a normal distribution, t-tests were conducted to compare pH, alkalinity, TSS, COD, BOD_5, TN, NH_4-N and NO_x-N values between effluents of the two wetlands. The significance threshold was established at 0.05. Moreover, Pearson's correlational analysis was used to identify significant linear relationships between NH_4-N removal efficiency and NO_x-N production, as well as TN removal efficiency and BOD_5/COD ratio.

In relation to the abundance of genes copies identified by qPCR, the Shapiro-Wilk test was executed to identify the normalization of the data. According to a normal distribution, a t-test n analysis was performed including different combinations. Firstly, average values of the seven sampling campaigns conducted at each layer of two CW was utilized to verify whether there was a statistical difference between (i) different layers of the same wetland; (ii) the same layer of the different wetlands. Subsequently, to

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assess possible temporal-based significance, the number of gene copies (16S rRNA, *amoA*, *norB*, *nosZ*) quantified in each sampling campaigns (top and bottom layer; UVF and SVF wetland) were evaluated. The significance threshold of the t-test was established at 0.05. Moreover, relationships among the N functional genes and the ambient temperature significance were tested with the Pearson correlation coefficient. The variables were tested for normality before further analysis.

For evaluation of the diversity of the bacterial community, number of OTUs, Shannon index (H), Goods coverage and Chao1 richness estimator were calculated by using the Monthur software v.1.134.4. All the estimators were normalized to 50,000 reads within the range of the lowest number of reds among the different samples. All statistical analyses were performed by means of Statistic 12.0 software (California, USA).

Results and discussion

General treatment performance of the vertical flow constructed wetlands

In general, the SVF wetland showed a higher treatment performance than the UVF wetland (Table 1). TSS removal efficiency was similar in both wetlands (95 % for SVF and 92 % for the UVF wetland), showing no statistical significance. The COD removal efficiency was however significantly higher in the SVF wetland (90 %) than in the UVF wetland (85 %). This difference could be associated with the higher retention time of the SVF wetland effluent providing longer time for COD removal. In influent wastewater, most of the nitrogen was in the form of NH_4-N (Fig. 2a). The remotion of NH_4-N was similar in the SVF (62 %) and UVF wetland (58 %), showing no statistical difference. The main mechanism associated with the NH_4-N elimination was nitrification, due to NO_x-N production identified in effluent from both units (Fig. 2b). These results showed that the saturated layer of SVF wetland did not limit the nitrification process. This same behavior was identified in another SVF wetland (Pelissari *et al.,* 2018). Meanwhile, TN removal was significantly higher in the SVF (45 %) than the UVF wetland (34 %). This behavior showed a greater denitrification in the SVF wetland due to the presence of reducing environments provided by the saturated zone (Fig. 2a,b).

Table 1. Average (standard deviation) and mean removal efficiency of the unsaturated (UVF) and partially saturated vertical (SVF) flow constructed wetlands of the first year of operation.

Parameters n=29	Influent wastewater	UVF wetland effluent	SVF wetland effluent	Removal efficiency (%)	
				UVF wetland	SVF wetland
[a]pH	7.2 (0.2)	6.6 (0.3)	6.8 (0.4)	-	-
[a]Alkalinity (mg L^{-1})	282 (36)	82 (55)	118 (79)	-	-
TSS (mg L^{-1})	43 (18)	4 (5)	3 (6)	92 (11)	95 (10)
[a]BOD_5 (mg L^{-1})	280 (48)	40 (14)	28 (12)	86 (4)	90 (4)
[a]COD (mg L^{-1})	557 (149)	82 (55)	57 (54)	85 (8)	90 (7)
[a]TN (mg L^{-1})	75 (15)	52 (16)	41 (12)	34 (12)	45 (13)
NH_4-N (mg L^{-1})	72 (15)	29 (10)	22 (15)	58 (15)	62 (20)
[a]NO_x-N (mg L^{-1})	1 (3)	22 (11)	16 (10)	-	-

[a] Significant difference (t-test) between effluent concentrations of the UVF and SVF wetlands ($p < 0.05$).

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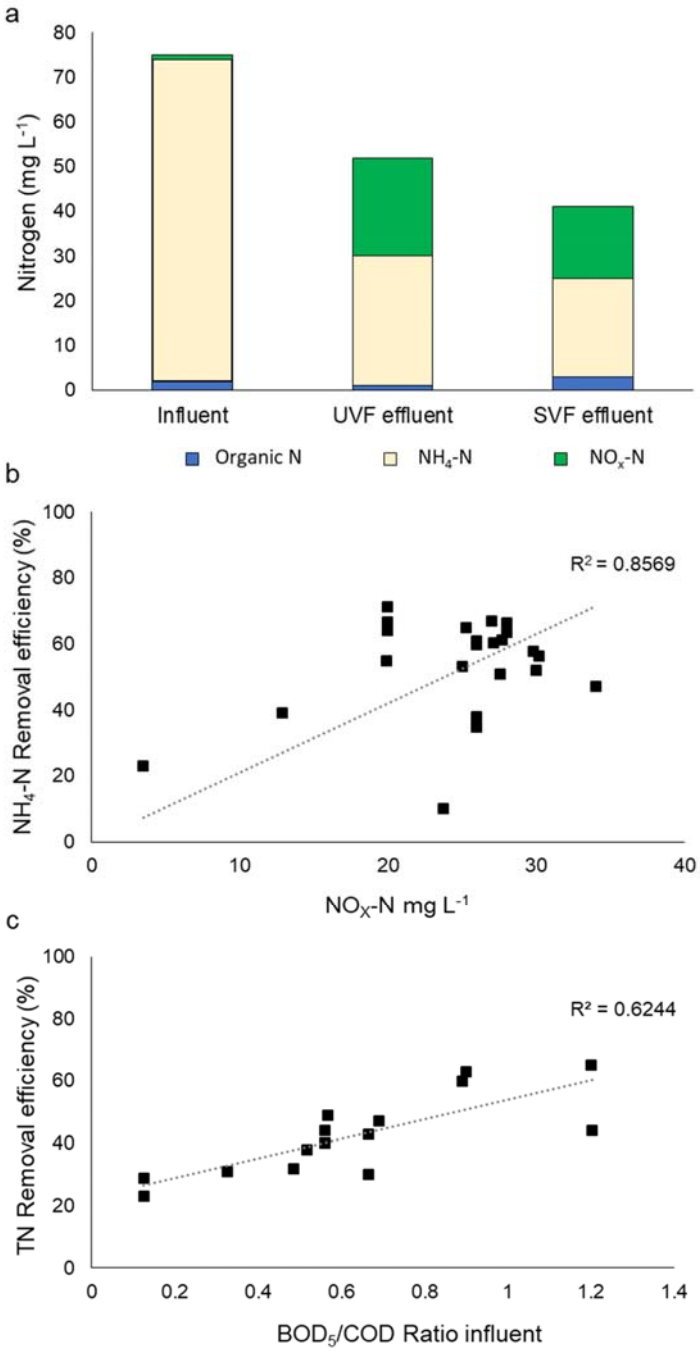

Figure 2. Nitrogen transformations in the unsaturated (UVF) and partially saturated vertical (SVF) subsurface flow wetlands. a) Nitrogen species concentrations in influent wastewater, and effluent of the UVF and SVF wetlands; b) Correlation plot of NH_4-N removal efficiency vs. effluent NO_x-N in the UVF wetland; c) Correlation plot of TN removal efficiency vs. influent BOD_5/COD ratio in the SVF wetland.

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The increase in the denitrification process due to the presence of the saturated layer in the SVF wetland has already been reported by previous studies (Carvalho Jr. *et al.,* 2018; Martínez *et al.,* 2018; Pelissari *et al.,* 2018). Nevertheless, NO_x-N concentrations at the effluent of the SVF were still high (16 mg L^{-1}), which indicates incomplete denitrification despite the saturation of 57 % of its total media depth. This could be explained by the lack of sufficient biodegradable matter for denitrifying bacteria to carry out the denitrification process during the early stage of the system's operation. Pelissari *et al.* (2018) showed complete denitrification process in a SVF wetland (<1 mg L^{-1} of NO_x-N at the effluent), which had been in continuous operation for more than 5 years. Low BOD_5/COD ratios in influent wastewater (about 0.5) might have also contributed to the low denitrification rates, as depicted in Fig. 2c, and being in accordance with Sun and Saeed (2009).

According to Ye and Li (2009) it is necessary 2.86 g of BOD_5 to transform 1 g of NO_3-N to N_2. In this way, low organic carbon availability may have impaired the denitrification process, which might improve as the system's ages and organic matter accumulates in the filter media. This same tendency was reported in a previous study conducted in a SVF wetland where TN removal was very variable (29 to 42 %) due to the limiting conditions for denitrification by the lack of carbon in the medium (Saeed and Sun, 2017). Moreover, Martínez *et al.* (2018) showed greater TN removal efficiency in a SVF wetland (73 %) when operating with internal solid source of organic carbon (corncob), compared to the SVF wetland without any external carbon source (60 %).

Spatial and temporal bacterial dynamics within the depth profile of the vertical flow constructed wetlands

Global nitrifying and denitrifying functional genes quantification

Eubacterial population showed a different behavior between the layers and CW units (Fig. 3a). In the top layer, bacterial abundance was one order of magnitude higher at the SVF wetland (10^{11} 16S rRNA copies gene g^{-1}) than at the UVF wetland (10^{10} 16S rRNA copies gene g^{-1}). On the other hand, in the bottom layer the bacterial profile was inverted (Fig. 3a), being three orders of magnitude higher at the UVF wetland (10^9 vs. 10^6 16S rRNA copies gene g^{-1} in the UVF and SVF, respectively). This behavior can be associated with the operational configuration of each wetland type. In the first place, the greater bacterial abundance at the bottom in relation to the top of the UVF wetland may be associated with the oxygen availability throughout the depth of this wetland bed when operating under relatively low OLR (35 g COD m^{-2} d^{-1}). This is in accordance with Pelissari *et al.* (2017), which reported higher bacterial abundance at the bottom when operating an UVF under a lower OLR (80 vs. 130 g COD m^{-2} d^{-1}). Meanwhile, the lower bacterial abundance identified in the bottom layer of the SVF wetland in relation to the top could be related to the low oxygen concentrations at the saturated layer. Pelissari *et al.* (2018) showed that the implementation of the partial saturation of a UVF wetland resulted in a decrease in the eubacterial abundance. In this way, the oxygen availability present in the filter media seems to exert a direct influence on the bacterial community stratification in VF wetlands.

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Regarding AOB, *amoA* showed a higher abundance at the top layer of both CW (Fig. 3b), being one order of magnitude greater at the SVF than at the UVF unit (10^6 vs. 10^5 copies g^{-1}, respectively). On the other hand, the abundance of AOB was significantly lower at the bottom layer of both wetlands, particularly at the SVF unit (10^4 vs. 10^2 *amoA* copies g^{-1} in UVF and SVF, respectively) (Fig. 3b). The higher AOB abundance at the top layer of the SVF in relation to the UVF wetland could be attributed to a displacement of these populations to the upper layer due to the anoxic/anaerobic conditions of the saturated zone. Conversely, in the UVF wetland, where oxic conditions predominated within the filter media, AOB populations showed a fairly uniform distribution throughout the vertical profile. This is also in agreement with Pelissari *et al.* (2018), which identified higher AOB abundance at the top of the SVF in respect to the UVF wetland, when operating under similar OLR (40 g COD m^{-2} d^{-1}). Obtained results indicate that the saturated conditions of the SVF wetland influenced the AOB community structure, suggesting that nitrification occurred in a larger magnitude in the first 15 cm of this wetland type, whereas within the UVF wetland the nitrification process took place even at deeper layers of the unit.

Furthermore, denitrifying populations (*norB* and *nosZ*) were identified throughout the vertical profile of both wetlands (Fig. 3c and d). In the UVF wetland, *norB* gene, which is associated with N_2O production, was one order of magnitude higher (10^2 g^{-1} copies in both layers) than the *nosZ* gene (10^1 g^{-1} copies in both layers), which is linked to N_2 accumulation. These results point out the predominance of incomplete denitrification (Lu *et al.,* 2014). This fact in the UVF wetland can be related to the high oxygen content of its filter media, as oxygen availability inhibits this process by providing a better electron acceptor for denitrifying populations to generate energy (Lu *et al.,* 2014).

In addition, nitrous oxide reductase (expressed by the *nosZ* gene) is the most sensitive enzyme to oxygen compared with the other upstream reductases, resulting in transient N_2O accumulation under aerobic conditions (Lu and Chandran, 2010). However, it is important to take into account that incomplete denitrification can also be the result of nitrifiers-denitrification. AOB populations are able to conduct nitrifier-denitrification by means of nitrite reductases (*nirS* genes) and nitric oxide reductases (*norB* genes), favoring the transformation of NO_2-N to N_2O under low oxygen environments (Kampschreur *et al.,* 2009; Kozlowski *et al.,* 2016). Oppositely, in the SVF wetland, the *norB* gene (10^1 and 10^2 g^{-1} copies in top and bottom layers, respectively) was two orders of magnitude lower than the *nosZ* gene (10^3 and 10^4 g^{-1} copies in top and bottom layers, respectively) (Fig. 3c and d), being both genes one order of magnitude higher at the bottom in respect to the top layer. The higher abundance of *norB* gene in the bottom of this wetland can be associated with the nitrifier-denitrification metabolism, both given the presence of AOB populations (Fig 3b) together with the limited availability of oxygen. Meanwhile, the high occurrence of the *nosZ* gene reaffirms the trend of complete denitrification until N_2, especially in the saturated layer, where anaerobic/anoxic conditions are predominant.

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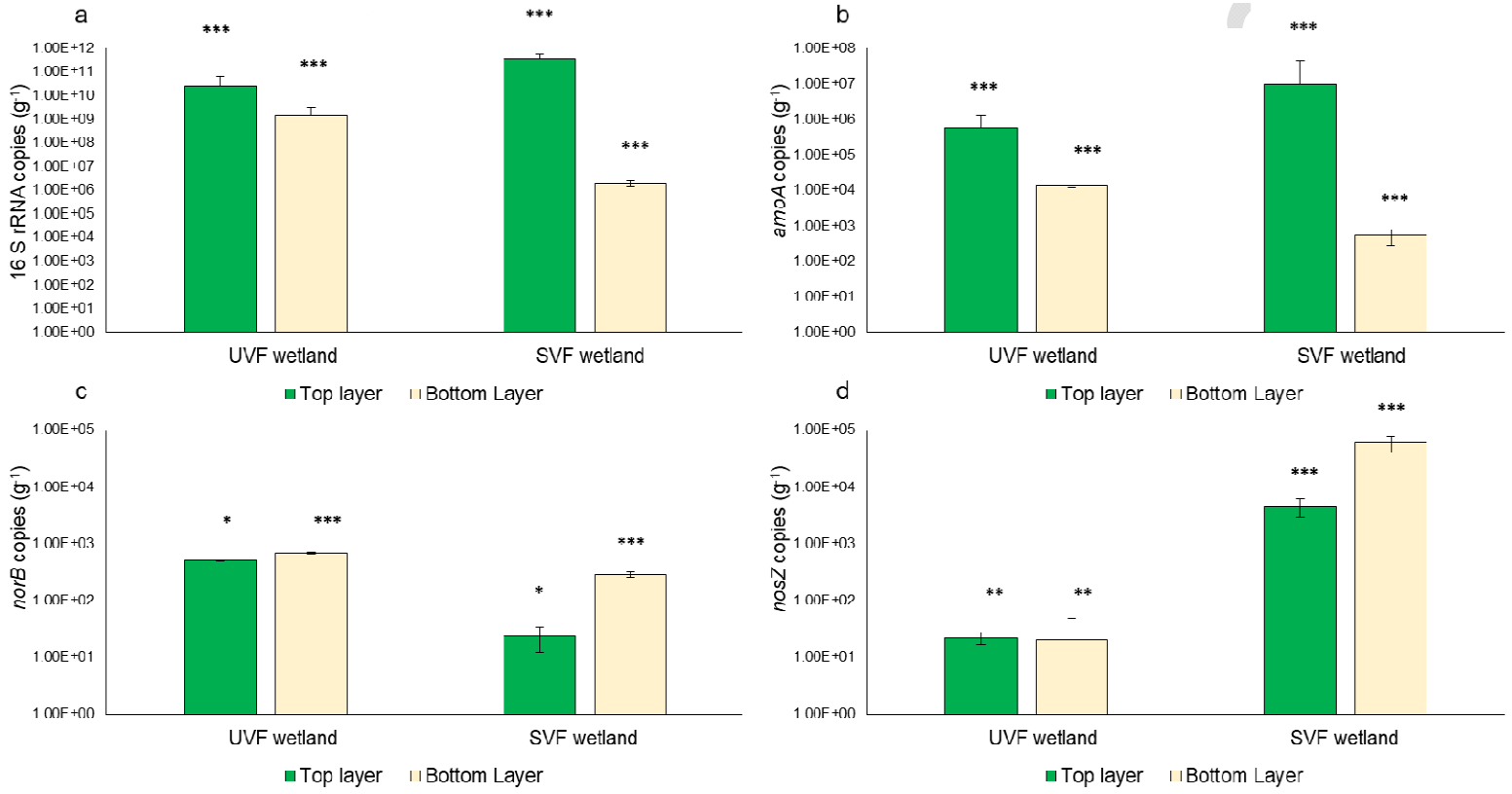

Figure 3. Nitrogen functional genes average from seven sampling campaigns, identified in the biofilm from top (0-15 cm) and bottom (60-70 cm) layers of the unsaturated vertical (UVF) and partially saturated vertical (SVF) flow constructed wetland, during the first year of operation. a) Average of 16S rRNA copies abundance; b) Average of *amoA* copies abundance; c) Average of *norB* copies abundance; c) Average of *nosZ* (clade I) copies abundance. *Presented values are the mean and SD of independent triplicates from seven sampling campaigns. * statistically differences between top and bottom layer of the same wetland (p <0.05); ** statistically differences between the same layer in different wetlands (p <0.05); *** statistically differences between the top and bottom layer of the same wetland and the same layer in different wetlands (p <0.05).*

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Nitrifying and denitrifying bacterial temporal dynamics

The bacterial populations revealed an interesting behavior during the first year of operation although the first sampling was statistically different from the others ($p \leq 0.05$) (Fig. 4a). After the first month of operation the lowest eubacteria abundance of the study was found, in both layers and CW (10^7 and 10^8 16S rRNA copies g^{-1} in the top, and 10^5 and 10^4 16S rRNA copies g^{-1} in the bottom layer of the UVF and SVF wetlands, respectively). After three months of operation (Sep. 2015), an increment of three orders of magnitude took place in the upper layer (10^{10} and 10^{11} 16S rRNA copies g^{-1} of the UVF and SVF wetlands, respectively), and four and two orders of magnitude were increased in the bottom layer in the UVF and SVF wetland, respectively (10^9 and 10^6 16S rRNA copies g^{-1}). At this point, similar eubacteria abundance was found between the two CW. Based on the depth profile of the UVF and SVF wetlands, bacterial stability was reached about 90 d after starting operation of the units. This finding is similar to a study conducted in a tidal flow CW, which indicated that the biomass can take up to 100 d to stabilize during batch (fill and draw) operation (Ragusa *et al.,* 2004), and is also in agreement with Weber and Legge (2011), which showed bacterial steady-state at 75 d. In addition, another study conducted in VF wetland showed that The bacterial teh 16S rRNA gene abundance increased rapidly and reached its maximum by day 85 (Truu *et al.,* 2019).

On the other hand, AOB populations exhibited a different temporal behavior between both units (Fig.4b). In the top layer of the two CW a large variation of the nitrifying population's abundance was depicted throughout the study. Statistical significance ($p \leq 0.05$) was found among the samplings collected over time at this layer for both CW. The lowest abundance of these populations was identified after 30 d of operation (at the first sampling) in both wetlands (10^3 and 10^4 *amoA* copies g^{-1} in the UVF and SVF wetlands, respectively). Thenceforth, the AOB abundance increased in the UVF (10^4 to 10^6 *amoA* copies g^{-1}) and in the SVF wetland (10^5 to 10^6 *amoA* copies g^{-1}) (Fig. 4b). Studies conducted in different wastewater treatment plants showed that a relative AOB abundance increased after 90 d of operation (Wang *et al.,* 2012). Similar results were shown by Truu *et al.* (2019) in VF wetlands where the nitrifying activity increased after 78 days of operation. Nitrifiers grow very slowly and even the fastest growing AOB, *Nitrosomonas europaea*, has a specific growth rate up to only about 2 d, equivalent to a doubling time of 8 h (Prosser, 1990). In this way, the increase of AOB after 30 d of operation observed in the current study may be associated to the period of establishment of these populations in the biofilm during the start-up phase. Thereafter, there was a large variation of the AOB abundance over time in the top layer of the two wetlands. AOB are known to be highly sensitive to several environmental factors, including temperature, pH, dissolved oxygen, and a wide variety of chemical inhibitors (Prosser, 1990). To this respect, a positive correlation was observed between air temperature and *amoA* copies from the top layer of both CW ($R^2 = 0.86$). This behavior elucidates the possible influence of air temperature variation (17.6 to 25.8 ºC) on the AOB abundance.The optimal temperature for nitrifying bacteria ranges from 28 to 36 °C (Hammer and Hammer, 2001). However, nitrifying communities can adapt to temperature changes and may maintain their activity at lower temperatures by metabolic adaptation (Cookson *et al.,* 2002).

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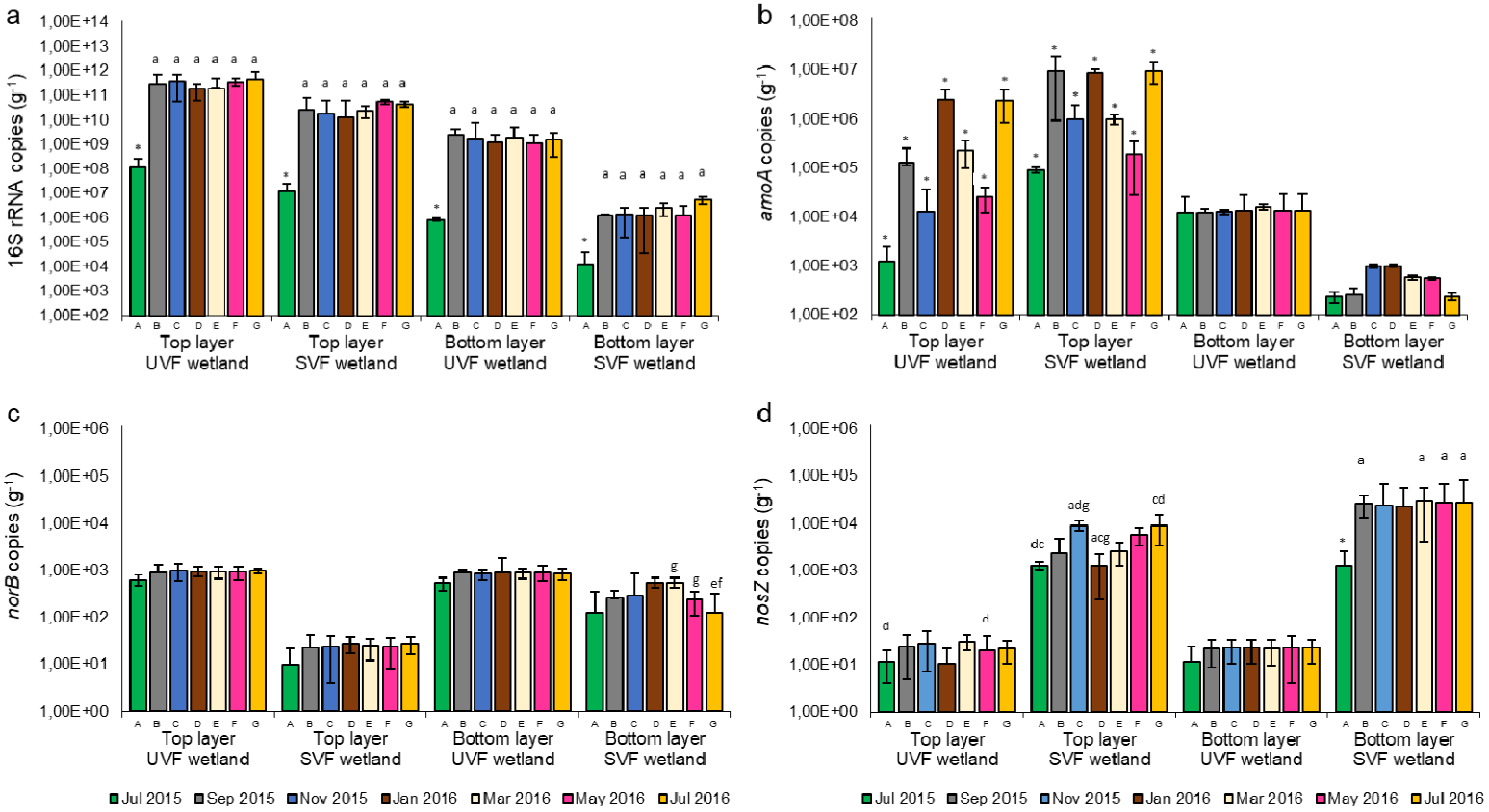

Figure 4. Nitrogen functional genes average identified in the biofilm from top (0-15 cm) and bottom (60-70 cm) layers of the unsaturated vertical (UVF) and partially saturated vertical (SVF) flow constructed wetland over the first year of operation. a) abundance of 16S rRNA copies; b) abundance of *amoA* copies; c) abundance of *norB* copies; c) abundance of *nosZ* (clade I) copies. *Presented values are the mean and SD of independent triplicates.* statistically differences between all sampling from the same layer from de the same wetland (p <0.05); Different letters above the columns indicate significant differences in gene abundance between different sampling over time in the same layer in the same wetland (p <0.05).*

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Moreover, higher AOB abundance was identified in the top than the bottom layer of both wetlands (Fig. 3b). The same trend was shown by Silveira *et al.* (2020). Oppositely, in the bottom layer of both wetlands, AOB populations showed similar abundances throughout time, being of 10^4 and 10^2 *amoA* copies g^{-1} in the UVF and SVF wetlands, respectively. This finding indicates that the nitrifying populations present in the bottom layer of CW are less susceptible to seasonal variations, as well as to the characteristics of influent wastewater.

In relation to denitrifying bacteria, *norB* gene showed no significant changes over the monitored period in both CW, with an abundance in the order of 10^2 copies g^{-1} in both layers of the UVF wetland, and of 10^1 and 10^2 copies g^{-1} in the top and bottom layers of the SVF, respectively (Fig. 4c). The key process conditions for N_2O production (*norB* expression) are associated with pH, dissolved oxygen, nitrite concentrations and carbon sources (Law *et al.,* 2012). In this way, the low variation on *norB* abundance over time can be associated with little fluctuations in environmental conditions of the filter media from both CW. Moreover, greater *norB* gene abundance was identified in the bottom layer than in the top layer of the SVF wetland, showing greater denitrification potential in this layer of the unit. For the *nosZ* gene there was much greater temporal abundance fluctuation in the top layer of both units (10^1 and 10^3 *nosZ* copies g^{-1} in the UVF and SVF wetland, respectively) finding statistical significance ($p \leq 0.05$) among the sampling campaigns for both CW (Fig. 4d). This fluctuation can be associated with the dissolved oxygen concentration within this layer. Oxygen influence on denitrification process by providing a better electron acceptor for denitrifying populations to generate energy affected the nitrous oxide reductase action (Lu *et al.,* 2014). Studies carried out with mathematical simulation in VF wetlands showed that up to 30 cm of depth, there is a great variation in the dissolved oxygen concentrations present in the filter media, whereas after that depth, oxygen concentrations remain constant (Langergraber and Simunek, 2005). Oppositely, in the bottom layer, after the first month of operation, the *nosZ* gene abundance (10^1 and 10^3 *nosZ* copies g^{-1} in the UVF and SVF wetland, respectively) in both CW depicted an increase and remained rather stable over time, showing little fluctuation. In this way, the temporal behavior of nosZ indicates that the denitrification process occurred over time in greater magnitude in the SVF wetland that in the UVF unit.

Bacterial population diversity

The alpha diversity of the bacterial populations in the biofilm of the top layer of both CW (Shannon index of 6.15 – 6.68) was clearly higher than that found in the bottom layer (Shannon index of 4.12 – 4.89) (Table 2). Whereas the bacterial diversity structure was similar in the top part of both units (H 6.1 – 6.6; 6.2 – 6.5 and Chao1 4.500 – 4.600; 4.400 – 4.600 for UVF and SVF wetlands, respectively), the bottom zone of the SVF wetland (H 4.2 - 4.4 and Chao 1 3.400 – 3.600) showed a lower diversity over the whole study period than the UVF (H 4.1 - 4.8 and Chao 1 3.500 – 3.600).

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Table 2. Alpha diversity indexes for bacteria populations in the microbial biofilm established on the filter media of the unsaturated (UVF) and partially saturated (SVF) vertical flow wetlands during the first year of operation in the top and bottom layers (mean ± SD).

Sampling campaign	CW unit	Reads (contigs)	Coverage[1]	OTUs[1]	Chao1[1]	Shannon[1]
Top layer - Jul 2015	UVF wetland	71024	0.9761 (0.0003)	3586 (15)	4523 (38)	6.153 (0.0003)
	SVF wetland	71223	0.9700 (0.0002)	3579 (12)	4419 (32)	6.225 (0.0006)
Top layer - Nov 2015	UVF wetland	70125	0.9876 (0.0004)	3613 (16)	4635 (40)	6.585 (0.0002)
	SVF wetland	69128	0.9805 (0.0003)	3620 (18)	4628 (42)	6.529 (0.0008)
Top layer - Jan 2016	UVF wetland	70125	0.9733 (0.0001)	3815 (19)	4689 (51)	6.689 (0.0009)
	SVF wetland	69256	0.9801 (0.0003)	3798 (18)	4658 (47)	6.523 (0.0006)
Top layer - May 2016	UVF wetland	69375	0.9852 (0.0003)	3701 (10)	4602 (42)	6.680 (0.0005)
	SVF wetland	70124	0.9981 (0.0004)	3856 (15)	4691 (39)	6.540 (0.0002)
Top layer - Jul 2016	UVF wetland	69874	0.9832 (0.0001)	3823 (12)	4692 (42)	6.659 (0.0008)
	SVF wetland	69689	0.9842 (0.0002)	3872 (13)	4685 (47)	6.526 (0.0003)
Bottom layer - Jul 2015	UVF wetland	59288	0.9881 (0.0004)	3452 (11)	3545 (42)	4.126 (0.0003)
	SVF wetland	59835	0.9832 (0.0002)	3356 (15)	3489 (48)	4.256 (0.0005)
Bottom layer - Nov 2015	UVF wetland	63895	0.9765 (0.0002)	3456 (16)	3556 (38)	4.385 (0.0009)
	SVF wetland	63256	0.9832 (0.0001)	3345 (15)	3425 (34)	4.316 (0.0002)
Bottom layer - Jan 2016	UVF wetland	64589	0.9895 (0.0003)	3458 (14)	3550 (36)	4.552 (0.0004)
	SVF wetland	65895	0.9834 (0.0003)	3478 (12)	3529 (37)	4.468 (0.0003)
Bottom layer - May 2016	UVF wetland	64375	0.9702 (0.0002)	3525 (11)	3619 (38)	4.856 (0.0002)
	SVF wetland	63568	0.9823 (0.0004)	3458 (10)	3549 (39)	4.495 (0.0006)
Bottom layer - Jul 2016	UVF wetland	64528	0.9872 (0.0003)	3532 (11)	3610 (38)	4.896 (0.0003)
	SVF wetland	65893	0.9845 (0.0002)	3585 (12)	3600 (40)	4.475 (0.0003)

Data normalized by using contigs close to the sample with the lowest number of contigs (50,000 reads).

This finding indicates that low oxygen and redox conditions seem to decrease bacterial diversity, being in accordance with previous observations (Pelissari *et al.,* 2018). Moreover, the structure of the eubacterial community showed a great variation throughout the first year of operation within both CW (Fig. 5a and b). During the first five months of operation, biofilms from the top and bottom layers of both units were dominated by *Bacillales*, *Enterobacteriales*, *Bacteroidales* and *Xanthomonadales,* being the latter only present in the SVF wetland. The cited orders accounted for 15 to 70 % of the relative abundance (RA) of the classified sequences. After that period, the bacterial diversity clearly increased in both layers of the two wetlands (Table 2), although depicting clearly different structures. In the top layer of the two CW, *Bacillales*, *Burkholderiales*, *Rhizobiales*, *Enterobacteriales*, *Bacteroidales*, *Xanthomonadales and Actinomycetales* became dominant with RA between 7 and 25 %. Meanwhile, the structure of the bottom layer clearly differed between the two units. *Burkholderiales* (40 % RA), *Rhizobiales* (15 % RA), *Bacillales* (7 % RA) and *Xanthomonadales* (9 % RA) predominated in the UVF wetland, whereas *Rodhocyclales* (40 % RA), *Lactobalilalles* (18 % RA), *Pseudomonadales* (18 % RA) and *Xanthomonadales* (13 % RA) prevailed in the SVF wetland, showing a specialization of populations that survive under anaerobic / anoxic conditions. This population shift may be associated with the higher carbon availability in the biofilm, since the majority of OTUs are associated with

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heterotrophic bacteria. After one year of operation, eubacterial community structure was clearly specialized in each wetland configuration. In the UVF biofilms *Xanthomonadales* (19 and 31 % RA in the top and bottom layer), *Rodhocyclales* (7 and 20 % RA in top and bottom), *Rhizobiales* (8 % RA in both layers), *Pseudomonadales* (31 and 3 % RA in top and bottom) *and Actinomycetales* (10 and 6 % RA in top and bottom) were the predominant orders.

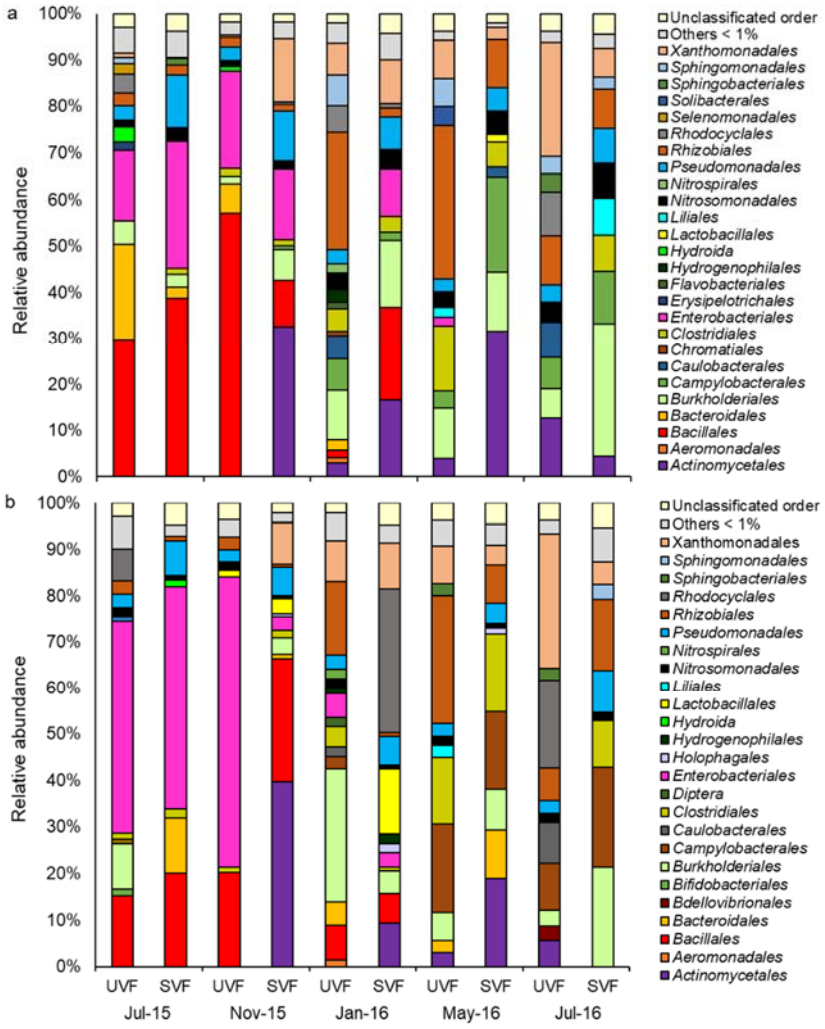

Figure 5. Taxonomic assignment of sequencing reads at order level of the bacterial populations from biofilm of the filter media of unsaturated (UVF) and partially saturated vertical (SVF) flow constructed wetlands during the first year of operation (sampling collected in July 2015, November 2015, January 2016, May 2016 and July 2016). a) Top layer of both wetlands. b) Bottom layer of both wetlands. *Relative abundance was defined by the number of reads (sequences) affiliated with any given taxon, divided by the total number of reads per sample. Phylogenetic groups with relative abundance lower than 1% were categorized as others.*

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On the other hand, *Burkholderiales* (20 and 12 % RA in top and bottom)*, Actinomycetales* (55 and 56 % RA in top and bottom)*, Campylobacteriales* (12 % RA only in the bottom layer) *and Rhizobiales* (6 and 9 % RA in top and bottom) were the most abundant orders in the SVF wetland.

As regards to the diversity related to the nitrogen cycle, AOB populations were represented by OTUs belonging to *Nitrosomonadales (Nitrosomonas* and *Nitrosospira genus).* In the top part of both CW, AOB populations showed an interesting behavior. The first seven months of operation, the AOB community was dominated by *Nitrosospira*. Thereafter, coinciding with the rise of air temperature during the summer season (January), the occurrence of *Nitrosomonas* was identified in the two CW, generating an increase of the AOB abundance in the top zone (from 1.5 % and 3 % to 3.5 % and 5% RA in the UVF and SVF wetlands, respectively). Despite this, *Nitrosospira* were the predominant AOB order throughout the study. In the bottom layer of both wetlands, *Nitrosospira* was dominant with 2% and 1% RA in the UVF and SVF wetlands, respectively. This behavior is associated with the metabolism of each AOB population. *Nitrosospira*-like bacteria are postulated as K-strategist with a higher affinity than *Nitrosomonas* to ammonia and oxygen (Schramm *et al.,* 1999). Moreover, the temperature is regarded as one of the most important factors that affect the balance between *Nitrosospira* and *Nitrosomonas* in wastewater treatment plants, where low temperature (< 20 Cº) seems to favor *Nitrosospira* (Cydzik-Kwiatkowska and Zielinska, 2016; Siripong and Rittman 2007). In this way, *Nitrosospira* presents advantages in the competition with *Nitrosomonas*, and showed to be more resistant and stable in both wetlands.

Nitrospirales (*Nitrospira* genus) and *Rhizobiales* (*Nitrobacter* genus) were the NOB populations found in CW' biofilms. *Nistrospira* was identified just in the UVF unit after seven months of operation (2% RA in both layers). Meanwhile, *Nitrobacter* populations showed a stable behavior over time in the UVF wetland, being more abundant in the bottom (3% RA at all samplings) than in the top layer (2% RA). The abundance of *Nitrobacter* was also slightly higher in the top (2% RA) than in the bottom zone of the SVF wetland. The higher predominance and stability of *Nitrobacter* in relation to *Nitrospira* in both CW may be indicative of the availability of high nitrite concentrations, due to the higher affinity of *Nitrospira*-like bacteria (K-strategist) to nitrite and oxygen, reaching high densities under substrate limiting conditions (Schramm *et al.,* 1999). This hypothesis implies that the relative population size of *Nitrobacter* would be larger than that of *Nitrospira* under high nitrite concentrations and vice versa (Blackburnea *et al.,* 2007). Moreover, higher *Nitrobacter* abundance in the bottom than the top zone of the UVF wetland can be associated with lower organic carbon availability in the bottom, which favors the autotrophic metabolism. In addition, previous studies have shown that *Nitrobacter* species could survive even in anoxic conditions (Kim and Kim, 2006).

Denitrifying bacteria were found along the vertical profile of both CW. In the UVF wetland lower diversity and abundance of denitrifying populations were found in relation to the SVF (Fig. 5).

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Moreover, many of the bacteria associated with the denitrification process are heterotrophic, therefore their occurrence may be associated with the availability of carbon especially in the UVF wetland due to higher NO_x-N in the effluent. *Pseudomonadales* (*Pseudomonas* genus), with 3 % RA in both layers of the UVF wetland and 8 % to 18% RA in the two layers of the SVF, was the most stable denitrifying organism identified at all samplings in both CW. Furthermore, it is important to note the predominance of *Actinomycetales* (*Streptomyces* genus) after five months of operation in the SVF wetland (28 % and 32 % RA in top and bottom, respectively). *Streptomyces* was found with a large proportion in this period and are known to be denitrifying organisms. (Cheneby *et al.,* 2000). However, a further decrease of this population was observed thenceforward, reaching a RA of 3 % and 6 % in top and bottom layers. This finding is in agreement with the physico-chemical data, elucidating the denitrification decrease over time in the SVF wetland, due to the decline of organic carbon availability.

Conclusions

Based on the assessment of nitrogen transformations linked with bacterial community dynamics during the first year of operation of an unsaturated (UVF) and a partially saturated vertical (SVF) subsurface flow CW operated in parallel:

- The SVF wetland showed a higher treatment performance of organic matter and solids removal (around 90 % for SST, COD and BOD_5) than the UVF wetland (around 85 % for SST, COD and BOD_5);
- Low availability of organic carbon in the filter media of the SVF wetland due to the early stage of the wetland resulted in incomplete denitrification, releasing the final effluent with high concentrations of oxidized nitrogen (16 mg L^{-1}). Despite that fact, TN and NH_4-N removal efficiencies were greater in this wetland (45 % and 62 %, respectively) than in the UVF unit (34 % and 58 %, respectively);
- Bacterial stability was reached about 90 days after the start-up of the wetlands. AOB populations (*amoA* gene) exhibited a different temporal behavior between wetlands, especially in the top layer. In the bottom layer AOB populations were less susceptible to seasonal variations, as well as the characteristics of the wastewater influent;
- Saturated conditions of the SVF wetland influenced the AOB populations (*Nitrosomonas* and *Nitrosospira* genus), suggesting that nitrification occurred in a larger magnitude in the first 15 cm of this wetland, whereas within the UVF wetland the nitrification process took place even at deeper layers of the unit;
- Denitrifying bacteria (*nosZ* gene) showed a great temporal abundance fluctuation in the top layer of both wetlands. In the bottom layer, after the first month of operation, denitrifiers depicted an increase and remained rather stable over time, showing little fluctuation. Meanwhile, *norB* gene showed no significant changes;

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- NOB populations (*Nitrobacter* and *Nitrospira* genus) were identified throughout the monitoring period. In the UVF wetland *Nitrobacter* was more abundant in the bottom zone, while in the SVF wetland the higher abundance was found in the top zone;
- The predominance of incomplete denitrification in the SVF wetland was identified. The denitrification process could be improved by increasing the supply of organic carbon in the medium by increasing the organic load rate applied.

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