NITROGEN TRANSFORMING BACTERIA WITHIN A FULL-SCALE PARTIALLY SATURATED VERTICAL SUBSURFACE FLOW CONSTRUCTED WETLAND

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Abstract

The aim of this study was to characterize the nitrogen transforming bacterial communities within a partially saturated vertical subsurface flow constructed wetland (VF) treating urban wastewater from a residential condominium in southern Brazil. The VF had a surface area of 3,141 m^2, and was divided into four wetland units, out of which two were operated while the other two rested, alternating cycles of 30 days. The wetland had a depth of 0.7 m, out of which 0.2 m were saturated at the bottom. The bed media was sand, and was planted with *Cyperus papyrus*. The nitrifying and denitrifying bacterial communities were characterized in wetland unit 3 (763.6 m^2 surface area) over a period of 12 months by using the FISH technique. Samples were collected monthly (February 2014 to February 2015) from different layers within the vertical profile, during operation and rest periods, comprising a total of 6 sampling campaigns while the unit was in operation and another 6 when the unit was at rest. This wetland unit 3 operated with an average organic loading rate (OLR) of 4 g COD m^{-2} d^{-1} and a hydraulic loading rate of 24.5 mm d^{-1}. The rest periods of the wetland unit presented influences on the abundance of ammonia-oxidizing bacteria (AOB) (8% and 3% for feed and rest periods, respectively), and nitrite-oxidizing bacteria (NOB) (5% and 2% for feed and rest periods, respectively). However, there was no influence of the rest periods on the denitrifying bacteria. AOB were only identified in the top layer (AOB *β-proteobacteria*) in both operational and rest periods. On the other hand, the NOB (*Nistrospirae* and *Nitrospina gracilis*) were identified in the operational periods just in the top layer and during rest periods just in the intermediate layer. The denitrifying bacteria (*Pseudomonas* spp.and *Thiobacillus denitrificans*) were identified from the intermediate layer downwards, and remained stable in both periods.Based on the bacterial dynamics identified, VF wetland partially saturated operated with low OLR promoted conditions for simultaneous nitrification and denitrification.

Keywords: nitrogen removal, bacterial community structure, low organic loading rate, treatment wetland.

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1. INTRODUCTION

Constructed wetlands (CW) are systems designed and constructed in order to simulate the conditions of processes occurring in natural wetlands to treat wastewater (Kadlec and Wallace, 2009). The use of this technology has developed rapidly over the last decades for the treatment of wastewater in decentralized areas and small communities both in industrialized and low-income countries due to their various advantages over conventional wastewater treatment systems (García et al., 2010). These include low to no energy consumption, ease of maintenance and operation, and good integration into the landscape and promotion of biodiversity, among others.

Despite the latest advancements on the CW technology, subsurface flow CWs still show a deficiency in total nitrogen (TN) removal, being in fact one of the fundamental issues for the further development of this technology (Adrados et al., 2014). The pathways for nitrogen transformation in horizontal subsurface flow (HF) and vertical subsurface flow (VF) wetlands when operated as separate technological arrangements generally do not completely remove TN but instead convert it into various nitrogen forms. These processes are complex and diverse, and include ammonification, sorption, plant and microbial uptake, ammonia volatilization, nitrification, and denitrification (Vymazal, 2007). The prevailing processes in a CW differ in magnitude depending on the design and mode of operation. In general, VF wetlands are unsaturated units generally fed intermittently with several pulses throughout the day, resulting in a high oxygen transfer capacity, thus favoring the nitrification process within the wetland bed (Platzer, 1999). On the other hand, HF wetlands operate mostly under anoxic/ana'erobic conditions due to the permanent saturation of the wetland bed, which makes it a likely environment for denitrification (Vymazal, 2007). In general, the limitations for TN removal in CWs can be associated with the competition for oxygen by heterotrophic and autotrophic bacteria (Saeed and Sun, 2011), and the absence of organic compounds available for the process of denitrification (Lavrova and Koumanova, 2010).

Various configurations and operational strategies have been investigated in order to provide complete removal of nitrogen, such as the use of tidal flow systems (Hu et al., 2014; Austin et al., 2006), the intermittent aeration (Foladori

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et al., 2013), the recirculation of the treated effluent (Foladori et al., 2013; Ayaz et al. 2012), the use of hybrid systems combining VF and HF wetlands (Vymazal, 2013), the use of filter media that promotes greater adsorption of the different forms of nitrogen (Saeed and Sun, 2011), the bioaugmentation of microbial populations (Zhao et al., 2016), and the promotion of aerobic and anaerobic environments within the same reactor (Silveira et al., 2015; Prigent et al., 2013; Dong and Sun, 2007).

In the latter strategy, the use of a partially saturated zone at the bottom of VF wetlands aims at creating anaerobic/anoxic conditions in the lower part of the bed, and aerobic conditions in the top part, thus promoting favorable conditions for simultaneous nitrification and denitrification within a single wetland unit (Silveira et al., 2015). Only a few examples of this configuration have been reported. Langergraber et al. (2008) evaluated two stages of VF wetlands in series and the average removal efficiency of TN was of about 50%. Dong and Sun (2007) evaluated in parallel a typical unsaturated VF (43.7 m²) with 0.80 m of depth, and a partially saturated VF wetland (28.07 m²) with 0.55 m of saturation. The partially saturated VF presented higher TN removal efficiency (37%) than the typical unsaturated wetland (25%). Moreover, Silveira et al. (2015) studied two partially saturated VF beds (2 m² each) operating with two different heights of saturation, 0.15 m and 0.25 m. Under an influent load of 14 g TKN m^{-2} d^{-1}, the TKN load removal was 45% for a saturation height of 0.15 m and increased up to 58% when the saturation height was of 0.25 m.

In general, all efforts directed towards maximizing the removal of pollutants in CW are directly associated with enhancing the activity of the microbial communities responsible for various nutrient transformations occurring in the biomass of the filter media and the rhizosphere (Mayo and Bigambo, 2005). Indeed, the understanding of the dynamics of bacterial communities in constructed wetlands and their relation to treatment performance has been the focus of many studies. Foladori et al. (2015) evaluated the viability and decay of bacteria in water and soil in a typical unsaturated VF wetland (2.25 m^2 surface area) and observed that the number of viable bacteria in the surface (0 to 0.10 m) layer was 3.7 times higher than in the deeper layer (0.40 to 0.50 m). What is more, Salomo et al. (2009) investigated the influence of plant roots and soil

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structure on the metabolic diversity of microorganisms in a VF wetland (48 m^2), and results show that in the surface of the bed easily degradable components such as carbohydrates were used, whereas in the deeper layers inert compounds were metabolized. Adrados et al. (2014) characterized the prokaryotic microbial community (bacteria and archaea) of VF, HF and biofilters and reported a higher bacterial than archaeal activity in all systems studied. Other studies have shown that shifts in the structure of bacterial communities can be associated with changes in a number of soil properties, including soil texture and soil nitrogen availability (Dong and Reddy, 2010). Calheiros et al. (2010) showed that the type of macrophyte seems to influence bacterial community dynamics in HF wetlands polishing high salinity tannery wastewater. In another study, Calheiros et al. (2009) indicated that the diversity of the bacterial community in CW systems might influence the final effluent quality. What is more, Button et al. (2015) showed that microbial metabolic functions identified in different CW types (VF, HF, VF + HF + aeration) are related to the design of each system, spatial position within the bed, and especially with levels of pretreatment.

Nonetheless, the knowledge particularly concerning nitrogen transforming bacteria in VF wetlands is still limited to a few studies (Adrados et al., 2014; Mayo and Bigambo, 2005). Tietz et al. (2007a) evaluated the community of ammonia-oxidizing bacteria (AOB) in three VF beds (18 m^2 , 0.50 m depth) treating municipal sewage after 2.5 years of operation and despite nitrification was stable, little AOB bacterial activity was identified. During the winter nitrification decreased, however not affecting the spatial distribution of AOB, being *Nitrosomonas europaea*, *Nitrosococcus mobilis* and *Nitrosospira* the dominant AOB. Zhi and Ji (2014) identified functional genes involved in nitrification and denitrification in a tidal flow CW showing nitrification activity performed by AOB and Annamox bacteria, as well as denitrification.

Thus, there is a clear need to gain further insight into the dynamics of nitrogen transforming bacteria in VF wetlands, and even more particularly in partially-saturated VF beds intended to promote TN removal within a single wetland bed, The aim of this study was therefore to characterize the nitrifying and denitrifying bacterial communities within a mature partially saturated vertical subsurface

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flow constructed wetland at full-scale used in the treatment of urban sewage and operated under low organic load.

2. MATERIALS AND METHODS

2.1 Description of the wastewater treatment plant

The research was conducted at a full-scale VF system located in a residential condominium in the city of Palhoça, Santa Catarina state, southern Brazil (latitude 27°45'4.82" and longitude 48°37'39.35"). This treatment plant started operation in 2006, and was designed for an estimated maximum demand of 2,200 P.E. However, during the time of the current study the system received a contribution of approximately 100 people, due to the fact that the residential condominium was not totally inhabited. This resulted in low organic and hydraulic loading rates applied to the VF wetland.

Urban wastewater flowed into an anaerobic baffled reactor acting as primary treatment before it was conveyed to the VF wetland, which had a surface area of 3,141 m², a depth of 0.7 m and a saturated zone at the bottom of 0.20 m, kept constant by a level controller. The bed media consisted of 0.1m gravel of granite (3 - 8 mm) at the bottom of the drainage area, followed by 0.5 m of sand (Effective diameter d_{10} = 0.3 mm and Uniformity Coefficient of 4.84), and finally 0.1 m of gravel (3 - 8 mm) on the top. The planted macrophyte was *Cyperus papyrus*, which was very well developed at the time of this study.

The VF wetland had a surface area of the 3,144 m² divided into four wetland cells interconnected, which alternated cycles of feed and rest. In this way, while two of the wetland cells (crosswise) were being fed the other two were at rest, and vice-versa. These cycles were of 30 days each, and were applied so as to control the growth of the attached biomass, to maintain aerobic conditions within the filter bed and to mineralize the organic deposits accumulated on the bed surface (Molle et al., 2008). Distribution pipes were installed across the surface of the wetland and feeding was done intermittently on the surface of the unit by pumping every 6 hours, giving a total number of 4 pulses per day (4,516 L per pulse) (Fig. 1).

2.2 Sampling procedure

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From February 2014 to February 2015, collection of samples from the bed media of the saturated VF wetland was performed monthly, totaling 12 collections throughout the year. Sample collections were always completed in wetland cell 3 (763.6 m² surface area) at the end of each period of feed and rest. In this way, 6 sampling campaigns were carried out during the feeding periods, and another six during the rest periods (n=12).

For each sampling campaign, a complete vertical profile was collected from 3 points of the wetland cell 3 with the aid of a manual auger. These points were located along the length of the wetland coinciding with the holes of the distribution pipes of the unit (i.e. initial – point 1, central – point 2, and final - points 3 of portion the wetland) (Fig. 2). The vertical profile was divided in layers along the depth, namely 'top' (0 to 0.17m depth), 'intermediate' (0.17 to 0.34 m depth), 'unsaturated/saturated' (0.34 to 0.51 m depth), and 'saturated' layers (0.51 to 0.68 m).

Moreover, monitoring of the performance of the system in terms of conventional water quality parameters was performed, taking grab samples at the influent and effluent of the VF. Samples were collected just when the wetland cell 3 was being fed (n=10)The parameters evaluated in final effluent of VF were: pH, alkalinity, chemical oxygen demand (COD), total suspended solids (TSS), total nitrogen (TN), ammonia nitrogen (NH_4-N), nitrite nitrogen (NO_2-N), and nitrate nitrogen (NO_3-N). Their analysis followed APHA (2005) recommendations, with the exception of NH_4-N, which was determined following recommendations of Vogel (1981).

2.3 Bacterial analysis

2.3.1 Pre-treatment of samples

For bacterial analysis of nitrifying and denitrifying communities, aliquots of 20 g of bed media were collected from every point and depth, to which 50 mL of deionized water was added. The samples were mixing and sonicated in an ultrasound for 5 min in order to loosen the biofilm from the bed media. Then, the

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samples were centrifuged at 1500 G for 5 min. Absorbance of the samples before and after centrifugation and sonication process was performed to confirm the integrity of the extracted biofilms.

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2.3.2 Quantitative fluorescence in situ hybridization

For fixation of samples, a volume of 1 mL of each sample (mixed water and biofilm suspension) was centrifuged at 16000 G for 5 min, and after, the recovered pellet were fixed at 4 °C for 3 h by adding three volumes of 4% paraformaldehyde solution and one volume of Phosphate Buffered Saline (PBS), following recommendations of Amann et al. (1995). The fixed samples were washed twice with 1 mL of PBS and finally suspended in a solution of PBS and absolute ethanol and stored at −20 °C.

For the quantification of total bacterial cells, considering the Eubacteria domain, a volume of 10 µL of each sample (mixed water and biofilm suspension) was fixed in a PTFE-printed microscope slide and covered with 4',6-diamidino-2-phenylindole (DAPI) solution (1 µg mL^{-1}).

For the analysis of the nitrifying and denitrifying bacterial communities specific probes for each community were used (Table 1). All probes were labeled at 5′ position with the fluorochrome Cy3 (Biomers). To carry out the hybridization reactions, PTFE-printed microscope slides with 10 wells measuring 5 mm^2 in diameter were used. For the fluorescence in situ hybridization (FISH) reaction, 10 µL of each fixed sample was prepared in each slide. Quantification of nitrifying bacteria was performed by direct counting of 10 random fields in each well using an epifluorescence microscope (Olympus BX41, Tokyo, Japan).

For the estimation of the abundance of cells hybridized with the probe EUB mix, the cells stained with DAPI were considered as representatives of 100% of all microorganisms determined by digital image. For the rest of the probes 10 fields were randomly singled and cells stained with the probe EUB mix were considered as being 100% of all bacteria determined by digital image. The relative abundance of nitrifying and denitrifying bacteria from the total DAPI-stained were calculated using DAIME software and split into individual color channels before image segmentation (Daims et al., 2006).

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2.3 Statistical data analysis

Kolmogorov–Smirnov test was utilized for water quality parameters for examining whether data distribution was approximated to normality. This analysis was performed by using software Statistic 7.0^{TM}.

With regards to bacterial abundance identified, statistical analyses were made by considering the operating variables of wetland, local climate conditions, and the different sampling collected. Firstly, *t* test was realized to identify if there was statistical difference between bacterial abundance identified in feed and rest periods. Secondly, one way analysis of variance - ANOVA test was used to (i) identify if there was statistical difference on bacterial abundance in different layers of wetland; (ii) evaluate if difference in bacterial abundance occurred in different samples cores along the length of wetland cell 3 (Points 1, 2, and 3); (iii) evaluated if occurs differences in bacterial community in different seasons. These analyzes were performed with a level of significance of α= 0.05, and was performed by using software Statistic 7.0^{TM}.

3. RESULTS AND DISCUSSION

3.1 Treatment performance of the constructed wetland

The wetland cell 3 operated with low organic (OLR) and hydraulic loading rates (HLR), with average values of 4 g COD m^{-2} d^{-1}, and a HLR of 24.5 mm d^{-1}. The nitrogen load was 1.5 g TN m^{-2} d^{-1}, resulting in C/N ratio of 3 (considering the average concentrations of COD and TN). The literature recommends an OLR in the range of 60 to 70 g COD m^{-2} d^{-1} and a HLR of 250 mm d^{-1} for unsaturated VF in warm climates (Hoffmann et. al., 2011), and of 41 g COD m^{-2} d^{-1}, 10.2 g NH_4-N m^{-2} d^{-1} and HLR of 230 mm d^{-1} in subtropical climates (Sezerino et al., 2012). Design parameters as OLR and HLR are directly associated to the TN removal process (Saeed and Sun, 2012). Firstly, OLR is linked to the C/N ratio of the middle. Low OLR applied can result in low availability of organic carbon for denitrification. Zhao et al. (2011) showed better TN removal efficiencies in VF, with C/N ranging between 5 to10, comparted to the C/N of 2.5. Secondly, HLR is related to the transfer of oxygen into the filter bed in VF wetland.

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Therefore, lower HLR results in lower dragging oxygen in bed midea the case of VF wetland with sand as bed midea (Platzer, 1999).

Table 2 shows the wastewater physico-chemical characterization of influent and effluent of the wetland cell 3 , relating the feeding periods.. In general, the VF presented a good removal of solids and carbonaceous organic matter, being of 86 ± 4% for TSS, and 89 ± 10% for COD. With regards to nitrogen, the removal efficiency of TN and NH_4-N was of 58 ± 12% and 93 ± 2% respectively. The good performance of nitrogen removal in this system may be associated with nitrification followed by denitrification, provided the bottom saturation, and the bacterial interactions in the medium. The large removal efficiency of total nitrogen in the wetland can be attributed to the saturation of the bottom of the unit, which may have enhanced the complete elimination of nitrogen through the promotion of denitrification (Silveira et al. 2015).

3.2 Influence of feed and rest periods on nitrifying and denitrifying bacterial communities

In general, periods of feed and rest largely influenced the structure of bacterial communities identified present in the wetland cell (Fig 3). Based in statistical analysis, rest periods influenced mainly in abundance of Eubacteria and AOB (*β-Proteobacteria*) and NOB (*Nitrospirae*) (Fig. 3). The composition, diversity, and abundance of microbial communities in CW depends on environmental factors, properties of wastewater, substrate types, macrophytes, and operational conditions of the treatment units (Meng et al., 2014).

The overall average relative abundance of Eubacteria (EUB mix) identified in the four layers of the vertical profiles of the VF, was of 59 ± 13% during feeding periods, and of 30 ± 21% during rest periods (Fig. 3). The decay of this domain in rest periods may be associated with the limited availability of organic matter and nutrients in the bed media during these periods, which is particularly magnified due to the low organic and hydraulic loads applied to the unit during feed periods. This is accordance with Johnson et al. (2014), which used the metatranscriptome technique applied in different sewage treatment plants, and observed that the functional richness and microbial taxonomy is directly associated with the environmental nitrogen variation and the availability of

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carbon. Via the FISH technique, various abundance values of Eubacteria are reported in CW. Krasnits et al. (2009) in a HF (130 m^2 surface area) operated for 7 years in the treatment of municipal sewage under a OLR larger (11 g BOD_5 m^{-2} d^{-1}) identified an abundance of 85%. In another study, Sawaittayothin and Polprasert (2007) reported an abundance of 49% in CW treating municipal landfill leachate, while according to the authors the remaining 51% may be associated with archaea or inactivated bacteria.

In general, the diversity of bacteria involved in nitrogen transformations was the same for periods of operation and rest. However, the abundance of nitrifying bacteria was lower in rest periods (Fig. 3). The decay of the abundance of this group could be a bioindication of environments with low concentration of substrate and/or oxygen, since ammonia-oxidizing bacteria (AOB) have a lower affinity for ammonium and oxygen than ammonia-assimilating heterotrophic bacteria (Hanaki et al, 1990; Verhagen et al., 1992).

On the other hand, the average relative abundance of AOB *β-proteobacteria* (NSO190) (*Nitrosomonas* and *Nitrosospira*) was of 8 ± 1% for feed periods and of 3 ± 1% for rest periods (Fig. 3). *Nitrosomonas* sp. (NEU 653) was however not identified in any of the samples. This could be attributed to the fact that *Nitrosomonas* spp. have lower affinity for substrate than *Nitrosospira* spp. (although higher activity) (Scharamm et al., 1996). In this study, *Nitrosospira* spp. was the dominant AOB, showing to prevail in environments with low NH_4-N concentrations, and a good capacity of withstanding the physico-chemical variations in the bed media (Purkhold et al., 2000).

In regards to the nitrite-oxidizing bacteria (NOB), the *Nitrospirae* phylum (NTSPA 662) was the most abundant of this group (5 ± 1 % during feeding periods and 2 ± 1 % during rest periods). The identification of this phylum in CW has been shown by previous studies. Guan et al. (2015) characterized the microbial community in three VF wetlands (1.2 m^2 surface area each) applied to river water treatment, with influent concentrations around 8 to 12 mg L^{-1} of dissolved organic carbon and 2 to 4 mg L^{-1} of NH_4-N. The wetlands presented an NH_4-N removal efficiency of 95%, and the main bacteria associated with nitrification, which was present in greater abundance, was *Nitrospira*-like.

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Besides the phylum *Nitrospirae,* the NOB species *Nitrospina gracilis* (NTSPN 693) was present and remained stable, although with low abundance (1 ± 0%) in operation and rest periods.

Despite the lower abundance of nitrifying bacteria during rest periods these were able to remain active along the whole study period, showing their ability to recover their enzymatic activities quickly after a period of stress. This is in accordance with various studies, such as that of Wilhelm et al. (1988), which observed how *Nitrosomonas europaea* was able to recover its oxidant activity in a few minutes after about one year under limited NH_4-N concentrations. Moreover, *Nitrosospira briensis* after a period of 2 weeks with substrate limitation reached their maximum potential for NH_4-N oxidation within 30 to 60 minutes after a pulse of ammonia (Bollmann et al., 2005). Autotrophic nitrifying bacteria can adapt to survival strategies with low substrate based on cellular components, and have a high capacity to generate power when new substrate becomes available (Geets et al., 2006). In the other study conducted in a aerated saturated VF wetland was showed that after two weeks of no aeration in the established wetland, nitrification recovered within two days, whereas nitrification establishment in a new wetland was previously observed to require 20 to 45 days. Based on these results, once established resident nitrifying microbial communities are quite robust (Murphy et al., 2016). For denitrifying bacteria the abundance and diversity was the same in the feed and rest periods. The identified average relative abundance was of 3 ± 2.4% for the genus *Pseudomonas* spp. (PAE 997), of 6 ± 4% for the species *Thiobacillus denitrificans* (TBD 1419) and of 3 ± 0.8% for the species *Paracoccus versutus* and *denitrificans* (PDV 163) (Fig. 3). The fact that rest periods showed no influence on this community can presumably be explained by the stability provided by the bottom saturation layer, which maintained the necessary conditions for the stability of this group. Moreover, rest periods due to low availability of oxygen seems to favor denitrifying bacteria.in a study performed in two microcosms simulating a vertical profile of wetland was identified greater abundance of denitrifying bacteria in rest periods than in feed periods (Pelissari et al., 2016).

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In addition to the classic denitrification carried out mainly by *Pseudomonas spp.* (Ahn, 2006), other pathways of denitrification were identified in this study. *Thiobacillus denitrificans* are organisms that use inorganic compounds as a carbon source, and compounds such as sulfates, nitrates or nitrites as electron donors. Therefore, this species is associated to autotrophic denitrification. The presence of *Thiobacillus* in CW has previously been reported by Zhong et al., (2015) in a HF bed (HLR = 100 mm d^{-1}) treating urban wastewater. According to the authors this genus was present in great abundance (680 sequences) due to the anaerobic conditions of the HF. Furthermore, *Paracoccus denitrificans* was also identified in the current study, a species that is associated with aerobic denitrification and heterotrophic nitrification (Richardson, 2000). Austin et al. (2006) in a tidal flow VF wetland (10 m^2 divided into 6 cells, under flow of 1.5 to 2.3 m^3), reported a nitrification rate of 95%, related to a load of 104 g TKN m^{-3} $d^{-1}$and C/N ratio of 3.3:1. During the study period, two characterizations of microbiota through the FISH technique revealed that after 9 months of operation 9% of the bacterial community was composed of *Paracoccus denitrificans,* while after 30 months of operation colonization of this bacterium increased up to 15%.

3.3 Nitrifying and denitrifying bacteria distribution along the vertical profile of the filter media

According to the ANOVA test performed with bacterial abundance identified in each layer of VF wetland cell 3, was identified statistical difference ($p \leq 0.05$) in all layers of wetland. This difference could be explained due to difference bacterial community structures for each layer.*3.3.1 Top layer (0 to 0.17 m*)

The highest average relative abundance of Eubacteria in the VF wetland profile was identified on the top layer (76 ± 7% for feed periods, and 30 ± 1% for rest periods) (Fig. 4). This behavior has already been observed by other studies. Tietz et al. (2007b) in a VF bed (1 m^2 surface area) with sand as bed media applied in the treatment of municipal wastewater reported that more than 50% of the bacterial biomass and activity was concentrated in the top 0.10 m of the unit. Moreover, Foladori et al. (2015) in a VF wetland (2.25 m^2 surface area) detected that the highest amount of viable bacteria were found in the top from 0 to 0.10 m deep, mainly owed to the vertical feed which causes increased

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availability of organic matter and nutrients on the upper layer of the bed, thus stimulating microbial growth.

AOB bacteria were identified in both feed and rest periods. AOB β-*proteobacteria (Nitrosospira* and *Nitrosomonas)* presented an abundance of 8 ± 1% in the feed periods, and of 3 ± 1% in rest periods. Despite the decay of abundance of AOB β-*proteobacteria* during rest periods at the top layer this group remained active throughout the study (Fig. 4).

Conversely, the NOB were identified only in feed periods, with a relative abundance of 5 ± 0.3% for *Nitrospirae* phylum, and of 1 ± 0.2% for *Nitrospina gracilis* species. *Nitrospira sp.* have high affinity for substrate, even in low concentrations (Andrews and Haris 1986). Therefore *Nitrospira* sp. are well suited to low concentrations of oxygen and nitrite (Kim and Kim, 2006). In this way, the absence of *Nitrospirae*, and, as well as, feeding in rest periods can be a bioindicator of absence of substrate adsorbed in the bed media.

The occurrence of the genus *Paracoccus* of the species *denitrificans* and *versutus* was identified throughout the whole study at this layer with an average relative abundance of 3 ± 0.8% for the feed and rest periods. The presence of P. *denitrificans* can be associated both with heterotrophic oxidation of NH_4-N and aerobic denitrification, as heterotrophic nitrification can occur in media with a C/N ratio as low as 2 (Kuenen and Robertson, 1994).

3.3.2 Intermediate layer (0.17 to 0.34 m)

The average relative abundance of Eubacteria decreased to 40 ± 3% for feed periods and to 10 ± 12% for rest periods at this layer (Fig. 4). As previously discussed, the depth has great influence on bacterial distribution, due to the differing environmental conditions of the bed media along the vertical profile (Foladori et al., 2015; Tietz et al., 2008).

AOB were not identified in this layer (Fig. 4). Instead, the AOB community in this study was concentrated on the top layer, presumably since the low organic loadings applied to the wetland resulted in low oxygen competition between heterotrophic and autotrophic bacteria (faster heterotrophic organic degradation depletes dissolved oxygen availability (Saeed and Sun 2011)), but also due to

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increased availability of oxygen at the upper part of the bed media. This finding agrees with other studies conducted in VF wetlands operated with higher OLR, which show a stratification of AOB along the vertical profile owing to the higher amount of organic matter available on the surface of VF wetland, which results in highest abundance of heterotrophic bacteria. Tietz et al. (2007a) studied the AOB in three VF operated with 27 g COD m^{-2} d^{-1}, C/N ratio of 8.4, and HLR of 43 mm d^{-1}, and identified AOB species up to 0.50 m depth from bed surface.

Although AOB were not found in this layer, NOB were identified just in rest periods, with average relative abundance for *Nitrospirae* phylum of 2 ± 0.8% and 1 ± 0.2% for the species *Nitrospina gracilis*. The NOB colonization may be associated with the presence of adsorbed nitrite in the filter material.

The colonization of denitrifying bacteria of the genus *Pseudomonas* spp. was observed at the intermediate layer, with an average relative abundance of 5 ± 0.2% for feed periods. Although the intermediate layer presumably has aerobic conditions during operation periods, the colonization of this genus may be associated with the availability of carbon at this bed depth. According to Salomo et al. (2009), this layer of VF beds are predominantly ideal for bacterial use of organic substrates, due to the penetration of the roots of macrophytes which provide an additional source of easily degradable carbon. The average relative abundance of *Pseudomonas* spp. increased to 10 ± 1% during rest periods. The lower availability of oxygen due to reduced oxygen transfer capacity during rest periods should explain the increase in abundance of *Pseudomonas* spp., indicating the occurrence of the denitrification process in these periods.

3.3.2 Semi-saturated layer (0.34 to 0.51 m)

At this depth, during feed periods an increase in the abundance of Eubacteria (60 ± 3%) in relation to the intermediate layer (40 ± 3%) was identified. This could be associated with the moisture in the filter media, since this layer is half saturated with water. During rest periods the relative abundance of the bacterial community was of 20 ± 2% (Fig. 4). Despite the larger bacterial abundance in the feed periods, AOB and NOB were not identified at this layer, which may be explained by an environment with low oxygen concentrations.

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Furthermore, a greater diversity of denitrifying bacteria was observed in comparison with the intermediate layer. Besides the genus *Pseudomonas spp.*, as identified in the intermediate layer, the species *Thiobacillus denitrificans* was also observed at this depth (Fig. 4).

The average relative abundance was the same for feed and rest periods, being of 5 ± 1% and 2 ± 0.2% for *Pseudomonas spp.* and *Thiobacillus denitrificans*, respectively. The presence of these two denitrifying genus in the same layer may indicate distinct dynamics regarding the use of carbon. The denitrifying bacteria are usually heterotrophic, however, several bacteria, among them the genus *Pseudomonas* are facultative chemolithoautotrophic, capable of growing in the absence of organic matter, using molecular hydrogen as electron donor, and CO_2 as inorganic carbon source. They can also switch between chemolithotrophic or chemoorganotrophic metabolism, depending on the nutritional status of their habitats (Madigan et al., 2010). However, *Thiobacillus denitrificans* is an obligatory chemolithotrophic example, since it uses only inorganic compounds as electron donors for energy, and CO_2 as a carbon source. These findings are in accordance to those reported by Salomo et al. (2009), which evaluated the microbial metabolic diversity in a VF wetland (48 m^2 surface area) that had been for 13 years in operation applied in the treatment of domestic wastewater, and in the third layer in depth (where anoxic conditions equivalent to the unsaturated/saturated layer of this study prevailed) none of the tested carbon sources were metabolized by the microbial groups present in the medium. Since anoxic bacteria are dependent on oxidized electron acceptors, such as NO_x, SO_4^{2-} or Fe_3^{+}, it is hypothesized that the denitrification process may be associated with autotrophic bacteria, as indicated by the presence of the species *Thiobacillus denitrificans.*

3.3.3 Saturated layer (0.51 to 0.68 m)

In the bottom of the wetland bed the abundance of Eubacteria remained stable (60 ± 4 %) throughout the whole study (both in feed and rest periods), which can be attributed to the saturation of the bed. Bacteria growth seems rather favored in water films along the preferential water flow directions. Foladori et al. (2015) evaluated the relationship between viable and dead bacteria in the soil of

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a VF wetland, and in the influent of the unit. According to the authors, dead bacteria were prevalent in the soil of VF (viable/dead bacteria ratio of 0.52). In the same direction, Rajabzadeh et al. (2015) VF mesocosms reported that the flow of organic matter necessary for the growth of heterotrophic bacteria may be limited in dead zones of the unit.

At this layer only denitrifying bacteria were identified. The diversity of this group in the saturated layer was the same as in the unsaturated/saturated layer. However, the abundance was higher. The average relative abundance was of 5 ± 2% for Pseudomonas spp. in feed and rest periods, and of 10 ± 3% for *Thiobacillus denitrificans* in feed and rest periods, respectively.

3.4 Spatiotemporal variation of nitrifying and denitrifying bacteria

Considering the results of ANOVA test performed with average relative abundance of bacteria identified during periods of feed and rest, and in the different points and depth layers of the wetland cell 3, the total, nitrifying and denitrifying bacterial communities were not influenced by seasonal variations (Fig. 5). The bacterial stability identified in this study may be associated with the climatic conditions of the study site, since temperatures in this part of the country are mild even during the winter (14 ± 2 °C). Bouali et al. (2014) in a HF wetland showed variation a microbial community in different temperatures (summer average water temperature of 23.9±3 °C and average winter temperature of 11.7±1 °C). Microbial activity is related to temperature, and growth rate and metabolic activity of bacteria are greatly reduced with low temperatures (Atlas and Bartha, 1998). In general, nitrifying activity is inhibited in a temperature range of 6 to 10 °C, and denitrifying active was detected a 5 °C (Brodric et al., 1988; Werker et al., 2002).

.

Based on an ANOVA test, no variation of spatial distribution of the total, nitrifying and denitrifying bacterial communities were observed for the three sampling points (1, 2 and 3) along the length of wetland unit 3 ($p \geq 0.05$). The bacterial community structure does not vary due the fact that wastewater is uniformly distributed across the wetland surface, resulting in the evenly availability of carbon and nutrients across the media. Conversely, in HF wetlands, where

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565 feeding is provided by a single distribution pipe at an end of the wetland, a
566 decrease in biomass and microbial activity along the distance from the inlet
567 zone has been observed due to decreased availability of organic matter and
568 nutrients (Samsó and García, 2014; Nguyen, 2000; Nurk et al., 2005).

569

570 **4. CONCLUSIONS**

571 In this study the nitrifying and denitrifying bacterial communities were
572 characterized at different depths of the vertical profile of a partially-saturated
573 vertical subsurface flow constructed wetland (0.20 m of saturation) at full-scale
574 treating urban wastewater from a residential area in south Brazil. The wetland
575 operated under low organic and hydraulic loads and was divided into four
576 wetland cells, two of which were operated while the other two rested, alternating
577 cycles of 30 days. Samples were taken monthly from February 2013 to
578 February 2014 from one wetland unit. The main conclusions are:

579 - The average abundance of Eubacteria was higher in the feed (59%) than in
580 the rest periods (30%). However, nitrifying and denitrifying bacteria remained
581 active even during the rest periods.

582 - Ammonia-oxidizing bacteria (AOB β-*proteobacteria*) were identified in the top
583 layer (0 to 0.17 m deep) of the wetland, both during feed (8%) and rest (3%)
584 periods.

585 - The identified nitrite-oxidizing bacteria (*Nistrospirae* and *Nitrospina gracilis*)
586 were present in the top and intermediate layers (0.17 to 0.34 m deep) of the
587 unit, but showing different dynamics in the feed and rest periods. When the
588 wetland unit was in operation these bacteria were present just in the top layer,
589 however when it was at rest they were also present in the intermediate layer.

590 -Denitrifying bacteria (*Pseudomonas* spp., *Paracoccus denitrificans* and
591 *versutus*, and *Thiobacillus denitrificans*) were identified from the top layer
592 downwards and were not affected by the rest periods.

593 - No seasonal or spatial influences were observed on bacteria abundance or
594 diversity.

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- In addition to the classical nitrification and denitrification, the occurrence of species such as *Paracoccus denitrificans*, *Paracoccus versutus* and *Thiobacillus denitrificans* was identified, which are associated with heterotrophic nitrification and aerobic and autotrophic denitrification.

- The saturated zone (0.51 to 0.68 m deep) of the filter was beneficial for the abundance of the bacterial domain as well as for the abundance of denitrifying bacteria.

-Different wetland layers provided distinct transformations of nitrogen. Nitrification was performed only in top (feed and rest periods) and intermediate (only in rest periods) layers. On the other hand, denitrification occurred from top layer in both periods. Nitrifying bacteria showed greater variation in relation to rest period, while, denitrifying bacteria remained stable in this periods. Based in bacteria community identified in wetland cell 3, simultaneous nitrification and denitrification occurred in this unit treatment.

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ACKNOWLEDGEMENTS

The authors would like to thank the National Council of Scientific and Technological Development (CNPq) for financial support, and Rotária do Brasil Company for operational support.

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844 **FIGURES**

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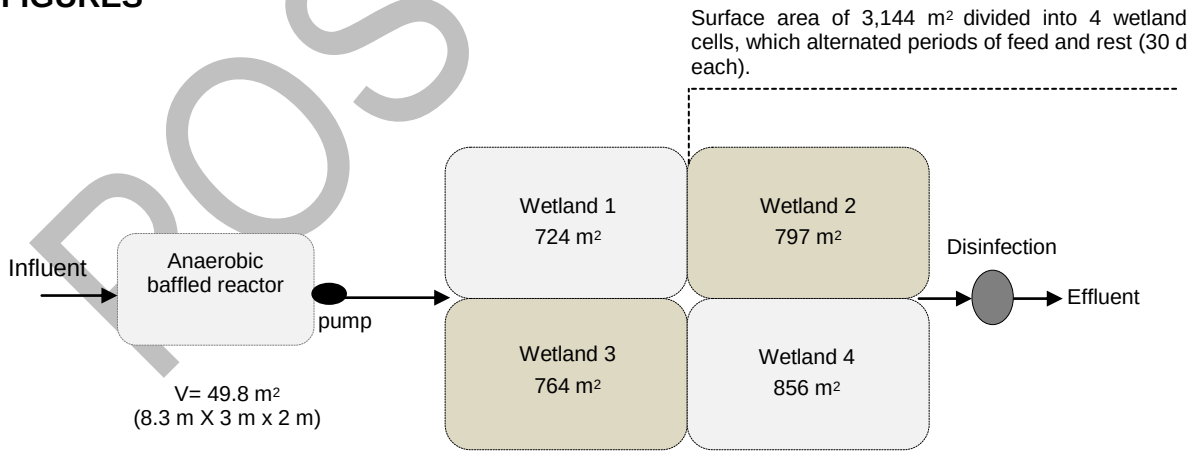

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859 **Figure 1.** Diagram of the partially-saturated vertical subsurface flow wetland.
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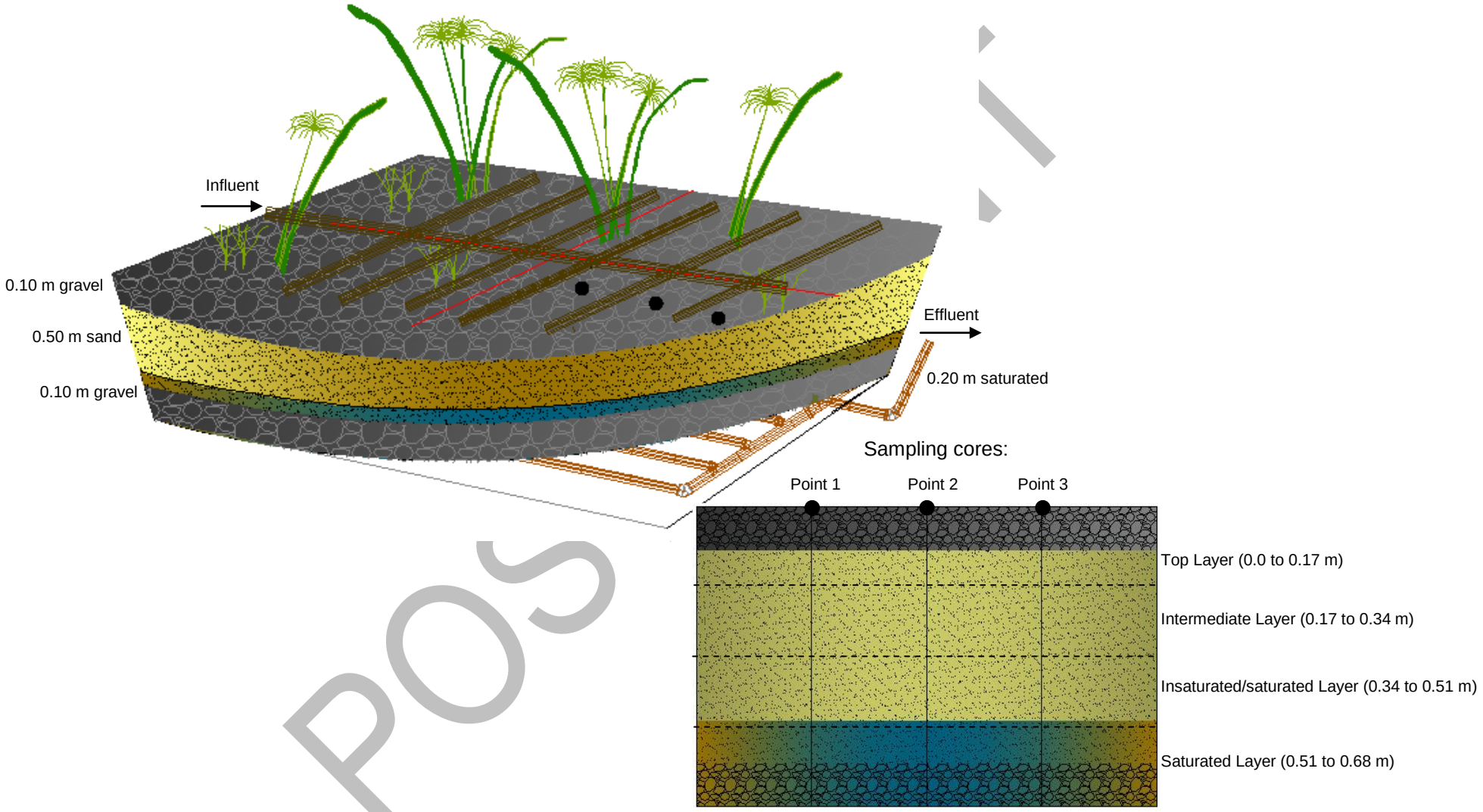

Figure 2. Representation of the sampling points within the bed media of the partially saturated vertical subsurface flow constructed wetland cell 3 at different depths and at different locations along the length of the unit.

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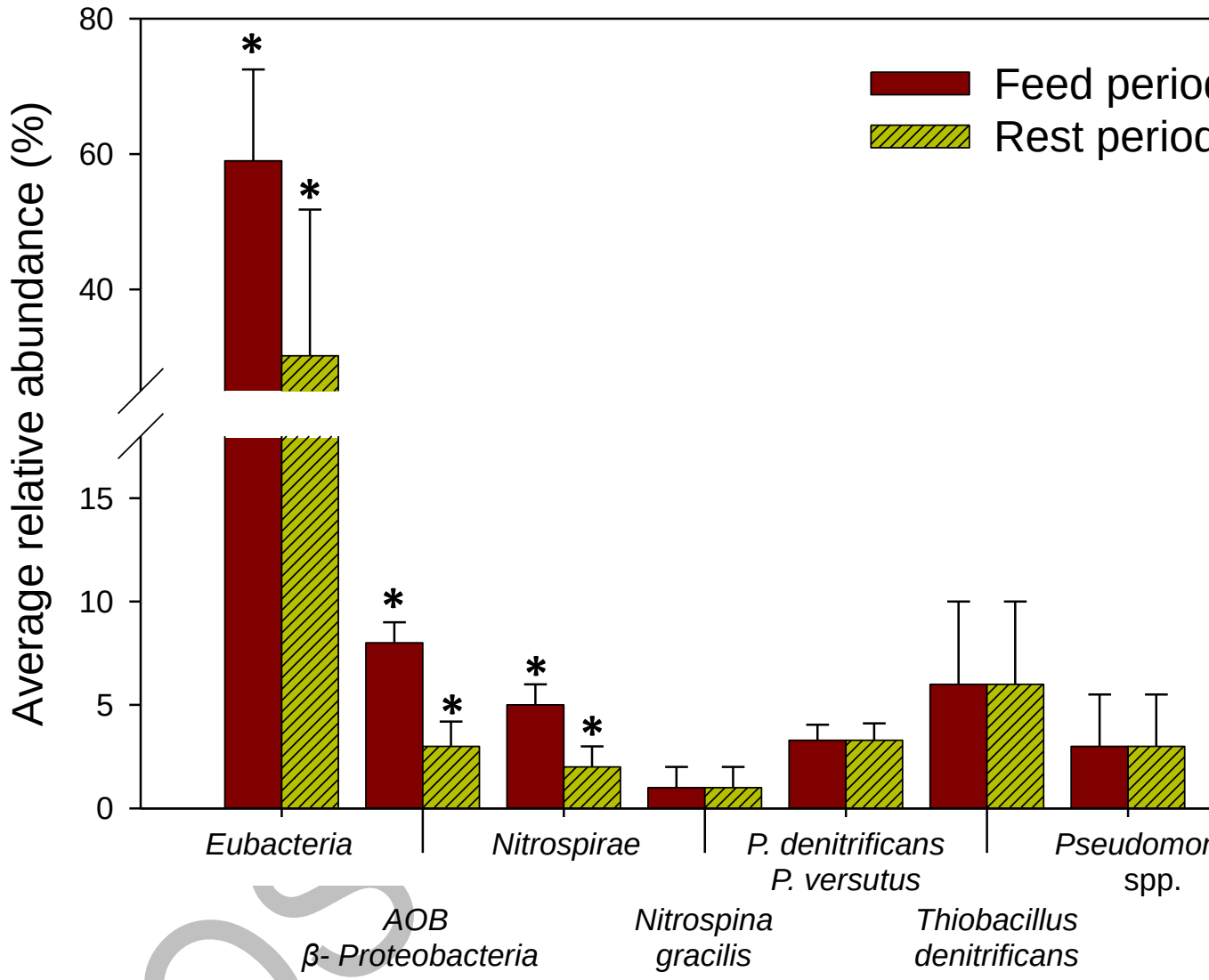

Figure 3. Mean (±SD) relative abundance of bacteria in the partially-saturated vertical subsurface flow wetland cell 3 in feed and rest periods

*Statistically significant : ($p<0.05$)

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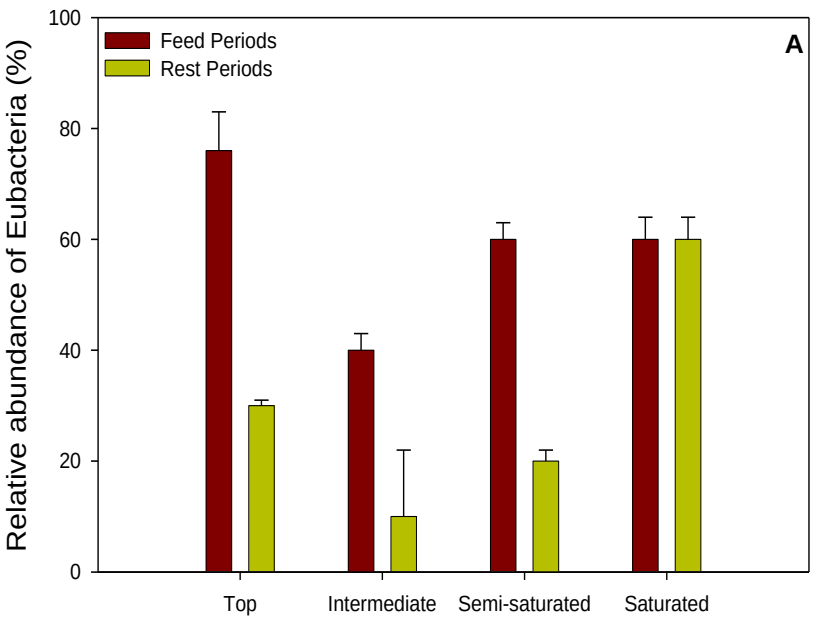
Feed Periods
Rest Periods
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Relative abundance of Eubacteria (%)
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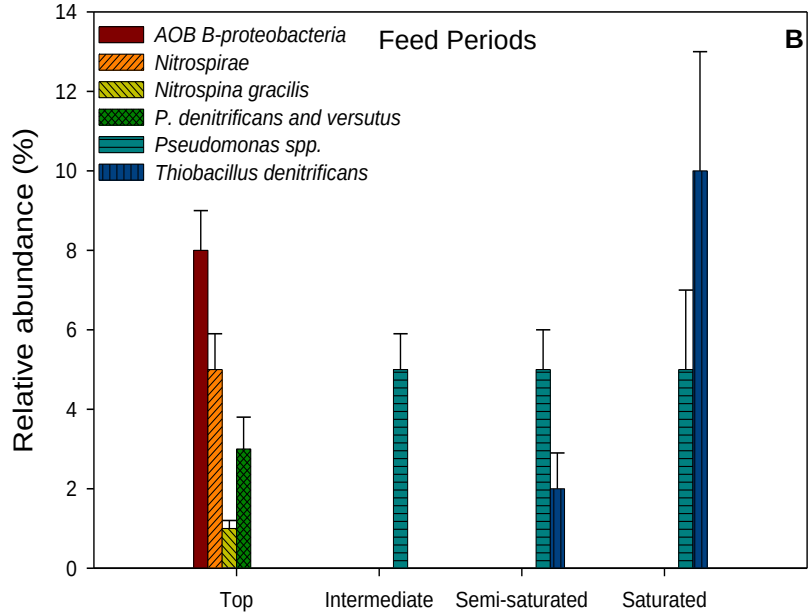
Semi-saturated
Saturated


AOB B-proteobacteria
Nitrospirae
Nitrospina gracilis
P. denitrificans and versutus
Pseudomonas spp.
Thiobacillus denitrificans
Feed Periods
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Relative abundance (%)
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Intermediate
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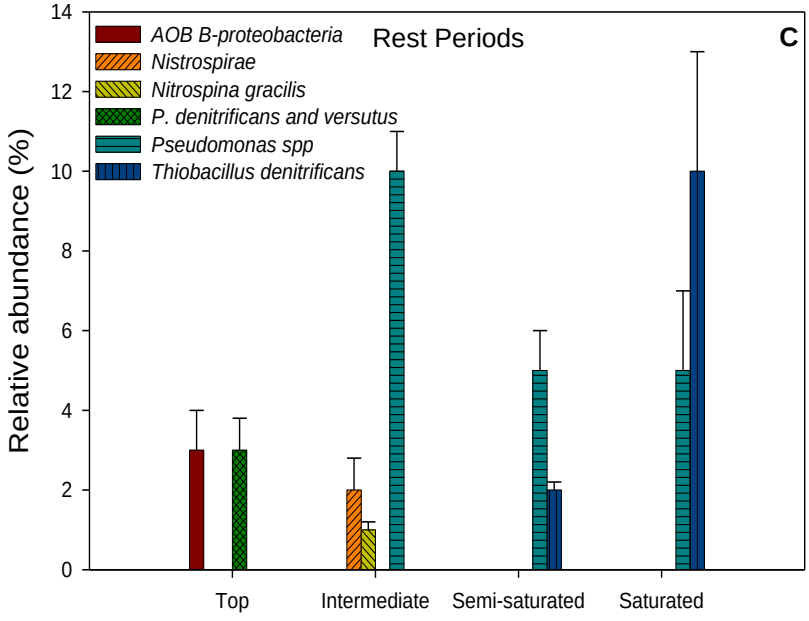
AOB B-proteobacteria
Nistrospirae
Nitrospina gracilis
P. denitrificans and versutus
Pseudomonas spp
Thiobacillus denitrificans
Rest Periods
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Top
Intermediate
Semi-saturated
Saturated

Figure 4. Mean (±SD) relative abundance of studied bacteria in different layers of wetland unit 3 during feed and rest periods. A) Relative abundance of Eubacteria in feed and rest periods at different layers. B) Nitrifying and denitrifying bacteria in different layers during feed periods. C) Nitrifying and denitrifying bacteria in different layers during rest periods.

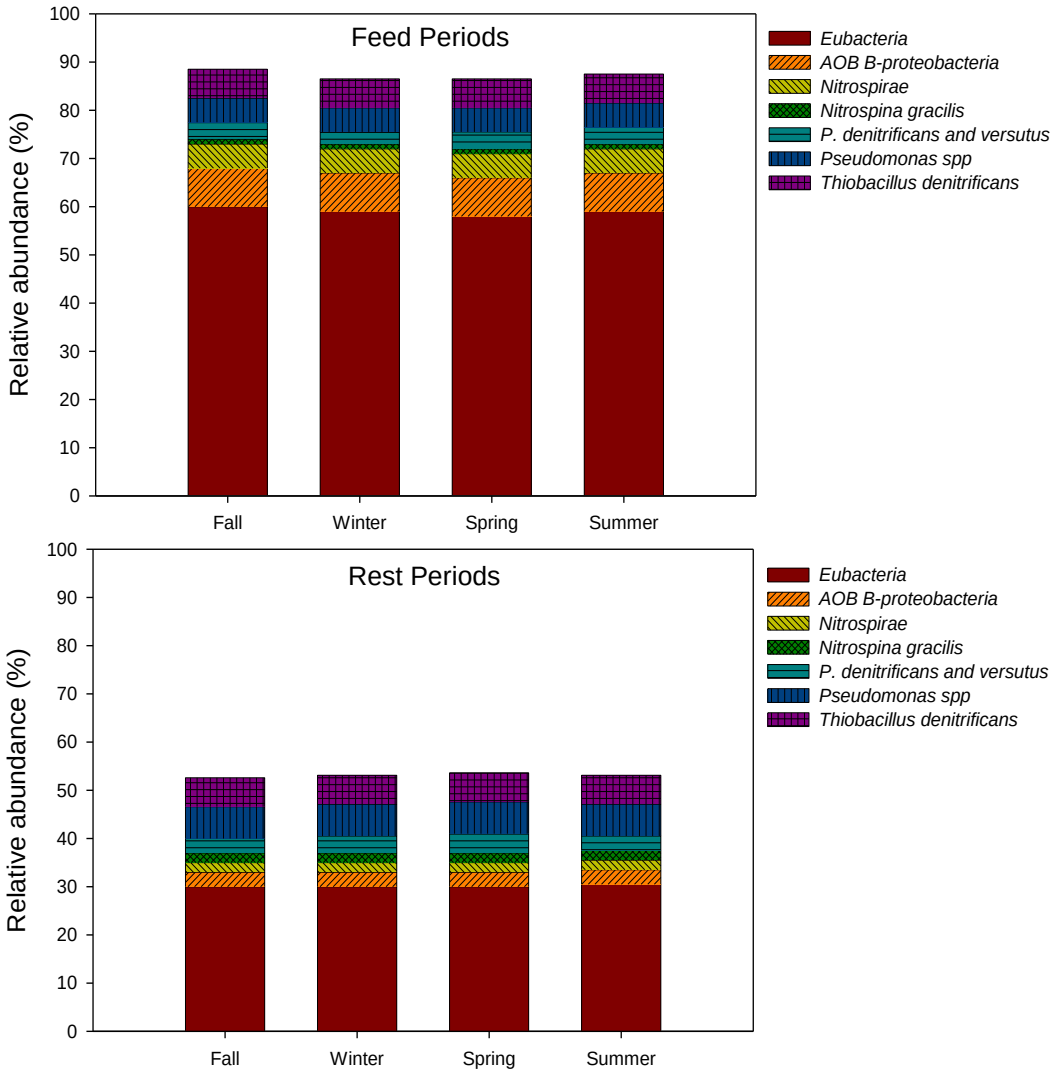

Figure 5. Average relative abundance of bacteria in the four layers of wetland unit 3 at the different seasons.

962 **TABLES**

963 **Table 1.** Probes used for identification of nitrifying and denitrifying bacterial communities.

Probes	Target group	Sequence (5'- 3')	Reference
EUB mix (I+II+III)	*Eubacteria*	I- CTGCCTCCCGTAGCA II- CAG CCACCCTAGGTGTCTG III- CCACCCGTAGGTGT	I – Amann *et al.* (1990) II e III – Daims *et al*. (1999)
PAE 997	*Pseudomonas* spp..	TCTGGAAAGTTCGCATCA	Amann *et al.* (1996)
NTSPA 662	*Nitrospirae*	CGCCTTCGCCACCGGCCTTCC	Daims *et al.* (2001)
NTSPN 693	*Nitrospina gracilis*	TTCCCAATATCAACGCATTT	Juretschko (2000)
NSO 190	*AOB β-Proteobacteria*	CGATCCCCTGCTTTTCTCC	Mobarry *et al.* (1996)
NEU 653	*Nitrosomonas* sp	CCCCTCTGCTGCACTACTCTA CCTGTGCTCCATGCTCCG	Wagner *et al*. (1995)
NIT 3	*Nitrobacter sp*	CCTGTGCTCCATGCTCCG	Wagner *et al*. (1996)
AMX 820	*Candidatus Brocadia* *Candidatus Kuenenia stuttgartiensis*	AAAACCCCTCTACTTGCCAGTC	Schmid *et al*. (2001)
PDV 163	*Paracoccus denitrificans* *Paracoccus versutus*	CTAATCCTTTGGCGATAAATC	Neef *et al.* (1996)
TBD 1419	*Thiobacillus denitrificans*	ACTTCTGCCAGATTCCAC	Fernández *et al.* (2008)

964

965 **Table 2**. Mean, median and standard deviation of wastewater quality parameters at the influent
966 and effluent wastewater of the partially saturated vertical subsurface flow constructed wetland,.

Parameter n=10	Influent VF			Effluent VF			Removal efficiency (%)
	Mean	Median	SD	Mean	Median	SD	
pH	6.9	7.0	0.4	6.5	6.5	0.3	-
Alkalinity (mg L^{-1})	206	212	68	120	115	36	-
TSS (mg L^{-1})	21	14	11	3	3	3	86
COD (mg L^{-1})	154	174	65	17	15	14	89
TN (mg L^{-1})	52	50	18	22	18	10	58
NH_4-N (mg L^{-1})	42	46	21	2	3	2	93
NO_2-N (mg L^{-1})	<LOD	-	-	<LOD	-	-	-
NO_3-N (mg L^{-1})	<LOD	-	-	10	10	7	-

967 <LOD: below limit detection

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