Unraveling the active microbial populations involved in nitrogen utilization in a vertical subsurface flow constructed wetland treating urban wastewater

Catiane Pelissari¹, Miriam Guivernau², Marc Viñas², Samara Silva de Souza³, Joan García⁴, Pablo Heleno Sezerino¹, Cristina Ávila⁴,⁵

¹GESAD - Decentralized Sanitation Research Group, Department of Sanitary and Environmental Engineering, Federal University of Santa Catarina, Trindade, Florianópolis, Santa Catarina, 88040-900, Brazil.

²GIRO Joint Research Unit IRTA-UPC, Research and Technology, Food and Agriculture (IRTA), Torre Marimon, E-08140, Caldes de Montbui, Barcelona, Catalonia, Spain.

³INTELAB - Integrated Technologies Laboratory, Chemical and Food Engineering Department, Federal University of Santa Catarina, Trindade, Florianópolis, Santa Catarina, 88040-900, Brazil.

⁴GEMMA - Environmental Engineering and Microbiology Research Group, Department of Civil and Environmental Engineering, Universitat Politècnica de Catalunya-BarcelonaTech, c/ Jordi Girona, 1-3, Building D1, E-08034, Barcelona, Spain.

⁵ICRA, Catalan Institute for Water Research, Scientific and Technological Park of the University of Girona, Emili Grahit, 101, E-17003 Girona, Spain

*Corresponding author: Cristina Ávila

Tel: +34 972183380
Fax: +34 972183248
Email: cavila@icra.cat
Abstract

The dynamics of the active microbial populations involved in nitrogen transformation in a vertical subsurface flow (VF) constructed wetland (VF) treating urban wastewater was/were?? evaluated. Aquí hay que decir que se consideraron 2 periodos que se diferenciaron en .... The VF wetland (1.5 m²) operated under average loads of 130 g COD m⁻² d⁻¹ and 17 g TN m⁻² d⁻¹ in Period I, and of 85 g COD m⁻² d⁻¹ and 19 g TN m⁻² d⁻¹ in Period II. The mean hydraulic loading rate was 375 mm d⁻¹ and C/N ratio was 2 in both periods. Samples for microbial characterization were collected from the filter medium (top and bottom layers) of the wetland, and from water inflow and outflow at the end of Periods I (Jun-Oct) and II (Nov-Jan). Decir los meses cuando se presentan los dos periodos (lo que he escrito arriba) The combination of qPCR and high throughput sequencing (NGS, MiSeq) assessment at DNA and RNA level of 16S rRNA genes and nitrogen-based functional genes (amoA and nosZ-clade I) revealed that nitrification was associated both with ammonia-oxidizing bacteria (AOB) (Nitrosospira) and ammonia-oxidizing archaea (AOA) (Nitrososphaeraceae), and nitrite-oxidizing bacteria (NOB) such as Nitrobacter. Considering the active abundance (based in amoA transcripts), the AOA population revealed to be more stable than AOB in both periods and depths of the wetland, being less affected by the organic loading rate (OLR). Although denitrifying bacteria (nosZ copies and transcripts) were actively detected in all depths, but the denitrification process was lower (removal of 2 g TN m⁻² d⁻¹ for both periods) as shown by NOx-N accumulation in the effluent. Overall, AOA, AOB and denitrifying bacteria (nosZ) were observed to be more active in the bottom than in the top layer at lower OLR (Period II). A proper design of OLR and hydraulic loading rate (HLR) seems to be crucial to control the activity of microbial biofilms in VF wetlands on the basis of oxygen, organic - carbon and NOx-N forms, to improve their capacity for total nitrogen removal.

Keywords: ammonia oxidizing bacteria, ammonia oxidizing archaea, metabolically-active populations, urban wastewater, High Throughput Sequencing.
1. INTRODUCTION

Constructed wetlands (CW) are engineered systems designed to simulate the conditions that occur in natural systems to treat wastewater (Kadlec and Wallace, 2009). This technology is under continuous development worldwide as a sustainable alternative for decentralized wastewater treatment in small communities or remote areas, due to its low energy consumption, ease of operation and provision of ecosystem services, and it has been widely employed for the treatment of different types of wastewater (García et al., 2010). Vertical subsurface flow (VF) constructed wetlands (VF) are one of the configurations of subsurface CW which holds greater oxygen transfer capacity due to its design (unsaturated bed) and operational mode (intermittent feeding), and require a smaller land area compared to other types of CW operating without air induction (Cui et al., 2010). Given their large oxygen transfer capacity, VF wetlands are mainly employed for nitrification and removal of organic matter (Platzer, 1999).

It has been proven that the nitrification capacity of VF wetlands is directly related to the applied organic loading rate (OLR), since the excess of organic compounds can affect the oxidation of ammonia due to the competition of oxygen between heterotrophic and autotrophic organisms (Saeed and Sun 2012; Sun et al., 1998). On the other hand, the presence of biodegradable organic compounds seems to promote the growth of denitrifying organisms (Headley et al., 2005). Therefore in general, nitrogen removal is associated with nitrification of ammonia nitrogen followed by denitrification of nitrate. In this way, nitrogen transformation in VF wetland is accomplished by ammonia-oxidizing bacteria (AOB), ammonia-oxidizing archaea (AOA), nitrite oxidizing bacteria (NOB) and to a lesser extent by denitrifying bacteria enriched in the biofilm of filter media, which are metabolically active depending on the specific linked to certain environmental conditions.

Several studies have been conducted to elucidate the bacterial dynamics involved in the nitrogen cycle in VF wetlands. In a VF wetland operated under OLR of 27 g COD m\(^{-2}\) d\(^{-1}\) was identified that *Nitrosomonas europaea*, *N. mobilis* and *Nitrosospira* were dominant AOBs in the filter media (Tietz et al., 2007). Guan et al. (2015) evaluated in three VF wetlands (1.2 m\(^2\)) the influence of
different substrates (sand, zeolite and gravel) and showed that the bacterial community was significantly influenced by substrate type. However, *Nitrospira* one of the NOB, was abundant in all units showing no influence of substrate type. Wu et al. (2016) using fluorescence in situ hybridization (FISH), reported that the growth of AOB and NOB in VF wetlands was enhanced by the use of intermittent aeration and of a specific substrate (sludge-ceramsite). Pelissari et al. (2016) showed how lower OLR (41 vs. 104 g COD m\(^{-2}\) d\(^{-1}\)) and hydraulic loading rates (HLR) favored simultaneous nitrifying and denitrifying bacteria in two VF wetland microcosms (microcosm 1, 41 g COD m\(^{-2}\) d\(^{-1}\), HLR of= 72 mm d\(^{-1}\); microcosm 2, 104 g COD m\(^{-2}\) d\(^{-1}\), HLR of 170. 5 mm d\(^{-1}\)). Recently, Pelissari et al., (2017) showed in a partially saturated full scale VF (full scale) showed that nitrifying bacteria are presents in the first layers of the filter bed (until 34 cm of depth), while denitrifying bacteria since top layer. Coban et al. (2015) described that anaerobic ammonia-oxidizing bacteria were marginal in CW running with urban wastewater.

Despite the progress achieved with modern molecular techniques, microbial dynamics involved in nitrogen transformations in VF wetlands are still unclear. Firstly, many of the microbiological studies carried out in CW refer to microbial abundance and do not demonstrate the active abundance microbial, which actually acts in the removal and transformation of nitrogen. Secondly, microbial processes in CW depend on environmental factors, properties of wastewater, substrate type, and operational conditions of the treatment units (Meng et al., 2014).

It has been well established in the literature that the autotrophic oxidation of ammonia is not only limited to the bacteria domain, but it is also performed by archaenal domain (Angnes et al., 2013; Konneke et al., 2005). Current, studies conducted in CW have demonstrated that bacterial diversity is greater than archaenal in VF and horizontal subsurface flow wetlands (HF), but ammonia-oxidizing archaea (AOA) and taxonomic assignment of archaea were not assessed (Adrados et al., 2014). The same behavior was observed in a free water surface (FWS) wetland, where archaenal communities showed lower richness and diversity than bacterial communities (Fan et al., 2016). Zhi and Ji (2014) reported in a tidal flow CW that archaea were not dominant in the
microbial community during the entire operation period. Oppositely, Sims et al. (2012) showed that AOA were found to be generally in higher abundance than AOB in FWS soils and water in both summer and winter over a period of two years. On the other hand, Paranychianakis et al. (2016) showed in planted and unplanted HF wetlands, the abundance of amoA genes of AOA was lower than that of AOB and plant species showed to have a weak effect on the abundance of AOA.

In spite of the knowledge gained in the abovementioned studies in regards to the dynamics between AOA and AOB, the contribution fraction of ammonia oxidizers (AOA vs. AOB) and their amoA gene expression between kingdoms in VF wetlands is still unknown (You et al., 2009). In addition, there are no studies evaluating the effect of operational conditions (different OLR) on nitrifying and denitrifying microbial populations. To our knowledge, the microbial community structure of active microbial populations (eubacteria and archaea) involved in nitrogen cycle in CW wetlands is scarcely known in the literature. The present study aims at gaining insight into the dynamics of active microbial populations during a nitrification-based process in a vertical flow constructed wetland treating urban wastewater under high OLR.

2. MATERIALS AND METHODS

2.1. Description of the wastewater treatment plant

This study was conducted in a VF wetland which was part of a hybrid CW system. The hybrid system was comprised of a primary treatment performed by an Imhoff tank, followed by a VF wetland stage, a HF wetland, and a FWS wetland in series. The experimental treatment plant is set outdoors at the experimental facility of the GEMMA group (Department of Civil and Environmental Engineering of the Universitat Politècnica de Catalunya-BarcelonaTech, Spain) in a Mediterranean climate. The treatment plant was commissioned in 2010, and up to the time of the current study the treatment system operated in a continuous mode under different organic and hydraulic loads over the years of operation (Ávila et al., 2016, 2014, 2013).
The VF wetland stage had a surface area of 3 m², divided into two cells with 1.5 m² of surface area each (1.0 W × 1.5 L × 1.3 D), operating alternatively in cycles of 3.5 days, in order to control the growth of attached biomass, maintain aerobic conditions within the filter bed and mineralize the organic deposits accumulated on the bed surface (Molle et al., 2008). The filter media was composed by a 0.1 m sand layer (ø = 1-2 mm) in the top, and 0.7 m layer of fine gravel (ø = 3-8 mm) underneath (Fig.1). The VF cells were constructed in polyethylene tanks, and a polyethylene pipe distributed the pumped water 0.1 m above the top of the bed. This pipe contained 5 perforations with diffusers that provided a true 360° radial horizontal water pattern, thus ensuring an evenly distribution of the wastewater over the whole surface of the filter. Water was pumped from the effluent of the Imhoff tank to the VF bed in operation in an intermittent mode, providing about 22 pulses per day (about 50 L pulse⁻¹). Each VF container had a metal tramex 0.1 m above floor level and a number of holes situated underneath it so as to allow for passive aeration of the bed. The aquatic macrophyte planted in all wetland units was Phragmites australis.

During the period of this study (Jun 2015 to Jan 2016), the hybrid system operated with a recirculation strategy, with the purpose of enhancing the removal of total nitrogen (see Ávila et al., submitted). A parcel of the final effluent of FWS was recycled back to the Imhoff tank by means of a peristaltic pump in a recirculation flow rate of 50% (RFR = daily recirculated effluent volume/daily raw wastewater volume x 100) (Fig 1). The performance of the hybrid system varied substantially during the implementation of the recirculation strategy owed to the poor performance of the FWS during the fall season, which was attributed to the senescence stage of macrophytes. The decay and decomposition of the plant biomass caused a steep increase in the concentration of organic matter and many other contaminants in the water table, which generated a high OLR applied in the VF wetlands (considered as Period I). As a remediation measure the aboveground biomass was harvested in this unit, and the pollutant loads recycled back to the Imhoff tank from the final effluent decreased, resulting in a lower OLR applied in the VF beds (considered as Period II). The determination of the microbial community structure and activity was carried out in one of the
two VF beds at the culmination of these two periods. In Period I (Jun-Oct) the VF wetland operated with an OLR of 130 g COD m\(^{-2}\) d\(^{-1}\), whereas in Period II (Nov-Jan) the OLR decreased to 85 g COD m\(^{-2}\) d\(^{-1}\). In both periods the VF wetland operated under a flow of 1.125 m\(^3\) d\(^{-1}\), resulting in a HLR of about and a HLR of 375 mm d\(^{-1}\) (taking into account the area of the two VF beds). Table 1 shows the operational conditions of each period.

An electromagnetic flow meter (Sitrans FM Magflo\®) was installed at the inlet and outlet of the VF wetland, so as to assist on the follow up of the flow values entering the treatment system, which allowed expressing the results on a mass balance basis. Physicochemical data from influent and effluent samples from the VF beds were determined twice a week throughout the whole study period. Some water quality parameters (i.e. temperature, pH, dissolved oxygen –DO-, electrical conductivity –EC- and redox potential –\(E_\text{H}^{-}\)) were determined onsite at the time of sample collection, and grab water samples were taken to the adjacent laboratory for the immediate analysis of the following parameters: total suspended solids (TSS), chemical oxygen demand (COD), biochemical oxygen demand (BOD\(_5\)), total organic carbon (TOC), total nitrogen (TN), ammonium nitrogen (NH\(_4\)-N), nitrate and nitrite nitrogen (NO\(_x\)-N).

Onsite measurements of water temperature, DO, pH and EC were taken by using a Checktemp-1 Hanna thermometer, a Eutech Ecoscan DO6 oxymeter, a Crison pH-meter and a EH CLM 381 conductivity meter, respectively. \(E_\text{H}^{-}\) was also measured onsite by using a Thermo Orion 3 Star redox meter and values were corrected for the potential of the hydrogen electrode. The determination of conventional wastewater quality parameters, including TSS and NH\(_4\)-N was done by following the Standard Methods (APHA, 2012). TN and TOC were analyzed using a Multi N/C (2100 S) analyzer. BOD\(_5\) was measured by using a WTW\® OxiTop\® BOD Measuring System. NO\(_x\)-N was analyzed using a DIONEX ICS-1000 chromatography system.

**2.2. Microbial community assessment**

To elucidate the microbial community dynamics involved in nitrogen transformation in the VF wetland, a DNA- vs RNA-based assessment of
functional genes (qPCR of amoA and nosZ genes versus 16S rRNA both for eubacteria and archaebacteria) was performed in order to quantify active microbial populations during nitrification and denitrification processes. Moreover, active eubacterial and archaeal microbial communities were deeply assessed by means of 16S rRNA-based high throughput sequencing (rRNA-based MiSeq) to identify the most predominant microbial key players which were enriched and active in the different depths of the VF wetland and during different OLR (Period I vs Period II). Nucleic acid extracts such as DNA, RNA and complementary DNA (cDNA) of functional genes and 16S rRNA genes were stored frozen at -80°C until analysis.

2.2.1. Sample collection and RNA/DNA extraction

In order to identify the microbial community involved in nitrogen utilization in the VF wetland and the effect of the OLR, water inflow, water outflow and filter media samples (gravel and sand) from the top (0-15 cm depth) and bottom (70-80 cm depth) layers were collected at two sampling campaigns. The first campaign took place at the end of Period I (October), after 5 months of VF wetland operation (OLR = 130 g COD m⁻² d⁻¹), and the second one was performed at the end of Period II (January), after 3 months of operation under lower OLR (85 g COD m⁻² d⁻¹). Samples were immediately submerged and mixed with 2 mL of LifeGuard Reagent (MO BIO, Inc., Carlsbad, CA) to prevent RNA degradation according to manufacturer’s instructions.

Simultaneous RNA + DNA extraction from approx. 0.25 g of filter media and 1mL pellet of water samples (20,000g/5' at 4°C) were extracted in triplicate for each period by using an adapted protocol of PowerMicrobiome™ RNA Isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA). The RNA extracts were treated during 10 minutes at 25°C with 10 units of DNase I (a room temperature stable DNase enzyme provided by the PowerMicrobiome Isolation kit) to remove any contamination of genomic DNA. All of the DNase I-treated RNAs were subjected to 16S rRNA-based PCR amplification as previously described (Prenafeta Boldú et al., 2012) to verify their purity. RNAs were subsequently transcribed to cDNA by means of PrimeScript™ RT reagent Kit (Perfect Real
Time, Takara) following the manufacturer's instructions. cDNA and DNA extracts were kept frozen at -80°C until further analysis.

2.2.2. Quantitative assessment of total, nitrifying and denitrifying microbial populations

- **Quantitative Polymerase Chain Reaction (qPCR)**

Quantitative analysis of total eubacterial population was conducted on the V3 hypervariable region of 16S rRNA (Prenafeta-Boldú et al., 2012). The denitrifying population was quantified by nosZ (clade I), the encoding gene of catalytic subunit of nitrous oxide reductase, as previously reported in Calderer et al. (2014). AOB and AOA population were quantified by means of ammonia monooxygenase α-subunit encoding genes (amoA_AOB (eubacteria) and amoA_AOA (archaea) genes, respectively). amoA_AOB abundance genes was performed as previously reported by Rotthauwe et al. (1997), whereas a new combination of primers for amoA_AOA genes was applied in the present study in order to include the known amoA-related AOA lineages (group I.1a: Nitrosopumilus cluster; group I.1a-associated: Nitrosotalea cluster; group I.1b: Nitrososphaera cluster; and ThAOA group: Nitrosocaldus cluster): CamoA19Fw 5’-ATGGTCTGGYTWAGACG-3’ (Pester M. et al., 2012) and Arch_amoAF_Rv 5’-GATGTCCARGCCCARTCAG-3’ (Wuchter et al., 2006). The reaction was performed in 10 µl volume containing 1 µl of DNA template, 400 nM of each primer, 5 µl of the ready reaction mix (Brilliant II SybrGreen qPCR Master Mix, Stratagene) and 30 nM of ROX reference dye. qPCR reaction was operated with the following protocol: 10 min at 95°C, followed by 40 cycles of denaturation at 95°C for 30 s; annealing for 30 s at 52°C, extension at 72°C for 45 s and the image capture was performed at 75°C for 35 s to exclude background fluorescence from the amplification of primer dimmers. All qPCR reactions were conducted in a Real Time PCR System MX3000P (Stratagene, La Jolla, CA). All samples were analyzed in triplicate by means of three independent cDNA and DNA extracts.

For the standard curve of each target gene, it was designed by using FunGene data base (http://fungene.cme.msu.edu/) five gBlocks® Gene Fragments (IDT, Integrated DNA Technologies). Ten-fold serial dilutions from synthetic genes
were subjected to qPCR assays in duplicate showing a linear range between $10^1$ and $10^8$ gene copy numbers per reaction to generate standard curves. qPCR reactions fitted quality standards: efficiencies were between 90-110% and $R^2$ above 0.985. All results were processed by MxPro™ QPCR Software (Stratagene, La Jolla, CA) and were treated statistically.

2.2.3 Active microbial community abundance and diversity

- Next Generation Sequencing (NGS)

A 16S rRNA based metabarcoding assessment through MiSeq platform was performed to study the diversity of active microbial populations. Transcribed 16S rRNA libraries targeting V1-V3 and V3-V4 regions from eubacterial and archaeal population, respectively, were sequenced by utilizing MiSeq Illumina sequencing platform at Molecular Research DNA following manufacturer’s instructions. For the eubacterial and archaeal libraries, the primer set 27F (5’-AGRGTGGATCMTGGCTCAG-3’)/519R (5’-GTNTTACNGCGGCKGCTG-3’) and 349F (5’-GYGCASCAGKCGMGAAW-3’)/806R (5’-GGACTACVSGGCTATCTAAT-3’) were used, respectively.

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 further bioinformatic processing. Trimming of the 16S rRNA barcoded sequences into libraries was carried out using QIIME software version 1.8.0 (Caporaso et al., 2010). Quality filtering of the reads was performed at Q25, prior to the grouping into Operational Taxonomic Units (OTUs) at a 97% sequence homology cutoff. The following steps were performed using QIIME: Denoising using Denoiser (Reeder and Knight, 2010); reference sequences for each OTU (OTU picking up) were obtained via the first method of UCLUST algorithm (Edgar, 2010); for sequence alignment and chimera detection the algorithms PyNAST (Caporaso et al., 2010b) and ChimeraSlayer (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).
Data from MiSeq NGS assessment were submitted to the Sequence Read Archive (SRA) of the National Center for Biotechnology Information (NCBI) under the accession number SRP090290.

2.3 Statistical data analyses

Normality of the data of water quality parameters was tested by Kolmogorov – Smirnov test. Furthermore, Student’s \( t \) test \((p< 0.05)\) was used to test whether there were statistically significant differences on (i) water quality of samples between Periods I and II; (ii) bacterial and archaeal abundance between the top and bottom layer samples within the same period; (iii) bacterial and archaeal abundance between Periods I and II. For statistical analysis Statistic 7.0 software was used (Statsoft Inc, 2004).

3. RESULTS AND DISCUSSION

3.1 Treatment performance of the vertical subsurface flow constructed wetland

Recommendations regarding design OLR loads to be applied in VF wetlands vary in relation to climatic conditions. For warm climates, Hoffmann et al. (2011) recommends a range of 60 to 70 g COD m\(^{-2}\) d\(^{-1}\), for subtropical climates the recommendation is 41 g COD m\(^{-2}\) d\(^{-1}\) (Sezerino et al., 2012), and under cold climate the indicated OLR decreases to 20 g COD m\(^{-2}\) d\(^{-1}\) (Winter and Goetz, 2003). The VF wetland in the current study operated under high OLR (average of 130 g COD m\(^{-2}\) d\(^{-1}\) and 80 g COD m\(^{-2}\) d\(^{-1}\) in Periods I and II, respectively) as can be observed by influent COD and BOD\(_5\) values in Table 2. However, the TN load applied to the VF unit was very similar in the two periods. Despite the high HLR, the performance of the VF wetland was generally high and stable overtime, showing a great capacity of the VF wetland to handle large loads, observing no signs of clogging of the filter bed throughout the whole study period.
Average load removal efficiencies were very similar in Periods I and II, exhibiting values of about 50% COD and BOD$_5$, 70% NH$_4$-N and 20% TN. High organic load removal rates were achieved, observing mean values of 73 g COD m$^{-2}$ d$^{-1}$ and 45 g BOD$_5$ m$^{-2}$ d$^{-1}$ in Period I, and 32 g COD m$^{-2}$ d$^{-1}$ and 21 g BOD$_5$ m$^{-2}$ d$^{-1}$ in Period II. These results are in accordance with other studies which report that, the higher the organic load, the greater the removal of organic carbon in CW (Calheiros et al., 2007; Saeed and Sun, 2012).

The removal of the ammonium nitrogen load was also similar in both periods (4 g NH$_4$-N m$^{-2}$ d$^{-1}$). Nitrification is the main mechanism associated with the elimination of NH$_4$-N in VF wetlands (Kadlec and Wallace, 2009). Although effluent NOx-N values were similar in both periods (10 ± 4 mg NOx L$^{-1}$ in Period I and 15 ± 3 mg NOx L$^{-1}$ in Period II) (Fig. 2). The average TN removal rate was of 2 g TN m$^{-2}$ d$^{-1}$ (20% load removal) in both periods (Fig. 2), which is in agreement with other studies in VF wetlands, owed to the low denitrification capacity of these systems due to the prevailing aerobic conditions within the filter bed, which hinder the establishment of denitrifying microorganisms (Saeed and Sun 2012; Vymazal, 2013).

### 3.2 Microbial community assessment

#### 3.2.1 Quantification of nitrifying and denitrifying population

Eubacterial populations exhibited variability on the metabolic activity and active diversity as a function of the filter depth and OLR applied in the VF wetland (Fig. 3a). In Period I (130 g COD m$^{-2}$ d$^{-1}$) eubacteria were more active in top than in the bottom layer of the wetland ($10^{12}$ and $10^{10}$ 16S rRNA transcripts g$^{-1}$, respectively). Greatest microbial abundance has been reported to occur in the top layer of VF wetlands by previous studies, being attributed to the higher availability of organic matter and nutrients in the surface of the unit (Foladori et al., 2015; Tietz et al., 2008). However, in the current study, the activity of eubacteria decreased in the top layer ($10^{10}$ 16S rRNA transcripts g$^{-1}$) and increased in bottom layer ($10^{12}$ 16S rRNA transcripts g$^{-1}$) when the OLR decreased (Period II). This stratification may be associated with greater
availability of oxygen along the vertical profile of the filter medium promoted by
the lower OLR applied in this period.

Nitrification was identified as an active process in the top and bottom layers of
the VF wetland throughout the study, where ammonia oxidizing bacteria (AOB)
and archaea (AOA), and phylotypes related to nitrite oxidizing bacteria (NOB)
belonging to *Nitrobacter* genus were actively detected (Fig. 3 b, c and 5).

Independently of the applied OLR, total AOB were more abundant than AOA
populations in both periods and depths, being $10^6$ *amoA*~AOB~ copies g$^{-1}$
quantified in Period I, and $10^7$ *amoA*~AOB~ copies g$^{-1}$ in Period II, in both layers;
and $10^5$ *amoA*~AOA~ copies g$^{-1}$ for Periods I and II, in both layers.

Paranychianakis et al. (2016) showed higher abundance of *amoA*~AOB~ gene
copies in respect to *amoA*~ AOA~ in pilots 6 units of CW with conditions of
horizontal flow (planted and unplanted) fed with synthetic wastewater. Lower
abundance of AOA has been reported to be presumably caused by a lack of
ecological niche variables in CW (Correa-Galeote et al., 2013). However, the
previous studies conducted in CW were not performed at gene expression level
of *amoA*, and therefore no information regarding the effect of environmental
variables such as the OLR on the metabolic activity of ammonia-oxidizers has
been described so far.

Current results concerning the active biomass at gene expression level (*amoA
transcripts*) showed different dynamics of the ammonia-oxidizing population
than those previously observed. The active AOA community showed a stable
throughout the study, showing high resilience to changes in organic load.
Moreover, in Period I, when the VF wetland operated under higher OLR, alike
active archaeal and bacterial abundance was recorded in top and bottom layers
($10^6$ and $10^5$ *amoA* transcripts g$^{-1}$ in top and bottom respectively). However, in
Period II at lower OLR, AOB activity decreased in the top layer ($10^5$ *amoA
transcripts g$^{-1}$) and increased in the bottom layer ($10^6$ *amoA* transcripts g$^{-1}$),
while, AOA activity remained stable ($10^6$ *amoA* transcripts g$^{-1}$ in both layers).

Environmental conditions seem to be fundamental in the growth and
development of stable and specialized ammonia-oxidizing communities (Fan et
al., 2016). AOA have been detected over a wide pH range, whereas AOB are
neutrophilic and their highest growth rate occurs at pH 7 to 7.5 (Prosser and
Nicol, 2012). qPCR results show how at higher oxygen availability across the filter bed (under lower ORL) and more availability of carbon in top layer, AOB exhibited their highest activity at the bottom layer. Differently, metabolically active AOA remained more stable. As previously described, increasing oxygen concentrations enhanced enrichment of AOB, whereas the archaeal population was almost oxygen-insensitive.

In relation to the denitrification, *nosZ* gene abundance (clade I) was similar in both periods and along the depth of the filter bed (10^7 *nosZ* copies g^-1). Nevertheless, *nosZ* gene copies were always lower in respect to *nosZ* gene transcripts (Fig. 3d). In Period I greater activity of denitrifying bacteria was identified in the top (10^6 *nosZ* transcripts g^-1) than in the bottom (10^4 *nosZ* transcripts g^-1) of the wetland. When the OLR was decreased (Period II), the activity of denitrifying bacteria showed a similar behavior than AOB, decreasing in the top layer (10^5 transcripts g^-1) and increasing in the bottom layer (10^6 transcripts g^-1). These results suggest that under conditions of high carbon concentrations the denitrification could occur in the surface layers at low oxygen availability (Period I). When the OLR was decreased (Period II), a higher oxygen transfer capacity would displace the denitrifying community to the bottom of the wetland. This community may also be linked to the activity of the nitrifying community in this part of the wetland, which would help decreasing oxygen availability in the filter, thus promoting the denitrification activity both inside the biofilms and in planktonic cells.

Globally, ammonia oxidizers (eubacteria and archaea), as well as denitrifying bacteria (*nosZ*) were detected to be more active in the bottom layer during Period II at lower OLR (Fig 3 b, c, and d), compared with top layer and Period I, which would confirm the occurrence of higher simultaneous active nitrifying-denitrifying process in the VF wetland at a specific range of organic load.

Figure 4 shows the ratio of genes transcripts vs. genes copies of bacterial and archaeal *amoA* and *nosZ* in top and bottom layers of the VF wetland at the two sampling campaigns. Regardless of OLR applied in the VF wetland, AOA was the active nitrifying community more abundant along the vertical profile of wetland, whereas AOB activity was highly dependent on the OLR. Interestingly, AOB could be also be influenced by the availability of carbon. High carbon
availability resulted in higher specific growth rate of heterotrophic organisms (compared to autotrophic) and promoting a rapid consumption of available oxygen (Saeed and Sun, 2012). This also would also end up promoting the displacement of the nitrifying bacteria to the lower part of the filter, where the availability of organic carbon compounds would be lesser (Salomo and Roske, 2009). Transcript levels of nosZ gene were lower than amoA gene in both periods, which was in accordance the low denitrification potential observed in VF wetlands (Vymazal, 2013).

3.2.2 Active microbial community diversity

High-throughput bacterial 16S rRNA (cDNA) sequencing detected 3,263 and 112 OTUs with 48,525-92,518 and 62,921-55,563 reads, for eubacteria and archaea, respectively. Fig. 5 and 6 shows the relative abundance (RA) of the active eubacterial and archaeal populations, present in water inflow and the biofilm established at gravel samples in Period II.

Active microbial community from water inflow is dominated by Gammaproteobacteria (40% RA), Epsilonbacteria (19% RA) and Flavobacteria (16% RA) classes, whereas the active biofilm from the filter media of the VF wetland presented a different microbial community, showing more diverse and similar between layers (see SM). In the top layer Deltaproteobacteria (21% RA) was the predominant class followed by Alphaproteobacteria (18% RA), Planctomycetia and Actinobacteria (both at 12% RA). The profile of active bacteria in the bottom layer was slightly different being Alphaproteobacteria the most active class (22% RA), followed by Gammaproteobacteria, Planctomycetia, Acidobacteria_Gp4, Betaproteobacteria (all classes at 9% RA). Interestingly Deltaproteobacteria was marginal at the bottom layer accounting for 6% of RA.

NGS results revealed that eubacterial populations of biofilms attached to bed material (gravel-sand) were clearly represented by metabolically active families linked to the nitrogen cycle (Graf et al., 2014). Regarding the active AOB population, Nitrosomonadaceae family (1% and 0.8% RA at top and bottom layers, respectively) were represented by OTUs belonging to the genus Nitrosospira (see supplementary material- Figure S1) that could accumulate
nitrite in the oxygenated layers of the biofilm and CWs. Interestingly, recently it has been described that all known AOB are able to conduct nitrifier-denitrification by means of nitrite reductases (nir genes) and nitric oxide reductases (nor genes), favoring the transformation of NO$_2^-$ to N$_2$O under low O$_2$ environments (Kozlowski et al., 2016; Zhu et al., 2013).

Active NOBs were represented by OTUs belonging to the genus *Nitrobacter* (4% RA at both layers) that belongs to the order *Rhizobiales* (*Bradyrhizobiaceae* family) (Fig. 6). The high revealed activity of *Nitrobacter* could be related with the accumulation of nitrate observed in the present study and enhanced at lower OLR. *Nitrobacter* have a low-nitrite affinity, high growth rate and develop large populations when nitrite is present at high concentrations (Andrews and Harris, 1986). In this way, the active presence of AOB and AOA populations in the VF wetland could favor the nitrite accumulation and the potential activity of *Nitrobacter* genus even at the bottom layer.

Active archaeal community structure and diversity was completely different between inflow and filter media. Active methanogenic archaea were highly specialized in the biofilms accounting for 99.4% of active archaeal population in water inflow and for less than 5% in biofilms in the top layer and 20% in the bottom layer. Archaeal active biofilms on filter media were clearly dominated by AOA with a relative abundance of *Nitrososphaeraceae* accounting for 97.5% at top layer and 78.4% at bottom layer. It is obviously the establishment and activity of AOA population, becoming an important group of the filter media biofilm as previously observed by qPCR quantification (Fig 3b). The main representative OTU of the AOA family was OTU2 that belongs to the genera *Nitrososphaera* (see supplementary material- Figure S1). That sequence showed 100% of similarity (NCBI Blastn) with Archaeon G61 (KR233006.1), belonging to the new genus *Candidatus Nitrosofontus exaquare* (*Nitrososphaeraceae*), that were found in a municipal wastewater treatment plant. Despite the fact that AOA could accumulate nitrite, Sauder et al. (2016) found the important role of available nitric oxide (NO) as a key player of *Thaumarchaeotal* ammonia oxidizing pathway. In The microbial community from the biofilm that could generate NO i.e. *Planctomycetia*, could enhance the ammonia-oxidizing activity of AOA.
Jin et al. (2010) showed that AOB community was more sensitive than of AOA to operational conditions, such as ammonia loading rate and dissolved oxygen in a nitrogen-removing reactor. Lower active abundance of AOB in relation AOA can be associated with higher OLR applied in the wetland, which favored the activity of heterotrophic bacteria, such as *Myxococcales* that was identified as active biomass in higher abundance (20% in top and 5% in bottom layers) (Fig. 6). *Myxococcales* live in environments with lots of decomposed organic matter, and are gliding bacteria commonly found in soils and activated sludge that are thought to significantly impact biomass carbon (Luerders et al., 2006).

On the other hand, denitrifying bacteria were found in low abundance and activity in the filter bed of the VF wetland (Fig. 6) observing accumulation of NO$_3$-N in the effluent. The active abundance of *Pseudomonadales* (*Pseudomonadaceae* family; *Pseudomonas* genus – see supplementary material- Figure S1) was higher in the influent wastewater (38% RA). However, in the filter bed of the wetland the active abundance of *Pseudomonadaceae* was significantly lower (2% in top and 5% in bottom layers). The same behavior was identified for other well-known denitrifying bacteria in wastewater such as *Opitutus* (*Opitutaceae* family; *Opitutus* genus – see supplementary material-Figure S1) and *Clostridiales* (*Peptostreptococcaceae* family; *Clostridium XI* genus), which indicated a low stability of active denitrifying bacteria in the filter bed, probably due the overall high oxygen availability. However, they were detected in both periods, when active methanogenic bacteria were identified.

### 4. CONCLUSION

This study showed the microbial population dynamics involved in nitrogen transformation of a vertical flow constructed wetland operated under high OLR (Period I: 130 g COD m$^{-2}$ d$^{-1}$; Period II: 85 g COD m$^{-2}$ d$^{-1}$). COD and BOD$_5$ removal load rates were higher in Period I (73 g COD m$^{-2}$ d$^{-1}$ and 45 g BOD$_5$ m$^{-2}$ d$^{-1}$) than Period II (32 g COD m$^{-2}$ d$^{-1}$ and 21 g BOD$_5$ m$^{-2}$ d$^{-1}$), demonstrating the great capacity of the wetland to handle large organic loads. NH$_4$-N –an TN removal rates were similar in both periods (4 g NH$_4$-N m$^{-2}$ d$^{-1}$ and 2 g TN m$^{-2}$ d$^{-1}$).
The combination of qPCR and NGS at RNA level revealed that the nitrification process was associated with AOB (*Nitrosospira*), AOA (*Nitrososphaeraceae*) and NOB (*Nitrobacter*). AOB populations were observed more abundant (at DNA level) than AOA in both layers. However, considering the active abundance (based in amoA transcripts) the ammonia oxidizing population dynamics was inverted being AOA population more stable in both periods and depths. Although denitrifying bacteria (*nosZ* copies and transcripts) were detected active in the filter bed, it was not enough to minimize NO$_2$-N accumulation in the water effluent.

Ammonia oxidation was performed mainly by AOB (*Nitrosospira*) and AOA (*Nitrososphaeraceae*). Nitrite oxidation was accomplished by NOB (*Nitrobacter*) both in top and bottom layers. Although the denitrifying community was metabolically active in the CW, denitrifying microbial populations were not highly enriched in the biofilm of VF wetland.

A proper design of OLR and HLR becomes crucial in VF wetlands to control the activity of microbial biofilms on the basis of oxygen, organic carbon and NO$_x$-N available forms in the water phase and the biofilm, in order to promote adequate conditions for an efficient nitrification-denitrification processes to enhance total nitrogen removal from wastewater.

**ACKNOWLEDGEMENTS**

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Figure 1. Diagram of the treatment system. a) Sampling points of inflow and outflow wastewater. b) Sampling collects of filter media from vertical subsurface flow constructed wetland.
Figure 2. Nitrogen transformation in the vertical subsurface flow constructed wetland in Period I (130 g COD m⁻² d⁻¹) and II (85 g COD m⁻² d⁻¹). a: Statistical significance between the two periods (p<0.05).
Figure 3. Average of nitrogen functional genes identified in top (0-15 cm) and bottom (70-80 cm) layers of the filter bed of the vertical subsurface flow constructed wetland in the two microbiological sampling campaigns: Period I (ORL= 130 g COD m$^{-2}$ d$^{-1}$) and Period II (ORL= 85 g COD m$^{-2}$ d$^{-1}$). a) Abundance of 16 S rDNA and 16 S rRNA; b) Abundance of bacterial $amoA$ genes and transcripts; c) Abundance of archaeal $amoA$ genes and transcripts; d) Abundance of $nosZ$ genes and transcripts.

a: Statistical significance observed in the layer between the two periods (p<0.05);
b: Statistical significance observed between top and bottom layers within the same period (p<0.05);
Figure 4. Ratio of genes transcripts and genes copies of bacterial and archaeal amoA and nosZ in top (0-15 cm) and bottom layers (70-80 cm) of the vertical flow wetland at the two microbiological sampling campaigns: Period I (ORL= 130 g COD m\(^{-2}\) d\(^{-1}\)) and Period II (ORL= 85 g COD m\(^{-2}\) d\(^{-1}\)).
Figure 5. Taxonomic assignment of sequencing reads (MiSeq) from the active eubacterial community (16S rRNA-based cDNA) of water inflow, and filter media from top and bottom layers of Period II at order level. 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 a relative abundance lower than 1% were categorised as ‘others’. Taxonomic assignment of individual datasets using the RDP Bayesian Classifier with a bootstrap cut-off of 80%.
Figure 6. Taxonomic assignment of sequencing reads from the active archaeal community (16S rRNA based cDNA) of water inflow, and filter media from top and bottom layers of Period II at family level. 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 a relative abundance lower than 0.5% were categorised as ‘others’. Taxonomic assignment of individual datasets using the RDP Bayesian Classifier with a bootstrap cut-off of 80%
**TABLES**

Table 1. Operational conditions of the vertical subsurface flow constructed wetland in Periods I and II.

<table>
<thead>
<tr>
<th>Operational conditions</th>
<th>Period I (Jun-Oct)</th>
<th>Period II (Nov-Jan)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration (months)</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Air temperature °C</td>
<td>25</td>
<td>14</td>
</tr>
<tr>
<td>Flow (m³ d⁻¹)</td>
<td>1.125</td>
<td>1.125</td>
</tr>
<tr>
<td>HLR (mm d⁻¹)</td>
<td>375</td>
<td>375</td>
</tr>
<tr>
<td>OLR (g COD m² d⁻¹)</td>
<td>130</td>
<td>85</td>
</tr>
<tr>
<td>TN (g m² d⁻¹)</td>
<td>17</td>
<td>19</td>
</tr>
<tr>
<td>*C/N Ratio influent</td>
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*Ratio performed between TOC/TN

Table 2. Average (±SD) concentration and loads of water quality parameters at the influent and effluent of the vertical subsurface flow constructed wetland in Periods I and II.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Period I (Jun-Oct)</th>
<th>Period II (Nov-Jan)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Influent</td>
<td>Effluent</td>
</tr>
<tr>
<td></td>
<td>Mean concentration removal</td>
<td>Influent</td>
</tr>
<tr>
<td>T (°C)</td>
<td>23 ± 5</td>
<td>23 ± 5</td>
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<tr>
<td>DO (mg L⁻¹)</td>
<td>0.5 ± 0.2</td>
<td>2.5 ± 1</td>
</tr>
<tr>
<td>EC (mS cm⁻¹)</td>
<td>2 ± 0.5</td>
<td>2 ± 0.4</td>
</tr>
<tr>
<td>E₄₇₃ (mV)</td>
<td>-101 ± 66</td>
<td>+181 ± 67</td>
</tr>
<tr>
<td>pH</td>
<td>7.5 ± 0.3</td>
<td>7.5 ± 0.3</td>
</tr>
<tr>
<td>TSS (mg L⁻¹)</td>
<td>72 ± 31</td>
<td>55 ± 65</td>
</tr>
<tr>
<td>COD (mg L⁻¹)</td>
<td>347 ± 104</td>
<td>207 ± 88</td>
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<tr>
<td>BOD₅ (mg L⁻¹)</td>
<td>223 ± 88</td>
<td>91 ± 50</td>
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<tr>
<td>TOC (mg L⁻¹)</td>
<td>90 ± 30</td>
<td>45 ± 21</td>
</tr>
<tr>
<td>TN (mg L⁻¹)</td>
<td>46 ± 10</td>
<td>36 ± 9</td>
</tr>
<tr>
<td>NH₄-N (mg L⁻¹)</td>
<td>18 ± 7</td>
<td>6.5 ± 3</td>
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<tr>
<td>*NO₃-N (mg L⁻¹)</td>
<td>&lt;LOD</td>
<td>10 ± 4</td>
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<table>
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<tr>
<th>Parameters</th>
<th>Load applied (g m² d⁻¹)</th>
<th>Load removal (g m² d⁻¹)</th>
<th>Load removal %</th>
<th>Load applied (g m² d⁻¹)</th>
<th>Load removal (g m² d⁻¹)</th>
<th>Load removal %</th>
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<tbody>
<tr>
<td>COD (g m² d⁻¹)</td>
<td>130 ± 39</td>
<td>73 ± 36</td>
<td>50 ± 24%</td>
<td>80 ± 8</td>
<td>32 ± 9</td>
<td>51 ± 10%</td>
</tr>
<tr>
<td>BOD (g m² d⁻¹)</td>
<td>79 ± 38</td>
<td>45 ± 39</td>
<td>58 ± 10%</td>
<td>46 ± 9</td>
<td>21 ± 6</td>
<td>46 ± 8%</td>
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<tr>
<td>TN (g m² d⁻¹)</td>
<td>17 ± 4</td>
<td>2 ± 2</td>
<td>23 ± 12%</td>
<td>19 ± 5</td>
<td>2 ± 2</td>
<td>21 ± 6%</td>
</tr>
<tr>
<td>NH₄-N (g m² d⁻¹)</td>
<td>7 ± 2</td>
<td>4 ± 2</td>
<td>71 ± 8%</td>
<td>6 ± 1</td>
<td>4 ± 1</td>
<td>67 ± 7%</td>
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</table>

<LOD: below limit of detection
* Statistical significance between the periods (p<0.05)