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

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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~~ers~~ (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 NO_x-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 NO_x-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.CITA

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 present~~s~~ 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 archaeal domain (Angnes et al., 2013; Konneke et al., 2005). Current, studies conducted in CW have demonstrated that bacterial diversity is greater than archaeal 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 archaeal 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

136 microbial community during the entire operation period. Oppositely, Sims et al.
137 (2012) showed that AOA were found to be generally in higher abundance than
138 AOB in FWS soils and water in both summer and winter over a period of two
139 years. On the other hand, Paranychianakis et al. (2016) showed in planted and
140 unplanted HF wetlands, the abundance of *amoA* genes of AOA was lower than
141 that of AOB and plant species showed to have a weak effect on the abundance
142 of AOA.

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

154

155 ## 2. MATERIALS AND METHODS

156

157 ### 2.1. Description of the wastewater treatment plant

158 This study was conducted in a VF wetland which was part of a hybrid CW
159 system. The hybrid system was comprised of a primary treatment performed by
160 an Imhoff tank, followed by a VF wetland stage, a HF wetland, and a FWS
161 wetland in series. The experimental treatment plant is set outdoors at the
162 experimental facility of the GEMMA group (Department of Civil and
163 Environmental Engineering of the Universitat Politècnica de Catalunya-
164 BarcelonaTech, Spain) in a Mediterranean climate. The treatment plant was
165 commissioned in 2010, and up to the time of the current study the treatment
166 system operated in a continuous mode under different organic and hydraulic
167 loads over the years of operation (Ávila et al., 2016, 2014, 2013).

168 The VF wetland stage had a surface area of 3 m^2, divided into two cells with 1.5
169 m^2 of surface area each (1.0 W × 1.5 L × 1.3 D), operating alternatively in
170 cycles of 3.5 days, in order to control the growth of attached biomass, maintain
171 aerobic conditions within the filter bed and mineralize the organic deposits
172 accumulated on the bed surface (Molle et al., 2008). The filter media was
173 composed by a 0.1 m sand layer (ø = 1-2 mm) in the top, and 0.7 m layer of fine
174 gravel (ø = 3-8 mm) underneath (Fig.1). The VF cells were constructed in
175 polyethylene tanks, and a polyethylene pipe distributed the pumped water 0.1 m
176 above the top of the bed. This pipe contained 5 perforations with diffusers that
177 provided a true 360 ◦ radial horizontal water pattern, thus ensuring an evenly
178 distribution of the wastewater over the whole surface of the filter. Water was
179 pumped from the effluent of the Imhoff tank to the VF bed in operation in an
180 intermittent mode, providing about 22 pulses per day (about 50 L $pulse^{-1}$). Each
181 VF container had a metal tramex 0.1 m above floor level and a number of holes
182 situated underneath it so as to allow for passive aeration of the bed. The
183 aquatic macrophyte planted in all wetland units was *Phragmites australis*.

184 During the period of this study (Jun 2015 to Jan 2016), the hybrid system
185 operated with a recirculation strategy, with the purpose of enhancing the
186 removal of total nitrogen (see Ávila et al., submitted COMO LO VAN A VER SI
187 NO ESTÁ PUBLICADO???). A parcel of the final effluent of FWS was
188 recycledback to the Imhoff tank by means of a peristaltic pump in a recirculation
189 flow rate of 50% (RFR = daily recirculated effluent volume/daily raw wastewater
190 volume x 100) (Fig 1). The performance of the hybrid system varied
191 substantially during the implementation of the recirculation strategy owed to the
192 poor performance of the FWS during the fall season, which was attributed to the
193 senescence stage of macrophytes. The decay and decomposition of the plant
194 biomass caused a steep increase in the concentration of organic matter and
195 many other contaminants in the water table, which generated a high OLR
196 applied in the VF wetlands (considered as Period I). As a remediation measure
197 the aboveground biomass was harvested in this unit, and the pollutant loads
198 recycled back to the Imhoff tank from the final effluent decreased, resulting in a
199 lower OLR applied in the VF beds (considered as Period II). The determination
200 of the microbial community structure and activity was carried out in one of the

201 two VF beds at the culmination of these two periods. In Period I (Jun-Oct) the
202 VF wetland operated with an OLR of 130 g COD m^{-2} d^{-1}, whereas in Period II
203 (Nov-Jan) the OLR decreased to 85 g COD m^{-2} d^{-1}. In both periods the VF
204 wetland operated under a flow of 1.125 m^3 d^{-1}, resulting in a HLR of about and a
205 HLR of 375 mm d^{-1} (taking into account the area of the two VF beds). Table 1
206 shows the operational conditions of each period.

207 An electromagnetic flow meter (Sitrans F M Magflo®) was installed at the inlet
208 and outlet of the VF wetland, so as to assist on the follow up of the flow values
209 entering the treatment system, which allowed expressing the results on a mass
210 balance basis. Physicochemical data from influent and effluent samples from
211 the VF beds were determined twice a week throughout the whole study period.
212 Some water quality parameters (i.e. temperature, pH, dissolved oxygen –DO-,
213 electrical conductivity –EC- and redox potential –E_H) were determined onsite at
214 the time of sample collection, and grab water samples were taken to the
215 adjacent laboratory for the immediate analysis of the following parameters: total
216 suspended solids (TSS), chemical oxygen demand (COD), biochemical oxygen
217 demand (BOD_5), total organic carbon (TOC), total nitrogen (TN), ammonium
218 nitrogen (NH_4-N), nitrate and nitrite nitrogen (NO_x-N).

219 Onsite measurements of water temperature, DO, pH and EC were taken by
220 using a Checktemp-1 Hanna thermometer, a Eutech Ecoscan DO6 oxymeter, a
221 Crison pH-meter and a EH CLM 381 conductivity meter, respectively. E_H was
222 also measured onsite by using a Thermo Orion 3 Star redox meter and values
223 were corrected for the potential of the hydrogen electrode. The determination of
224 conventional wastewater quality parameters, including TSS and NH_4-N was
225 done by following the Standard Methods (APHA, 2012). TN and TOC were
226 analyzed using a Multi N/C (2100 S) analyzer. BOD_5 was measured by using a
227 WTW® OxiTop® BOD Measuring System. NO_x-N^- was analyzed using a
228 DIONEX ICS-1000 chromatography system.

229

230 **2.2. Microbial community assessment**

231

232 To elucidate the microbial community dynamics involved in nitrogen
233 transformation in the VF wetland, a DNA- vs RNA-based assessment of

234 functional genes (qPCR of *amoA* and *nosZ* genes versus 16S rRNA both for
235 eubacteria and archaea) was performed in order to quantify active microbial
236 populations during nitrification and denitrification processes. Moreover, active
237 eubacterial and archaeal microbial communities were deeply assessed by
238 means of 16S rRNA-based high throughput sequencing (rRNA-based MiSeq) to
239 identify the most predominant microbial key players which were enriched and
240 active in the different depths of the VF wetland and during different OLR (Period
241 I vs Period II). Nucleic acid extracts such as DNA, RNA and complementary
242 DNA (cDNA) of functional genes and 16S rRNA genes were stored frozen at -
243 80ºC until analysis.
244

245 *2.2.1. Sample collection and RNA/DNA extraction*
246

247 In order to identify the microbial community involved in nitrogen utilization in the
248 VF wetland and the effect of the OLR, water inflow, water outflow and filter
249 media samples (gravel and sand) from the top (0-15 cm depth) and bottom (70-
250 80 cm depth) layers were collected at two sampling campaigns. The first
251 campaign took place at the end of Period I (Oct~~ober~~), after 5 months of VF
252 wetland operation (OLR = 130 g COD m^{-2} d^{-1}), and the second one was
253 performed at the end of Period II (Jan~~uary~~), after 3 months of operation under
254 lower OLR (85 g COD m^{-2} d^{-1}). Samples were immediately submerged and
255 mixed with 2 mL of LifeGuard Reagent (MO BIO, Inc., Carlsbad, CA) to prevent
256 RNA degradation according to manufacturer's instructions.

257 Simultaneous RNA + DNA extraction from approx. 0.25 g of filter media and
258 1mL pellet of water samples (20.000g/5' at 4ºC) were extracted in triplicate for
259 each period by using an adapted protocol of PowerMicrobiome™ RNA Isolation
260 kit (MO BIO Laboratories, Inc., Carlsbad, CA). The RNA extracts were treated
261 during 10 minutes at 25°C with 10 units of DNase I (a room temperature stable
262 DNase enzyme provided by the PowerMicrobiome Isolation kit) to remove any
263 contamination of genomic DNA. All of the DNase I-treated RNAs were
264 subjected to 16S rRNA-based PCR amplification as previously described
265 (Prenafeta Boldú et al., 2012) to verify their purity. RNAs were subsequently
266 transcribed to cDNA by means of PrimeScript™ RT reagent Kit (Perfect Real

267 Time, Takara) following the manufacturer's instructions. cDNA and DNA extracts
268 were kept frozen at -80ºC until further analysis.

269 *2.2.2. Quantitative assessment of total, nitrifying and denitrifying microbial*
270 *populations*

271

272 *- Quantitative Polymerase Chain Reaction (qPCR*)
273 Quantitative analysis of total eubacterial population was conducted on the V3
274 hypervariable region of 16S rRNA (Prenafeta Boldú et al., 2012). The
275 denitrifying population was quantified by *nosZ* (clade I), the encoding gene of
276 catalytic subunit of nitrous oxide reductase, as previously reported in Calderer
277 et al. (2014). AOB and AOA population were quantified by means of ammonia
278 monooxygenase α-subunit encoding genes (a*moA*_AOB (eubacteria) and
279 *amoA*_AOA (archaea) genes, respectively). *amoA*_AOB abundance genes was
280 performed as previously reported by Rotthauwe et al. (1997), whereas a new
281 combination of primers for *amoA*_AOA genes was applied in the present study
282 in order to include the known *amoA*-related AOA lineages (group I.1a:
283 *Nitrosopumilus* cluster; group I.1a-associated: *Nitrosotalea* cluster; group I.1b:
284 *Nitrososphaera* cluster; and ThAOA group: *Nitrosocaldus* cluster): CamoA19Fw
285 5'-ATGGTCTGGYTWAGACG-3' (Pester M. et al., 2012) and Arch_amoAF_Rv
286 5'-GATGTCCARGCCCARTCAG-3' (Wuchter et al., 2006). The reaction was
287 performed in 10 µl volume containing 1 µl of DNA template, 400 nM of each
288 primer, 5 µl of the ready reaction mix (Brilliant II SybrGreen qPCR Master Mix,
289 Stratagene) and 30 nM of ROX reference dye. qPCR reaction was operated
290 with the following protocol: 10 min at 95°C, followed by 40 cycles of
291 denaturation at 95°C for 30 s; annealing for 30 s at 52ºC, extension at 72°C for
292 45 s and the image capture was performed at 75°C for 35 s to exclude
293 background fluorescence from the amplification of primer dimmers. All qPCR
294 reactions were conducted in a Real Time PCR System MX3000P (Stratagene,
295 La Jolla, CA). All samples were analyzed in triplicate by means of three
296 independent cDNA and DNA extracts.

297 For the standard curve of each target gene, it was designed by using FunGene
298 data base (http://fungene.cme.msu.edu/) five gBlocks® Gene Fragments (IDT,
299 Integrated DNA Technologies).Ten-fold serial dilutions from synthetic genes

300 were subjected to qPCR assaysin duplicate showing a linear range between 10^1
301 and 10^8 gene copy numbers per reaction to generate standard curves. qPCR
302 reactions fitted quality standards: efficiencies were between 90-110% and R^2
303 above 0.985. All results were processed by MxPro™ QPCR Software
304 (Stratagene, La Jolla, CA) and were treated statistically.
305
306 *2.2.3 Active microbial community abundance and diversity*
307
308 - Next Generation Sequencing (NGS)
309 A 16S rRNA based metabarcoding assessment through MiSeq platform was
310 performed to study the diversity of active microbial populations. Transcribed
311 16S rRNA libraries targeting V1-V3 and V3-V4 regions from eubacterial and
312 archaeal population, respectively, were sequenced by utilizing MiSeq Illumina
313 sequencing platform at Molecular Research DNA following manufacturer's
314 instructions. For the eubacterial and archaeal libraries, the primer set 27F (5'-
315 AGRGTTTGATCMTGGCTCAG–3') / 519R (5'-GTNTTACNGCGGCKGCTG-3')
316 and 349F (5'-GYGCASCAGKCGMGAAW-3') / 806R (5'–
317 GGACTACVSGGGTATCTAAT-3') were used, respectively.
318 Downstream MiSeq data analysis was carried out by using QIIME software
319 version 1.8.0. The obtained DNA reads were compiled in FASTq files for further
320 bioinformatic processing. Trimming of the 16S rRNA barcoded sequences into
321 libraries was carried out using QIIME software version 1.8.0 (Caporaso et al.,
322 2010). Quality filtering of the reads was performed at Q25, prior to the grouping
323 into Operational Taxonomic Units (OTUs) at a 97% sequence homology cutoff.
324 The following steps were performed using QIIME: Denoising using Denoiser
325 (Reeder and Knight, 2010); reference sequences for each OTU (OTU picking
326 up) were obtained via the first method of UCLUST algorithm (Edgar, 2010); for
327 sequence alignment and chimera detection the algorithms PyNAST (Caporaso
328 et al., 2010b) and ChimeraSlayer (Haas et al., 2011) were used. OTUs were
329 then taxonomically classified using BLASTn against GreenGenes and RDP
330 (Bayesian Classifier) database and compiled into each taxonomic level
331 (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.

363 Average load removal efficiencies were very similar in Periods I and II,
364 exhibiting values of about 50% COD and BOD_5, 70% NH_4-N and 20% TN. High
365 organic load removal rates were achieved, observing mean values of 73 g COD
366 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
367 m^{-2} d^{-1} in Period II. These results are in accordance with other studies which
368 report that, the higher the organic load, the greater the removal of organic
369 carbon in CW (Calheiros et al., 2007; Saeed and Sun, 2012).

370 The removal of the ammonium nitrogen load was also similar in both periods (4
371 g NH4-N m^{-2} d^{-1}). Nitrification is the main mechanism associated with the
372 elimination of NH_4-N in VF wetlands (Kadlec and Wallace, 2009). Although
373 effluent NOx-N values were similar in both periods (10 ± 4 mg NO_x L^{-1} in Period
374 I and 15 ± 3 mg NO_x L^{-1} in Period II) (Fig. 2).The average TN removal rate was
375 of 2 g TN m^{-2} d^{-1} (20% load removal) in both periods (Fig. 2), which is in
376 agreement with other studies in VF wetlands, owed to the low denitrification
377 capacity of these systems due to the prevailing aerobic conditions within the
378 filter bed, which hinder the establishment of denitrifying microorganisms (Saeed
379 and Sun 2012; Vymazal, 2013).

380

381 **3.2 Microbial community assessment**

382

383 *3.2.1 Quantification of nitrifying and denitrifying population*

384 Eubacterial populations exhibited variability on the metabolic activity and active
385 diversity as a function of the filter depth and OLR applied in the VF wetland (Fig.
386 3a). In Period I (130 g COD m^{-2} d^{-1}) eubacteria were more active in top than in
387 the bottom layer of the wetland (10^{12} and 10^{10} 16S rRNA transcripts g^{-1},
388 respectively). Greatest microbial abundance has been reported to occur in the
389 top layer of VF wetlands by previous studies, being attributed to the higher
390 availability of organic matter and nutrients in the surface of the unit (Foladori et
391 al., 2015; Tietz et al., 2008). However, in the current study, the activity of
392 eubacteria decreased in the top layer (10^{10} 16S rRNA transcripts g^{-1}) and
393 increased in bottom layer (10^{12} 16S rRNA transcripts g^{-1}) when the OLR
394 decreased (Period II). This stratification may be associated with greater

395 availability of oxygen along the vertical profile of the filter medium promoted by
396 the lower OLR applied in this period.

397 Nitrification was identified as an active process in the top and bottom layers of
398 the VF wetland throughout the study, where ammonia oxidizing bacteria (AOB)
399 and archaea (AOA), and phylotypes related to nitrite oxidizing bacteria (NOB)
400 belonging to *Nitrobacter* genus were actively detected (Fig. 3 b, c and 5).
401 Independently of the applied OLR, total AOB were more abundant than AOA
402 populations in both periods and depths, being 10^6 *amoA*_AOB copies g^{-1}
403 quantified in Period I, and 10^7 *amoA*_AOB copies g^{-1} in Period II, in both layers;
404 and 10^5 *amoA*_AOA copies g^{-1} for Periods I and II, in both layers.
405 Paranychianakis et al. (2016) showed higher abundance of *amoA*_AOB gene
406 copies in respect to *amoA*_ AOA in pilots 6 units of CW with conditions of
407 horizontal flow (planted and unplanted) fed with synthetic wastewater. Lower
408 abundance of AOA has been reported to be presumably caused by a lack of
409 ecological niche variables in CW (Correa-Galeote et al., 2013). However, the
410 previous studies conducted in CW were not performed at gene expression level
411 of *amoA*, and therefore no information regarding the effect of environmental
412 variables such as the OLR on the metabolic activity of ammonia-oxidizers has
413 been described so far.

414 Current results concerning the active biomass at gene expression level (*amoA*
415 transcripts) showed different dynamics of the ammonia-oxidizing population
416 than those previously observed. The active AOA community showed a stable
417 throughout the study, showing high resilience to changes in organic load.
418 Moreover, In Period I, when the VF wetland operated under higher OLR, alike
419 active archaeal and bacterial abundance was recorded in top and bottom layers
420 (10^6 and 10^5 *amoA* transcripts g^{-1} in top and bottom respectively). However, in
421 Period II at lower OLR, AOB activity decreased in the top layer (10^5 *amoA*
422 transcripts g^{-1}) and increased in the bottom layer (10^6 *amoA* transcripts g^{-1}),
423 while, AOA activity remained stable (10^6 *amoA* transcripts g^{-1} in both layers).
424 Environmental conditions seem to be fundamental in the growth and
425 development of stable and specialized ammonia-oxidizing communities (Fan et
426 al., 2016). AOA have been detected over a wide pH range, whereas AOB are
427 neutrophilic and their highest growth rate occurs at pH 7 to 7.5 (Prosser and

428 Nicol, 2012). qPCR results show how at higher oxygen availability across the
429 filter bed (under lower ORL) and more availability of carbon in top layer, AOB
430 exhibited their highest activity at the bottom layer. Differently, metabolically
431 active AOA remained more stable. As previously described, increasing oxygen
432 concentrations enhanced enrichment of AOB, whereas the archaeal population
433 was almost oxygen-insensitive.

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

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

455 Figure 4 shows the ratio of genes transcripts vs. genes copies of bacterial and
456 archaeal *amoA* and *nosZ* in top and bottom layers of the VF wetland at the two
457 sampling campaigns. Regardless of OLR applied in the VF wetland, AOA was
458 the active nitrifying community more abundant along the vertical profile of
459 wetland, whereas AOB activity was highly dependent on the OLR. Interestingly,
460 AOB could be also be influenced by the availability of carbon. High carbon

461 availability resulted in higher specific growth rate of heterotrophic organisms
462 (compared to autotrophic) and promoting a rapid consumption of available
463 oxygen (Saeed and Sun, 2012). This also would also end up promoting the
464 displacement of the nitrifying bacteria to the lower part of the filter, where the
465 availability of organic carbon compounds would be lesser (Salomo and Roske,
466 2009). Transcript levels of *nosZ* gene were lower than *amoA* gene in both
467 periods, which was in accordance the low denitrification potential observed in
468 VF wetlands (Vymazal, 2013).

469

470 *3.2.2 Active microbial community diversity*

471 High-throughput bacterial 16S rRNA (cDNA) sequencing detected 3,263 and
472 112 OTUs with 48,525-92,518 and 62,921-55,563 reads, for eubacteria and
473 archaea, respectively. Fig. 5 and 6 shows the relative abundance (RA) of the
474 active eubacterial and archaeal populations, present in water inflow and the
475 biofilm stablished at gravel samples in Period II.

476 Active microbial community from water inflow is dominated by
477 *Gammaproteobacteria* (40% RA), *Epsilonbacteria* (19% RA) and *Flavobacteriia*
478 (16% RA) classes, whereas the active biofilm from the filter media of the VF
479 wetland presented a different microbial community, showing more diverse and
480 similar between layers (see SM). In the top layer *Deltaproteobacteria* (21% RA)
481 was the predominant class followed by *Alphaproteobacteria* (18% RA),
482 *Planctomycetia* and *Actinobacteria* (both at 12% RA). The profile of active
483 bacteria in the bottom layer was slightly different being *Alphaproteobacteria* the
484 most active class (22% RA), followed by *Gammaproteobacteria*, *Plantomycetia*,
485 *Acidobacteria*_Gp4, *Betaproteobacteria* (all classes at 9% RA). Interestingly
486 *Deltaproteobacteria* was marginal at the bottom layer accounting for 6% of RA.

487 NGS results revealed that eubacterial populations of biofilms attached to bed
488 material (gravel-sand) were clearly represented by metabolically active families
489 linked to the nitrogen cycle (Graf et al., 2014). Regarding the active AOB
490 population, *Nitrosomonadaceae* family (1% and 0.8% RA at top and bottom
491 layers, respectively) were represented by OTUs belonging to the genus
492 *Nitrosospira* (see supplementary material- Figure S1) that could accumulate

493 nitrite in the oxygenated layers of the biofilm and CWs. Interestingly, recently it
494 has been described that all known AOB are able to conduct nitrifier-
495 denitrification by means of nitrite reductases (*nir* genes) and nitric oxide
496 reductases (*nor* genes), favoring the transformation of NO_2^- to N_2O under low
497 O_2 environments (Kozlowski et al., 2016; Zhu et al., 2013).

498 Active NOBs were represented by OTUs belonging to the genus *Nitrobacte*r
499 (4% RA at both layers) that belongs to the order *Rhizobiales*
500 (*Bradyrhizobiaceae* family) (Fig. 6). The high revealed activity of *Nitrobacter*
501 could be related with the accumulation of nitrate observed in the present study
502 and enhanced at lower OLR. *Nitrobacter* have a low-nitrite affinity, high growth
503 rate and develop large populations when nitrite is present at high concentrations
504 (Andrews and Harris, 1986). In this way, the active presence of AOB and AOA
505 populations in the VF wetland could favor the nitrite accumulation and the
506 potential activity of *Nitrobacter* genus even at the bottom layer.

507 Active archaeal community structure and diversity was completely different
508 between inflow and filter media. Active methanogenic archaea were highly
509 specialized in the biofilms accounting for 99.4% of active archaeal population in
510 water inflow and for less than 5% in biofilms in the top layer and 20% in the
511 bottom layer. Archaeal active biofilms on filter media were clearly dominated by
512 AOA with a relative abundance of *Nitrososphaeraceae* accounting for 97.5% at
513 top layer and 78.4% at bottom layer. It is obviously the establishment and
514 activity of AOA population, becoming an important group of the filter media
515 biofilm as previously observed by qPCR quantification (Fig 3b). The main
516 representative OTU of the AOA family was OTU2 that belongs to the genera
517 *Nitrososphaera* (see supplementary material- Figure S1). That sequence
518 showed 100% of similarity (NCBI Blastn) with Archaeon G61 (KR233006.1),
519 belonging to the new genus *Candidatus Nitrosofontus exaquare*
520 (*Nitrosophaeracea*e), that were found in a municipal wastewater treatment
521 plant. Despite the fact that AOA could accumulate nitrite, Sauder et al. (2016)
522 found the important role of available nitric oxide (NO) as a key player of
523 *Thaumarchaeotal* ammonia oxidizing pathway. In The microbial community from
524 the biofilm that could generate NO i.e. *Planctomycetia*, could enhance the
525 ammonia-oxidizing activity of AOA.

526 Jin et al. (2010) showed that AOB community was more sensitive than of AOA
527 to operational conditions, such as ammonia loading rate and dissolved oxygen
528 in a nitrogen-removing reactor. Lower active abundance of AOB in relation AOA
529 can be associated with higher OLR applied in the wetland, which favored the
530 activity of heterotrophic bacteria, such as *Myxococcales* that was identified as
531 active biomass in higher abundance (20% in top and 5% in bottom layers) (Fig.
532 6). *Myxococcales* live in environments with lots of decomposed organic matter,
533 and are gliding bacteria commonly found in soils and activated sludge that are
534 thought to significantly impact biomass carbon (Luerders et al., 2006).

535 On the other hand, denitrifying bacteria were found in low abundance and
536 activity in the filter bed of the VF wetland (Fig. 6) observing accumulation of
537 NO_x-N in the effluent. The active abundance of *Pseudomonadales*
538 (*Pseudomonadaceae* family; *Pseudomonas* genus – see supplementary
539 material- Figure S1) was higher in the influent wastewater (38% RA). However,
540 in the filter bed of the wetland the active abundance of *Pseudomonadaceae*
541 was significantly lower (2 % in top and 5% in bottom layers). The same behavior
542 was identified for other well-known denitrifying bacteria in wastewater such as
543 *Opitutus* (*Opitutaceae* family; *Opitutus* genus – see supplementary material-
544 Figure S1) and *Clostridiales* (*Peptostreptococcaceae* family; *Clostridium XI*
545 genus), which indicated a low stability of active denitrifying bacteria in the filter
546 bed, probably due the overall high oxygen availability. However, they were
547 detected in both periods, when active methanogenic bacteria were identified.
548

549 ## 4. CONCLUSION

550

551 This study showed the microbial population dynamics involved in nitrogen
552 transformation of a vertical flow constructed wetland operated under high OLR
553 (Period I: 130 g COD m^{-2} d^{-1}; Period II: 85 g COD m^{-2} d^{-1}). COD and BOD_5
554 removal load rates were higher in Period I (73 g COD m^{-2} d^{-1} and 45 g BOD_5 m^{-2}
555 d^{-1}) than Period II (32 g COD m^{-2} d^{-1} and 21 g BOD_5 m^{-2} d^{-1}), demonstrating the
556 great capacity of the wetland to handle large organic loads. NH_4-N –an TN
557 removal rates were similar in both periods (4 g NH_4-N m^{-2} d^{-1} and 2 g TN m^{-2} d^{-1}).
558

559 The combination of qPCR and NGS at RNA level revealed that the nitrification
560 process was associated with AOB (*Nitrosospira*), AOA (*Nitrososphaeraceae*)
561 and NOB (*Nitrobacter)*. AOB populations were observed more abundant (at
562 DNA level) than AOA in both layers. However, considering the active abundance
563 (based in *amoA* transcripts) the ammonia oxidizing population dynamics was
564 inverted being AOA population more stable in both periods and depths.
565 Although denitrifying bacteria (*nosZ* copies and transcripts) were detected
566 active in the filter bed, it was not enough to minimize NO_x-N accumulation in the
567 water effluent.

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

573 A proper design of OLR and HLR becomes crucial in VF wetlands to control the
574 activity of microbial biofilms on the basis of oxygen, organic carbon and NO_x-N
575 available forms in the water phase and the biofilm, in order to promote adequate
576 conditions for an efficient nitrification-denitrification processes to enhance total
577 nitrogen removal from wastewater.

578

579 **ACKNOWLEDGEMENTS**

580 C. A. acknowledges a postdoctoral grant from the Spanish Ministry of Economy
581 and Competitiveness (Plan Nacional de I + D + i 2008–2011, Subprograma
582 Juan de la Cierva (JDC) 2014). Authors are thankful to M.Sc. Stefania Spadola
583 for her support during sampling and analysis of water quality parameters.

584
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770
771

FIGURES

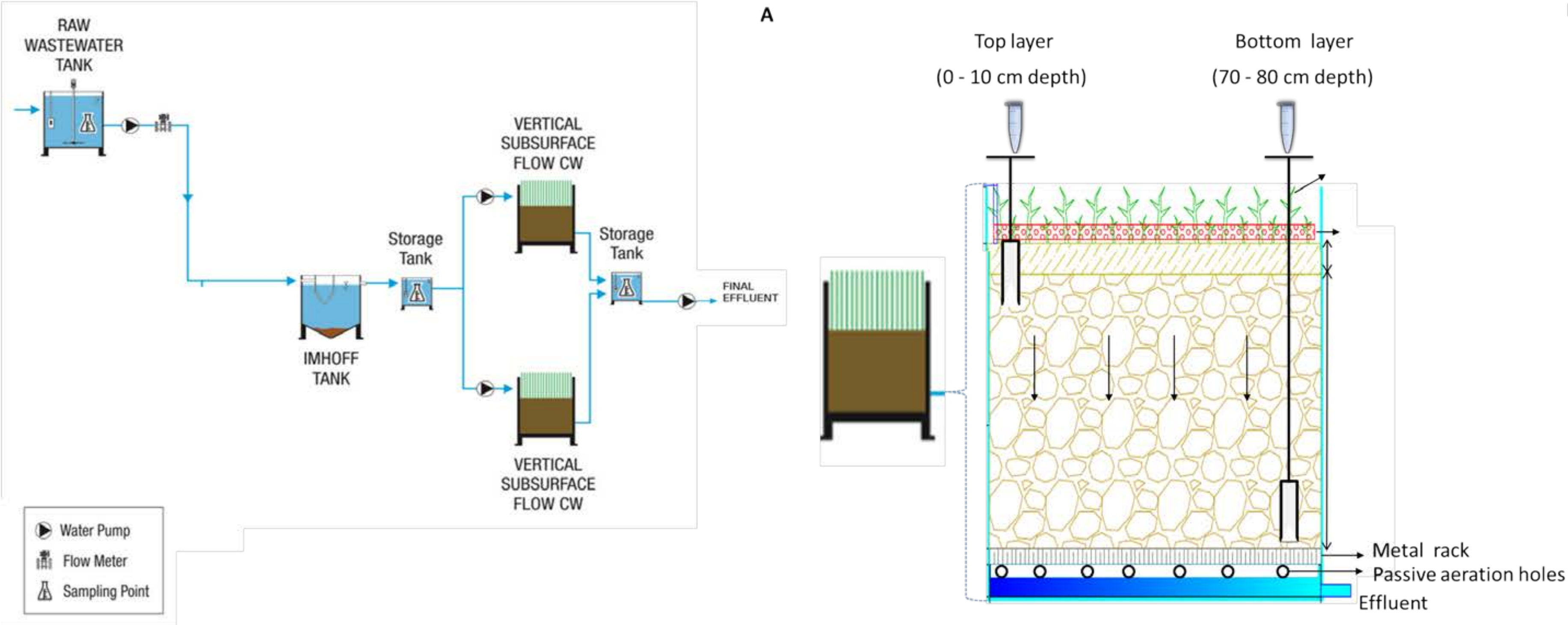

Figure 1. Diagram of the treatment system. a) Sampling points of inflow and outflow wastwater. b) Sampling collects of filter media from vertical subsurface flow constructed wetland.

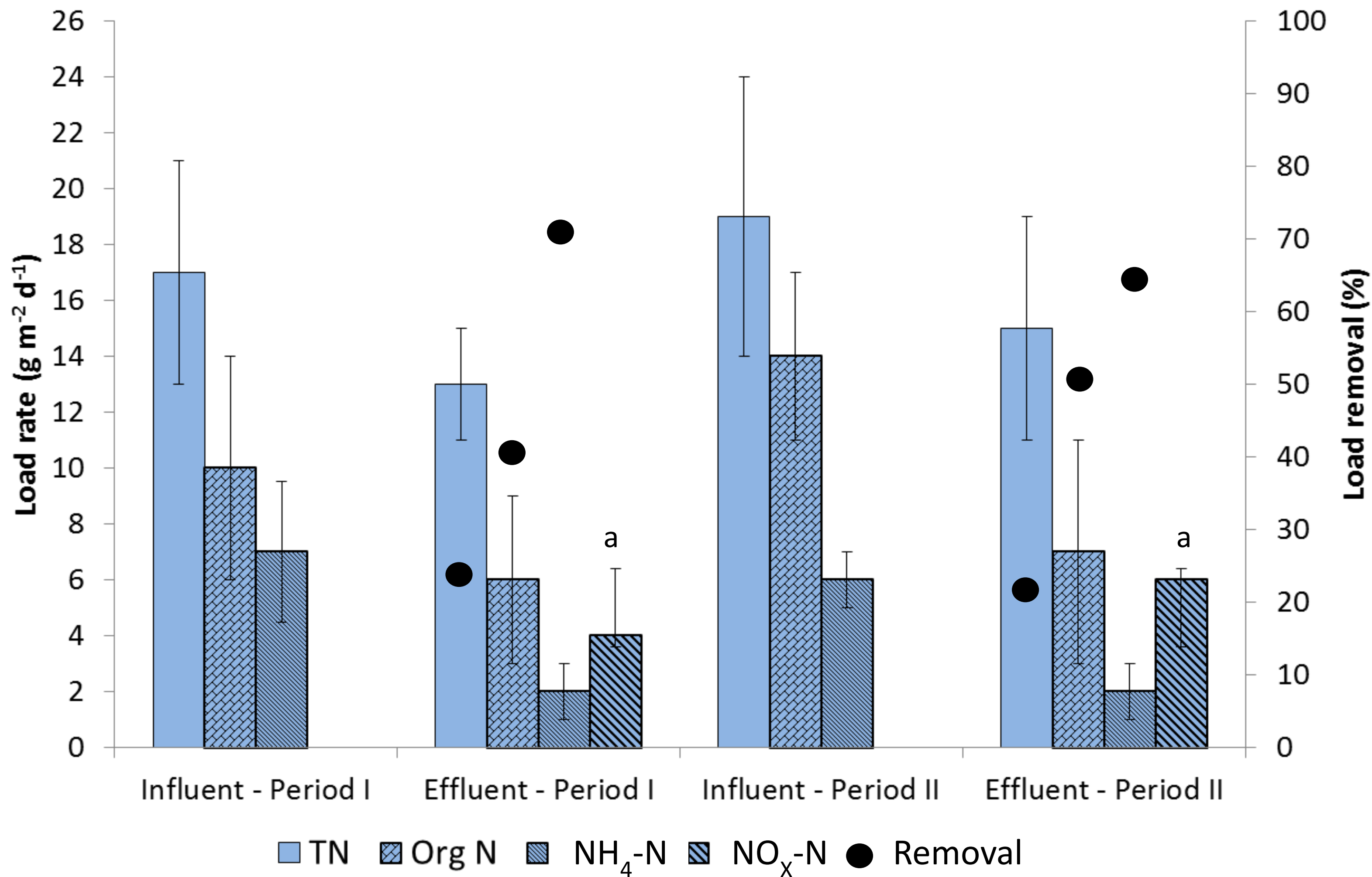

Figure 2. Nitrogen transformation in the vertical subsurface flow constructed wetland in Period I (130 g COD m^{-2} d^{-1}) and II (85 g COD m^{-2} d^{-1}). a: Statistical significance between the two periods ($p<0.05$).

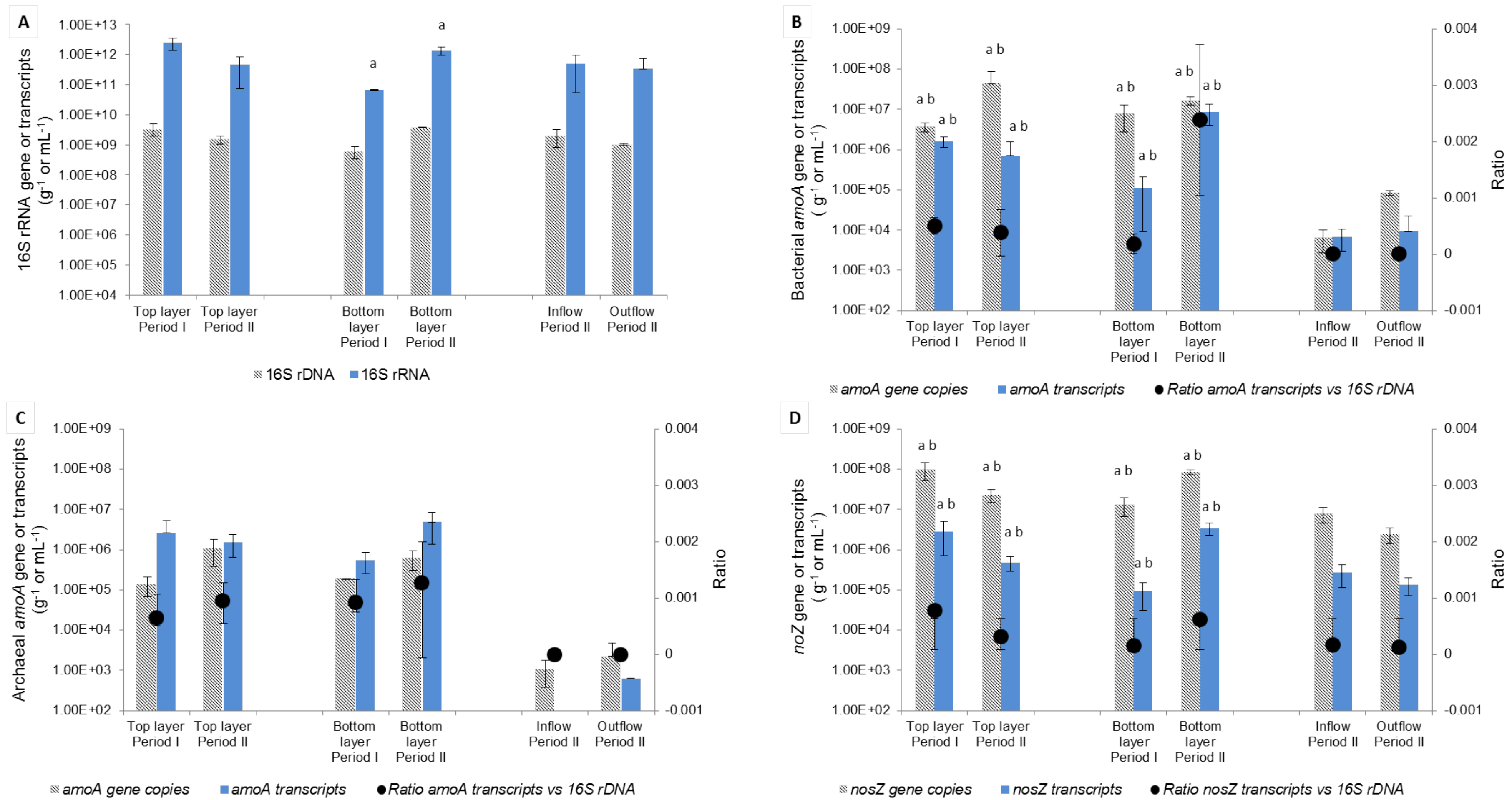

Figure 3. Average of nitrogen functional genes identified in top (0-15 cm) and bottom (70-80 cm) layers of the filter bed of the vertical subsurface flow constructed wetland in the two microbiological sampling campaigns: Period I (ORL= 130 g COD m^{-2} d^{-1}) and Period II (ORL= 85 g COD m^{-2} d^{-1}). 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 ($p<0.05$);

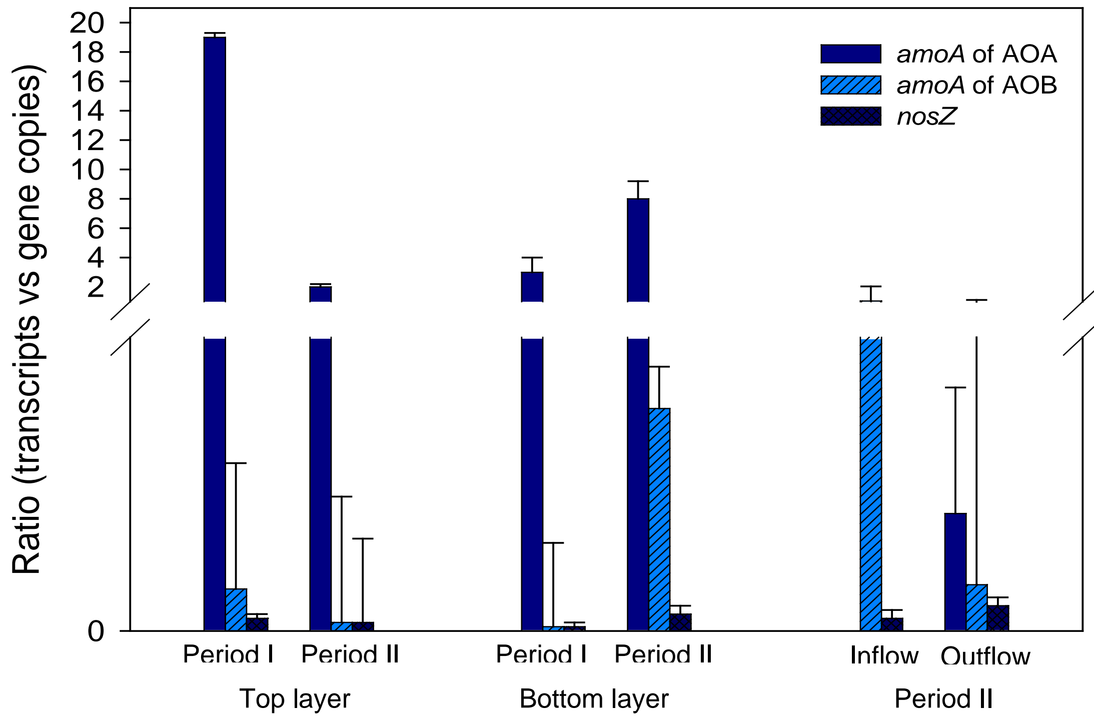

Figure 4. Ratio of genes transcripts and genes copies of bacterial and archaeal *amoA* and *nosZ* in top (0-15 cm) and bottom layers (70-80 cm) of the vertical flow wetland at the two microbiological sampling campaigns: Period I (ORL= 130 g COD m^{-2} d^{-1}) and Period II (ORL= 85 g COD m^{-2} d^{-1}).

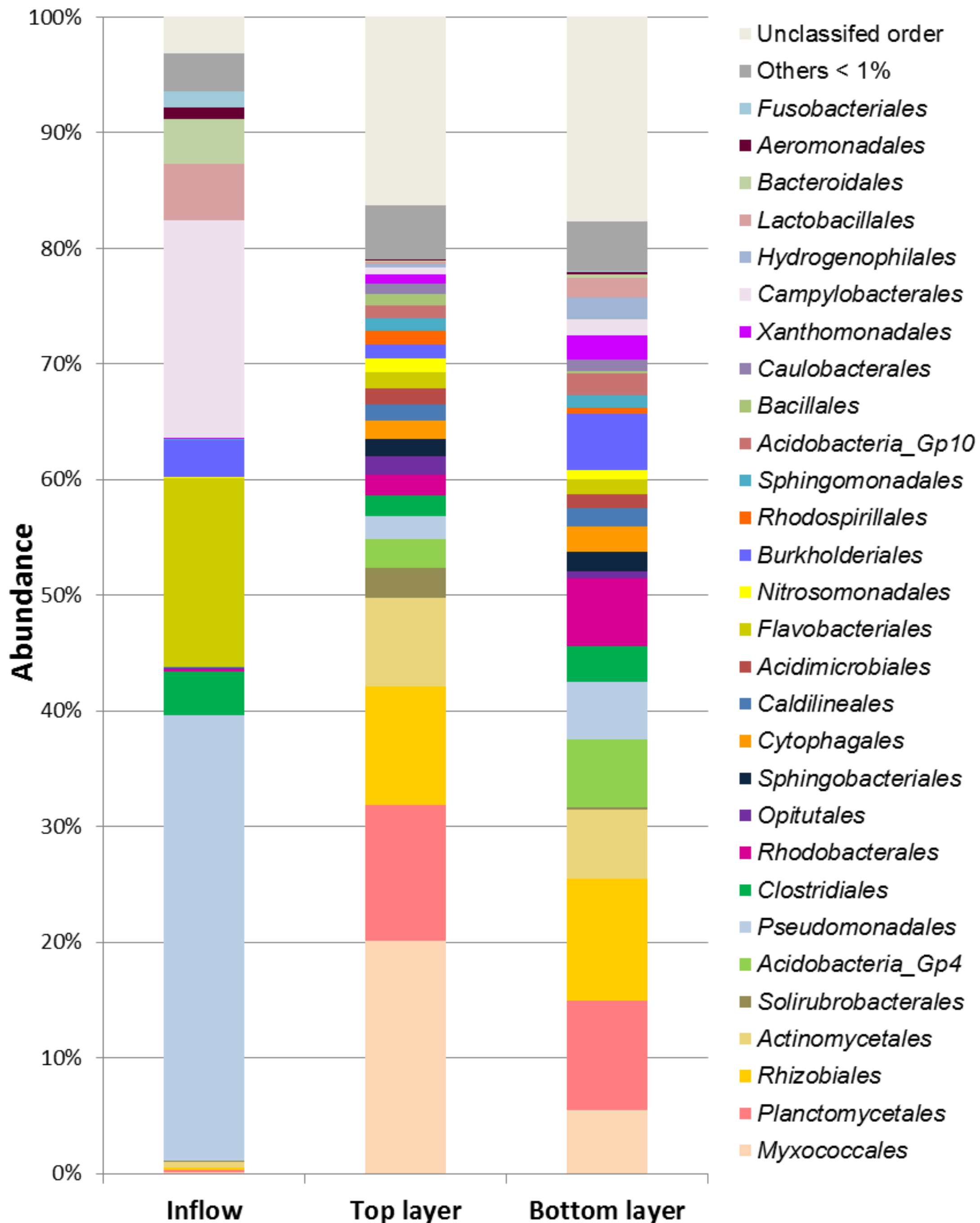

Figure 5. Taxonomic assignment of sequencing reads (MiSeq) from the active eubacterial community (16S rRNA-based cDNA) of water inflow, and filter media from top and bottom layers of Period II at order level. Relative abundance was defined by the number of reads (sequences) affiliated with any given 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%.

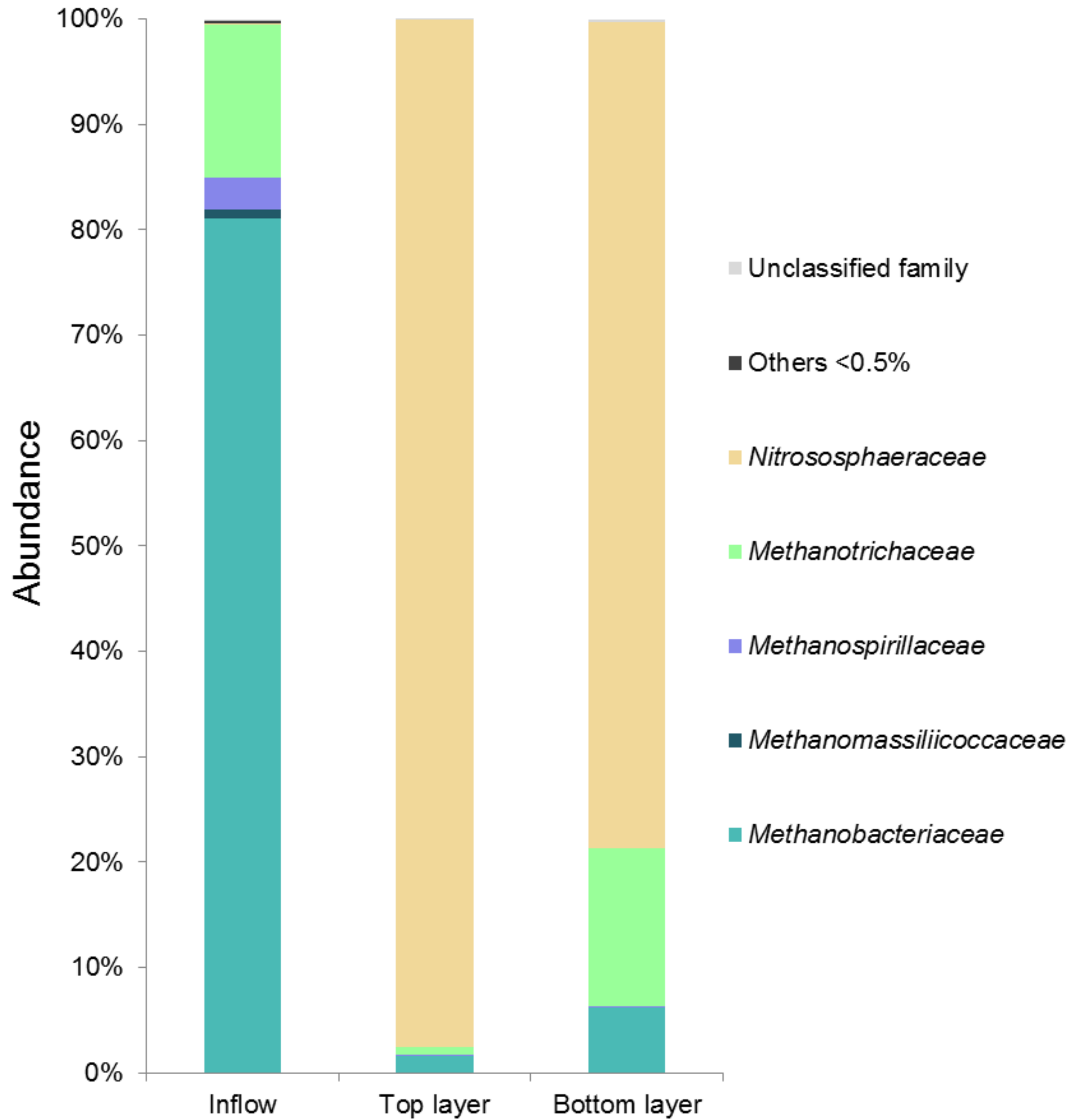

Figure 6. Taxonomic assignment of sequencing reads from the active archaeal community (16S rRNA based cDNA) of water inflow, and filter media from top and bottom layers of Period **II** at family level. Relative abundance was defined by the number of reads (sequences) affiliated with any given taxon, 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.

Operational conditions	Period I (Jun-Oct)	Period II (Nov-Jan)
Duration (months)	5	3
Air temperature ºC	25	14
Flow (m^3 d^{-1})	1.125	1.125
HLR (mm d^{-1})	375	375
OLR (g COD m^{-2} d^{-1})	130	85
TN (g m^{-2} d^{-1})	17	19
*C/N Ratio influent	2	2

*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.

Parameters	Period I (Jun-Oct) n = 26			Period II (Nov-Jan) n = 15		
	Influent	Effluent	Mean concentration removal	Influent	Effluent	Mean load removal
T (ºC)	23 ± 5	23 ± 5	-	11 ± 7	11 ± 9	-
DO (mg L^{-1})	0.5 ± 0.2	2.5 ± 1	-	0.6 ± 0.2	3.1 ± 1.1	-
EC (mS cm^{-1})	2 ± 0.5	2 ± 0.4	-	2 ± 0.2	2 ± 0.2	-
E_H (mV)	-101 ± 66	+181 ± 67	-	-71 ± 63	+112 ± 72	-
pH	7.5 ± 0.3	7.5 ± 0.3	-	7.8 ± 1.2	7.6 ± 0.3	-
TSS (mg L^{-1})	72 ± 31	55 ± 65	19 ± 72%	112 ± 53	40 ± 30	62 ± 24%
COD (mg L^{-1})	347 ± 104	207 ± 88	43 ± 14%	213 ±21	129 ±17	39 ± 10%
BOD_5 (mg L^{-1})	223 ± 88	91 ± 50	76 ± 14%	126 ± 23	71 ± 25	81 ± 7%
TOC (mg L^{-1})	90 ± 30	45 ± 21	50 ± 12%	91 ± 9	51 ± 28	44 ± 18%
TN (mg L^{-1})	46± 10	36 ± 9	20 ± 21%	50 ± 12	40 ± 10	15 ± 22%
NH_4-N (mg L^{-1})	18 ± 7	6.5 ± 3	62 ± 11%	15 ± 3	5 ± 2	61 ± 12%
*NO_x-N (mg L^{-1})	<LOD	10 ± 4	-	<LOD	15 ± 3	-
Parameters	Load applied (g m^{-2} d^{-1})	Load removal (g m^{-2} d^{-1})	Load removal %	Load applied (g m^{-2} d^{-1})	Load removal (g m^{-2} d^{-1})	Load removal %
COD (g m^{-2} d^{-1})	130 ± 39	73 ± 36	50 ± 24%	80 ± 8	32 ± 9	51 ± 10%
BOD (g m^{-2} d^{-1})	79 ± 38	45 ± 39	58 ± 10 %	46 ± 9	21 ± 6	46 ± 8 %
TN (g m^{-2} d^{-1})	17 ± 4	2 ± 2	23 ± 12%	19 ± 5	2 ± 2	21 ± 6%
NH_4-N (g m^{-2} d^{-1})	7 ± 2	4 ± 2	71 ± 8%	6 ± 1	4 ± 1	67 ± 7%

<LOD: below limit of detection

* Statistical significance between the periods ($p<0.05$)