

1 **NITROGEN TRANSFORMING BACTERIA WITHIN A FULL-**
2 **SCALE PARTIALLY SATURATED VERTICAL SUBSURFACE**
3 **FLOW CONSTRUCTED WETLAND**

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43 **Abstract**

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45 The aim of this study was to characterize the nitrogen transforming bacterial
46 communities within a partially saturated vertical subsurface flow constructed
47 wetland (VF) treating urban wastewater from a residential condominium in
48 southern Brazil. The VF had a surface area of 3,141 m², and was divided into
49 four wetland units, out of which two were operated while the other two rested,
50 alternating cycles of 30 days. The wetland had a depth of 0.7 m, out of which
51 0.2 m were saturated at the bottom. The bed media was sand, and was planted
52 with *Cyperus papyrus*. The nitrifying and denitrifying bacterial communities were
53 characterized in wetland unit 3 (763.6 m² surface area) over a period of 12
54 months by using the FISH technique. Samples were collected monthly
55 (February 2014 to February 2015) from different layers within the vertical profile,
56 during operation and rest periods, comprising a total of 6 sampling campaigns
57 while the unit was in operation and another 6 when the unit was at rest. This
58 wetland unit 3 operated with an average organic loading rate (OLR) of 4 g COD
59 m⁻² d⁻¹ and a hydraulic loading rate of 24.5 mm d⁻¹. The rest periods of the
60 wetland unit presented influences on the abundance of ammonia-oxidizing
61 bacteria (AOB) (8% and 3% for feed and rest periods, respectively), and nitrite-
62 oxidizing bacteria (NOB) (5% and 2% for feed and rest periods, respectively).
63 However, there was no influence of the rest periods on the denitrifying bacteria.
64 AOB were only identified in the top layer (AOB *β-proteobacteria*) in both
65 operational and rest periods. On the other hand, the NOB (*Nitrospirae* and
66 *Nitrospina gracilis*) were identified in the operational periods just in the top layer
67 and during rest periods just in the intermediate layer. The denitrifying bacteria
68 (*Pseudomonas* spp. and *Thiobacillus denitrificans*) were identified from the
69 intermediate layer downwards, and remained stable in both periods. Based on
70 the bacterial dynamics identified, VF wetland partially saturated operated with
71 low OLR promoted conditions for simultaneous nitrification and denitrification.

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

76

77 **1. INTRODUCTION**

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

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

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

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

117 In the latter strategy, the use of a partially saturated zone at the bottom of VF
118 wetlands aims at creating anaerobic/anoxic conditions in the lower part of the
119 bed, and aerobic conditions in the top part, thus promoting favorable conditions
120 for simultaneous nitrification and denitrification within a single wetland unit
121 (Silveira et al., 2015). Only a few examples of this configuration have been
122 reported. Langergraber et al. (2008) evaluated two stages of VF wetlands in
123 series and the average removal efficiency of TN was of about 50%. Dong and
124 Sun (2007) evaluated in parallel a typical unsaturated VF (43.7 m²) with 0.80 m
125 of depth, and a partially saturated VF wetland (28.07 m²) with 0.55 m of
126 saturation. The partially saturated VF presented higher TN removal efficiency
127 (37%) than the typical unsaturated wetland (25%). Moreover, Silveira et al.
128 (2015) studied two partially saturated VF beds (2 m² each) operating with two
129 different heights of saturation, 0.15 m and 0.25 m. Under an influent load of 14
130 g TKN m⁻² d⁻¹, the TKN load removal was 45% for a saturation height of 0.15 m
131 and increased up to 58% when the saturation height was of 0.25 m.

132 In general, all efforts directed towards maximizing the removal of pollutants in
133 CW are directly associated with enhancing the activity of the microbial
134 communities responsible for various nutrient transformations occurring in the
135 biomass of the filter media and the rhizosphere (Mayo and Bigambo, 2005).
136 Indeed, the understanding of the dynamics of bacterial communities in
137 constructed wetlands and their relation to treatment performance has been the
138 focus of many studies. Foladori et al. (2015) evaluated the viability and decay of
139 bacteria in water and soil in a typical unsaturated VF wetland (2.25 m² surface
140 area) and observed that the number of viable bacteria in the surface (0 to 0.10
141 m) layer was 3.7 times higher than in the deeper layer (0.40 to 0.50 m). What is
142 more, Salomo et al. (2009) investigated the influence of plant roots and soil

143 structure on the metabolic diversity of microorganisms in a VF wetland (48 m²),
144 and results show that in the surface of the bed easily degradable components
145 such as carbohydrates were used, whereas in the deeper layers inert
146 compounds were metabolized. Adrados et al. (2014) characterized the
147 prokaryotic microbial community (bacteria and archaea) of VF, HF and biofilters
148 and reported a higher bacterial than archaeal activity in all systems studied.
149 Other studies have shown that shifts in the structure of bacterial communities
150 can be associated with changes in a number of soil properties, including soil
151 texture and soil nitrogen availability (Dong and Reddy, 2010). Calheiros et al.
152 (2010) showed that the type of macrophyte seems to influence bacterial
153 community dynamics in HF wetlands polishing high salinity tannery wastewater.
154 In another study, Calheiros et al. (2009) indicated that the diversity of the
155 bacterial community in CW systems might influence the final effluent quality.
156 What is more, Button et al. (2015) showed that microbial metabolic functions
157 identified in different CW types (VF, HF, VF + HF + aeration) are related to the
158 design of each system, spatial position within the bed, and especially with levels
159 of pretreatment.

160 Nonetheless, the knowledge particularly concerning nitrogen transforming
161 bacteria in VF wetlands is still limited to a few studies (Adrados et al., 2014;
162 Mayo and Bigambo, 2005). Tietz et al. (2007a) evaluated the community of
163 ammonia-oxidizing bacteria (AOB) in three VF beds (18 m² , 0.50 m depth)
164 treating municipal sewage after 2.5 years of operation and despite nitrification
165 was stable, little AOB bacterial activity was identified. During the winter
166 nitrification decreased, however not affecting the spatial distribution of AOB,
167 being *Nitrosomonas europaea*, *Nitrosococcus mobilis* and *Nitrosospira* the
168 dominant AOB. Zhi and Ji (2014) identified functional genes involved in
169 nitrification and denitrification in a tidal flow CW showing nitrification activity
170 performed by AOB and Anammox bacteria, as well as denitrification.

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

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

178

179 **2. MATERIALS AND METHODS**

180 **2.1 Description of the wastewater treatment plant**

181 The research was conducted at a full-scale VF system located in a residential
182 condominium in the city of Palhoça, Santa Catarina state, southern Brazil
183 (latitude 27°45'4.82" and longitude 48°37'39.35"). This treatment plant started
184 operation in 2006, and was designed for an estimated maximum demand of
185 2,200 P.E. However, during the time of the current study the system received a
186 contribution of approximately 100 people, due to the fact that the residential
187 condominium was not totally inhabited. This resulted in low organic and
188 hydraulic loading rates applied to the VF wetland.

189 Urban wastewater flowed into an anaerobic baffled reactor acting as primary
190 treatment before it was conveyed to the VF wetland, which had a surface area
191 of 3,141 m², a depth of 0.7 m and a saturated zone at the bottom of 0.20 m,
192 kept constant by a level controller. The bed media consisted of 0.1m gravel of
193 granite (3 - 8 mm) at the bottom of the drainage area, followed by 0.5 m of sand
194 (Effective diameter $d_{10} = 0.3$ mm and Uniformity Coefficient of 4.84), and finally
195 0.1 m of gravel (3 - 8 mm) on the top. The planted macrophyte was *Cyperus*
196 *papyrus*, which was very well developed at the time of this study.

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

207

208 **2.2 Sampling procedure**

209

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

216 For each sampling campaign, a complete vertical profile was collected from 3
217 points of the wetland cell 3 with the aid of a manual auger. These points were
218 located along the length of the wetland coinciding with the holes of the
219 distribution pipes of the unit (i.e. initial – point 1, central – point 2, and final -
220 points 3 of portion the wetland) (Fig. 2). The vertical profile was divided in layers
221 along the depth, namely 'top' (0 to 0.17m depth), 'intermediate' (0.17 to 0.34 m
222 depth), 'unsaturated/saturated' (0.34 to 0.51 m depth), and 'saturated' layers
223 (0.51 to 0.68 m).

224 Moreover, monitoring of the performance of the system in terms of conventional
225 water quality parameters was performed, taking grab samples at the influent
226 and effluent of the VF. Samples were collected just when the wetland cell 3 was
227 being fed (n=10)The parameters evaluated in final effluent of VF were: pH,
228 alkalinity, chemical oxygen demand (COD), total suspended solids (TSS), total
229 nitrogen (TN), ammonia nitrogen (NH₄-N), nitrite nitrogen (NO₂-N), and nitrate
230 nitrogen (NO₃-N). Their analysis followed APHA (2005) recommendations, with
231 the exception of NH₄-N, which was determined following recommendations of
232 Vogel (1981).

233

234 **2.3 Bacterial analysis**

235

236 *2.3.1 Pre-treatment of samples*

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

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

244 .

245 *2.3.2 Quantitative fluorescence in situ hybridization*

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

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

257 For the analysis of the nitrifying and denitrifying bacterial communities specific
258 probes for each community were used (Table 1). All probes were labeled at 5'
259 position with the fluorochrome Cy3 (Biomers). To carry out the hybridization
260 reactions, PTFE-printed microscope slides with 10 wells measuring 5 mm² in
261 diameter were used. For the fluorescence in situ hybridization (FISH) reaction,
262 10 µL of each fixed sample was prepared in each slide. Quantification of
263 nitrifying bacteria was performed by direct counting of 10 random fields in each
264 well using an epifluorescence microscope (Olympus BX41, Tokyo, Japan).

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

273

274 **2.3 Statistical data analysis**

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

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

289

290 **3. RESULTS AND DISCUSSION**

291 **3.1 Treatment performance of the constructed wetland**

292 The wetland cell 3 operated with low organic (OLR) and hydraulic loading rates
293 (HLR), with average values of $4 \text{ g COD m}^{-2} \text{ d}^{-1}$, and a HLR of 24.5 mm d^{-1} . The
294 nitrogen load was $1.5 \text{ g TN m}^{-2} \text{ d}^{-1}$, resulting in C/N ratio of 3 (considering the
295 average concentrations of COD and TN). The literature recommends an OLR in
296 the range of 60 to $70 \text{ g COD m}^{-2} \text{ d}^{-1}$ and a HLR of 250 mm d^{-1} for unsaturated
297 VF in warm climates (Hoffmann et. al., 2011), and of $41 \text{ g COD m}^{-2} \text{ d}^{-1}$, 10.2 g
298 $\text{NH}_4\text{-N m}^{-2} \text{ d}^{-1}$ and HLR of 230 mm d^{-1} in subtropical climates (Sezerino et al.,
299 2012). Design parameters as OLR and HLR are directly associated to the TN
300 removal process (Saeed and Sun, 2012). Firstly, OLR is linked to the C/N ratio
301 of the middle. Low OLR applied can result in low availability of organic carbon
302 for denitrification. Zhao et al. (2011) showed better TN removal efficiencies in
303 VF, with C/N ranging between 5 to 10, compared to the C/N of 2.5. Secondly,
304 HLR is related to the transfer of oxygen into the filter bed in VF wetland.

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

307 Table 2 shows the wastewater physico-chemical characterization of influent and
308 effluent of the wetland cell 3, relating the feeding periods. In general, the VF
309 presented a good removal of solids and carbonaceous organic matter, being of
310 $86 \pm 4\%$ for TSS, and $89 \pm 10\%$ for COD. With regards to nitrogen, the removal
311 efficiency of TN and $\text{NH}_4\text{-N}$ was of $58 \pm 12\%$ and $93 \pm 2\%$ respectively. The
312 good performance of nitrogen removal in this system may be associated with
313 nitrification followed by denitrification, provided the bottom saturation, and the
314 bacterial interactions in the medium. The large removal efficiency of total
315 nitrogen in the wetland can be attributed to the saturation of the bottom of the
316 unit, which may have enhanced the complete elimination of nitrogen through the
317 promotion of denitrification (Silveira et al. 2015).

318

319 **3.2 Influence of feed and rest periods on nitrifying and denitrifying** 320 **bacterial communities**

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

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

338 carbon. Via the FISH technique, various abundance values of Eubacteria are
339 reported in CW. Krasnits et al. (2009) in a HF (130 m² surface area) operated
340 for 7 years in the treatment of municipal sewage under a OLR larger (11 g BOD₅
341 m⁻² d⁻¹) identified an abundance of 85%. In another study, Sawaittayothin and
342 Polprasert (2007) reported an abundance of 49% in CW treating municipal
343 landfill leachate, while according to the authors the remaining 51% may be
344 associated with archaea or inactivated bacteria.

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

352 On the other hand, the average relative abundance of AOB *β-proteobacteria*
353 (NSO190) (*Nitrosomonas* and *Nitrosospira*) was of 8 ± 1% for feed periods and
354 of 3 ± 1% for rest periods (Fig. 3). *Nitrosomonas* sp. (NEU 653) was however
355 not identified in any of the samples. This could be attributed to the fact that
356 *Nitrosomonas* spp. have lower affinity for substrate than *Nitrosospira* spp.
357 (although higher activity) (Scharamm et al., 1996). In this study, *Nitrosospira*
358 spp. was the dominant AOB, showing to prevail in environments with low NH₄-N
359 concentrations, and a good capacity of withstanding the physico-chemical
360 variations in the bed media (Purkhold et al., 2000).

361 In regards to the nitrite-oxidizing bacteria (NOB), the *Nitrospirae* phylum
362 (NTSPA 662) was the most abundant of this group (5 ± 1 % during feeding
363 periods and 2 ± 1 % during rest periods). The identification of this phylum in CW
364 has been shown by previous studies. Guan et al. (2015) characterized the
365 microbial community in three VF wetlands (1.2 m² surface area each) applied to
366 river water treatment, with influent concentrations around 8 to 12 mg L⁻¹ of
367 dissolved organic carbon and 2 to 4 mg L⁻¹ of NH₄-N. The wetlands presented
368 an NH₄-N removal efficiency of 95%, and the main bacteria associated with
369 nitrification, which was present in greater abundance, was *Nitrospira*-like.

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

373 Despite the lower abundance of nitrifying bacteria during rest periods these
374 were able to remain active along the whole study period, showing their ability to
375 recover their enzymatic activities quickly after a period of stress. This is in
376 accordance with various studies, such as that of Wilhelm et al. (1988), which
377 observed how *Nitrosomonas europaea* was able to recover its oxidant activity in
378 a few minutes after about one year under limited $\text{NH}_4\text{-N}$ concentrations.
379 Moreover, *Nitrospira briensis* after a period of 2 weeks with substrate
380 limitation reached their maximum potential for $\text{NH}_4\text{-N}$ oxidation within 30 to 60
381 minutes after a pulse of ammonia (Bollmann et al., 2005). Autotrophic nitrifying
382 bacteria can adapt to survival strategies with low substrate based on cellular
383 components, and have a high capacity to generate power when new substrate
384 becomes available (Geets et al., 2006). In the other study conducted in a
385 aerated saturated VF wetland was showed that after two weeks of no aeration
386 in the established wetland, nitrification recovered within two days, whereas
387 nitrification establishment in a new wetland was previously observed to require
388 20 to 45 days. Based on these results, once established resident nitrifying
389 microbial communities are quite robust (Murphy et al., 2016). For denitrifying
390 bacteria the abundance and diversity was the same in the feed and rest
391 periods. The identified average relative abundance was of $3 \pm 2.4\%$ for the
392 genus *Pseudomonas* spp. (PAE 997), of $6 \pm 4\%$ for the species *Thiobacillus*
393 *denitrificans* (TBD 1419) and of $3 \pm 0.8\%$ for the species *Paracoccus versutus*
394 and *denitrificans* (PDV 163) (Fig. 3). The fact that rest periods showed no
395 influence on this community can presumably be explained by the stability
396 provided by the bottom saturation layer, which maintained the necessary
397 conditions for the stability of this group. Moreover, rest periods due to low
398 availability of oxygen seems to favor denitrifying bacteria. In a study performed
399 in two microcosms simulating a vertical profile of wetland was identified greater
400 abundance of denitrifying bacteria in rest periods than in feed periods (Pelissari
401 et al., 2016).

402 In addition to the classic denitrification carried out mainly by *Pseudomonas spp.*
403 (Ahn, 2006), other pathways of denitrification were identified in this study.
404 *Thiobacillus denitrificans* are organisms that use inorganic compounds as a
405 carbon source, and compounds such as sulfates, nitrates or nitrites as electron
406 donors. Therefore, this species is associated to autotrophic denitrification. The
407 presence of *Thiobacillus* in CW has previously been reported by Zhong et al.,
408 (2015) in a HF bed (HLR = 100 mm d⁻¹) treating urban wastewater. According to
409 the authors this genus was present in great abundance (680 sequences) due to
410 the anaerobic conditions of the HF. Furthermore, *Paracoccus denitrificans* was
411 also identified in the current study, a species that is associated with aerobic
412 denitrification and heterotrophic nitrification (Richardson, 2000). Austin et al.
413 (2006) in a tidal flow VF wetland (10 m² divided into 6 cells, under flow of 1.5 to
414 2.3 m³), reported a nitrification rate of 95%, related to a load of 104 g TKN m⁻³ d⁻¹
415 and C/N ratio of 3.3:1. During the study period, two characterizations of
416 microbiota through the FISH technique revealed that after 9 months of operation
417 9% of the bacterial community was composed of *Paracoccus denitrificans*, while
418 after 30 months of operation colonization of this bacterium increased up to 15%.
419

420 **3.3 Nitrifying and denitrifying bacteria distribution along the vertical** 421 **profile of the filter media**

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

426 **3.3.1 Top layer (0 to 0.17 m)**

427 The highest average relative abundance of Eubacteria in the VF wetland profile
428 was identified on the top layer ($76 \pm 7\%$ for feed periods, and $30 \pm 1\%$ for rest
429 periods) (Fig. 4). This behavior has already been observed by other studies.
430 Tietz et al. (2007b) in a VF bed (1 m² surface area) with sand as bed media
431 applied in the treatment of municipal wastewater reported that more than 50%
432 of the bacterial biomass and activity was concentrated in the top 0.10 m of the
433 unit. Moreover, Foladori et al. (2015) in a VF wetland (2.25 m² surface area)
434 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

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

437 AOB bacteria were identified in both feed and rest periods. AOB β -
438 *proteobacteria* (*Nitrospira* and *Nitrosomonas*) presented an abundance of $8 \pm$
439 1% in the feed periods, and of $3 \pm 1\%$ in rest periods. Despite the decay of
440 abundance of AOB β -*proteobacteria* during rest periods at the top layer this
441 group remained active throughout the study (Fig. 4).

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

449 The occurrence of the genus *Paracoccus* of the species *denitrificans* and
450 *versutus* was identified throughout the whole study at this layer with an average
451 relative abundance of $3 \pm 0.8\%$ for the feed and rest periods. The presence of *P.*
452 *denitrificans* can be associated both with heterotrophic oxidation of $\text{NH}_4\text{-N}$ and
453 aerobic denitrification, as heterotrophic nitrification can occur in media with a
454 C/N ratio as low as 2 (Kuenen and Robertson, 1994).

455

456 3.3.2 Intermediate layer (0.17 to 0.34 m)

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

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

467 increased availability of oxygen at the upper part of the bed media. This finding
468 agrees with other studies conducted in VF wetlands operated with higher OLR,
469 which show a stratification of AOB along the vertical profile owing to the higher
470 amount of organic matter available on the surface of VF wetland, which results
471 in highest abundance of heterotrophic bacteria. Tietz et al. (2007a) studied the
472 AOB in three VF operated with $27 \text{ g COD m}^{-2} \text{ d}^{-1}$, C/N ratio of 8.4, and HLR of
473 43 mm d^{-1} , and identified AOB species up to 0.50 m depth from bed surface.

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

478 The colonization of denitrifying bacteria of the genus *Pseudomonas* spp. was
479 observed at the intermediate layer, with an average relative abundance of $5 \pm$
480 0.2% for feed periods. Although the intermediate layer presumably has aerobic
481 conditions during operation periods, the colonization of this genus may be
482 associated with the availability of carbon at this bed depth. According to Salomo
483 et al. (2009), this layer of VF beds are predominantly ideal for bacterial use of
484 organic substrates, due to the penetration of the roots of macrophytes which
485 provide an additional source of easily degradable carbon. The average relative
486 abundance of *Pseudomonas* spp. increased to $10 \pm 1\%$ during rest periods. The
487 lower availability of oxygen due to reduced oxygen transfer capacity during rest
488 periods should explain the increase in abundance of *Pseudomonas* spp.,
489 indicating the occurrence of the denitrification process in these periods.

490

491 3.3.2 Semi-saturated layer (0.34 to 0.51 m)

492 At this depth, during feed periods an increase in the abundance of Eubacteria
493 ($60 \pm 3\%$) in relation to the intermediate layer ($40 \pm 3\%$) was identified. This
494 could be associated with the moisture in the filter media, since this layer is half
495 saturated with water. During rest periods the relative abundance of the bacterial
496 community was of $20 \pm 2\%$ (Fig. 4). Despite the larger bacterial abundance in
497 the feed periods, AOB and NOB were not identified at this layer, which may be
498 explained by an environment with low oxygen concentrations.

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

503 The average relative abundance was the same for feed and rest periods, being
504 of $5 \pm 1\%$ and $2 \pm 0.2\%$ for *Pseudomonas spp.* and *Thiobacillus denitrificans*,
505 respectively. The presence of these two denitrifying genus in the same layer
506 may indicate distinct dynamics regarding the use of carbon. The denitrifying
507 bacteria are usually heterotrophic, however, several bacteria, among them the
508 genus *Pseudomonas* are facultative chemolithoautotrophic, capable of growing
509 in the absence of organic matter, using molecular hydrogen as electron donor,
510 and CO_2 as inorganic carbon source. They can also switch between
511 chemolithotrophic or chemoorganotrophic metabolism, depending on the
512 nutritional status of their habitats (Madigan et al., 2010). However, *Thiobacillus*
513 *denitrificans* is an obligatory chemolithotrophic example, since it uses only
514 inorganic compounds as electron donors for energy, and CO_2 as a carbon
515 source. These findings are in accordance to those reported by Salomo et al.
516 (2009), which evaluated the microbial metabolic diversity in a VF wetland (48 m²
517 surface area) that had been for 13 years in operation applied in the treatment of
518 domestic wastewater, and in the third layer in depth (where anoxic conditions
519 equivalent to the unsaturated/saturated layer of this study prevailed) none of the
520 tested carbon sources were metabolized by the microbial groups present in the
521 medium. Since anoxic bacteria are dependent on oxidized electron acceptors,
522 such as NO_x , SO_4^{2-} or Fe_3^+ , it is hypothesized that the denitrification process
523 may be associated with autotrophic bacteria, as indicated by the presence of
524 the species *Thiobacillus denitrificans*.

525

526 3.3.3 Saturated layer (0.51 to 0.68 m)

527 In the bottom of the wetland bed the abundance of Eubacteria remained stable
528 ($60 \pm 4\%$) throughout the whole study (both in feed and rest periods), which
529 can be attributed to the saturation of the bed. Bacteria growth seems rather
530 favored in water films along the preferential water flow directions. Foladori et al.
531 (2015) evaluated the relationship between viable and dead bacteria in the soil of

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

537 At this layer only denitrifying bacteria were identified. The diversity of this group
538 in the saturated layer was the same as in the unsaturated/saturated layer.
539 However, the abundance was higher. The average relative abundance was of 5
540 $\pm 2\%$ for *Pseudomonas* spp. in feed and rest periods, and of $10 \pm 3\%$ for
541 *Thiobacillus denitrificans* in feed and rest periods, respectively.

542

543 **3.4 Spatiotemporal variation of nitrifying and denitrifying bacteria**

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

558 .

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

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 (*Nitrospirae* 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.

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

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

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

609

610 **ACKNOWLEDGEMENTS**

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

614

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844 FIGURES

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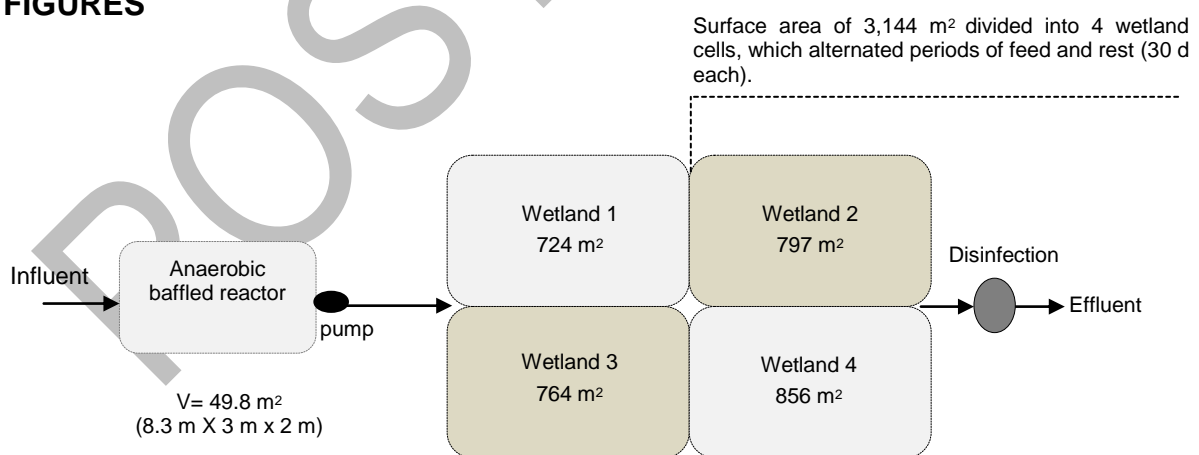
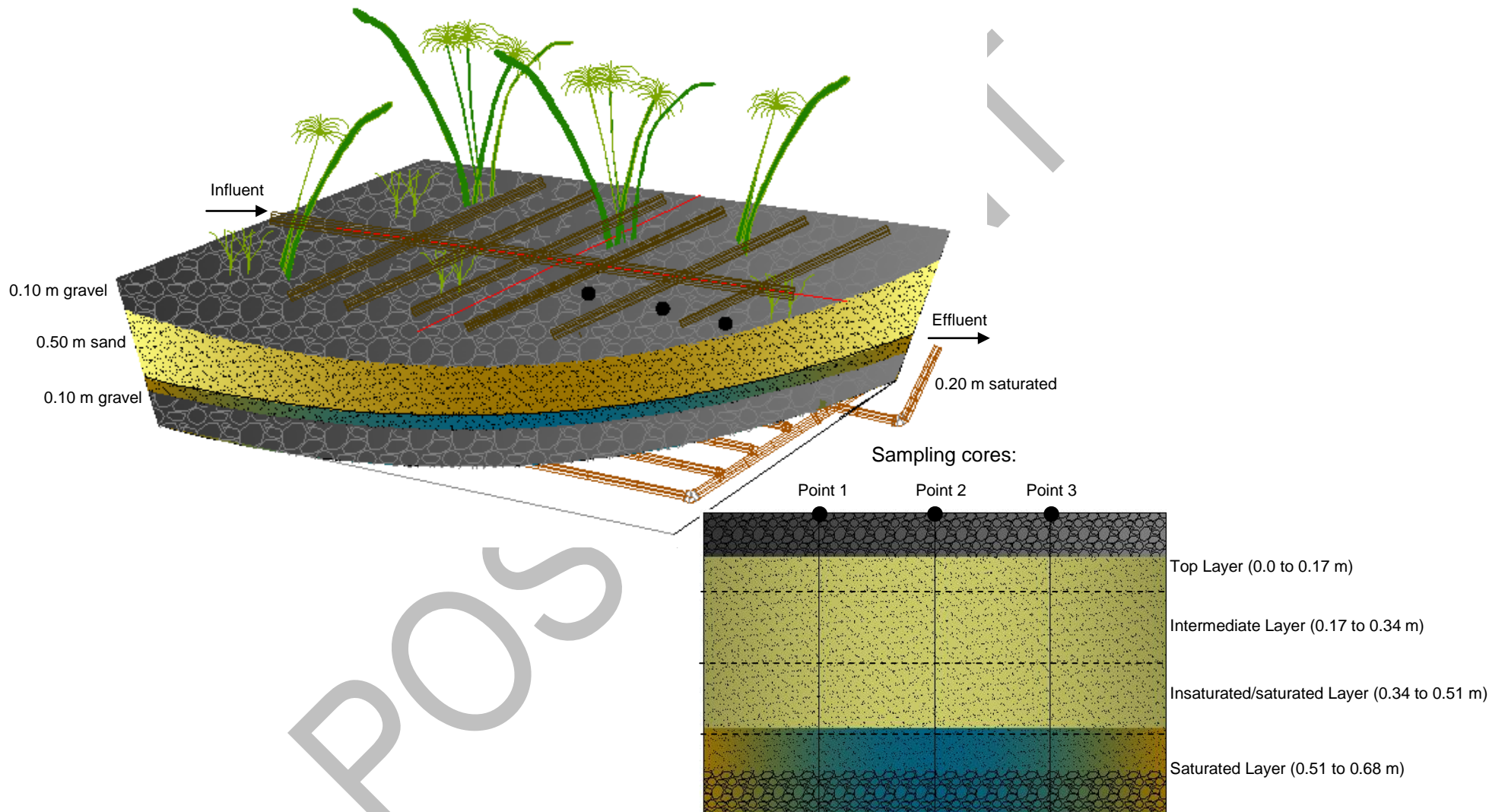


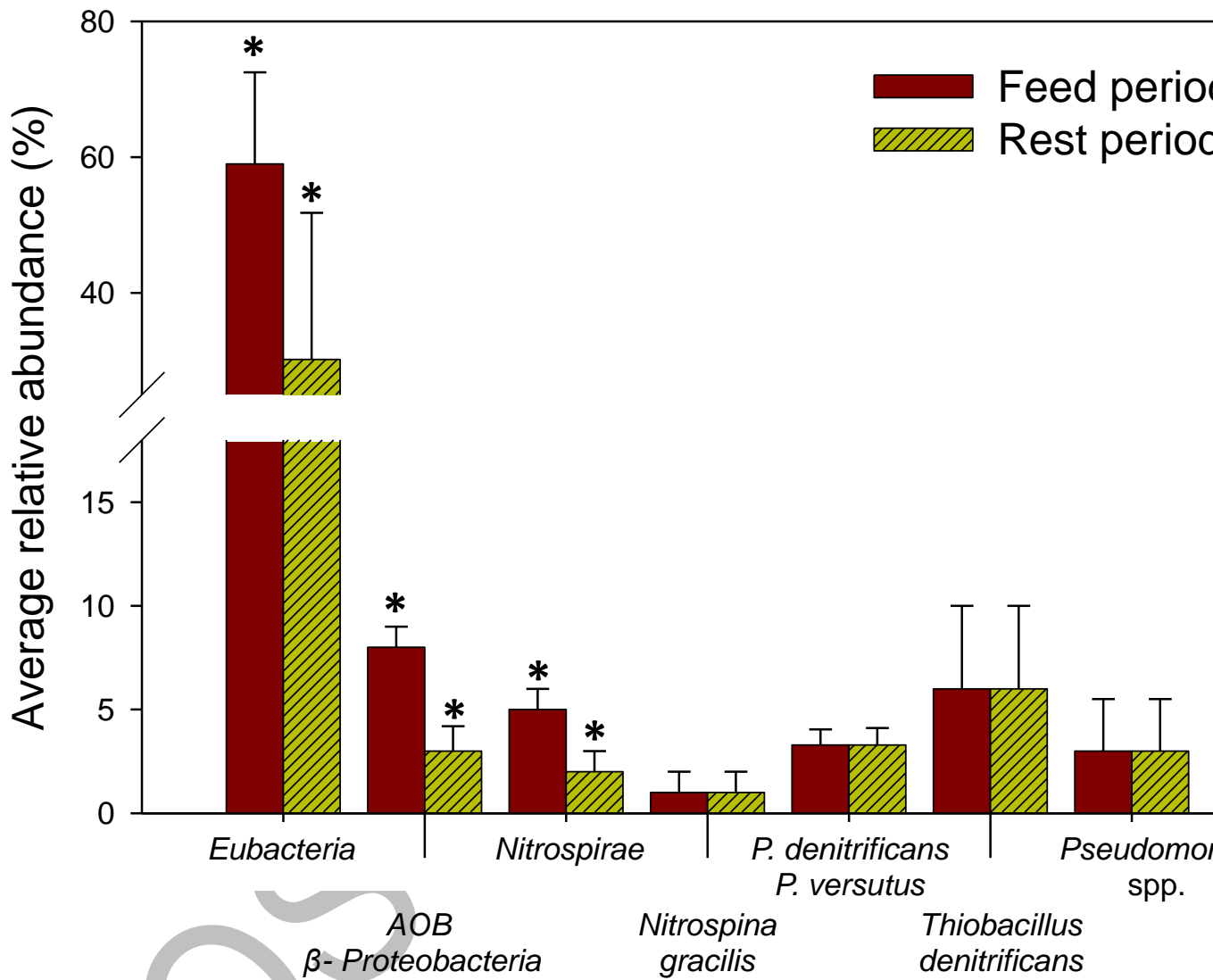
Figure 1. Diagram of the partially-saturated vertical subsurface flow wetland.



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

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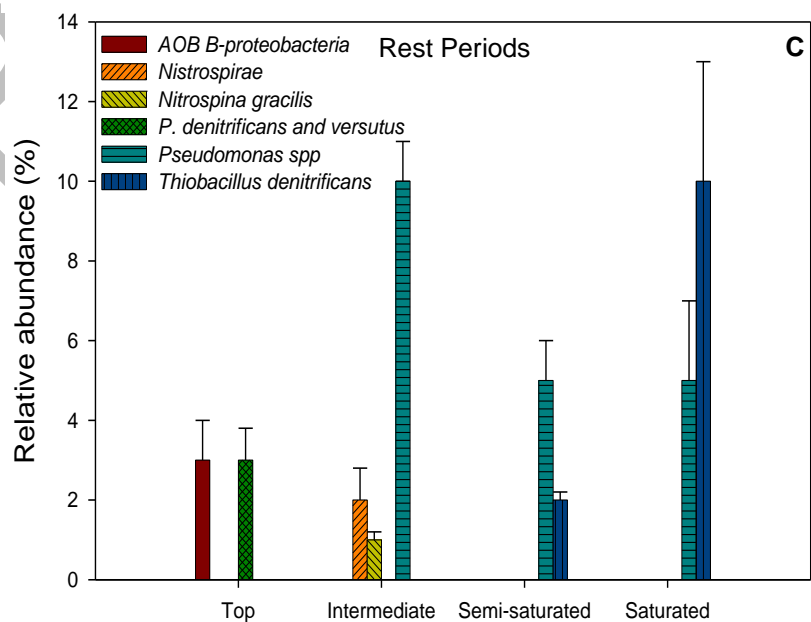
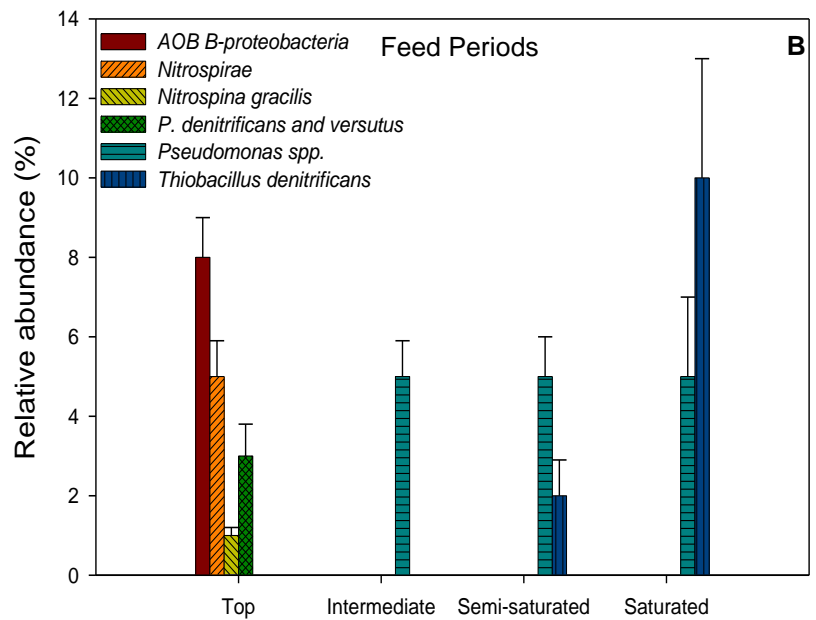
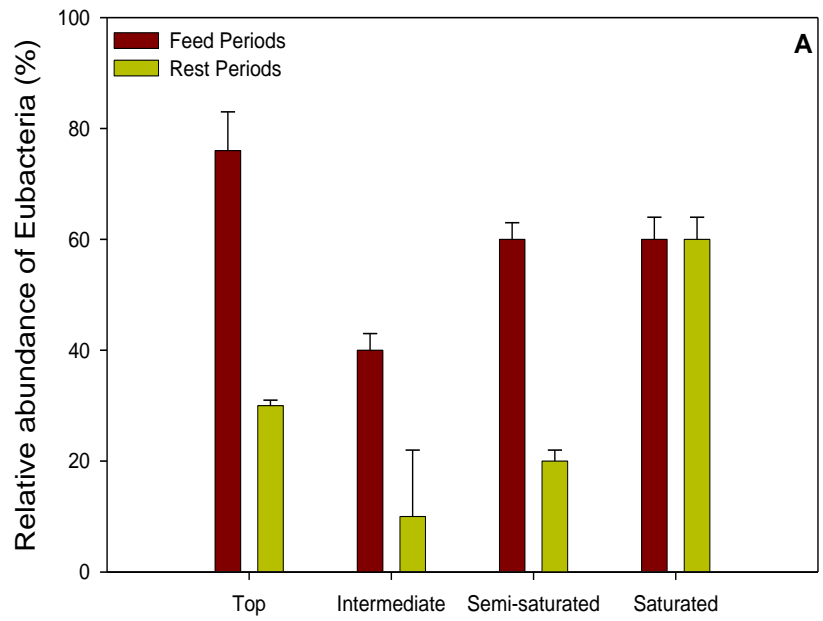


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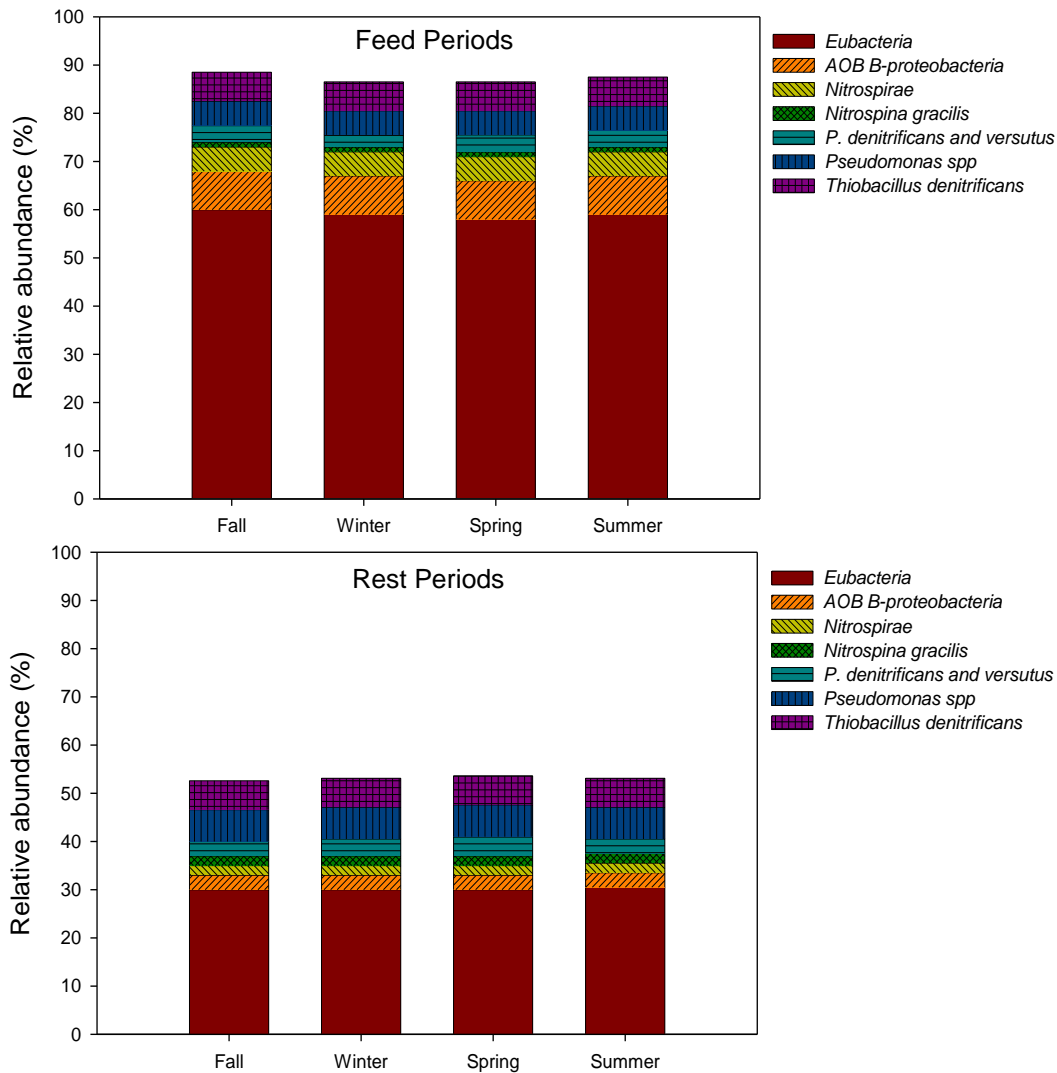
Figure 3. Mean (\pm SD) relative abundance of bacteria in the partially-saturated vertical subsurface flow wetland cell 3 in feed and rest periods

*Statistically significant : ($p < 0.05$)

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946 **Figure 4.** Mean (\pm SD) relative abundance of studied bacteria in different layers of wetland unit 3
 947 during feed and rest periods. A) Relative abundance of Eubacteria in feed and rest periods at
 948 different layers. B) Nitrifying and denitrifying bacteria in different layers during feed periods. C)
 949 Nitrifying and denitrifying bacteria in different layers during rest periods.



950 **Figure 5.** Average relative abundance of bacteria in the four layers of wetland unit 3 at the
 951 different seasons.
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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)	<i>Eubacteria</i>	I- CTGCCTCCCGTAGCA II- CAG CCACCCTAGGTGTCTG III- CCACCCTAGGTGT	I – Amann <i>et al.</i> (1990) II e III – Daims <i>et al.</i> (1999)
PAE 997	<i>Pseudomonas spp.</i>	TCTGGAAAGTTCGCATCA	Amann <i>et al.</i> (1996)
NTSPA 662	<i>Nitrospirae</i>	CGCCTTCGCCACCGGCCTTCC	Daims <i>et al.</i> (2001)
NTSPN 693	<i>Nitrospina gracilis</i>	TTCCCAATATCAACGCATT	Juretschko (2000)
NSO 190	<i>AOB β-Proteobacteria</i>	CGATCCCCTGCTTTTCTCC	Mobarry <i>et al.</i> (1996)
NEU 653	<i>Nitrosomonas sp</i>	CCCCTCTGCTGCACTACTCTA CCTGTGCTCCATGCTCCG	Wagner <i>et al.</i> (1995)
NIT 3	<i>Nitrobacter sp</i>	CCTGTGCTCCATGCTCCG	Wagner <i>et al.</i> (1996)
AMX 820	<i>Candidatus Brocadia</i> <i>Candidatus Kueneria stuttgartiensis</i>	AAAACCCCTCTACTTGCCAGTC	Schmid <i>et al.</i> (2001)
PDV 163	<i>Paracoccus denitrificans</i> <i>Paracoccus versutus</i>	CTAATCCTTTGGCGATAAATC	Neef <i>et al.</i> (1996)
TBD 1419	<i>Thiobacillus denitrificans</i>	ACTTCTGCCAGATTCCAC	Fernández <i>et al.</i> (2008)

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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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