The Effects of Plants on Pollutant Removal, Clogging, and Bacterial Community Structure in Palm Mulch-Based Vertical Flow Constructed Wetlands

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Abstract: In this study, the effects of plants on the performance and bacterial community structure of palm mulch-based vertical flow constructed wetlands was studied. The wetlands were built in August 2013; one of them was planted with *Canna indica* and *Xanthosoma* sp., and the other one was not planted and used as a control. The experimental period started in September 2014 and finished in June 2015. The influent was domestic wastewater, and the average hydraulic surface loading was 208 L/m^2d, and those of COD, BOD, and TSS were 77, 57, and 19 g/m^2d, respectively. Although the bed without plants initially performed better, the first symptoms of clogging appeared in December 2014, and then, its performance started to fail. Afterwards, the wetland with plants provided better removals. The terminal restriction fragment length polymorphism (T-RFLP) analysis of *Enterococci* and *Escherichia coli* in the effluents suggests that a reduction in their biodiversity was caused by the presence of the plants. Thus, it can be concluded that the plants helped achieve better removals, delay clogging, and reduce *Enterococci* and *E. coli* biodiversity in the effluents.

Keywords: Constructed wetland; mulch-based substrate; clogging; plants; T-RFLP

1. Introduction

Constructed wetlands (CWs) are particularly well suited for wastewater treatment in small communities, i.e., those with a <2.000 p.e. (person equivalent) because of their low cost, easy maintenance, high treatment efficiency, and visual appeal [1]. Similarly, CWs are environmentally friendly and a real alternative for the treatment of different types of wastewaters [2]. CWs are open, shallow reactors composed basically of an impervious layer, a mineral substrate (usually gravel or sand), helophytes, water, and the associated microbes. According to the water flow, CWs can be classified as horizontal flow (HF) or vertical flow (VF) and surface or subsurface flow. One of the main constraints of CWs is their high surface area, usually 3–5 m^2/p.e. VF CWs demand less surface and are more efficient than HFs because of the higher aeration of the substrates [3]. However, VF CWs usually include smaller-sized substrates, such as sand, to achieve a good surface influent distribution

and appropriate hydraulic retention times. One of the main problems associated with the use of sand in VF CWs is the risk of clogging [4]. A remarkable exception is the so-called "French system", which can treat raw domestic wastewater without primary settling [5].

CWs are considered to be more sustainable than conventional wastewater treatments, such as activated sludge [6,7]. However, a relevant proportion of the environmental impact of CWs is associated with the construction phase and, particularly, the use of gravel and/or sand as porous media. A conventional VF for 200 p.e. (3 m^2/p.e. with depth: 80 cm) would require 480 m^3 of gravel and/or sand Fuchs et al. [8] claimed that the construction phase impact could be significantly reduced by using local materials to minimize transport. Sand extraction poses serious threats to the environment in terms of erosion, loss of land and biological diversity, and the increase of poverty among people [9]. Additionally, some regions face supply constraints due to the overexploitation of natural aggregates in construction [10]. In the particular case of the Canary Islands, most of the sand used in the construction sector and beach regeneration has serious legal, environmental, economic, and political implications, because it is imported from the near Western Sahara in the African coast. This region, a former Spanish colonial territory, is still in disputes with Morocco and the Polisario Front [11]. The volume of sand and gravel devoted to the construction of CWs is a small proportion of the total amount extracted, but the point is to understand how sustainable CWs should be, ideally.

Over the last decade, many studies have been devoted to finding cost-effective substrates to increase the treatment efficiency of and/or minimize clogging in CWs [12]. The environmental impact of CWs can be drastically reduced by using wastes as substrates. Different materials, such as construction wastes, dewatered alum sludge [13], bamboo rings [14], palm tree mulch [15,16], and rice straw [17], have been tested.

Plants are considered to be an essential part of CWs. They help stabilize the bed surface, prevent clogging and channeled flow [18], and remove pollutants (N, P, heavy metals, etc.). Another important role of plants in CWs is their ornamental value [19] and the improvement of the CW's ecological significance, including wildlife habitat creation, cooling by means of evapotranspiration, recreation, and landscaping [20]. Additionally, plants can accelerate the development of microbial communities by promoting alternate aerobic and anaerobic micro-environments in roots [21]. Thus, not only the presence of plants, but also plant biodiversity plays a key role in the microbial communities and the CW's ability to remove pollutants.

The study of the bacterial communities allows us to analyze the contribution of these organisms to CWs and, to a large extent, to understand CWs work appropriately. Similarly, the study of bacterial communities could determine how different substrates, in combination with the presence of plants, affect the efficiency of CWs. From myriad of bacteria, fecal indicator bacteria (FIB) are the most commonly analyzed as a consequence of their involvement in European regulations and their impact on human wellness. FIB are represented by *Escherichia coli* and *Enterococci* [22,23]. The understanding of the whole bacterial community requires a detailed screening to unveil their diversity and distribution. Unlike bacteria culture methods, culture-independent methods based on molecular markers, such as 16s rRNA, and analysis of terminal restriction fragment length polymorphisms (T-RFLP) are an excellent approach to studying the bacterial community structure of CWs [24–27]. The analysis of T-RFLP patterns from different CWs and the comparison among them allows us to unveil to what extent a bacterial community can change and how far experimental conditions (flow, substrate type, presence of plants) would increase the performance of CWs.

The main goal of this work was to study the effect of plants on the performance of palm mulch-based VF CWs treating domestic wastewater. The role of plants was analyzed through both chemical and microbiological parameters. Furthermore, we aimed to compare the evolution over time of the structure of bacterial communities in the effluents of planted and unplanted mulch-based VF CWs.

2. Material and Methods

2.1. Location of Constructed Wetland and Sampling

The studied CW was designed to treat raw wastewater from a group of 4 households located in a rural zone of the Island of Gran Canaria, Spain. The households have a permanent population of 5 people, which is increased to 15 people during holidays and weekends.

Wastewater was discharged directly into a partially clogged, non-waterproofed, 36 m^3 seepage pit (Figure 1) with an approximate hydraulic retention time of 1.5 days. Two submergible, 700-W pumps, which were timer-controlled to function for 1 min each hour, were installed. Influent pulses were of approximately 15–20 L. The pit was supposed to provide partial sedimentation and homogenization. Thus, the influent can be considered to be partially decanted domestic wastewater.
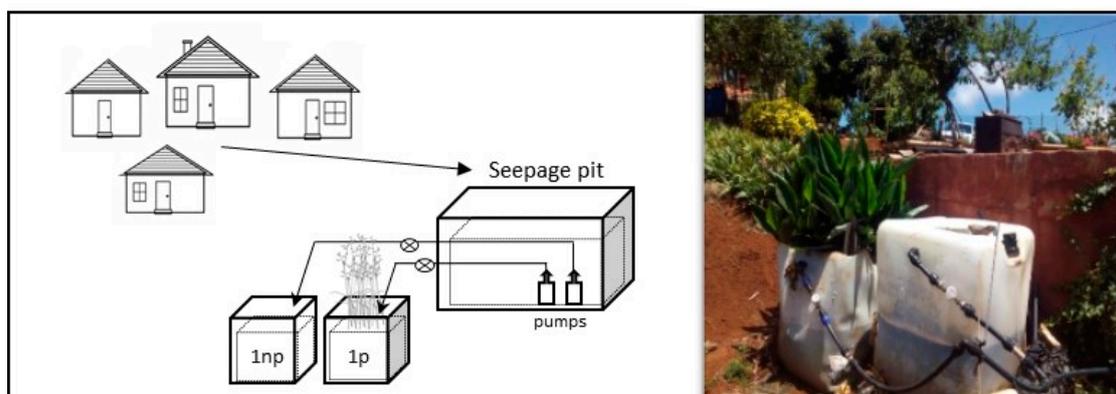
Figure 1. Layout and photograph of the constructed wetlands (CWs). 1p: planted vertical flow (VF) CW; and 1np: unplanted VF CW.

The CW was placed at 650 m above sea level. The average temperature is 18 °C, with January being the coldest month with an average temperature of 12 °C and August the warmest month with an average temperature of 23 °C. The average relative air moisture is 80% and the predominant wind direction is NNE. The average pluviosity is 485 mm/year, with the rainy season being between October and March.

The two VF CWs were constructed with 1.080-L plastic, cubic-like recipients. A layer of washed gravel with a height of 10–15 cm and an average diameter of 1 cm was deposited at the bottom of the recipients. The organic substrate was placed directly above the gravel and consisted of approximately 1-m-high mulch made of dry branches of Canarian palm tree (*Phoenix canariensis*). Additionally, two flow meters and valves were placed at the inlet of each reactor to measure and control the inflow. The influent was distributed on the surface of the VF CWs by means of 5 perforated tubes with a 12-mm diameter.

In order to value the contribution of the plants, one VF CW was planted with *Canna indica* and *Xanthosoma* sp. and compared with another VF CW (the control), which was not planted. These species of plant are characterized by large roots and big tubers, respectively. The analyses were started one year after the construction of the VF CWs, because the mulch substrate had been reduced by degradation and compaction.

Chemical and microbiological analyses were completed in unfiltered and homogenized samples of the influent and the effluents of the planted (1p) and unplanted (1np) VF CWs.

2.2. Analytical Methods

2.2.1. Water Analysis

Sampling was performed in the morning at 08:00 h, coinciding with when the pumps started a working period. The influent sample was taken during pumping from the influent outlet. The volume sampled was 1 L for each sampling point. The sampling recipients were 1-L plastic bottles for standard parameters, sterilized 50-mL glass bottles for *E. coli* and total *E. coli* enumeration, and sterilized 1-L amber glass bottles for DNA studies.

The water quality parameters were measured according to standard methods (APHA, 2005). BOD_5 (Biological Oxygen Demand, henceforth BOD) and Chemical Oxygen Demand (COD) were measured in homogenized, unfiltered samples. Ammonia-N and Na ions were determined with selective electrodes from Crison (Spain). Ca^{2+} and Mg^{2+} ions were determined by the EDTA titrimetric method. Anions (PO_4^{3-}, Cl^-, F^-, NO_2^-, NO_3^-, and SO_4^{2-}) were analyzed with a 792 Basic IC ionic chromatograph from Metrohm, equipped with a Metrosep A sup 4 column. The mobile phase and suppression solutions were 1.8 mM Na_2CO_3/1.7 mM $NaHCO_3$ buffer and 20 mM H_2SO_4, respectively.

The concentrations of *Escherichia coli* and total coliforms (TC) were determined by the membrane filter method with incubation at 37 °C for 24 h with a chromogenic agar (Panreac, Spain). *E. coli* displayed dark blue to violet colonies, while TC were assumed to be the sum of the *E. coli* colonies plus the salmon-red colonies.

2.2.2. Molecular Method

In order to analyze molecularly the bacterial community structure, a terminal restriction fragment length polymorphism (T-RFLP) analysis was carried out in the effluents of both CWs. The analyses were performed in December 2014, when the initial clogging symptoms were observed in 1np (light clogging), and in May 2015, when the clogging in 1 np was severe (severe clogging).

DNA Isolation

First, 1 liter of water of each sample was centrifuged, and pellets pooled to extract genomic DNA. The DNA isolation was performed according to the CTAB method described by Murray and Thompson [28]. In detail, CTAB was made of CTAB 0.1% (*w/v*), PVPP 0.1% (*w/v*), TRIS-HCl pH 8.6, SDS 10, EDTA 0.5M pH 8, NaCl 4M, and β-Mercaptoethanol 2% (*v/v*). To obtain bacterial samples, 800-μg pellets were introduced into microtubes. Then, an extraction buffer (800 μL) was added to a microtube containing the source of the DNA template. The sample was held for 1 h in a bath at 65 °C, and mixed gently by inversion approximately every 20 min. Later, 800 μL of CIA (chloroform: isoamyl alcohol, 24:1 *v/v*) was added and centrifuged for 20 min at 3000 rpm in a Beckman centrifuge.

Successive washes in CIA and centrifugations were carried out until the supernatant became whitish. To continue, 2/3 of isopropyl alcohol at −20 °C was added, and a centrifugation for 30 min at 15,000× *g* was carried out. Afterwards, the isopropyl alcohol was removed, and 20 μL of ethanol (80%) was added. Finally, the samples were centrifuged for 5 min at 15,000× *g*. The supernatant was then discarded, and the pellets were resuspended in 15 μL of H_2O and stored at 4 °C until used.

The yield and purity of genomic DNA were calculated from the A260/A280 ratio measured using a NanoDrop spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). For each VF CW type and date, an assay was repeated three times with two replicates of each one.

T-RFLP Analysis

The bacterial 16s rRNA gene of genus-specific *Enterococcus* sp. [29] was amplified by using a HEX fluorescent-labeled forward primer E1 (5′-TCA ACC GGG GAG GGT-3′) and an unlabeled reverse primer E2 (5′-ATT ACT AGC GAT TCC GG-3′). The sequence encoding *lacZ* for *E. coli* [30] was amplified using a fluorescent-labeled 6 FAM forward primer ZL-1675 (5′-ATG AAA GCT GGC TAC AGG AAG GCC-3′). The corresponding unlabeled reverse primer sequence was ZR-2548

(5′-CAC CAT GCC GTG GGT TTC AAT ATT-3′). DNA (5 µL) was thus amplified in a PCR reaction mix (50 µL of total volume) containing 10 µL of the 5X PrimeSTAR^{TM} buffer (Takara Bio Inc), 0.5 µL of PrimeSTAR^{TM} HS DNA Polymerase (2.5 U/µL) (Takara Bio Inc), 4 µL of dNTPs mix (2.5 mM each one, Takara Bio Inc), and 2 µL of each oligonucleotide (10 µM).

The amplifications were performed in a thermocycler MyCyclerTM (Biorad, Hercules, CA, USA), with an initial denaturation at 96 °C for 4 min followed by 40 cycles at 94 °C for 1 min, 45 °C for 1 min, and 72 °C for 5 min. The final elongation was carried out at 72 °C for 10 min. The PCR-amplification products were checked by agarose (1%) electrophoresis at 75 V, resulting in gene fragments of 733 pb and 876 pb for *Enterococcus* sp. and *E. coli*, respectively. The amplifications were carried out by triplicate.

PCR-amplification products were separately purified using a Wizard$^{®}$SV Gel and PCR Clean-Up System (Promega, Madison WI, USA). Purified products from *E. coli* and *Enterococcus* sp. were checked by electrophoresis on a 2% agarose gel. The concentrations were estimated as previously mentioned.

T-RFLP analysis was carried out based on the previous enzyme digestion assay. The NEBcutter v2.0 (New England BioLabs) software program was used to estimate a range of terminal restriction fragments (T-RFs) of varying sizes in silico. The restriction enzyme MseI (New England BioLabs) was selected in silico according to the yielded T-RFs of the respective PCR amplicons for *E. coli* and *Enterococcus* sp.

Enzyme digestion was performed with 100 ng of purified PCR amplicon for 3 h at 37 °C and inactivated at 65 °C for 20 min. Amplicon was then cut with 0.5 U of the restriction enzyme MseI (recognition site: T'TAA) according to the manufacturer's instructions (BioLabs, New England BioLabs). Restriction digestion was carried out 3 times for the *E. coli* and *Enterococcus* sp., respectively.

Sequencing was carried out on an ABI 3730 capillary sequencer (Life Technologies) by Secugen (Madrid, Spain), and the resulting sequences were analyzed using GeneMarker v1.85 (SoftGenetics). Optimizing was performed with different dilutions according to Secugen.

2.3. Statistics

2.3.1. Chemical and Microbiological Analysis

Average values of concentrations, surface loadings, and removals were compared by means of ANOVA if the data were homoscedastic (Bartlett test) and normally distributed (Shapiro–Wilk test). If these conditions were not met, the Kruskal–Wallis non-parametric test was used. In all cases, a significance level of 95% (p-value > 0.05) was utilized.

2.3.2. T-RFLP Analysis

The T-RFLP fragment sequences were obtained in .fsa format and GeneMarker software (SoftGenetics) was used for data scoring. The analyses of the polymorphic bands were only scored when they were highly reproducible and according to the peak height. Thus, small T-RFs ranging from 0 to 100 bp were excluded from the analysis, as they were considered to be PCR artifacts. Moreover, those T-RFs with intensities lower than 1% florescence, regarded as background interference, were also excluded from the matrices. Next, relative peak areas were recalculated according to the removal of artifactual peaks. The relative abundance of T-RFs was determined by calculating the ratio between the peak height of each peak and the total peak height of all the peaks within 1 sample and assuming that unambiguous T-RFs ranged in size from 100 to 850 bp.

For a comparative analysis of T-RFLP profiles, alignment was carried out through T-Align software with a default value threshold of 0.5 [31]. This software allowed for the compiling of peak profiles of different electropherograms, which corresponded to separate sampling days and sampling sites. In addition, the resulting consensus profile (henceforth overall consensus electropherogram) valued the presence or absence of T-RFs through a binary matrix by comparing with the other consensus profile of each of the other sites. For the purposes of qualitative valuation, the number of peaks in the consensus electropherograms was interpreted to be the operational taxonomic units (OTUs) [27], although

non-sequencing was carried out. Statistical comparisons of a number of T-RFs were performed using R software [32]. A one-way ANOVA followed by post hoc Tukey HSD and Dunnett T3 tests was used to detect significant differences ($p \leq 0.1$) between 1p and 1np under light and severe clogging. Additionally, Venn diagrams were performed with Venn Diagram Plotter (Integrative Omics, Pacific Northwest National Laboratory) [33].

3. Results and Discussion

3.1. Features of the Influent

As mentioned above, wastewater from the households was discharged directly into the seepage pit. The pit acted as a primary settler, making the influent more stable and reducing the concentration of the total suspended solids (TSS) as compared with other systems without a primary settler [16]. Table 1 shows the chemical and microbiological parameters of the influent. The number of data points changes for each parameter because only analytically valid data and non-outliers are included. Other parameters such as Na, electrical conductivity, and Sodium Absorption Ratio (SAR)were analyzed less frequently with the goal to obtain the agronomic quality of the effluents. As can be observed, the biodegradability (BOD/COD) ratio was 71% and turbidity was 521 NTU. With these traits, the influent can be considered to range from medium to high strength for organic matter (COD and BOD), low strength for TSS, and medium strength for nutrients N and P [34].

Table 1. Chemical and microbiological parameters, expressed as average value $\pm$ SD, associated with influent from September 2014 to May 2015.

Parameter	Average $\pm$ Std. Dev.	Max–Min, Number of Data Points	Units
COD	390 ± 114	783–260, 26	mg/L
BOD	279 ± 89	470–140, 22	mg/L
Total Suspended Solids	94 ± 27	137–48, 26	mg/L
Turbidity	521 ± 132	723–266, 26	NTU
N-ammonia	31.1 ± 6.2	42.8–20.2, 25	mg/L
Escherichia coli	$1.5\ (\pm 0.92) \times 10^6$	3.0×10^6–3.6×10^5, 23	CFU/100 mL
Total coliforms	$3.1\ (\pm 2.1) \times 10^6$	8.0×10^6–8.0×10^5, 23	CFU/100 mL
Na$^+$	114 ± 39	157–41, 9	mg/L
Hardness (Ca^{2+}Mg^{2+})	3.0 ± 1.5	5.4–1.6, 10	meq/L
Sodium Absorption Ratio	6.2 ± 2.9	10.7–2.5, 9	
Electrical Conductivity	1945 ± 169	2190–1304, 25	µS/cm
pH	7.8 ± 0	8.0–7.5, 25	
N-Nitrites	0.09 ± 0.4	1.6–0.0, 17	mg/L
N-Nitrates	1.1 ± 0.83	2.6–0.0, 17	mg/L
P-Phosphates	10.4 ± 3.9	14.7–2.0, 17	mg/L
Sulfates	14 ± 17	76–4, 16	mg/L
Cl$^-$	99 ± 11	122–83, 17	mg/L
F$^-$	7.9 ± 1.2	10.23–6.27, 17	mg/L

3.2. Average Surface Loading Rates and Removals

The hydraulic loading rate (HLR) and organic loading rates (OLR) are represented in Table 2. The average BOD-LRs applied in the present study were 51 and 70 g/m^{2}d, for 1np and 1p, respectively. These values fall in the upper range of those found in the literature. For instance, the OLR applied to the French System's first stage ranged between 40 and 50 g BOD/m^{2}d [5].

The average HLR of 1p (233 L/m^{2}d) was significantly higher (p: 0.026) than that of 1np (189 L/m^{2}d). The same result was obtained for the BOD-LR (1p: 70 g/m^{2}d, 1np: 51 g/m^{2}d, p: 0.0233) and ammonia-N (1p: 9 g/m^{2}d, 1np: 7 g/m^{2}d, p: 0.0212). Non-significant differences were reported for the COD-LR (1p: 90 g/m^{2}d and 1np: 70 g/m^{2}d, p: 0.0965) and TSS-LR (1p: 22 g/m^{2}d and 1np: 18 g/m^{2}d, p: 0.129). Considering that in the cases of dissimilarity, the p-values were close to 0.05

(HLR, COD, and ammonia-N) and that there was no difference for COD and TSS, it can be concluded that both VF CWs received almost similar surface loadings and their differences in removal can be mainly ascribed to the presence of plants.

Table 2. Hydraulic loading rate (HLR, L/m^{2}d) and organic loading rates (OLR, g/m^{2}d) for 1p and 1np.

Parameters	Average ± Std. Dev. Max–Min	
	1p	**1np**
HLR	233 ± 54 323–146	189 ± 78 349–44
COD	90 ± 29 137–46	74 ± 40 171–18
BOD	70 ± 29 126–24	51 ± 23 98–6
TSS	22 ± 9 40–8	18 ± 10 41–4
Ammonia-N	9 ± 3 18–5	7 ± 3 16–1

Although for some parameters, 1p received higher surface loadings, its performance was equal to or better than 1np (Table 3). The removals were slightly higher for 1p, although not significantly (p > 0.1) for COD (1p: 58%, 1np: 50%), BOD (1p: 62%, 1np: 59%), TSS (1p: 77%, 1np: 72%), *E. coli* (1p: 79%, 1np: 73%), and TC (1p: 82%, 1np: 74%). The exception was turbidity removal (1p: 90%, 1np: 73%, p: 0.005) for which the presence of plants improved clearly. This result is of interest, because according to Spanish legislation, turbidity is a basic parameter for the reuse of treated wastewater [35].

Table 3. Average removals ± standard deviation, expressed as %, and range of maximum and minimum values.

Parameters	Average ± Std. Dev. Max–Min	
	1p	**1np**
COD	58 ± 17 77–8	50 ± 17 86–27
BOD	62 ± 28 97–(−3)	59 ± 30 92–(−34)
Turbidity	90 ± 10 98–62	73 ± 19 99–46
TSS	77 ± 20 99–20	72 ± 15 99–39
Ammonia-N	−39 ± 50 55–(−123)	−53 ± 59 48–(−192)
Phosphate-P	−38 ± 169 39–(−666)	−46 ± 163 36–(−675)
Sulfate	−297 ± 282 86–(−1071)	−175 ± 239 61–(−777)
E. coli	79 ± 26 99.9–11	73 ± 28 99.8–(−8)
Total coliforms	82 ± 26 99.6–(−20)	74 ± 25 99.9–5.5

The removals of ammonia-N, phosphate-P, and sulfate were negative in all cases. The increased concentrations of the ions can only come from the substrate, because it is an organic material and from the accumulated sludge. As mentioned above, high surface loadings were applied, and no rest periods were allowed. Additionally, the reactors were very efficient in removing TSS, which were retained

as sludge, mainly on the surface of the CWs. The negative removals of ammonia-N, phosphate, and sulfate ions are more likely to come from the leaching or degradation of the accumulated sludge, rather than from the mulch, which had been in operation for 1 year. In other works (e.g., [16]) with palm mulch, we have observed good physical and chemical stability of the substrate, both in vertical and horizontal CWs, once it became stabilized, after 2–3 months in operation. However, under the stringent conditions imposed to the reactors, a further degradation of the substrate cannot be rejected.

It can be thought that because the substrate was mainly of an organic nature, the release of these nutrients can be caused by its decomposition. In fact, Saeed and Sun [36] observed that HF CWs, which employed wood mulch and gravel-mulch media, released organic matter, phosphorus, and TSS. This behavior was not observed in the VF CWs tested in our study. Additionally, in the present research, the substrate height was reduced by 40% by compaction and degradation during the first few months of operation. However, the analyses were initiated 1 year after construction. Thus, it seems more probable that the released ions came from the fast mineralization of the particulate matter retained on the surface of the VF CWs under the appropriate conditions of temperature, moisture, and aeration.

3.3. Performance Evolution of the Wetlands

The evolution of parameters such as COD, BOD, turbidity, TSS, ammonia-N, *E. coli*, and TC are represented for the influent and the effluents of 1p and 1np (Figure 2). Missing points have been removed after the statistical analysis of outliers.

(**a**)

(**b**)
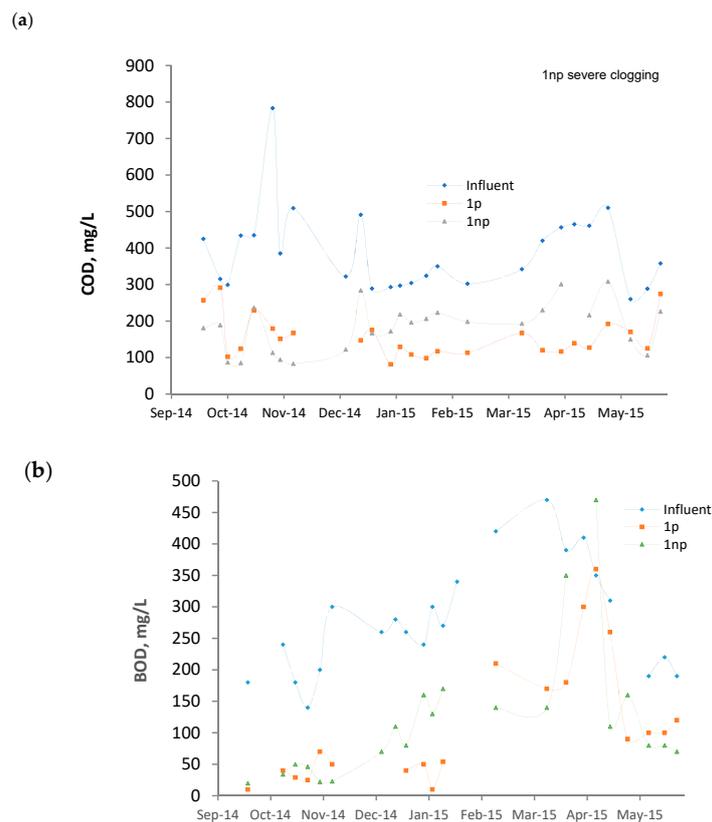
Figure 2. *Cont.*

(**c**)

(**d**)

(**e**)

(**f**)

Figure 2. *Cont.*

(**g**)
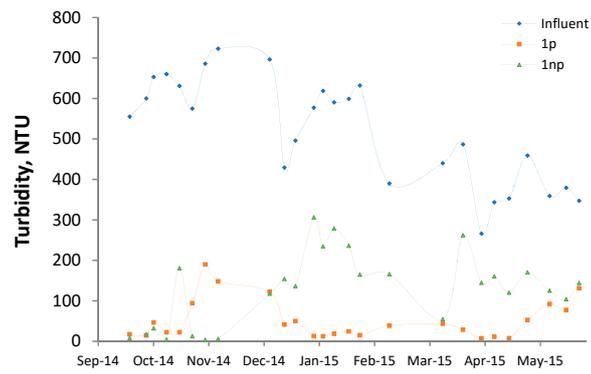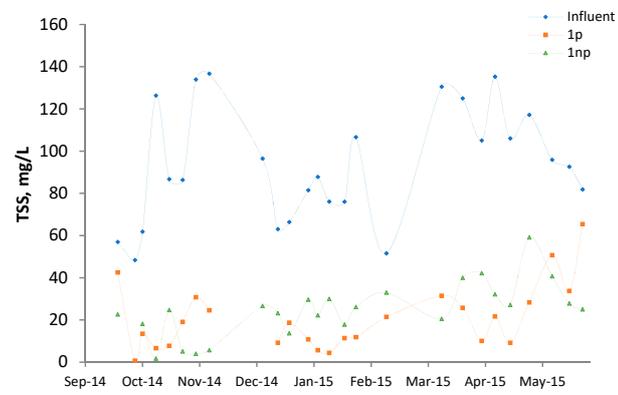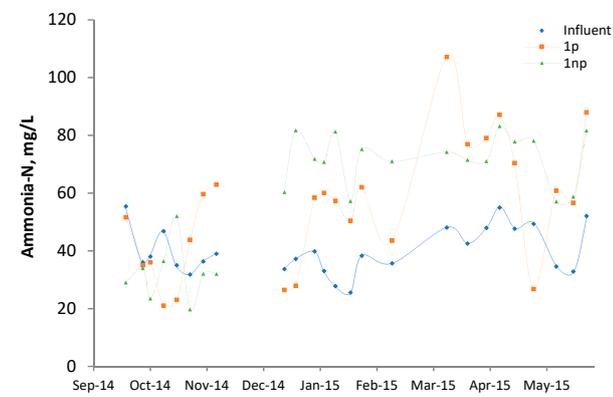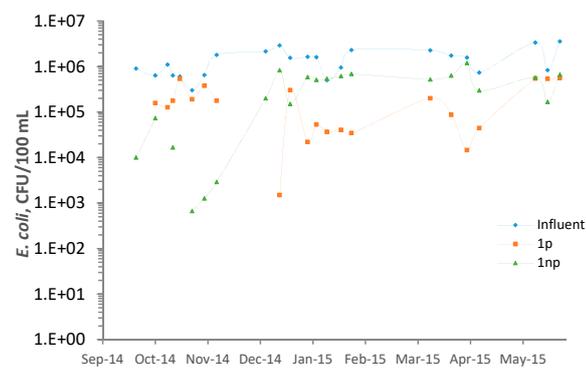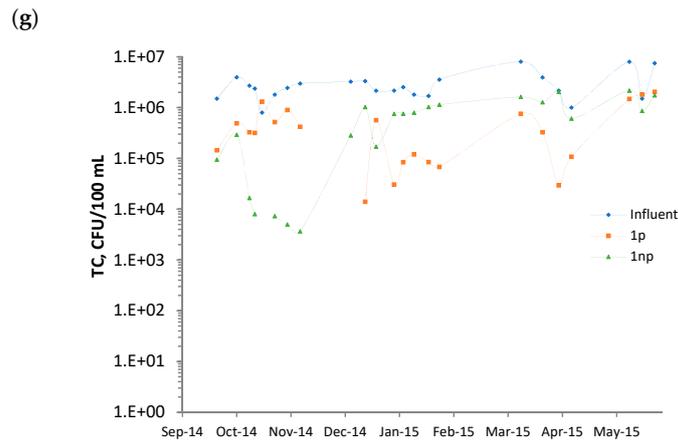
Figure 2. Concentrations in the influent (-◆-) and effluents of 1p (-■-) and 1np (-▲-) of (**a**) COD, (**b**) BOD, (**c**) turbidity, (**d**) TSS, (**e**) ammonia-N, (**f**) *E. coli*, and (**g**) TC.

Figure 3 shows the timeline of removals. During December 2014, the first clogging symptoms were observed in 1np. After that, the relative performance of the wetlands changed dramatically. Table 4 shows the average removals of chemical and microbiological parameters before and after clogging. Before clogging, 1np performed better than 1p with respect to turbidity (1np: 95% and 1p: 90%; p: 0.028), *E. coli* (1p: 59%, 1np: 97%, p: 0.007), and TC (1p: 82%, 1np:98 %, p: 0.001). Nevertheless, the removals of BOD (1p: 83%, 1np: 85%), COD (1p: 64%, 1np: 62%), and TSS (1p: 84%, 1np: 80%) were similar (p > 0.288). After clogging, 1np performance was significantly damaged (p < 0.006) for COD, BOD, turbidity, *E. coli*, and TC, while that of 1p was improved or remained unaltered.

(a)

(b)
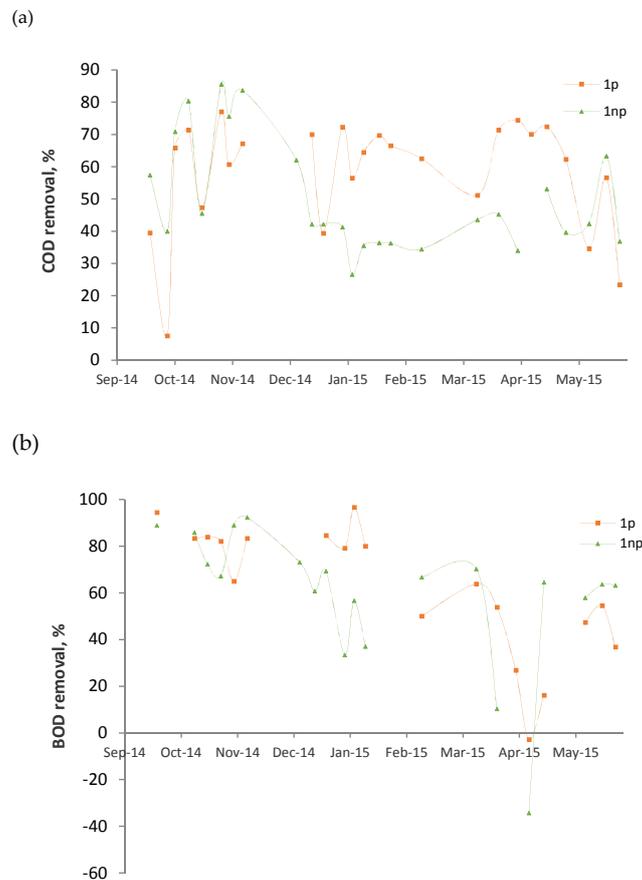
Figure 3. *Cont.*

(c)

(d)

(e)

(f)

Figure 3. *Cont.*

(g)
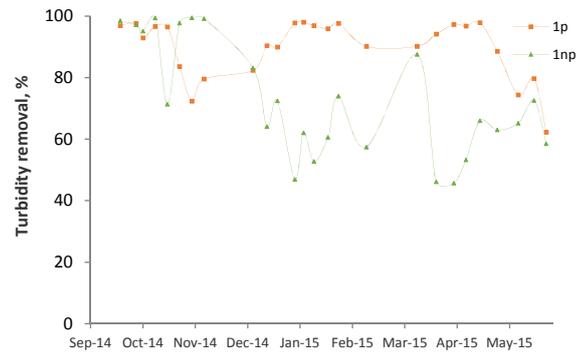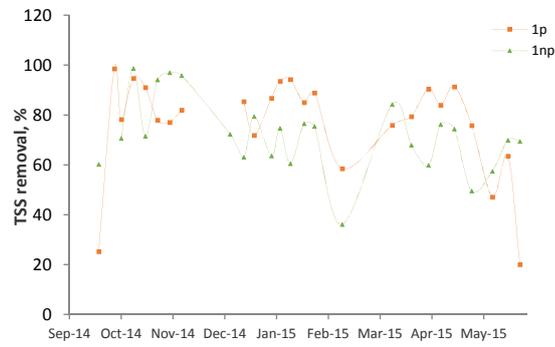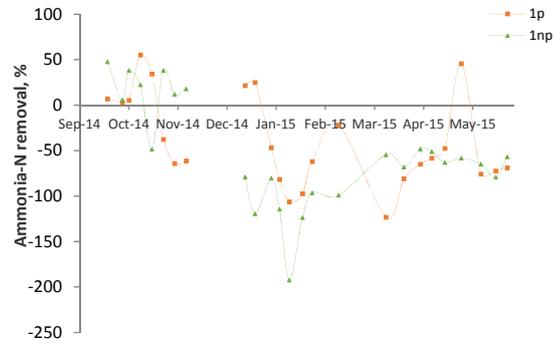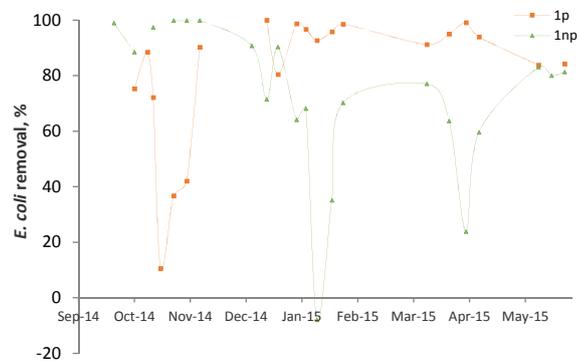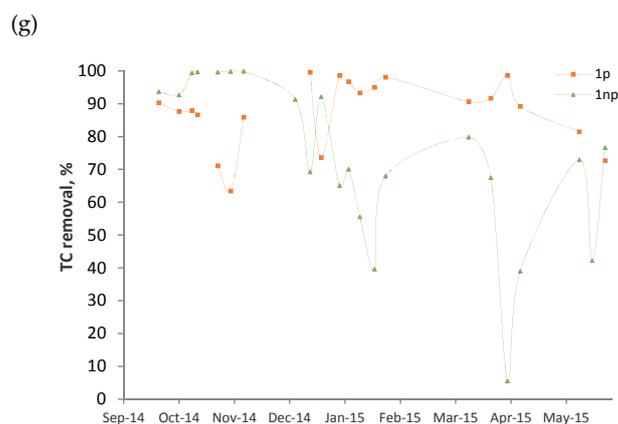
Figure 3. Evolution of the removal of (**a**) COD, (**b**) BOD, (**c**) turbidity, (**d**) TSS, (**e**) ammonia-N, (**f**) *E. coli*, and (**g**) TC for 1p (-■-) and 1np (-▲-).

The better performance of 1np before clogging can be explained by the stronger media compaction in the absence of plants and the consequent increase in the hydraulic retention time (HRT). In 1p, plant roots would be able to keep the media unclogged and, as a consequence, reduce the HRT and removal efficiency. In fact, it was observed that the effluent of 1np exited more slowly than that of 1p. Torrens et al. [37] observed a similar behavior in planted VF CWs, because the effluent from the first batches flowed through the beds more quickly than through unplanted ones. The authors claimed that the plant rhizomes could create preferential pathways. The preventing effect of plant roots regarding clogging has been discussed for a long time. Some studies have suggested that plants can make the substrate more porous [38], while others, such as Teixeira et al., [39] found a reduction in the porosity of the medium of HF CWs caused by the development of roots of Vetiver and Tifton 85 grasses. Yet, they considered that the low root volume, only 3.07% and 4.11% of the total pore space, could not have a great influence on clogging. However, Hua et al. [40] claimed that the role of plants varied throughout the clogging process. In the early stages, plant roots restricted water flow, while in the later stages, growing roots opened new pore spaces in the substrate. Our results are in agreement with those of Hua et al. [40] for the later stage but are the opposite for the early stages. This illustrates the complexity of the clogging process and the role of plant rhizomes.

Table 4. Average removals (%) achieved by 1p and 1np, before and after the light clogging of 1np.

	Wetland	Before	After
COD	1p	55	63
	1np	67	40
BOD	1p	82	85
	1np	83	55
Turbidity	1p	90	94
	1np	95	65
TSS	1p	75	87
	1np	84	71
Ammonia-N	1p	−7	−50
	1np	17	−115
E. coli	1p	59	95
	1np	97	60
Total coliforms	1p	82	94
	1np	98	69

The fact that 1np became clogged in such a short time, after about two years in operation, was unexpected. Additionally, in the case of 1p, a negative trend in performance along time can be observed, particularly for BOD (Figure 3). To understand these results, the following must be considered:

(i)　The mulch employed was not fully stable. Although the analyses were made 1 year after the construction of the beds, the results indicate that the substrate was not fully stabilized. The palm branch is basically composed of a central rachis on which leaflets and spines are inserted. Once the branch is dead, the leaflets degrade relatively fast and release color, organic matter, and ions. Hence, their presence in palm mulch for CW substrate should be minimized.

(ii)　The average surface loadings were relatively high (BOD: 59–62 g/m^{2}d, TSS: 18–22 g/m^{2}d, Table 2).

(iii)　The reactors were in continuous operation without any rest period. In a survey of 169 full-scale VF CWs treating raw domestic wastewater, Paing et al. [5] claimed that the nominal BOD load calculated on the first stage was generally 40–50 g/m^{2}d, which is a similar value to that applied in this study. However, the success of the French system is partially justified by the good aeration of the substrate. This was achieved by different measures that include the application of rest periods, allowing the sludge accumulated on the surface to become mineralized.

The reduction in the performance of 1np caused by clogging underlines the importance of the substrate aeration. Regarding pathogen removal, Headley et al. [41] observed that aeration improved *E. coli* removal in horizontal flow wetlands. The better performance of 1p could be caused by the accumulation of sludge on the bed surface and the consequent HRT increase. At the same time, the presence of the roots would keep the substrate unclogged.

These results show that the effect of plants in mulch-based VF CWs was not univocal. In the early stages of the CWs, the presence of the plants reduced the efficiency of the removal of pollutants by augmenting the substrate porosity with their roots. However, in the long term, they helped to retard clogging and, as a consequence, to achieve better removals.

3.4. Analysis of T-RFLP

Terminal restriction fragment length polymorphism (T-RFLP) is a rapid, highly reproducible, and robust molecular tool for the study of bacterial community structure [27,42]. It is known that bacteria communities are affected by several factors, such as physicochemical properties, climate conditions, and the presence of plants [43,44]. Hence, bacterial communities of 1p and 1np were analyzed via T-RFLP in order to ascertain the effects of the presence of plants.

Bacterial diversity, as estimated by the number of T-RFs, showed reproducible patterns across the MseI enzyme. In addition, the size distribution of the T-RFs generated by the restriction enzyme was consistent with that of the T-RFs derived from in silico digestion.

Interestingly, shifts in the sizes of the T-RFs occurred as a result of two different factors: the presence of plants and the progressive clogging of the substrate, as can be inferred from the analysis of the operational taxonomic units (OTUs). The two VF CWs shared 31 T-RFs for *E. coli* and 19 for *Enterococcus* sp. (Figure 4). The shared T-RFs indicate the presence of a common bacteria population regardless of the presence of plants (Figure 4). Furthermore, the size of the T-RFs ranged from 117 to 810 bp for *E. coli* and from 115 to 714 bp for *Enterococcus* sp.

In addition, 1np showed a significant increase ($p < 0.1$) in T-RFs compared with that of 1p for both *E. coli* and *Enterococcus* sp. (Figure 5).
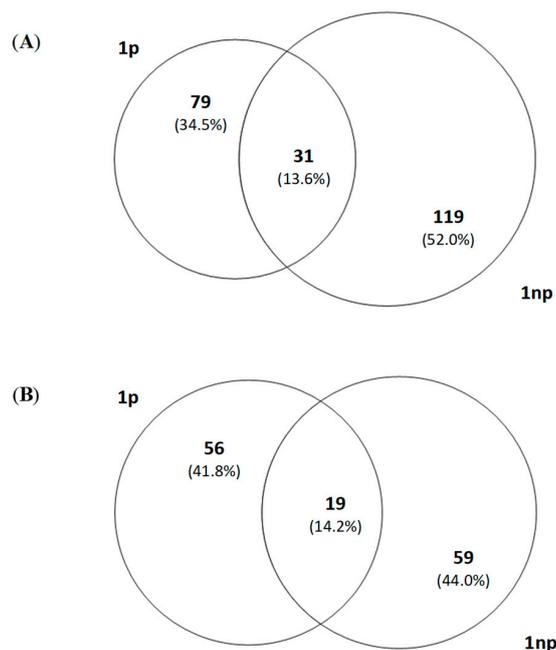
Figure 4. (**a**) Venn diagram of the size of the *Escherichia coli* terminal restriction fragments (T-RFs) shared between 1p and 1np. The number of T-RFs of 1p = 79; the number of T-RFs of 1np = 119; common number of T-RFs shared between both VF CWs = 31; (**b**) Venn diagram of the size of the *Enterococcus* sp. T-RFs shared between 1p and 1np. The number of T-RFs of 1p = 56; the number of T-RFs of 1np = 59; the number of T-RFs shared between both VF CWs = 19.

In addition, the results from the light and severe clogging periods showed that 1p reported a significant diminution ($p < 0.1$) in the T-RFs of *E. coli* over time (22.22% in light clogging versus 17.68% severe clogging). This behavior was also shown for 1np (38.89% light clogging versus 21.21% severe clogging; Figure 5a). This difference was more significant ($p < 0.01$) when relative abundance of T-RFs in 1np (38.89%) were compared to that in 1p (22.22%) at light clogging (Figure 5a). This result may infer that 1np was slightly clogged in December 2014 and continued to clog over time. In this sense, the chemical parameters sustain the T-RFs results (Table 4).

Additionally, the same trend in relative abundance of T-RFs was reported for *Enterococcus* sp., although the lower biodiversity of 1np under severe clogging was unexpected (Figure 5b). Hua et al. [26] claimed that clogging reduced bacterial diversity. Hence, an approach to explain low diversity in 1np was attempted by comparing shared T-RFs in 1p and 1np over time (Figure 6). Venn diagrams showed that 1np shared a major number of T-RFs between two sampled periods (1.8% light clogging versus 7.8% severe clogging). The presence of low shared-T-RFs, namely OTUs, in 1p would highlight the role of plant roots in combination with palm mulch substrate at retaining FIB bacteria. Kadam et al. [27] argued that the diminution of T-RFs in the planted VF CWs was committed to the diminution of genetic features, which could be correlated with the presence of root-associated microbiota. In addition, we assumed that root presence and microfauna might affect 1p performance.

The results obtained in this work indicate that plants increase the substrate porosity, but the results can be quite different depending on the level of substrate clogging. During the early stage of the experimental period, when the substrate was far from being clogged, the effect was negative as the CW with plants demonstrated worse pollutant removal. In this case, an increase in porosity led to shorter HRT. The sludge accumulated on the substrate would help to increase the HRT and thus the removals. However, later, when the sludge accumulation becomes excessive and clogging starts to develop, the consequences were very positive, because the plants were able to notably delay clogging by increasing the substrate porosity.
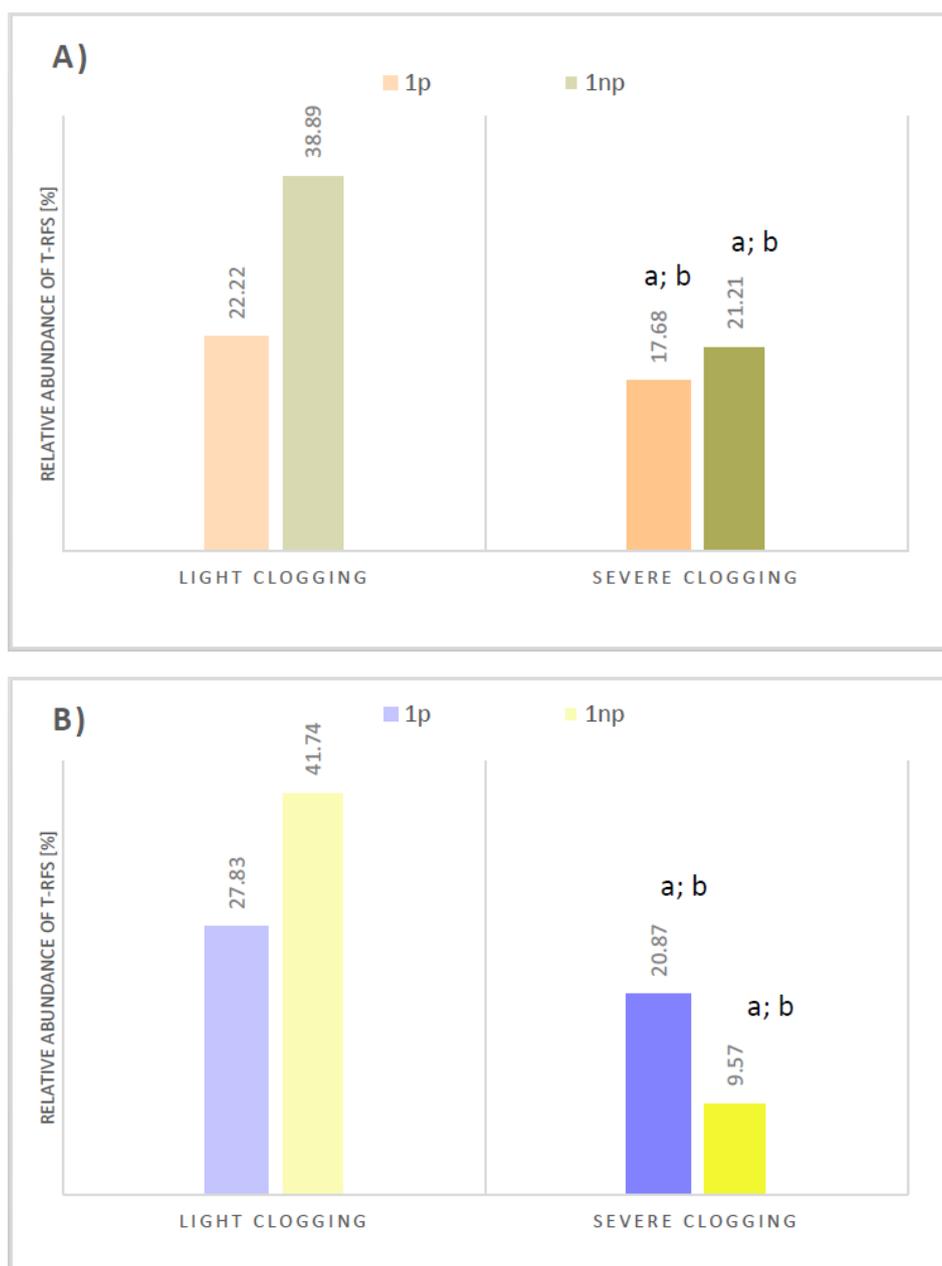
Figure 5. Relative abundance (%) of T-RFs at light and severe clogging for 1p and 1np. (**a**) Corresponds to the relative abundance of *Escherichia coli*. The 100% was assumed as the ∑ T-RFs (198 T-RFs) obtained for both VF CWs (1p and 1np) and both stages (light and severe clogging); (**b**) Corresponds to the relative abundance *Enterococcus* sp. The 100% was assumed as the ∑ T-RFs (114 T-RFs) obtained for both VF CWs (1p and 1np), and both stages (light and severe clogging); [a] indicates differences over time; [b] indicates differences between 1p and 1np.

The strong reduction of the palm mulch during the first year of operation indicates the importance of the substrate selection. The application of rest periods is highly recommended to delay clogging and improve the pollutant removals and the accumulated sludge mineralization. Similarly, the constructed wetland performance was optimized by the presence of plants as the reduction in the number of OTUs demonstrated.
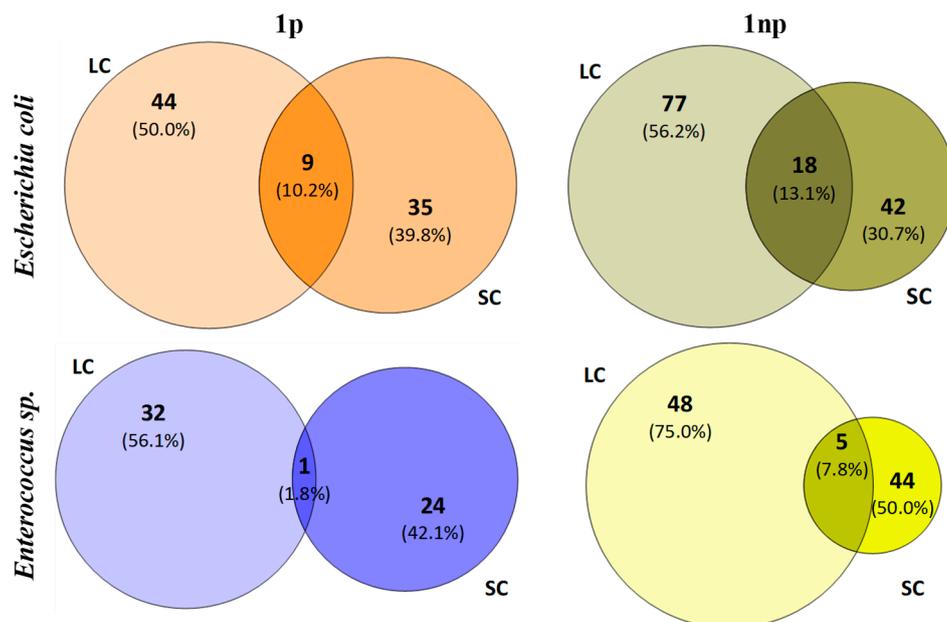
Figure 6. Venn diagrams showing the numbers of *Escherichia coli* T-RFs and *Enterococcus* sp. T-RFs in light and severe clogging and the number of T-RFs shared between both stages in each case. LC = light clogging; and SC = severe clogging.

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