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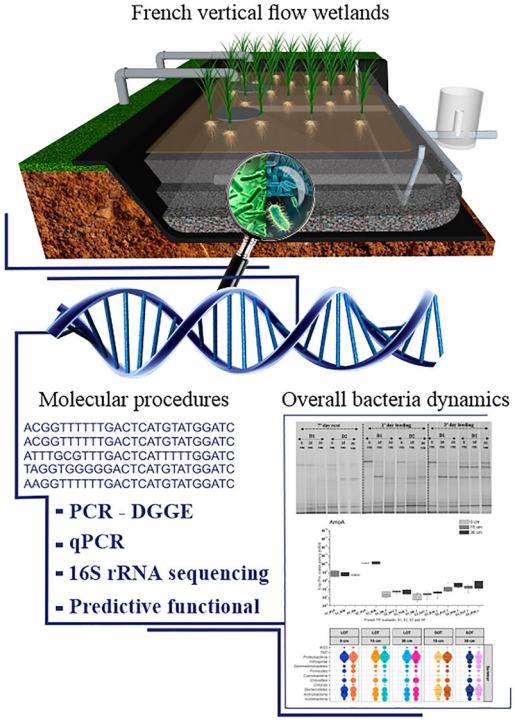
ABSTRACT

A multiphase study was proposed to examine microbial communities linked to the nitrogen cycle in the first stage of four full-scale French vertical flow treatment systems. To this end, denaturing gradient gel electrophoresis (DGGE) was performed for structural assessment and quantitative PCR (qPCR) to enumerate the abundance of ammonia-oxidizing (AOB). 16S rRNA sequencing was used to assess the taxonomic profile followed by putative assessment of functional genes. The samples were collected under different conditions, such as operational time (presence/absence of sludge layer on the surface of the filters), season (winter and summer), sampling depth (0, 15 and 30 cm) and operation cycle (rest and feed periods). A structural disparity was noted in the upper layers, whereas higher similarity at 30 cm was observed highlighting the effect of organic matter on bacterial diversity. The 7th rest day was highlighted by an apparent decline in the microbial community abundance. Additionally, qPCR indicated that the largest amount of AOB was found at 30 cm depth and during the feeding period. From the taxonomic profile, *Mycobacterium*, *Acinetobacter*, *Flavobacterium*, and *Nitrospira* were the most abundant genre found in all systems. The functional prediction results showed predicted genes linked to the denitrification process. The results suggested that operating time and season were responsible for the pattern of the microbial community behavior. This study allowed us to further understand the bacterial dynamics and to advance the idea of design modifications made in the first stage of the classical French system to improve nitrogen removal are promising.

KEYWORDS

French VF wetland; Microbial community structure; Nitrifying bacteria; Taxonomic profile; Functional prediction



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1. Introduction

French vertical flow (VF) wetlands are biological systems where raw wastewater [1] percolates through a porous medium creating a sludge deposit layer on the top surface; hence, these systems treat sludge and wastewater in a single step (a specificity of French VF wetlands). In order to mitigate the clogging in the system, rest and feed periods are implemented, creating highly variable conditions of both water quality and oxygen content. Consequently, this might induce spatially (horizontally and with depth) [2] and temporal heterogeneity in the microbial population and, by extension, in microbial activity. This activity is the main factor responsible for the treatment performance and durability of the system. Furthermore, the French VF wetland has been adapted in a number of climates, such as cold [3,4] and tropical climates [5–7], which would add another layer of variability to the microbiota distribution. Improving knowledge of microbial diversity and dynamics, together with the physicochemical results and their relationship with systems operation and efficiency, could help inform the system design and operation mode, thus improving the performance of French VF wetland. To date, there is little knowledge on the microbial dynamics in French VF wetlands opting instead to focus on global indicators (stability of nitrification, low rate of deposit accumulation, oxygen content within the filter material, etc.) [1] to evaluate performance.

From very early on, scientists have paved the way for microbial community assessment and provided evidence of the strong impact that the microorganisms have on pollutant degradation in treatment wetlands (TWs). Beginning in the 1980s, the literature started to report the use of activity and enumeration techniques and, from 2008, structural assessments and functional studies started to become more popular [8].

Weber [8] further points out that TW microbial community research has increased from 2013 to 2016, and microbial community structure was the most commonly used methodological type followed by activity, function, and enumeration, respectively.

More recently, great strides are being made in the field of microbial community assessment in TWs, with functional assessment methods being developed, better utilized, and is related directly to water treatment. The use of next-generation sequencing and biological computational tools have allowed cost-effective, large-scale multiplexing analyses, which have transformed our understanding of the interactions between microbial communities and their corresponding niches [8,9].

In this sense, several studies have benefited from these techniques to evaluate the microbiota of TWs [10–21]. Some authors [22–24] have also reported on the dynamics and heterogeneity of TWs and emphasize that changes in environmental conditions and other factors such as layer depth and operational time [25,26], wastewater quality characteristics [27] and flow [28], can result in variations in composition, bacterial structure and influence the quality of the final effluent.

All of these studies have provided valuable information on the microbial community's behavior and identification. However, since microbiota are known to be heterogeneous, temporally dynamic, and structurally and functionally diverse, especially in wetland systems that possess a vast array design modifications, additional efforts are required to gain a solid understanding of microbial community dynamics, diversity, and potential functional under different operational conditions [8]. Thus, further studies should be performed, especially in full-scale systems, which operate over longer periods with well-defined parameters and an established bacterial community. To our knowledge, this study is the first to address this issue to improve our understanding of the microbial community in classical French VF wetland at a full-scale. Due to the importance of improving nitrogen performances of the first stage of the French system, this study is mainly focused on the bacteria involved in the nitrogen cycle in order to better inform design and operation modes in the future. In this framework, this paper proposes a multiphase study aimed to assess microbial community through identification of the dominant genes, highlighting the bacteria linked to the nitrogen cycle, and understand their structural and potential function dynamics spatially and temporally on the first stage of French VF treatment systems.

2. Materials and methods

2.1. French vertical flow wetlands – description of the systems and physicochemical analyses

In this study, the first stage of four classical French vertical flow wetlands (denoted as S1 through S4) treating domestic wastewater at a full-scale was used for the analysis of microbiota from the filters (Figure 1). The selected French VF wetlands are located in different cities (see map in Figure 1a) and were all designed according to the design standards for the classical two-stage French vertical flow wetland design (Figure 1b) [1,29,30].

The classical French systems are comprised of two stages, and each stage contains alternately operated cells. More specifically, the first stage is divided into three parallel cells (total of 1.2 m^2 / PE) where the sludge treatment, partial removal of organic matter

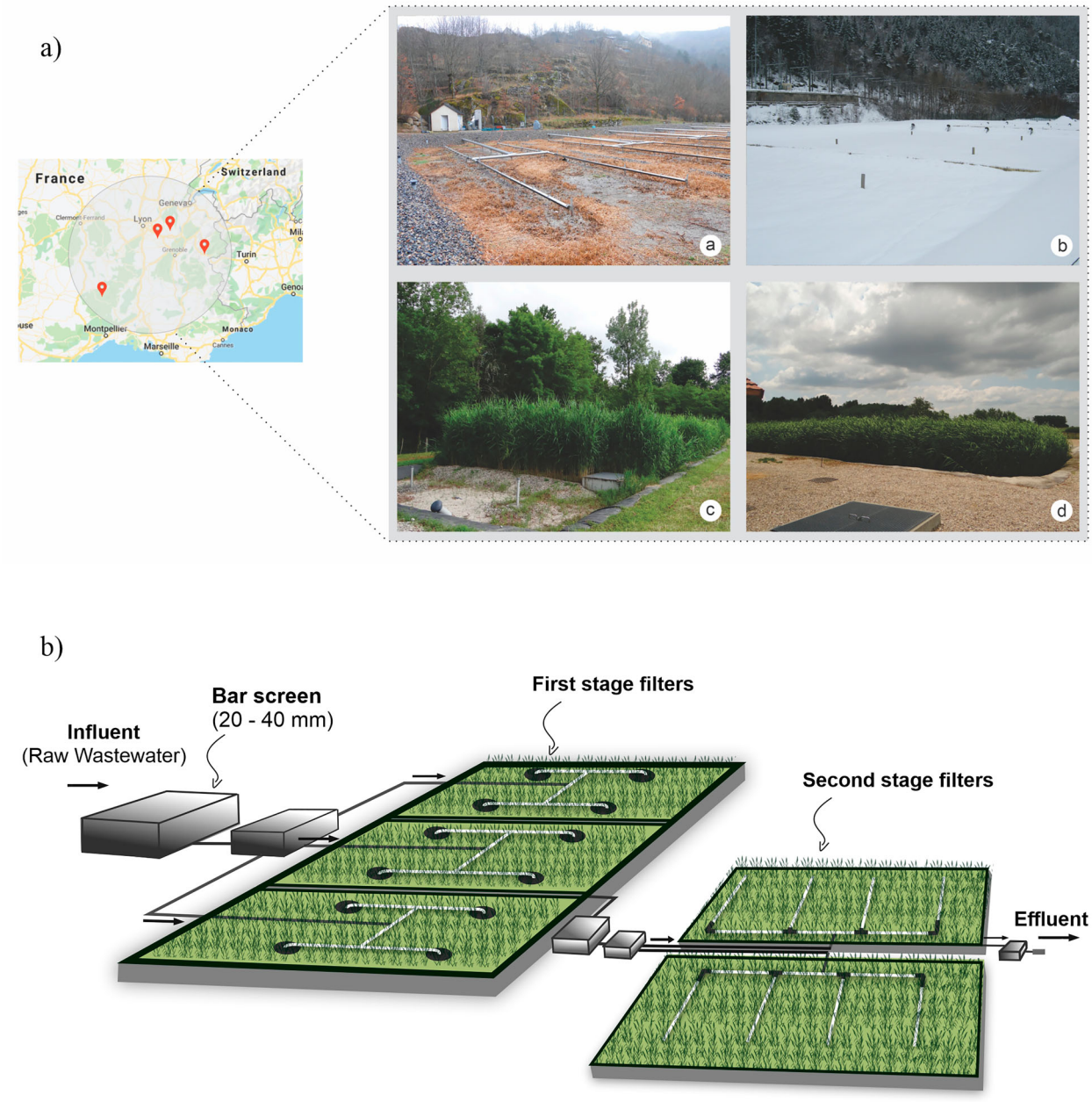

Figure 1. First stage French VF wetlands on a full-scale. a) Location map and evaluated systems: a) S1 (1800 m^2); b) S2 (1204 m^2); c) S3 (240 m^2); d) S4 (600 m^2). b) Schematic of the classical French VF design highlighting the first stage [adapted from 30].

and nitrification occur. Raw wastewater initially passes through a simple screen of 20–40 mm mesh before being fed into the system. The filters are fed for 3.5 days and then rest for 7 days. The first stage is composed of three layers: the upper layer (the main layer responsible for ensuring aerobic conditions) composed of 2/6 mm gravel; a transition or intermediate layer (5/15 mm gravel) and a drainage layer, which consists of coarse gravel (20/60 mm) at the bottom of the bed. Note that only one of three parallel cells and the upper layer was evaluated in this study and all filters had the same filter material (2/6 mm).

Average influent pollutant concentrations and removal efficiencies for the first stage of the VF wetlands are presented in Table 1. Also, the average hydraulic and organic loads on the filter during operation are included (m/d). All removal rates refer to first stage treatment performances [1], and systems can be considered as similar in terms of performances regarding the variation of flow and climatic conditions.

Table 1. Average influent pollutant concentrations and removal efficiencies.

	S1		S2		S3		S4	
	Value	Removal rate (%)	Value	Removal rate (%)	Value	Removal rate (%)	Value	Removal rate (%)
Organic (COD) load ($g/m^2/d$)	181	–	162	–	226	–	128	–
Hydraulic load (m/d)	0.28	–	0.30	–	0.46	–	0.21	–
BOD_5 (mg/l)	304	87	200	78	197	76	252	82
COD (mg/l)	649	83	541	74	493	68	611	78
TSS (mg/l)	275	89	281	85	193	75	238	81
TKN (mg/l)	65	59	73	45	78.2	55	64	53
Temperature (°C)	6°C	–	3°C	–	22°C	–	24°C	–

2.2. Molecular analyses of the samples

2.2.1. Sampling characteristics

The four full-scale systems evaluated in this study had been under long-term operation (at least two years), and therefore, the microbial communities were considered stable.

The objective was to select French VF wetlands with similar designs, but different operational times (short operation time – SOT, operating approximately for two years, and long operation time – LOT, i.e. with different deposit layer heights of organic matter) and sample them across two seasons, winter and summer. Two of the systems (one SOT and one LOT), which are well described [3], were selected in a mountainous area (S1 and S2), whereas the other two wetlands were located in plains (S3 and S4).

Additionally, different sampling points were selected to conduct the temporal and spatial evaluation including different depths (0, 15 and 30 cm), distance from a feeding point (1 and 2 m). For S3 and S4 systems, additional sampling was conducted to examine the influence of the operation cycle (feeding/rest period) on the microbial communities. Table 2 summarizes the conditions and sampling strategy of each site.

We used a stainless-steel core drill (SDEC France – a soil sampler sold commercially) that was 100 cm in length and 5 cm in diameter to sample the filter material. Two individual plexiglass tubes of 30 cm were cored from each system, at 1 (d1) m and 2 (d2) m from the feeding point respectively, to extract the filter material. This core was then sampled at 0 cm (deposit layer), 15, and 30 cm depths. However, in the SOT systems where no sludge was present, collection occurred only at depths of 15 and 30 cm. A minimum of 60 g was collected at each sample point.

Ultimately, regarding the molecular techniques used in this study, denaturing gradient gel electrophoresis (DGGE) analysis was chosen to visualize the bacterial community structure. Owing to the focus on the nitrogen cycle in the first stage, the DGGE analysis was centered on the ammonia-oxidizing bacteria (AOB), which are responsible for nitrification, to verify the bacterial temporal and spatial profiles. In total, 70 samples (4 samples for S1, 6 samples for S2, 36 samples for S3 and 24 samples for S4) were characterized by DGGE analysis.

After the analysis of the results from DGGE, for the taxonomic analysis and functional potential of the microbiota, only samples from d1 were selected and a sample pool was considered for feeding and rest periods. Following this, a total of 15 samples were amplified, 2 for S1, 3 for S2, 6 for S3 and, 4 for S4. Table 3 details the codes for the sampling points for these analyses and a sampling schematic plan is shown in Supplementary Figure 1

Table 2. The conditions and sampling strategy of each system evaluated.

Variables	Description			
Systems and location	S1 (Pont de Montvert) Mountain	S2 (Orelle) Mountain	S3 (Evieu) Plain	S4 (Culin) Plain
Operational time - Young: short operation time (SOT) - Old: long operation time (LOT)	SOT	LOT	LOT	SOT
Deposit height (cm)	0	>10	>10	0
Measurement season	Winter	Winter	Summer	Summer
Sampling distance from a feeding point	1 and 2 meters			
Sampling depth from the surface of each distance (cm)*	15–30	0–15–30	0–15–30	15–30
Sampling operation cycle	-	-	Feeding/rest period	Feeding/rest period

* 0 (deposit layer), first 15 cm to 30 cm depth of the first layer of gravel (2/6 mm)

Table 3. Information on the sampling points used to collect and display data.

French VF wetlands	Code	Information
S1	S1_D15	S1 = System 1
	S1_D30	D15 = 15 cm depth
		D30 = 30 cm depth
S2	S2_D0	S2 = System 2
	S2_D15	D0 = 0 cm – deposit layer
	S2_D30	D15 = 15 cm depth
		D30 = 30 cm depth
S3	S3_D0_f	S3 = System 3
	S3_D15_f	D0 = 0 cm – deposit layer
	S3_D30_f	D15 = 15 cm depth
	S3_D0_r	D30 = 30 cm depth
	S3_D15_r	f = feeding period
	S3_D30_r	r = rest period
S4	S4_D15_f	S4 = System 4
	S4_D30_f	D15 = 15 cm depth
	S4_D15_r	D30 = 15 cm depth
	S4_D30_r	f = feeding period
		r = rest period
For all systems	d1	d1 = distance from the feeding point – 1 m

2.2.2. DNA extraction, PCR and profiles of the amplifications by DGGE

Genomic DNA was extracted from pellets of concentrated effluent using the Power Soil™ DNA Isolation Kit (Mobio™, laboratories, Inc., Carlsbad. CA. EUA) according to the manufacturer's protocol. Then, the DNA was amplified by polymerase chain reaction (PCR) using an ammonia-oxidizing community (AOB)-specific primer set: CTO 189f (5´-GGAGRAAAGCAGGG-GATCG-3´), CTO 189f GC (5´-GGAGGAAAGTAGGG-GATCG-3´) and the reverse primer CTO 654R (5´-CTAGCYTTGTAGTTTCAAACGC-3´). The size of the amplified region was 465 bp [31], corresponding to the V3–V4 region of the 16S rRNA gene using the following procedure: activation (hot start) at 95°C for 15 min, 35 cycles of three steps (denaturation at 94°C for 30 s, hybridization at 57°C for 30 s, and extension at 72°C for 1 min). Followed by a final extension at 72°C for 5 min. The PCR mixtures contained 2.5 µl DNA, 250 µM dNTP, 1.5 mM MgCl2, 0.32 µM CTO 189F, 0.16 µM CTO 189F, 0.48 µM CTO 654R, 0.625 U Taq polymerase and 1X buffer.

After PCR, the standardized samples were placed on an acrylamide/bisacrylamide gel at a concentration of 6% (v/v) with denaturing gradients of urea and formamide at concentrations of 15% and 55% for the DGGE gels. The run characteristics were as follows: 200 V for 6 h at 60°C. The profiles of the amplifications were analyzed using the program GelCompar II version 6.5 (Bio-Systematica™, Wales. UK) with hierarchical clustering using the Jaccard index and the UPGMA clustering model.

2.2.3. Quantitative PCR

To quantify the AOB the primers amoA 1f (GGGGTTTCTACTGGTGGT) and amoA 2r (CCCCTCKG SAAAGCCTTCTTC) [32] were used and total bacteria 16S rRNA was assessed using the primers 1055f (ATGG CTGTCGTCAGCT) and 1392r (ACGGGCGGTGTGTAC) [33,34]. Quantitative PCR (qPCR) was carried out in duplicate with an IQ™ (Bio-Rad, CA. EUA) and 2 µl DNA (5 ng/µl) in a CFX96 Real-Time System C1000™ Thermal Cycler using the following run conditions for total bacteria: 95°C for 3 min; 40 cycles of three steps, denaturation at 95°C for 30 s, hybridization at 60°C for 30 s, and extension at 75°C for 30 s; and a final step at 65°C – 95°C for 10 min. For AOB, the run conditions were 95°C for 3 min; 40 cycles of three steps, denaturation at 95°C for 30 s, hybridization at 56°C for 30 s, and extension at 75°C for 45 s; and a final step at 65°C – 95°C for 10 min. The data were automatically recorded on a qPCR Bio-Rad™ CFX Manager Quantity One device. Standard curves were obtained using a serial dilution of synthetic plasmids containing the amoA gene (the gene that carries out the first nitrification step, $NH_4^+ \rightarrow NO_2^-$) or rrs gene (the gene coding for 16S rDNA, which allows the assessment of the total bacterial community) and ranged from $1E^{+07}$ to $1E^{+02}$ gene copies/µl. The qPCR efficiency ranged between 85 and 110%.

2.2.4. High-throughput sequencing and statistical analyses

For High-throughput 16S rRNA sequencing analysis, all 16S rRNA reads were analyzed using sequencing of the V3–V4 region on the extracted DNA. The universal primers 341F 5´-CCTACGGGRSGCAGCAG-3´ [35] and 806R 5´-GGACTACHVGGGTWTCTAAT-3´ [36] were utilized since this pair has good taxonomic coverage in *Bacteria* and *Archaea* [37]. Purified products were sent to the company Neoprospecta Microbiome Technologies, Inc. (Florianópolis, Brazil) for sequencing using the Miseq platform (Miseq™, Illumina Inc., USA). The data was processed using mothur, version 1.33.3 [38], following the MiSeq standard operating procedure (SOP) (http://www.mothur.org/wiki/MiSeq_SOP) [39]. Sequences were clustered into operational taxonomic units (OTUs) at 0.03 (97% similarity) and classified using SILVA Database release 119 [40].

The count table and metadata from taxonomic annotation from mothur was imported as phyloseq object., In the R Statistical environment, a complete workflow was developed for data exploration, statistical analyses, and graphics. Alpha diversity (observed richness, Chao1,

and Shannon), beta diversity (Principal Coordinates Analysis – PCoA) and rare curve function were obtained after the normalization process.

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (version 1) was used to predict the metabolic dynamics of the communities [41] from the 16S rRNA gene using the recommendation obtained in the online protocol (http://picrust.github.io/picrust/). For that, OTUs were closed-reference picked against the May 2013 Greengenes database using QIIME (version 1.9.1) with 0.97 similarities [42]. The functional predictions were assigned to KEGG Ortholog, and the data were explored using KEGG modules (nitrogen conversion).

Sequencing data yielded in this study has been deposited in the European Nucleotide Archive under the accession number PRJEB31770.

3. Results and discussion

3.1. Bacterial biofilm detachment

The detachment technique of bacterial communities from biofilms is as critical as the techniques used for the detection of bacteria. It must be carefully performed so that a maximum number of bacteria can be detected to make the sample as representative as possible. Thus, to evaluate the best bacterial biofilm detachment method, several procedures were tested in this study. The method chosen included two steps: bacterial biofilm detachment and DNA extraction. The procedure that obtained the highest DNA concentration (ng/µl) (variation from 2% to 12% more DNA concentration) and that had the highest degree of replicability was then chosen as the method to be applied in this study. The procedure is as follows: approximately 60 g of the collected material was placed in a circular vessel, followed by the addition of ultrapure autoclaved water, and finally, the samples were placed on a shaking platform for 3 h at 200 rpm. After that, the liquid was transferred to individual centrifuge tubes by filtering with the aid of a 2 mm sieve to remove only large particles. Subsequently, the samples were centrifuged for 10 min at 12 000 rpm. Once the supernatant had been removed, the pellet weight was noted, and the biomass was stored at −20 ° C until the moment of use.

3.2. Microbial community assessment

3.2.1. Bacterial community structure

As a result of the structural assessment, in system 1 (S1), the statistical values determined by UPGMA indicated a high similarity among different depths and distances

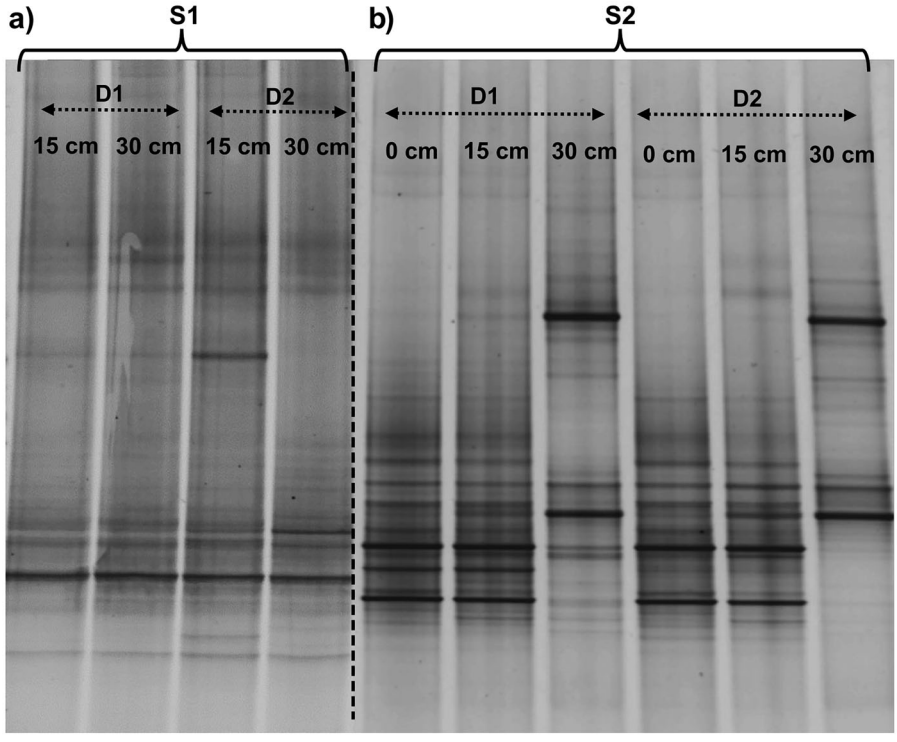

Figure 2. DGGE gel banding temporal and spatial profiles of nitrifying (AOB) bacterial communities in systems (a) S1 and (b) S2. d1 = 1 m from the feeding point; d2 = 2 m from the feeding point; 0, 15 and 30 cm depth. Each black line represents a band that corresponds to a group of AOB.

(≈80%) (Figure 2). This homogeneity may be linked to the fact that S1 is a short operation time (SOT) system and because there was a good distribution of the effluent in the area above the filter during feeding. As for the S2, the differences between the depths – high disparity in the upper layers and greater similarity (85%) in 30 cm was observed. This result highlighted the effect of organic matter deposition on bacterial diversity. This is understandable since organic deposition offers good conditions for the development of bacteria due to the presence of food supply and humidity [2]. This observation is consistent with the fact that—provided there is an adequate oxygen concentration—aging of the system is beneficial for treatment efficiency. Moreover, the presence of AOB at 30 cm may indicate that aerobic conditions are prevalent throughout the filter even under various conditions, such as low temperatures and the presence of snow.

For S3 system, a temporal evolution was observed through the feeding and rest periods (Figure 3). We uniformly noted a reduction in diversity and intensity throughout the rest period and an increase during the feeding period. The results obtained by UPGMA statistical analysis confirmed – 80% similarity for 1st and 2nd rest days, between the 15 and 30 cm depths and between distances. On the 4th rest day, we visually observed a lower bacterial community intensity, which was 59% similar to the 1st rest day, indicating a change in the bacterial profile. The 7th rest day showed an apparent decline in the number and intensity of bands compared to the first rest days. Thus, it can be concluded that this rest time (seven days) would be enough to avoid an excessive decline in the AOB community, thus optimizing the efficiency of the treatment.

The feeding period showed similarities above 75%, and its distances (d1 and d2) presented a higher similarity above 80%. This result may indicate a good homogenization of the wastewater distribution in the system (two feeding points for a 35 m^2 area with a 1 $m^3/h/m^2$ feed flow) as the filters had good operational behavior. Among the depths analyzed, the bacterial community was noted to be more similar (above 90%) at 15 and 30 cm.

The molecular fingerprint of the S4 system is shown in Figure 4. In general, it appears that in the SOT system, a lower diversity of AOB may be found. This result could be related to the fact that it is a young system.

3.2.2. Quantification of ammonia-oxidizing bacteria and total bacteria

To quantify the functional potential, qPCR was performed based on the number of copies of amoA/g pellet of AOB and the number of copies of rrs/g pellet. The gene concentrations for all systems are shown in Figure 5.

Regarding the S1 and S2 systems, it was possible to observe that at S1 (which does not contain organic deposits) the largest amount of AOB was found at the 15 cm depth (log1.00E + 09); however, at S2 (which has organic deposits), a greater amount was found at 30 cm (log1.00E + 11). This result corroborated the results of DGGE gels, which showed that the deposit layer, snow and the presence of an aeration drain (promoting aeration from the bottom) seem to influence the spatial structure and functional potential of the bacterial community, favoring nitrifying bacteria in the layers below the surface. For the total bacteria, the number of gene copies/g pellet showed a little variation (values were on the order of log1.00E + 11) for S2.

The qPCR results for system S3, in general, showed that the amount of AOB and total bacteria increased with depth (log1.00E + 06 and log1.00E + 07,

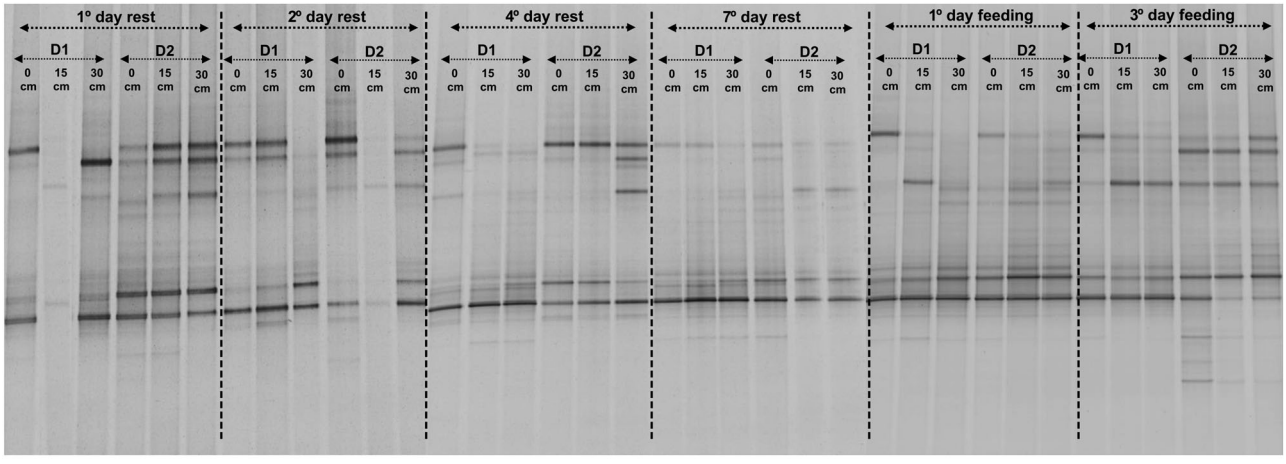

Figure 3. Temporal and spatial DGGE profiles of a nitrifying (AOB) bacterial community in S3. d1 = 1 m from the feeding point; d2 = 2 m from the feeding point; 0, 15 and 30 cm depth.

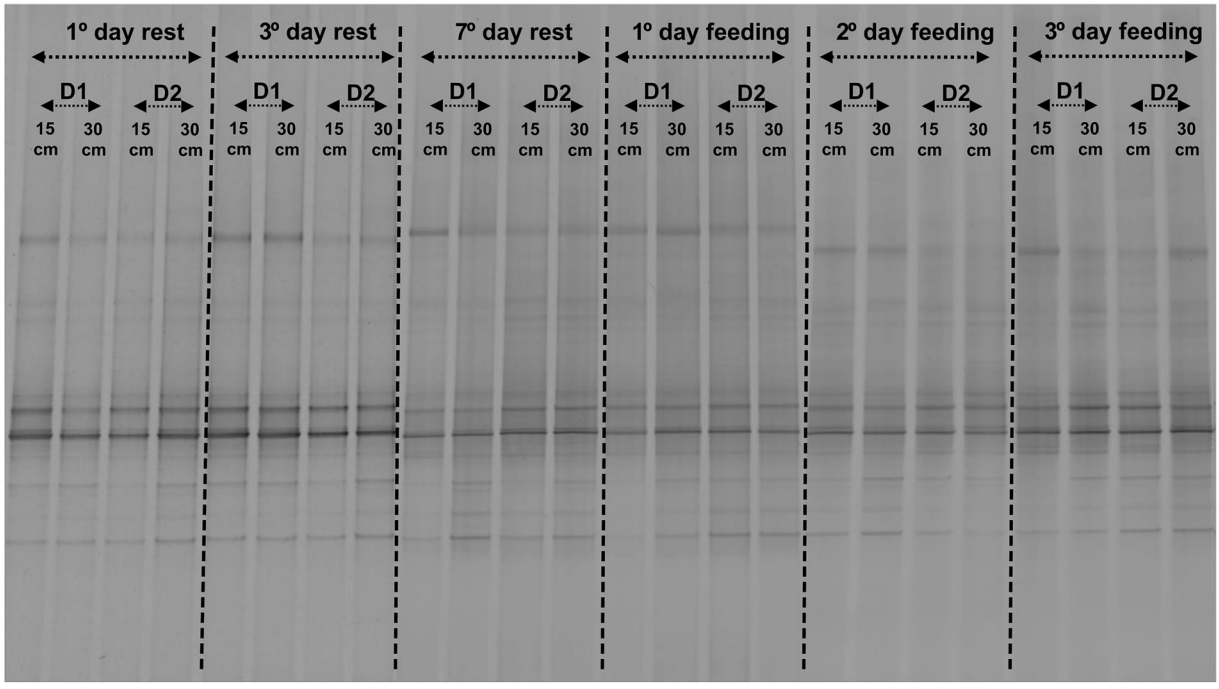

Figure 4. DGGE gel banding showing the temporal and spatial profiles of a nitrifying bacterial community in S4. d1 = 1 m from the feeding point; d2 = 2 m from the feeding point; 0, 15 and 30 cm depth.

respectively, at 30 cm). In this way, the influence of organic deposits on the spatial distribution of bacterial communities is reinforced. Considering only the operating cycle, a greater number of gene copies were found during the feeding period, from which we can infer a greater functional potential of these bacteria in this period. System S4 also presented a higher number of gene copies /g pellet for AOB and total bacteria at the 30 cm depth, however with higher values in the rest period. These results may be related to the oxygen content at the surface during rest and the transfer of nutrients to deeper layers during feeding impacting bacterial metabolism.

3.2.3. 16s rRNA sequencing of the dynamics of microbial community

To explore the microbial community structure and identify the main genera present in VF wetlands, we used a more exhaustive analysis based on High-throughput 16S rRNA sequencing. This technique is highly effective in assessing shifts in microbial communities and consequently, in understanding microbial dynamics over space and time [9]. Besides, if the enzymatic/functional capabilities of a microbial community are considered, one could generate an idea of potential function [8]. For this reason, PICRUSt was applied to understand the potential of microbial function linked to the nitrogen cycle.

A total of 640,663 sequences were retrieved from 15 samples from all four full-scale systems by the High-throughput 16S rRNA Illumina MiSeq™ sequencing platform. After quality control by mothur and chimera to remove low quality reads (Phred <24), a total of 258,590 high-quality sequences remained for further analysis.

Chao1 was use used to estimate richness in a sample and is based upon the number of rare classes (i.e. OTUs observed). Among the samples evaluated, the effluent sample S2_D0 showed the highest estimated richness (224.20), and the sample containing the lowest richness was S3_D15_r (119.55). The same was observed for the number of bacterial OTUs (Supplementary Table 1). The microbial community richness illustrated the remarkable spatial variation in each French VF wetland system; however, the highest values were found in systems with longer operating time (LOT), specifically in the summer with decreasing in depth. This pattern of behavior can be observed in Supplementary Figure 2, which shows the result of the Alpha Diversity indices.

Rarefaction analysis (Supplementary Figure 3) was employed to evaluate the sample coverages. All samples showed asymptotic curves, indicating that the sampling effort was sufficient to cover the overall richness. These results are similar to the Good's coverage values (> 0.98), reinforcing that the samples were sequenced at a high depth.

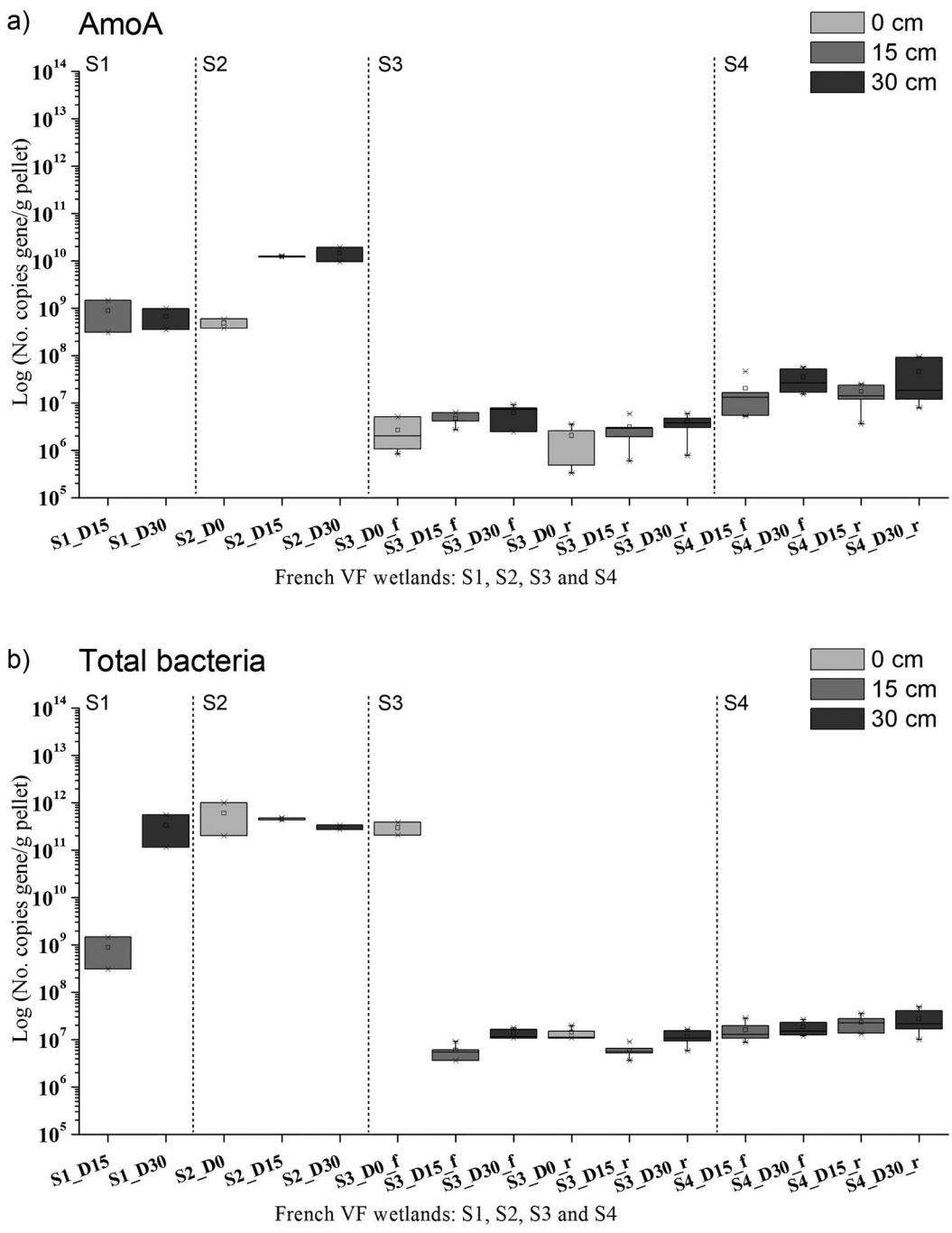

Figure 5. qPCR of the tested French vertical flow wetlands (S1; S2; S3; and S4). Absolute values. (a) Nitrifying bacteria (amoA, primers 1F, and 2R) and (b) Total bacteria (primers 1055F and 1392R).

According to the Shannon index, the microbial diversity also varied significantly, probably due to the specific factors of each system evaluated, such as operating time, operating cycle, temperature, and oxygenation. However, the highest Shannon index (3.79) was observed for a LOT system (S2_D0) sampled in winter during the rest period. This result can indicate that despite the presence of snow in the mountain station, little change in the microbial diversity was observed in this system. Interestingly, the sample that showed the lowest Shannon index (3.10) comes from the same system at 30 cm (Supplementary Table 1). This result coincides with the visually observed results for the DGGE, where greater diversity was seen in the first depth layers, in the summer, and in the deeper layer prevailed the nitrifying bacteria.

Principal coordinates analysis (PCoA), based on the Bray–Curtis distance metric, and was applied to evaluate

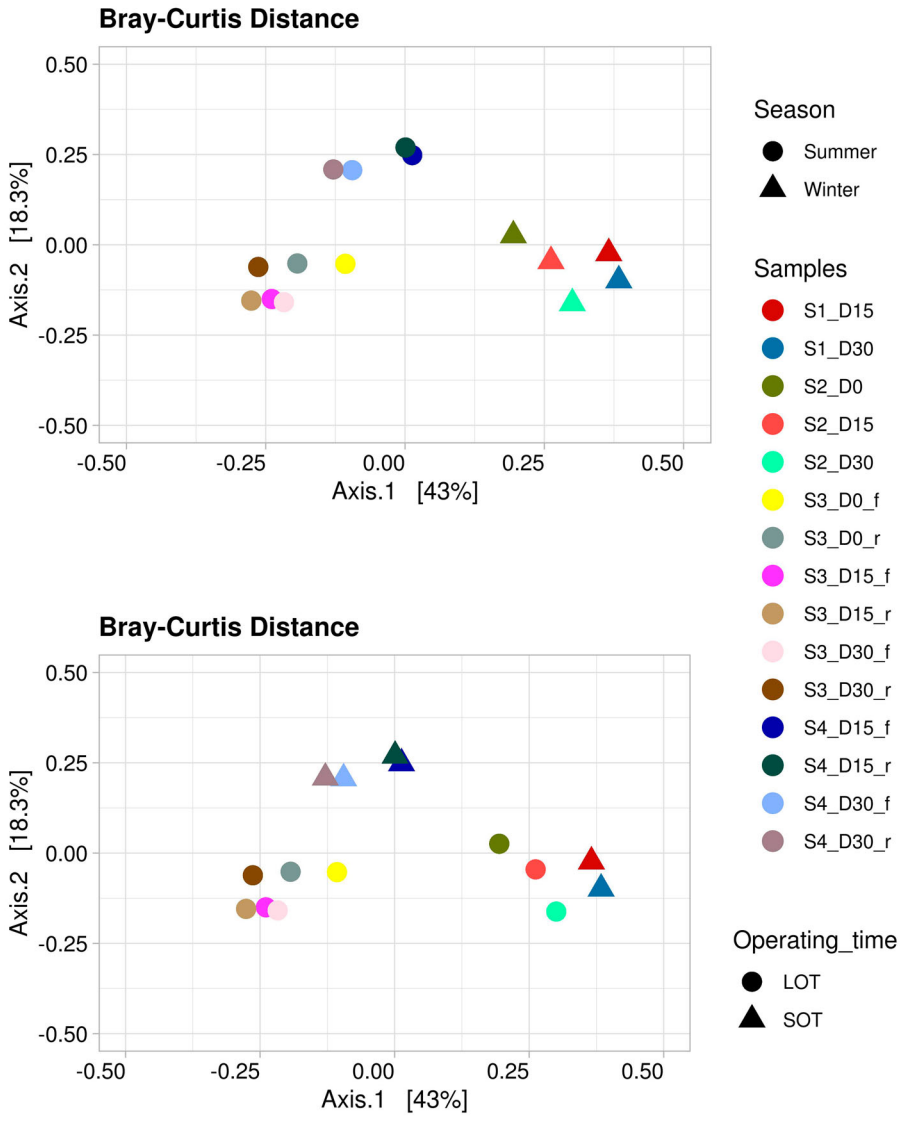

Figure 6. Principal coordinate analysis (PCoA) plot using Bray-Curtis distances.

the patterns of similarities of the samples and how the samples group according to metadata information (Figure 6). The results of PCoA corroborated the results of other analyses performed in this study. The samples showed a pattern of distribution according to the season of the year in which they were collected – the systems S3 and S4 in the summer and systems S1 and S2 in the winter (Figure 6a). A subdivision within the samples collected in summer and winter was observed about the variable LOT and SOT systems (Figure 6b). Other metadata information was tested, but the pattern of distribution was not conclusive. In this way, the PCoA results indicate that the variables, operating time and measurement season, were responsible for the similarity pattern of the samples and, consequently, microbial community behavior present in the systems.

3.2.3.1. Taxonomies and functional prediction. Sequences derived from all samples were distributed among 12 distinct phyla (Supplementary Figure 4). The most abundant phyla in all samples were Proteobacteria, Bacteroidetes, Actinobacteria, Nitrospirae, Chloroflexi, Acidobacteria, and Gemmatimonadetes. A higher relative abundance of all the phyla identified in the summer was clearly observed. However, concerning the other variables analyzed, no pattern was found, and the dynamics of these phyla seemed to be more related to their characteristics.

At the genus level, *Mycobacterium, Acinetobacter, Flavobacterium,* and *Rhodanobacter* were the most abundant genera in S1 and S2 systems (SOT and LOT, respectively, in the winter) (Figure 7). In the samples collected in the summer period (S3 and S4), two of the most

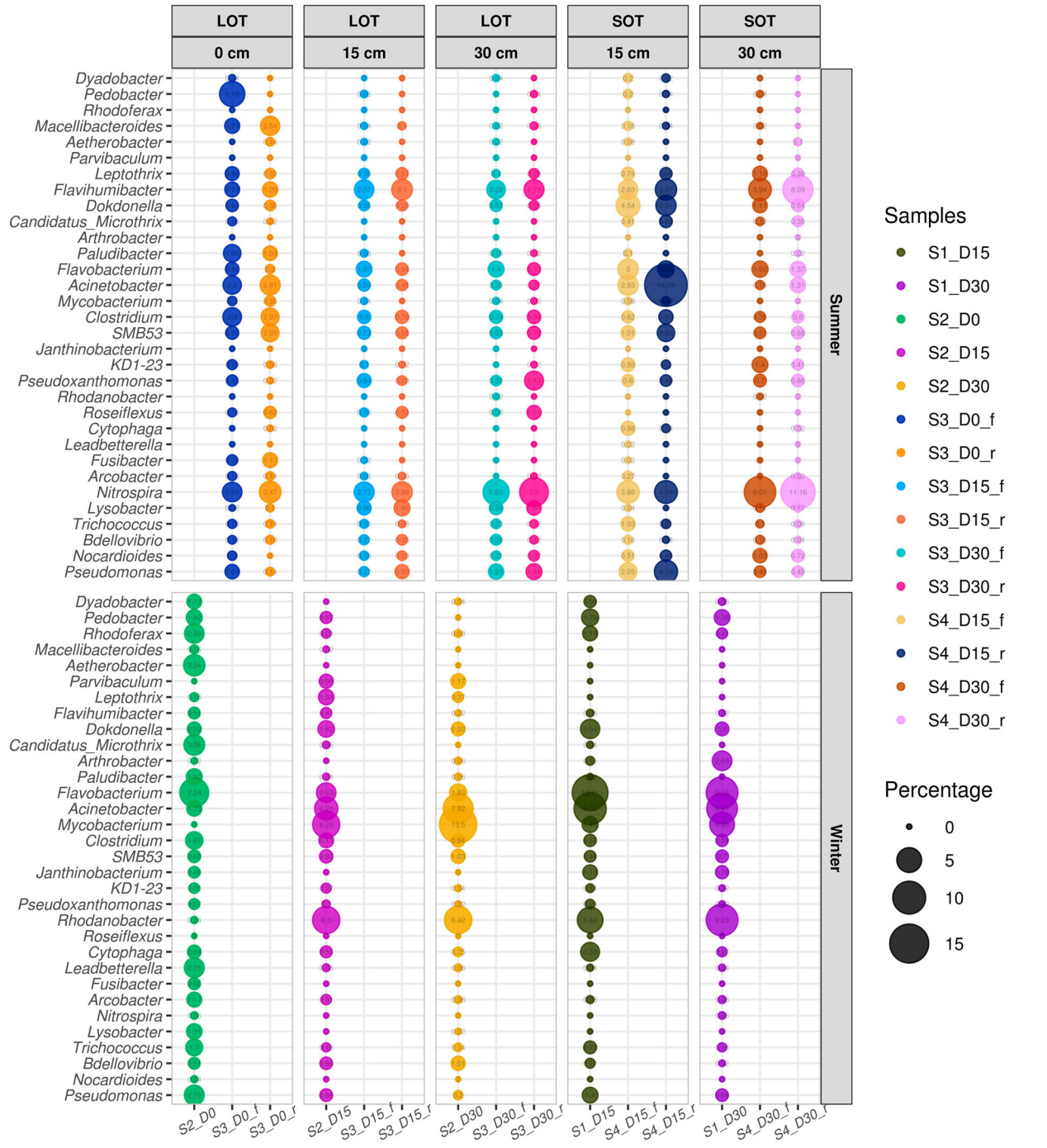

Figure 7. Relative abundance at the genus level found in the datasets of the French VF wetland.

abundant genera highlighted, *Flavihumibacter* and *Nitrospira*.

The *Mycobacterium* genus is a strict aerobic organism and gram-positive, associated with carbon degradation and can support nitrification; species that promote heterotrophic nitrification produce NO_3^-N [43,44]. An exponential increase in the relative abundance concerning depth was observed, with higher values at 30 cm (Figure 7). This result may be related to the presence of aeration drains located at the bottom of the filter. In winter, aeration from the bottom is essential since the deposit layer can stay saturated for several days. Finally, temperature was the variable that affected the community structure of this genus since for the S3 and S4 systems (in the summer) the genus had no significant presence. The same was observed for the *Rhodanobacter* genus. The same was observed for the *Rhodanobacter* genus. This genus is considered a denitrifying bacterium, beyond, *Cytophaga, Pseudomonas, Dokdonella* and *Pedobacter* also appeared, although with a lower relative abundance.

Regarding *Flavobacterium*, in contrast to *Mycobacterium*, a decrease in relative abundance with depth for all systems was observed (except in system S3, with higher value in 15 cm). It can also be noted that the values were higher in the period of feeding than in the period of rest. This behavior can be justified due to its characteristics. Members of this group are autochthonous soil bacteria [45] and are closely linked with the biodegradation of a variety of organic pollutants and nitrification [23,46]. Some studies have reported that this genus is associated with aerobic denitrification and helps to shorten the duration required for this process, thus providing a new route for denitrification [47]; furthermore, it has been well detected in vertical flow systems [10] and at low temperatures, such as 10 °C [45]. Additioanlly, *Acinetobacter* has also recently been cited as able to perform the heterotrophic nitrification and aerobic denitrification [48]. Compared to the others, it was found with a significant abundance in all winter samples, although the highest relative abundance was in the sample S4_D15_r (16.7%).

As shown in Figure 7, the genus that stood out in systems S3 and S4 (analyzed in summer) was *Nitrospira*. This genus notably increased with depth and the highest relative abundance was found at 30 cm during the rest period. The sample that obtained the highest percentage value was D30_f (8.8%) and D30_r (10.8%). The predominance of this genus in the rest period (period with small substrate concentrations) may be associated with some survival strategies, such as their low Ks. This makes them K-strategic bacteria, which means that they are well adapted to low concentrations of nitrite and oxygen. It was also reported by other studies [49,50] that states that the genus *Nitrospira* can increase under these conditions. Concerning the depth, the fact that there was a greater abundance at a 30 cm may be linked to several factors, such as the presence of aeration drains at the bottom of the filter that, coupled with the coarse drainage layer, promote good aeration from the bottom.

The genus *Nitrospira* comprises facultative autotrophic chemolithotrophic organisms and is composed of gram-negative bacteria. This genus is one of the most diverse known nitrifier groups and colonizes all oxic ecosystems, including high-temperature environments. For over a century, this genus has been described as responsible for the second step of nitrification (nitrites to nitrates) [51]. However, a remarkable study [52] reported the capacity of a bacterium from the genus *Nitrospira* to catalyze complete nitrification. The authors noted that a complete nitrifier ('comammox,' complete ammonia oxidizer) might be competitive under conditions that favor the maximization of growth yield rather than growth rate but still reported that metagenomic studies had revealed the dominance of *Nitrospira* over AOB. Another study [17] also reported the predominance of Nitrospira over AOB, possibly suggesting the presence of the comammox process. This may be able to partially explain the behavior of this genus in our study.

Concerning the nitrification process and the variable seasonality, the results of High-throughput sequencing, as well as the results obtained by qPCR and DGGE, showed the influence of temperature on the microbial community structure of both nitrifying bacteria and total bacteria. The increase in the genus *Nitrospira* in the systems evaluated in the summer period (S3 and S4) indicates an increase nitrification rate, which is sought in such aerobic systems. However, even at lower temperatures, the filters analyzed in this study had good nitrification efficiency. From this, we can suggest that depending on other factors that interfere with the system and that help to maintain bacterial metabolism, good nitrogen removal can also occur in winter. Some studies observed no difference in removal rates in treatment wetlands between summer and winter [53,54,55]. This trend has also been observed in French VF wetlands in cold climates [3].

Finally, results from PICRUSt highlights the functional dynamics of genes associated with nitrogen conversion, including nitrification, denitrification, and dissimilatory and assimilatory nitrate reduction (Figure 8).

Regarding the genes of the nitrogenase complex responsible for the conversion of atmospheric nitrogen (N_2) to other forms of nitrogen such as ammonia – *nifK, nifH*, and *nifD* – a higher percentage was observed in the summer period without a significant difference to depth. Looking at the genes related to the metabolism of AOB (hao, hydroxylamine oxidoreductase), a low prediction in all analyzed samples was found. The other techniques used in this study also showed a lower relative abundance of AOB compared to other bacterial groups.

Nitric oxide reductase genes related to denitrification as the *norB* and *norC* showed higher predicted abundances in winter samples, with the *norB* gene being more expressed than *norC*. No difference was observed in their predictions for the other variables. Other genes linked to denitrification such as nitrate reductase, *norA*, *narL*, *narH, narG, nirK*, and *nosZ* (nitrous oxide reductase) [56] were observed. Due to the good efficiency of the nitrification documented in the evaluated systems, we can infer that the significant presence of denitrifying genes in vertical systems is related to the conditions of each system and temperature. The well-established sludge layer on the surface of the filters and feeding

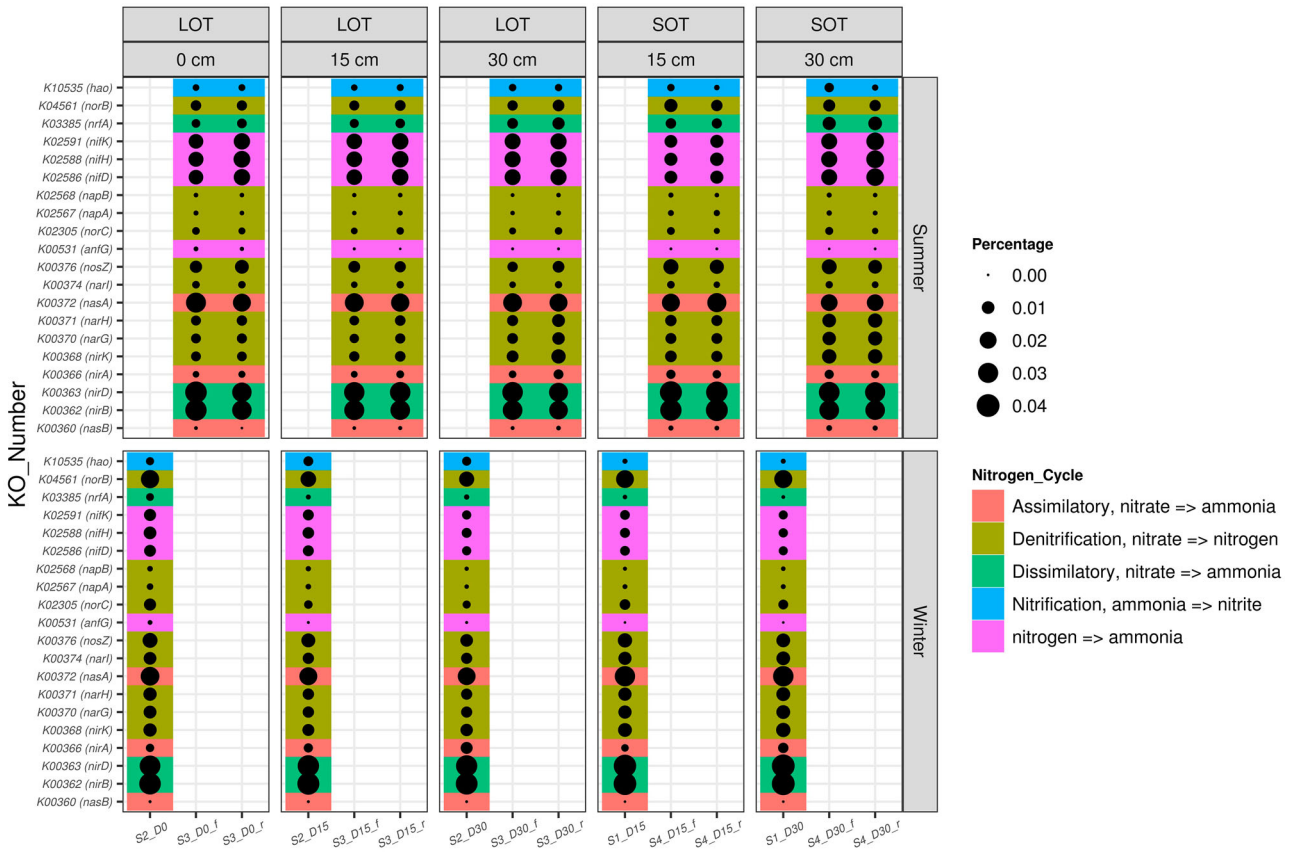

Figure 8. Functional genes involved in biological nitrogen cycling found in the datasets of the French VF wetland as predicted by PICRUSt.

periods, where the oxygen decreases, may also have contributed to this result.

Functions associated with nitrite reductase were evidenced by the genes *nrfA*, *nirD,* and *nirB*. The results indicated a high presence of these genes in comparison to the others under any condition the dissimilatory process (nitrate → ammonia) was observed.

The results of functional prediction still showed genes related to the assimilatory process of nitrate, mainly enzymes for rhizobial denitrification. The *nasA* gene is considered to be a NO_3^-/NO_2^- transporter, i.e. required for the assimilatory NO_3^-/NO_2^- reductase apparatus and NO_2^- reductase (*nasB*). Both had a very similar percentage in all the samples evaluated, with smaller values for the *nasB* gene. In addition, the *nirA* gene (assimilatory nitrite reductase) was found, with the highest percentage in winter samples and a slight increase at 30 cm depth. This genus is considered Key for NO_3^- assimilation and is immediately downstream of genes recently reported to code for a NO_3^-/NO_2^- responsive regulatory system (asS-NasT) [56].

In summary, it seems that the behavior microbial community from the functional prediction was more affected by temperature. Another important point to note is that even if the systems have a vertical flow prioritizing nitrification the taxonomies and predicted genes for the denitrification process were found. This factor allowed us to further understand the bacterial dynamics and to be able to advance the idea that modifications made in the first stage of the classical French system to improve nitrogen removal are promising. In this sense, the importance of this study was reinforced. As an example, the use of the saturated bottom layer in the first stage (French VF wetlands) [57] promoted the total simultaneous treatment of nitrogen in the same filter, thereby reducing the required area and operating costs of the treatment. This modification would have create a different microbial community profile to achieve these results.

4. Conclusions

Based on the results of the molecular analyses of the microbiological communities of four classical French vertical flow wetlands treating domestic wastewater at a full-scale. The main conclusions are as follows:

- The sludge layer significantly interferes in the filter operation, not only in a hydraulic sense but also in the microbial community providing food for the bacteria that perform nitrification and denitrification throughout the verticality of the filter.
- Greater diversity was seen in the first depth layers, and in the deeper layer prevailed the nitrifying bacteria. Regardless of the presence of snow in the mountain station, little change in the bacterial diversity was observed. This result could be indicated that even in winter, good nitrification efficiency can be achieved with a well-established community.
- An apparent decline in the diversity was observed throughout the cycle of operation; thus, the classical 7-day rest period was reinforced.
- qPCR indicated that the largest amount of AOB was found at 30 cm depth. Concerning the operating cycle, a greater number of copies were found during the feeding period, from which we can infer a greater functional potential of these bacteria in this period.
- *Mycobacterium, Acinetobacter, Flavobacterium, Rhodanobacter, Flavihumibacter,* and *Nitrospira* were the abundant genera highlighted. *Nitrospira* stood out in the summer, at 30 cm depth with a slight increase in the resting period and overcame AOB.
- The presence of denitrifying genes in vertical systems is related to the conditions of each system and temperature, with a more significant presence in the winter, as expected. However, they were detected to the depth of 30 cm.
- Integrated analysis of the results revealed that the variables, time of operation (presence/absence of sludge layer) and temperature, were responsible for the distribution pattern of the samples based on taxonomic profile and, consequently, for the behavior of the microbial community present in the systems.

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Disclosure statement

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