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Structural and functional spatial dynamics of microbial communities in aerated and non-aerated horizontal flow treatment wetlands

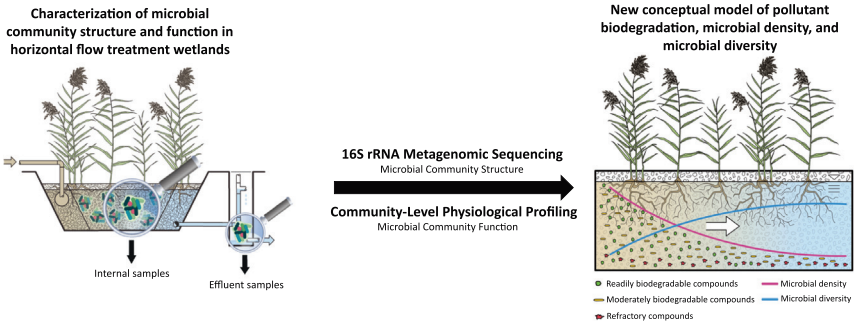
D.D. Silveira [a], A.J. Farooq [b], S.J. Wallace [b], F.R. Lapolli [a], J. Nivala [c,*], K.P. Weber [b]

[a] Federal University of Santa Catarina (UFSC), Campus Universitário, Trindade, CEP 88040-900 Florianópolis, SC, Brazil
[b] Department of Chemistry and Chemical Engineering, Royal Military College of Canada, Kingston, ON, Canada
[c] INRAE, UR REVERSAAL, 5 rue de la Doua, CS 20244, 69625 Villeurbanne, France

HIGHLIGHTS

- A conceptual model links microbial dynamics to interstitial pollutant concentration.
- Microbial community profiles of internal and effluent water samples are different.
- Microbial activity and density decrease along the flow path in HF treatment wetlands.
- Microbial richness and diversity decrease with increasing DNA concentration.

GRAPHICAL ABSTRACT



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ABSTRACT

A multiphasic study using structural and functional analyses was employed to investigate the spatial dynamics of the microbial community within five horizontal subsurface flow treatment wetlands (TWs) of differing designs in Germany. The TWs differed in terms of the depth of media saturation, presence of plants (*Phragmites australis*), and aeration. In addition to influent and effluent water samples, internal samples were taken at different locations (12.5 %, 25 %, 50 %, and 75 % of the fractional distance along the flow path) within each system. 16S rRNA sequencing was used for the investigation of microbial community structure and was compared to microbial community function and enumeration data. The microbial community structure in the unaerated systems was similar, but different from the aerated TW profiles. Spatial positioning along the flow path explained the majority of microbial community dynamics/differences within this study. This was mainly attributed to the availability of nutrients closer to the inlet which also regulated the fixed biofilm/biomass densities. As the amount of fixed biofilm decreased from the inlet to the TW outlets, structural diversity increased, suggesting different microbial communities were present to handle the more easily utilized/degraded pollutants near the inlet vs. the more difficult to degrade and recalcitrant pollutants closer to the outlets. This study also confirmed that effluent water samples do not accurately describe the microbial communities responsible for water treatment inside a TW, highlighting the importance of using internal samples for investigating microbial communities in TWs. The results of this study reinforce an existing knowledge gap regarding the potential for TW design modifications which incorporate microbial community spatial dynamics (heterogeneity). It is suggested that utilizing step-feeding could allow for improved water treatment within the same areal footprint, and modifications enhancing co-metabolic processes could assist in improving the treatment of more difficult to degrade or recalcitrant compounds such as micropollutants.

* Corresponding author.
E-mail address: jaime.nivala@inrae.fr (J. Nivala).

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1. Introduction

Treatment wetlands (TWs) are considered an economical and practical nature-based technology for decentralized wastewater treatment (Kadlec and Wallace, 2009). There are a wide range of treatment wetland designs, ranging from passive horizontal flow (HF) systems to designs with moderate energy requirements, such as unsaturated vertical flow (VF) wetlands with pulse loading. Treatment wetland designs can also be intensified through the use of water pumps to control water level fluctuation or through air pumps to provide aeration (Nivala et al., 2013). Compared to traditional passive horizontal flow wetlands, aerated treatment wetlands are capable of higher removal rates for carbon and nitrogen (Wallace et al., 2008), micropollutants (Nivala et al., 2019), and mixture effects (Sossalla et al., 2021). Microbial communities are responsible for the majority of pollutant biodegradation in treatment wetlands (Button et al., 2015; He et al., 2015; Weber, 2016). Factors such as choice of filter material, presence of plants, filter depth, loading regime, saturation status, and hydraulic retention time lead to the development of different microenvironments within these systems which will support and promote microbial communities of varying structure and function.

Many studies have evaluated the functional or structural dynamics and heterogeneity of microbial communities in treatment wetlands (Truu et al., 2009; Adrados et al., 2014; Ansola et al., 2014; Button et al., 2015; Babatunde et al., 2016; Button et al., 2016; Lv et al., 2017a; Zhang et al., 2018; Zhang et al., 2019). Although these studies provide essential knowledge towards improving the understanding of microbial communities in treatment wetlands, only recently have investigations started to use both structural and functional assessment together (e.g. Ruppelt et al., 2020; Silveira et al., 2020; Zheng et al., 2021; Yang et al., 2021). Structural information is useful in microbial community-based research; however, if it is not paired with functional analysis, assumptions must be made when interpreting the impact of microbial communities on pollutant transportation in treatment wetlands. Multiphasic studies which utilize both structural and functional measures are preferable (Weber, 2016).

The dynamics of microbial communities are an important and defining aspect of treatment wetlands. Previous research has shown that vertical gradients of microbial activity and functional diversity exist (Weber and Legge, 2013), that horizontal dynamics of microbial community function have been observed in field-scale treatment wetlands of various designs (Button et al., 2015), and that microbial community functional fingerprints can be associated with the degradation of specific pollutants (Lv et al., 2017b; Ruppelt et al., 2020; Zhang et al., 2018, 2019). Microbial community functionality has been shown to be dynamic and either controlling or correlated with pollutant removal in treatment wetlands. It is, however, not yet known if microbial community structure can explain the same pollutant removal dynamics or spatial microbial community trends observed when assessing microbial community function in field-scale treatment wetlands of different designs.

Microbial community investigations at the larger scale are uncommon. Weber (2016) summarized that less than 5 % of treatment wetland microbial community studies are completed at the field scale. In addition, the majority of those studies use influent and effluent water samples to draw conclusions with respect to microbial communities inside of treatment wetlands. Although influent and effluent samples are easier to collect, they do not necessarily represent the microbial community which interacts and influences pollutants in a treatment wetland. Previous work has shown that microbial communities from biofilm samples in treatment wetlands are different than those communities found in the interstitial water (e.g., pore water found beside and interacting with biofilms), having activities and densities of at least an order of magnitude greater (Weber and Legge, 2013). Therefore, biofilm samples are preferred for microbial community analysis in TWs because they are more representative of the whole system (Weber and Gagnon, 2014). Often, these samples are challenging to obtain at the field scale as destructive sampling is often possible. As biofilm can be detached when enough shear force is applied (Rajabzadeh et al., 2015), a practical solution proposed in Button et al. (2015) and employed in this study, utilized a high flow-rate pump to shear biofilm alongside interstitial water through pre-installed sampling ports to obtain representative samples without the need for destructive sampling. In this previous study, the authors observed that the microbial community activity (substrate utilization rate) and metabolic richness (number of carbon sources utilized) decreased dramatically with increasing distance along the flow path for field-scale saturated treatment wetlands of several different designs. This emphasizes the spatial functional dynamics/heterogeneity of the microbial community in field-scale TW systems. Understanding the correlation between function and structure in these same systems could help understand if microbial community structure and function are correlated along internal transects in field-scale TWs, and therefore inform if assuming function from structural data can be appropriate in interpretation. Such a data set could also help inform optimized design or operational modes to improve the performance of treatment wetland systems of different designs.

The objective of this study was to gain a deeper understanding of the spatial dynamics of microbial community structure in horizontal flow treatment wetlands through a comparison across multiple systems of different designs. This was done in a three-phase approach including: (i) investigation of the spatial variability (structure and diversity) of the representative biofilm microbial community both between and within different horizontal flow treatment wetlands; (ii) investigating the potential correlations between microbial community structure and microbial community function; (iii) comparison of the microbial community profiles of internal samples versus effluent water samples.

2. Materials and methods

2.1. Langenreichenbach Research Facility

In order to gain an understanding of the overall microbial dynamics in HF systems, five field-scale pilot treatment wetland systems were used in this study. They were located at the wetland research platform in Langenreichenbach, Germany (Nivala et al., 2013). The systems had been in steady-state operation for three years at the time this study was conducted. The dimensions of each system were 4.7 m by 1.2 m (length × width) and were fed with primary treated municipal wastewater. The wetland systems differed in terms of the depth of media, presence of plants (*Phragmites australis*), and aeration (Table 1).

The nomenclature designated for the non-aerated horizontal flow wetland systems is based on saturation depth (one pair at 25 cm depth: H25 and H25p and one at 50 cm depth: H50 and H50p). The hydraulic loading rate was approximately 36 mm/d for H50/H50p and 18 mm/d for H25/H25p, resulting in a nominal hydraulic retention time (nHRT) of approximately 5.5 days. The aerated planted horizontal flow system (HAp) was constructed with an integrated aeration system (aerated with electric air blowers), with a density of aeration orifices of approximately 0.07 m^2/orifice. The aeration system in HAp was run continuously (24 h per day). HAp had a hydraulic loading rate of approximately 65 mm/d, which resulted in a hydraulic retention time of approximately 3.5 days. A complete description of all systems is provided in Nivala et al. (2013).

Table 1
Operational details of the treatment wetland systems in this study.

Abbreviation[a,b]	Effective depth[c] (cm)	Surface area (m^2)	Design flow (L/d)	Hydraulic loading rate (L/m^2-d)
H25, H25p	25	5.6	100	18
H50, H50p	50	5.6	200	36
HAp	100	5.6	730	65

[a] "H" refers to horizontal flow systems; "A" refers to the system that is aerated.
[b] "p" indicates systems planted with *Phragmites australis*.
[c] Effective depth refers to the depth of the media involved in treatment.

2.2. Microbial community sampling

Samples were collected on October 21, 2013. A peristaltic pump and polyethylene tubing connected to a small stainless-steel pipe was used for sample collection. Initially, the pipe was flushed with fresh tap water before inserting the tube into the sampling tee. Following this, 500 mL of interstitial water was flushed through the tubing before the microbial community sample was collected in a sterile 50 mL vial. The peristaltic pump flow rate was approximately 1 L/min to encourage the maximum detachment of the surrounding biofilm through sheer stress providing a mix of interstitial water and loose biofilm (Button et al., 2015). Biofilm detached was assessed as part of sample TOC measurements. TOC measurements here represent the organic carbon from the water collected in the sample as well as the organic carbon from the biofilm detached and collected. In this sense TOC is regarded as an indirect assessment of the microbial biomass at that specific point along the flow path in this study.

Additionally, effluent samples (described as outlet samples, or samples taken at 100 % of the fractional length) were collected to assess the structural differences between the microbial community in the effluent and interstitial water samples. Effluent samples were collected in a central control building just prior to the flow measuring device for each system. All samples were stored in a cooler with ice for transport to the laboratory for analysis. 16S rRNA samples were stored at −20 °C until analyzed.

2.3. Community-level physiological profiling

In this study, community-level physiological profiles (CLPP) were generated for each sample using 96-well BIOLOG™ Ecoplates (Biolog Inc. Hayward CA, USA) to assess heterotrophic microbial function. Each plate contains 31 different carbon substrates and a blank, in triplicate. Together with the carbon substrate, each well contains tetrazolium violet, a redox dye indicator, and when a mixed microbial community sample is inoculated into the well, the production of NADH via cell respiration reduces the tetrazolium dye to formazan. This results in a color change that can be measured photometrically. The mixed microbial samples were prepared according to methods described in Button et al. (2015) and Weber and Legge (2010). Briefly, samples were first diluted 10:1 with sterile phosphate buffer solution (10 mM, pH 7.4, 8.5 g/L NaCl) before 100 μL was added to each well of the BIOLOG™ Ecoplates. The plates were incubated at 20 °C on an orbital shaker (Edmund Buehler, Germany) at 100 rpm in the dark. Absorbance was measured at regular intervals for a total of 96 h using a microplate absorbance reader (VICTOR2 TM, WALLAC Oy, Finland) at a wavelength 590 nm. All plates were inoculated within 3 h of sample collection. Given the methodology used in this study, only aerobic CLPPs were obtained. These CLPPs may represent higher overall activity than what would be expected in the TWs due to a higher DO content used during CLPP incubation. The microbial activity can be determined by the average well color development (AWCD), which is based on the carbon substrates utilization pattern (CSUP) of a given sample. The AWCD time point (70 h) was chosen to obtain the most variation within the data set while minimizing the number of over-saturated wells (absorbance units >2.0) (Weber and Legge, 2010). The carbon substrates can be divided into five groups (guilds), as suggested by Weber and Legge (2009): polymers, carbohydrates, carbonic acids and acetic acids, amino acids and amines/amides to obtain a better overview of the substrates and to better understand specific functional differences in the microbial community.

2.4. 16S rRNA metagenomic sequencing

16S rRNA metagenomic sequencing was conducted on all samples to assess and determine the microbial community structure. DNA extracts were obtained by subsampling the same treatment wetland samples used for CLPP analysis. Subsampling for DNA extractions was completed after the 10:1 dilution step with phosphate buffer. 50 mL of each subsample was passed through an individual 0.22 μm filter. Filters were then processed using the FastDNA Spin Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) following the manufacturer's protocol. The DNA concentrations were measured using a Qubit fluorometer and Qubit dsDNA HS assay kit (Invitrogen, ON, CAN). The variable V3-V4 regions of the 16S ribosomal RNA gene (16S rRNA) were amplified from 5 ng/μL DNA using the universal primers 341F 5′-CCTACGGGRSGCAGCAG-3′ (Wang and Qian, 2009) and 806R 5′-GGACTACHVGGGTWTCTAAT-3′ (Caporaso et al., 2011) (~80 % coverage for Bacteria and ~50 % for Archaea; Quast et al., 2013) following the Illumina 16S Metagenomic Sequencing Library Preparation guide (version B, Illumina Canada Ulc., Victoria, BC, Canada).

Following the multiple amplification and bead purification steps, the concentration of each sample library was measured on a Qubit fluorometer and normalized to 4 nM. Sample libraries were pooled together, denatured, and diluted to 2 pM for sequencing with the MiSeq (Illumina Canada Ulc., Victoria, BC, Canada) using the MiSeq Reagent v3 600 cycle kit to achieve 2 × 300 bp reads. A 10 % PhiX control library spike in was included as a positive control for sequencing quality.

Fastq files were de-multiplexed with the MiSeq software according to their index and analyzed using QIIME 2, version 2 (2019.10) (Bolyen et al., 2019), on VirtualBox (6.1.10 version). Sequencing reads were filtered, denoised, merged, and chimeras were removed using DADA2 (Callahan et al., 2016) for quality control. DADA2 is one of the most recently developed filtering methods with significantly advanced quality control measures by denoising sequences into Amplicon Sequence Variants (ASVs) to better discriminate between true sequence diversity and sequencing errors (Nearing et al., 2018). Subsequently, sequences were taxonomically classified using the Greengenes database 13_8 (DeSantis et al., 2006) and mitochondria or chloroplast related features were removed. The median frequency was 19,380 (min: 12,159–max 26,262) reads so the number of sequences were rarefied to 12,000 reads for each sample for further diversity analyses. The align-to-tree-mafft-fast tree pipeline from q2-phylogeny was used for phylogenetic dependent analyses. Alpha diversity analyses were performed to assess the complexity of microbial diversity for each sample including observed Operational Taxonomic Units (OTU) to measure observed species richness, Shannon index to identify community diversity, Pielou evenness index to measure evenness, Faith's phylogenetic diversity to measure phylogenetic richness, and Good's coverage index to characterize sequencing depth.

2.5. Data analysis

Analysis of the CLPP data was performed as described by Weber et al. (2007) and Weber and Legge (2010). The AWCD and the specific carbon source trends, with respect to microbial community structure identified in this study, were evaluated through correlation analysis using XLSTAT statistical software (2020.1.2, Addinsoft). Principal component analysis (PCA) was performed using the covariance matrix of the CSUP data to further assess for differences between systems. Datasets were processed prior to the PCA following the recommendations of Weber et al. (2007).

PCA ordinations were also generated in XLSTAT using the genera abundance data set to compare the differences in the microbial communities within and between each system type. This was followed by Agglomerative Hierarchical Clustering (AHC) of the metagenomic data to further identify differences in the microbial communities using the Unweighted Pair Group Method with Arithmetic mean (UPGMA). One-way permutational analysis of variance (PERMANOVA) was performed in QIIME 2 with both beta diversity matrices Bray-Curtis and Euclidean distance to assess the differences in microbial composition among samples from different systems. The percent of reads in each sample matching to the top 20 abundant genera were plotted and compared among designs in a heatmap using an average Bray-Curtis metric. All alpha diversity indices and the beta diversity analysis were performed using QIIME 2 software (2019.10).

Pearson's correlation analysis was carried out to examine the significant relationships between microbial community metrics (DNA concentration, Shannon diversity, taxon, AWCD, and guild utilization) and the different water metrics (TOC, TN, DO, water temperature, EC, pH). The correlation coefficient r was interpreted as a strong correlation when $r \geq |0.9|$ and a

Table 2
Water quality data for the five treatment wetland systems on October 21, 2013. Effluent water quality data are shown in italics.

Sample name	Water temperature (°C)	Electrical conductivity (μS/cm)	Dissolved oxygen (mg/L)	TOC (mg/L)	TN (mg/L)	pH
Influent[a]	14.8	1527	0.5	112	73	7.1
H25						
12.5 %	13.3	1520	0.7	1280	195	7.2
25 %	13.1	1522	2.4	1571	252	7.2
50 %	13.0	1455	0.7	30	66	7.3
75 %	12.9	1182	2.5	18	51	7.3
Effluent	*13.4*	*1511*	*3.6*	*22*	*60*	*7.1*
H25p						
12.5 %	13.9	1529	1.5	1225	182	7.2
25 %	13.4	1512	1.0	1420	201	7.2
50 %	13.0	1497	1.0	32	58	7.1
75 %	13.2	1495	0.8	24	51	7.1
Effluent	*13.1*	*1468*	*4.0*	*23*	*50*	*7.6*
H50						
12.5 %	13.5	1539	1.0	1580	259	7.1
25 %	13.2	1531	1.1	1702	295	7.2
50 %	12.9	1486	0.9	46	71	7.3
75 %	12.7	946	1.0	16	44	7.3
Effluent	*12.7*	*1470*	*4.0*	*21*	*60*	*7.4*
H50p						
12.5 %	14.0	1257	0.8	601	124	7.1
25 %	13.6	1240	0.9	1267	192	7.1
50 %	13.1	1516	1.3	879	143	7.1
75 %	13.0	1497	1.1	37	60	7.1
Effluent	*12.7*	*1546*	*2.6*	*24*	*56*	*7.2*
HAp						
12.5 %	14.1	1495	1.7	742	142	7.5
25 %	14.1	1434	4.6	178	84	7.4
50 %	14.3	1205	8.8	50	42	7.3
75 %	13.9	1218	9.7	46	40	7.5
Effluent	*13.1*	*1243*	*11.0*	*8*	*35*	*7.7*

[a] Septic tank effluent.

moderate correlation when $|0.5| \leq r < |0.7|$ (Milton et al., 2011; Zhang et al., 2018). All statistical tests involving physicochemical parameters were conducted using the XLSTAT Pro® statistical software (XLSTAT, Paris, France), and $p < 0.05$ was considered significant.

3. Results and discussion

3.1. Water quality

Water quality data for influent, internal, and effluent samples for all systems are shown in Table 2, Table S1 (for October 21, 2013) and Table 3 (mean values for 2013). The non-aerated wetlands H25, H25p, H50, and H50p maintained internal dissolved oxygen (DO) concentrations generally near or below 1 mg/L (e.g., anaerobic conditions). Note that effluent DO concentrations for these systems increased between the internal sample at 75 % and the effluent sample, which was collected as a grab sample in the control building. The internal DO profile in the aerated wetland HAp, per design, differs from its non-aerated counterparts with higher DO concentrations along the flow path (up to 9.7 mg/L DO at 75 % fractional distance of the wetland). Water samples collected from the inside of the wetlands contained a mixture of interstitial water and detached biofilm, which is reflected in a dramatic spike in TOC and TN concentrations, compared to the influent wastewater. It is interesting to note, that the presence of plants in H25p and H50p shows a noticeable improvement in the treatment of TN as compared to their unplanted counterparts (Table 3). Similar results were observed by Maltais-Landry et al. (2009) who noted that the presence of plants could improve TN removal up to 128 % due to plant-mediated oxygenation through the rhizosphere, hence assisting the nitrification step. TOC and TN concentration profiles decrease sharply between 25 % and 50 % fractional distance for all systems.

3.2. Microbial community diversity

The spatial dynamics of the microbial density in each system were assessed through variations in the DNA concentration and interstitial TOC concentrations. Given the sampling methodology used in this study, the TOC in interstitial water samples represents a combined measure of TOC in the water and the detached biomass. Both the DNA concentration and the TOC measures here are used to estimate microbial biomass along the flow path (Fig. 1a, b), while still remembering that some of the TOC dynamics are related to water treatment itself. For most systems, the DNA concentrations peaked in the inlet region, up to 25 % of the fractional distance along the flow path, before decreasing with increased distance along the bed (Fig. 1a). Although not a direct measure, DNA concentrations have been used in previous research successfully as a proxy for microbial density (Faulwetter et al., 2009). Given that, across all systems, the DNA concentrations were positively correlated with the interstitial TOC concentrations (Fig. 1a, b, Table S2), we can infer that these interstitial measurements can also act as a proxy for microbial biomass in this study. Previous studies have reported spatial variation in microbial density, decreasing both from the inlet towards the outlet regions and with depth in subsurface flow treatment wetland systems (Deng et al., 2011; Krasnits et al., 2009; Nguyen, 2000). This is primarily attributed to the change in nutrient availability along the flow path of systems influencing the growth rate and steady-state biomass density of microbial communities.

For the alpha diversity indices, community species richness (OTU), and community diversity (Shannon index), a positive correlation with distance along the flow path is observed (Fig. 1c, d). This indicates a more diverse and richer microbial community with increasing distance along the flow path.

Microbial density (OTU) and microbial diversity (Shannon index) have a general negative correlation with DNA concentration (Fig. 2). The opposing internal microbial dynamics observed across the systems in this study suggest that the microbial density is highest near the front end due to the increased nutrient and pollutant concentrations; however, as the pollutant concentrations and availability decrease, the diversity of the microbial community increases.

Further investigations into the microbial structural patterns within all systems using beta diversity were conducted through a Principal Component

Table 3
Mean water quality data for the five treatment wetland systems in 2013 (number of samples n = 28–37). Outlet parameters are presented for the different treatment wetland systems. Means and standard deviations are shown.

Sample name	Water temperature (°C)	Electrical conductivity (μS/cm)	Dissolved oxygen (mg/L)	TOC (mg/L)	TN (mg/L)	pH
Influent[a]	12.9 ± 2.5	1613 ± 185	0.8 ± 0.3	140 ± 19	79 ± 9	7.2 ± 0.1
H25	11.1 ± 3.1	1538 ± 154	4.1 ± 1.3	23 ± 7	63 ± 9	7.5 ± 0.2
H25p	11.1 ± 3.1	1649 ± 235	4.5 ± 0.7	23 ± 7	56 ± 7	7.3 ± 0.1
H50	10.6 ± 3.2	1563 ± 116	3.5 ± 0.5	22 ± 4	66 ± 7	7.4 ± 0.1
H50p	10.7 ± 3.3	1667 ± 121	2.9 ± 1.4	24 ± 7	61 ± 7	7.3 ± 0.1
HAp	10.9 ± 3.3	1249 ± 107	10.5 ± 1.2	12 ± 4	41 ± 5	7.6 ± 0.1

[a] Septic tank effluent.

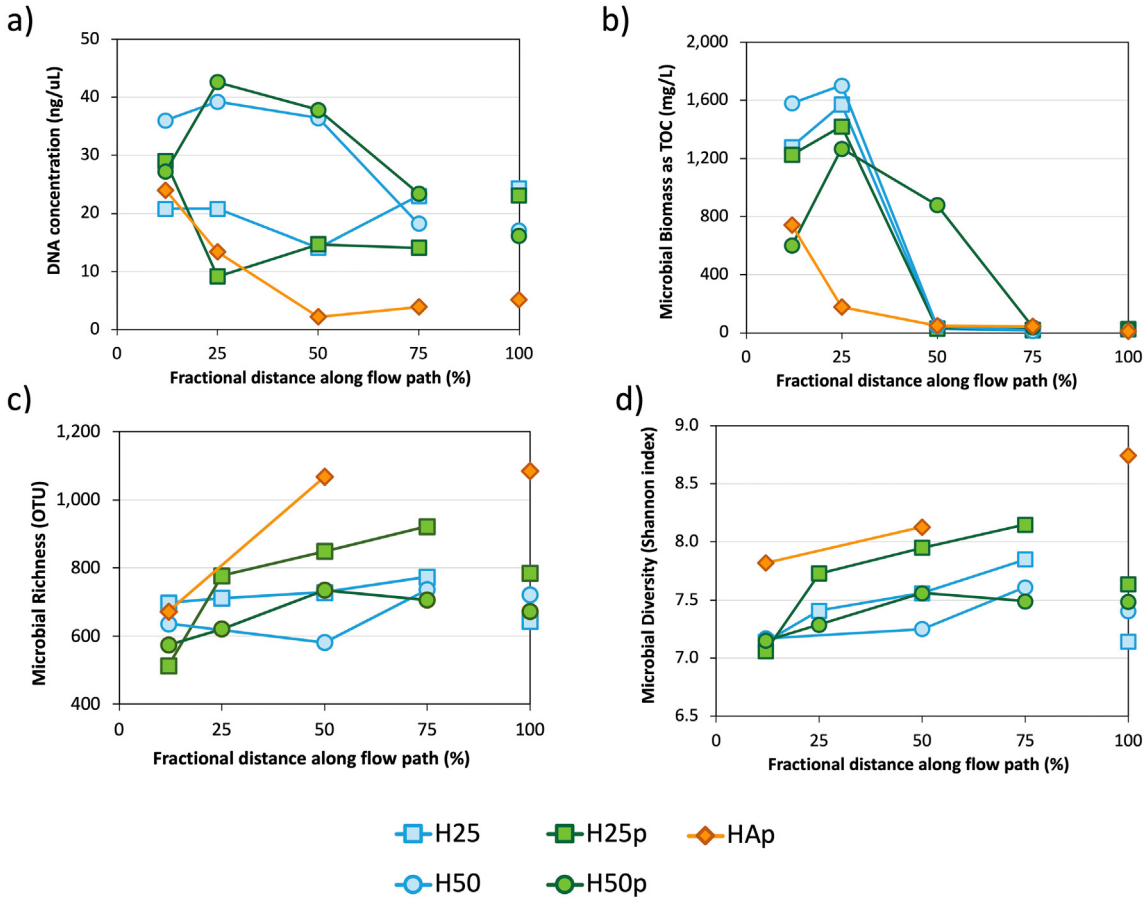

Fig. 1. Characterization of the microbial communities inside the treatment wetlands in this study, (a) DNA concentration, (b) microbial biomass as TOC, (c) microbial richness (number of unique species, (OTU)), (d) microbial diversity (Shannon index).

Analysis (PCA) (Fig. 3a). The two principal components (F1 and F2) in the PCA described 47.8 % of the variance of the normalized microbial community structure data (i.e., abundance profiles). Specifically, both the PCA and the dendrogram based on the Agglomerative Hierarchical Clustering (AHC) (Fig. 3b) showed that the microbial communities were grouped ($p < 0.05$, PERMANOVA) in four categories: (1) samples from the inlet region (12.5 %–25 % along the flow path), (2) samples from 50 %–75 % along the flow path, (3) effluent water samples and (4) samples from HAp.

Samples taken at 12.5 % of the fractional length for all systems likely grouped together because the systems received effluent from a common septic tank. A PERMANOVA analysis ($p > 0.05$; Table S4) revealed a significant difference in microbial abundance between HAp and the non-aerated systems; however, no difference was noted between the H25/H25p and H50/H50p pairs themselves. The first principal component (F1) accounted for the variation due to the presence of aeration as HAp diverges from its non-aerated counterparts at 25 % of the fractional distance and is grouped

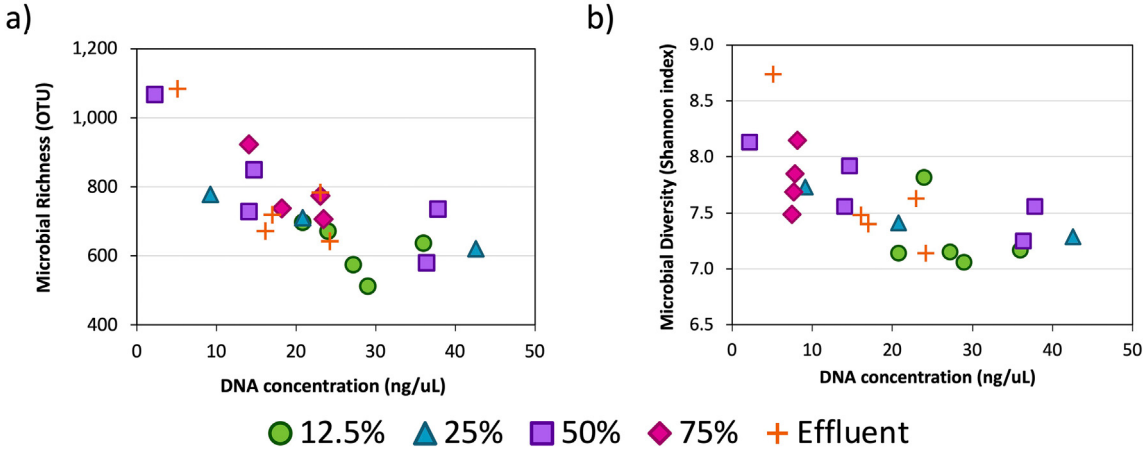

Fig. 2. (a) Microbial richness versus DNA concentration and (b) microbial diversity versus DNA concentration at various locations within the wetland systems (12.5 %, 25 %, 50 %, 75 % fractional length) and in the final effluent.

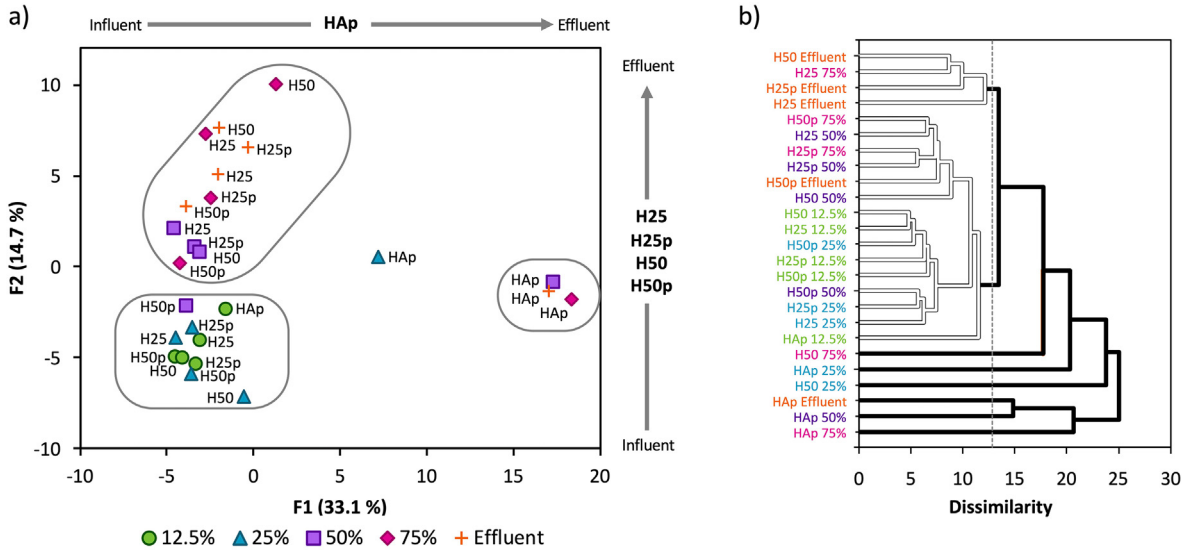

Fig. 3. a) PCA ordination using the genera abundance data. Oval groupings were based on PERMANOVA analysis. b) Clustering analysis dendrogram by AHC using UPGMA.

separately, indicating that aeration caused a shift in its taxonomic abundance profile. The variation for the non-aerated HF systems is explained primarily through their spatial position along the F2 axis.

The groupings from the PCA (Fig. 3a), supported by the PERMANOVA, denoted the spatial heterogeneity across the flow path, and shows that the microbial communities have adapted and are statistically different between the inlet and outlet regions. The dissimilarity between the internal and effluent water samples can be also clearly observed (Fig. 3a). Further support for this observation is the AHC clustering, which shows separate clusters with outlet samples from H25, H25p, H50, H25_75 (75 %), and HAp_75 (75 %) (Fig. 3b). This reinforces the conclusions of the Button et al. (2015) that using effluent samples for interpreting microbial communities dynamics in treatment wetlands could lead to erroneous interpretations and, as such, internal sampling of interstitial water is required in order to accurately represent the microbial communities observed in saturated treatment wetland systems.

3.3. Microbial community structure

Taxonomic analysis revealed variations at the genus level in the most abundant taxa (microorganisms) along the flow path (Fig. 4). This was expected because the microbial diversity indices indicated such dynamics; however, Fig. 4 focuses on the 31 most abundant genera. Recalling that the samples from these systems had between 500 and 1000 OTU (Fig. 1c), a small subset of genera accounted for a large portion of the microbial community in most samples.

For H25, H25p, H50 and H50p, the main identified taxa with the highest relative abundance in the inlet region (12.5–25 %) were under the phyla Firmicutes (described as heterotrophic denitrifying microorganisms; Si et al., 2020), Euryarchaeota (archean microorganisms linked to methanogenic activities; He et al., 2015), and Bacteroidetes (frequently observed in anaerobic processes linked to nitrogen and phosphorus removal; Fu et al., 2019; Li et al., 2021). Among the most prevalent were the anaerobic bacteria from the genus *Blautia*, unknown genera in the order Clostridiales, *Trichococcus*, *Methanosaeta*, and *Macellibacteroides*, respectively (highlighted in green in Table S5). These microbial taxa followed the same decreasing trends along the flow path as observed with the interstitial TOC and TN concentrations. It is likely these genera were therefore involved in and potentially the main genera responsible for pollutant treatment within the front end of the four non-aerated treatment wetland systems.

The remaining abundant genera identified for the non-aerated wetlands H25, H25p, H50 and H50p were positively correlated with the flow path (i.e., higher relative abundance in 50 %–75 %) particularly some of the facultatively anaerobes that belong to the phyla Bacteroidetes, Proteobacteria, Firmicutes, and Euryarchaeota. This includes an unknown genus in the order Bacteroidales, *Sulfuricurvum*, *Sulfurimonas*, *Geobacter*, unknown genus in the family Christensenellaceae, and *Methanocorpusculum* (highlighted in yellow in Table S5). The higher abundance of the genera found in the 50 %–75 % fractional distances was most likely due to the reduced competition for nutrients in these regions, but also pollutant profiles which contained more difficult to degrade compounds. The more readily biodegradable compounds would have been utilized already in the high microbial density regions at the inlet of the treatment wetland systems. Further along the flow path, more functionally adaptable microbial communities are likely to develop. Some of the genera identified as increasing in abundance towards the back end of the treatment wetland systems have been previously correlated with the autotrophic denitrification process in (Tang et al., 2020) and also play important roles in sulfur autotrophic denitrification (SAD); examples of which include *Sulfuricurvum* and *Sulfurimonas* (Chen et al., 2016; Di Capua et al., 2019).

With respect to the physicochemical properties, the temperature and electrical conductivity (EC) decreased along the flow path (Table 2). No statistically significant trend was seen between the genera and DO in the non-aerated systems H25, H25p, H50 and H50p (Table S5); however, an overall positive correlation between DNA quantity with DO was observed, being more prevalent for H25/H25p than H50/H50p (Table S5). For the aerated wetland HAp, there was a significant shift in the microbial community when compared to the non-aerated systems after the first internal sampling point (12.5 %) as shown in Fig. 4. A total of 58 % of the most abundant genera identified in the HAp system were positively correlated with DO (highlighted in yellow on Table S4b) and showed higher relative abundance in the internal samples taken from 25 %–50 % fractional distances (Fig. 4). Among them, the class ZB2, ABY1, and *Candidatus Rhabdochlamydia* genus (order Chlamydiales) had a notable increase in HAp compared to non-aerated systems. These microorganisms are often related to the oxygen content in wastewater systems, described as having the ability to degrade complex carbon compounds, and are linked to sulfur cycling (Kantor et al., 2013; Yadav et al., 2014; L. Lv et al., 2017a, 2017b; Capson-Tojo et al., 2021). Since HAp had higher interstitial DO concentrations and lower TOC concentrations (analogous to lower microbial biomass) compared to the non-aerated systems H25, H25p, H50, H50p (Fig. 1b; Table 2), both oxygen availability and microbial biomass could

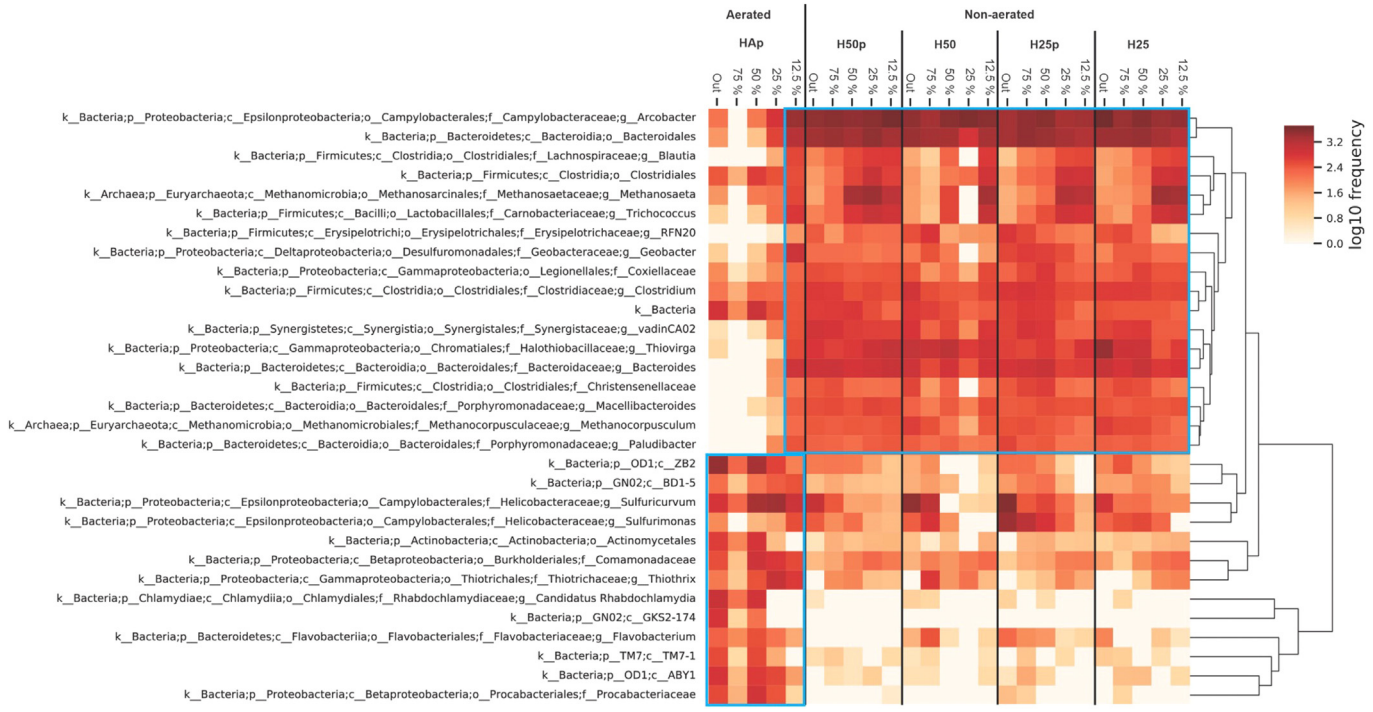

Fig. 4. Taxonomy of the most abundant genera across all samples grouped based on the average Bray-Curtis distance metric. The top corresponds to the fractional distance along the flow path (%). The dendrogram on the right represents the clustering microbial to relative abundance. On the frequency scale, the darker the color, the more abundant the genus in the sample. *Note blue boxes identify that the samples taken at the 12.5 % fractional distance for HAp (aerated) shared some common genera with the non-aerated systems.

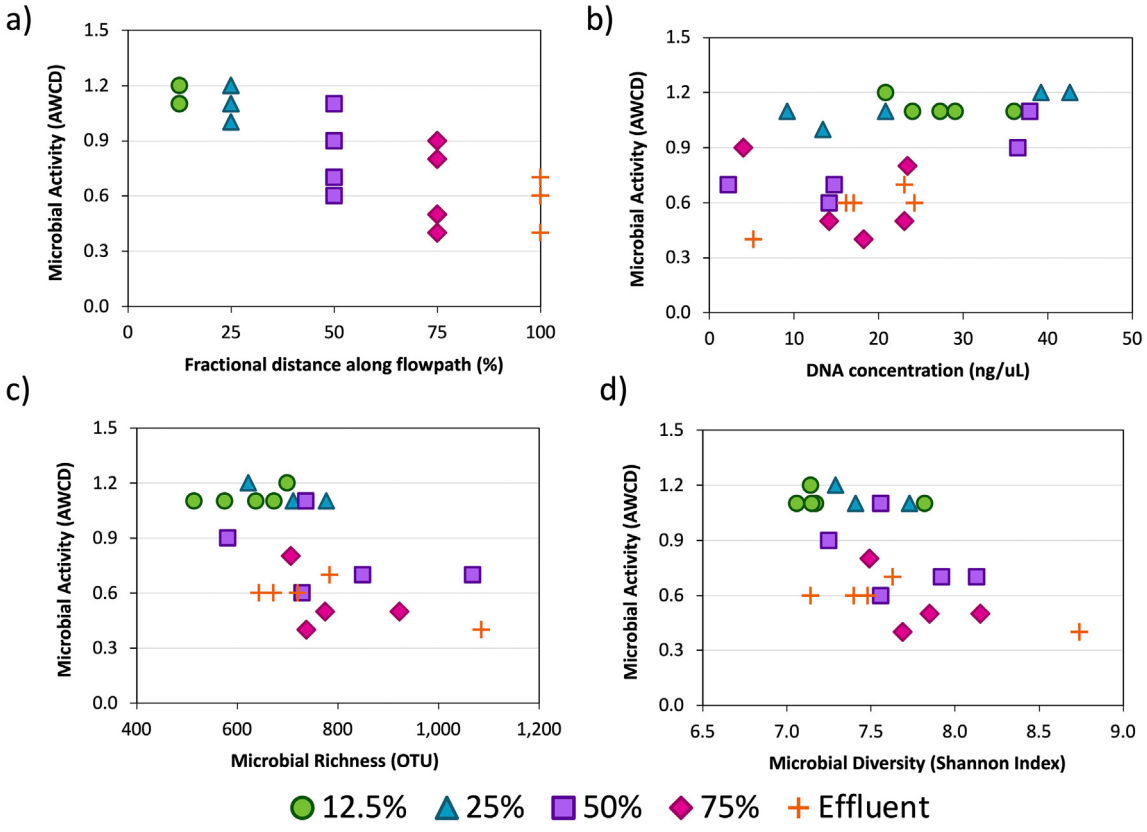

Fig. 5. Microbial activity (AWCD) versus a) fractional distance along the flowpath, b) DNA concentration; c) microbial richness (number of unique species, OTU); and d) microbial diversity (Shannon index).

be considered, at least partially, to be responsible for the observed spatial variability of the microbial communities in HAp.

3.4. Relationship between microbial community structure and function

Higher microbial activity, as measured by Average Well Color Development (AWCD), was observed in the inlet regions (12.5 %–25 % fractional distance) (Fig. 6a), before decreasing along the flow path. Notable exceptions were H50p in which microbial activity remained elevated up to 50 % along the flow path, and HAp, which showed an increase in microbial activity again at 75 % along the flow path (Fig. 5a). Subsequently, AWCD had a positive correlation with DNA concentration as it follows the same decreasing trend along the flow path (Fig. 5b; Table S5) seen with the most abundant microbial genera in the inlet region (12.5–25 %) for all systems (highlighted in blue in Table S5).

These findings are supported by the results of other studies which reported decreases in microbial biomass (C and N) and activity with increased distance along the flow path in horizontal flow treatment wetland systems (Nguyen, 2000; Truu et al., 2009; Button et al., 2015). Furthermore, a negative correlation between microbial activity (AWCD) and structural diversity (OTUs and Shannon index) (Fig. 5c, d; Table S2) was observed.

Upon examining the specific carbon guild utilization patterns for the non-aerated horizontal systems (H25, H25p, H50, H50p), there is an observable gradient along the flow path of these systems in the utilization of carboxylic acids, amino acids, and amine/amides (highlighted in blue in Table S5). Trends towards the use of polymers were less evident, whereas the carbohydrates showed a positive correlation with the most abundant microorganisms around 50 %–75 % along the flow path (highlighted in yellow in Table S5). This suggests that alongside the shift in the microbial community composition in the non-aerated HF wetland pairs, their ability to utilize specific contaminants has adapted to their specific location along the flow path. In the case of HAp, there were no specific trends were noted in the utilization of polymers or carbohydrates along the flow path (Table S5). However, positive correlations were observed after the 25 % fractional distance along the flow path for amino acids indicating increased utilization of this specific guild. Aerated wetland systems have often reported improved treatment performance when compared to conventional (non-aerated) wetland designs (Nivala et al., 2020). The treatment capacity of HAp might be associated with the unique microbial structure and function established along the flow path (the increase in activity in the front-end through 75 %), which is not observed in the non-aerated systems.

3.5. Correlation between pollutant availability and microbial community dynamics

Combined with the results presented earlier in this study, internal concentration profiles of Emerging Organic Contaminants (EOCs) (Fig. 6) support the development of a conceptual visualization of pollutant degradation superimposed on microbial density and activity in a treatment wetland. Fig. 6 shows internal pollutant profiles for carbamazepine, benzotriazole, naproxen, and $CBOD_5$ in the horizontal flow aerated wetland HAp. Fig. 6 shows that readily biodegradable contaminants are removed within the first portion of the wetland (25 % of the fractional distance), whereas naproxen, a moderately biodegradable compound, was removed to a lesser extent, further along the flow path. Fig. 6 also demonstrates the behavior of recalcitrant compounds in treatment wetlands (carbamazepine). The high concentrations of organic matter (as measured by TOC and/or $CBOD_5$), can promote co-metabolic processes, which have been shown in recent studies to enhance the removal of readily degradable EOCs (Lv et al., 2017a; Lv et al., 2017b; Zhang et al., 2018; Zhang et al., 2019).

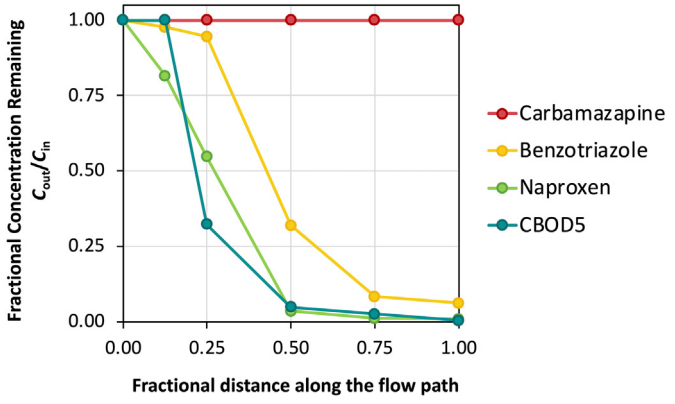

Fig. 6. Internal pollutant concentration profiles in the horizontal flow aerated wetland HAp (number of samples per point, n = 9–10).

Based on the findings of this study, a conceptual visualization of the pollutant biodegradation profile is proposed in horizontal flow treatment wetlands (Fig. 7). This profile is dictated by the structural microbial community profile, which, in turn, is influenced by the quantity of microbial biomass along the flow path. This conceptual model describes what has been anecdotally observed in horizontal flow treatment wetlands, where readily biodegradable compounds are rapidly removed in the inlet region of the system and refractory compounds persist along the length of the system. In this study, the microbial community structural profile in the inlet region (12.5–25 % fractional distance) was primarily composed of heterotrophic anaerobic bacteria that positively correlated with high interstitial TOC and TN concentrations (Fig. 4; also highlighted in blue in Table S5). These bacteria obtain energy from readily available carbon sources; hence, the microorganisms rapidly utilizing readily biodegradable pollutants. In contrast, the genera at 50–75 % along the flow path display a higher number of unique species (as seen with increasing OTU, Fig. 1c), which signify a more diverse microbial community capable of utilizing more difficult to biodegrade compounds.

Other studies have also highlighted that different environment and physicochemical conditions within treatment wetlands systems can promote the spatial heterogeneity of microbial diversity structure that consequently promotes different microbial community functions (Button et al., 2015; Weber, 2016). Therefore, multiphase studies that assess both structure and function improve the understanding microbial dynamics both within and between different treatment wetland systems.

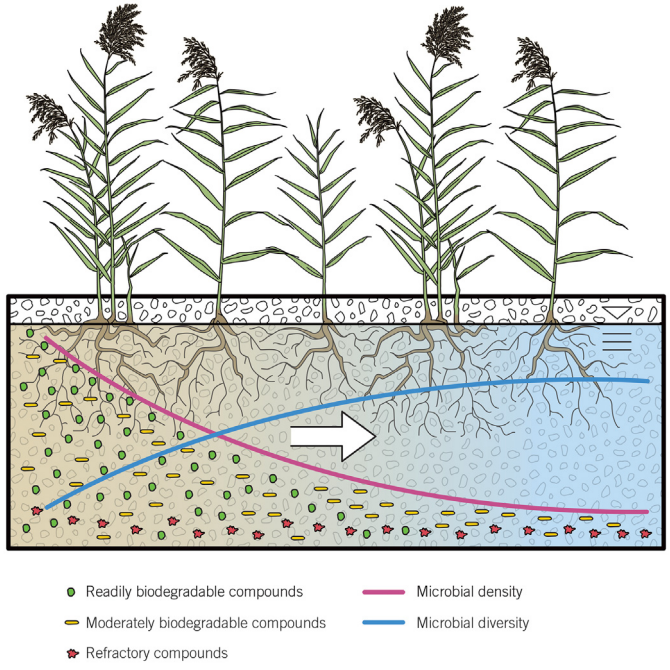

Fig. 7. Conceptual visualization of pollutant biodegradation, microbial density, and microbial diversity in a horizontal flow treatment wetland system.

Furthermore, the results of this study reinforce the concepts presented by Button et al. (2015), where certain operational modifications, such as step-feeding, could promote the development of microbial communities further along the flow path of the systems, allowing for either increased overall loading using the same system footprint, or a smaller system footprint for the same pollutant loading. This operational modification could potentially stimulate microbial activity in less active areas, minimizing clogging, and increasing the lifespan of a horizontal flow treatment wetland system. If the goal is to remove only pollutants that are readily biodegradable, a decrease in the required area in non-aerated horizontal flow wetlands could be useful; however, in order to remove refractory compounds, recirculation could be employed. For example, in HAp, the introduction of recirculation with an additional carbon source to areas where there was low dissolved oxygen, could aid denitrification, since carbon was predominately consumed in the inlet region.

4. Conclusions

The multiphasic approach used in this study allowed the comparison of spatial trends in microbial community activity, diversity, and density in different horizontal flow treatment wetland systems. The results of this study confirm the hypothesis of the conceptual model proposed: as pollutant concentrations and microbial biomass/density decreases along the flow path in horizontal flow treatment wetland systems, microbial diversity increases. The overall profile of biodegradability of pollutants gives insight into metabolic and co-metabolic processes along the flow path and indicates a functional potential for degradation of the most difficult contaminants towards the end of the systems. This suggests that alongside the shift in the microbial community composition in HF systems, the ability of microbial communities to utilize compounds is adapted to their specific location along the flow path. The observations of this study have broad applicability to treatment wetland design, identifying a potential knowledge gap regarding design modifications that aim to stimulate microbial activity in different areas within a treatment wetland system.

CRediT authorship contribution statement

Daniele Damasceno Silveira: Software, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization. **Anbareen J. Farooq**: Validation, Formal analysis, Investigation, Data curation, Writing - review & editing. **Sarah J. Wallace**: Software, Formal analysis, Writing - review & editing. **Flávio Rubens Lapolli**: Writing - review & editing, Supervision. **Jaime Nivala**: Validation, Investigation, Resources, Writing - review & editing, Conceptualization, Methodology, Supervision, Project administration, Funding acquisition. **Kela. P. Weber**: Validation, Investigation, Resources, Writing - review & editing, Conceptualization, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.156600.

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