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Assessment of bacterial communities and prediction of their metabolic functions in vegetation-activated sludge process treating landfill leachate

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ABSTRACT

Optimizing the structure and function of microbial communities is crucial in the design and operation of treatment systems, particularly because the species composition of biomass determines the metabolic pathways, which ultimately impacts the treatment quality. As the impact of landfill leachate (LL) on microbial communities in treatment systems remains poorly understood, this study aimed to analyze the composition of bacterial communities and their variability over time in vegetation-activated sludge process (V-ASP) and modified ASP systems. The predicted metabolic functions of microorganisms and their relationship with nitrogen removal from LL were also determined. Treatment efficiency was associated with the composition of microbial communities. The dominant bacterial phyla in all the treatment systems were Pseudomonadota and Bacteroidota. This study confirmed that landfill leachate supports a unique microbiome that is distinct from other microbiomes. The most abundant bacterial phylum in the LL was Patescibacteria, which reached a maximum abundance of over 30%. However, only a small proportion of Firmicutes (from 1.6% to 3.2%) was recorded in the LL. Biofilms on plant roots favored the occurrence of aerobic denitrifying microorganisms, such as Halomonas, which reached an abundance of up to 20% and may be linked to increased nitrogen removal efficiency. Functional prediction based on PICRUSt2 and KEGG indicated that dissimilatory nitrate reduction and denitrification were the dominant predicted nitrogen-related pathways in all treatment variants, with the highest abundance in Phase II. Microbiological characterization provides new information to help understand the role of bacteria involved in LL treatment and is important for its management.

1. Introduction

Landfilling remains one of the main methods of waste management, and as a result, landfill leachate (LL) is generated [1]. If not properly managed, LL may pose a threat to human health and the environment. Uncontrolled LL leakage may cause groundwater contamination and impair the quality of drinking water [2,3]. As a result of surface runoff, LL can cause contamination of surface water reservoirs [4], and when it penetrate the soil environment, they can pose a threat to agricultural activities [5,6]. Due to the fact that landfills and generated LL constitute a global environmental problem [7], many studies have been conducted in recent years on the properties and treatment of LL and a high-efficiency method for their treatment is still being sought [8].

An interesting solution is a method that combines the activated sludge process (AS) with hydrophyte systems (CW), that is, the ecological vegetation-activated sludge process (V-ASP) [9]. The limited number of studies conducted so far has shown that it has high efficiency in removing pollutants from municipal wastewater and is resistant to high and variable pollutant loads [10]. Recently, the effectiveness of removing selected pollutants from LL has also been tested in V-ASP systems operating as a sequencing batch reactors (SBRs), where laboratory tests achieved a reduction of up to approximately 99% of ammonium nitrogen and up to a maximum of 74% of total nitrogen was achieved [11,12].

The physicochemical complexity of LL complicates the selection of appropriate treatment methods.

LL is a highly polluted wastewater with a variable physicochemical composition that depends on factors such as landfill age, waste type, and management practices [13]. Furthermore, LL contains a wide range of contaminants, typically including inorganic salts, nitrogen compounds, and various toxic and emerging pollutants [14]. The most common contaminants found in leachates are ammonium nitrogen, chlorides, and

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sulfates. LL also usually contain high concentrations of organic matter and phosphorus [15]. Less common contaminants include humic acids, dioxins, furans, pesticides, phthalates, and compounds used in drug production [16].

The heterogeneous composition of LL and the numerous compounds dissolved in it may potentially negatively affect the activated sludge [17]. Studies conducted thus far have emphasized that optimizing the structure and function of the microbial community should be the main goal of design and operation a treatment systems [18]. Therefore, it is crucial to identify the types and numbers of bacteria and the responses of bacterial communities that comprise activated sludge. This is particularly true because the species composition of the biomass determines the metabolic pathways, which ultimately affect the quality of the treatment [19].

Nitrogen removal in CW is a complex process that usually involves nitrification–denitrification and uptake by plants, substrate adsorption, N_2O emissions, and volatilization [20]. Nitrogen in CW can also be removed by simultaneous nitrification and denitrification (SND) and anaerobic ammonia oxidation (ANAMMOX) [21]. However, nitrification-denitrification usually accounts for more than 50% of nitrogen removal [22], with microorganisms playing important roles [23]. Nitrogen transformations and mass balance in CWs treating wastewater have been studied extensively [22,24]. However, the nitrogen removal process in different CWs often differs significantly, which can be explained by differences in system design [25].

The substrate used is important not only for plant growth but also because it influences biological transformations by microorganisms and plants [26] and microbial adhesion [27]. The type of substrate and its porosity influence the dissolved oxygen conditions, which in turn affect microbial activity and the structure of microbial communities in the systems [28]. The structure of bacterial communities can be influenced by vegetation and microbiomes introduced by vegetation. Many unknowns remain regarding plant-microbe partnerships under unfavorable conditions, such as exposure to chemical pollutants [29]. However, little research has been conducted on this topic to date, which requires further systematic monitoring of pollutant removal mechanisms and investigation of the complex interactions between activated sludge and vegetation [30]. New molecular biology techniques, such as high-throughput sequencing, can be used to investigate the relationships between microorganisms responsible for removing pollutants from wastewater. This method has been found to be reliable and cost-effective for analyzing the species composition of environmental samples [19,31]. High-throughput sequencing techniques, such as MiSeq analysis, allow for the acquisition of larger sequence counts and more accurate comparisons of bacterial phylogenetic affiliations in complex systems [32].

High-throughput sequencing analysis has been used extensively to characterize bacterial communities in various CW systems, including bacterial community composition in CWs treating municipal sewage [33–35], combined sewer overflows [36], surface waters [37], and agricultural runoff [38]. However, to date, few studies have examined the bacterial community structure in CWs treating LL [39–41].

Recent studies on V-ASP systems have mainly focused on treatment efficiency and operational parameters [11], whereas microbial communities in V-ASP systems have been analyzed only in a few studies, such as [10,42].

Considering that landfilling is still one of the most commonly used methods of municipal waste management worldwide [43], and the generated LL constitutes a global environmental problem, real leachate from an active municipal waste landfill was treated in V-ASP systems. The experiment was conducted in eight systems operating under laboratory conditions as SBRs. Depending on the variant used, the constructed models were filled with either sunflower husk biochar or zeolite.

As the impact of LL on the microbial community in wastewater treatment systems remains poorly understood, the primary objective of this study was to analyze the composition of bacterial communities and their temporal variations in V-ASP and modified SBR systems. To address this gap, we analyzed 24 metagenomic samples of landfill leachate, activated sludge, and plant root sludge from different systems. We also determined potential bacterial interactions within the systems and predicted the metabolic functions of microorganisms and their relationship with nitrogen removal from LL. Microbiological characterization provides information that help clarify the role of bacteria involved in LL treatment and is important for its management. The information obtained may be helpful not only in facility design but also in improving system performance, optimizing design, and understanding the causes of system failure or inefficiencies.

2. Materials and methods

2.1. Reactor operation

Eight models with dimensions of 23 × 28 × 25 cm and a capacity of 10 L were prepared for the tests. Four operated as vegetation-sequencing batch reactors (V-SBR) and four as sequencing batch reactors (SBR). The reactors were operated in a closed room at temperatures ranging from 20 to 22 °C. The duration of the experiment was 138 days. The experiment was divided into a preliminary phase (Phase I) and the main experimental phase (Phase II). Phases I and II differed in the composition of landfill leachate dosed into the systems, the characteristics of which are presented in Table 1.

The experimental system was operated as an SBR on a 24 h cycle consisting of five stages (Fig. 1): 0.5 h of feeding, 3 h of mechanical mixing using a power-driven agitator, 19 h of aeration using a fine-bubble diffuser, 1 h of activated sludge sedimentation, and 0.5 h of automatic decantation of treated leachate into an outflow collection tank for analysis. All operations were automated and the order in which the elements were activated was controlled by a timer.

Depending on the variant, landfill leachate (LL) or tap water (T) was dosed into the prepared systems. The following experimental variants were obtained: unplanted systems with zeolite filling: 1-ZT (tap water), 3-ZL (leachate), and unplanted systems with biochar filling: 2-BT (tap water), 4-BL (leachate); planted with vegetation, with zeolite filling: 5-ZTP (tap water), 7-ZLP (leachate); and biochar: 6-BTP (tap water), 8-BLP (leachate). Details regarding the experimental design are presented in the publications [11,12].

2.2. Sampling and physicochemical analysis

Leachate samples were collected from a municipal waste landfill located in Poland that has been in operation for almost 50 years. The samples were collected twice from a retention pond with a capacity of 2440 m^3 (51°14′14.9"N, 16°11′07.0″E). Detailed information on the landfill has been presented in a previous publication [44].

For analysis, the samples were transported to the laboratory at the Environmental Quality Analysis Center of the University of Environmental and Life Sciences in Wrocław.

Table 1
Physicochemical characteristics of LL before treatment and tap water used in experiment [12][a].

Parameter	Unit	Leachate		Tap water
		LL1	LL2	
pH	–	8.3 ± 1.7	8.3 ± 0.2	7.4 ± 1.5
EC	µS/cm	7150 ± 715	6020 ± 120	590 ± 59
COD	mg/l O_2	1100 ± 165	620 ± 124	<25
TN	mg/l	154 ± 21	198 ± 40	<0.5
ON	mg/l	30.5 ± 6.1	21 ± 4	<0.5
AN	mg/l	80 ± 8	108 ± 22	<0.010
TP	mg/l	2.21 ± 0.44	1.85 ± 0.37	2.7 ± 0.54

[a] ± Uncertainty.

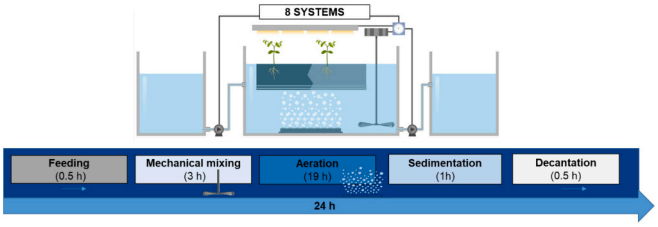


Fig. 1. Experimental setup, the reactor operating cycle is described in detail in the text.

During the experiment, the physicochemical composition of the outflow from the eight systems was verified once a week for the following: pH, electrical conductivity (EC), total phosphorus (TP), chemical oxygen demand (COD), ammonium nitrogen (AN), organic nitrogen (ON), and total nitrogen (TN) (17 series of tests were performed). All parameters were determined using standard methods, which are presented in detail in the Supplementary Materials (Table S1). All reagents used for the analyses were supplied by MERCK (Germany). The physicochemical characteristics of LL before treatment (Phase I: LL1; Phase II: LL2) and tap water used in the experiment are presented in Table 1.

2.3. DNA extraction, amplification and sequencing of the V3-V4 region of the 16S rRNA

DNA was extracted from 24 samples in three technical replicates using the GeneMatrix Environmental DNA/RNA Extraction Kit (Eurx, Gdańsk, Poland), according to the manufacturer's instructions. The quality of the obtained DNA was verified by electrophoresis on a 1% agarose gel. DNA concentration was determined using the Qubit High Sensitivity DNA Kit and a Qubit 3.0 fluorimeter (Thermo Scientific). Following extraction, DNA obtained from the three technical replicates of each sample was pooled prior to PCR amplification to minimize extraction-related variability and to obtain a representative sample. The primers used for 16S rRNA gene sequencing targeting the hypervariable V3–V4 regions followed previously published recommendations [45]. Amplification was performed in triplicate for each pooled sample using an ABI 9700 thermocycler (Life Technologies) and KAPA HiFi PCR Mix (Roche).The obtained amplicons (~450 bp) were verified on a 1% agarose gel and purified using AMPure XP magnetic beads (Beckman Coulter). Amplicon libraries were then pooled in equimolar ratios and indexed according to the Nextera indexing strategy (Illumina). Sample indexing enabled multiplexing of amplicons in a single sequencing run and subsequent demultiplexing of reads. Sequencing was performed using next-generation sequencing (NGS) of the V3–V4 region on a MiSeq platform (Illumina) with the v3 600-cycle MiSeq Reagent Kit at the DNA Sequencing and Oligonucleotide Synthesis Laboratory, Institute of Biochemistry and Biophysics, Polish Academy of Sciences (Warsaw, Poland). Paired-end sequencing (2 × 300 bp) enabled full coverage of the target region and reconstruction of complete V3–V4 fragments of the bacterial 16S rRNA gene for downstream analysis.

For further analysis, samples were categorized into three groups:

1) raw sample: landfill leachate (two sampling series: LL1, LL2) and activated sludge (AS);
2) I samples collected after Phase I and Phase II treatment in individual reactors, described in detail in Section 2.1. reactor operation: 1-ZT (I/ II), 2-BT (I/ II), 3-ZL (I/ II), 4-BL (I/ II), 5-ZTP (I/ II), 6-BTP (I/ II), 7-ZLP (I/ II), 8-BLP (I/ II);
3) Plant roots (PR): PR (before experiment - control sample), and after completion of the experiment: 5-PR (5-ZTP), 6-PR (6-BTP), 7-PR (7-ZLP) and 8-PR (8-BLP).

2.4. Bioinformatic analysis, taxonomic classification, functional prediction and statistical analysis

Sequence reads were quality-checked using the FastQC toolkit (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and processed using the QIIME2 pipeline [46]. Paired-end reads were merged, quality filtered, and denoised using the DADA2 algorithm implemented in QIIME2. Resulting amplicon sequence variants (ASVs) were taxonomically classified using a Bayesian naïve classifier pre-trained on the SILVA and MiDAS databases [47]. Downstream analyses were conducted in the R v. 4.3.3 environment. Microbial community analyses and visualization were performed using the following packages: phyloseq (https://joey711.github.io/phyloseq/), microbiome (https://microbiome.github.io/tutorials/), vegan (https://github.com/vegandevs/vegan), qiime2R (https://github.com/jbisanz/qiime2R), ggplot2 (https://ggplot2.tidyverse.org/) and Ampvis2 (https://github.com/KasperSkytte/ampvis2) [48]. Alpha diversity was assessed using the Chao1 richness estimator and the Shannon diversity index. Differences in alpha diversity between sample groups were evaluated using the Kruskal–Wallis test, followed by pairwise Wilcoxon rank-sum tests with Benjamini–Hochberg correction for multiple comparisons. For selected taxonomic groups differences in relative abundance between experimental phases were assessed using paired Wilcoxon signed-rank tests, accounting for matched sampling across systems. *P*-values were adjusted using the Benjamini–Hochberg method. Functional prediction of microbial communities was performed using PICRUSt2 [49] based on 16S rRNA gene sequences and the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Predicted KEGG ortholog (KO) abundances were further analyzed and visualized using MicrobiomeAnalyst 2.0 [50] and the ComplexHeatmap package. PICRUSt2 predictions are based on reference genomes and phylogenetic inference and therefore represent inferred functional potential rather than direct measurements of gene abundance or expression.

Data preprocessing and statistical analyses were conducted using Microsoft Excel 2016 (Redmond, WA, USA) and related figures were drawn using OriginPro 2025 (OriginLab, Northampton, MA, USA). The laboratory test results were subjected to further analysis. Basic statistics (i.e., minimum, maximum, mean, median, standard deviation, and coefficient of variation) were carried out for selected physicochemical parameters at the outflow of the four systems with LL (3-ZL, 4-BL, 7-ZLP, and 8-BLP). A nonparametric rank-based test, the Kruskal–Wallis test, was used to determine whether there were statistically significant differences between independent groups for a continuous or ordinal dependent variable. This test does not require the assumption of normal distribution of the analyzed data [51]. When statistically significant differences were detected ($p < 0.05$), multiple comparisons were conducted using Dunn's post-hoc test with Bonferroni correction. The Bonferroni-adjusted significance level was $\alpha = 0.0125$. The sample size for each system was $N = 11$.

3. Results and discussion

3.1. Characterization of selected physicochemical parameters in the outflows from leachate treatment systems

The pH value of the outflow from the systems ranged from 6.8 to 8.8. The lowest pH values were recorded in the outflow from the ZP system (Fig. 2a), where a decrease in the pH of the outflow was observed compared to that of the inflow (pH 8.3). Differences between phases were evaluated using the Kruskal–Wallis test. Statistically significant differences ($p < 0.05$) between system variants, depending on the type of filling used or the presence of plants, are indicated in Fig. 2 (*), as confirmed by Dunn's post hoc test. Statistically significant differences in pH were observed between the 3-ZL and 4-BL systems, as well as between the 7-ZLP and 8-BLP systems. The lower pH value of the outflow compared to the inflow can be explained by increased nitrification,

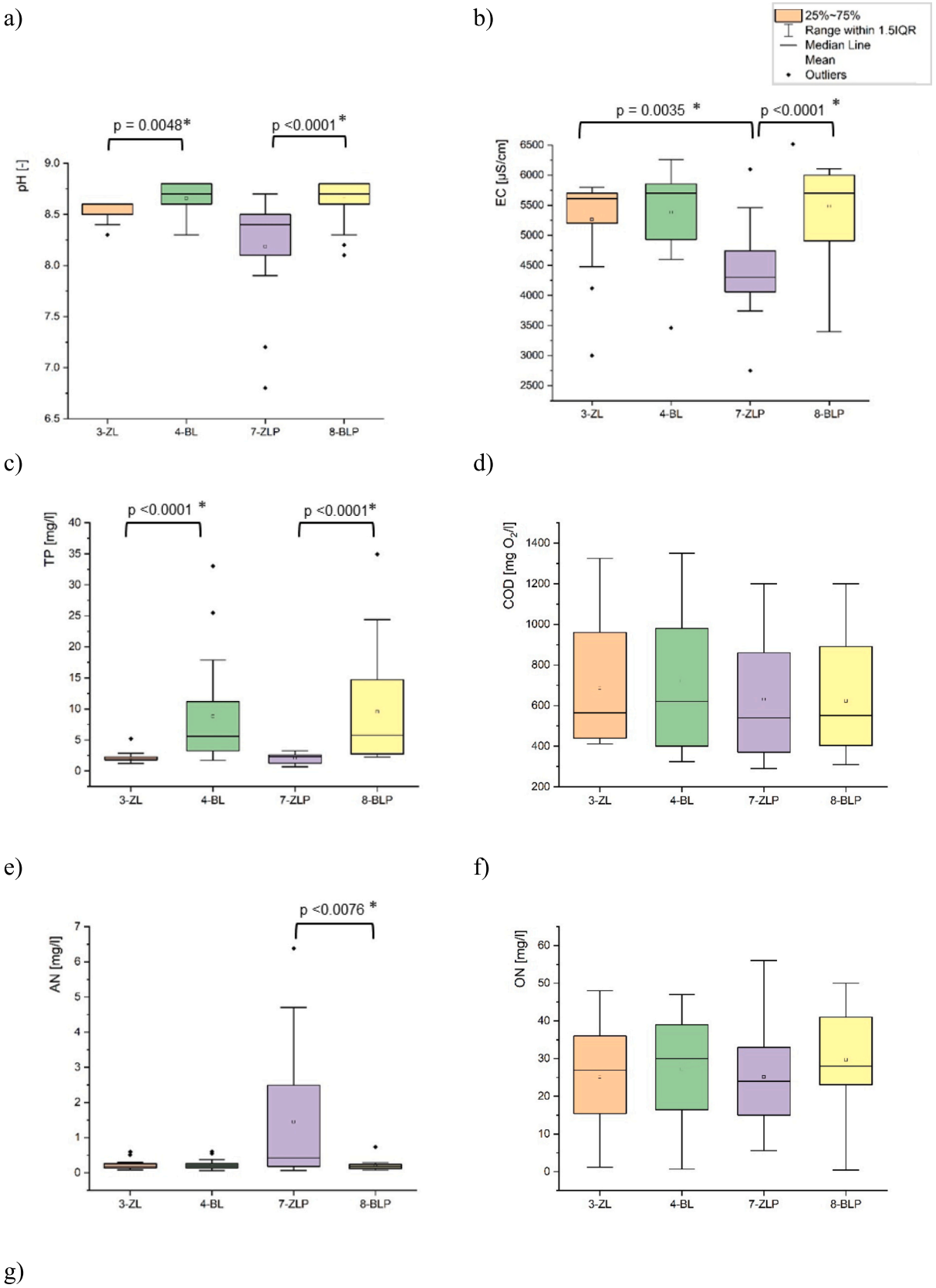


Fig. 2. Boxplots for physicochemical parameters at the outflow from the systems: a) pH, b) EC, c) TP, d) COD, e) AN, f) ON and g)TN.

which resulted in the production of hydrogen ions [52]. The optimal pH for nitrification is reported in the literature to be between 7.5 and 8.5, while a pH of around 7 favors denitrification [53].

EC is an important parameter that can affect the structure and

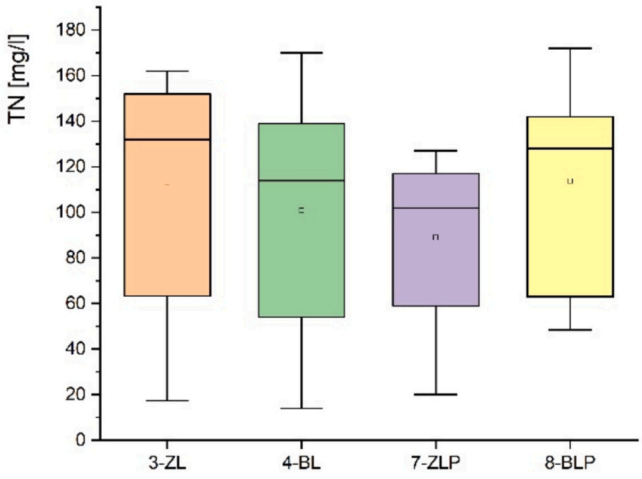


Fig. 2. (*continued*).

settling properties of sludge flocs. High EC values can adversely affect or even inhibit nitrification and denitrification processes occurring in systems [54]. The EC at the outflow from the systems ranged from 3781.7 μS/cm to 5829.2 μS/cm (Fig. 2 b). However, the raw leachate ranged from 6020 μS/cm to 7150 μS/cm. Statistically significant differences ($p < 0.05$) were observed between the 3-ZL and 7-ZLP systems and between the 7-ZLP and 8-BLP systems. The lowest EC was recorded in the outflow of the ZP system. A lower EC value may indicate a reduction in the levels of cations and anions such as nitrate, sulfate, ammonia, potassium, sodium, and chloride [55,56].

The TP in the inflow of the systems was low and ranged from 1.9 mg/L to 2.2 mg/L. In the outflow of the systems, there was no significant difference in TP reduction between the vegetated and unvegetated systems (Fig. 2 c). During the experiment, plants were not harvested or removed from the systems; therefore, phosphorus taken up by the plants could be released back into the systems during biomass decomposition [57,58]. The results of the physicochemical analysis of plant dry matter have been presented in a previous publication [12].

Therefore, no significant difference in the TP levels was observed between the vegetated and unvegetated systems. However, greater differences were observed depending on the type of filler used (biochar and zeolite). Statistically significant differences ($p < 0.05$) were observed between the 3-ZL and 4-BL systems and between the 7-ZLP and 8-BLP systems. This is consistent with the observations of other authors, who also noted that phosphorus removal from LL occurs primarily through substrate adsorption [59]. The larger specific surface area of zeolite (31.5 m^2/g) compared to biochar (1.19 m^2/g) enabled more stable and better TP removal in zeolite-based systems compared to biochar-based systems, confirming that TP adsorption by calcium- and aluminum-rich substrates is more effective than by other substrates [27].

The COD in the raw leachate at the inflow remained at a low level, ranging from 620 mg O_2/L to 1100 mg O_2/l, which may indicate reduced biodegradability of the leachate [60]. The low biodegradability or recalcitrance of the organic matter in the leachate could be the reason for the low COD reduction observed in this study (Fig. 2d). Lower COD values were observed in vegetated systems than in unvegetated systems, although other researchers observed significant differences in COD levels in the outflow of vegetated and unvegetated CWs [54].

The AN concentration in the raw leachate ranged from 80 to 108 mg/L. These values are lower than those typically reported for mature landfill leachates. In the literature, ammonium nitrogen concentrations in mature leachates are often reported to exceed 1000 mg/L [61]. In the outflows from all systems, AN remained at low levels (Fig. 2 e), regardless of the filling variant used. Statistically significant differences were noted only between the 7-ZLP and 8-BLP systems. This can be explained by the fact that the most important role in AN degradation is to ensure sufficient dissolved oxygen (i.e., above 3 mg/L) [30]. A higher AN value was recorded only in the outflows from the 7-ZLP system, which could be related to the higher overall stimulation of biological activity during the experiment in this reactor [62].

For ON, no significant differences were observed between the systems planted with vegetation and those without (Fig. 2 f). One might expect higher levels of ON removal in vegetated systems, because plants support ON reduction through uptake and filtration. Moreover, microorganisms in the rhizosphere facilitate the transformation and degradation of nitrogen [63]. However, the observed ON levels in the outflows may also be influenced by decaying plant litter, which could explain the lack of significant differences between the analyzed systems.

The average TN concentration in the inflow of the system ranged from 326 to 429 mg/L. The systems achieved an average TN reduction of 38–54%.This could be because, in addition to oxygen availability, carbon availability is a key factor in denitrification, which is a mechanism for TN removal [11].

Although the lowest TN concentrations were expected to be achieved in planted systems because plants provide organic carbon through root exudates, thus aiding TN reduction [64], no significant differences were observed between systems. Only the outflow from the 7-ZLP system showed lower TN values than the other systems.

3.2. *Analysis of the microbiological composition in systems*

3.2.1. *Microbiome diversity in samples*

In addition to the composition and structure of microorganisms, the diversity of microbial communities can influence the pollutant removal efficiency [65]. Therefore, the richness and diversity of bacterial communities in raw samples of leachate, plant root sludge, and samples collected from the eight treatment systems were analyzed and are presented in Fig. 3. Additionally, the alpha diversity of microbial communities at three different time points (raw samples, after phase I treatment, and after phase II treatment) is presented in the Supplementary Materials (Fig. S1).

Alpha diversity indices were calculated from 16S rRNA gene amplicon sequences using QIIME2 and the phyloseq package in R [66]. The Shannon index was selected because it is positively correlated with microbial diversity and is the most widely used alpha diversity index [67]. The Chao estimator was used to determine the richness of the microbial community [68].

In all analyzed samples, the Shannon index was above 5.0 for the 16S rRNA gene amplicon data, except for the raw landfill leachate samples (LL1 and LL2), where it was lower. A Shannon index above 5 indicates a

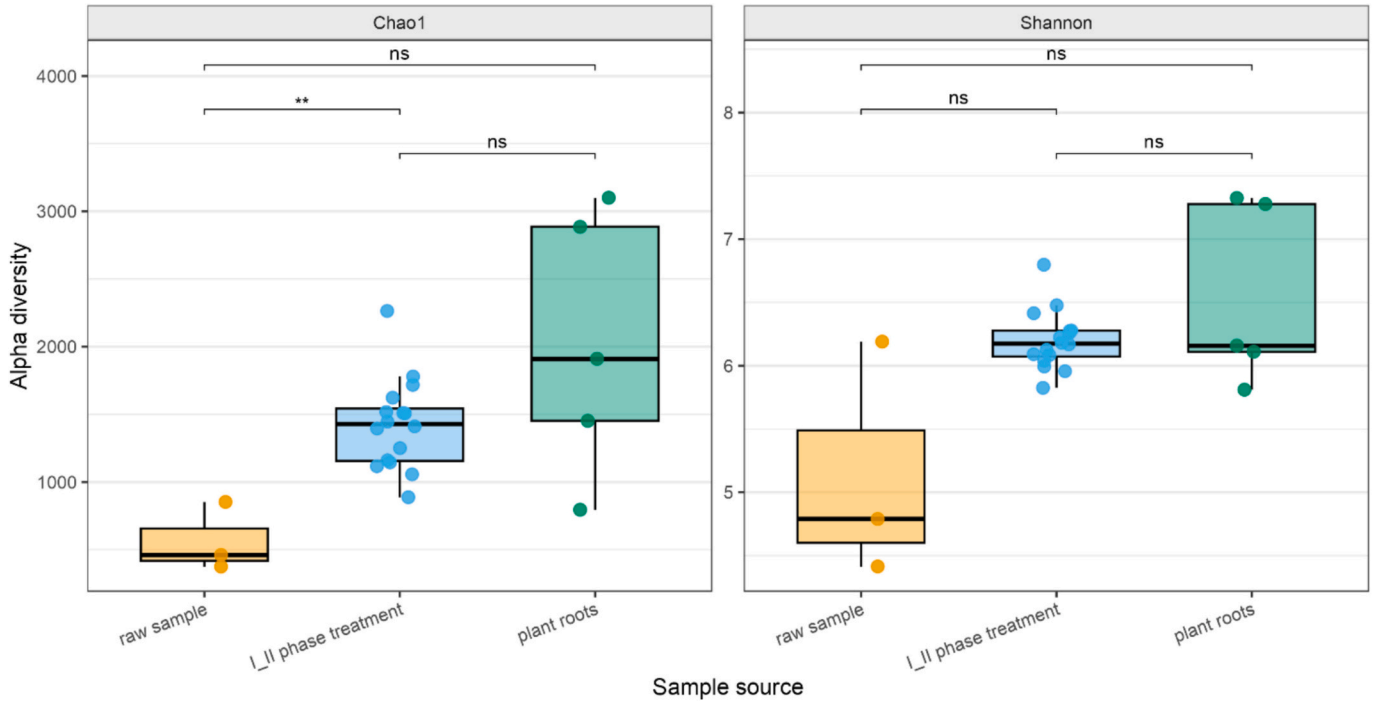


Fig. 3. Alpha diversity of microbial communities across sample sources. Alpha diversity was assessed using Chao1 richness estimator and the Shannon diversity index for samples grouped by isolation source (raw sample, I-II Phase treatment, and plant root). Boxplots represent the median and interquartile range (IQR), with individual points showing sample-level values. Statistical differences were assessed using the Kruskal-Wallis test followed by pairwise Wilcoxon rank-sum tests with Benjamini-Hochberg correction. Significance levels are indicated as ns (not significant), $p \leq 0.05$ (*) and $p \leq 0.01$ (**).

high level of microbial richness and uniformity in most of the analyzed samples. High alpha diversity values indicate that the samples are composed of phylogenetically diverse microorganisms, regardless of the presence of dominant phyla [69].

The lowest Shannon and Chao indices were recorded for raw landfill leachate samples (LL1 and LL2). Low diversity in landfill leachate may be due to high pollutant loads that can inhibit the growth of microorganisms [70]. Other authors have also observed that microbial richness is severely reduced in environments with high pollutant concentrations, which is explained by the fact that microorganisms in extreme environments are eliminated or suppressed, resulting in fewer microbial species [71,72].

In all cases, the samples taken from the reactors showed higher Shannon index and Chao estimator values than those in the raw leachates, which can be explained by the fact that when the appropriate amount of oxygen and the appropriate concentration of organic matter are provided, a greater diversity and richness of microorganisms can be obtained [70]. However, statistical analysis revealed that most of these differences were not statistically significant (Kruskal–Wallis test, $p > 0.05$). Only the Chao1 index showed a statistically significant difference between raw leachate samples and Phase II treatment samples (pairwise Wilcoxon test, $p < 0.01$).

The highest Chao values were recorded for the plant rhizosphere samples (6-PR and 5-PR), indicating the highest microbial richness. The highest Shannon index, indicating diversity, was also recorded in these samples (6-PR and 5-PR). The Shannon index and Chao estimator indicated that the most diverse bacterial communities occurred in rhizosphere sediment samples, which is consistent with the results obtained by other authors [32,73], who reported that plant roots have an obvious influence on the spatial distribution of microbial communities in sediment.

In the AS samples, a high Shannon index was observed, indicating high diversity, whereas a lower Chao estimator value indicated lower microbial richness in this sample. Other authors analyzing the microbial community in CW observed higher Shannon diversity index and Chao estimator values in planted CW systems compared to unplanted CW systems, indicating greater diversity and abundance of microbial species [74]. However, no such differences were observed in the present study. No significant differences were observed between the reactors packed with biochar and zeolites. Other researchers have indicated that the addition of biochar results in a richer bacterial community and greater microbial diversity than the control [75].

However, differences were observed between Phases I and II of the experiment. Higher values of the Chao estimator were observed in Phase II compared to Phase I; however, these differences were not statistically significant, suggesting only a trend toward increased microbial richness over time during the wastewater treatment process, as also reported in previous studies [76].

To assess similarities and differences in microbial community structure among the evaluated groups, beta diversity was analyzed using Principal Coordinate Analysis (PCoA) and statistically tested by PERMANOVA. The results are presented in Fig. 4.

Global analysis showed significant shifts in the overall microbial community composition depending on the sample source across all evaluated metrics: Bray-Curtis ($R^2 = 0.179$, $p = 0.001$), Jaccard ($R^2 = 0.118$, p = 0.001), Unweighted UniFrac ($R^2 = 0.152$, $p = 0.002$), and Weighted UniFrac ($R^2 = 0.234$, p = 0.001). PCoA plots demonstrated distinct clustering patterns. The incorporation of phylogenetic distance metrics indicated that the sample source significantly influenced both the presence of rare microbial lineages (Unweighted UniFrac) and the shifts in dominant taxa abundance (Weighted UniFrac). Pairwise PERMANOVA comparisons further confirmed these observations. Specifically, the microbial structure of the I_II phase treatment group was significantly distinct from the plant roots group in terms of both quantitative community structure (Bray-Curtis: $R^2 = 0.112$, adjusted $p = 0.009$) and evolutionary lineage composition (Weighted UniFrac: $R^2 = 0.160$, adjusted $p = 0.0015$). Furthermore, the initial 'raw sample' community differed significantly from both the treated wastewater and the plant root communities across all tested distance metrics (adjusted $p < 0.05$ for all pairwise comparisons), indicating a clear succession in the

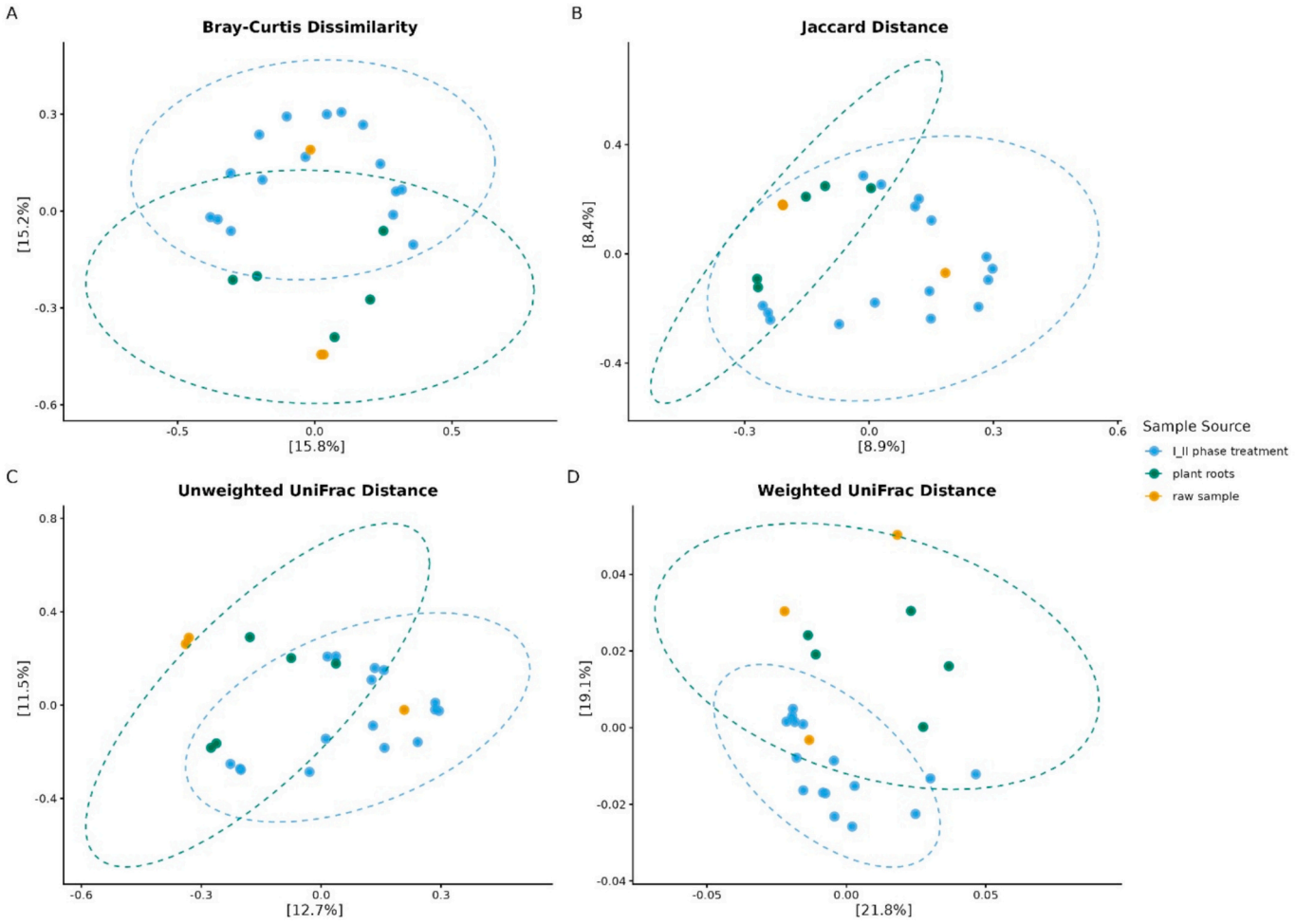


Fig. 4. Beta diversity of microbial communities across different sample sources. PCoA plots illustrating the dissimilarities in microbiome composition based on four different distance metrics: (A) Bray-Curtis dissimilarity, (B) Jaccard distance, (C) Unweighted UniFrac distance, and (D) Weighted UniFrac distance. Each point represents a single sample, color-coded by the sample source (orange: raw sample, blue: I_II phase treatment, green: plant roots). The percentages on the axes indicate the proportion of total variance explained by the first two principal coordinates. Dashed lines represent 95% confidence ellipses for the sample groups.

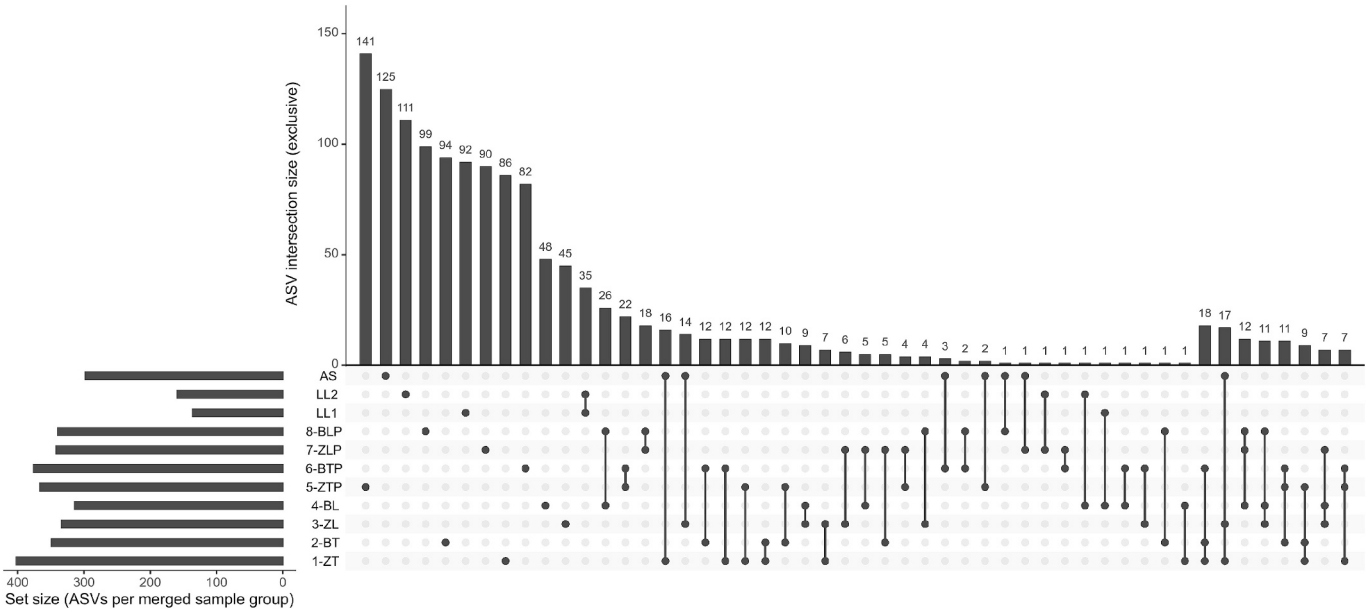


Fig. 5. UpSet plot of ASVs presence across merged sample groups.

microbiome over the treatment process.

3.2.2. Comparison of bacterial composition in different samples

Fig. 5 shows the UpSet plot of amplicon sequence variants (ASVs) presence across merged sample groups, that is, in raw leachates, activated sludge from plant roots, and samples taken from eight system Phases I and II of purification. Replicates "I/II" were OR-merged into group-level samples (1-ZT, 2-BT, 3-ZL, 4-BL, 5-ZTP, 6-BTP, 7-ZLP, 8-BLP); LL1, LL2, and AS are shown individually. Vertical bars ("Intersection size") show the number of ASVs present exclusively in each indicated combination of groups (filled dots = groups in the intersection). Horizontal bars on the left ("Set size") show the total number of ASVs present in each group. Presence was determined per sample using a relative abundance threshold of $\geq$0.1%. The highest number of ASVs (141) was found in the sample taken from the 5-ZT reactor and in the AS sample (125); in both cases, they occurred exclusively in these samples, which means that they are unique to these conditions.

The greatest similarities were found between the raw leachate samples (LL1 and LL2) (35 ASVs). This may be due to the fact that landfills and their leachates support a unique microbiome, fundamentally distinct from any previously studied ecosystem [77]. Another high similarity of 26 ASVs was found between reactors 8-BLP and 4-BL, where the fill was biochar. Many values ranged from 1 to 12, indicating the presence of a large number of rarely co-occurring ASVs. Intersections connecting mainly two samples and, in a few cases, three samples were also observed, indicating common ASVs groups.

No common ASVs were recorded for all the analyzed samples, indicating that the microbial communities in each reactor were distinct, likely because they arose independently. Other researchers have also observed significant differences in the presence of ASVs across reactors, indicating that only approximately 25% of the microbial community ASVs were common to all analyzed reactors and samples [78].

3.2.3. Microbial community composition

Previous studies on activated sludge have shown that the main bacterial types vary depending on the wastewater treatment plant and operating conditions [79]. The 20 dominant bacterial genera from all samples collected constituted 94.3–99.5% of the total bacterial population. The 20 dominant genera in the raw LL samples constituted between 98.6% and 99.5% of the total bacterial population, and in the AS samples, 97.2%. The AS samples collected after purification in Phase I of the experiment ranged from 94.3% to 98.9%, and after Phase II, from 97.2% to 99.2%. The 20 dominant genera in the plant root sludge constituted 97.4–98.9% of the total bacterial population. A total of 52 bacterial genera were identified in all the analyzed samples, of which 27 bacterial genera were present in relative abundance >1% in at least one sample.

In the raw leachate samples injected into the systems (LL1,LL2), Pseudomonadota were dominant, with an abundance ranging from 25.3% (LL2) to 42.4% (LL1), and Bacteroidota, with an abundance ranging from 20% (LL2) to 33% (LL1) (Fig. 6a). In addition to these two phyla, bacteria belonging to the phylum Actinobacteriota were observed in the leachate samples (from 6.63% in LL2 to 11.49% in LL1). Although Actinobacteriota are primarily considered soil bacteria, they can be abundant in water and are characterized by metabolic versatility [80]. Actinobacteriota have been confirmed to have the ability to decompose complex organic matter [41]. Similarly, the phyla Pseudomonadota and Bacteroidota play major roles in organic matter degradation and carbon turnover [81].

The most abundant bacterial phylum in raw leachate (LL2) was Patescibacteria, which reached an abundance of 30.55%. Such a high abundance of these bacteria has not been observed in any previous studies conducted to date. To date, only Sauk et al. have found the presence of Patescibacteria at a high level, averaging approximately 34%, during their study of microorganisms in municipal waste landfills in Canada [69].

The remaining bacterial phyla, whose average number was >1% and dominated in the 2 series of collected leachates, included Firmicutes (from 1.56% in LL2 to 3.17% in LL1), Dependentiae (from 1.03% in LL1 to 1.25% in LL2), Verrucomicrobiota (0.86% LL1, 4.19% LL2), Planctomycetota (0.56% LL1, 3.34% LL2), Spirochaetota (1.71% LL2), Chloroflexi (0.28% LL1, 1.21% LL2), and Bdellovibrionota (0.27% LL1, 1.19% LL2). Previous studies have shown that the most abundant bacterial phyla found in landfill leachates are Bacteroidota, Pseudomonadota, and Firmicutes [82–84]. These three phyla are also typically found in the highest abundance in landfills [69,72,77], although the bacterial phyla present in leachates may differ significantly from those in waste. For example, the phylum Pseudomonadota, which constituted approximately 55% of the sequences in leachate, accounted for less than 3% of the solid waste [82]. Observations by other researchers are partially consistent with our results; the dominant phyla in the leachate were Bacteroidota and Pseudomonadota, while bacteria belonging to Firmicutes, which play a major role in the decomposition of cellulose in landfills, were not as numerous as reported in the literature [72,81].

Although previous studies have shown that, regardless of the geographic location of the landfill or the identification method, Firmicutes is the dominant bacterial phylum in landfill leachate [81,85], in the present study, they constituted a small share, ranging from 1.56% to 3.17%.

It can be observed that the types of bacteria in leachates change over time and depend on the period of leachate collection, which is confirmed by the conducted leachate studies (LL1, LL2) and earlier studies on leachates collected from the same landfill [86]. The literature emphasizes that landfills are microbiological systems that are subject to constant changes and disturbances, resulting in a high diversity of the microbiological community [69].

In activated sludge (AS) collected from a wastewater treatment plant, 16 main bacterial phyla were detected, with an average abundance of >1% and a total of over 97% of the AS community.

The most abundant phyla in the AS from the municipal wastewater treatment plant were Bacteroidota (28%), Pseudomonadota (24%), Patescibacteria (10%), and Myxococcota (7%). Other phyla present in the AS included Planctomycetota, Acidobacteriota, and Verrucomicrobiota, each accounting for 4%, and Actinobacteriota, Chloroflexi, and Firmicutes, each accounting for 3%.

Bacteria belonging to the phyla Bacteroidota and Pseudomonadota have also been indicated as the dominant bacterial types in activated sludge in other studies [87–89], and play an important role in the decomposition of organic carbon in sewage [79,90]. Among these two phyla, Pseudomonadota are the dominant phylum in most municipal wastewater treatment plants [89,91,92], unlike Bacteroidota, as observed in the present study.

Fig. 6b shows the 20 most abundant bacterial genera identified in activated sludge samples collected from the wastewater treatment plant (AS), treatment systems after Phases I and II of the experiment, plant roots, and raw landfill leachate samples (LL1 and LL2).

In the raw LL1, LL2, and AS samples, a very high proportion of reads were other reads (64–92%), indicating low-abundance taxa that collectively dominated the sample profile. Previous studies have shown that plants influence microbial communities in CW and the overall system performance. Biofilms on plant roots favored the occurrence of aerobic denitrifying microorganisms, such as Halomonas, as observed in root sludge samples (7-PR and 8-PR). These bacteria were particularly abundant in root-activated sludge samples collected from the 7-ZLP system with zeolite packing, where they occurred at levels as high as 20%. This system also had the lowest TN values in the outflow of all systems analyzed (Fig. 2 g). Other researchers have also observed that biofilms on plant roots favor aerobic denitrifying bacteria, including Halomonas, which can be related to increased N removal efficiency from wastewater [93,94].

Analyzing the types of bacteria present during Phase I and II of the treatment, differences in their abundance were observed depending on



Fig. 6. Microbial community composition of bacteria identified in activated sludge samples (AS), treatment systems after Phases I and II of the experiment, plant roots, and raw landfill leachate samples (LL1 and LL2), shown at the (a) phylum and (b) genus levels. Relative abundances are presented as percentages of total reads. The top 20 most abundant taxa across all samples are displayed, as determined using the Ampvis2 package, while remaining taxa are grouped as "Remaining taxa."

the system variant (Fig. 6). In the sludge samples collected after Phase II of treatment in all systems, the dominant phylum was Pseudomonadota (representing 24.1–33.4%), which plays an important role in nitrogen removal and organic matter decomposition [95]. Other researchers have also observed a dominant share of bacteria from the phylum Pseudomonadota at a much higher level than that in the present study. For example, in samples collected from CW, the proportion of Pseudomonadota was as high as 72%–74% [41]. However, after Phase I of treatment, Bacteroidota dominated the outflow in all cases (from 17.5% to 26.1%), except for System 7, where the dominant phylum was Pseudomonadota (24.3%). Pseudomonadota and Bacteroidota are abundant in aquatic environments and have been indicated as the dominant bacterial taxa in SBR by other researchers [67].

The dominant types of activated sludge bacteria in Phases I and II of the treatment are shown in Fig. 6a. Changes in relative abundance were observed for several phyla, including Acidobacteriota, Actinobacteriota, Bacteroidota, Chloroflexi, Nitrospira, Patescibacteria, Planctomycetota, Pseudomonadota, and Verrucomicrobiota. The most pronounced trends included an increase in Acidobacteriota (1–6% to 3–12%) and decreases in Actinobacteriota (2–9% to 2–4%) and Planctomycetota (15–23% to 8–13%). To further assess differences between Phase I and Phase II, selected dominant phyla were compared using paired statistical analysis (Fig. 7).

Significant changes were observed for Acidobacteriota, Bacteroidota, Planctomycetota, and Pseudomonadota ($p < 0.05$). Acidobacteriota showed a significant increase in Phase II compared to Phase I (median: 3.03% vs 6.00%, adjusted $p = 0.038$), while Pseudomonadota also increased significantly (18.93% vs 26.97%, adjusted $p = 0.042$). In contrast, Bacteroidota exhibited a significant decrease (23.63% vs 16.07%, adjusted $p = 0.038$), and Planctomycetota also decreased significantly between phases (17.55% vs 11.11%, adjusted $p = 0.038$). Differences for Actinobacteriota, Chloroflexi, Nitrospirota, Patescibacteria, and Verrucomicrobiota were not statistically significant.

A decrease in the abundance of Planctomycetota was observed (from 15 to 23% in Phase I to 8–13% in Phase II). This phylum includes bacteria involved in anaerobic ammonium oxidation (Anammox process)

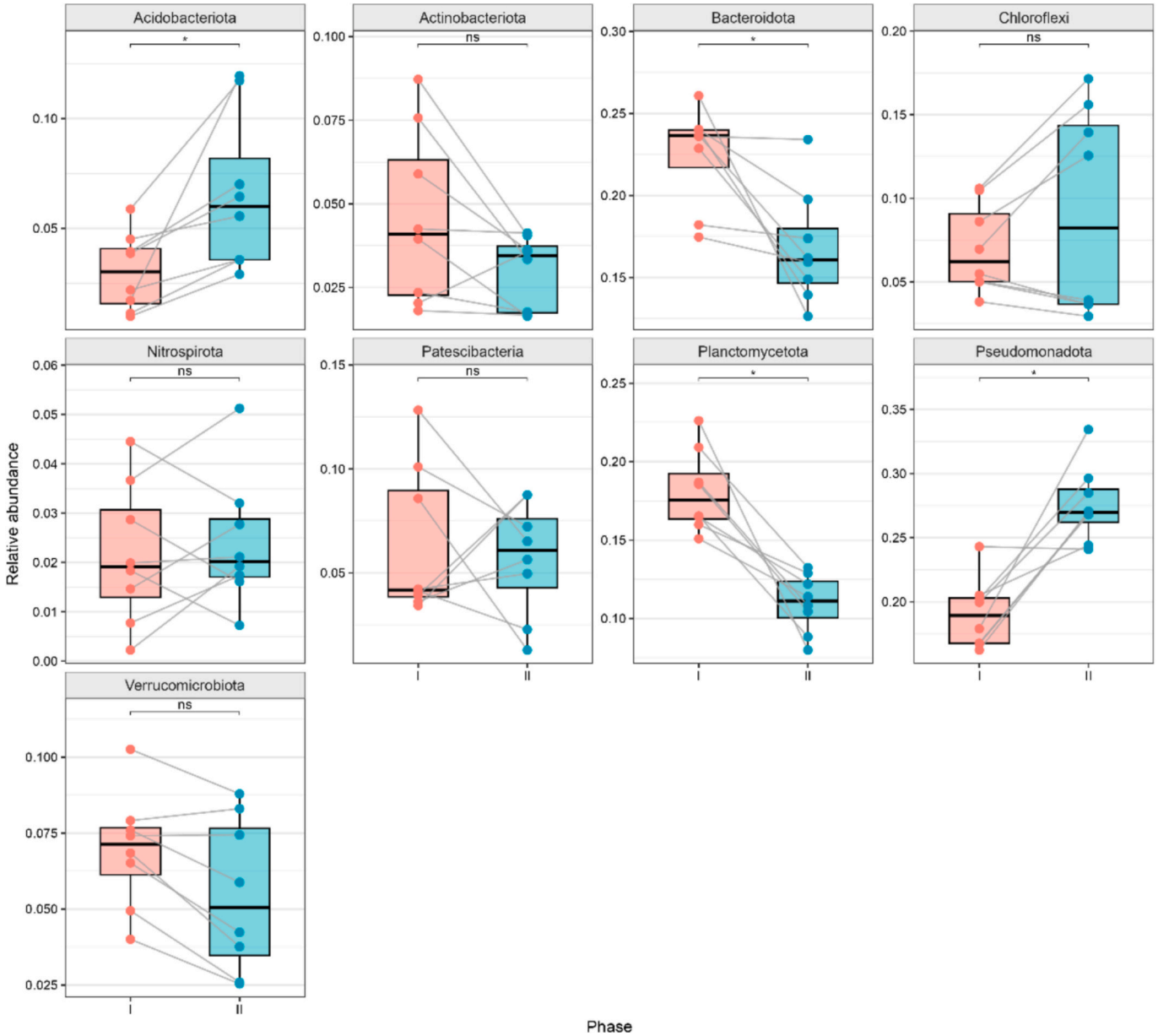


Fig. 7. Comparison of relative abundance of selected dominant bacterial phyla between Phase I and Phase II. Statistical significance was assessed using a paired Wilcoxon test. Significance levels are indicated as follows: ns, not significant ($p \geq 0.05$); *$p < 0.05$.

[96], suggesting a potential shift in nitrogen transformation pathways. These results confirm that, despite observable trends in several phyla, only selected groups contributed significantly to the shift in microbial community structure between treatment phases.

An almost two-fold increase in the number of Chloroflexi bacteria was also noted in Phase II compared to Phase I of purification in all LL reactors (i.e., 3-ZL, 4-BL, 7-ZLP, and 8-BLP). Chloroflexi bacteria are anaerobic photosynthetic bacteria involved in the carbon cycle that use organic matter as an energy source [97,98]. Despite their widespread occurrence, Chloroflexi bacteria have not been fully studied, but previous studies have shown that Chloroflexi have the ability to reduce nitrates to nitrites [99].

Other researchers have noted significantly higher numbers of Chloroflexi in ASP systems than in V-ASP [10], which was not observed in this study. However, in the reactors planted with plants (i.e., 7-ZLP and 8-BLP), the appearance of bacteria that were absent in the first Phase of treatment, such as Myxococcota (7-ZLP 0–3%; 8-BLP 0–2%), was observed. It can be observed that the composition of activated sludge collected from all reactors, even in control reactors where tap water was dosed at the phylum level, changed compared to activated sludge collected from a wastewater treatment plant. Other researchers have also confirmed that the diversity of bacterial communities in CW depends on the quality of the incoming water [97]. The observed changes in bacterial types in the analyzed samples can also be explained by the duration of the experiment, which was also observed in CW treatment studies [32].

In both the control variants and the leachate treatment systems, the dominant bacterial phylum in the plant root sludge was Pseudomonadota (from 32.9% to 49.8%), which in the plant root sludge in almost all samples (except for the 6-BTP system) constituted a higher share than in the sludge samples from reactors collected from the first and second Phases of treatment, and the differences reached up to 25.5%. Other researchers have also noted an increase in the abundance of the phylum Pseudomonadota in planted CWs compared to unplanted ones, as in the present study, but also observed a higher abundance of other phyla, such as Acidobacteriota, Firmicutes, and Actinobacteriota [32,100]. The phylum Pseudomonadota is characterized by an exceptionally high level of bacterial metabolic diversity associated with the global carbon, nitrogen, and sulfur cycles [74]. During the experiment, a variable Hydraulic Retention Time (HRT) was used (14, 7, 5, and 3 days). The effect of HRT on the bacterial community was not analyzed in the present study; however, other researchers have reported that changes in HRT have a limited effect on most microorganisms at the phylum level [101].

Wastewater treatment efficiency is strongly related to the presence of functional microorganisms. In this study, the dominant phyla were Pseudomonadota and Bacteroidota, which may indicate a high potential for the degradation and transformation of various organic compounds [88,90].

3.3. *Predicted metabolic functions of microbial communities*

Based on the KEGG database and PICRUSt2 analysis, predicted functional genes and key enzymes related to nitrogen metabolism were classified to further investigate nitrogen transformation pathways in the systems. Seven nitrogen metabolism modules were identified, with predicted abundances with predicted abundances varying across systems and analysis Phases (Fig. 8a). The highest abundance was observed for module M00530 (dissimilatory nitrate reduction), followed by M00529 (denitrification module), and M001804 (complete nitrification, comammox module). The lowest abundance was observed in modules M00175 (nitrogen fixation module), M00528 (nitrification module), and M00531 (nitrate assimilation reduction module). Studies on nitrogen removal from landfill leachates in various CW variants have revealed a similar trend in the abundance of individual modules [41].

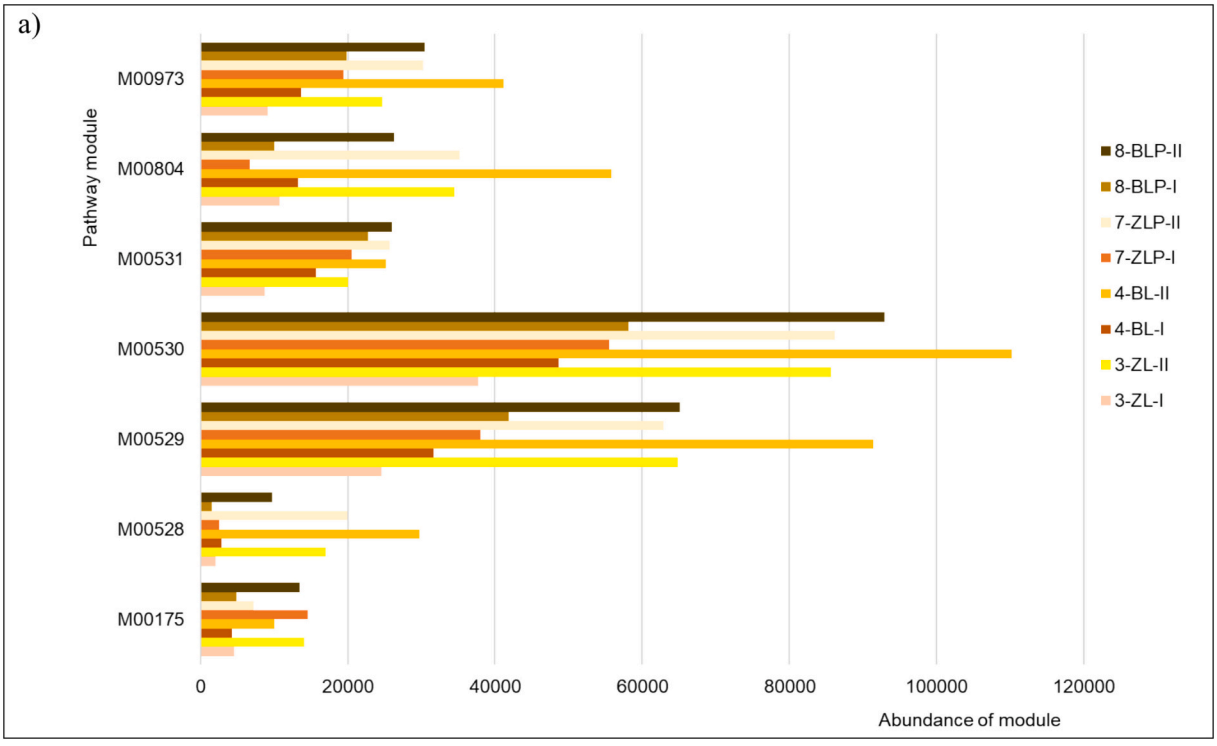


Fig. 8. Nitrogen metabolism pathway in different systems with LL (3,4,7,8) for Phases I and II. a) KEGG functional annotation, b) Abundance of key enzyme classifications (identified by their EC number) for each treatment in the nitrogen metabolism pathway, c) Heatmap of the standardized relative values of nitrogen-cycle functional genes. Values represent normalized relative abundances of predicted gene families, with color scaling from low (blue) to high (red). Rows are hierarchically clustered based on gene profiles, while columns are ordered according to experimental design (Phase I and Phase II). Gene annotations are shown alongside KO identifiers.



Fig. 8. (*continued*).

However, the authors observed almost no differences between the analyzed variants of CW systems, whereas in the present study, differences were noted between individual systems and between the two analyzed Phases.

The greatest differences in the abundance of the nitrogen removal modules were observed between Phases I and II. In almost all cases, Phase II was characterized by a higher predicted abundance of key nitrogen removal modules than that of Phase I. This may indicate a shift in microbial community composition and an increased potential for nitrogen-cycling functions over time, rather than a direct measure of microbial activity.

The highest abundance of all key modules responsible for nitrogen removal was identified in the 4-BL system (Phase II), and a high proportion was also observed in the 8-BLP system (Phase II), where biochar was used as a filler. This can be explained by the fact that biochar provides a favorable habitat for microorganisms [102,103] and can increase their denitrification capacity by facilitating electron transfer [104]. Other researchers have also observed that the addition of biochar improves denitrification in the case of AN [102,103] and promotes nitrate removal [104]. In the case of the nitrification module, in addition

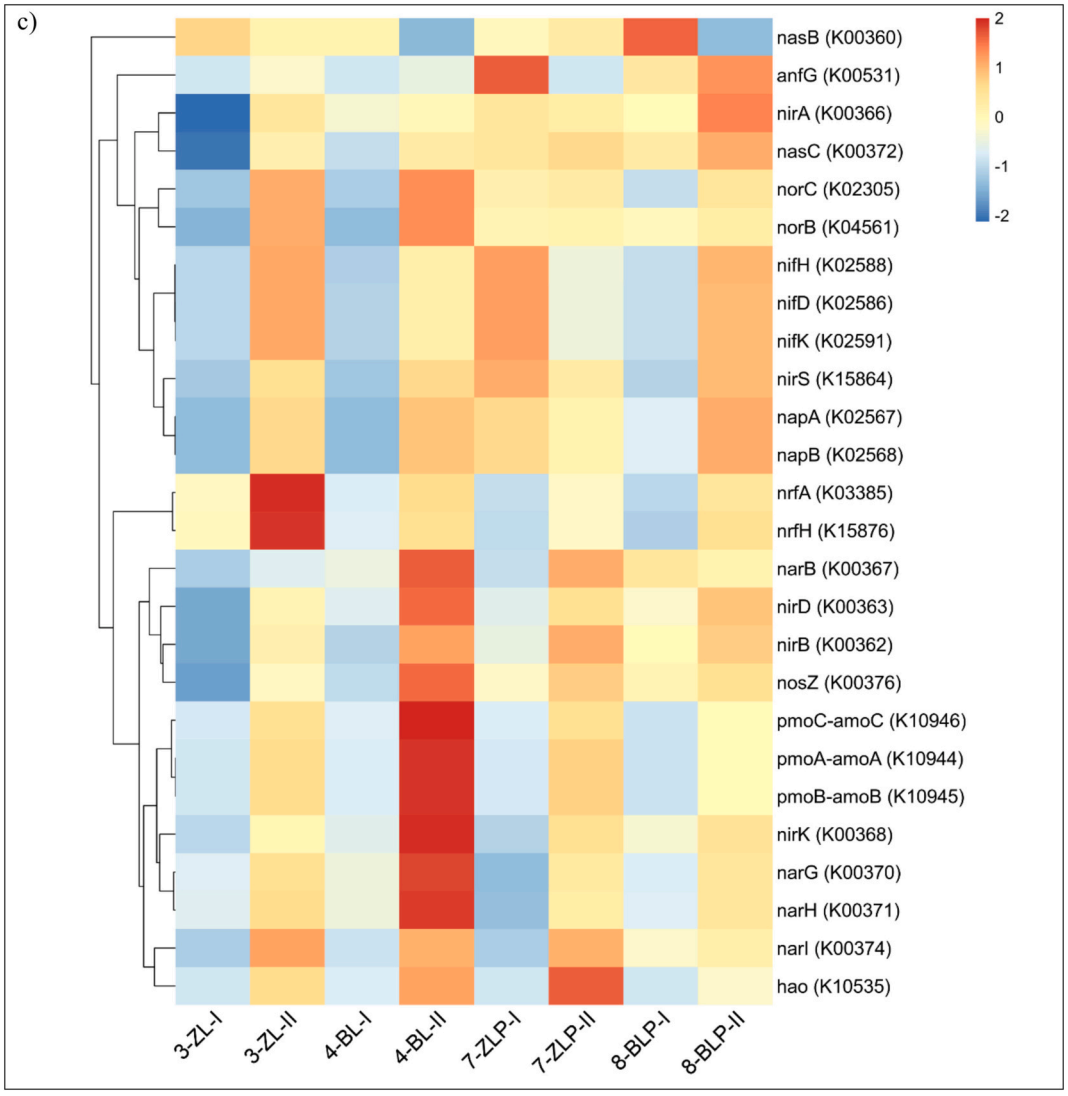


Fig. 8. (*continued*).

to high values for the biochar-filled system (4-BL-II), high values were observed for the zeolite-filled systems (7-ZLP-II and 3-ZL-II). Previous studies have shown that zeolites can provide an excellent binding surface for nitrifiers and allow for increased nitrification efficiency [105,106].

Dissimilatory nitrate reduction and denitrification were the main nitrogen transformation pathways in all system variants. However, the abundance of the Nitrogen Fixation module was low, indicating that this process is of secondary importance and does not contribute significantly to nitrogen balance. This is consistent with the observations of other researchers, who also noted that nitrogen fixation is significantly lower or even negligible compared with other nitrogen transformation processes [25].

Several key enzymes are involved in the nitrogen metabolism pathway (Fig. 8b), such as nitrate reductase (EC: 1.7.5.1) and nitrite reductase (EC: 1.7.2.1), which catalyzes the reduction of nitrite to nitric oxide; nitrite reductase (NADH) (EC: 1.7.1.15), and nitric oxide reductase (EC: 1.7.2.4), which are responsible for the reduction of nitric oxide to N_2; and nitric oxide reductase (EC: 1.7.2.5), which is involved in the reduction of nitric oxide to nitric oxide (I). The N metabolism pathways are illustrated in Fig. S2 in the Supplementary Materials.

PICRUSt2 analysis detected 26 KEGG orthologsassociated with N cycling, revealing differences in inferred functional potential across the systems. To support these observations, key nitrogen cycling genes (e.g. *amoA, amoB, amoC, hao, nirK, nirS, norB, nosZ*) were specifically examined in the PICRUSt2 predictions (Fig. 8c). Fig. 8c shows a heatmap of the predicted genes contribution to nitrogen metabolism, with blue indicating a relatively low contribution, yellow an intermediate contribution, and red a relatively high contribution of individual genes in the samples. In almost all cases, differences were observed between Phases I and II, with higher relative abundance of most nitrogen cycling genes observed in Phase II, indicating microbial population growth and development in the analyzed systems over time [76].

Among genes associated with nitrification, a predicted presence of genes encoding subunits of ammonia monooxygenase (*amoA, amoB, amoC*) and hydroxylamine oxidoreductase (*hao*) was identified. The highest relative predicted abundance of *amoA, amoB, and amoC* genes was observed in the 4-BL system in the second phase of the experiment. Among genes associated with denitrification, a predicted presence of genes encoding nitrate reductases *narG, narH, narI* (membrane-bound complex) and *napA, napB* (periplasmic form), responsible for the reduction of nitrate to nitrite ($NO_3^- \rightarrow NO_2^-$), was identified. The reduction of nitrite to nitric oxide ($NO_2^- \rightarrow NO$) was represented by the alternative genes *nirK* and *nirS*. The subsequent step ($NO \rightarrow N_2O$) was associated with the presence of *norB* and *norC* genes, while the final reduction of nitrous oxide to dinitrogen ($N_2O \rightarrow N_2$) was represented by

the *nosZ* gene (Fig. S2).

The relatively high predicted abundance of *nirB* and *nirD* genes in the 4-BL system (Phase II) suggests a potential for enhanced dissimilatory nitrite reduction, although this inference is based on functional prediction rather than direct gene quantification. The *nirB* and *nirD* genes are involved in nitrite degradation and convert it into ammonium ions, which can be used for bacterial cultivation or released into the environment, enabling other bacteria to survive under unfavorable environmental conditions [107]. In turn, the *nosZ* genes encode nitrous oxide reductase, which acts as a marker of complete denitrification [108,109].

The higher predicted abundance of *nosZ* in the 4-BL-II system may indicate an increased potential for heterotrophic denitrification [107,110], whereas the predicted abundance of nosZ varied considerably in the other systems, which may be related to the role of organic matter. However, a high abundance of *nirA*, compared to other systems, was observed in the 8-BLP-II system (Fig. 8c), which is essential for the complete denitrification process [111].

In the second Phase of the experiment, the 4-BL system had the highest predicted gene abundance in almost all nitrogen removal modules, indicating that this system had the greatest potential to promote nitrogen removal. Biochar filling created the most favorable environment for nitrogen removal. Interestingly, the highest gene abundance was observed in the system without vegetation, although previous studies have shown that plants improve N uptake and increase denitrification with root exudates [112].

4. Conclusion

This study is useful for understanding the composition and temporal variation of bacterial communities and the role of bacteria involved in LL purification in V-ASP and modified ASP systems. Basic phyla were identified in all samples, regardless of the treatment variant used (i.e., Proteobacteria and Bacteroidetes). Landfill leachate exhibited a unique microbiome distinct from other samples, which changed over time and depended on the leachate collection period, confirming the dynamic nature of the microbial communities in landfills. One of the dominant phyla in the leachate was Patescibacteria, which had not been previously detected in such high numbers. In contrast, Firmicutes, previously identified as the dominant phylum in leachate, accounted for a small proportion.

The most diverse bacterial communities were found in rhizosphere sediment samples, as confirmed by the Shannon index and Chao estimator, indicating that plant roots influence the spatial distribution of microbial communities. Higher microbial community richness was observed during Phase II, indicating an increase in the population and development of microorganisms in the analyzed systems over time. PICRUSt2/KEGG-based functional prediction indicated that dissimilatory nitrate reduction and denitrification were the main nitrogen-related pathways across all system variants, with higher predicted abundance in Phase II. The 4-BL system showed the highest predicted abundance of several nitrogen-cycling genes, while the enrichment of Halomonas in root-associated samples may have contributed to nitrogen removal. The results provide a basis for further studies on the role of microbial communities in landfill leachate treatment and may help guide future system optimization.

CRediT authorship contribution statement

Aleksandra Wdowczyk: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Jan Gawor:** Visualization, Software, Investigation, Formal analysis.

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cej.2026.178313.

Data availability

Data will be made available on request.

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