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Microbiome Structure of Activated Sludge after Adaptation to Landfill Leachate Treatment in a Lab-Scale Sequencing Batch Reactor

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Abstract: During adaptation to waters that are rich in xenobiotics, biological systems pass through multiple stages. The first one is related to the restructuring of communities, pronounced destruction of the structure, and multiplication of active biodegradants. The purpose of the present research was to describe the microbiome restructuring that occurs during the adaptation stage in landfill leachate treatment. In a model SBR (sequencing batch reactor), a 21-day purification process of landfill leachate was simulated. Wastewater was fed in increasing concentrations. When undiluted leachate entered, the activated sludge structure disintegrated (Sludge Volume Index—4.6 mL/g). The Chemical Oxygen Demand and ammonium nitrogen concentration remained at high values in the influent (2321.11 mgO_2/L and 573.20 mg/L, respectively). A significant amount of free-swimming cells was found, and the number of aerobic heterotrophs and bacteria of the genera *Pseudomonas* and *Acinetobacter* increased by up to 125 times. The *Azoarcus-Thauera* cluster (27%) and *Pseudomonas* spp. (16%) were registered as the main bacterial groups in the activated sludge. In the changed structure of the microbial community, *Gammaproteobacteria*, family *Rhizobiaceae*, class *Saccharimonadia* were predominantly represented. Among the suspended bacteria, *Microbactericeae* and *Burkholderiaceae*, which are known for their ability to degrade xenobiotics, were present in larger quantities. The enzymological analysis demonstrated that the ortho-pathway of cleavage of aromatic structures was active in the community. The described changes in the leachate-purifying microbial community appear to be destructive at the technological level. At the microbiological level, however, trends of initial adaptation were clearly outlined, which, if continued, could provide a highly efficient biodegradation community.

Keywords: landfill leachate; activated sludge adaptation; *Pseudomonas*; sludge disintegration; catechol dioxygenases

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1. Introduction

Municipal solid waste (MSW) generation has increased dramatically around the world during the past few decades as a result of human overpopulation, urbanization, and economic growth [1,2]. While innovative and effective solutions are still being developed and implemented to reduce, reuse, and recycle these huge amounts of waste materials, landfilling remains the most widely used concept in waste management and sanitary landfills are the most commonly used final disposal option around the world [3,4]. Although landfilling is a well-established technology, it has environmental consequences such as greenhouse gas emissions and leachate formation. The impact of waste disposal sites can be severe due

to improper operation and the release of leachate is commonly rated as the highest priority concern in the risk assessment of landfills. Among the 17 sustainable development goals (SDGs), at least 4 are related to the effective treatment of landfill leachate—clean water and sanitation, affordable and clean energy, sustainable cities and communities, and climate action [5,6]. Achieving these is impossible without managing the treatment of the highly polluted landfill leachate generated by the waste—a problem faced by every city.

Leachate is a highly concentrated organic liquid, produced by the percolation of rainwater during the decomposition of wastes, and is considered one of the most harmful types of wastewater [1,7]. It contains a wide variety of pollutants including heavy metals, inorganic salts, ammonium nitrogen, both biodegradable and refractory organic matter, and xenobiotic compounds [8]. The presence of xenobiotics poses an additional challenge due to the extremely concentrated and complicated composition of leachate. Leachate contains an unknown number of dangerous chemicals with varying chemical compositions and concentrations. Numerous studies have found different hydrophobic aliphatic and aromatic organic substances (benzene, toluene, ethylbenzene, and xylenes—BTEX), polyaromatic hydrocarbons, toxic metals, phenols, phthalates, pesticides, microplastics, polyethylene, plasticizers, per- and polyfluoroalkyl substances (PFASs and their derivates), and halogenated organic compounds such as PCBs and dioxins [9–12]. This nitrogen- and toxic compound-rich composition requires highly specialized treatment technologies aimed at solving specific problems—the removal of high nitrogen content—and emerging contaminants.

On-site treatment plants, transport to a wastewater treatment plant for co-treatment with municipal wastewater, and reinjection or recycling to a landfill cell are the options for leachate treatment [8]. Successful technologies for leachate treatment include physicochemical (coagulation/flocculation, advanced oxidation, precipitation, membrane separation, reverse osmosis, air stripping, filtration), biological (activated sludge processes, sequencing batch reactors, moving bed biofilm reactors, trickling filters, anaerobic processes), and hybrid processes (different combinations of above) [13–16]. Due to their simplicity, good-quality effluents, and low cost, biological methods for treatment are regarded as promising options for removing organic compounds and nitrogen species from landfill leachates. However, their successful application requires specially developed strategies to enhance the biological systems to achieve a high efficiency. These strategies include the use of different techniques and processes such as cometabolism, algal–bacterial symbiosis, quorum sensing, augmentation, bioaugmentation, granulation, and perhaps the most accessible and widely used: the prior adaptation of the biological system [17].

In general, a step-by-step adaptation of sludge to the toxic effect of leachate improves the breakdown rates of persistent organic components and reduces the risk of sludge disintegration or deactivation. There are several interconnected adaptation mechanisms including (i) the restructuring of microbial community with selective enrichment of some microbial species or groups, (ii) activation and/or inhibition of certain enzymes, and (iii) genetic modifications that result in novel metabolic capacities [18]. Usually, the shift in microbial populations is expressed in the predominance of active biodegradants and some functionally important genera from the groups of heterotrophic bacteria, denitrifying bacteria, ammonia-oxidizing bacteria, nitrite-oxidizing bacteria, and/or Anammox bacteria [18,19].

However, there is a knowledge gap regarding the restructuring and changes in the composition of microbial communities throughout the initial adaptation period to the leachate biodegradation process. This stage of development is crucial for the evolving community—the key biodegraders multiply when they are in an environment with abundant substrates while the non-biodegraders are inhibited or even eliminated from the community. Lab-scale modeling may be used to imitate the real full-scale adaptation process under controlled and defined conditions. In our study, we investigated the initial phase of activated sludge adaptation to leachate in a model sequencing batch reactor. For this aim, the activated sludge community was exposed to leachate in increasing concentrations and the changes in structure, composition, and enzymological profile were investigated.

2. Materials and Methods

2.1. Experimental Design

The modeling of the treatment of increasing concentrations leachate was carried out in a laboratory reactor-type SBR (sequencing batch reactor) with a volume of 4 L. It simulated the real purification process in a WWTP, where the same leachate goes through biological treatment in a full-scale SBR—the municipal waste treatment plant (MWTP) of Sofia, Bulgaria.

This MWTP is a municipal enterprise responsible for treating waste generated in the city of Sofia, Bulgaria. It is one of the largest plants of this type in Europe, processing around 1100 tons of waste daily. About 85% of the waste is recycled into various products—compost, RDF fuel, biogas, etc. The remaining 15% of the waste, which is not integrated into the city's circular economy, is deposited in a closed-type landfill. It generates 15,000 L of leachate per day, which enters 3 storage tanks. It is then fed to a dedicated wastewater treatment plant. There, with the help of 4 SBRs, the highly polluted leachate is treated biologically. A significant challenge for this plant is achieving complete purification due to the high degree of pollution of the leachate (COD is approximately 4776 mgO_2/L). One of the main approaches to managing such detoxification processes is the adaptation of microbial communities to pollutants and activating their biodegradation potential in the specific biotechnological situation.

Therefore, to study the biodegradation capacity of a biological system and its adaptation response to the pollutants in the leachate, a 21-day aerobic purification process was simulated. The treatment cycle in the model laboratory SBR lasted for 48 h (aeration 47 h, sedimentation 30 min, discharge of effluent 15 min, feeding influent 15 min) (Figure 1). Activated sludge from the MWTP was used. At each cycle, the wastewater was replaced with new water containing pollutants at a certain dilution. Landfill leachate dilutions (50- and 25-fold) were used during the first and second weeks of the experiment (days 0–7 and days 8–14). During days 15–21, undiluted leachate was fed into the reactor. Glycerol was added to the influent once a week until a ratio of 4:1 COD/BOD was reached, as is regularly performed in the MWTP.

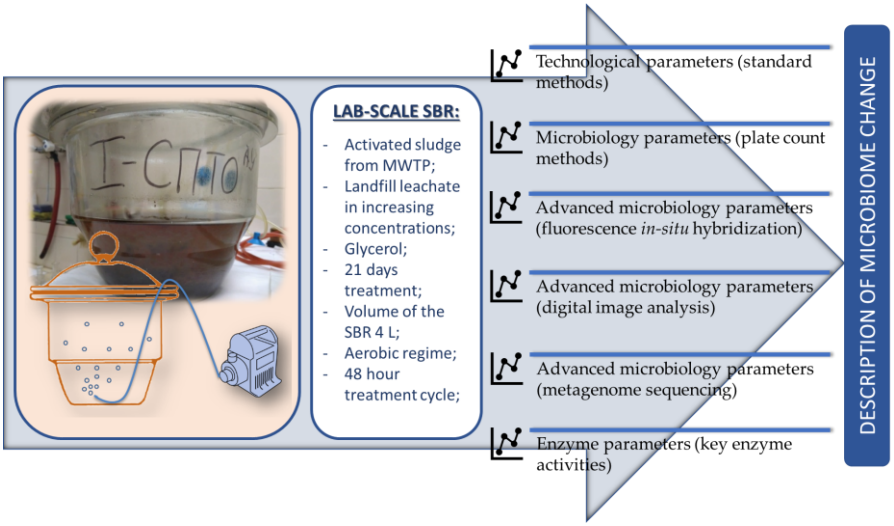

Figure 1. Experimental design.

Four critical control points (CCPs) were determined for analyzing the functioning of the system consisting of activated sludge and landfill leachate. The CCPs were at the beginning of the process (0 h) and at the end of the first (7th day), the second (14th day), and the third (21st day) week. The main technological, microbiological, and enzymological parameters were investigated at these CCPs.

2.2. Methods and Reagents

The following standards were used: technological indicators—SVI ISO 18749:2004 [20]; NH_4—ISO 7150/1 [21]; NO_2—BDS EN 26777 [22]; NO_3—ISO 7890-3 [23]; PO_4—BDS EN 1189 [24]; and COD—BDS 17.1.4.02-77 [25,26]. These parameters were determined after removing the suspended solids.

To study the microbiological characteristics of activated sludge, various methods for obtaining information about microbial biodiversity were applied including plate count techniques, fluorescence in situ hybridization with digital image analysis, and sequencing.

The nutrient media used for the cultivation microbiological analyses were nutrient agar (Merck, Darmstadt, Germany) for aerobic heterotrophs; glutamate starch pseudomonas agar (Merck, USA) for *Pseudomonas* sp.; Sellers agar (HiMedia Laboratories, Mumbai, India) for *Acinetobacter* sp.; and Hiltay agar (2 g/L KNO_3 (Honeywell, Charlotte, NC, USA), 1 g/L asparagine (Honeywell, USA), 5 g/L Na-citrate, 2 g/L KH_2PO_4 (Honeywell, USA), 2 g/L $MgSO_4 \cdot 7H_2O$ (Honeywell, USA), 0.2 g/L $CaCl_2 \cdot 6H_2O$ (Honeywell, USA), 0.08 g/L bromothymol blue, traces of $FeCl_3$ (Honeywell, USA), agar agar (Merck, USA), pH 6.8) was used for denitrifying bacteria. The bacteria were cultured for 24 h at 30 °C under aerobic conditions. The denitrifying microorganisms were incubated under anaerobic conditions (anaerobic jars with oxygen-adsorbing sachets (Merck, USA) for seven days at 30 °C). The bacteria from all the groups were determined in the mixed liquor of activated sludge. Before cultivation, the samples were homogenized by sonication at 22 Hz and 40% intensity using an ultrasonic disintegrator (Sonics VCX750; Sonics, Newtown, CT, USA).

To directly study the amount and spatial distribution of key groups of microorganisms in the activated sludge without destroying its structure, fluorescence in situ hybridization was used. The samples were preserved according to the method of Amann [27]. Table 1 shows the oligonucleotide probes applied in this study. They were labeled with Cy3 dye. The labeled oligonucleotides were supplied by Merck, USA. In situ hybridization was performed according to the Nielsen protocol [28]. A nonsense probe was used as a negative control.

Table 1. Oligonucleotide probes used in the experiments.

Target Microorganisms	Probes for FISH	Nucleotide Sequence	FA, %	Reference
Pseudomonas sp.	Ps	GCT GGC CTA GCC TTC	20	Schleifer, 1992 [29]
Paracoccus spp.	PAR1244	GGA TTA ACC CAC TGT CAC C	20	Neef, 1996 [30]
Azoarcus-Thauera cluster	AT1458	GAA TCT CAC CGT GGT AAG CGC CCG AAC CGC CTG CGC AC	50	Rabus, 1999 [31]
Alcaligenes spp.	ALBO577	Competitor—GCG AAC CGC CTG CGC AC	35	Friedrich, 2003 [32]
None (nonsense probe)	NON-EUB	ACT CCT ACG GGA GGC AGC	0–80	Wallner, 1993 [33]

The resulting fluorescence images were subjected to digital image analysis by the DAIME 2.0 software [34] using a custom segmentation criterion. The proportion of target microorganisms was calculated based on images of the corresponding DAPI-stained microorganisms.

The community structure at the end of the model process was also investigated by sequencing. Analyses of the V3–V4 region of the 16S rRNA gene were performed to study the microbial composition of the activated sludge. Since the activated sludge showed a high abundance of free-swimming bacteria at the final stage of the experiment, the sludge and the free-swimming bacteria were tested separately. First, the samples were allowed to sediment for 1 h. Then, DNA extraction using a Zymo Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, Irvine, CA, USA) was performed separately on the sludge and the supernatant. The quantity of DNA was checked using a QuickDrop™ UV-Vis Spectrophotometer (Molecular Devices, San Jose, CA, USA) and samples with more than 7 ng/μL DNA were sent for sequencing.

16S V3–V4 libraries were prepared using custom fusion primers including a P5/P7 Illumina adapter sequence, an 8 nt index sequence, and the gene-specific primer sequence

(Table 2). The libraries were purified with Agencourt AMPure XP (Beckman Coulter Diagnostics, Brea, CA, USA) beads and the sequencing was performed on an Illumina HiSeq 2500 (Illumina, San Diego, CA, USA) in the 2 × 300 bp mode.

Table 2. Gene-specific primer sequences.

Primer Name	Sequence
341F	ACTCCTACGGGAGGCAGCAG
806R	GGACTACHVGGGTWTCTAAT

The bioinformatic analysis was performed using omics2view.consulting GbR (Kiel, Germany). The sequencing data were demultiplexed and the quality of demultiplexed reads was checked with FastQC v0.11.7. The remaining contigs (ASVs—amplicon sequence variants) were taxonomically classified through the IDTAXA approach implemented in the R package DECIPHER v2.18.1 [35] using the GTDB database release 202 [36,37]. A neighbor-joining phylogenetic tree was constructed from aligned ASVs using the R package DECIPHER. The ASV copy numbers were normalized by dividing them by their respective copy number. The result was multiplied by the ratio of original to normalized ASV counts to preserve the total count per sample. Taxonomic data were taken from the OTU counts at the species level. The taxa were merged at the class level. Those with a frequency less than 0.5% were classified as "other". The results from the sequencing were deposited into the NCBI database under accession number PRJNA1056534.

The activity of the key biochemical pathways of biodegradation was investigated by determining the activities of (1) catechol-1,2-dioxygenase, an indicator of the ortho-pathway of decyclization of aromatic substances using the method of Willetts and Cain [38]; (2) catechol-2,3-dioxygenase, a good indicator of the activity of the meta-pathway of decyclization of aromatic substances, using the method of Farr and Cain [39]; (3) protocatechuate-3,4-dioxygenase, which reflects the decyclization activity of aromatic xenobiotics through a key metabolite (protocatechuate) using the method of Fujisawa and Hayashi [40]; and (4) total dehydrogenase activity (TDA), which reflects the enzymatic activity of dehydrogenases in cells and is an indication of the general metabolic activity of organisms in the community, and was determined using the method of Lenhard [41].

The analyses were performed in triplicates. Excel MS Office was used for data calculations and visualization.

3. Results

The results for the main technological parameters are shown in Table 3, with these parameters being assessed at four Critical Control Points (CCPs) during the leachate treatment—0 h, 7th day, 14th day, and 21st day. At the beginning of the process (0 h), a low sludge volume index (SVI) was found, indicating a deformation in the structure of the activated sludge which was characterized by pin-point flocs. Such a change is often found in communities treating waters with a high degree of toxicity. From the data shown, it can be seen that when the load was reduced (7th and 14th days), the index increased, reaching values considered normal for activated sludge (80–180 mL/g). This normalization suggests an improved purification process under conditions of reduced toxicity (50 and 25 times lower dilution than the landfill leachate). At this stage of the process, a decrease in COD to 309 mgO_2/L was also found at influent values of 2150 mgO_2/L. At the same time, the concentration of nitrates remained significant (154–452 mg/L), indicating a low degree of denitrification. This is related to the treatment of the landfill leachate in an aerobic regime. It allows for a limited elimination of nitrates—only through the conventional denitrification, taking place inside the flocs, where the oxygen concentration is lower—and through aerobic denitrification, a still poorly understood process. However, the concentration of ammonium ions was low (3–6 mg/L, Table 3), which showed that the activated sludge successfully performed nitrification.

Table 3. Main indicators of the landfill leachate treatment process at the end of the treatment cycles.

Control Point	Leachate Dilution	SVI, mL/g	COD, mgO_2/L	NH4, mg/L	NO_2, mg/L	NO_3, mg/L	PO_4, mg/L
0 h	-	30 ± 2	1858 ± 55	20.1 ± 2.1	0.02 ± 0.01	452.1 ± 24.3	0.25 ± 0.06
7th day	×50	66 ± 3	309 ± 22	2.8 ± 0.8	0.03 ± 0.02	154.4 ± 14.4	0.07 ± 0.02
14th day	×25	105 ± 5	890 ± 6	5.9 ± 1.1	0.02 ± 0.01	235.3 ± 18.7	0.07 ± 0.03
21st day	×1	5 ± 1	2321 ± 9	573.2 ± 18.3	0.97 ± 0.1	4.4 ± 0.9	15.27 ± 3.7

At the end of the model process, when undiluted leachate was fed into the system, a significant increase in pollutants in the effluent was found—the COD increased by 2.6 times, phosphates increased by 218 times, nitrites increased by 49 times, and ammonium ions increased by 98 times up to 573 mg/L. This high value indicates a strong inhibition of nitrification, which is also the reason for the low concentration of nitrates (4.39 mg/L), indicating that ammonium ions were not transformed into nitrates. At this CCP (21st days), an extremely low SVI (4.6 mL/g) was also registered, indicating an almost complete disintegration of the activated sludge structure. All the technological data indicated that when undiluted leachate was fed into the system, inhibition of the biological processes and intoxication of the microorganisms that support them occurred.

To elucidate the processes underlying the obtained data, it is important to analyze the community treating the landfill leachate, considering the technological indicators. Figure 2 illustrates the results of the cultivation analyses showing the number of bacteria from several key groups—aerobic heterotrophs, bacteria from the g. *Pseudomonas*, g. *Acinetobacter*, and denitrifying bacteria. The data demonstrated an increase in the number of aerobic heterotrophs at the end of the process by nearly 7 times compared the beginning. Such an increase was also registered in all the other studied groups. In the case of bacteria from the g. *Pseudomonas*, the increase was 58 times; in the case of g. *Acinetobacter*, it increased by 299 times; and in the case of denitrifying bacteria, it increased by 17 times. The substantial variations in the number of bacteria from the xenobiotic-degrading groups (*Pseudomonas* and *Acinetobacter*) indicated a specialization of the community towards the biodegradation of the specific pollutants in the landfill leachate. Notably, within these groups, an increase in CFU/mL was registered at each subsequent CCP (2 to 6 times), with the largest increase in *Acinetobacter* occurring after adding the first part of the diluted filtrate (×50) to the system (increase by 56 times).

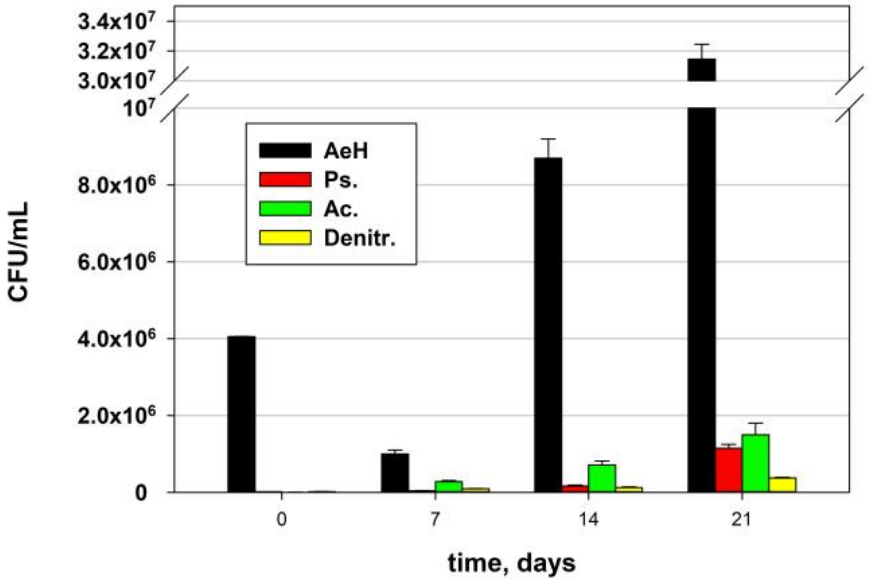

Figure 2. Number of the bacteria from the key groups, determined by plate counting techniques.

At the end of the model process, numerous free-swimming cells were found in the reactors. These were bacteria that were well adapted to the toxic conditions in the reactor. They used pollutants as a substrate for their development, which is why they had a competitive advantage and develop more rapidly. Since they were part of the biodegradation potential of the system that was capable of efficient adaptation, they were subjected to additional cultivation analyses. Thus, in the homogeneous phase, an especially high number of aerobic heterotrophs was found, constituting 71% of the total number of microorganisms on the 21st day of the process. In the same phase of the mixed liquor, 5% of the bacteria were a *Pseudomonas* sp., 11% of the microorganisms were an *Acinetobacter* sp., and 5% were bacteria capable of denitrification (Figure 2). The high difference in the part of the aerobic heterotrophs and narrower bacterial groups indicated that there are other microbial groups with important role in the treatment process. Thus, sequencing was performed to further elucidate the taxonomic structure of this adaptationally important segment.

The quantity of several more taxonomic groups that are also important in the biodegradation of xenobiotics in the leachate was investigated in situ. For this, the FISH method was used and the obtained data are presented in Figure 3.

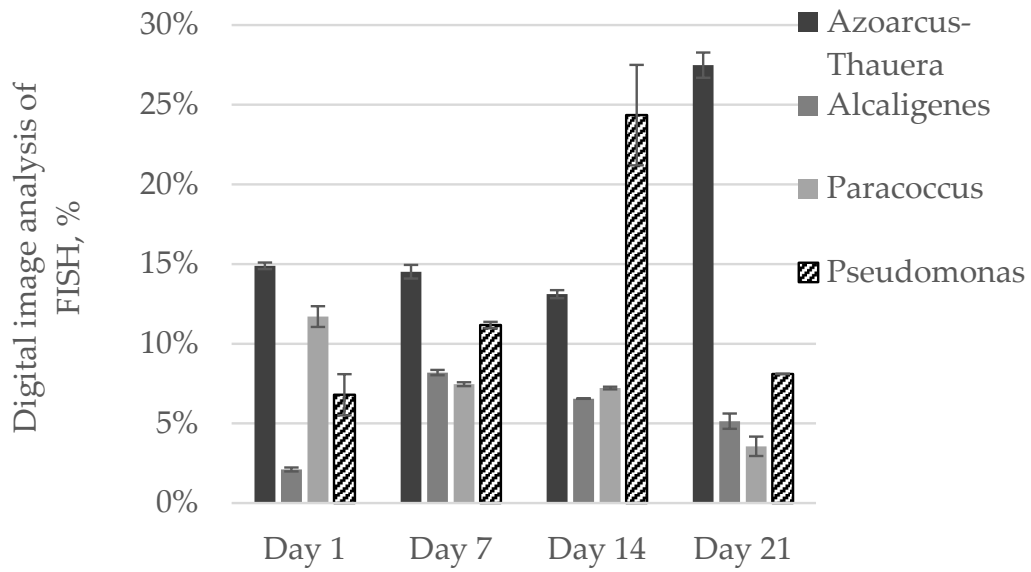

Figure 3. Digital image analysis of FISH images of samples at the four control points during the experiments.

The predominant portion of the community was occupied by bacteria from the *Azoarcus-Thauera* cluster (13–27%). Their average share for the process was 17.50%, which exceeded the other studied groups by 39% to 300%. The high proportion of these microorganisms was due to their ability to degrade toxic substances in landfill leachates. The gradually increasing concentration of the pollutants activated *Azoarcus-Thauera*, and their amount in the activated sludge rose nearly two-fold by the end of the model process and reached 27%.

Pseudomonas microorganisms exhibited a significant increase according to cultivation methods, which was confirmed by the data obtained from the in situ analyses. The proportion of *Pseudomonas* in the community ranged from 7% to 24% in the period 0 h–14th day. In the final stage, however, the proportion decreased to levels similar to those at 0 h (8%).

The FISH analyses also showed that g. *Paracoccus*, the key group for the detoxification and aerobic denitrification processes, decreased over the course of the model process from 12% to 4%. The bacteria from the g. *Alcaligenes* were activated during the model process of leachate treatment. At the end (21st day), this bacterial group increased its proportion by 2.4 times—from 2% at 0 h to over 5%.

The data from the sequencing analyses are presented in Figure 4. It can be seen that the proportion of *Alphaproteobacteria* was the largest one (32%). These ancient bacteria inhabit diverse niches and are well adapted to extreme conditions. Some of them are well-known biodegraders of xenobiotics, such as the bacteria of the genus *Paracoccus*, which were well represented in the activated sludge in the model SBR. These included *Paracoccus alkenifer* and unclassified *Paracoocus*, which were 4% of the established OTUs (operational taxonomic units). Additionally, the presence of the higher taxonomic group to which they belong, *Rhodobacteriaceae* (5%), was also observed. The bacterial group with the highest OTU share was the family *Rhizobiaceae* (13%). These organisms are common representatives of soil microbiomes. Since the leachate was generated in a closed-type landfill, there is a large amount of topsoil between the layers of waste to which these microorganisms had most likely adapted to.

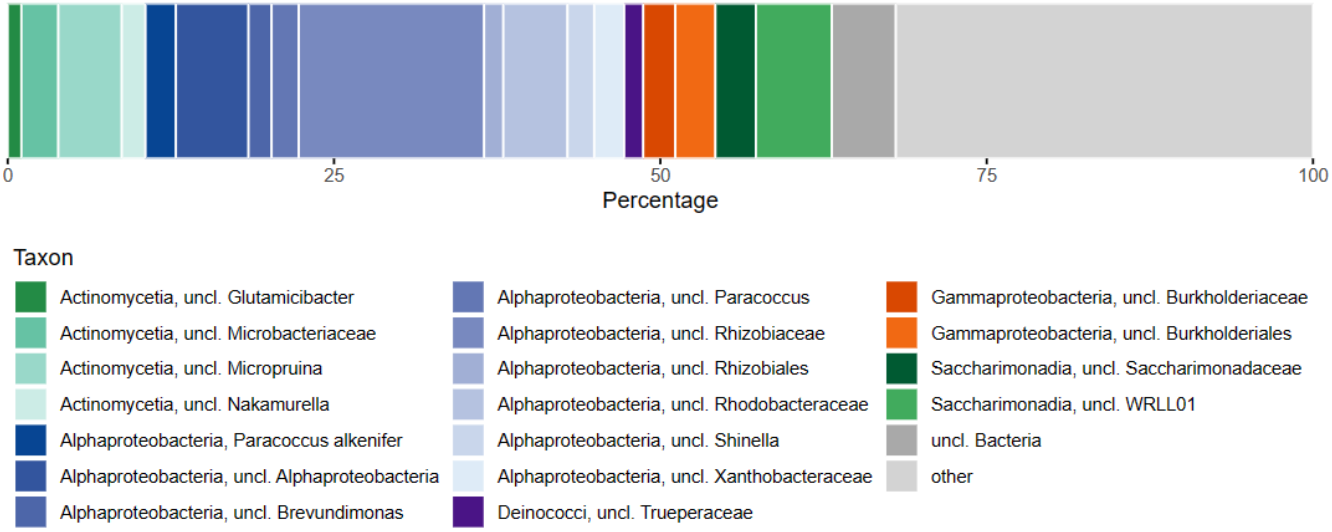

Figure 4. Metagenomic analysis of the activated sludge at the end of the model SBR treatment of landfill leachate (21st day).

Among the identified bacterial groups, the *Actinomycetia* class was represented by a significant percentage (10%). These microorganisms, which are also normal inhabitants of soils, include extremophiles that are capable of thriving in highly contaminated habitats, such as solid waste landfills and activated sludge leachate. Many of them form spores, which makes them particularly resistant to toxic influences. It is known that representatives of this class participate in the nitrogen cycle and, as shown in Table 3, the leachate used in the described experiments is extremely rich in different forms of nitrogen. The main representatives of this group were *Microbacteriaceae* (3%), *Nakamurella* (5%), and *Micropruina* (3%).

Gammaproteobacteria, which are known as the most active biodegraders of xenobiotics, were represented in 6% of the determined OTUs. They were identified as part of the order *Burkholderiales*. The bacteria in this group include xenobiotic-degrading and phosphate-accumulating bacteria. With the help of sequencing, their important role in the treatment of wastewater contaminated with toxicants is being increasingly recognized.

An interesting group found in 10% of the OTUs was the class *Saccharimonadia*. These microorganisms have never been isolated in pure cultures. They were discovered only thanks to sequencing methods. The reason for this is thought to be due to their incomplete biochemical pathways, which force them to exist only as symbiotic organisms. They are often associated with *Alphaproteobacteria* [42].

Figure 5 presents the results of the metagenomic analysis of the free-swimming cells found at the end of the model experiment. They appeared during the period of strong deterioration of the technological indicators. As mentioned, the presence of such cells was

detected microscopically and a metagenomic analysis was performed. In this segment of the biodegradation system, the main groups found in the sludge were *Actinomycetia*, *Alphaproteobacteria*, *Gammaproteobacteria*, and *Saccharimonadia*. *Actinomycetia* (23%) and *Alphaproteobacteria* were the most abundant (22%). *Microbacteriaceae* (13%) were found in the most OTUs. They are found in many soil and water habitats, but are not known to have particularly pronounced biodegradation properties. *Burkholderiaceae* were also found in a high percentage (8%), which was significantly higher than that recorded in the sludge (2%). Since these bacteria are known to be active biodegradants of xenobiotics, it is most likely that their multiplication within the free-swimming segment of the microbiome was related to the detoxification of pollutants. A significant share of the recorded OTUs were bacteria from the family *Rhizobiaceae*. They were most likely soil inhabitants of the landfill, and were adapted to the pollutants found there.

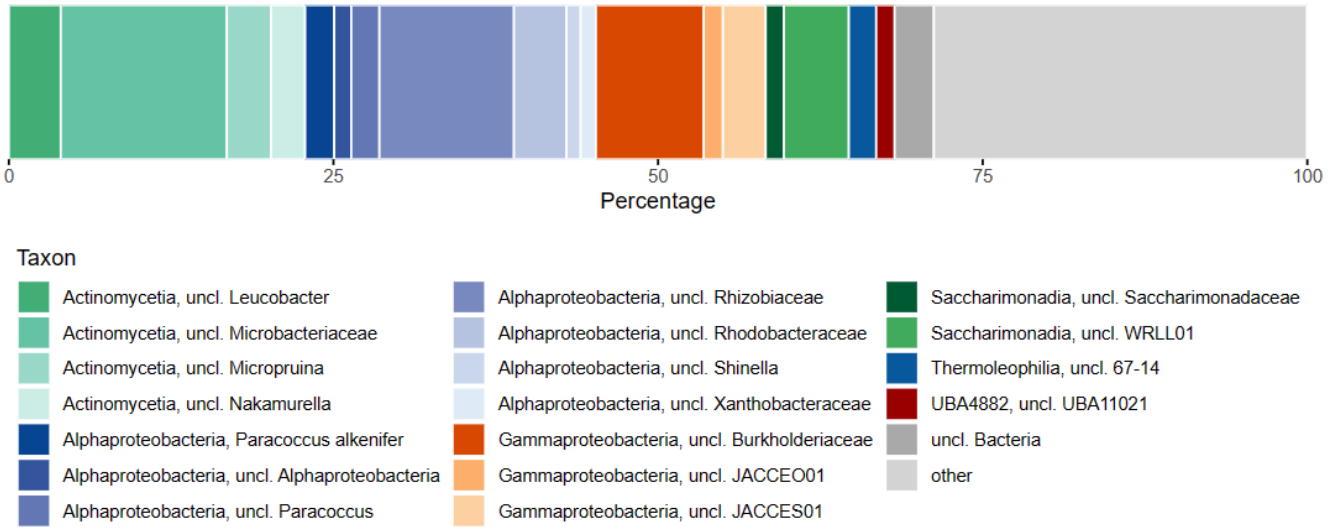

Figure 5. Metagenomic analysis of the free-swimming bacteria in the activated sludge at the end of the model SBR treatment of landfill leachate (21st day).

Also, among *Actinomycetia*, *Nakamurella* (4%), *Micropruina* (3%), and *Leucobacter* (4%) had significant OTU proportions. They are known to participate in the biodegradation of carbon-containing pollutants. The family *Rhodobacteraceae* was also registered with 4% of the OTUs, pointing to the key role of *Paracoccus* as its representative, *Paracoccus alkenifer*, constituted 2% of the OTUs.

Saccharimonadia was found as a considerable proportion (6%) of the free-swimming cells, indicating the development of a complex symbiont structure not only within the activated sludge but also among the free-swimming bacteria.

In addition to the taxonomic structure, analyses were also performed to determine the biodegradation potential of the landfill leachate community. The obtained data showed that at the beginning of the model process in the community, there were two pathways for the degradation of aromatic pollutants: an ortho-pathway through catechol metabolites (catechol-1,2-dioxygenase activity was 0.82 μg H+/mL/min/mgP) and an ortho-pathway through protocatechuate metabolites (protocatechuate-3,4-dioxygenase activity was 0.87 μg H+/mL/min/mgP) (Figure 6). After 48 h, in the conditions with reduced toxicity, the total dioxygenase activity (sum of the activity of the three tested oxygenases) decreased by 39%. At the same time, a decrease in COD was recorded, mostly related to the increased activity of the general metabolism of the cells and the accumulation of biomass in the system. The TDA and protein concentration per ml of mixed liquor increased 3-fold.

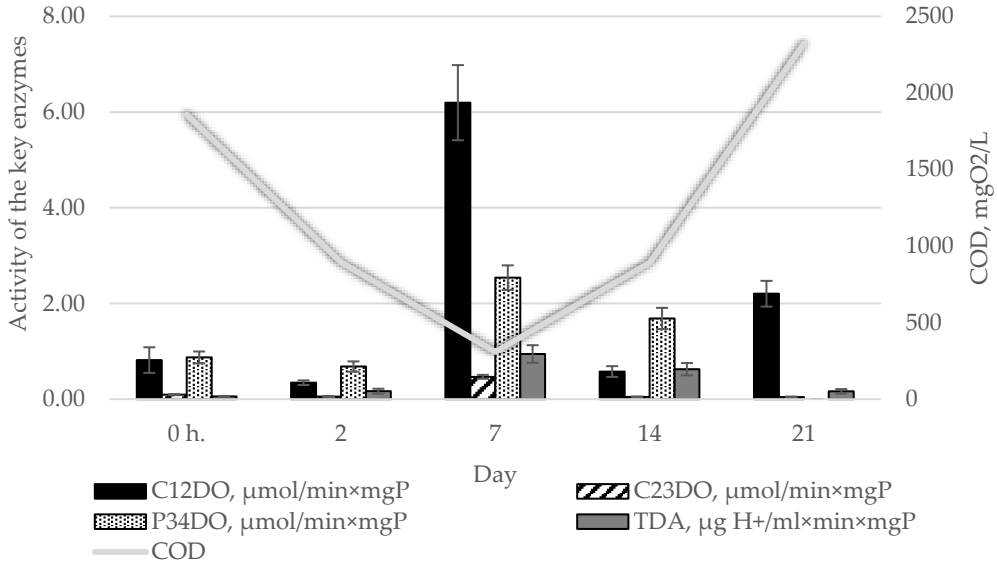

Figure 6. Activity of the key detoxifying enzymes (C12DO—catechol-1,2-dioxygenase; C23DO—catechol-2,3-dioxygenase; P34DO—protocatechuate-3,4-dioxygenase; TDA—total dehydrogenase activity) and the remaining pollutants which was measured as COD.

The highest dioxygenase activity was recorded on day 7 when the filtrate was diluted by 50 times (3.07 µg H+/mL/min/mgP). The highest dehydrogenase activity was also found at the same time point (0.94 µg H+/mL/min/mgP). This result corresponded to the release of the toxic pressure on the biological system and the activation of its biodegradation potential. At the end of the model process, at the highest concentration of pollutants, a C12DO activity of 2.2 µg H+/mL/min/mgP was recorded, which exceeded the initially demonstrated one (at 0 h) by 2.7 times (Figure 6). This was indicative of the community's adaptation and specialization toward the biodegradation of cyclic xenobiotics through the most active pathway in the community—using the key enzyme C12DO. The average activity for this enzyme was 14 times higher than that of C23DO and 75% higher than that of P34DO.

The high COD recorded at the end of the process was related to the inhibition of the general metabolism of the biodegradants; the TDA was reduced by 4 times, which was also accompanied by destructive changes in the structure of the activated sludge.

4. Discussion

Studies of a biological system treating increasing concentrations of leachate in a model SBR revealed a complex picture of an initial adaptive response. Despite significant deterioration in purification activity based on the technological indicators (Table 3), a detailed examination of the bacterial community showed an increase in the groups with a high biodegradation potential. The registered SVI of only 4.6 mL/g at the end of the process was related to the overall restructuring of the community—destruction of flocs and multiplication of active biodegradants that had found a suitable substrate for their development. Therefore, the multiplication of free-swimming bacteria up to 2.2×10^7 CFU/mL was also found. They accounted for 71% of all cells found per mL of the mixed liquor. Among them, bacteria from the xenobiotic-degrading groups—*Pseudomonas* (5.3×10^4 CFU/mL) and *Acinetobacter* (1.7×10^5 CFU/mL)—were well represented. Through the metagenomic analysis, *Actinomycetia* and *Alphaproteobacteria* were found to predominate in this segment of the biological system. Of the former, *Microbacteriaceae* (13%), *Nakamurella* (4%), *Leucobacter* (4%), and *Micropruina* (3%) were represented in the largest percentages. *Microbacteriaceae* are known to be a diverse group that occurs widely in soil [43] and aquatic [44] habitats. Therefore, they are likely to be found to a significant extent in leachate-treated samples as well. This type of wastewater is formed by the infiltration of water through landfills, which

inevitably trains the landfill's soil microorganisms. They are adapted to the pollutants emitted by the waste at the particular site. They are then successfully incorporated into the leachate treatment communities. A similar result was also found by González-Cortés et al. [45], who observed an increase in the same bacterial group after the adaptation of activated sludge to leachate treatment. The *Leucobacter* group was also identified as a key group by other scientific teams. Remmas et al. [46] demonstrated that this was the dominant group (41%) in the microbiome of leachate from a solid waste landfill. A study by Zou et al. [47] demonstrated that the amount of these organisms increased with a gradual increase in the leachate concentration in a model process, as *Leucobacter* is believed to have a high resistance to heavy metals, especially chromium. Bacteria of the *Nakamurella* group are little studied, but what is known about them is that they are capable of degrading large amounts of carbon-containing pollutants [48]. A high COD most likely favors the development of these organisms in the homogeneous phase of the system. Of interest were bacteria from the *Micropruina* group, which were found as a significant part of the microbiome of both the free-swimming microorganisms and sludge. They belong to the group of glycogen-accumulating organisms (GAOs) [49]. A high concentration of phosphates (25 mg/L) was detected in the effluent on the 21st day, which exceeded the initial concentration by 61 times. This was likely due to GAOs, which are competitors of phosphate-accumulating bacteria [50]. Their presence is often associated with a low phosphate elimination efficiency. Kong et al. showed such a role for *Micropruina* in a model SBR [51].

A significant proportion of *Alphaproteobacteria* was found both in the sediment (32%) and in the single, free-swimming bacteria in the water layer (22%). Different species of the genus *Paracoccus* were found in both fractions of mixed liquor (4% each). These bacteria were also detected by the FISH analysis (4%). Their important role in the biodegradation of xenobiotics, which are abundant in the leachate, is known [52,53]. Also, they were identified by Zheng et al. [54] as bacteria capable of aerobic denitrification and heterotrophic nitrification, which makes their development in waters rich in ammonium ions and xenobiotics very successful.

Rhizobiaceae had a high share of the identified OTUs—14% in the sediment and 10% in the homogeneous phase of the samples. Although bacteria from this taxonomic group are known as plant symbionts [55], they are also highly active biodegraders of various xenobiotics [56,57]. This is a prerequisite for their establishment in the community treating infiltrate.

Through the FISH analysis, the presence of key groups of the *Betaproteobacteria*, *Azoarcus-Thauera* cluster and *Alcaligenes* spp., was detected. The *Azoarcus-Thauera* cluster reached a significant 27% of the community, which was an indication of their leading role in contaminant purification. The role of g. *Azoarcus* in the biodegradation of xenobiotics in landfill leachate is known. Sun et al. showed their dominant role in the bioremediation of solid waste landfills [58]. The bacteria in g. *Thauera* are known as biodegraders of toxic substances, but they also belong to the group of aerobic denitrifying microorganisms [59]. The role of this group of organisms was important in the model treatment process in which aerobic conditions were maintained. Our results are also supported by Remmas et al. [60] who demonstrated a shift in the dominant group from *Gammaproteobacteria* to *Betaproteobacteria* when treated with glycerol-supplemented leachate.

The FISH analysis of *Gammaproteobacteria* was focused on the key group of bacteria of the genus *Pseudomonas*. In the course of the purification process, their proportion reached 24% (on 14th day), but in the end, at the highest applied concentrations, these microorganisms decreased 3-fold. This was an indication of the reduction in their biodegradation role in the final stage with high intoxication. These data supported the results for the metabolic activity of the community. Bacteria of the genus *Pseudomonas* have a proven high dioxygenase enzyme activity, using catechol and protocatechate as substrates [61]. Thus, at the initial activation of the genus on day 7 (increase in CFU/mL by 2.3 times) and at low concentrations of landfill leachate, the maximum activity of the three investigated dioxygenases was also detected (Figure 6).

The cultivation methods showed an increase in *Pseudomonas* sp. on the 21st day compared to the 14th day which is a 5-fold increase. However, a more detailed analysis showed that the bacteria of this genus reached 1.07×10^7 CFU/mL on day 17, after which, the number fell by 9-fold due to inhibition of this group of bacteria. The lower amount of *Gammaproteobacteria* was also observed in the metagenomic analysis results. These bacteria, generally considered the main bacteria leading the biodegradation of organic pollutants in the leachate [62,63], only accounted for 6% of the OTUs in the sludge and 12% in the suspended portion. In this final stage of the process, a decrease in the activity of dioxygenase enzymes characteristic of *Gammaproteobacteria* was also recorded. At the highest degree of toxicity, in aerobic conditions, *Actinomycetia*, *Alphaproteobacteria*, and some representatives of *Betaproteobacteria* acquire leading importance.

The other major group found in the leachate-treating activated sludge were representatives of *Saccharimonadia* (9%). Although bacteria of this class have been very little studied, more and more investigations are being published showing the presence of *Saccharimonadia* populations in reactors treating toxic wastewater and landfill leachate [64–66].

5. Conclusions

The obtained data in this research showed that with an increase in toxicity, the system undergoes disintegration changes (SVI—5 mL/g) and a decrease in the efficiency of the purification processes (COD—2321 mgO_2/L). At the same time, a segment of very active bacteria appeared, a large part of which floated freely outside the structure of the activated sludge. The number of aerobic heterotrophs and bacteria of the genera *Pseudomonas* and *Acinetobacter* increased by up to 125 times. In this stage, microorganisms from the groups *Actinomycetia*, *Alphaproteobacteria*, *Betaproteobacteria*, and especially the representatives of the genera *Paracoccus*, *Azoarcus*, and *Thauera*, and the families *Rhizobiaceae*, *Microbacteriaceae*, and *Saccharimonadaceae* played a key role. The ortho-pathway of cleavage of aromatic structures was found to be active in the community. The obtained results demonstrated that in the period when the treatment process was seemingly inefficient, dynamic changes in the microbiome structure took place in terms of increasing the overall bacterial numbers and especially the bacteria involved in xenobiotic degradation.

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