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Purposely Development of the Adaptive Potential of Activated Sludge from Municipal Wastewater Treatment Plant Focused on the Treatment of Landfill Leachate

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Abstract: Biological treatment is a key technology in landfill leachate treatment However, often its efficiency is not high enough due to the pollutants in concentrations above the critical ones. The present study aimed to investigate the adaptive responses that occur in activated sludge (AS) during landfill leachate purification. A model process with AS from a municipal wastewater treatment plant and landfill leachate in increasing concentrations was constructed. The data showed that when dilutions 25 and 50 times had been applied the structure of the AS was preserved, but the COD cannot be reduced below 209 mg O_2/L. The feed of undiluted leachate destroyed the AS structure as SVI was reduced to 1 mL/g, biotic index to 1, floc size was greatly reduced and COD remained high (2526 mg O_2/L). The dominant group of protozoa was changed from attached to free-swimming ciliates. An increase of the bacterial groups responsible for the xenobiotics elimination (aerobic heterotrophs, genera *Pseudomonas*, *Acinetobacter*, *Azoarcus*, *Thauera*, *Alcaligenes*) was registered. This was accompanied by a significant increase in free bacteria. The obtained data showed that for optimal treatment of this type of water it is necessary to include a combination of biological treatment with another non-biological method (membrane filtration, reverse osmosis, etc.).

Keywords: landfill leachate; xenobiotics; adaptation; activated sludge; micro- and metafauna; free-swimming ciliates; *Pseudomonas*; *Acinetobacter*

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Citation: Belouhova, M.; Yotinov, I.; Schneider, I.; Dinova, N.; Todorova, Y.; Lyubomirova, V.; Mihaylova, V.; Daskalova, E.; Lincheva, S.; Topalova, Y. Purposely Development of the Adaptive Potential of Activated Sludge from Municipal Wastewater Treatment Plant Focused on the Treatment of Landfill Leachate. *Processes* **2022**, *10*, 460. https://doi.org/10.3390/pr10030460

Academic Editor: Davide Dionisi

Received: 31 January 2022
Accepted: 21 February 2022
Published: 24 February 2022

1. Introduction

The global production of municipal solid waste (MSW) has been growing on a huge scale over the last few years. The world generation of MSW is estimated at 2.01 billion tons annually and is expected to reach the value of 3.40 billion tons in 2050 despite all efforts to reduce this growth [1]. The collection and processing of these enormous quantities of waste are one of the biggest management challenges everywhere in the world. Although that disposal and treatment is the less favorable option in the waste hierarchy and circular economy action plans, landfilling remains the most widely used solution in management strategies due to its low cost and easy maintenance [2–6]. One of the serious environmental threats from the operation of landfills is the production of leachate—highly concentrated organic and nitrogen-rich liquid with complex composition. The landfill leachate is a consequence of water infiltration, percolating through the waste, and different biotransformation processes that take place in landfills (aerobic hydrolysis and biodegradation, anaerobic hydrolysis and fermentation, acetogenesis, methanogenesis) [7–9]. The chemical content and volume of leachate can vary greatly depending on waste composition, landfill age, the technology used in landfill, phase of transformation processes, climate, and seasonal specifics. 50–200 kg leachate is generated on average from one ton of MSW [4]. The typical leachate contains a high concentration of organic matter (a wide range of easy biodegradable and recalcitrant compounds), nutrients with a predominance of ammonia nitrogen, inorganics (heavy metals and salts), acids, and chlorinated compounds. Table 1 presents a summary of leachate chemical characteristics according to the age of the landfill [7,9,10].

Table 1. Typical chemical characteristics of leachate from young and old landfills [7,9,10].

	Concentration in Recent Landfill Leachate (Age < Five Years)	Concentration in Old Landfill Leachate (Age > Five Years)
pH	6.5	>7.5
COD, mg O_2/L	>10,000 (20,000 ÷ 40,000)	<4000
BOD_5, mg/L	>5000 (10,000 ÷ 20,000)	<1000
BOD_5/COD	>0.3	<0.1
TOC, mg/L	1500 ÷ 15,000	100 ÷ 1000
Organic compounds	80% volatile fatty acids	humic and fulvic acids
Total Nitrogen, mg/L	200–10,000	200–1000
C/N ratio	>1.8	1

COD—chemical oxygen demand; BOD—five-day biochemical oxygen demand; TOC—total organic carbon; C/N—carbon/nitrogen ratio.

The specific problem associated with this highly concentrated and complex composition of leachate is the presence of toxic components. An unknown number of hazardous substances with different chemical structures and variable concentrations can be found in leachate. Many studies identify a high diversity of xenobiotic compounds—hydrophobic aliphatic and aromatic organic substances (benzene, toluene, ethylbenzene and xylenes—BTEX), polyaromatic hydrocarbons, toxic metals, phenols, phthalates, pesticides, microplastics, polyethylene, plasticizers, halogenated organic compounds like PCBs (polychlorinated biphenyls) and dioxins, PFAS (per-and polyfluoroalkyl substances) [11–14]. These dangerous substances have high synergetic toxicity and their release into the environment represents a serious ecological threat. Currently, each landfill management plan provides as an obligatory element, the different variants for leachate treatment and removal of toxic compounds. Many conventional technologies from the classic wastewater treatment portfolio are adapted to the specifics of leachate pollutant loading and different innovations are in development for the improvement of removal effectiveness. One summary of processes/methods used for leachate treatment is presented in Figure 1 [9,15–17].

In this variety of different processes and technological arrangements, one of the widely used, simple and cost-effective solutions is based on biological methods, applied as a standalone technology or in combination [4,6,7,18]. Principally, there are two main strategies for the biological treatment of leachate. The first strategy is on-site use of engineering facilities specially designed for effective removal of recalcitrant pollutants, for example—hybrid airlift bioreactors, up-flow anaerobic sludge bed reactors, baffled reactors, sequencing batch reactors, anaerobic migrating blanket reactors, and submerged and recirculated membrane bioreactors [19–23]. The biological systems used in these technologies are highly specialized for effective utilization of recalcitrant carbon and nitrogen fractions in different detoxification, biodegradation, denitrification/nitrification, Anammox/nitritation and other processes [4,24].

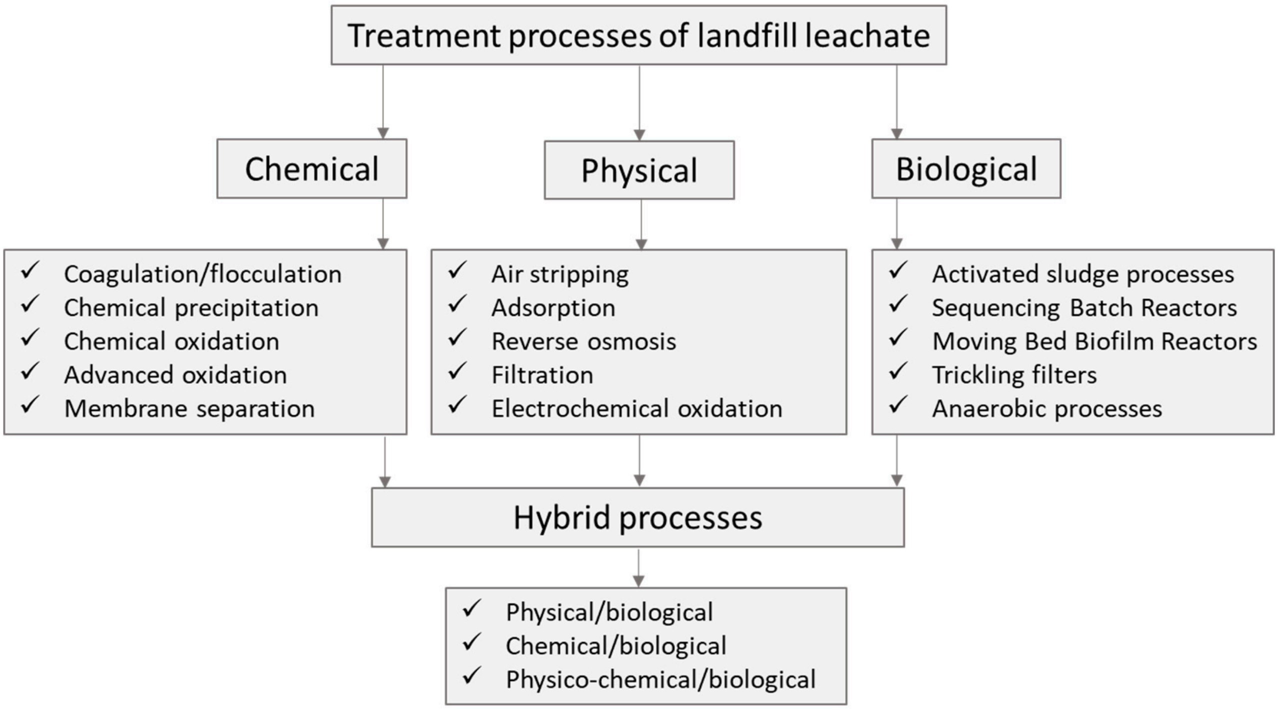

Figure 1. Treatment processes of landfill leachate.

The second strategy is based on co-treatment with other types of wastewaters (domestic, food) in common activated sludge processes [25–28]. The co-treatment has obvious economic advantages—use of existing wastewater treatment facilities, easy maintenance and low operating costs. But it should be taken into account that due to the toxicity of the leachate, an obligatory prerequisite for this type of treatment is the addition of the leachate in a volumetric ratio of 5–10% to the wastewater [9,25]. Despite achieving a high COD removal effectiveness (up to 90%) and sufficient nitrogen removal in many case studies, the co-treatment mode has serious concerns and put important questions about the fate of recalcitrant compounds and whether it is real biodegradation or simple dilution [28,29]. In this context, it is clear that the successful co-treatment of the complex mixture of hazardous constituents in leachate requires a preliminary assessment of the biodegradation potential of activated sludge and specialized assistance for the improvement of its biodegradability [30]. One of the ways to achieve this is by gradually increasing the ratio of leachate to wastewater, which will allow the adaptation of the biological system to the specific pollutants and the monitoring of adaptive changes.

Regarding the adaptation process of activated sludge from municipal treatment plants to leachate biodegradation, there is relatively little information in the literature. Most studies discuss variations in the technological and chemical parameters during the process of co-treatment but there is not enough data on adaptive changes in the structure of activated sludge and how these changes are related to the effectiveness of the process. Generally, the input of xenobiotic compounds in the common activated sludge process restructures the microbial communities and fauna complex—the genera *Pseudomonas* and *Acinetobacter* increase their quantitative share, the attached and crawling ciliates reduce their numbers, the abundance of flagellates and the free-swimming ciliates also increases [31,32]. There are two scientific and applied issues that are addressed in this article: where are the adaptation limits of AS as an important synergistic biological system to purify water biologically in the actual facility of the Municipal Enterprise for Waste Treatment and the second question is at what values of the treatment process and AS parameters other modules must be included to implement the hybrid algorithm to completely eliminate degradable, non-degradable, and toxic pollutants.

The current research aimed to assess the adaptive changes of activated sludge from municipal wastewater treatment plant towards the treatment of landfill leachate. The study presents a complex assessment of chemical, technological and biological parameters of activated sludge in the simulated adaptation process with a step-by-step increase of the landfill leachate.

2. Materials and Methods

2.1. Experimental Design

The analogous modeling approach was used to study the adaptive changes in the activated sludge.

The model adaptation process was carried out in specially designed laboratory reactors of the type of sequencing batch reactors (SBR), with the following phases: filling phase, reaction phase, precipitation phase, decantation phase, and rest phase, after which the new purification cycle starts (Figure 2). The working volume of the reactor was 4 L. In the model SBR, the reaction phase was aerobic with an oxygen supply maintaining a DO of about 3 mg/L. The experiments were performed at ambient temperature (approx. 22 °C). It is known from the literature that xenobiotics with aromatic structures, which are also found in landfill leachate, undergo detoxification and mineralization at a higher rate and efficiency under aerobic conditions compared to anoxic or anaerobic conditions [33,34]. The reason for this is that the main enzymes of detoxification, the oxygenases—strongly depend on the presence of oxygen. They include directly molecular oxygen in the molecules of the toxic pollutants. The duration of the aerobic phase was 46 h, followed by the sedimentation phase lasting 2 h. The choice of the reactor and the conditions of the treatment process of the landfill leachate were as close as possible to the conditions under which it takes place in the real facility—the Municipal Enterprise for Waste Treatment (Sofia), from where the landfill leachate was taken for the present study.

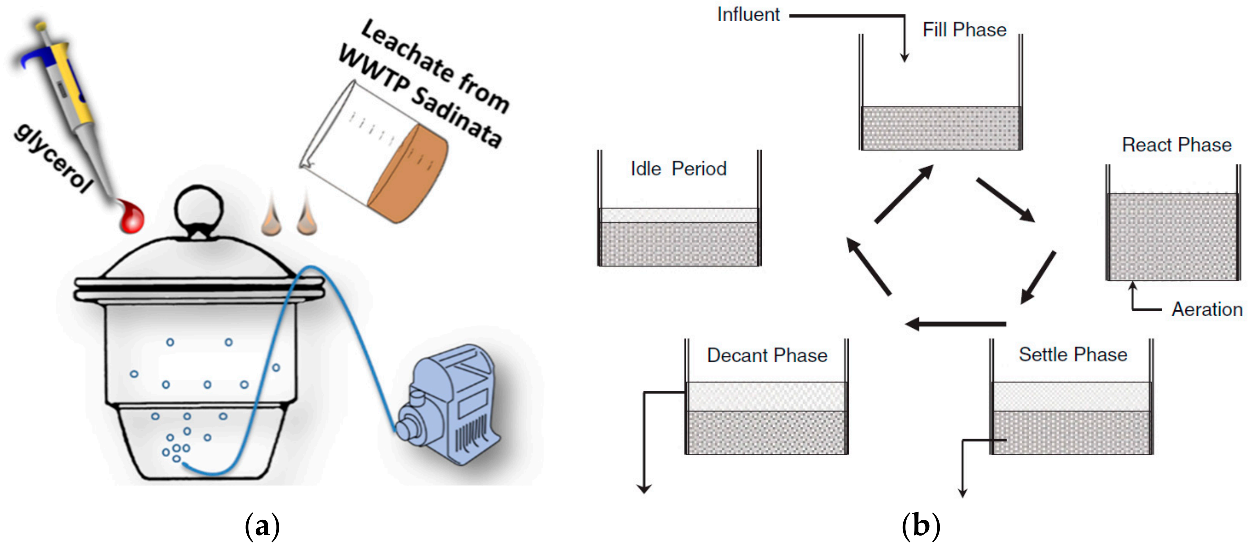

Figure 2. Model sequencing batch reactor (**a**) and phases of functioning (**b**).

The experiments were carried out with activated sludge taken from the Kubratovo wastewater treatment plant, which mainly treats municipal wastewater in Sofia, and to a lesser extent industrial wastewater in the industrial enterprises located in the area. The purification there is on the principle of denitrification/nitrification, with intensive elimination of phosphorus. The activated sludge was taken from the denitrification zone of the aeration tank. The concentration of activated sludge at the start of the experiment was 3 g/L, measured as dry matter.

Model experiments included a gradual adaptation of the activated sludge, for which purpose the concentration of landfill leachate was increased step by step. The duration of the modeled processes was three weeks or twenty-one days (Figure 3). The processes started with a dilute amount of the landfill leachate (×50) (1433.3 mg O_2/L). The leachate concentration increased each week. Glycerol (0.79 g/L) was used as a source of easily degradable organic matter. On the seventh day from the start of the experiment, ×25 diluted landfill leachate was added, and on the 14th day, undiluted landfill leachate was added. Thus, the experiment was divided into three stages (Figure 3), which differed in the added leachate portion. During these steps, the process parameters changed as described below, and adaptive changes in the activated sludge were induced. They were monitored and diagnosed with a complex indicator approach described below.

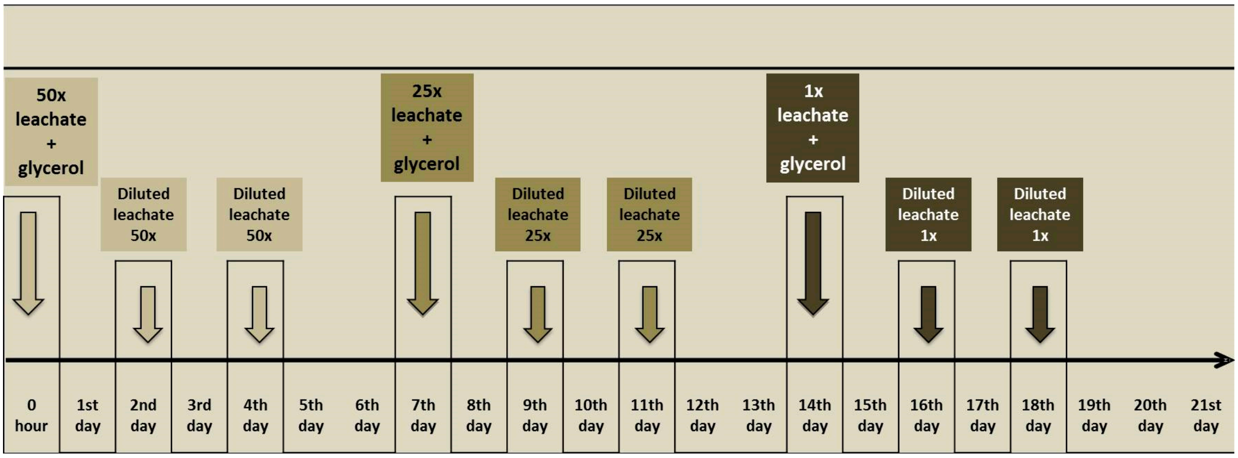

Figure 3. Duration of the modeling process and stages of purification with the addition of a new landfill leachate.

The first stage of the experiment took place from day zero to day six, and at the 0 hour, a 50 times diluted infiltrate and co-substrate glycerol was added to the activated sludge. The co-substrate was added until the ratio of COD:BOD 3:1 was reached, as it is done in the WWTP to Municipal Enterprise for Waste Treatment. Samples were taken for analysis of technological, hydrochemical, and biological indicators. On the 2nd and 4th days, the landfill leachate was replaced with a fifty-fold diluted landfill leachate, but without added glycerol co-substrate.

The second stage of the experiment lasted from the 7th day to the 13th day. On the 7th day, 25-fold diluted landfill leachate and glycerol co-substrate were added (COD was 1481.8 mg O_2/L). On the 9th and 11th days, the landfill leachate was replaced with a new one with a dilution of twenty-five times without the addition of glycerol.

The third stage of the experiment covered the days from the 14th to the 21st. On the 14th day, undiluted landfill leachate and co-substrate glycerol (3807.9 mg O_2/L) were added. Samples were taken before the addition of the new portion of the landfill leachate and glycerol. Technological, hydrochemical, and biological parameters were examined. On the 16th and 18th days, the landfill leachate infiltrate was replaced with a new one, but undiluted and without the addition of glycerol. Samples from these days were taken for technological and hydrochemical indicators.

2.2. Chemical and Technological Analyzes

The dry matter and sludge volume index (SVI) were studied. The latter provides information on structural deformations of the AS. The index was determined according to BDS EN 14702-1:2006 and represents the volume in ml, which occupies 1 g of sediment after 30 min of precipitation. The dry matter for the calculation of SVI was determined by a standardized weighting method [35].

The protein content of the samples from mixed liquor in the model SBR was determined by the microbiuret method [36]. The protein concentration was expressed as mg proteins per mL of the mixed liquor in the reactor. Ultrasonic disintegration with microscopic control of the sample destruction was applied for disruption of the flocs and biomass. A UD-20 automatic Techpan ultrasonic disintegrator was used.

The chemical oxygen demand (COD) was studied also. It provides information about the content of organic matter in the landfill leachate and its elimination during the process. COD was determined by a standardized spectrophotometric method [35]. Biochemical oxygen demand (BOD5) is an indicator of the concentration of biodegradable organic matter. This indicator was analyzed by the method described in BDS EN ISO 5815-1:2019 with the addition of allylthiourea to inhibit nitrification.

The metal absorption by the activated sludge (AS) was assessed by the determination of the chemical composition of the initial leachate and the effluent after 48 h contact with the activated sludge (AS).

The evaluation of the AS adaptation to the leachate and saturation with metals was conducted by determination of the composition of a leachate added at the 18th day of the experiment and the effluent at the last 21st day of the experiment (after 72 h contact with the AS).

To minimize the effect of matrix interferences caused by the high and varying organic content in the samples, the influent and effluent samples were subjected to acid digestion. An aliquot of 30 mL of each sample was transferred to a beaker and was digested with 20 mL HNO_3 (67–69%, Fisher Chemicals, TraceMetal Grade) and 10 mL H_2O_2 (30% Fisher Chemicals, Trace Analysis Grade) by heating on a hotplate. The heating continued until transparent solutions were obtained and the volume was reduced to 0.5–1 mL. Next, the samples were quantitatively transferred to polypropylene tubes by rinsing several times with deionized water and diluted to 30 mL. Three parallel samples were prepared from each leachate and effluent.

The chemical analyses of the samples were carried out using ICP-MS (Perkin-Elmer SCIEX Elan DRC-e) with a cross-flow nebulizer. External calibration by multi-element standard solution was performed. The concentrations of the elements were determined using the isotopes as follows: macroelements (^{27}Al, $^{136,\,138}Ba$, $^{42,44}Ca$, $^{54,57}Fe$, ^{39}K, $^{24,25,26}Mg$, ^{55}Mn, ^{23}Na, ^{31}P and ^{28}Si) and the micro- and trace elements ($^{107,109}Ag$, ^{75}As, ^{209}Bi, $^{110,\,112,\,114}Cd$, ^{59}Co, ^{52}Cr, ^{133}Cs, $^{63,65}Cu$, ^{200}Hg, ^{139}La, $^{96,98}Mo$, $^{60,62}Ni$, $^{204,208}Pb$, $^{85,87}Rb$, $^{121,123}Sb$, $^{77,78}Se$, $^{118,120}Sn$, $^{86,88}Sr$, $^{46,48}Ti$, ^{232}Th, ^{238}U, ^{51}V and $^{64,66}Zn$). The simultaneous determination of all elements in the samples was achieved by reducing the signal of the macroelements thanks to the application of a dynamic bandpass tuning parameter RPa, as described in Lyubomirova [37]. The estimation of accuracy was done by the analysis of wastewater CRM (Landfill leachate—trace metals LGC6177).

Specific qualitative reactions were used to establish different classes of organic compounds. The presence of polyhydric alcohols was performed with freshly prepared $Cu(OH)_2$, which was added to the test sample. In the presence of polyhydric alcohols, a complex is formed which imparts an inky blue color to the solution. $FeCl_3$ was used to detect phenols. If there are phenols in the test sample, a violet color is obtained. A demonstration of short-chain monovalent alcohols was performed using iodine tincture, heating and then NaOH was added to discolor the solution and form yellow crystals of CHI_3. The detection of aldehydes was performed with Fehling's solution and Tolens reagent. In the first case, the so-called "Copper mirror" $CuSO_4$ and NaOH were added to the sample. Initially, a fluffy precipitate forms which dissolves after shaking. The resulting blue solution was heated. In the presence of an aldehyde group, the characteristic orange color of the obtained Cu_2O was observed. The Tolens reagent is a silver-ammonia complex. In case of aldehyde in the sample and when the water bath is heated, the "silver mirror" process is carried out, i.e., elemental silver is released. Bromine water and chlorinated lime were used to prove aromatic amines. In the presence of aromatic amines in the sample, discoloration of the bromine water and the formation of a white precipitate of 2,4,6-tribromoaniline was observed. On the other hand, when interacting with chlorinated lime, aromatic amines give a red-violet complex.

2.3. Microbiological Analyzes

Quantitative determination of microorganisms was performed by culturing on solid nutrient media according to the routine microbiological practice [38]. The studied groups of microorganisms, the nutrient media for their isolation, and the cultivation conditions are listed in Table 2.

Table 2. Studied groups of microorganisms, nutrient media, and culture conditions.

Microbiological Parameter	Nutrient Media	Manufacturer	Incubation
Aerobic heterotrophs (AeH)	Nutrient agar	HiMedia	24 h, 28 °C, aerobic
p. *Pseudomonas* (Ps.)	Glutamate Starch *Pseudomonas* Agar	HiMedia	24 h, 28 °C
p. *Acinetobacter* (Ac.)	Sellers Differential Agar	HiMedia	24 h, 28 °C

Microbiological analyzes were performed on activated sludge samples after pretreatment with a UD-20 automatic Techpan ultrasonic disintegrator, in triplicate for 10 s. The obtained results were represented as colony forming units per gram dry weight (CFU/g).

Microscopic analyzes were performed according to the rules of standard routine practice [39]. The identifiers of Sladka & Sladecek [40] and Foissner & Berger [41] were used to study micro- and metafauna organisms. The determination of the biotic index of AS was carried out by the method of Madoni [42].

The fluorescence in-situ hybridization analyses were performed according to Nielsen [43]. The samples were collected in the 0th hour, 7th day, 14th day, and 21st day of the model process and were fixed according to Amann [44]. The used oligonucleotide probes are described in Table 3. Key bacterial groups were studied with FISH—Anammox, *Pseudomonas* spp., *Paracoccus* spp., *Alcaligenes* spp., cluster *Azoarcus-Thauera*. The probe NON-EUB was used as a negative control. The samples were counterstained with DAPI.

Table 3. Oligonucleotide probes used in the experiments.

Target Group	Probe	Sequence	Reference
Alcaligenes spp.	ALBO577	CCG AAC CGC CTG CGC AC	[45]
cluster *Azoarcus-Thauera*	AT1458	GAA TCT CAC CGT GGT AAG CGC	[46]
Pseudomonas spp.	Ps	GCT GGC CTA GCC TTC	[47]
Domain *Bacteria* (EUB mix)	EUB338	GCT GCC TCC CGT AGG AGT	[48]
	EUB338 II	GCA GCC ACC CGT AGG TGT	[49]
	EUB338 III	GCT GCC ACC CGT AGG TGT	[49]
Non-specific	NON-EUB	ACT CCT ACG GGA GGC AGC	[50]

Digital image analysis was performed on the fluorescence images from FISH. The software *daime 2.2* (University of Vienna, Vienna, Austria) was used for the purpose [51]. The part of the community of each of the studied bacterial groups was calculated on the base of the images of the DAPI staining. The threshold of the segmentation was chosen manually.

All the analyses were made in three independent repetitions. The results and standard deviations were calculated with the software products MS Excel (Microsoft Corp., Redmond, WA, USA) and Sigmaplot 11 (Systat Software Inc., San Jose, CA, USA).

3. Results

The study monitored two groups of parameters—residual amounts and type of pollutants in the landfill leachate and the reaction of activated sludge in the process of the adaptation and elimination of pollutants.

The effectiveness of metal absorption and removal in the course of the model process is demonstrated in Figure 4. The measured elements were presented in descending order of the element removal. The experimental results were in very good agreement with the certified values.

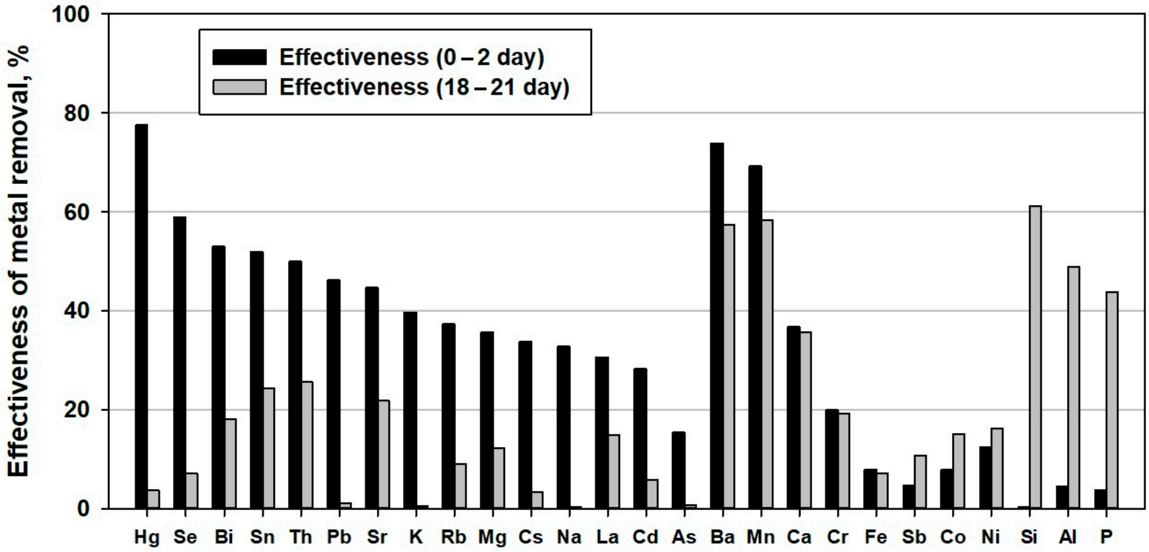

Figure 4. Effectiveness of metal removal at the first two days and the last three days of the process.

The data indicated that the degree of removal varied from 0.4% (for Si) to 77.6% (for Hg) in the first stage and from 0.36% (for Na) to 61% (for Si) in the second stage.

According to the degree of metal removal, three groups of elements can be identified. The first group comprises the elements starting from Hg to As, in which, regardless of the initial element concentration in the influent, metal removal was achieved predominantly in the first stage in the range from 15% to 77.6%. The reduction in concentration in the second stage was negligible, especially for the macroelements Na, K and the potentially toxic elements As, Pb, Hg and Cd.

The comparison of the efficiency of metal removal in the two stages showed different tendency for the elements Ba, Mn, Ca, Cr, Fe, Sb, Co and Ni for which comparable percentages were established in the two stages varying in the interval from 5% to 74%.

Of great interest were the elements of the third group (Si, P and Al), for which a reduction of the concentrations from 44% to 62% was observed in the interval 18th to 21st day. The significant decrease in the concentration of these elements at the end of the experiment could be explained by their absorption due to the sharp increase of the quantity of the activate sludge after the 17th day of the experiment. For the rest of the elements (Ag, Cu, Mo, Ti, U, V and Zn), no decrease in the concentrations was observed during the experiment.

The results of the qualitative organic analysis showed that all analyzed classes of organic compounds were present in the samples of the influent at the beginning of the experiment and on the 18th day (Table 4). In the sample from the first treatment cycle (second day), low molecular weight and aromatic amines, short-chain monovalent alcohols and aldehydes were not detected, except for very small amounts of phenols and ketones.

In the effluent from the last treatment cycle (21st day), a positive reaction was obtained for short-chain monovalent alcohols, phenols, aldehydes and ketones after concentration of the sample.

Table 4. Results of the conducted qualitative analysis for the presence of different classes of organic compounds in wastewater samples.

Sample	Low Molecular Weight Amines	Aromatic Amines	Short-Chain Monovalent Alcohols	Polyhydric Alcohols	Phenols	Aldehydes	Ketones
Influent first treatment cycle	+	+	+	+	+	+	+
Effluent first treatment cycle	+	-	-	-	+ *	-	+ *
Influent last treatment cycle	+	+ *	+ *	+ *	+ *	+	+
Effluent last treatment cycle	+	-	+ *	-	+ *	+ *	+*

*—very low concentrations (positive reaction obtained after concentration of the sample).

The removal of organic matter (toxic and easily degradable) during the modeling process was studied by the reduction of the chemical oxygen demand (Figure 5). The process was divided into three stages, which started on the days 0, 7 and 14, respectively, as described above (Figure 3). At the beginning of each of the three stages, an increase in COD values was found as a result of the new portions of landfill leachate with glycerol, which further increased the COD value (Figure 5).

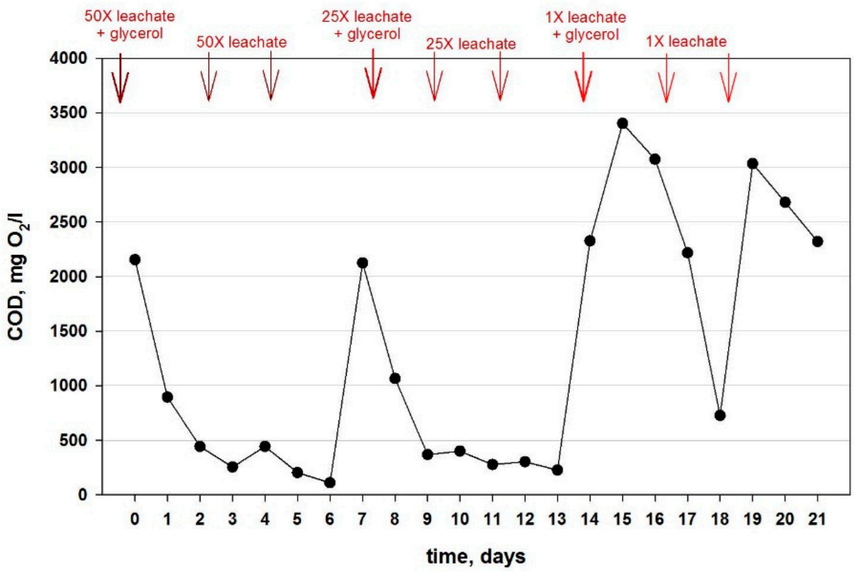

Figure 5. Dynamics of organic matter measured as chemical oxygen demand (COD) during the experiment.

During the first stage of the experiment, the COD decreased from 2614.5 mg O_2/L to 1173.8 mg O_2/L on the first day due to the rapid absorption of glycerol and its use by activated sludge as an easily accessible source of carbon and energy. The added ×50 diluted landfill leachate on the 2nd and 4th days increased COD to about 440.2 m gO_2/L, then COD decreased to 210.1 mg O_2/L in 24 h. The results obtained showed that ×50 diluted infiltrate and added glycerol were absorbed by the unadapted activated sludge. At the end of the first stage (6th day), COD reached 221.7 mg O_2/L. The lack of COD reduction below 189.9 mg O_2/L indicated that the wastewater contained biodegradable organic compounds. We found experimentally that the treated infiltrate contained aromatic amines and phenols (Table 4).

The established trend during the first stage of the experiment was maintained in the second (7th–13th days). Then, after removal of the liquid, ×25 diluted landfill leachate with glycerol was added to the activated sludge. COD increased again to 1048.7 mg O_2/L.

COD on the 14th day decreased about two times in 24 h, from 2125.5 mg O_2/L to 1048.7 mg O_2/L. On the 9th and 12th days, new portions of ×25 diluted landfill leachate were added, but without glycerol, which increased COD by an average of about 375.3 mg O_2/L. At the end of the second stage, the COD decreased to 237.4 mg O_2/L.

The third stage of the experiment began with a COD of about 2300 mg O_2/L with the addition of undiluted landfill leachate and glycerol. On the 15th day, COD increased to 3794.4 mg O_2/L, unlike the previous two stages, where there was a twofold decrease in COD. This clearly showed that in this case the activated sludge was strongly inhibited by the contaminants contained in the undiluted landfill leachate (Figure 4 and Table 4). On the 16th and 18th days, new portions of undiluted landfill leachate were added, which aggravated the inhibition of activated sludge and affected its ability to degrade both xenobiotics and trivial organics. At the end of the third stage of the process, the COD remained high and stable above 2300 mg O_2/L.

It was of interest to gather data for the parameters of the model process, which are directly related to the structural and functional characteristics of the activated sludge. These parameters in a complex allow the assessment of the extent of the capability of biological purification to treat the landfill leachate in the used technological setting. The structure of the floccules in the AS was monitored in parallel, which is important not only for the decomposition of pollutants but also for the sedimentation of the suspended solids and the clarification of the effluent. While the structure of the bacterial complex assessed the possibility of biodegradation of pollutants, the segment of micro- and meta-fauna as structure and functions allowed assessment of other characteristics and properties of AS—its self-renewal, regulation of age and activity, sedimentation capacity, and even more technological parameters that are important for the complex water purification process. In the presented case, to assess the adaptive potential of the community, the focus was put on the following key parameters of the process and AS—sludge volume index, biotic index, amount of microorganisms from key groups for the treatment process, assessment of the structure and activity of the microbial segment through FISH analysis and, of course, the quantitative and qualitative relationship of the bacterial segments and the segments of the micro- and meta-fauna. It should be emphasized that the bacterial segment has wider and more reactive adaptive capabilities due to the haploid nature of bacteria and their rapid multiplication after past genetic changes. At the same time, the segment of micro and meta fauna is more vulnerable due to their diploid nature, their low tolerance to toxic pollutants, and the limit of their adaptation, all of which are key to the structure and functions of complex activated sludge and the ultimate possibilities of biological treatment of landfill leachate [52–55].

Immediately after monitoring the dynamics of residual pollutants in the water, the dynamics of the floccular structure of the activated sludge were monitored by SVI and microscopic control.

The key technological indicator SVI in the first stage of the simulated model process decreased gradually and after the third day the value of the index was below 70 mL/g, and at the end of the first stage (the sixth day), it reached 27 mL/g. The data showed that the sludge from strongly bulking after the addition of new portions of ×50 diluted landfill leachate without glycerol turned into deformation of the pinpoint flocculation type, which is an indicator of starving sludge (Figure 6). In the second step, after the addition of readily degradable glycerol (7th–13th days), SVI data showed an improvement in sludge structure, with the index varying between 68 and 104 mL/g.

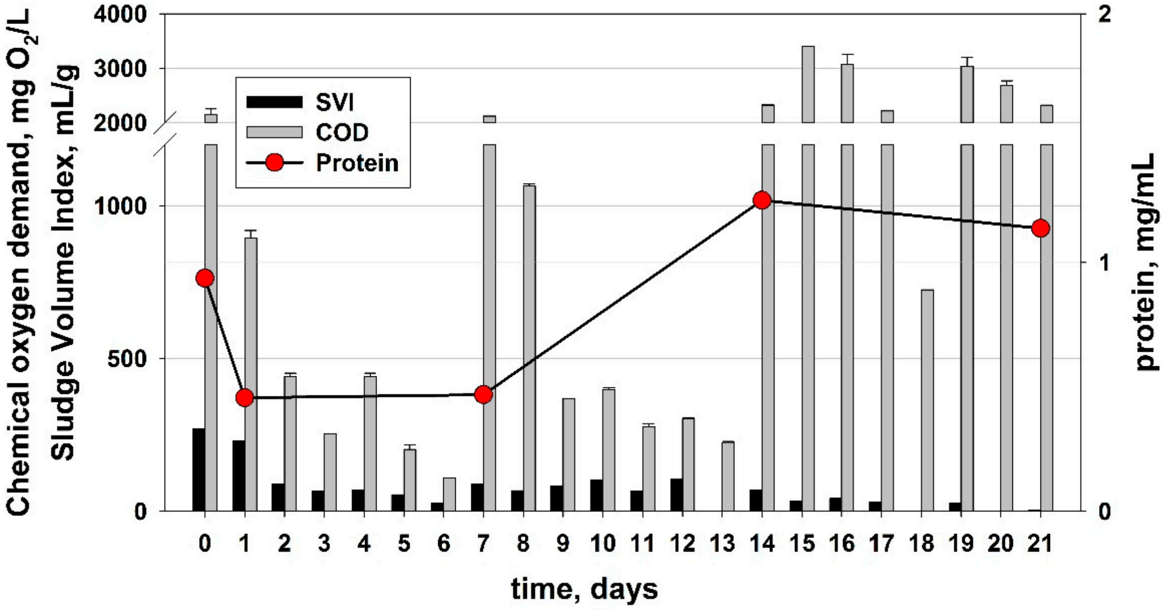

Figure 6. Dynamics of the sludge volume index (SVI) and amount of activated sludge biomass measured as protein content.

During the third stage of the process (14th–21st day), the SVI again began to gradually decrease, and from 70.65 mL/g on the 14th day reached 3.94 mL/g on the 21st day. This again showed a strong inhibition of AS, manifested in small destroyed flocs and impaired sedimentation capacity (Figure 6). At the end of the experiment (21st day), when COD remained high at about 2300 mg O_2/L, BOD5 was 160.0 mg O_2/L or biodegradable organic matter represented less than 7% of the total organic content.

Depending on the construction of the aeration tanks and the technology of the treatment plants, the dry matter usually varies widely between 1 and 20 g/L. According to the literature on aerobic processes with activated sludge, the biomass is about 3 g/L [53]. At the beginning of the experiment, the values of the indicator decreased, reaching about 1.63 g/L on the fourth day, and then began to rise. In general, for the two experimental periods, the first and the second, it can be concluded that the biomass values varied between 1 and 3.19 g/L, which showed the process of adapting the AS. In the last stage, a significant rise in dry matter was observed, and it reached 7.66 g/L. The parameter was strongly affected by the concentration of the landfill leachate.

The dry weight increased, but this was not due to an increase in the biomass of activated sludge, but to the accumulated heavy metals, inorganic and toxic pollutants, which was supported by the data presented in (Figures 4 and 5 and Table 4). Figure 6 shows that between the 13th and14th day of the adaptation period, during the purification of landfill leachate 25 times diluted and enriched with glycerol, a biological purification process can be regulated with average purification efficiency of about 50%. The residual amount of pollutants measured as COD in the aqueous phase was about 240 mg O_2/L. The further increase of the AS load by applying a concentrated infiltrate led to its complete inhibition and to a decrease of the parameters of the water purification process. However, in the model experiment, the process and AS were additionally loaded with landfill leachate in higher concentrations, including undiluted leachate to investigate the mechanisms of this inhibition.

The microscopic analysis of the experiment registered dynamic changes in the structure of the activated sludge (Figure 7). Large flocs with a normal structure were observed during the first stage of adaptation. A greater presence of filamentous microorganisms was registered—between 6–20 filaments of a floccule (first and third days). In the picture from the third day, you can see a representative of the genus *Vorticella*. In the second and third stages of the adaption, a slow deconstruction of the floccules in the activated sludge was registered. At the same time, the number of homogeneous cells in the activated sludge increased. In the images from the seventh and ninth days (same stage of the process), nematode worms can be seen. The reduction in floc size was particularly pronounced in the third stage of adaptation when undiluted landfill leachate from the waste treatment plant was added. At the end of the experiment, small, tightened flocs with dark deposits were observed (the 18th day).

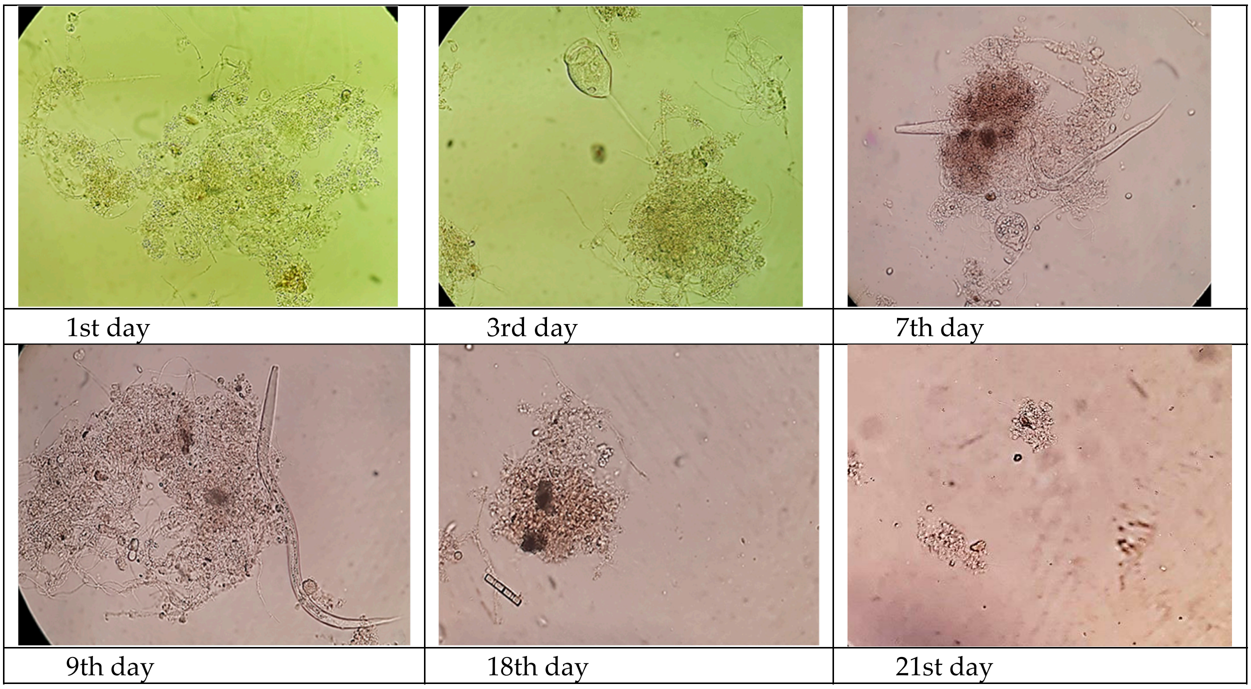

Figure 7. Microscopic images of activated sludge samples at different time points of the model process /400×/.

Changes in the structure and quantitative indicators of micro- and meta-fauna supported the results shown above about the processes in the various stages of adaptation of AS.

From the data on the total number of macroorganisms, it can be seen that during the first stage of the experiment there was a decrease. During the second stage, and in particular, on the ninth day, a significant increase was registered (Figure 8). This, however, cannot be accepted unequivocally as positive. It was largely associated with the increase in the number of small flagellates from 1.88×10^6 ind/mL on the seventh day to 10.36×10^6 ind/mL on the ninth day. If present in large quantities, they are an indicator of an inefficient purification process. In addition, these organisms increase their number when the structure of the AS is disturbed, which can also be seen from microscopic photographs. A similar increase in the total number of micro- and metafauna organisms was found at the 18th and 21st days. The reason for this increase was similar to that of the ninth day and was again linked to the sharply increased number of small flagellates and free-swimming ciliates. These two groups are also an indicator of destruction in the macrostructure of the AS. The total number of macro-organisms showed that the fauna had the ability to adapt through the change in the dominant groups and the increase in the number of organisms from them.

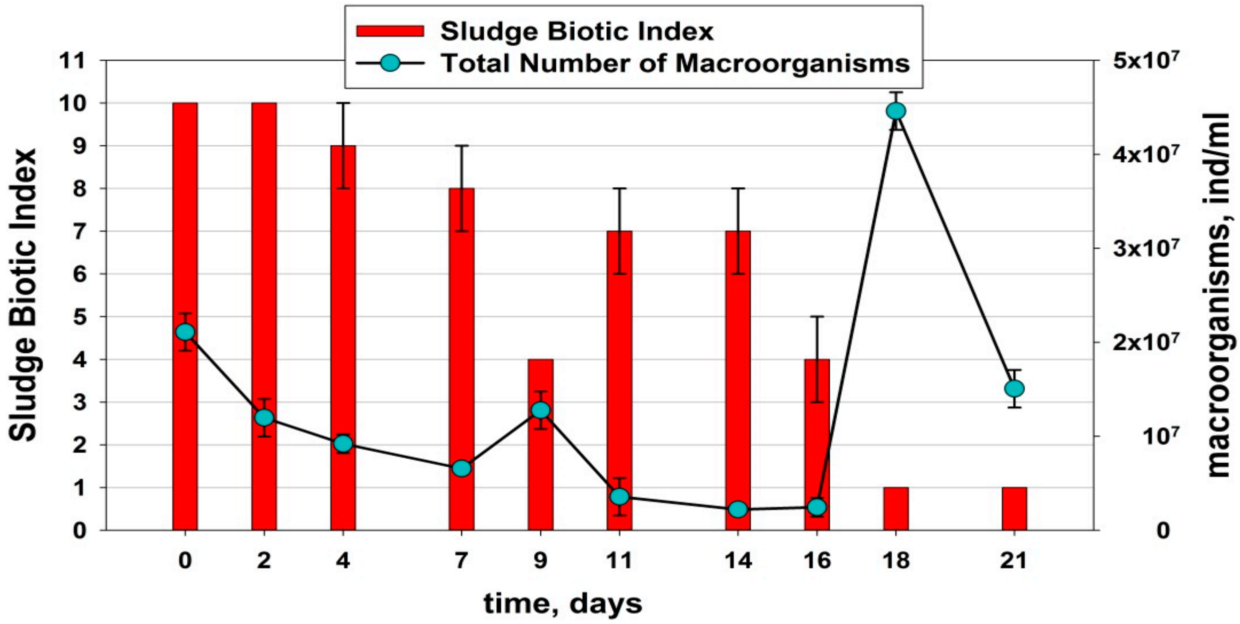

Figure 8. Total number of macroorganisms/micro- and metafauna/. Max. value for SBI—10; min. value for SBI—0 [41].

The data on the sludge biotic index (SBI) of AS showed a tendency to decrease its values during the process. This index is based on quantitative and qualitative information about the protozoans in the activated sludge (micro-fauna). Its interpretation according to Madoni [42] is illustrated in Table 5. This decline was most significant in the second and third stages. At 216 h, after the addition of landfill leachate with a dilution of $\times 25$, SBI decreased from 8 to 4. At the 264th and 336th hours, there was a slight increase in the index, reaching values of 7. After the 384th hour, there was a decrease again, and at the 432nd and 504th hours, SBI decreased to 1.

Table 5. Interpretation of the sludge biotic index.

Conversion of SBI Values into Four Quality Classes		
SBI Value	**Class**	**Estimation**
8–10	I	Very well colonized and stable sludge; excellent biological activity; very good performance.
6–7	II	Well colonized and stable sludge; biological activity on decrease; good performance.
4–5	III	Insufficient biological purification in the aeration tank; mediocre performance.
0–3	IV	Poor biological purification in the aeration tank; low performance.

The number and structure of the fauna complex are bound and dependent on the number of bacteria on the one hand, as this is the main nutrient substrate for the fauna. On the other hand, the functions and group distribution of fauna in AS depend on a number of microbiological and chemical parameters of the process: the amount of homogeneous bacterial cells, the density of flocs, their size, and surface layers. All of these parameters affect the functions of the fauna. However, the accumulation of pollutants that strongly inhibit their function, such as heavy metals and xenobiotic pollutants, shouldn'tbe ignored. If the changes in the bacterial segment in the course of adaptive changes and the load of the AS with increasing landfill leachate concentrations are considered, the following can be said. The bacterial complex had far greater abilities for adaptation compared to the fauna, flocs structure, and the complex purification potential of AS.

The results for aerobic heterotrophs showed a clear decrease in their number on the seventh day (Figure 9a). On the 14th day, they increased their number, reaching values of 1.55 × 10^7 CFU/g. At the end of the study, they slightly reduced their amount, but in general, it remained high. The bacteria of the genus *Pseudomonas* and genus *Acinetobacter* had a similar trend to heterotrophic bacteria (Figure 9b). Their values fell sharply on the seventh day, followed by an increase on the 14th day. On the 14th day, the pseudomonads reached values of 8.06 × 10^6 CFU/g. Bacteria of the genus *Acinetobacter* maintained high, with values of 1.78 × 10^6 CFU/g on the 21st day, while bacteria of the genus *Pseudomonas* reduced their number to 0.06 × 10^6 CFU/g. In general, from the study of cultured microorganisms, it can be concluded that at the beginning of the process, aerobic heterotrophs, pseudomonads, and *Acinetobacter* decreased by the end of the second stage. This can be explained by two parallel processes: on the one hand, the fact that many of these microorganisms were involved in well-functioning flocs and therefore they enter into synergistic relationships. Thus, these relationships do not allow them to be registered with classical cultivation techniques. On the other hand, the high amount and good structure of the fauna complex had high trophic pressure on the free-floating bacteria and those on the surface of the flocs. Thus, cultivation techniques take into account the low number of bacteria in the most biodegradable phases. With the increasing concentration of the landfill leachate, a strong increase in the number of homogeneous bacteria from all groups studied after 14 h was found. This fact, which at first glance contradicts the purification logic, has a biological explanation. Bacteria, which are mainly *Pseudomonas* and *Acinetobacter*, had the greatest potential to adapt to resistance and biodegradation to toxic xenobiotics due to their ability to use alternative energy sources—polyhydroxybutyrate and polyhydroxyacetate. In this case, however, it was an adaptation to resistance and multiplication of free-swimming *Pseudomonas* and *Acinetobacter*. This was confirmed by the low degree of elimination of toxic pollutants and the high COD at the end of the process. The high amount of homogeneous bacteria resistant to toxic substances was a trophic basis for increasing the number of fauna organisms. The fauna complex was more abundant at the end of the process, not only because of the large amount of easily available food but also because of its quality. Homogeneous bacteria had enzymes that overcame intoxication at the end of the process, but the fauna eating them could acquire bacterial detoxification (external) enzymes. This is a good process from the point of view of the richness of the activated sludge, but not from the point of view of the purification of the landfill leachate. AS had a destroyed structure and developed resistance to pollutants, but with the following adverse properties: it had accumulated heavy metals and other toxic pollutants, had developed resistance of certain bacterial groups that weren't in flocs, but were free-floating; its biodegradation activity was greatly reduced and its sedimentation capacity was impaired. Such AS cannot perform a purification process.

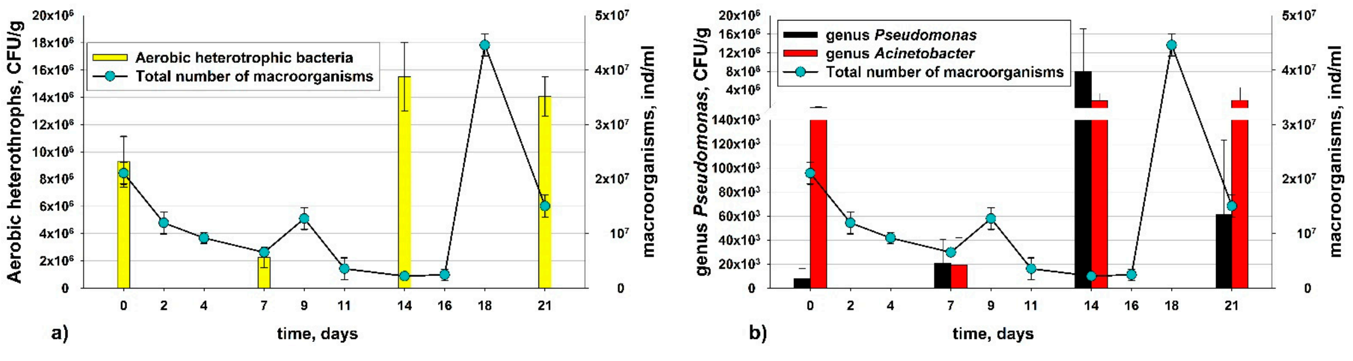

Figure 9. Quantity of aerobic heterotrophic bacteria (**a**) and quantity of bacteria of the genus *Pseudomonas* and bacteria of the genus *Acinetobacter* (**b**) compared with total number of macroorganisms (micro- and metafauna).

These intermediate conclusions were confirmed by in situ hybridization analysis.

The groups *Azoarcus-Thauera*, *Alcaligenes*, *Pseudomonas* were studied. Bacteria of the genus *Pseudomonas* were represented in the largest number of the studied groups (8.1% on average for the process). In the first week, they were about 5% of the community. However, when the concentration of pollutants increased, the number of bacteria of the genus also increased 2.3 times and remained high until the end of the process (Table 6).

Table 6. Digital image analysis of FISH of key bacterial groups.

	Azoarcus-Thauera Cluster	g. *Alcaligenes*	g. *Pseudomonas*
Hour 0	3.25% ± 0.21%	0.69% ± 0.12%	5.37% ± 1.29%
Day 7	4.58% ± 0.43%	0.60% ± 0.17%	5.20% ± 0.20%
Day 14	4.64% ± 0.25%	1.31% ± 0.01%	13.24% ± 3.15%
Day 21	2.61% ± 0.79%	2.77% ± 0.48%	8.66% ± 0.00%
Mean	3.77% ± 0.42%	1.34% ± 0.19%	8.12% ± 1.16%

The representatives of the *Azoarcus-Thauera* cluster increased their share from 3.2% in the activated sludge to 4.6% in 14 days. This was most likely related to the potential of these microorganisms to degrade xenobiotics. The bacteria from the genus *Alcaligenes* also increased in number, but in general, their share remained low (average 1.3%) (Table 6).

From the presented results it became clear that in the course of the model process the bacterial structure changed, displaying adaptive reactions. It was the most important factor confirming the conclusions made so far and is shown in Figure 9 and on the fluorescent images presented above (Figure 10). After loading the treatment system with undiluted landfill leachate, even though all studied groups of microorganisms were preserved, their ratio changed quantitatively. After the 14th day, at high loads, the number of bacteria of the genus *Pseudomonas* increased and to a lesser extent so did those of the genus *Acinetobacter*. This reaffirms that polyhydroxybutyrate and polyhydroxyacetate, which are alternative energy sources, were becoming more important as toxic pollutants increased. The relationships of bacteria in AS were interesting. By the 14th day, denser accumulations of pseudomonads were found, which means that they enter into more strong synergistic and symbiotic relationships. This is always reflected in higher biodegradation activity—the COD was lowered by 40% during the second week of the experiment. These relationships were most pronounced on the 14th day, but on the 21st day, after a strong reduction in the biodegradation activity of the bacterial segment, a reduction in dense cell clusters, and a large number of predominantly free-floating pseudomonad cells, were found. These were those homogeneous resistant pseudomonads that were registered by cultivation methods and that had inhibited biodegradation activity.

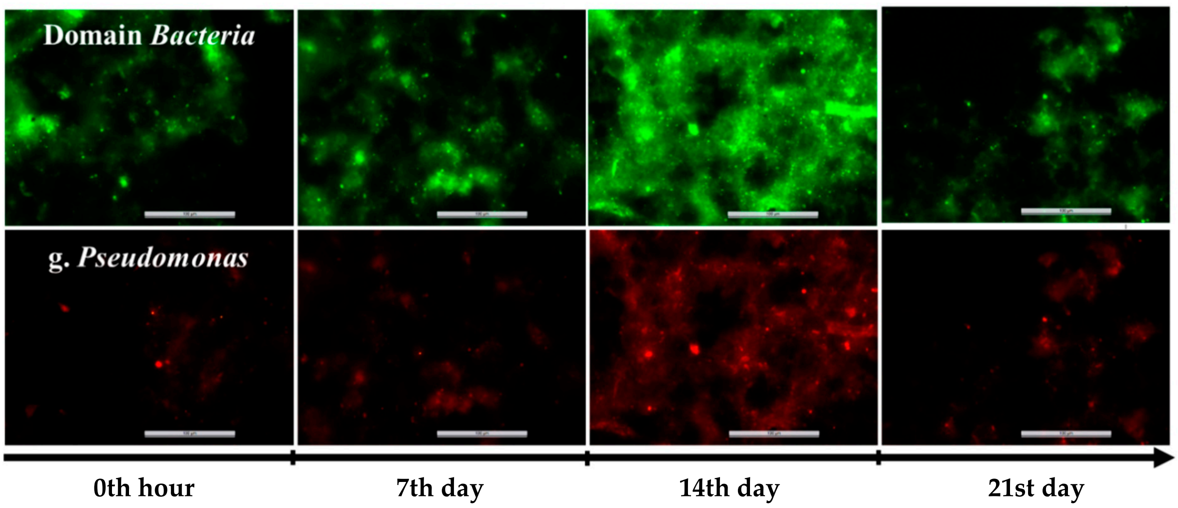

Figure 10. FISH images for domain *Bacteria* (green) and g. *Pseudomonas* (red)—the two bacterial groups that were affected most during the infiltrate treatment (the bar indicates 100 µm).

4. Discussion

The data from the hydrochemical analyses showed that in the process of purification performed by AS from the urban wastewater treatment plant, COD remained high (average 1231.2 mg O_2/L). The lowest values reached were about 200 mg O_2/L, which was an indication that the landfill leachate, although diluted, contained biodegradable organic compounds that do not allow further reduction of COD. This was confirmed by the qualitative analysis of the different classes of organic compounds (Table 4). When diluting the leachate $\times$25, the concentration of contaminants in the leachate approached the critical level for the activated sludge. Although the purification process continued at this concentration of contaminants, the residual COD remained relatively high (above 200 mg O_2/L). When the landfill leachate was applied without dilution, the COD in the effluent remained very high (average 2525.9 mg O_2/L). This showed a deepening of the inhibition of the biodegradation abilities of the activated sludge. In the applied conditions, this was the limit of the adaptive capabilities of AS in the used process parameters and in the purification of landfill leachate. After this limit, residual contaminants must be eliminated by including other modules such as membrane filtration or ultrafiltration to completely eliminate non-degradable, recalcitrant, or toxic contaminants.

Activated sludge developed resistance to these residual concentrations of non-degradable pollutants and operated under these conditions. This resistance was manifested at the following levels:

(1) The floccular structure of the activated sludge was disturbed, and pin-point flocs and many homogeneous cells increased;

(2) The dry weight of AS increased during the process, which is associated with the accumulation of heavy and other metals in the cells of organisms and the remaining small flocs. Toxic and degradable pollutants accumulated in the same biological structures, blocking the biodegradation activity of the bacterial segment and restructuring the micro- and metafauna segment, with shell amoebae and flagellates beginning to predominate. They are indicators of poorly functioning activated sludge;

(3) This was also confirmed by the strong decrease of the SVI. The index provides information on the settling capacity of the activated sludge and together with the microscopic analysis of the macrostructure of the microbial community can be used to identify deformations at the structural level [53]. In the case of a normally functioning sludge, the values of the index vary between 100 and 120 mL/g, while in the case of "starvation" sludge with pinpoint flocs the index has values below 70 mL/g [53]. Despite the fluctuations in the value of the index, it was preserved to about 100 mL/g during the adaptation process up to day 14. After that, the high concentrations of landfill leachate led to its reduction. This was associated with the impaired sedimentation capacity and the decreased purification activity of AS.

(4) In the third stage of the process (14th–21st day) the structure of the sludge was most strongly deformed as a result of the lack of sufficient biodegradable organic matter (7% of the total organic content) and the presence of toxic pollutants. Reaching the critical concentration of the pollutants in the landfill leachate was related to the raised concentration of pollutants fed into the reactor and to the adsorption and absorption of toxic pollutants in the flocs and their subsequent accumulation. We registered similar effects in other processes with activated sludge at the entry of dyes, fuel oil, nitrophenols, chlorophenols, and others into treatment plants [32,54,56,57]. These accumulated contaminants in the flocs of the sludge can also be seen in the photos (Figure 7).

(5) From the results for the micro- and meta-fauna organisms (Figure 8), it was established that at the beginning of the study the attached ciliates dominated. This was an indication of an effective purification process and well-functioning activated sludge. In the second stage of the study, the group of small flagellates became dominant, together with free-swimming ciliates. This change in the groups led to a very sharp deterioration in the qualities of AS. These results also showed the relatively difficult adaptation of macro-organisms to changing conditions. This can be seen until the end of the study, with

the only change being that the group of swimming ciliates became dominant. This was mostly a kind of adaptive reaction of the representatives of the micro- and metafauna in the presence of xenobiotics in higher concentrations. At the end of the study, the attached ciliates significantly reduced their share, which revealed the extreme inability of micro- and metafauna organisms to adapt to the addition of pure landfill leachate.

(6) The data for the biotic index of AS (Figure 8) highlighted once again the situation that can be seen from the distribution by groups of the micro- and meta-fauna. During the first stage of the study, there were very high values that were at the maximum for this index. This means that a well-functioning AS was present. After the addition of landfill leachate with a dilution of x50, the values of the index began to decline, which was due to the change of the dominant key groups of the fauna. In the third stage, the index dropped to critically low values, which was a clear indicator of deterioration in the properties of AS and difficulty in the purification process.

(7) From the data for aerobic heterotrophs it was established that on the seventh day there was a sharp decrease in the number, which may have been a momentary reaction to the added landfill leachate with a dilution of $\times$25. This reduction of the culturable microorganisms showed that the non-culturable ones with clearly expressed synergetic properties and with increased biodegradation and purifying activity were beginning to prevail. This was illustrated by FISH analysis. Elevated values on the 14th day showed that heterotrophs responded to increased xenobiotics amounts with a rapid increase in their number. However, this was related to the free-floating bacteria that developed resistance to the available toxic pollutants. In general, their biodegradation activity was low. This was confirmed by the high residual amounts of pollutants in the aqueous phase after the 14th hour. The high adaptive potential of bacteria of the genus *Pseudomonas* and the genus *Acinetobacter* was confirmed, which in high toxic pressure developed high resistance to it and increased their amount based on their ability to use alternative energy sources—polyhydroxybutyrate and polyhydroxy acetate.

(8) In parallel and with a small shift in time, with the increase of homogeneous free-floating bacteria, the total number of organisms from the complex of micro- and meta-fauna increased. The reason was that most of the bacterial biomass was available as food, as well as the fact that this bacterial food was of the genus *Pseudomonas* and genus *Acinetobacter*, which developed resistance to xenobiotics and most likely contained protective enzymes that helped fauna that consumed "food with detoxifying properties".

(9) Regardless of the ongoing processes of restructuring of AS after increasing the concentration of landfill leachate and toxic substances in it, these structural changes were not associated with increasing the purification potential of the biological system. The result was a deteriorated purification process after the 14th hour, high residual contaminants in the aqueous phase, impaired precipitation of activated sludge, and high concentration of accumulated contaminants in the flocs.

(10) This was confirmed by the study of key bacterial groups in AS through FISH analysis. The obtained results showed that among the groups studied with FISH with the highest share were those of the genus *Pseudomonas* (average 8.1%). When applying the high concentrations of the landfill leachate from SPTO, their amount increased by 2.3 times when the leachate was diluted $\times$25 and by 60% when applying the undiluted landfill leachate. This effect was probably related to the very well-developed biodegradation functions of these bacteria towards different xenobiotic molecules [58–62]. Numerous studies have shown the important role of the genus in the treatment of leachate from solid waste landfills [63–65]. Other studies have shown the role of the genus in C12DO production during the biodegradation of pollutants in landfill leachate [64,66]. This enzyme is key in the biodegradation of xenobiotics and was leading in the activated sludge from the present study.

An interesting result was the twofold increase in the amount of *Alcaligenes* spp. during the modeling process. The role of these microorganisms is central in microbial communities in the environment and especially in those performing purification processes [67–69].

However, the role of *Alcaligenes* spp. in the treatment of leachate from solid waste landfills is not so significant and they have been identified as a major factor in the treatment of such waters only in individual studies [70]. This was confirmed by the results obtained in the present study—in the first seven days these microorganisms were only 0.6−0.7%. At the end of the treatment, their percentage still remained relatively low (2.77%), but its increase compared to the beginning is probably related to its participation in biodegradation processes. Heang et al. [10] added a strain of the genus *Alcaligenes* to increase the biodegradation of pollutants in landfill leachate. It was likely that the increase in the amount of these microorganisms was an adaptive response of the community to the treatment of the leachate. This was also found in the *Azoarcus-Thauera* cluster, whose percentage of the community increased from 3% to 5% by the 14th day. The considered bacterial group is characteristic of biotechnological processes of landfill leachate purification [71–73]. This was the most probable reason for their increase when putting the sludge in an environment with such. The decrease to below 3% at the end of the process was probably related to the overall inhibition of the community.

(11) After the 14th day the share of the genus *Pseudomonas* increased, but the diffuse location of these bacteria was confirmed, which showed a decrease in the symbiotic and synergistic relationships and hence their biodegradation activity. This again confirmed that the limit of the adaptive biodegradation properties of AS in such a composition of the biological purification process had been reached. Further elimination of residual pollutants can only be done by completing the biological water treatment module with other ones—membrane modules for the complete elimination of pollutants. This would create an effective hybrid technology, starting with a biological module to reduce major pollutants and a membrane or ultrafiltration system to purify those pollutants that cannot be removed biologically.

The technologies in the full-scale plants that treat landfill leachate are based either on the biological treatment of pollutants or on their physico-chemical removal. The present study demonstrated that the optimal approach to eliminate pollutants from these heavily polluted waters is a hybrid one: biodegradation of pollutants to be carried out as much as possible, and the remaining pollutantsto be removed by a physicochemical approach (e.g., reverse osmosis, ultrafiltration, etc.).

5. Conclusions

The presented study aimed to assess the adaptive changes of activated sludge from a municipal wastewater treatment plant towards the treatment of landfill leachate. The obtained results demonstrated the adaptive capabilities of activated sludge and the limit of pollutant concentration, beyond which biological purification was impossible. It was found that when the landfill leachate was diluted fifty times, the AS community was well structured and the elimination of contaminants was active. When the landfill leachate portion was increased to 25-fold dilution, the activity and structure of the AS remained preserved, although the accumulation of intoxication changes began (increased number of active biodegradants, decrease in the biotic index, and floc size). The use of undiluted landfill leachate led to the destruction of the floccular structure of the AS, the proliferation of free cells, the change of the dominant group of protozoa, and the blocking of the purification process. The obtained data showed that the optimal approach for the treatment of this type of highly polluted wastewater was the combination of biological treatment with physico-chemical treatment. This will allow an environmentally friendly treatment of the leachate according to the capabilities of the biological system and removal of the remaining non-biodegradable pollutants by physico-chemical methods. In this way effective hybrid technologies directed to the smart protection of the environment can be constructed.

Author Contributions: Conceptualization, Y.T. (Yana Topalova), S.L.; methodology, I.Y., M.B., N.D., I.S., V.L., V.M., Y.T. (Yovana Todorova); data collection—M.B., I.Y., I.S., V.L., V.M., N.D., S.L., E.D.; investigation, I.Y., M.B., I.S., N.D., V.L., V.M., E.D.; data curation, M.B., I.Y., I.S., V.L., V.M.; writing—original draft preparation, Y.T. (Yana Topalova) I.Y., M.B., I.S., V.L., V.M., Y.T. (Yovana Todorova);

writing—review and editing, Y.T. (Yana Topalova), I.Y., M.B.; supervision, Y.T. (Yana Topalova), I.S.; project administration, Y.T. (Yana Topalova); funding acquisition, Y.T. (Yana Topalova). All authors have read and agreed to the published version of the manuscript.

Funding: This investigation has been supported financially by Operational Program 'Science and education for smart growth', co-financed by the European Union through the European structural and investment funds, project BG05M2OP001-1.002-0019: 'Clean Technologies for Sustainable Environment—Waters, Waste, Energy for a Circular Economy'.

Data Availability Statement: Data is contained within the article.

Conflicts of Interest: The authors declare that they have no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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