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A Biological Method of Treating Surface Water Contaminated with Industrial Waste Leachate

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Abstract: The progressive chemicalization of all areas of everyday life and the development of the industry cause the appearance of various types of pollutants, both in groundwater and surface waters. Kalina Pond (Świętochłowice, Poland) is an example of a degraded water reservoir as a result of many years of activity, among others hard coal mines, storing metallurgical waste by zinc plants, and the activities of the Hajduki Chemical Plants from Chorzów. Inadequate securing of waste heaps resulted in the penetration of pollutants, i.e., phenol, petroleum compounds, PAHs, cyanides, and heavy metals. The aim of the research was to determine the suitability of biopreparations for the removal of pollutants. The research used a bacterial biopreparation from BioArcus, "DBC plus type R5", to remove petroleum compounds and phenol. Then, in order to restore the microbiological balance, "ACS ODO-1" from the biopreparation was used. The research was carried out in laboratory conditions, using three variants: direct dosing of biopreparations, dosing of biopreparations previously activated by multiplication on the medium, and dosing of biopreparations into water after filtration on a diatomite bed. The optimal method of recultivating water from a reservoir was to filter this water through a diatomite bed and then dose the multiplied bacteria. After the filtration process, the obtained percentage of TOC reduction allowed for the rapid development of microorganisms from the biopreparation, despite the 100 times lower dose used. In addition, the application of lyophilized biopreparation to contaminated water resulted in a very fast biodegradation effect of pollutants, despite the high concentration of numerous toxic compounds.

Keywords: biopreparation; FCM; ATP; industrial waste

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1. Introduction

Water is a crucial resource for the ecosystem and human existence that determines life on Earth. The problem of surface and groundwater pollution affects both poorly and highly developed countries. This is mainly due to the progressive chemicalization of all areas of everyday life and the widely developed industry. This results in the appearance of various types of contaminants, often unidentified, and therefore causing risks that are not fully known. For this reason, maintaining a good condition of water resources should be one of the most important objectives of human economic activity. Many sources adversely affect water resources, including agricultural and industrial activities, wastewater treatment, and waste disposal. Leachate from industrial landfills may pose a severe threat to the aquatic environment. In the event of inadequate or insufficient insolation of the mass of stored waste, various organic and inorganic pollutants may penetrate into the water–soil environment, which in consequence will directly reflect in the deterioration of the landscape values of the immediate surroundings, the quality of ground, and surface waters, and constitute a real threat to living organisms.

Kalina pond, in Świętochłowice, is an example of such a degraded water reservoir. The unsatisfactory water condition of the reservoir is the result of many years of activity, i.e., coal mines, storage of metallurgical waste by zinc plants located near the reservoir,

and the activities of the Hajduki Chemical Plants from Chorzów, responsible for the production of coal derivatives, such as crude benzol–phenol and pyridines, crude tar–anthracene, naphthalene, chemical reagents or resins, and then storing post-production waste in heaps in Świętochłowice. Subsequently, inadequate protection of the waste heaps resulted in the contamination of the Kalina pond. As a consequence of these activities, the reservoir water was contaminated with phenol, petroleum compounds, isomers of benzene, ethylbenzene, toluene, and xylene (compounds from the BTEX group), polycyclic aromatic hydrocarbons, cyanides, thirates, and heavy metals. In the following years, the first preventive and recultivation measures were taken, including the installation of a waterproof screen around the landfill, installation of horizontal drainage to collect rainfall and groundwater, deployment of drainage ditches system, pumping the discharged water to a sewage treatment plant, creating a so-called "small pond" filled with neutral material and humus with plants. Recultivation was completed in 1995. In the following years, many attempts were made to restore the good condition of the waters in the Kalina reservoir, but the treatments that were carried out turned out to be insufficient. Currently, due to the fact that the area around the reservoir is planned to be used for housing development, it has become necessary to restore the biological balance of the reservoir.

Most of these chemicals are characterized by high toxicity, persistence in the environment, and the ability to move. They also bioaccumulate in living organisms [1]. Phenolic compounds are detected in groundwater, wastewater, and effluents of much industrial wastewater. Nowadays, the degradation of phenol is of great importance due to its toxicity and high solubility. These compounds are contaminants in environmental matrices, food, and medical products that can easily be absorbed through humans' and animals' skin or mucous membranes. Phenols significantly inhibit microbial activity due to membrane damage at higher phenol concentrations, and toxicity to biological treatment systems and bacterial cell disruption was observed in high phenol concentrations. Adaptation of the microbial community to phenol as a sole carbon source led to an increase in the ability of the community to degrade m-cresol, m-aminophenol, and p-chloropheno [2]. In the environment, phenol can be aerobically degraded to catechol using a single microbe or a combination of microorganisms, such as *Trichosporon cutaneum*, *Brevibacterium fuscum*, *Hormodendrum bergeri*, *Fusarium oxysporum* and, *Aspergillus flavus var. coulmnaris* [3]. Accumulation of these pollutants may adversely affect fauna and flora as well as human health, as these compounds are toxic, mutagenic, and also carcinogenic [4]. The European Union and other countries around the world have placed most phenols on the Priority List of Pollutants [5,6].

One of the most common organic pollutants in the aquatic environment is hydrocarbons. Hydrocarbons are poorly soluble or insoluble in water, so they are found in surface water and wastewater as an oil film or water–hydrocarbon emulsion. The degree of their dispersion is a significant factor in choosing a method for their removal [7,8]. The leakage of petroleum hydrocarbons into the environment may cause serious consequences due to these substances' highly toxic and carcinogenic character. Petroleum compounds are classified in the IRIS (Integrated Risk Information System by the United States Environmental Protection Agency) database as toxic, which may cause risks to human health as a result of environmental exposure to these substances [9].

Several organic compounds, including petroleum-based compounds, pose a risk to human health because of their genotoxicity, mutagenic effects, and carcinogenicity [1]. Concentrations of many of these compounds can become toxic due to the accumulation of metabolic products, i.e., PAHs (Polycyclic Aromatic Hydrocarbons) and cyclic aromatic compounds. These components affect the lipid bilayer membrane, which causes transduction through biological membranes [10–13].

Cyanides and thirates generally do not occur in natural waters. Pollution is caused by industrial sewage (electroplating plants, coking plants, gasworks, metal processing plants, etc.). These are very toxic compounds, and the sewage in which they are present should not be discharged into surface water reservoirs [14–17].

With the development of civilization and human activities, the level of heavy metal pollution has intensified. This phenomenon is particularly evident in inland surface waters as well as in shallow groundwater. Water pollution is of particular importance because of the role that water plays in the turnover of elements in diverse environments. The concentration of heavy metals in water depends mainly on their physicochemical properties, including solubility and impurities in water, pH, oxidation-reduction potential, and ability to form soluble complexes [17]. In the aquatic environment, heavy metals occur in the form of soluble and insoluble compounds, distributed in water, sediments, and in the tissues of organisms. The toxic properties of metals appear in the organisms when they come into contact with a bioavailable form of the metal at a concentration that causes an adverse reaction in the organism. The toxic potential of a trace metal to aquatic organisms depends on many factors. These include the physicochemical characteristics of the water and sediment, the composition and health of the population of living organisms, and trace metal concentration and availability. Most trace metals play an important role as micronutrients in maintaining the life of aquatic organisms. Their toxic properties are only manifested when the concentration accessible to the organism exceeds the value necessary to meet its nutritional needs [18].

Natural self-purification processes occur in surface water reservoirs. However, with the significant contamination by many chemical and toxic compounds, natural mechanisms are not sufficient. Current methods of water treatment can be divided into chemical, mechanical, biological and biotechnological, and bioremediation. Chemical methods consist of pollutant removal using coagulants, sorbents, and flocculants, often combined with mechanical sediment removal dredging scraping after emptying the reservoir [19]. The photocatalytic process was put forward as an outstanding elimination system, which could remove pharmaceutical pollutants and decline the toxicity of the treated solution [20,21]. The second group consists of microbiological bioremediation methods, such as the biotechnological method supported by ecotone planting or natural sorbents (kaolin clays, sorbents made of different types of zeolites) [22]. The last one, which is microbial bioremediation, is one of the most popular and promising methods for the restoration of contaminated surface waters and other environmental aspects [23–26]. Compared to other technologies, bioremediation methods are non-invasive and do not disturb the trophic relations in the food cycle of aquatic ecosystems [23]. These technologies belong to the environmental biotechnology industry and benefit from recent advances in this science. Companies offering water treatment services have different types of biopreparations in solid and liquid form. The selection of microorganisms and composition are very often considered as trade secrets (know-how) of a particular company. Biopreparations are based on the consortia of microorganisms selected for a specific type of pollution. The advantage of the discussed solution is the very fast and effective biodegradation of pollutants existing in waters [27]. For example, biodegradation of contaminated hydrocarbon mixtures with different chain lengths, saturation degrees, and configurations requires the interaction of a complex of microorganisms belonging to different taxonomic groups. There are complex ecological and physiological relationships between them [28]. The most valuable source of this type of microorganisms are natural environments with a certain level of hydrocarbon contamination. The conditions of temperature, pH, metal ion concentration, and oxygenation are similar to those in which they can be used later [29]. The effects of the microbial revitalization process are very poorly described in the scientific literature, although they have been applied in recent years.

The literature reports practical applications of biopreparations for the recultivation of surface water reservoirs in Poland. The process of microbiological revitalization has shown a positive influence on the improvement in water quality parameters in the Słoneczko reservoir [30]. Bottom sediments have been virtually eliminated, creating a sandy bottom. Water clarity has significantly improved, and the microorganisms introduced into the reservoir waters have also reduced the level of organic pollution to a safe state for aquatic ecosystems. The applied biopreparations ("ACS ODO-1") in the waters of the bathing

site on Lake Kórnik made it possible to improve many surface water parameters and to restore the recreational activity of the bathing site [31]. The process of microbiological bioremediation brought visible effects of improved water quality in the dam reservoir in Głuchów [32].

The aim of the study was to determine the usefulness of biopreparations for removing contaminants polluting the waters of the Kalina pond. BioArcus bacterial biopreparation for the removal of petroleum compounds was used in this study. "ACS ODO-1" biopreparation was then applied to restore the microbial balance. The use of biopreparation in water contaminated with many toxic compounds is a novelty in the conducted research. Another novelty in the conducted research is the use of flow cytometry to assess the development of microorganisms from the biopreparation in treated water.

2. Materials and Methods

2.1. Research Material

The research material was water from the Kalina pond located in Świętochłowice (50°16′49″ N, 18°55′38″ E). The area of the pond is around 0.053 km^2, which is approximately 5.3 ha. The depth of the reservoir varies from 1.5 m in the western part and not exceeding 3.5 m in the eastern part. Kalina Pond was formed as a result of filling with water sedimented post-mining areas, created because of the exploitation of hard coal deposits before the First World War. The Kalina reservoir, initially used for recreational purposes, also plays the role of a retention reservoir for rainwater from a catchment area of about 80 ha, accumulated around the reservoir [33].

In the second half of the 20th century, which is characterized by significant industrial development, the reservoir waters were polluted, therefore becoming a significant threat to the environment. The poor condition of the water resulted from the location of the reservoir in a depression and the storage of post-production and coke-chemical waste from chemical plants in heaps near the reservoir. In the years 1888–1990, near the pond, the already mentioned Hajduki Chemical Plants in Chorzów, collected various types of lime sludge phenolized after soda caustification (about 13,000 tons per year) tar product wastes, including approximately 6000 tons per year of post-refining acids from benzol and naphthalene refining, sludge from the on-site sewage treatment plant and slag from the on-site boiler house [34,35]. It is estimated that the annual stream of waste deposited at the landfill was about 25,000 tons. Storage of post-production waste near the Kalina Pond was conducted until 1990, when the landfill was closed.

The lack of proper protection of the waste heap resulted in the leakage of a number of pollutants to the adjacent Kalina pond, which contaminated the pond water with phenol, isomers of benzene, ethylbenzene, toluene, polycyclic aromatic hydrocarbons, and heavy metals. In addition, at the bottom of the pond, there are sediments with highly alkaline pH (8.5 to 10.2), containing volatile phenols in concentrations ranging from several hundred to several thousand mg/l and COD above 10,000 mg O_2/L [35–37]. The poor condition of the water in Kalina Reservoir is evidenced by its chemical composition shown in Table 1 (the results made available by the organizer of the tender for the bioremediation of the reservoir).

Table 1. The chemical composition of the water from the Kalina pond.

Parameter	Unit	Surface Water Taken from 30 Different Places of Different Depth		
		Minimum	Maximum	Average
TOC	ppm	308	310	309
ATP	RLU	3,018,500	3,018,905	3,018,703
Turbidity	NTU	175	185	180
Arsenic (As)	µg/L	16.10	19.90	17.64
Barium (Ba)	µg/L	53.60	86.20	70.39
Chromium (Cr)	µg/L	13.80	20.70	16.39
Zinc (Zn)	µg/L	7.10	21.20	9.96
Nickel (Ni)	µg/L	8.20	11.50	9.55
Lead (Pb)	µg/L	3.10	10.70	4.58
Free cyanides	µg/L	9.90	18.00	11.99
Concentration of thirates	µg/L	3900	4600	4190
Cyanides total	µg/L	240	260	247
Phenol index	µg/L	33,000	68,000	51,900
O-xylene	µg/L	7.24	49.70	16.90
Benzene	µg/L	2.34	97.70	44.76
Toluene	µg/L	1.05	64.00	26.85
Etylobenzene	µg/L	1.84	22.90	7.15
(m + p)-xylene	µg/L	18.10	117	43.61
Acenaphthene	µg/L	0.49	21.20	5.13
Phenanthrene	µg/L	1.02	41.40	8.59
Fluoranthene	µg/L	0.29	15.20	2.86
Fluoren	µg/L	0.19	12.80	2.06
Naphthalene	µg/L	3.22	350	99.01
Pyrene	µg/L	0.07	3.85	0.93
PAH sum	µg/L	8.73	453	120.36
Index mineral oil/petroleum hydrocarbons	mg/L	1.20	8.90	2.73
pH	pH	7.15	7.19	7.17

2.2. Methodology Biotechnological Research

Two preparations were used for the research:

1. "DBC Plus Type R5" Biopreparation (BioArcus Company, Warsaw, Poland)
   This biopreparation was used in two forms:
   - As granule—lyophilised bacteria;
   - As bacteria previously multiplied on nutrient broth (the bacterial population was determined by the culture method and amounted to 82×10^7).
2. "ACS ODO-1" Biopreparation (ACS Company, Chęciny, Poland).

Mother biomass compositions were:

"ACS ODO-1" biopreparation: Water, a consortium of lactic acid bacteria, phototrophic bacteria, yeast, ecological molasses from sugar cane, fermented wheat bran, minerals. The additional ingredients of biopreparation at the micro-level: Phytosterols (sitosterol, taraxasterol), phytohormones, triterpenes (lupeol, betulin, betulinic acid), flavonoids (hyperoside, quercetin, kaempferol), ellagic acid, pyrocatechic acid, brevofolin (ellagic acid derivative), vitamins (C, PP, P, B3, B5, B8, B11, B1, B2, A, E, F), and tannins.

Studies on the effectiveness of biopreparations were conducted in open flasks of 1500 mL capacity. The volume of raw water was 1000 mL. The research was conducted in three variants:

Variant 1. "DBC" Plus Type R5 granule biopreparation was added directly to the raw water in the following doses: 0.5 g, 1.0 g, 2.0 g (dose per 100 mL). After 10 days, the biopreparation "ACS ODO-1" in a liquid form was added to the water in the same dose of 0.5 mL per 100 mL of treated water.

Variant 2. Microorganisms cultivated on nutrient broth were added to the raw water in doses of 100, 500, and 1000 µL per 100 mL of water. After 10 days, biopreparation "ACS ODO-1" was added to the water in a dose of 0. 5 mL per 100 mL of treated water.

Variant 3. The water was subjected to the filtration process. A filter filled with diatomite (fraction 1–1.2 mm) was used for the filtration process; the contact time between water and the deposit was 25 min. "DBC" biopreparation was added to the filtered water using the cultivated bacteria in the doses of 100, 500, and 1000 µL. After 10 days, the biopreparation "ACS ODO-1" was added.

The experimental samples were open, mixed daily, incubated at 22 °C. Each sample was carried out three times.

2.3. Methodolody for Determining Physicochemical and Microbiological Properties of Water

The following water parameters were controlled (Table 2.):

Table 2. Scope and methodology for determining physicochemical and microbiological properties of water.

Tested Parameter	Method/Standard
Turbidity	Nephelometric method; PN-EN ISO 7027: 2003
Total Organic Carbon (TOC)	Sievers TOC analyzer 5310 C; PN-EN 1484: 1999
ATP (Adenosine triphosphate) concentration	Luminometric determination; www.promega.com/protocols (accessed on 10 June 2021)
Enumeration of microorganisms	Partec Cube 6 flow cytometer (FCM)

The total number of microorganisms present in the tested waters was determined using flow cytometry. Cells were counted using Partec Cube 6 flow cytometer (Sysmex-Partec, Görlitz, Germany). The dye is perfect for excitation by the blue argon laser line at 488 nm: SYBRGreen I nucleic acid stain (10,000× diluted in DMSO) [38]. Determinations were performed for the sample of 100 µL. The results obtained, i.e., the number of particles in µL was converted to the number of particles present in 1 mL of the tested water, taking into account the dilutions used earlier. The final result was the number of particles—bacteria with a high content of nucleic acids (HNA-high DNA content). FCM analysis with fluorescence dye was performed according to the method described in [39,40].

A Glomax luminometer (Promega, Madison, WI, USA) was used to determine ATP (Adenosine triphosphate) concentration in microbial cells. In the first stage, the reagent for the determination was prepared. The two solutions, BacTiter-GloTM Buffer and BacTiter-GloTM Substrate reached room temperature, and then the appropriate volume of BacTiter-GloTM Buffer was added to the bottle containing BacTiter-GloTM Substrate. The resulting reagent was gently stirred to obtain a homogeneous solution. The prepared reagent was stored at −18 °C. Prior to the determination, the reagent was placed in a sand bath at 37 °C. For the determination of total ATP (intracellular and extracellular), 100 µL of tested water was taken into a tube. The collected water was then placed in a sand bath for 30 s. After heating, 100 µL of reagent was added to the tube. The sample was mixed with a vortex motion, placed in a luminometer and, the RLU (Relative Light Unit) was interpreted. The value (RLU/100 µL) was reported as the final result.

Every determination was performed three times. The final score reported in the article was the arithmetic mean of these results.

3. Results

3.1. Variant 1

By analyzing the TOC (Total Organic Carbon) values at different doses of the biopreparation, an increase in the concentration of TOC values in the tested samples can be observed

TOC concentration in raw water was 309 ppm). In the first days of the experiment, the maximum values reached 457 ppm, 523 ppm, and 840 ppm for the doses of 0.5 g, 1.0 g, and 2.0 g, respectively. Since a decrease in TOC values was observed in the analyzed samples (in the sample with the highest applied dose of biopreparation "DBC" from the 7th day of the experiment), the second biopreparation used in the study was added to the samples. After the addition of biopreparation "ACS ODO-1", an increase in TOC values was observed for another 5 days. In the samples with higher doses of the biopreparation, a faster increase in the TOC values was again observed, reaching maximum values of 1168 ppm for the doe of 2.0 g, 848 ppm for the dose of 1.0 g, and 570 ppm for the dose of 0.5 g (Figure 1). This suggests faster dose-dependent growth of microorganisms from the biopreparation. The water changed colour from brown to yellow. Turbidity was very high at 180 NTU. Visually, the samples showed sedimentation of larger suspensions, and the turbidity decreased to the value of 24 NTU. From the 15th day of the experiment, a decrease in the value of TOC was observed. This was most likely related to the depletion of biogenic compounds in the permanent culture. The final percentages of reduction in TOC values in the samples were: 75.7% for the dose of 0.5 g, 69.6% for the dose of 1.0 g, and 59.9% for the dose of 2.0 g of the "DBC" biopreparation.

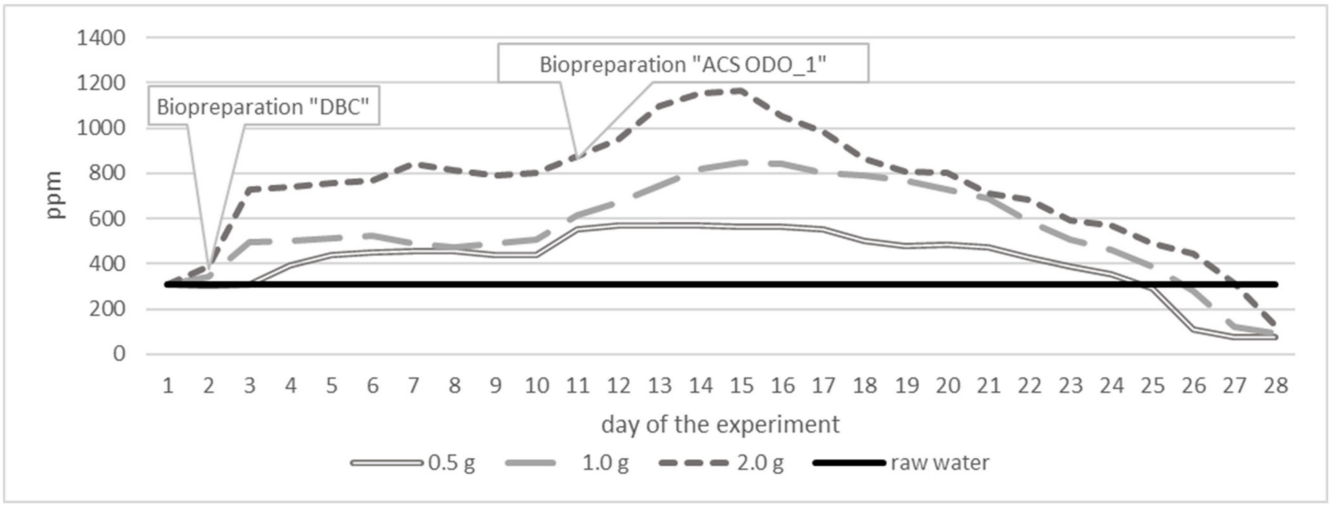


Figure 1. TOC values—variant 1 of the research.

Analyzing the RLU values in the studied samples, very fast growth of the microbial population from the biopreparation can be observed. At the dose of 2.0 g, there was an increase in RLU values to 380,883,520. At 1.0 and 2.0 g doses, after a rapid increase in RLU value, on the 4th day of the experiment, a decrease in these values was observed, respectively, to the level of approx. 38 mil and 42 mil Adding the biopreparation "ACS ODO-1" resulted in the renewed increase in the RLU value, however, after 3 days, flotation and sedimentation of the suspensions were observed in the samples, and these values decreased again (Figure 2). On the final days of the experiment, the RLU values in the tested samples were approx. 8 mil for the dose of 0.5 g, 6 mil for the dose of 1.0 g, and 3 mil for the dose of 2.0 g, respectively.

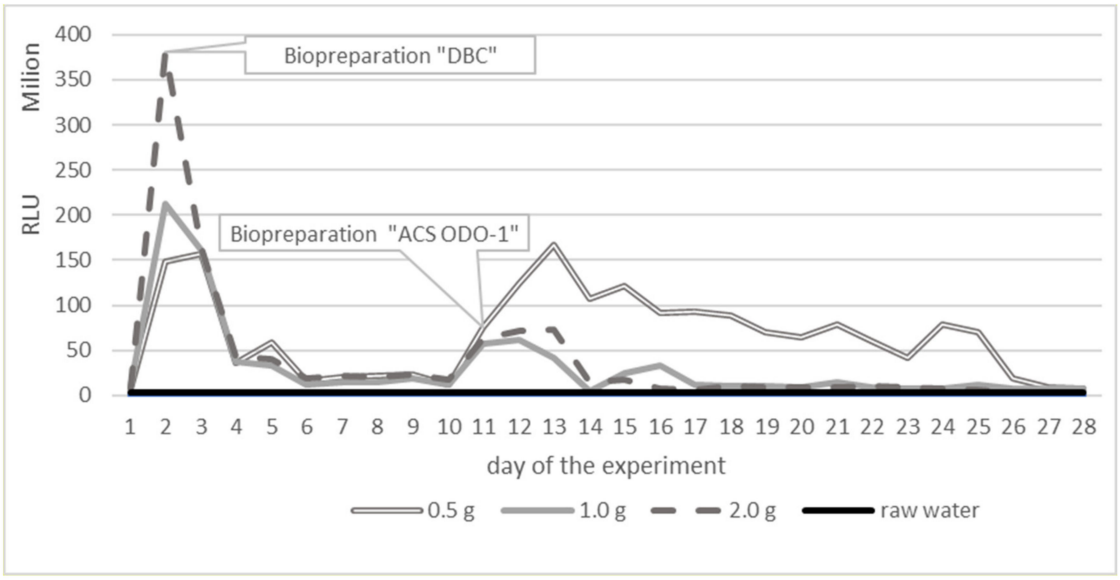


Figure 2. RLU values—variant 1 of the research.

The results of the flow cytometric analysis (FCM)—the number of particles measured—show similar trends. The size of the population gradually increased during the first days of the experiment; a decrease in the number of particles was observed from the 6th day for the sample with the highest dose relevant to the study. The addition of the biopreparation "ACS ODO-1" on day 10 of the experiment resulted in a renewed increase in the number of particles, which in this case clearly translates into population sizes (Figure 3). The size of HNA across individual samples varied with the duration of the experiment. For the dose of 0.5 g, compared to raw water (HNA-30,368), the HNA size increased to a maximum of 831,749 particles (mL) to decrease to 495,861 particles at the end of the experiment. Similarly, in subsequent samples, the maximum size of the HNA population reached 1,092,509 particles (mL) for 1.0 g and 918,461 particles (mL) for 2.0 g to finally decrease to 474,877 particles (mL) (1.0 g) and 575,712 particles (mL) (2.0 g).

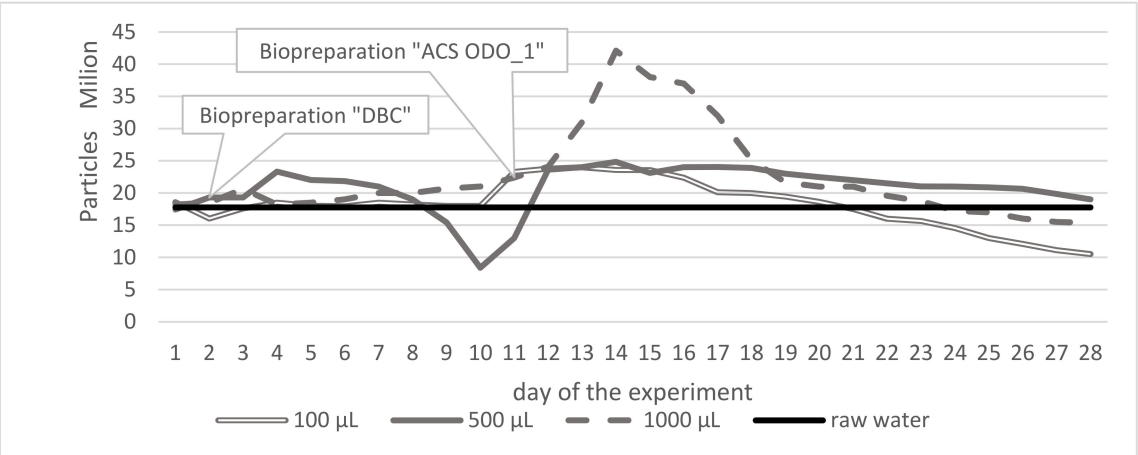


Figure 3. FCM values–variant 1 of the research.

Looking at the raw water graph (Figure 4.), one can see a separated region of LNA and HNA, with the LNA region having significantly more particles: LNA 1,028 549 (68%) and HNA 30,368 (2%). The graphs from the 5th and 25th after dosing the "DBC" biopreparation are presented as exemplary graphs from the cytomeric analysis. When analyzing two-dimensional (FL1/SSC) diagrams (Figure 4b–g), two populations can be distinguished, but the HNA group (which was mostly bacteria) is the dominant group. In the sample with

1.0 g of biopreparation, two clearly separated populations were observed at the beginning of the study. Over time, one of the groups began to disappear, and the remaining bacteria began to form one single, enlarging group (Figure 4c,f). The dose of 0.5 g sample looks similar. However, in the second image (after 25 days), although the second population cannot be clearly separated, the LNA remnant is still visible—in the form of a thin streak (Figure 4b,e). Throughout the study, only one population was visible in the sample with 2.0 g of biopreparation (Figure 4d,g). This indicates the fastest dominance of bacteria from the biopreparation used at this dose.

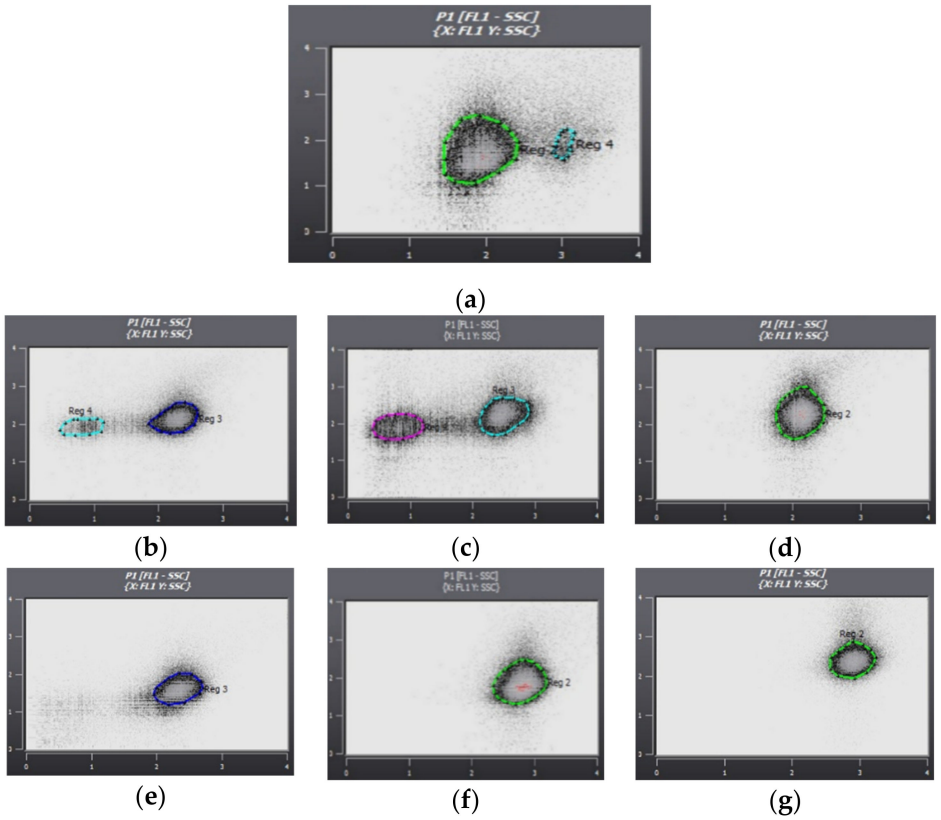


Figure 4. FCM analysis of raw water and raw water with the addition of 0.5, 1.0, and 2.0 g of biopreparation. FCM analysis of raw water, on collection day (FL1 (x): SSC (y)), HNA-Reg.4 (**a**) FCM analysis of raw water with the addition of 0.5 g of biopreparation (5 days) HNA-Reg. 3 (**b**) FCM analysis of raw water with the addition of 1.0 g of biopreparation (5 days) HNA-Reg. 3 (**c**) FCM analysis of raw water with the addition of 2.0 g of biopreparation (5 days) HNA-Reg. 2 (**d**) FCM analysis of raw water with the addition of 0.5 g of biopreparation (day 25) HNA-Reg. 3 (**e**) FCM analysis of raw water with the addition of 1.0 g of biopreparation (day 25) HNA-Reg. 2 (**f**) FCM analysis of raw water with the addition of 2.0 g of biopreparation (day 25) HNA-Reg. 2 (**g**).

3.2. Variant 2

Dosing the multiplied bacteria to the raw water resulted in a gradual decrease in the TOC value in the examined water regardless of the dose applied. This indicates the gradual biodegradation of organic compounds in the water, while their dose did not allow the natural microflora to dominate.

As no change in colour or turbidity was observed in the tests (turbidity was 45 NTU—a value comparable to raw water), it was decided to administer the "ACS ODO-1" biopreparation. TOC values started to increase slowly over a period of 6 days and then declined. The final result is a TOC reduction: 36.9% for the dose of 100 µL, 40.8% for the dose of 500 µL, and 42.1% for the dose of 1000 µL (Figure 5). The water began to change from brown to orange. A suspension appeared in the water and quickly sedimented to

the bottom. This is confirmed by the analysis of the RLU value (Figure 6) and the FCM analysis (Figure 7).

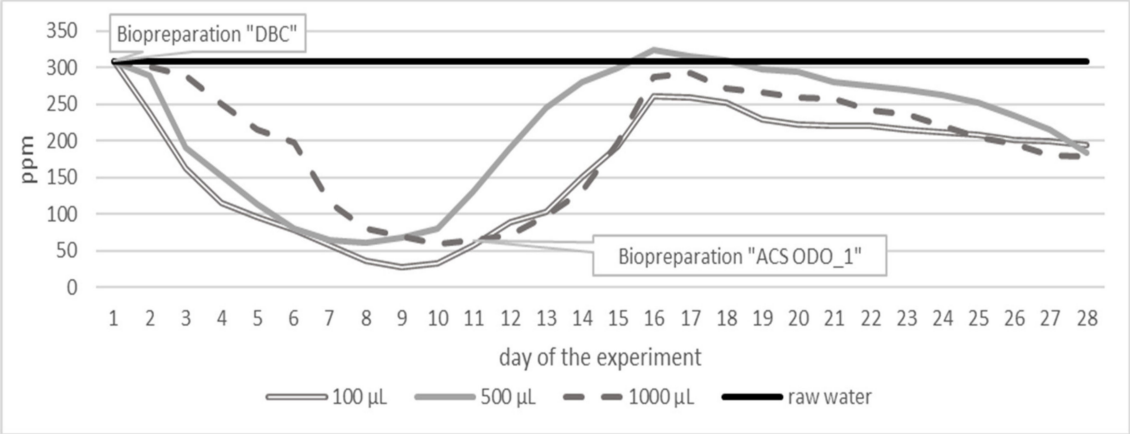


Figure 5. TOC values—variant 2 of the research.

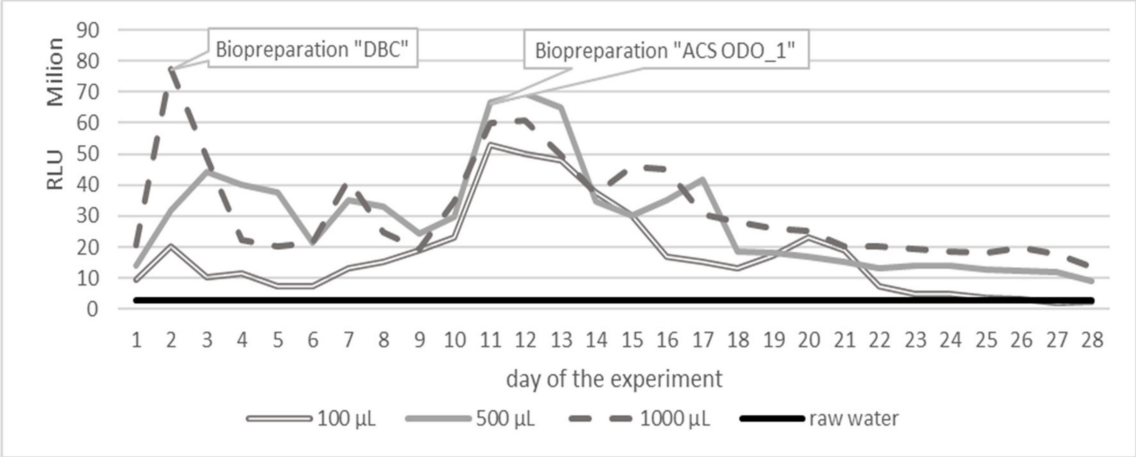


Figure 6. RLU values—variant 2 of the research.

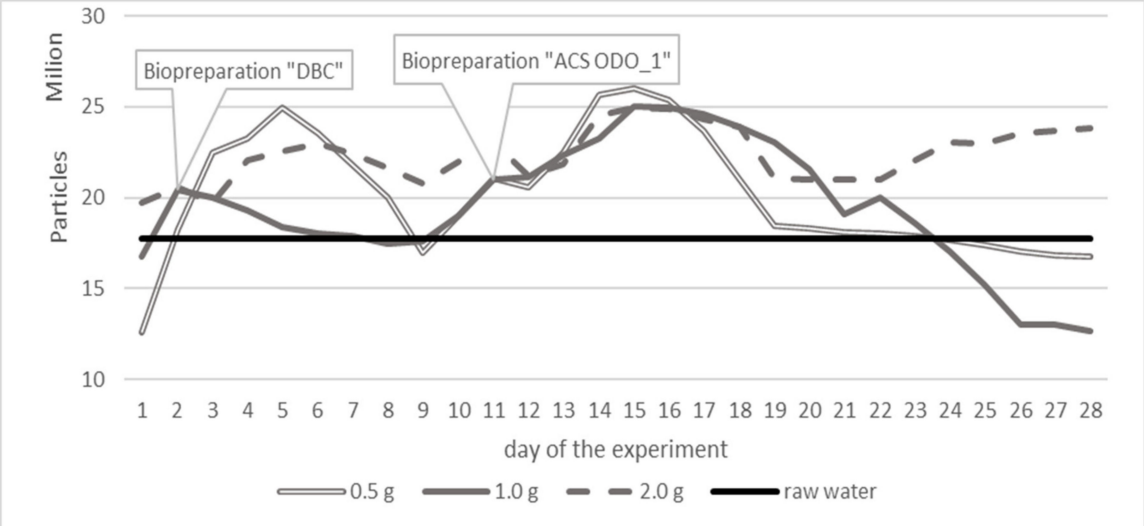
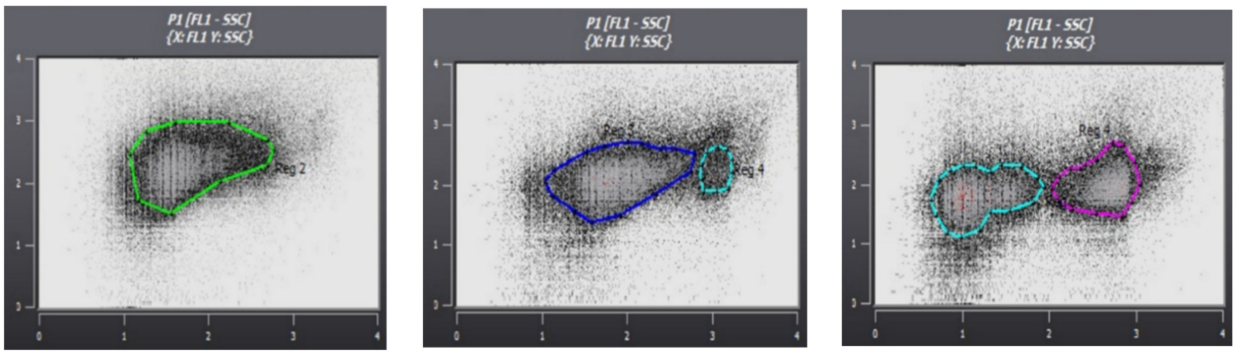


Figure 7. FCM values—variant 2 of the research.

The addition of the "DBC" biopreparation to the analyzed samples resulted in a sharp but short-term increase in the microbial content of the tested liquid. It can be observed in the 1000 µL sample, where the RLU value increased in the first days of the study from 3,018,703 (Raw Water RLU) to approx. 77 mil on the second day of the experiment. At smaller doses (500 and 100 µL in turn), a temporary increase in RLU was also observed, but it was not as high as at the highest dose applied. The increase was again recorded around the 11th day of the study, where RLU values doubled for all three doses. From day 14, RLU values started to decline (Figure 6).

When analyzing the image of dot plots from cytometric analysis in the tested samples with the dose of 100 µL of biopreparation, the formation of only one group of the bacterial population can be observed (image from the 5th day of the experiment). Over the next few days, the shape of the population did not change. However, the number of bacteria changed—an increase in the number of bacteria in the study population was recorded. Compared to raw water (HNA-30,368 particles), the addition of 100 µL of multiplied bacteria resulted in the increase in the size of the population, reaching a maximum size equal to HNA-675,742 particles (Figure 8a).



(**a**) (**b**) (**c**)

Figure 8. FCM analysis of raw water with the addition 100, 500, and 1000 µL of biopreparation (5th day of the experiment) FCM analysis of raw water with the addition of 100 µL of biopreparation HNA-Reg.2 (**a**) FCM analysis of raw water with the addition of 500 µL of biopreparation. HNA-Reg.4 (**b**) FCM analysis of raw water with the addition of 1000 µL of biopreparation. HNA-Reg.4 (**c**).

A higher dose of biopreparation (500 µL) resulted in the separation of a second bacterial population. In Figure 8b, a large group of LNAs can be observed, with relatively small HNAs. Such values could indicate the toxic effects of chemicals in the water. In addition, fluctuations in RLU values would confirm the lack of monotonous bacterial growth from the biopreparation. Visually, the image from the analysis of the sample with the dose of 500 µL resembles raw water, but the size of the HNA, even at the beginning of the study, was almost three times bigger (HNA-76.713).

Doubling the biopreparation dose (from 500 to 1000 µL) resulted in a clear separation of the second population. However, the abundance in the HNA and LNA group can be considered similar. (Figure 8c), which indicates the lack of dominance of bacteria from the applied biopreparations.

3.3. Variant 3

The diatomite bed filtration process resulted in a nearly 3-fold reduction in organic carbon concentration (from 309 ppm for raw water to 121 ppm for filtered water). The decrease in the value of TOC was 60.8%. Because of the addition of bacteria multiplying on the broth, the value of this parameter increased (Figure 9). After adding the "ACS ODO-1" biopreparation, there was an intensive development growth of microorganisms. To the samples to which the dose of 1000 µL of bacteria was added, the TOC value was 697 ppm.

After two weeks, there was a decrease in the TOC value and the RLU value, which can indicate the death of the microflora. The final TOC values in the investigated waters were 58 ppm at the dose of 100 µL, 31 ppm at the dose of 500 µL, and 64 ppm at the dose of 1000 µL. The water changed its colour from brown to straw yellow. The suspensions sank to the bottom.

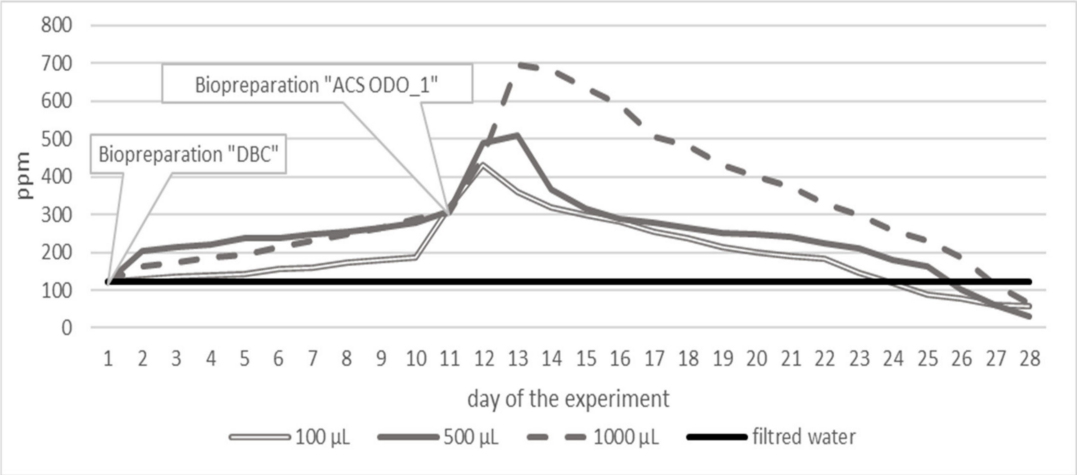


Figure 9. TOC values—variant 3 of the research.

The results of the TOC analysis confirm the RLU values. The water filtration resulted in a decrease in RLU values from 3,018,703 for raw water to 2,001,635 RLU. The next days of the experiment show a slow development of the microflora from the biopreparation "DBC" and a second, clearer that started on the 11th day of the experiment—after the addition of the biopreparation "ACS ODO-1" (Figure 10). At the peak of the study, RLU values reached, respectively: 133 mil at the dose of 100 µL, 115 mil RLU at the dose of 500 µL, and 158 mil at the dose of 1000 µL.

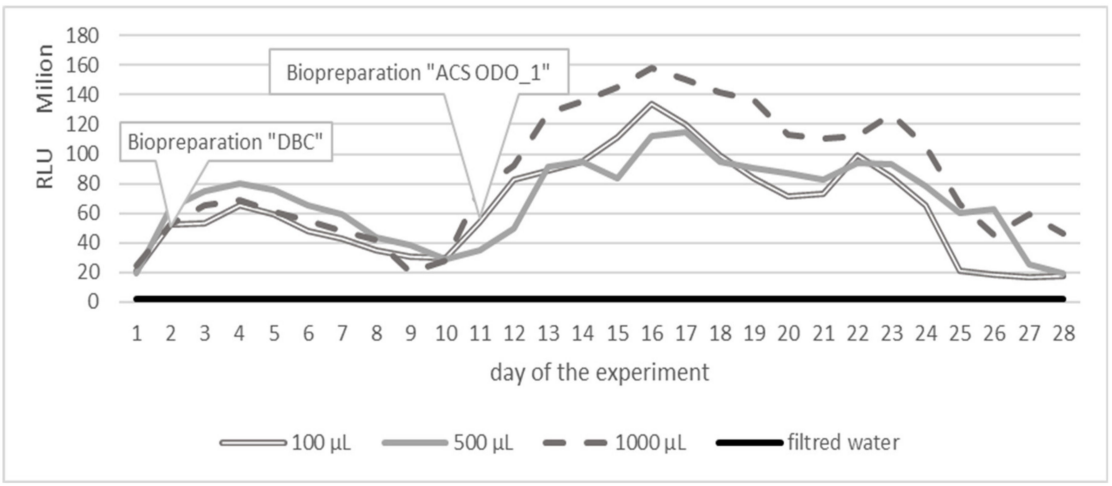


Figure 10. RLU values—variant 3 of the research.

The FCM analysis also confirms the above trends. By cytometric analysis of filtered water with biopreparations, respectively: "DBC" and "ACS ODO-1", both on the 5th and 25th day of the study, irrespective of the applied dose (100, 500, or 1000 µL), only one HNA population was isolated. Its size, counted in particles, increased in the first days of the experiment, then decreased and increased again around day 12 (Figure 11). The change in population size was due to the addition of another biopreparation.

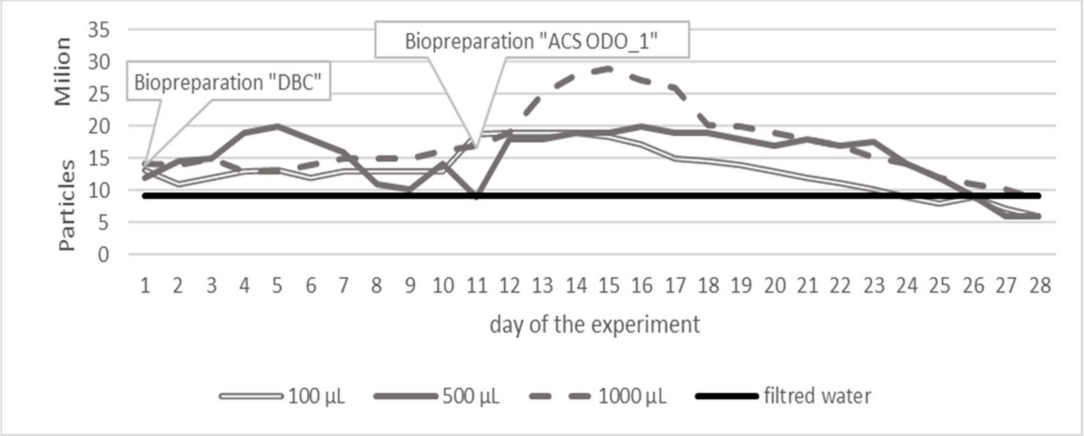


Figure 11. FCM values—variant 3 of the research.

When analyzing images from dot plots (Figure 12), one separated group of HNA can be distinguished. Visually, the image of the population did not change over the survey days. Compared to the 100 and 500 µL samples, the population isolated in the 1000 µL sample was slightly larger from the start of the study, and at the time of the largest "jump" (around day 14–15), the size difference between the 100 and 500 µL and 1000 µL populations was as much as 10,000 particles.

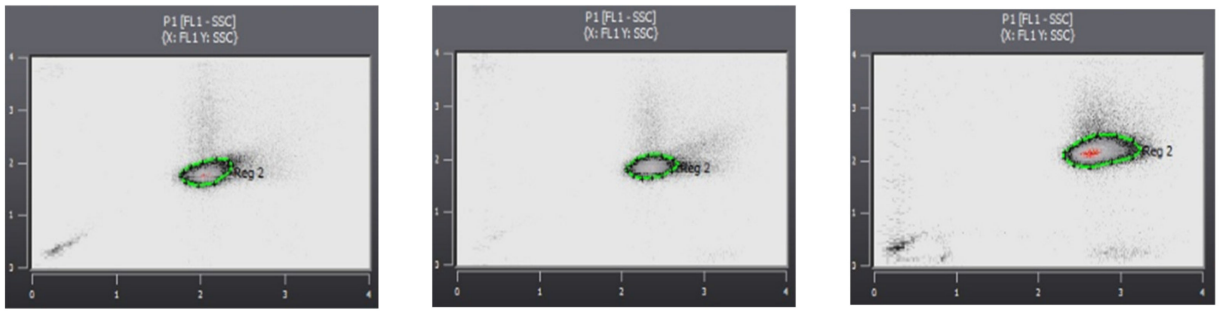


Figure 12. FCM analysis of filtered water with the addition 100, 500, and 1000 µL of biopreparation (5th day of the experiment) FCM analysis of filtered water with the addition of 100 µL of biopreparation HNA-Reg.2 (**a**) FCM analysis of filtered water with the addition of 500 µL of biopreparation HNA-Reg.2 (**b**) FCM analysis of filtered water with the addition of 1000 µL of biopreparation HNA-Reg.2 (**c**).

4. Discussion

Water could be treated by other methods. Physical, chemical, and electrochemical methods could be used [20]. However, given the size of this reservoir and the fact that it is a still water reservoir, these methods would be expensive. The diatomite bed filtration method already used was very effective. However, performing such a process in a large surface water body is not easy. The dosage of microorganisms that will effectively metabolize the pollutants seems most practical in this case, especially since it has been shown to be effective in studies.

Biological recultivation has all the advantages. It turned out that there are microorganisms that are able to metabolize such a toxic chemical mixture—a simple and relatively cheap method for this volume of water. A bacterial biopreparation was selected for the research, which according to the manufacturer, is to conduct the process of biodegradation of phenol and petroleum compounds. The current offer of companies did not include biopreparations that would eliminate cyanides and thirates, which were present in the tested water in high concentrations. The "DBC type R-5" biopreparation contained mi-

croorganisms that decompose phenol and its derivatives, aromatic hydrocarbons, benzene, toluene, and other aromatic hydrocarbons (hydroxylated and nitrated). Due to the high toxicity of such compounds, longer acclimatization periods are often required to achieve adequate levels of biological activity [41].

The basic criteria for the selection of strains of microorganisms used for the biodegradation of chemical pollutants were: the ability to quickly adapt the strain to grow in a contaminated environment, substrate absorption rate, and non-toxic biodegradation products [5]. As microbial decomposition of pollutants may be hindered by the hydrophobic nature of other substances present and the associated limited availability for bacterial cells, a desirable feature of bioaugmentation candidates is their ability to synthesize surfactants, i.e., biosurfactants [42,43]. Taking into account the fact that the RLU values in the raw water indicated the presence of microflora (values characteristic for waters moderately polluted with organic matter), the natural microflora was adapted to the living conditions in the presence of many toxic compounds.

Amongst them, very high phenol concentrations were recorded in this water. According to Nwanaanwu and Abu, 2013 [44], phenol may affect the metabolic processes of microorganisms, which can often lead to their death. If they are able to acclimatize, they will conduct phenol biodegradation processes [45].

Phenol toxicity has often been tested on microorganisms. In *Pseudomonas* sp. and *Escherichia* sp., phenol gradually inhibited dehydrogenase activity at concentrations of 200–1400 mg/l throughout the exposure. Toxicity and phenol concentration limits vary depending on bacterial strains and exposure time and indicate that bacteria can acclimate to phenol as exposure time increases. Phenol has also shown negative effects on the nitrification process in wastewater treatment [46–50].

High concentrations of PAH (polycyclic aromatic hydrocarbons) were also detected in the treated water. Of the PAHs, eight hydrocarbons were listed (anthracene, fluoranthene, naphthalene, benzo (a) pyrene, benzo (b) fluoranthene, benzo (k) fluoranthene, benzo (ghi) perylene, indeno (123 cd) pyrene. Microorganisms are usually not capable of directly degrading xenobiotics, such as PAHs. Therefore, what is necessary is the adaptation of the microflora to biodegrade hydrocarbons present in a given environment. Microorganisms, in order to produce the appropriate enzymes, need a certain time, which depends on the type of organisms and the properties of the hydrocarbon. Many bacteria and fungi have the ability to degrade PAHs, but any species has the ability to produce enzymes that could degrade all the pollutants in this group. Biodegradation of PAHs is, therefore, a multi-step process involving many microorganisms that often exhibit synergistic effects towards each other. PAH mineralization can be carried out by pure bacterial strains, mixed populations, fungi, actinobacteria, cyanobacteria, and algae. Bacteria have the highest capacity to biotransform PAHs. In PAH decomposition processes, the degradation products can be significantly more toxic and more difficult to biodegrade than the original substances. Such a mechanism would explain the fluctuations in microbial population growth from the biopreparation at appropriate doses. Adaptation to the appearance of a new toxic compound may have resulted in temporary inhibition of bacterial growth and its slow development [50,51].

Despite the toxicity of cyanide, many organisms, including bacteria, fungi, plants, and some animals, synthesize cyanide, which is usually a defense mechanism (cyanogenic organisms), and some microorganisms can assimilate cyanide, using it as a nitrogen source for growth (cyanotrophic organisms). These microorganisms have different cyanide degradation pathways that are based on hydrolytic, reductive, oxidative, or substitution/addition reactions [52–55]. That is why cyanide biodegradation has become a suitable alternative to less efficient and more expensive chemical treatment methods for cyanide removal from industrial waste. Water containing cyanide ions is treated by the action of sulfur dioxide (hydronadoxysulfuric acid), chlorination process and/or aeration. Chlorination-based techniques are only effective for free cyanides and weak metal complexes. Other methods, such as ozonation or reverse osmosis, are very expensive or ineffective. Biological

treatment of water from cyanide is possible thanks to the microorganisms, such as fungi (e.g., *Fusarium solani*) and bacteria (e.g., *Pseudomonas fluorescens*). Under aerobic conditions and in the presence of glucose, microorganisms use ferrocyanide as a source of nitrogen and carbon. Under both aerobic and anaerobic conditions, ammonia, carbon dioxide, and formate are formed as end products. The best biodegradability conditions of cyanide ions were observed with glucose at a concentration of 0.0465 g/L and pH = 5 [56,57].

In the first variant of the study, the biopreparation was applied according to the manufacturer's instructions. Such studies were the first to be conducted due to the fact that the raw water contained a number of highly toxic compounds that could theoretically prevent the microorganisms from operating. However, despite the very high concentrations of many toxic compounds, microorganisms grew very quickly, and a reduction effect TOC (68%) was achieved after only 14 days, at the lowest biopreparation dose applied. The literature provides the use of biopreparations for biodegradation of petroleum compounds in the aquatic environment (3 items from the very end), but the doses used are the secret of companies engaged in their production and/or distribution.

In the conducted research, the highest dose gave the fastest organic compound reduction effect. The obtained final concentration of TOC and the visual effect of treated water indicated that the microorganisms actioned. After 20 days, the water was clear, and it changed its colour from dark brown to straw yellow. Visible suspensions were sedimented. The water no longer had the characteristic chemical smell. The used "DBC" biopreparation, when applied to the surface water with significant volumes, generates enormous costs. That is why doses of half, and a quarter lower were also used in the studies. Half the dose was equally effective. The dose reduced to a quarter was no longer as effective as half the dose. Microorganisms developed much slower in this water, as evidenced by the RLU values. TOC reduction occurred but amounted to only 40%. The literature provides a few examples of the practical application of the "ACS ODO-1" biopreparation used in the research. Microorganisms from biomass mixtures introduced into the reservoir water also reduced the level of organic pollution to a safe state for aquatic ecosystems [5]. The cytometric analysis confirmed the rapid bacterial dominance in the samples with the highest dose used in the study, while at lower doses, this dominance was not so well visible.

Due to the enormous cost of using the "DBC" biopreparation in the Kalina reservoir, an attempt was made to multiply the bacteria earlier on the broth. The applied dose of the biopreparation was about 100 times lower than the dose used in the first variant of the study. Both the TOC and RLU values, as well as the flow cytometry image, indicated that there was no rapid bacterial growth from the biopreparation used. Such fluctuations in the number of microorganisms prove that it was difficult for bacteria to acclimatize to water with such a large number of toxic compounds. Microorganisms exposed to unfavorable factors are subject to metabolic stress. It is a condition, also described as VBNC (viable but nonculturable), into which bacteria go spontaneously by activating certain defense mechanisms. Cells in this condition are characterized by reduced physiological and metabolic activity, synthesis of specific proteins that help to increase resistance to a stress factor, modifications of the cell membrane, or changes in the shape of cells [58]. Metabolic stress can be activated by various factors, such as osmotic pressure, temperature, pH, the presence of impurities, and the availability of nutrients (carbon, nitrogen, and phosphorus).

In the second variant of the study, only after 3 weeks from the application of biopreparations for phenol decomposition and biopreparation "ACS ODO-1", the development of microflora can be observed. Analyzing the RLU values, it can be observed that a higher dose of the multiplied biopreparation resulted in faster development of microorganisms. The end result was an approximate 60% TOC reduction. Visually, the water did not differ from the water with the biopreparation applied in loose form.

The third variant of the research was the filtration through the diatomite bed. Numerous literatures indicates filtration on zeolite beds as an effective method of removing petroleum compounds and phenols. Therefore, another variant of water treatment

from Kalina pond was filtration through a diatomite bed and then adding a biopreparation. Research under dynamic conditions allowed assessing the biosorption of petroleum substances on the bed, and that bed can be considered good for bacteria decomposing petroleum compounds [59]. Zeolites are molecular sieves that allow for the separation of some substances from others; they have the ability to exchange ions, which are a part of their structure, with others present in the environment. They are also excellent sorbents for many chemical compounds, often dangerous pollutants in our environment. These specific features of zeolites are due to their structure, which consists of a system of chambers and channels. That system makes these minerals porous and characterized by a very large specific surface area, which determines the effective course of the above-mentioned processes. An important aspect of zeolites, especially natural ones, is their availability and price. In addition, they can regenerate and can be used multiple times, which further reduces the cost of their use. An additional advantage of using such a technology would be the possibility of using the biodegradation process on the bed. The development of bacteria on the bed conducting the biosorption process allowed for the use of lower doses of the biopreparation into the water after the filtration process. In the conducted research, a 45% reduction in TOC was obtained after the filtration process through the diatomite bed. After the addition of biopreparation, microorganisms multiplied on the broth which resulted in the rapid growth of bacteria and an increase in the RLU value. In this variant of the research, fluctuations in the RLU value were also observed, similarly to variant 2; however, changes in the colour of the water and sedimentation of the formed suspensions were observed much faster [60].

5. Conclusions

In all three tested variants of biodegradation of pollutants into the water from the Kalina reservoir, a good cleaning effect was obtained, which was manifested in the reduction in TOC, change in the watercolour from brown to straw yellow, and reduction in turbidity.

The optimal way to rehabilitate water from a reservoir contaminated with leachate is to filter that water. After the filtration process, the obtained percentage of TOC reduction allowed for the rapid growth of microorganisms from the biopreparation, despite the 100 times lower dose used. Then, biopreparations can be applied to the water after the filtration process. The required doses will be significantly lower, taking into account the fact that a biological membrane should form on the bed during the filtration process.

The use of a biopreparation in the form of direct dosing of microorganisms to polluted water gives a very quick effect of biodegradation of pollutants. Despite the high concentration of numerous toxic compounds, this form of biopreparation application is effective.

The use of biopreparation in the form of multiplied bacteria was not really effective. The bacteria could not acclimatize, and the biodegradation effect could only be observed 4 weeks after administration.

Both the luminometry and flow cytometry techniques allow for a quick assessment of the development of microorganisms. Flow cytometry, however, seems to be more useful for determining the optimal dose of the biopreparation. This technique allows for a quick assessment of the development of microorganisms from the dosed biopreparation.

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