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Modeling of Effect of *Pseudomonas aureofaciens* AP-9 on Bioremediation of Phenol-Contaminated River Sediments

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Abstract: One of the most widespread and risky pollutants in the environment is phenol. It is a by-product of many industrial, agricultural, and other anthropogenic activities. Microbial-assisted transformation, known as bioremediation, is an effective and cheap method for treating groundwater, soil, and sediments contaminated with phenol and its derivates. This study aims to assess the effect of the addition of a selected, pre-adapted bacterial strain *Pseudomonas aureofaciens* AP-9 on key kinetic, microbiological, and enzymological parameters of simulated bioremediation processes for the removal of phenol (250 mg/kg). The early effect of adding this microbial biodegradant in contaminated sediments is insignificant. The effect of added bacteria is manifested at the 48th hour by a restructuring of the microbial sediment communities and an increase in the number of cultivated microorganisms. This preparation of the sediment communities for a prolonged detoxification process is also confirmed by the repeated induction and very high increase in the activity of the enzymes directly involved in the cleavage of the benzene ring. The effectiveness of phenol removal at the 48th hour is increased by 15%, too. Considering this stimulation of a sustainable long-term bioremediation process, we can conclude that microbiological pre-adapted inoculants are an important mechanism for the management of bioremediation detoxification processes and can increase their effectiveness.

Keywords: biodetoxification; bioremediation; sediments; phenol-degraded bacteria; pollutants

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1. Introduction

Xenobiotic pollutants such as phenolics, polycyclic aromatic hydrocarbons, halogenated compounds, pesticides, azodyes, nitroaromatic compounds, and per- and polyfluoroalkyl substances have a serious negative impact when entering ecosystems due to their high toxicity, recalcitrance, and slow or nonexistent degradation [1,2]. Microbial-assisted transformation which relies on the metabolic capacities of microorganisms is one of the most effective and cheap methods for treating groundwater, soil, and sediments contaminated with high concentrations of these hazardous chemicals [3–6]. These transformation processes are used in bioremediation–biological decontamination of the environment. In a broader sense, bioremediation includes natural self-purification processes running based on the biochemical activity of the indigenous microbial population and special-designed technologies applied "in-site" and "ex-site" [7–12]. In these bioremediation technologies, an important issue is the possibility of stimulating and increasing the rate limiting steps of removal processes by bioaugmentation. The bioaugmentation is a mechanism for managing pollutant biodegradation by the addition of selected, pre-adapted microorganisms or enzymes with high biodegradation ability to specific pollutants [13–24]. The augmenting biological system usually increases the effectiveness of pollutant removal by unlocking or facilitating some biochemical pathways and expanding the metabolic profile of the

whole microbial community. During the accelerated biotransformation, the structure of the microbial community may change too and some species become dominant, while other populations persist as minorities [25].

Among the huge diversity of hazardous contaminants where the application of bioaugmentation has the potential to lead to the real improvement of bioremediation technologies, phenol and its derivatives are the chemicals of great scientific interest. Phenol is one of the most frequently used model pollutants for studying the biodegradation of xenobiotics because: (1) The global phenol market volume is about 12 million metric tons for 2023 [26]; (2) Phenol is widely used in different industries, agricultural, and municipal sectors; it has relatively good water solubility and has a harmful effect on biota; (3) The mechanisms of phenolic biodegradation have been studied in detail [27]; (4) Microorganisms that degrade phenol are well studied (e.g., *Achromobacter* sp., *Pseudomonas* sp., *Acinetobacter* sp., *Bacillus* sp., *Arthobacter* sp., *Halomonas* sp., *Scenedesmus* sp., *Chlorella* sp., *Rhodosporidium* sp.) [28,29]; (5) Phenol is a relatively easily degradable xenobiotic for microbial degradants, but at a higher concentration it can inhibit the growth of microbial cultures and communities [19]; (6) Microbial degradation is an economically efficient and ecological method for removing phenol [19,27]. Phenolics have a common structure comprising an aromatic ring/rings with one or more hydroxyl substituents. These organic compounds have good chemical resistance but their structure is well known to living organisms due to its natural occurrence. Phenol biodegradation in aerobic conditions consists of three stages; the first stage is the enzymatic conversion of phenol to catechol by adding one oxygen atom. Different dioxygenase enzymes cleave the aromatic ring in the second stage, which is characterized by two distinct pathways for catechol metabolism: ortho- and meta-mechanisms. Key metabolic products of the central metabolic pathways are the result of the benzene ring cleavage as well as several subsequent transformation reactions from the third stage [19,30,31].

In this work, we studied the effect of the addition of a selected bacterial strain with a high aryl-biodegradable potential into phenol-contaminated sediments. The used bacterial strain *P. aureofaciens* AP-9 belongs to one of the well-studied biodegradants—the genus *Pseudomonas*. Phenol biodegradation in lab conditions using pure or mixed cultures of pseudomonads has been studied in detail but there is still a need to model the processes of biological stimulation of biodegradation in conditions close to the complexity of real ecosystems [19,32]. This study aims to assess the effect of the *P. aureofaciens* AP-9 strain on key kinetic, microbiological, and enzymological parameters of simulated bioremediation processes for the removal of phenol.

2. Materials and Methods

2.1. Experimental Design of Model Bioremediation Sites

The experiment aimed to evaluate the effect of the *P. aureofaciens* AP-9 strain on the phenol elimination processes when a model toxic pollutant was used in laboratory conditions on model bioremediation sites (Figure 1).

The working hypothesis of the present study is based on the understanding that to reveal the bioaugmentation mechanisms of new promising modulators on detoxification processes occurring in a real environment, it is necessary to apply a new combination of established approaches in laboratory practice. This combination is based on analog modeling resembling real detoxification processes and classical bioremediation techniques. A long-term task is to propose algorithms for managing biodegradation processes or bioremediation technologies for sediment detoxification.

The design of the analog model of the biodetoxification process of phenol-contaminated sediments and the bioaugmenting factor *P. aureofaciens* AP-9 includes the creation of two bioremediation simulation sites. Bioaugmentation with a bacterial culture of the genus (*P. aureofaciens* AP-9) provides an opportunity to further accelerate the biodetoxification processes in river sediments.

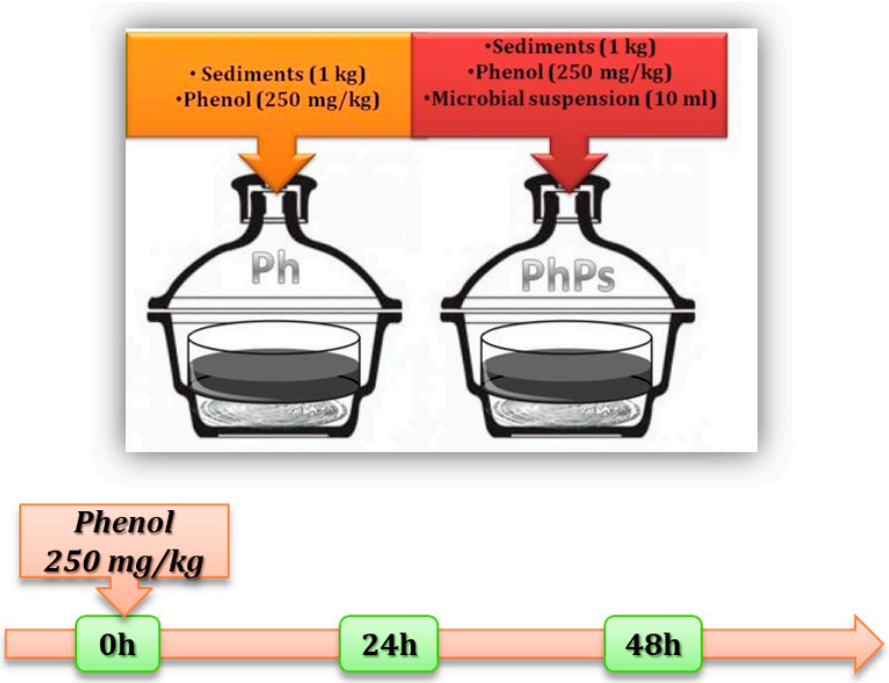

Figure 1. Experimental design with two variants of model bioremediation sites.

In a vessel with a large volume, river sediment from the Cerovo dam (MWPPs Middle Iskar) and a quartz sand were mixed. After good mixing, a synthetic saline medium was added to moisten the sediment.

In the first model system (Variant Ph), a laboratory bioremediation site containing 1 kg of sediment and a quartz sand with added phenol was made in a crystallizer.

In the second model system (Variant PhPs), a laboratory bioremediation site containing 1 kg of sediment and quartz sand with added phenol and microbial suspension (*P. aureofaciens* AP-9) was made in a crystallizer.

Phenol was added once at the 0th hour of the experiment and critical control points were used to monitor: 1. Whether there was a change in the microbial community at the very moment of intoxication; 2. The qualitative and quantitative distribution of culturable microorganisms; 3. System recovery time after phenol blast loading.

In order to track the efficiency and speed of phenol elimination, chemical, microbiological, and kinetic analyses of the sediments from the model system were carried out, as well as a complex of rationally selected enzymological indicators.

The aim of this model bioremediation is to track:

- The biodetoxification and adaptation processes of the autochthonous microbial community in a variant with the model pollutant phenol (Ph), (when simulating a critical situation with explosive addition of phenol with a concentration of 250 mg/kg, determined as critical).
- The biodetoxification and adaptation processes in a variant with phenol and modulating factor strain *P. aureofaciens* AP-9 (PhPs) (when simulating a critical situation with explosive addition of phenol with a concentration of 250 mg/kg).

Models of bioremediation sites for the treatment of heavily polluted sediments from the Tserovo dam at the sHPP Tserovo were simulated. The processes were monitored through a variety of chemical (residual concentration of phenol), microbiological (total number of aerobic heterotrophs, bacteria from genus *Pseudomonas*, and phenol-degrading bacteria), and kinetic (effectiveness of phenol eliminations and rate of phenol biodegradation) indicators.

2.2. Added Bacterial Culture

The bacterial cell culture of *P. aureufaciens* AP-9 added in Variant PhPs was selected based on its biodegrading potential in the Laboratory of 'Biological Water Treatment and Environmental Biotechnology' at the Faculty of Biology of Sofia University, Bulgaria. It was added to the model bioremediation sites in the form of a 24 h culture, cultured on nutrient agar, and washed with 0.9% solution of NaCl. It was added as 5% *v/v* as a microbial suspension with a density of 21.30 $\times$ 10^9 cells/mL (at 0th hour).

2.3. River Sediments

Sediment sampling was carried out from the surface bottom layer of the micro-dam at the "Tserovo" MWPP. Each of the two bioremediation simulation sites contains 1.5 kg of sediments. In addition to sediment from the Tserovo dam, each of the two bioremediation sites also contains 1.5 kg sterilized quartz sand (with a size of 0.8–1.6 mm).

2.4. Addedphenol and Sediment Moisture

At the 0th hour, 5 mL phenol (Ph) of 5% stock solution was added sterilely. This amount corresponds to a concentration of 250 mg/kg.

For the optimal course of phenolic biodetoxification, a humidity of 45% was maintained in the sediments of the two bioremediation sites using a synthetic salt medium. The synthetic salt medium (SSM) has the following composition: NaH_2PO_4−3.5 g/L, K_2HPO_4−5.0 g/L, $(NH_4)_2SO_4$−2.5 g/L, $MgSO_4$.7H2O−0.3 g/L, $FeSO_4$−0.05 mg/L, $CuSO_4$−0.01 mg/L, $ZnSO_4$−0.005 mg/L, $CoCl_2$−0.005 mg/L, $MgCl_2$−0.005 mg/L, $CaCl_2$−0.005 mg/L, Na_2MoO_4−0.005 mg/L, 3% nutrient solution (NaCl−5 g/L; peptone−10 g/L; yeast extract−5 g/L).

In addition to maintaining the necessary moisture, SSM is also used to regulate the C:N:P ratio, which ensures a minimum mineral background of the environment.

2.5. Chemical Indicators

Residual phenol was measured by the spectrophotometric method. After preliminary distillation, the samples were analyzed according to the specifications described in BDS and EPA methods [33,34]. The absorption was measured spectrophotometrically/Spectrophotometer-Pharmacia Biotech Ultrospec 3000/. All chemical reagents used in the measurement of residual phenol were supplied by Merck, Inc. Kenilworth, NJ, USA.

2.6. Microbiological Indicators

Assays were performed using the nutrient media described in Table 1. The Koch method was used for cultivation in Petri dishes with solid nutrient media that contain selective components that stimulate the development of certain groups of microorganisms [35,36]. Classical cultivation methods were used to determine the quantitative indicators of the microbial communities, by sowing a 0.1 mL sample of the corresponding dilution in a Petri dish. Cultivations were made in a minimum of three replicates for each dilution. Microbiological data were obtained as the arithmetic mean of two independent replicates and are presented as the number of CFU/g (colony forming units/gram).

Table 1. Conditions for the cultivation of functional and taxonomic groups of microorganisms.

Microbiological Indicator	Nutrient Media	Producer	Incubation Conditions
Aerobic heterotrophic bacteria (**AeH**)	Nutrient agar in aerobic conditions	HiMedia	24 h, 37 °C
Genus *Pseudomonas* (**Ps.**)	Glutamate Starch *Pseudomonas* Agar	HiMedia	24 h, 37 °C
Phenol-degrading bacteria (**Ph. degr.**)	Synthetic salt medium. The concentration of phenol to be added	According to Furukawa [37]	2–7 days, 28 °C

2.7. Kinetic Indicators

When studying the biodegradation activity of the microbial dominants and the microbial community in the sediments, after establishing the specific concentrations of phenol, the kinetic parameters of the process were calculated according to the following formulas:

Effectiveness of phenol biodegradation (Eff):

$$Eff = \frac{Ct_1 - Ct_2}{Ct_1} \cdot 100[\%],$$

where Ct_1 is phenol concentration at time t_1 and Ct_2 is phenol concentration at the next time t_2.

Rate of phenol biodegradation (RB):

$$SRB = \frac{Ct_1 - Ct_2}{t_2 - t_1} \text{mg/gxh},$$

Specific rate of phenol biodegradation (SRB):

$$SRB = \frac{Ct_1 - Ct_2}{(t_2 - t_1).Bm} \text{mg/gxh},$$

where Bm is biomass in $g \cdot L^{-1}$ dry weight.

2.8. Enzymological Indicators

Phenol 2-monooxygenase (EC.1.14.13.7) activity (**P2MO**) was assessed according to Neujahr and Gaal [38]. The reaction was followed up by measuring a decrease in absorption at 340 nm. Catechol-1,2-dioxygenase (EC 1.13.11.1) activity (**C12DO**) was determined according to the method of Wilets and Cain [39]. The accumulation of the ring-fission product *cis–cis* muconic acid was measured spectrophotometrically at 260 nm. Catechol- 2,3-dioxygenase (EC 1.13.11.2) activity (**C23DO**) was determined according to the method of Cain and Farr by measuring the accumulation of the ring-fission product 2-hydroxymuconic semialdehyde at 375 nm [40]. Protocatechuate-3,4-dioxygenase (EC 1.13.11.3) activity (**P34DO**) was determined according to the method of Fujisawa and Hayaishi by measuring the decrease in the protocatechuate at 290 nm [41]. Succinate dehydrogenase activity (**SDH**) was measured according to Veeger et al. [42]. The protein content of the cell extracts was determined by the micro-biuret method [43].

2.9. Data Analysis

The obtained data are average arithmetic values from the measurements of the various chemical, microbiological, and enzymological indicators made with three independent repetitions. Standard deviations are calculated with a 95% confidence level. The graphs presented in this paper were made using the software product SigmaPlot 12.5. Statistical analysis was performed using a *t*-test in Sigma Stat (version 4.0). Differences were considered statistically significant at the $p < 0.05$ level.

3. Results

3.1. Kinetic Parameters

Figure 2a shows the dynamics of phenol as residual concentrations at different times of the detoxification process. The phenol (250 mg/kg) was added at the 0th hour and thus a concentration of 254 mg/kg for variant Ph and 202 mg/kg for variant PhPs was reached. The lower value for the variant with added *P. aureofaciens* AP-9 is probably due to the very rapid absorption of phenol by the microorganisms. This also leads to a rapid decrease in its concentration at the beginning of the process.

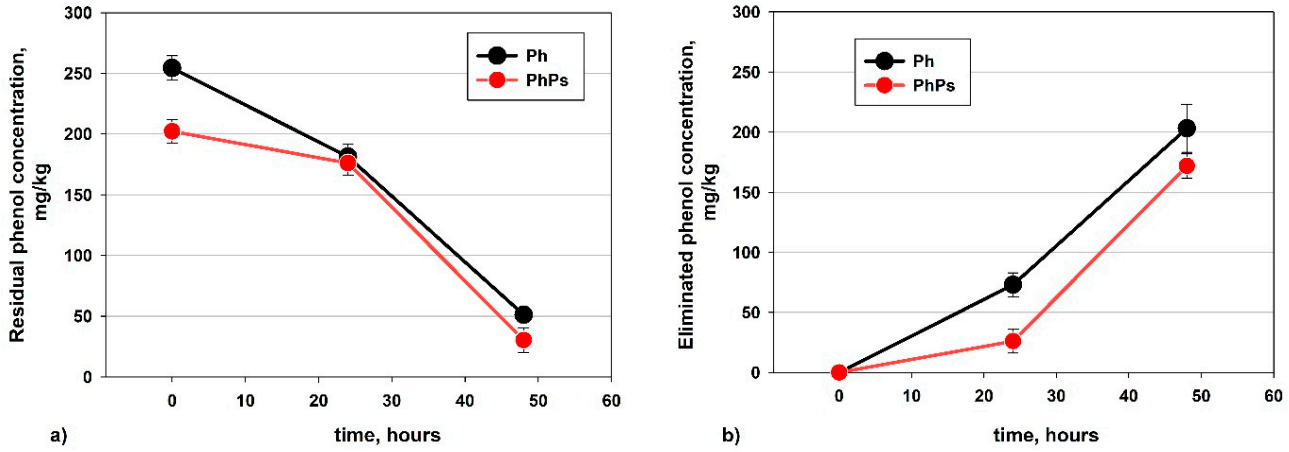

Figure 2. Dynamics of the residual phenol concentration ($p = 0.005$) (**a**) and eliminated phenol ($p = 0.006$) (**b**) in the two tested Ph and PhPs variants. Whiskers represent ± SD (standard deviation).

Figure 2b follows the dynamics of the eliminated phenol from 0th to 48th hours. In the figure, it can be seen that in the first phase of the process (0–24th hours) the values of the eliminated phenol in the control variant Ph are higher (72.95 mg/kg), compared to the variant with *P. aureofaciens* AP-9 (26.06 mg/kg). At the end of the process (48th hour), the values of the eliminated phenol for the Ph variant were 203.23 mg/kg, compared to 171.96 mg/kg for the control variant with the added *Pseudomonas* strain.

Table 2 reports that the efficiency of the phenol elimination in the Ph variant in the 24th hour is 28.67%, and in the variant with *P. aureofaciens* AP-9, it is 12.88%. The lower efficiency of phenol elimination in the variant with the added strain is due to the already mentioned rapid absorption of phenol at the beginning of the process. This phenomenon indirectly affects the efficiency of phenol elimination, which is also the reason for the reported lower efficiency at the 24th hour from the start of the experiment. At the end of the process at the 48th hour, the efficiency for the Ph variant is 71.81%, and for PhPs, 82.80%. Of all the investigated parameters, only the phenol elimination efficiency increased by 15.3% in the variant with the added allochthonous biodegradant.

Table 2. Kinetics parameter of phenol biodegradation ($p = 0.003$).

Parameter	Variant	24th hour	48th hour
Rate of Phenol Biodegradation (RB) [mg/g.h]	**Ph**	3.04 ± 0.42	7.42 ± 0.12
	PhPs	1.09 ± 0.25	6.08 ± 0.11
Effectiveness of Phenol Biodegradation (Eff) [%]	**Ph**	28.67 ± 3.67%	71.81 ± 2.56%
	PhPs	12.88 ± 2.55%	82.80 ± 2.43%

Table 2 presents the overall rate of phenolic biodegradation in experimental variants Ph and PhPs. The total phenolic biodegradation rate at the 24th hour is 3.04 mg/kg.h in the Ph variant and 1.09 mg/kg.h in the PhPs variant. At the end of the process at

the 48th hour, an increase in speed is found in the variant with the added *Pseudomonas* strain—6.08 mg/kg.h compared to the control variant Ph (5.42 mg/kg.h).

3.2. Microbiological Parameters

Figure 3a presents separately the results for the quantity of the three groups of key microorganisms in variant Ph. The highest values of aerobic heterotrophs, pseudomonads, and phenol-degrading bacteria are reported at the 48th hour. At the 24th hour, *Pseudomonas* bacteria decreased in number compared to the zero hour. At the 48th hour, their highest value is recorded. Here, most likely, the specific mechanisms are activated, which are in response to the past adaptation of the community.

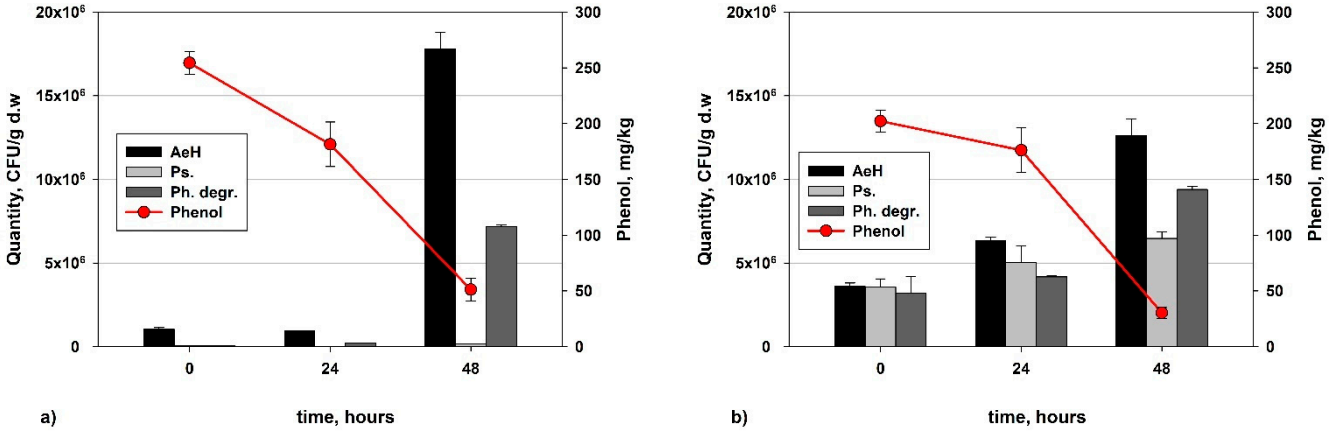

Figure 3. Comparison between the dynamics of the residual phenol and the key groups of microorganisms in the Ph variant ($p = 0.036$) (**a**) and the PhPs variant ($p = 0.029$) (**b**). Whiskers represent ± SD.

Phenol-degrading microorganisms increase significantly during the biodetoxification process. For them, the only carbon source of energy is phenol, and its explosive entry into the system has a positive effect on the amount of this group of bacteria. This group of bacteria reached their highest amounts, and probably their highest activity, at the end of the process after the logarithmic phase passed.

Figure 3b presents a comparison between the abundance of the three key microorganism groups in variant PhPs. The highest values of aerobic heterotrophs, pseudomonads, and phenol-degrading bacteria are reported at 48 h.

In all three studied groups, a slight increase is observed throughout the studied process, which is also directly related to the biodegradation of phenol. During the 48 h process, the studied groups of microorganisms almost doubled their amounts compared to the 0th hour. A clear linear increasing trend is seen in aerobic heterotrophs. Knowing that anoxic/anaerobic processes occur in sediments under natural conditions, this group should decrease with time. But we still have to consider the fact that a large part of the aerobic heterotrophs are facultative anaerobes by their metabolism, and this allows their growth in aerobic conditions. In the course of the process, an additional stimulation of the processes was carried out, in which the sediments were stirred at every 24th hour and thus activated processes close to those occurring in aerobic/anoxic conditions. In addition, according to the literature data, it is known that the 24th and 72nd hours are the most active for reduction in oxygen, and therefore the processes are fastest [19,27].

The quantity of *Pseudomonads* almost overlaps with the phenol-degrading bacteria, which supports the thesis that about 60–70% of the latter is composed of pseudomonads. In this experimental variant, the added strain *P. aureofaciens* AP-9 occupies a key place as the main biodegrader in the sediment microbial community. The exact effect of the addition of *P. aureofaciens* AP-9 is calculated as a percentage and will be discussed in the next task of the paper.

3.3. Enzymological Parameters

In Figure 4, the specific enzyme activities in the variants Ph and PhPs are compared. Specific enzyme activities of key enzymes such as the phenol catabolite pathway (phenol-2-monooxygenase, catechol-1,2-dioxygenase, catechol-2,3-dioxygenase, protocatechut-3,4-dioxygenase, and succinate dehydrogenase) are analyzed at the beginning of the model bioremediation detoxification process (0th hour).

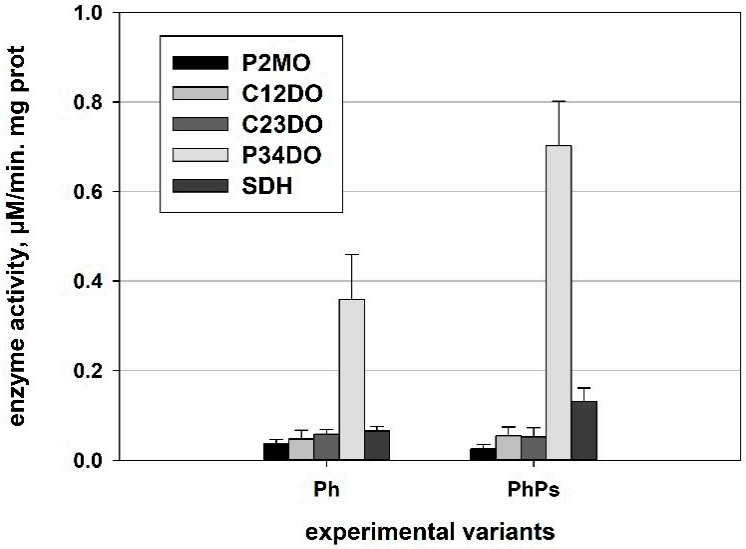

Figure 4. Key enzyme activities of phenol biodegradation in the 0th hour (p = 0.043). Whiskers represent ± SD.

In variant Ph, the highest enzyme activity is measured for the enzyme protocatechuate-3,4-dioxygenase 0.36 µM/min. mg prot (Figure 4), and the lowest CEA is for the enzyme phenol-2-monooxygenase 0.04 µM/min. mg prot (Figure 4). In variant PhPs, the highest CEA of protocatechuate-3,4-dioxygenase is 0.71 µM/min. mg prot. The lowest CEA value in the variant PhPs variant is for the enzyme phenol-2-monooxygenase 0.04 µM/min. mg prot.

In Figure 5, the specific enzyme activities in the variants Ph and PhPs are compared (48th hour). The specific enzyme activities are phenol-2-monooxygenase, catechol-1,2-dioxygenase, catechol-2,3-dioxygenase, protocatechuate-3,4-dioxygenase, and succinate dehydrogenase in the phase of active biodegradation of the xenobiotic–phenol model.

In variant Ph, the measured highest activity of the enzyme phenol-2-monooxygenase is 0.08 µM/min. mg prot, and in variant PhPs48, 0.8 µM/min. mg prot.

In variant Ph, the higher catechol-1,2-dioxygenase is 2.98 µM/min. mg prot., while in the variant with added bacterial culture, CEA is the lowest at 1.85 µM/min. mg prot (Figure 5).

Figure 5 shows the enzyme activity of the catechol-2,3-dioxygenase enzyme. The highest CEA is measured in variant PhPs at 0.12 µM/min. mg prot. In the variant with the added model, the xenobiotic phenol is 0.11 µM/min. mg prot.

The enzyme activity of protocatechuate-3,4-dioxygenase in the phase of active biodegradation of phenol is the highest in variant Ph at 8.68 µM/min. mg prot., while the variant PhPs variant is the lowest at 3.94 µM/min. mg prot (Figure 5).

An interesting result is that in the sediment communities with and without added pseudomonads, the highest oxygenase activity was recorded for protocatechuate 3,4 dioxygenase. This indicates that the ortho-mechanism of the benzene ring opening goes through protocatechuate. Also, there is a very active process of carboxylation of the benzene ring in the sediments. This is related to the intensive accompanying degradation of other substrates by the autochthonous and allochthonous sediment microbial communities.

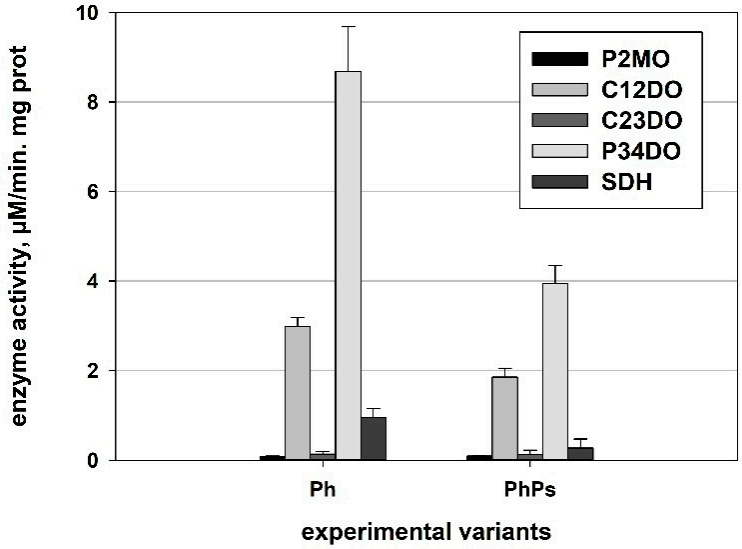

Figure 5. Key enzyme activities of phenol biodegradation in 48th hour (p = 0.016). Whiskers represent ± SD.

In Figure 5, the values of the enzyme activity of succinate dehydrogenase enzyme are compared. The highest enzyme activity is measured in variant Ph 0.95 µM/min. mg prot. In variant PhPs, the CEA is 0.27 µM/min. mg prot.

3.4. Effects of Added P. aureofaciens AP-9 on Kinetic Parameters

In Figure 6, the effect of the added strain *P. aureofaciens* AP-9 is presented. The strain had the greatest positive effect on the residual concentration of phenol at the 48th hour—40.75%. A strong positive effect is also found at the 0th hour with 20.49%. This is directly related to the higher efficiency and rate of biodegradation of phenol measured at the 48th hour. At the 48th hour from the experimental set-up, the microbial communities with the added strain pass through a short adaptation phase and are in their most active phase, which is also reflected in the higher effect compared to the residual concentration of phenol.

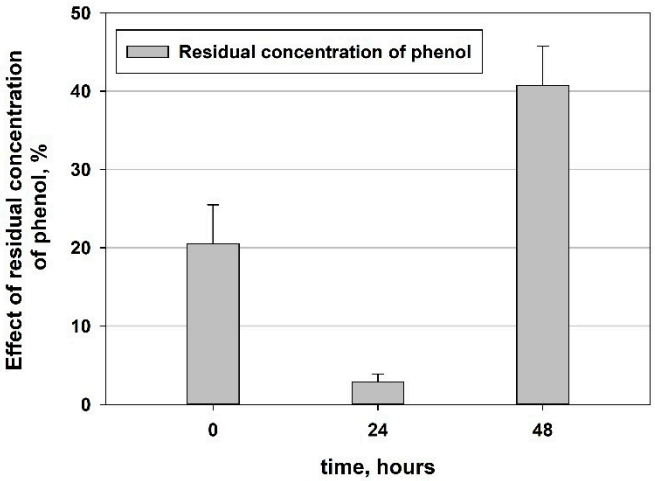

Figure 6. Effect of *P. aureofaciens* AP-9 on the residual concentration of phenol on the PhPs variant (p = 0.035). Whiskers represent ± SD.

In Figure 7, the effect of the added strain on the total biodegradation rate of phenol is presented. The data indicate that at the 24th hour, the total phenolic biodegradation rate of

the *P. aureofaciens* AP-9 spiked variant is 65% lower than that of the Ph control variant. At the 48th hour, a 12% increase in overall speed is found for the PhPs variant.

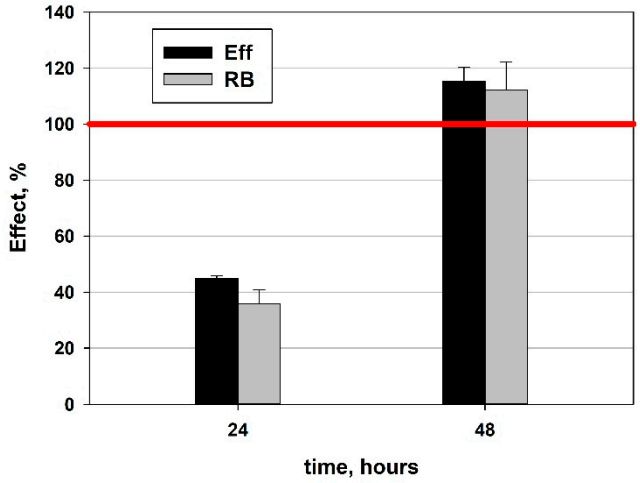

Figure 7. Effect of *P. aureofaciens* AP-9 on the total rate of phenol biodegradation in the PhPs variant and on phenol elimination efficiency in PhPs variant/control 100%-Ph variant/(p = 0.006). The red line is the data from the control variant Ph, which we have taken as 100%. Whiskers represent ± SD.

Figure 7 also presents the effect of the added strain *P. aureofaciens* AP-9 on the efficiency of phenol biodegradation. It is found that at the 24th hour of the model process, the phenol elimination efficiency of the PhPs variant is 55% lower than that of the Ph variant, while at the 48th hour, we find an increase in efficiency of + 15%.

4. Discussion

4.1. Kinetic Parameters

Figure 2a shows the dynamics of phenol as residual concentrations at different times of the detoxification process. Phenol was added at the 0th hour at 250 mg/kg and thus a concentration of 254 mg/kg for variant Ph and 202 mg/kg for variant PhPs was reached. The lower value for variant c added *P. aureofaciens* AP-9 is probably due to the very rapid absorption of phenol by the microorganisms. This also leads to a rapid decrease in its concentration at the beginning of the process. Between the 0th and 24th hours, a light decrease in the concentration of phenol is established in both operating options. This is the initial stage of phenol biodegradation, in which the microorganisms are still in the lag phase, in which there is a period of adaptation and a subsequent growth enhancement phase begins. The residual concentration is up to 180 mg/kg in both systems. In the period 24th and 48th hours, a sharp decrease in the concentration of phenol is registered (Figure 2a). This is mostly because the microbial communities have reached a late logarithmic phase, where the number of cells increases exponentially with time and the rate of the biodegradation is enhanced, reaching an early stationary phase of the microbial community. In the 48th hour of the process, the concentration of phenol is measured in the Ph variant at 51 mg/kg, while in the PhPs variant, it is 30 mg/kg. Here, the positive effect of *P. aureofaciens* AP-9 on the biodegradation of phenol can be seen. In the following pages, this will be supported by the measured rates as well as the dynamics of the microbial co-communities in the sediments.

When studying the dynamics of residual phenol in correspondence with the modeled blast pollution, we can summarize (Figure 2a):

- The modulating effect of *P. aureofaciens* AP-9 is evaluated in close-to-real ecological situations of explosive loading of sediments with phenol. The ecological situation is encountered in real practice and is a challenge in bioremediation technologies.
- Explosive loading of the sediments with a xenobiotic close to the critical concentration (250 mg/kg–at 0th hour). In this situation, the microbial community quickly activates its biodegradation potential and develops it over time. In our case, it is the interval

between 0th and 24th hours in which the first group of kinetic parameters is also measured. In the interval between the 24th and 48th hours, the active phase of biodegradation of phenol is established. It corresponds to kinetic parameters in the biodegradation of a xenobiotic close to the critical concentration.

The addition of *P. aureofaciens* AP-9 to phenol-contaminated sediments under model conditions showed that this microbial culture, although a known biodegradant, had no significant effect on phenol elimination. The reasons are complex: the early phase of adaptation of the allochthonous microorganism to the complex sediment environment was investigated. It is seen that this microbial graft does not significantly affect the parameters of the phenol biodegradation process. Only the elimination efficiency changes. This may be because the added microorganism, in addition to being a prominent biodegradant, is also a factor accelerating the solubilization of adsorbed phenols on the sediment particles. Hence, the efficiency of phenol elimination is improved by as much as 15%. This also shows the enzymatic activity of oxygenases, which we will discuss below.

The lower values for the eliminated phenol in the PhPs variant are due to the already-stated statement that at the beginning of the process, active absorption of phenol takes place in the microbial cells, which also reduces the concentration of phenol. This results in a deformation of the result (Figure 2b).

These results prove a higher efficiency of elimination of the toxic pollutant in the variant with added microorganisms, which confirms the positive bioaugmentation effect of *P. aureofaciens* AP-9 (Table 2). The measured efficiency in this phase (24th to 48th hours) reflects the actual elimination of phenol in the PhPs variant, and here there is no distortion of the result such as occurs in the initial phase (0th to 24th hours).

The higher RPB at the 48th hour may be because at this time, the microbial communities are in the late logarithmic phase of growth and the rate of phenolic biodegradation is higher. Enzyme activity is probably highest here, which is responsible for the degradation of the xenobiotic. From the obtained results, it can be said that *P. aureofaciens* AP-9 is a negative modulator on the speed and efficiency of phenolic biodegradation at the beginning of the process (24th hour). At the end of the process (48th hour) after adaptation, the added pseudomonads have a positive effect on the overall rate of biodegradation and the efficiency of phenol elimination. The reasons for the obtained results should be sought in the enzyme activities directly responsible for the biodegradation of phenol, as well as in the difference in the numerous mechanisms of activation and manifestation of the biodegradation potential of the microbial sediment communities.

4.2. Microbiological Parameters

The addition of *P. aureofaciens* AP-9 leads to a restructuring of the microbial communities in the sediments. The obtained results clearly show at the 0th, 24th, and 48th hours of the model bioremediation, the amount of groups of cultivable microorganisms directly involved and their high dependence on the presence and biodegradation of phenol-pseudomonads and phenol-degrading bacteria. Moreover, this increase is almost proportional to the progress of the adaptation process. All of this is a clear sign that the microbial additive is preparing the community for a longer biodegradation process that would provide long-term and sustainable microbial remediation. In our case, we do not follow this long-term process, but the microbiological parameters provide sufficient evidence for it. At the same time, the activity of the enzymes that cleave the benzene ring by the ortho-mechanism (catechol-1,2-dioxygenase and protocatechuate-3,4-dioxygenase) increased from the 0th to the 48th hour by 23 times and 6 times, respectively. This is an indication that the restructuring of the microbial community is related to the development of biodegradation potential, which would be realized during a long-term detoxification bioremediation process.

Figure 3 presents the comparison of the results for the dynamics of the residual concentration of phenol and the amount of the key groups of microorganisms for variants Ph and PhPs. From the results for microorganisms, the large differences between the

two options are visible. In variant Ph, in which only the natural autochthonous sediment microbial communities from the Cerovo reservoir are present, the amounts of the key groups of microorganisms are several times lower than in the variant with the added strain. In the Ph variant, the high values of aerobic heterotrophs and phenol-degrading bacteria at the end of the investigated process at the 48th hour make an impression. *Pseudomonas* bacteria, which are one of the main biodegraders of aromatic organics, are present in very low amounts during the process [19,27,44]. Here, it is very likely that the other genera of active biodegradants such as *Acinetobacter* and *Bacillus* are involved in the biodegradation of phenol. These high values could be related to the fact that at this moment where the biodegradation of phenol has fully occurred, the microbial communities have completely degraded the incoming phenol and have gone through a logarithmic phase and adaptation in which their amount has increased by several orders versus the 24th hour.

In the PhPs variant, an increase in the amount of the key groups of microorganisms can also be reported during the process (Figure 3b). The highest values are recorded at the end of the process at the 48th hour for all the three groups—aerobic heterotrophic microorganisms, bacteria from the genus *Pseudomonas*, and phenol-degrading bacteria. The higher amounts of the investigated groups in the final phase are directly related to the higher biodegradation activity of the microorganisms at the end of the process. Here, the principle of a higher quantity of microorganisms and a more active xenobiotic-degrading ability is defended. Only further studies of the oxygenase enzyme activity, as well as the study of the non-cultivable microbial communities, would shed more light on the intimate mechanisms of the biodegradation process.

Despite the large differences in the amounts of the key groups of microorganisms, in favor of the PhPs variant, almost complete biodegradation of phenol occurred in the 48^{th} hour in both variants. That is why the biodegradation activity of natural sediment communities can be estimated as very high in the elimination of aromatic xenobiotics, such as phenol. These natural microbial communities are very well-adapted, as it is very likely that high concentrations of xenobiotics with an aromatic structure have repeatedly and constantly fallen into the sediments of the Iskar River.

4.3. Enzymological Parameters

In the variants Ph and PhPs, a noticeable decrease in enzyme activity is registered. The lower enzyme activity may be due to the inhibition of the autochthonous microflora in the sediments as a result of xenobiotic stress generated by the added model xenobiotic–phenol. Only for catechol-2,3-dioxygenase is a higher enzyme activity recorded in variants Ph (0.058 μM/min. mg prot) and PhPs (0.052 μM/min. mg prot). Catechol-2,3-dioxygenase is an enzyme that cleaves the aromatic ring by the meta-mechanism. The vast majority of oxygenases are inductive enzymes. The ortho-cleaving enzymes are expressed by sequential induction, while the biosynthesis of the meta-cleavage catalyzing enzymes is regulated by a coordinated induction. It is the difference in the mechanisms controlling the biosynthesis of the enzymes in ortho- and meta-cleavage that could explain the increase in the specific enzyme activity of catechol-2,3-dioxygenase in the variants Ph and PhPs (0th hour).

The highest enzyme activity measurements are of the enzymes protocatechuate-3,4-dioxygenase and catechol-1,2-dioxygenase, which mainly take part in the ortho-cleavage mechanism (Figure 5). Significantly lower are the CEAs of the catechol-2,3-dioxygenase, catalyzing a reaction from the meta-mechanism for aromatic ring cleavage, and phenol-2-monooxygenase (<0.2 μM/min. mg prot).

Figures 4 and 5 compare the specific enzyme activities of phenol-2-monooxygenase, catechol-1,2-dioxygenase, catechol-2,3-dioxygenase, protocatechuate-3,4-dioxygenase, and succinate dehydrogenase in the initial phase and the active biodegradation phase of phenol in the two variants' Ph and PhPs of the model bioremediation process. The highest increase in enzyme activity during the phase of active biodegradation is found for the enzyme protocatechuate-3,4-dioxygenase. In the variant with added model xenobiotic, the largest difference in CEA at the 0th hour (4.81 μM/min. mg prot) is found and, at the 48th hour,

8.68 μM/min. mg prot. The lowest change is recorded for the PhPs variant, where during the initial phase of biodegradation, the CEA of the protocatechuate-3,4-dioxygenase is 0.70 μM/min. mg prot, and during the active phase of biodegradation, it reaches 3.95 μM/min. mg prot (Figures 4 and 5). This may be due to the accumulation of the protocatechuate metabolite in the medium. Under anaerobic conditions, some sulfate-reducing bacteria carboxylate phenol to 4-hydroxybenzoate. The hydroxylation of 4-hydroxybenzoate to protocatechuate is catalyzed by the enzyme 3-hydroxybenzoate-4-monooxygenase [45,46]. In the model bioremediation sites, aeration is carried out by stirring through a certain time interval. It is possible that in the interval between the mixing of the sediments, due to the depletion of oxygen in the medium, an accumulation of protocatechuate occurs in the system, which induces protocatechuate-3,4-dioxygenase synthase.

A clear increase in the specific enzyme activity is also found in the catechol-1,2-dioxygenase enzyme. A high increase in CEA is found at variant Ph from 0.05 to 2980.25 to 2.02 μM/min. mg prot. In the PhPs variant, CEA increases from 0.05 to 1.83 μM/min. mg prot (Figures 4 and 5).

A trend is found in the increase in CEA of protocatechuate-3,4-dioxygenase and catechol-1,2-dioxygenase. The most significant increase in both enzymes compared to the beginning of the experiment is recorded in the variant with Ph. The smallest difference is in the variant with added model xenobiotic and *P. aureofaciens* AP-9. The two enzymes occupy a central place in the ortho-pathway for cleavage of the aromatic nucleus of phenol. Compared to the results presented in Figures 4 and 5, we can assume that ortho-cleavage is the predominant mechanism for phenol detoxification in all three key variants of the model detoxification process, being the most abundant in variant Ph and with the lowest activity in variant PhPs. It is also striking that the CEA of protocatechuate-3,4-dioxygenase is twice as high as the CEA of catechol-1,2 dioxygenase, suggesting a predominance of aromatic ring cleavage via the key metabolite of ortho-cleavage protocatechuate.

Figures 4 and 5 compare the CEA of the enzyme phenol-2-monooxygenase in the initial and active phase of biodegradation in the three key variants of a model bioremediation process. The slight change in CEA in both variants is impressive.

The greatest increase in catechol-2,3-dioxygenase activity is found at the PhPs variant of 0.05 μM/min. mg prot (0th hour) to 0.12 μM/min. mg prot (48th hour). The low CEA of catechol-2,3-dioxygenase in both variants suggests that biodegradation communities carry out phenolic decyclization predominantly via the ortho pathway in all the variants of the model detoxification process (Figures 3 and 4).

Figures 4 and 5 show the CEA of the succinate dehydrogenase enzyme. The greatest increase in the CEA of succinate dehydrogenase was found in the Ph variant of 0.06 μM/min. mg prot to 0.95 μM/min. mg prot, followed by the PhPs variant (0.13 μM/min. mg prot to 0.27 μM/min. mg prot). Succinate dehydrogenase is an enzyme of the tricarboxylic acid cycle and provides information for the general metabolic activity of microorganisms in sediments [47,48].

4.4. Effects of Added P. aureofaciens AP-9 on Kinetic Parameters

The high effect found at the 0th hour is most likely due to the already-stated assumption about the absorption of phenol in the microbial cells (Figure 6). Here, it can be argued that after the addition of phenol, in a very short time, a large number of *P. aureofaciens* AP-9 bacteria assimilate the phenol into their cells. Thus, phenol has a lower residual concentration at the 0th hour, but no actual biodegradation of phenol has occurred in the bioremediation sites.

This positive effect at the 48th hour, as already mentioned above, is because the sediment microbial community with added strain has passed a short adaptation phase and is in a phase with the most actively developed biodegradation potential (Figure 7); i.e., the system is ready to receive new portions of phenol, with an increased concentration. The results prove, although less pronounced, the positive effect of the addition of the strain *P. aureofaciens* AP-9 on the biodegradation of phenol in the sediments of the bioremediation

site. Here we should also research the efficiency of the biodegradation of phenol, which has its economic importance for the application of similar bioaugmenting factors.

These results once again confirm the positive effect of the added *P. aureofaciens* AP-9 bacteria, mainly at the end of the biodegradation process (Figure 7). This has its definite techno-economic benefit and could be used in the development of future real bioremediation sites for sediments and soils. The positive effect of the addition of the strain means that it could be used to deal with eventual contamination with aromatic xenobiotics. These results can form the basis of future, even more in-depth, studies of contaminated sediments.

The reported lower effect of the added pseudomonads at the 24th hour of the process is probably due to the already-stated thesis about the period of adaptation of the microbial communities as well as the fact that at the beginning of the process, the added bacteria absorb phenol into their cells, which indirectly affects the kinetic indicators, despite the measured lower concentration at the 24th hour.

5. Conclusions

The results of the addition of the *P. aureofaciens* AP-9 at the 0th hour of the model bio-time-dating process of sediment contaminated with phenol give reason to draw the following conclusions. The early effect of adding the outer microbial graft is insignificant. It manifests itself only at the 48th hour as a real result of stimulating the effectiveness of phenolic elimination. At the same time, the allochthonous biodegradant leads to the restructuring of microbial communities in sediments by indicating a degree of increase in cultivated microorganisms from physiological groups that underlie future sustainable and large-scale detoxification processes—phenol-degrading bacteria and pseudomonads. This preparation of the sediments for a prolonged detoxification process is also confirmed by the repeated induction and increase in the activity of the enzymes directly involved in the cleavage of the benzene ring—catechol-1,2-dioxygenase by 23 times and protocatechuate-3,4-dioxygenase by 6 times per 48th hour. This is also associated with the fact that the effectiveness of phenol-elimination at the 48th hour is increased by 15%. All this gives us reason to claim that the early stimulatory effect of the microbial grafts from *P. aureofaciens* AP-9 is insignificant and can hardly be relied upon to accelerate bioremediation up to the 24th hour. However, the long-lasting effect is related to the restructuring of the microbial communities in the direction of increasing the number of resistant biodegradants with induced enzyme activities (oxygenases), which reflects in persistent and long-lasting detoxification bioremediation processes. Considering this stimulation of a sustainable long-term bioremediation process, we can conclude that microbiological grafts are an important mechanism for the management of bioremediation detoxification processes. In this management, however, it is important to monitor and fine-tune the stages of adaptation of sedimentary communities.

The addition of pseudomonads enhances the biodegradation of phenol, preparing the microbial communities for a prolonged bioremediation process. This is of important practical importance for the bioremediation technologies of the contaminated sediments. This has its techno-economic benefit and could be used in the development of future experimental pilot bioremediation sites for sediments, soils, and waters.

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