Prospects for the use of the phytoremediant *Phragmites australis* (Cav.) Trin. ex Steud. for obtaining green fertilizers

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Abstract. The article presents data on the accumulation of potassium, calcium, iron, phosphorus and sulfur in the tissues of the green vegetative mass of *Phragmites australis* (Cav.) Trin. ex Steud. when using the species in the phytopurification system of contaminated wastewater from a mining enterprise. The dynamics of changes in 3D plant leaf area and normalized chlorophyll index (NCPI) are shown during systematic exposure to mine wastewater with simultaneous inoculation with microorganisms: a consortium of *Bacillus* bacteria; *Bacillus subtilis* 26D, *Azotobacter vinelandii* IB-4. The most promising symbiotic microbial-plant complexes with a high accumulating capacity of Ca, K, Fe, P and S have been identified. Based on the experimental data obtained, the fundamental possibility of using the green vegetative mass of *Phragmites australis* to obtain green fertilizers enriched with these elements has been shown.

1 Introduction

Treatment of wastewater from mining enterprises using the method of phytoremediation is beginning to be widely introduced into production at metallurgical enterprises of the Russian Federation [1-3]. Theoretically developed systems are implemented and implemented in industrial conditions [4-7]. However, issues related to the utilization of plant raw materials, which are used as biofilters and bioaccumulators of pollutants, still remain completely unresolved.

Since mine wastewater from mining enterprises is highly mineralized and contains dissolved divalent iron in high concentrations, as well as other nutrients (potassium, calcium, iron, phosphorus and sulfur), the idea arose of using and processing plant raw materials phytoremediants to obtain green fertilizers and soil enrichment .

The first step in solving this problem should be the assessment of the accumulation of nutrients in plants with control of their condition as a result of the systematic action of technogenically polluted wastewater from mining enterprises.

Our previous studies suggest using *Phragmites australis (*Cav.) Trin as a plant that has proven itself quite well in the process of phytoremediation of wastewater. ex Steud. [8, 9], which has a number of advantages over other types. Due to the fact that the species belongs

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to the pluriregional (cosmopolitan) species, it has a wide range of resistance to unfavorable environmental factors and is quite well adapted to the agroclimatic conditions of the study region [10]. In addition, the species is a rhizomatous perennial that does not require additional costs for planting since it is able to independently spread in planting areas, as well as form stable symbiotic microbial-plant complexes [11]. At the same time, the most important advantage of *Phragmites australis* is the possibility of its clonal micropropagation, which opens up wide opportunities for the selection of technogenically resistant plants and their targeted selection for marker traits that are associated with the accumulation of pollutants.

Purpose of the work: to study the morpho-physiological characteristics and the degree of accumulation of biogenic elements of *Phragmites australis* during inoculation with symbionts, in the conditions of technogenically polluted wastewater from a mining enterprise.

2 Materials and methods

The study was carried out on the basis of the laboratory of experimental botany, the laboratory of biotechnology and the laboratory of physical and chemical methods of plant research of the Botanical Garden of the National Research University Belgorod State University using the equipment of a unique scientific installation (Botanical Garden of the Belgorod State National Research University, https://ckp- rf.ru/usu/200997/).

The introduction of Phragmites australis plants into in vitro culture was carried out through seeds and took 3 months. Seed germination occurred within a week, with a second week added to control sterility. The resulting microclones did not require additional time for adaptation and growth on the nutrient medium, or this time was reduced to two passages of 2 weeks each, after which the plants were used in the experiment (Figure 1).

Fig. 1. *Phragmites australis in vitro* culture.

Inoculation of plants with cultures of microorganisms was carried out on the 72nd day from the moment of their introduction into the in vitro culture. The following were used in the experiment:

- Consortium of Bacillus bacteria: *Bacillus subtilis* strain DSM 32424, Bacillus amyloliquefaciens strain VKPM B-10642 (DSM 24614) and *Bacillus amyloliquefaciens* strain VKPM B-10643 (DSM 24615) at a concentration of at least 1×10^6 CFU/g.

- Bacterial strain *Bacillus subtilis* 26D at a concentration of at least 2×10^9 CFU/g.
- Bacterial strain *Azotobacter vinelandii* IB-4 at a concentration of at least $2\text{-}3\times10^9$ CFU/g.

The plants were watered with tap water for six days, and on the 7th day they were treated with technogenically contaminated wastewater from mining enterprises according to the experimental design for 28 days:

- Control.
- Technogenically polluted wastewater from a mining enterprise.
- Consortium of *Bacillus* bacteria.
- Technogenically polluted wastewater from a mining enterprise + consortium of *Bacillus* bacteria
- Bacterial strain *Bacillus subtilis* 26D.
- Technogenically polluted wastewater from a mining enterprise + bacterial strain *Bacillus subtilis* 26D.
- Bacterial strain *Azotobacter vinelandii* IB-4.
- Technogenically polluted wastewater from a mining enterprise + bacterial strain *Azotobacter vinelandii* IB-4.

In laboratory conditions, plants were cultivated on phytoracks with LED lamps ECOLED-60-LX Fito IP 65 60W with a wavelength of 450-730 nm (LED) [12].

Determination of the content of Ca, K, Fe, P and S in plants was carried out on an AVIO 220 Max optical emission spectrometer after the end of the experiment; the results are presented as % of the dry weight of the sample.

The assessment of the dynamics of morpho-physiological parameters of plants was carried out every 3-4 days on 8 same-aged and morphologically similar specimens using a multispectral 3D installation PlantEye F500 ("Phenospex B.V.", the Netherlands) according to approaches developed previously [13] for the following parameters:

- 3D plant leaf area (3D Leaf Area), mm^2;
- Normalized pigment chlorophyll ratio index (NPCI) range of values from -1 to 1.

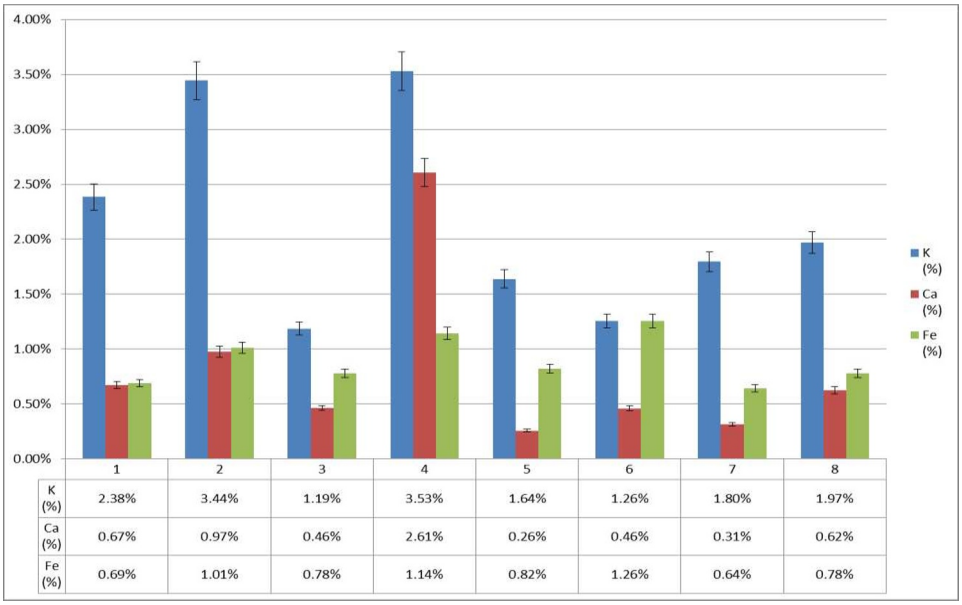
PlantEye F500 HortControl software was used to process the obtained data.

Statistical processing of data was carried out using Microsoft office Excel with calculation of the arithmetic mean (M) and confidence interval (±CI) at a significance level of $p = 0.05$. The growth rate based on digital biomass and normalized differential vegetation index was calculated using the formula: $\Delta TR = (Pk - Mon) / Mon \times 100\%$ (where Pk – final values of the indicator; Mon – initial values of the indicator).

3 Results

The results obtained on the accumulation of Ca, K, Fe, P and S in the green vegetative mass of *Phragmites australis* plants under the influence of technogenically polluted wastewater from a mining enterprise and inoculation with symbionts are shown in Figures 2-3.

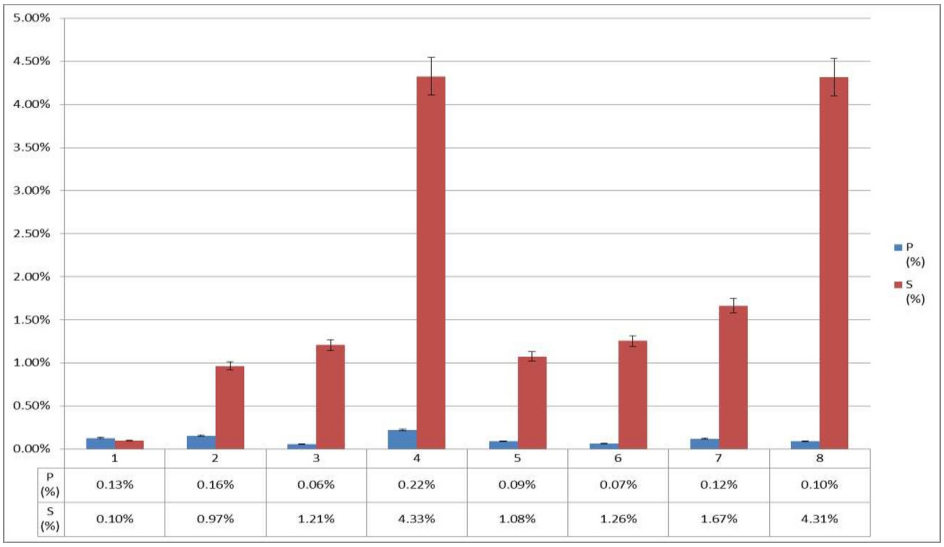
From the presented histogram it is clear that the accumulation of metals in tissues naturally increases under the influence of technogenically polluted wastewater from a mining enterprise (No. 2). The formation of symbiotic microbial-plant complexes with all strains of microorganisms (No. 3, 5, 7) in plants leads to a decrease in the accumulation of K and Ca in tissues compared to the control, and the supply of Fe increases when using a consortium of bacteria *Bacillus* and *Bacillus subtilis* 26D (No. 3, 5) compared to control.



	1	2	3	4	5	6	7	8
K (%)	2.38%	3.44%	1.19%	3.53%	1.64%	1.26%	1.80%	1.97%
Ca (%)	0.67%	0.97%	0.46%	2.61%	0.26%	0.46%	0.31%	0.62%
Fe (%)	0.69%	1.01%	0.78%	1.14%	0.82%	1.26%	0.64%	0.78%

Fig. 2. The level of accumulation of potassium, calcium and iron in the green vegetative mass of *Phragmites australis* according to the experimental scheme.

The effect of technogenically polluted wastewater from a mining enterprise on symbiotic microbial-plant complexes is ambiguous; for example, in the case of a symbiotic relationship between *Phragmites australis* and a consortium of *Bacillus* bacteria (No. 4), a significant increase in the level of Ca, K and Fe occurs. At the same time, when inoculated with *Bacillus subtilis* 26D (No. 6), there is a decrease in the accumulation of Ca and K, but an increase in the accumulation of Fe. Treatment of plants with the *Azotobacter vinelandii* IB-4 culture led to a decrease in the concentration of Ca, K and Fe in the green vegetative mass (No. 8).



	1	2	3	4	5	6	7	8
P (%)	0.13%	0.16%	0.06%	0.22%	0.09%	0.07%	0.12%	0.10%
S (%)	0.10%	0.97%	1.21%	4.33%	1.08%	1.26%	1.67%	4.31%

Fig. 3. The level of accumulation of phosphorus and sulfur in the green vegetative mass of *Phragmites australis* according to the experimental scheme.

From the presented histogram it is clear that the accumulation of phosphorus and especially sulfur in tissues naturally increases under the influence of technogenically polluted wastewater from a mining enterprise (No. 2). At the same time, during the formation of symbiotic microbial-plant complexes with all strains of microorganisms (nos. 3, 5, 7), plants experience a decrease in the accumulation of P and an increase in the accumulation of S in tissues compared to the control.

As a result of the action of technogenically polluted wastewater from a mining enterprise on symbiotic microbial-plant complexes *Phragmites australis* with a consortium of Bacillus bacteria (No. 4), a significant increase in the level of P and S occurs, and when inoculated with *Bacillus subtilis* 26D (No. 6), a decrease in the accumulation of P occurs, but increased S accumulation. Treatment of plants with *Azotobacter vinelandii* IB-4 also led to a decrease in P concentration, but a significant increase in S in the green vegetative mass (No. 8).

The peculiarities of the accumulation of technogenically polluted wastewater from a mining enterprise when using symbiotic microbial-plant complexes indicate complex biochemical interactions between plants and bacteria, and therefore of particular interest is the assessment of the dynamics of the morpho-physiological parameters of the development of *Phragmites australis* plants during the experiment.

Determining leaf area is a task that must be solved during the assessment of the physiological characteristics of plants, since this parameter affects the productivity of photosynthesis, pigment content, intensity of transpiration, respiration, etc. Of particular practical interest is a comprehensive study of the influence of mine wastewater and symbionts on the dynamics of changes in 3D area. Table 1 shows the dynamics of changes in this parameter during the experiment.

Table 1. Dynamics of changes in 3D leaf area of *Phragmites australis*, cm^2.

No.	Days of measurements								Growth rate, %
	1	4	8	12	16	20	24	28	
1	19.15	22.22	23.59	24.35	27.70	28.71	29.30	30.10	57.18
2	17.78	18.92	20.81	21.92	22.01	23.04	21.14	21.09	18.64
3	21.62	24.24	26.92	28.45	28.06	28.64	32.67	30.09	39.20
4	23.04	23.93	27.35	30.20	31.12	32.24	33.16	32.41	40.66
5	23.63	26.36	26.47	29.54	30.21	30.01	31.15	32.26	36.52
6	21.41	26.56	26.09	25.87	27.84	27.66	27.32	28.42	32.78
7	18.31	22.74	22.00	26.79	31.12	32.05	32.06	34.11	86.27
8	16.70	21.90	22.34	25.52	27.90	28.19	29.16	30.95	85.36

In *Phragmites australis*, there is a natural increase in 3D leaf area, both in the control and under the influence of technogenically polluted wastewater from a mining enterprise, while the rate of increase in 3D leaf area by the end of the experiment in the control for *Phragmites australis* was 57.18% (No. 1), and under the influence of mine wastewater 18.64% (No. 2), which indicates inhibition of growth processes in plants. The growth rate of 3D leaf area is lower compared to the control when inoculated with a consortium of bacteria Bacillus (No. 3) and *Bacillus subtilis* 26D (No. 5). At the same time, the culture of *Azotobacter vinelandii* IB-4 increases the growth rate of plants by 86.27% (7), which is even more than in the control.

The combined effect of technogenically polluted wastewater from a mining enterprise on symbiotic microbial-plant complexes, compared to the effect of mine wastewater alone (No. 2), has a positive effect when treated with microorganisms. The growth rate of 3D leaf area under the combined action of technogenically polluted wastewater from a mining enterprise and bacterial cultures in the case of using a consortium of *Bacillus* bacteria (No. 4) was 40.66%, which is unreliably higher than with the action of only symbionts. The

result of using *Bacillus subtilis* 26D (No. 6) was a plant growth rate of 32.78%, which is significantly lower in the case of the formation of symbiotic microbial-plant complexes. In the case of inoculation with *Azotobacter vinelandii* culture IB-4 and the action of technogenically polluted wastewater from a mining enterprise, approximately the same increase in 3D leaf area was observed: 86.27% (No. 7) and 85.36% (No. 8), which is 30% more than in control (No. 1).

The normalized chlorophyll ratio index (NPCI) is an index measured at a fluorescent wavelength excited by blue light (UV rays) that is compared to a fluorescent wavelength excited by red light. Chlorophyll fluorescence occurs at both wavelengths. As a result of the experimental data obtained, the conclusion is substantiated that using the difference between these two wavelengths using the formula: (RED − BLUE)/(RED + BLUE) it is possible to calculate values correlating with the chlorophyll content [14-15].

Table 2 displays the dynamics of changes in NPCI of *Phragmites australis* plants when exposed to mine wastewater in a model experiment.

Table 2. Dynamics of changes in NPCI *Phragmites australis*.

No.	Days of measurements								Growth rate, %
	1	4	8	12	16	20	24	28	
1	0.07	0.08	0.10	0.10	0.09	0.09	0.08	0.07	-5.72
2	0.04	0.07	0.07	0.08	0.11	0.12	0.10	0.10	190.64
3	0.08	0.09	0.09	0.10	0.13	0.14	0.07	0.14	77.75
4	0.07	0.07	0.09	0.06	0.07	0.08	0.07	0.08	8.59
5	0.08	0.09	0.09	0.09	0.09	0.10	0.10	0.10	36.04
6	0.10	0.11	0.12	0.12	0.13	0.13	0.13	0.14	40.96
7	0.07	0.10	0.10	0.09	0.10	0.10	0.12	0.12	73.29
8	0.04	0.07	0.06	0.05	0.06	0.07	0.07	0.07	90.87

During the experiment, it was found that the effect of technogenically polluted wastewater from a mining enterprise and symbionts leads to an increase in the growth rate of the NPCI index (No. 2), and in the control (No. 1), the photosynthetic activity of plants is inhibited. At the same time, the influence of only one symbionts (nos. 3, 5, 7) significantly increases the growth rate of the chlorophyll ratio index. The combined effect of mine wastewater on symbiotic microbial-plant complexes in the case of using a consortium of Bacillus bacteria gives a lower growth rate (No. 4), and in the case of inoculation with *Bacillus subtilis* 26D and *Azotobacter vinelandii* IB-4 - a higher one.

4 Discussion

The study suggests that technogenically contaminated wastewater from mining enterprises with high contents of Ca, K, Fe, P and S can be extracted using green vegetative mass of *Phragmites australis*. This allows us to consider it not only as a successful phytoremediant plant, but as a promising object for producing green fertilizers. The use of strains based on a consortium of *Bacillus* bacteria increases the degree of biogenic migration of Ca, K, Fe and S into the vegetative mass of plants. To increase the sulfur content, according to the results of our study, we can also recommend the use of the *Azotobacter vinelandii* strain IB-4, which increases the growth rate of 3D leaf area.

It has been established that technogenically polluted wastewater from mining enterprises inhibits physiological processes associated with plant growth, which is reflected in a decrease in the growth rate of leaf area. At the same time, symbionts increase the adaptive potential of plants to the action of anthropogenic factors. Mine wastewater contains a sufficient microelement composition, which is necessary for the effective

occurrence of photosynthetic processes in plants, which is confirmed by the results of an analysis of the growth rate of the NPCI index. Inoculation with plant symbionts reduces the growth rate of the index, which is apparently explained by the distribution of nutrients in the microorganism-plant system.

5 Conclusion

In the course of the experimental study, it was established that under the influence of technogenically polluted wastewater from mining enterprises, growth processes are inhibited and the photosynthetic activity of *Phragmites australis* plants is increased. At the same time, there is an active accumulation of Ca, K, Fe and S in their vegetative mass, which is confirmed by the results obtained on an AVIO 220 Max optical emission spectrometer.

Microbiological preparations reduce the degree of impact of technogenically polluted wastewater from a mining enterprise on the morpho-physiological state of plants: the growth rate of 3D leaf area and the NPCI index, which is confirmed by the results obtained using the PlantEye F500 multispectral 3D installation when assessing the dynamics of plant development in the experiment.

The combined use of *Phragmites australis* and cultures of the consortium of bacteria *Bacillus* and *Azotobacter vinelandii* IB-4 under the influence of technogenically polluted wastewater from a mining enterprise makes it possible to expand the ecological amplitude and sustainability of the species, as well as to increase the degree of accumulation of the elements Ca, K, Fe and S in the green vegetative mass. Therefore, *Phragmites australis* plants can be further used in the production of green fertilizers based on them.

Reproduction of *Phragmites australis* by the in vitro method opens up the possibility of selecting plant genotypes that have a high accumulating ability to certain types of pollutants, which provides new opportunities for targeted selection in relation to obtaining plant populations that are resistant to specific technogenic impacts.

Acknowledgement

The study was supported by: grant from the Ministry of Science and Higher Education of the Russian Federation No. FZWG-2023-0007 "Adaptive reactions of microorganisms: theoretical and applied aspects."

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