Microbiological stimulation of phytoremediation process using *Salvinia natans* to mercury contamined water

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Abstract. An alternative to traditional cleaning methods of heavy metals in the water environment is phytoremediation. They efficiency depends on used technological process conditions as well as plant species. One of the most dangerous metallic elements mercury plays a particular role, which is a trace element and a physiologically foreign in living organisms. Mercury has a high degree of toxicity with strong affinity to thiol groups. This may cause an adverse effect on the enzymatic processes and consequently inhibiting the physiological functions. Because of high risk for human health, water environment treatment from mercury is essential proecological action. Mercury removal studies were conducted using *Salvinia natans* pleustofit, sampled from its natural water environment. In the first step, epiphytic bacteria, which was resistant to high concentrations of mercury (0,6 mgHg/l), was isolated from the plant and than selected by the tiles gradient mthod. In the next step, the identification using molecular biology methods was made. In the following step plant *Salvinia natans* was exposure to high levels of mercury in the presence of the three isolated *Pseudomonas* strains with exceptional resistance characteristics to environmental factors. Has been found a positive bacteria effect on the plant condition because the selected strains belong to *Pseudomonas* species producing materials supporting plant growth. The use of microbial stimulation to phytoremediation by hyperaccumulator *Salvinia natans* can multiply the effectiveness of the process.

1 Introduction

Environmental pollution by heavy metals, i.e., Pb, Cu, Fe, R, Ni, Zn, Cr, Cd, Mo and metalloids (Se, As) is a serious global problem resulting from the intensive progress of civilization [1–3]. One of the most dangerous elements on Earth is mercury, since it occurs in liquid, gas or a solid form and is an element of a high volatility, easily spreading in the atmosphere [4]. It is necessary to control the presence of mercury in various parts of the environment, due to its emissions from a variety of industries, including chloralkaline,

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electrical, pharmaceutical, as well as from the process of combustion of coal and oil [5, 6]. Aquatic ecosystems are worthy of attention. They are particularly vulnerable to contamination with heavy metals, including mercury, as a result of surface runoff, discharges and dry or wet deposition from the atmosphere, because in this environment, as a result of microbiological changes, mercury can be present in a particularly toxic form [7, 8]. Due to the toxicity of mercury, it is necessary to control and effectively remove it. Therefore, the ions of the element should be removed from the water before the water treatment. The used methods could be of biological nature, such as accumulation in tissues of plants, for example in a known hyperaccumulator *S. natans*. In addition, the literature review reports the removal of mercury from the water by bacteria of the genus *Psuedomonas* and *Kleibsiella* [9].

Therefore, the aim of this study was to clarify the role of bacteria accompanying *S. natans* in the process of phytoremediation of water contaminated with mercury. It was assumed that bacteria *S. natans* can support the process of phytoremediation. The objective was to explain the process.

2 Materials and methods

The research material was the floating fern *Salvinia natans*, collected at the estuary of the Oława river to the Odra river (Wrocław, Polska). The endophytic bacteria from the plants were collected according to scheme no. 1.

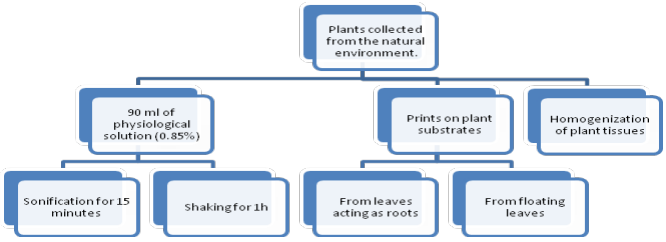


Fig.1. The scheme of bacteria isolation from *S. natans*.

The preliminary identification of Gram-negative strains resistant to mercury was performed with biochemical methods in accordance to the Bergey scheme [10].

The identification was carried out with the help of methods determining the physiological and biochemical traits of microorganisms and methods of molecular biology. The evaluation scheme for taxonomical identity is shown in fig. 2.

Selected Gram-negative bacteria resistant to high concentrations of mercury have been subjected to preliminary identification using the API 20E test (bioMerieux). The test strip consists of 20 microtubes containing dehydrated substrates and integrated oxidase assay. The test has been conducted according to manufacturer's instructions. The chamber with the microtubes has been incubated for 18h in temperature of 26 degrees Celsium, as those were the best growth conditions for the isolated bacteria. The results were interpreted with the help of apiweb software.

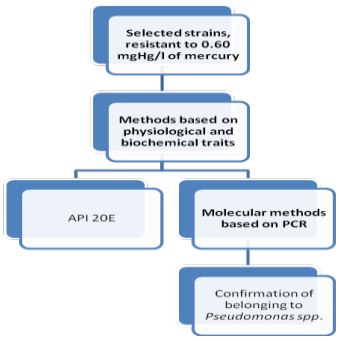


Fig. 2. Scheme of identification of strains resistant to 0.60 mgHg/l of mercury.

Isolation of the nucleic acids strains was performed using a set of regents Genomic Mini (A&A Biotechnology) according to the manufacturer's instructions. Amplification was performed using specific primers PA-GS-F of the sequenceGACGGGTGAGTAATGCCTA and PA-GS-R of the sequence CACTGGTGTTCCCTTCCTATA, yielding products of approximately 618bp [11]. The reaction mixture (25 µl) consisted of 1U BIO-X-ACT Short Polymerase (Bioline), 250 µM a mixture of DTP, 0,4 µM of each primer, 5 µl OptiBuffer, 10ng isolated DNA and 2 µM $MgCl_2$. Conditions of the reaction have been shown in table no.1.

Table 1. Amplification conditions [12].

Reaction phase	Temperature [°C]	Time [s]	Number of cycles
Initial denaturation	95	120	1
Denaturation	94	20	25
Hybridization	54	20	
Elongation	72	40	
Final extension	72	60	1

Control of the obtained PCR products has been carried out using electrophoretic separation (40min, 100V) on agarose gel 1%) in presence of the intercalating agent (ethidium bromide 0.5 µl/ml).

Phytoremediation has been conducted in reactors containing 500 ml of modified Hoagland medium with a mercury concentration of 0.3 mgHg/l with 5 g of plant biomass of *S.natans*.

To maintain a instable conditions, the experiment was carried out in FD 147 Inox phytotron of the Biosell company, equipped with fluorescent bulbs 18W/965 Biolux of the OSRAM company, with the day/night cycle (12h/12h), at the temperature of 22°C/15°C and air humidity of 40% (Fig. 14).The analyses were carried out on the 7th, 14th, and the 21st day.

Before the evaluation of the effect of epiphytic bacteria on the process of phytoremediation, the plants were washed with 70% ethanol and then placed for 3 hours in the bacterial solution of the selected strain (5 in the McFarland scale).After that time, the plants were transferred to the prepared culture media inoculated with 5 ml of bacterial solution. The control were plants incubated in the medium without mercury, and the positive control were plants exposed to mercury, but without the tested microorganisms.

To evaluate the toxicity of mercury (II) to the bacteria, the Microtox test was used, utilizing concentrations: 0.15; 0.2; 0.3; 0.4; 0.5; 0.6 mgHg/l. During an initial assessment of

the impact of mercury on the condition of *S. natans*, the plants were placed in the Hoagland medium containing 0.035; 0.075; 0.150; 0.200; 0.300; 0.350; 0.400; 0.500 mgHg/l.

In the culture vessels, 5 specimens with similar morphology were place. The specimens were incubated for 14 days in optimal conditions. After that time, a photographic documentation of chlorotic and necrotic changes of the leaves was taken.

The total protein content in the tested plants was measured using the Lowry method modified by Eggsteinand Kreutz [13] in homogenates of plant tissues obtained from 0.1 g of fresh tissues suspended in 7 ml 1M NaOH (homogenizer of the Ultra-TurraxTube Driver type). The amount of protein was read from the calibration curve at a wavelength of 750 nm on a spectrophotometer T80+ UV/VIS (PG Instruments Ltd). The amount of assimilation pigments was determined in extracts obtained through homogenization of 0.1 g of fresh plant tissues in 7 ml 90% acetone with the use of Ultra-TurraxTube Driver homogenizer. The amount of *a* chlorophyll has been read from respective calibration curve at a wavelength of 663 nm, and 645 nm, and for the chlorophyll *b* 645 nm on a spectrophotometer T80+ UV/VIS (PG Instruments Ltd).

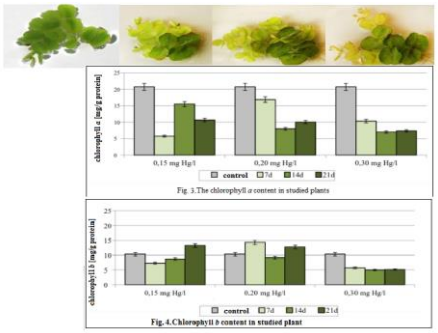
In order to control the growth of the biomass, the dry weight of the plants was measured after the experiment. The plants were dried at room temperature to the air-dry state. Because the determination of the dry mass of plants before the experiment is not possible, a mean dry mass has been experimentally determined. For this purpose, 10 samples with 5 g of fresh plants with different physiological condition have been prepared, which were weighted, then dried at room temperature to a solid weight. Obtained results were averaged, thus obtaining a mathematical converter. The mercury content was analyzed by atomic absorption spectrometer AMA 254 Altem company [14]. This method does not require sample preparation. Analysis was carried out in aqueous solution and on air-dry weight of the plant.

3 Results

Although *S. natans*is considered to be a hyperaccumulator of heavy metals, under the influence of high concentrations of mercury chlorosis and necrosis were observed, especially after 14 days of exposing the plant to the tested element in concentrations above 0.3 mgHg/l (fig. 3.). It should also be noted that the fist tested concentration is a value close to the one recorded by [15] in lakes Miedwie and Reko. Therefore, an assessment was made of the influence of that concentration on physiological parameters of *S. natans* (after 7, 14 and 21 days of exposition). There was a significant decrease of protein content in plants relative to control samples such that after 21 days for the concentration 0.15 mgHg/l it was 75%, for concentration of 0.2 mgHg/l 60%, and for concentration of 0.3 mg/l 53%. The toxic effects manifested also in the decrease of the amount of assimilation pigments with simultaneous disturbance of the proportions of chlorophyll *a/b* (figures 4 and 5), as a higher concentration of chlorophyll b was observed than the concentration of chlorophyll a, what can be clearly observed for most of the tested samples after 7 and 21 days of experiment. At the same time, for the concentration of 0.3 mgHg/l a very significant decrease of both chlorophyll *a* and *b* has been observed, what shows an advanced process of chlorosis.

Since the objective was also to stimulate the process of phytoremediation by the bacteria characterized by a high (from 0.3 to 0.6 mgHg/l) resistance to mercury, firstly the assessment of the toxicity of mercury was performed using Microtox test with bacteria *Vibrio fischeri* (fig. 6). As can be seen, the concentration of 0.3 mgHg/l has induced an effect of fluorescence decay, exceeding the limit for the III hazard class ($50\% < PR < 100\%$), because after 5 minutes of the process, the inhibition was 68.68, and after 15 min, 96.26.

Accompanying microorganisms obtained by cavitation in physiological solution of prolonged (1 h) shaking in physiological solution, direct print of floating leaves and those acting as roots and as a result of homogenization of tissues, were inoculated on a nutrient medium containing mercury at the concentration of 0.15 mgHg/l.



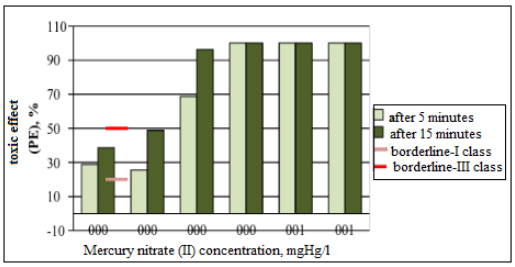


Fig. 5. Reduction of *Vibrio fischeri* bacteria bioluminescence.

Accompanying microorganisms obtained by cavitation in physiological solution of prolonged (1 h) shaking in physiological solution, direct print of floating leaves and those acting as roots and as a result of homogenization of tissues, were inoculated on a nutrient medium containing mercury at the concentration of 0.15 mgHg/l. As a result, 51 strains of bacteria were obtained (fig. 6). During selection of strains on gradient plates 0.00–0.3 mgHg/l 14 strains were obtained. Resistance to mercury in the range of 0.3–0.6 mgHg/l of 14 strains was evaluated with the gradient plates and disc-diffusion method. It was found, that in the pool of tested strains, 5 showed a remarkable tolerance to mercury and those were selected for the stimulation of the phytoremediation process. It needs to be emphasized that those strains have been obtained as a result of one-hour-long shaking of *S. natans* in a physiological solution. For the purpose of preliminary identification of the bacteria, first the API E20 test was used, on the basis of the result of the Gram staining method, which has shown that those are G(-)bacilli. The results of growth on selective-differentiating and chromogenic media shown their belonging to *Pseudomonadaecae*.

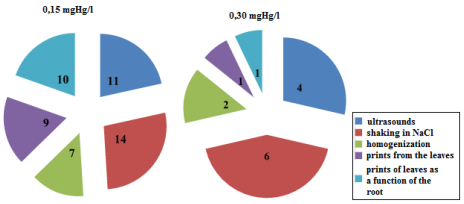


Fig. 6. Effectiveness of methods used to isolate mercury resistant bacteria in different concentrations.

For epiphytic strains: 1-26-27, 4-8-22, and st.pr. the highest degree of similarity to the reference species was obtained for *Pseudomonas aeruginosa* and they were one 77.5%, 67.1% and 43.6%, respectively. Because the bacterium belongs to the clinical pathogens, if these results were confirmed by genetic studies, the applicability of this species in water treatment would not be possible due to the epidemiological threat. Strain 3-22-22 was identified as *Pseudomonas fluorescens* or *Pseudomonas putida* in equal similarity (44.5%), and only 27.9% of traits pointed to *Pseudomonas aeruginosa*.

Based on the results obtained in the biochemical tests, the identification of the two strains (3-22-22, 1-26-27) has been recognized by the program as "a good for a genus", while the other three (2-26, st.pr., 4-8 -22) for "questionable for a genus" in connection with the discrepancy of the results obtained in biochemical tests. The API 20E method is based on the basic biochemical features designed for the detection of most enterobacteria, its application to environmental microbes may not take into account the high volatility of the so-called wild strains for which in most biochemical assays there is no reference data. Moreover, the test is designed for low-maintenance bacillus from the family *Pseudomonaceae*, what could also influence the accuracy of the measurement. The results of API 20E can be considered illustrative, and helpful in choosing the conditions for further analyses, as well as in choosing primers for PCR reactions. From five strains resistant to the highest concentration of mercury, DNA was isolated, which then was amplified by PCR reaction with the use of appropriate primers. For all tested DNA samples, the result of the reaction was positive, yielding products of the size 618bp (fig. 7). It confirms the belonging of the tested strains to the genus *Pseudomonas.*

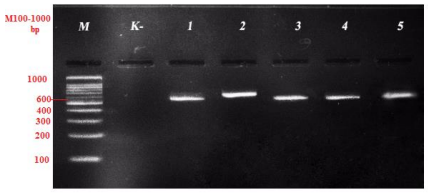


Fig. 7. Photo of agarose gel with PCR products: M - size marker (100-1000 bp); "K-" - negative control; 1-st.pr. strain, 2-strain 2-26; 3 -strain 3-22-22; 4 - strain 1-26-27; 5 - strain 4-8-22.

This genus has 245 species, and a detailed analysis of each strain requires further studies with the use of techniques of sequencing the order of DNA nucleotide base pairs.

Visual evaluation of the plants carrying the process of phytoremediation, stimulated microbiologically by bioaugmentation, in the presence of the strains 4-8-22, and 3-22-22 showed significant deterioration in their condition compared to controls (fig. 8). Many necrotic changes have been observed already in the 7th day of the experiment. This result prompted us to reject these two strains in further studies on phytoremediation process because, despite the high resistance to mercury concentration (0.6mgHg / l), there was no positive influence on *S. natans*.

Different results were obtained for the process of stimulating the process of phytoremediation involving strains 2-26, st.pr., 1-26-27.Control plants showed very high growth of dry mass. In the last day of experiment, its value increased to 1.97 g, which is more than seven-fold increase compared to the initial weight (0.26 g), what confirms the optimum conditions for the experiment.

In the case of the control plants exposed to mercury already at the 7th day of the experiment (Fig. 72) progressive chlorotic and necrotic changes in leaf could be observed. However, in *Salvinia natans* in the presence of selected microorganisms (2-26, 1-26-27, st.pr.) the necrosis of tissues was minor and concerned individual leaves.

The increase in weight of plants stimulated by the presence of bacteria 2-26 was similar as in the control, which suggests the reduction of the toxic effect presumably as a result of microbiological transformation of mercury. Also, for strain st.pr after 21 days of culture, the increase in biomass was the same as in the control against the strain 2-26 which in this case suggests the adaptation of bacteria to such a high concentration of mercury, and then their participation in the transformation of the element. The least favorable effect on plant growth was observed for strain 1-26-27.

S. natans against the tested bacterial strains maintained a correct ratio of the amount of chlorophyll a and b, whereas the amount of total chlorophyll gradually decreased, but only slightly differed from the amounts in controlled organisms. By contrast, without microbial stimulation, after 21 days this amount was lower by 50% compared to control.

From the graphs it can be seen that the correct proportions of chlorophyll a to chlorophyll b were preserved (approx. 1:2). In addition, in both cases similar trends can be observed. On the 14th day of the experiment, the pigment content significantly decreased in almost all the studied plants.

The exceptions being tissues of the control plants and with the addition of strain st.pr. For *S. natans* control, the content of chlorophyll a decreased proportionally from 74.81 mg/g of protein (day 7) to 57.74 mg/g of protein (day 21), and chlorophyll *b* from 40.88 mg/g of protein (day 7) to 32.73 mg/g protein (day 21).

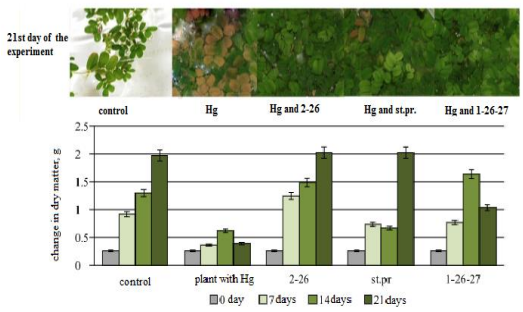


Fig. 8. Increase *S.natans* dry matter in the presence of mercury and strains.

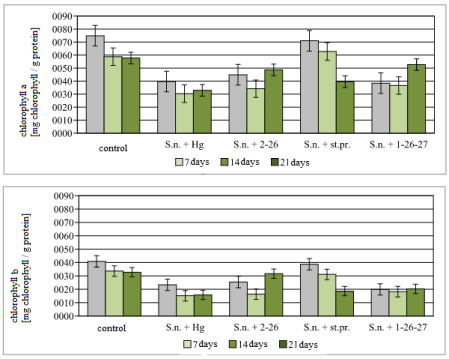


Fig. 9. Amount of chlorophyll in tested plant.

In contrast, in *S. natans* tissues exposed to mercury with the strain st.pr., the amount of chlorophyll content decreased from 71.03 mg/g of protein to 39.58 mg/g of protein forchlorophyll *a*, and from 38.71 mg/g of protein to 18.58 mg/g of protein for chlorophyll b.

As is apparent from the obtained results, the protein synthesis is not translated into a number of photosynthetically active pigments. This indicates a decrease in the living activity of plants, because the inhibition of photosynthesis process may result in a shift in the energy balance of the cell.

The highest content of chlorophyll a on the last day of the experiment characterized the control plants (57.74 mg/g of protein) and *Salvinia natans* in the presence of microorganism 1-26-27 (52.67 mg/g of protein).In the case of chlorophyll b, it was the highest in control plants (40.88 mg/g of protein), and plants in the presence of strain 2-26 (31.68mg/g of protein).The lowest value of the pigmentin both cases characterized the plants without the bacterial help grown in the presence of mercury.For chlorophyll a it amounted to 32.89 mg/g of protein, and for chlorophyll *b* – 15.77 mg/g of protein. This indicates a possible impairment of photosynthesis under the influence of mercury ions.

In the case of plants containing bacterial strains, an increase of the content of the element in tissues along with prolonged exposure time as compared to plants without the microorganisms can be seen (Fig. 10). The greatest accumulation of mercury was found for *Salvinia natans* in the presence of the strain on the 2-26 in the 21st day – 693.2 mg/kg DM, which is approx. 22% more than in the case of plants without the help of bacteria, which fully emphasizes the nature of the stimulus effect of the tested bacterial strains on process of mercury accumulation by *S. natans*. It should be emphasized that although the accumulated element impairs such physiological processes as protein and chlorophyll synthesis, it did not have a significant impact on the growth of biomass. In the case of the

other variants, after 21 days, the cumulative amount of mercury amounted in the presence of 1-26-27 to581.9 mg/kg DM and 632.4 mg/kg DM in st.pr. (Fig. 11).

4 Summary

The study has shown that in the case of used strains of bacteria resistant to high concentrations of mercury, not only measurable parameters as biomass growth and assimilation pigment content per gram of protein show its stimulating effect on plants. Visual assessment and condition of plants can allow to determine only minor in necrotic and chlorotic changes caused by mercury. Therefore, the significant reduction in toxic effects in *S. natans* by strains of *Pseudomonas,* with simultaneous increase by more than 20% of its accumulation capacity should be emphasized.

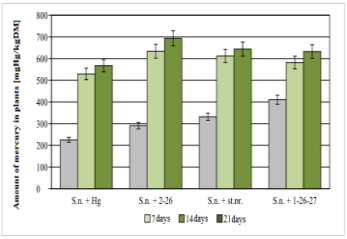


Fig.10. Amount of mercury in plants.

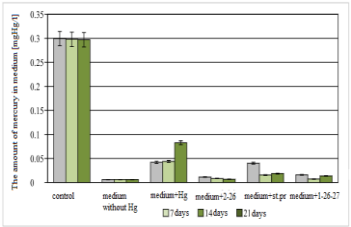


Fig. 11. The amount of mercury in Hoagland's medium.

In conclusion, it should be emphasized that, in the environment, especially among epiphytic microflora, bacterial strains with very specific characteristics can be found, the presence of which is difficult to explain with the influence of processes such as adaptation to high concentrations of anthropogenic pollution. Used *S. natans* was taken from the Oława river. A river, whose waters are used for the purposes of water supply for Wroclaw and in which the permissible concentrations of heavy metals are not exceeded. Nevertheless, among its epiphytic bacteria were *Pseudomonas* strains showing resistance to 0.6mgHg/l. The results indicate that in the use of stimulated phytoremediation processes of waters contaminated with mercury, it may be possible to find technology to remove that biologically very toxic element from water used for the purposes of water supply. Moreover, it seems that the relatively safe disposal of plant biomass contaminated by mercury can be achieved through drying and thermal utilization.

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