

Original Research

Assessment of *in vitro* Multiplication of *Lemna minor* in the Presence of Phenol: Plant/Bacteria System for Potential Bioremediation – Part I

Olga Radulović^{1*}, Marija Petrić², Martin Raspor², Vojin Tadić³,
Petar Jovanović⁴, Vladan Zečević⁵

¹Institute for Chemistry, Technology and Metallurgy, Njegoševa 12, Belgrade, Serbia

²Department for Plant Physiology, Institute for Biological Research “Siniša Stanković”,
Bulevar Despota Stefana 152, Belgrade, Serbia

³Mining and Metallurgy Institute Bor, Zeleni bulevar 35, Bor, Serbia

⁴Institute of General and Physical Chemistry, Studentski Trg 12-16, Belgrade, Serbia

⁵Faculty of Engineering and International Management, Carigradska 28, Belgrade, Serbia

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Abstract

The aim of this work was to examine the multiplication of the common duckweed (*Lemna minor*), an aquatic plant species widespread in European stagnant waters, in two different media (Murashige – Skoog and Hoagland) with and without phenol supplementation. In order to quantify plant multiplication we have used relative growth rate and tolerance indices on both tested media and at five phenol concentrations (10, 15, 20, 30 and 100 mg/L). Furthermore, we examined the possibility of phenol removal from aqueous media containing different phenol concentrations, by using plant/bacteria system consisting of the duckweed and its naturally occurring microbial populations. After 7 days, number of newly formed fronds was approximately four times higher than at the beginning of the experiment on both tested media. The most important result in this study was removal of 70% of phenol from the highest initial concentration of 100 mg/L, in mixed cultures of duckweed and bacteria. By comparison, aseptic duckweed cultures removed approximately 50% of phenol at the same initial concentration. Our duckweed specimen showed a fast reproduction rate, high tolerance to phenol and a possible cooperation with rhizosphere-associated bacteria. All of these traits can be ultimately utilized for bioremediation purposes.

Keywords: phytoremediation, bioremediation, phenol, rhizosphere, *Lemna minor*

Introduction

Duckweed (Lemnaceae) is a monocotyledonous aquatic plant endemic to water bodies such as lakes and ponds on most continents. Among five genera with 37 species, the genus *Lemna* is comprised of 13 species usually described as tiny floating aquatic plants with simplified morphology and a high biomass production rate [1]. Duckweed is an example of neoteny in higher plants, implying that they do not develop the shoot apical meristem and consequently form a frond, a hybrid tissue derived from leaf and stem precursor tissues [2]. In *Lemna* spp., each frond is connected to a single root [3]. Reproduction is dominantly vegetative and highly efficient, whereby each frond produces 4-12 daughter fronds during its 4-5-week-long life, which helps duckweed spread quickly over water surfaces [4]. Fast vegetative reproduction, along with the ability to accumulate inorganic and organic toxic substances, provides duckweed with great potential for remediation of waters polluted with macronutrients, organic xenobiotics, and inorganic pollutants such as heavy metals, arsenic, and selenium [3] which is a process defined as bioremediation or, specifically, phytoremediation. Duckweed biomass can be harvested after bioremediation and used for biofuel, fertilizer, or fodder, depending on its previous exposure to pollutants or nutrients. It has been suggested that duckweed has greater potential for application in wastewater bioremediation and biomass production than other fast-spreading water species such as water hyacinth (*Eichhornia crassipes*) [5].

Interplay of Bacteria and Duckweed in Bioremediation of Phenol Contamination

There have been attempts to assess the phytoremediation potential of duckweed [6], and while some of the remediation potential can be attributed exclusively to its metabolic activity, an important role in detoxification of wastewaters has been credited to the microorganisms inhabiting its rhizosphere – most prominently bacteria. Bioremediation may be conducted by the bacteria alone [7-8] or conversely by plants alone; however, the process is greatly enhanced with plants and bacteria combined [8-9], which implies the cooperative nature of interactions between the bacteria and their duckweed host. Additionally, Toyama et al. have proven that phenolic exudates of the giant duckweed attract phenol-degrading bacteria regardless of the extrinsic phenol in the bulk water [10], prompting the researchers even further to find the appropriate phenol-degrading bacteria within the rhizosphere.

Potential of Biological Decontamination of Phenolic Pollution in Ambient Waters

Phenolic compounds are very toxic industrial products that are hazardous to all aquatic species and to human health as well. In fact, even diluted phenol solutions can

cause acute poisoning [11]. Phenol elimination was definitely confirmed using two strains of rhizosphere-associated bacteria: *Acinetobacter calcoaceticus* and *Pseudomonas fluorescens* [8, 12-13]. Wastewater of industrial origin (pharmaceuticals, plastics, dyes, paper) usually contains high levels of phenolic compounds. Therefore, phenol-contaminated industrial wastewaters are strictly forbidden from being released into free river flow without prior treatment and decontamination, although the constant leakage of small amounts of phenolic compounds remains an alarming problem worldwide, especially in developing countries (including the Balkans) [13-15]. Standard physical and chemical methods of decontamination (Fenton oxidation, UV radiation, ozonization, etc.) are effective but expensive, and may produce other toxic byproducts. In light of this restriction, biological methods of decontamination are considered to be a preferable substitute and/or a highly desirable finishing polishing step: biological decontamination is more sustainable and effective long-term [16-18]; additionally, the utilization of autochthonic, non-invasive, and non-pathogenic organisms preserves and restores the natural balance of the contaminated site [16]. Consequently, further studies on the potential application of European-widespread species, common duckweed (*L. minor*) in wastewater remediation is needed. This work represents the first step toward constructing a biculture-based plant-bacterial system for *in vitro* analysis of bioremediation of contaminated surface waters. To this end, we conducted: (1) surface sterilization and *in vitro* cultivation of sterilized *L. minor* plants and (2) test of phenol removal using both surface-sterilized and non-sterile fronds.

Experimental Procedure

Preparing Plant Material

Duckweed plants (*Lemna minor* L.) were collected from a pond in the garden of the Siniša Stanković Institute for Biological Research, University of Belgrade. Plants were washed with tap water for 20 min and afterward surface-sterilized in commercial bleach solution containing 5% (v/v) sodium hypochlorite for 5 min, followed by washing with sterile distilled water three times. Plants (2-4) were placed on two standard nutrient media for *in vitro* plant cultivation: Murashige and Skoog medium with complete micro- and macronutrient formulation [19] or on typical Hoagland-Arnon medium [20], both made with 30 g/L sucrose and pH 6.2, to allow frond regeneration. Nutrient medium was made without agar (liquid medium). For non-aseptic cultures, plants were taken directly from the pond using instruments for aseptic culture, and washed three to five times in Petri dishes with sterile water before being transferred to sterile media without performing surface sterilization, in order to keep the original rhizosphere microorganisms while washing off casual contaminants. Both aseptic

and non-aseptic plants were grown at $+24\pm 2^\circ\text{C}$ (under fluorescent light of $40\ \mu\text{mol m}^{-2}\text{s}^{-1}$ 16 h light / 8 h dark photoperiod). Daughter fronds derived from a single mother frond were kept and maintained as stock cultures. The number of new plants was measured on both nutrient media. The experiment was performed in three replicates for each medium. Bacterial cultures associated with the rhizosphere were subsequently isolated by submerging approximately 10 fronds into 5 mL of lysogeny broth (LB) liquid medium, which was then streaked and re-streaked on LB solid agar until purity of the colony has been achieved [21]. Pure strains were transferred to MacConkey agar and cultivated at ambient temperature overnight or until growth was observed [22].

Effect of Media Composition on *in vitro* Growth of *L. minor*

Each replicate contained sterile fronds grown in cylindrical bottles (4 cm in diameter) containing 40 mL of liquid medium with randomly chosen 4 fronds. The number of fronds was observed every two days for 7 days. Biomass production was calculated after 20 days of growth on both nutrient media. Prior to biomass measuring, plants were transferred onto filter paper and left to dry for 1 minute.

L. minor relative growth rates, RGR ($\text{g g}^{-1}\text{day}^{-1}$) were calculated according to the equation (Eq. 1):

$$(\ln W_2 - \ln W_1) / (T_2 - T_1) \quad (1)$$

...where W_1 , T_1 , and W_2 , T_2 are initial and final dry weights and times for each treatment, respectively [23]. Tolerance index (T_i) was calculated according to (Eq. 2) [24]:

$$\frac{\text{Dry plant weight (phenol grown)}}{\text{Dry plant weight (control)}} \quad (2)$$

Study of the Effect of Phenol on *Lemna minor* Growth and Possibility of Phenol Removal from the Medium

Nutrient medium was supplemented with phenol: final concentrations of 10, 15, 20, 30, and 100 mg/l, respectively. Control plants were grown in medium without the addition of phenol. Non-sterile plants (picked directly from pond) were used as a model for investigating phenol removal with the natural "as is" rhizosphere community. Cultivation was performed at $24\pm 2^\circ\text{C}$. Samples (2 mL) of liquid nutrient medium were collected every day during five days of cultivation. Pure nutrient medium without phenol was used as blank control. Determining intrinsic phenol concentration was done in medium without artificially added phenol after 1 day. Further, aseptic plants and non-sterile plants were added to sterile phenol-supplemented media. Phenol

concentration was determined every day over the following five days.

Determining Phenol Concentration in Nutrient Medium

Determining the concentration of phenol in water was done spectrophotometrically with the use of 4-aminoantipyrine (4-AAP). Samples were treated with phosphoric acid at pH 4.0, and then distilled prior to spectrophotometric measurement. All measurements were performed at $+25^\circ\text{C}$.

The mixture prepared for the measurement of the concentration of phenol in water contained a distilled sample, buffer (16.9 g ammonium chloride dissolved in 143 mL of ammonium hydroxide, pH 10), 2 mL of 4-AAP solution (2 g of 4-AAP dissolved in 100 mL of distilled water), and 2 mL of potassium ferricyanide solution (8 g $\text{K}_3\text{Fe}(\text{CN})_6$, dissolved in 10 mL of distilled water). The reaction solution was mixed and after 15 min absorbance was measured at 460 nm, which is the absorbance maximum for the quinone – imide adduct formed in the reaction of 4-AAP and phenol. Standards were measured at the same wavelength, and the dilutions of phenol were prepared in the concentration range from 0 to 1000 $\mu\text{g/L}$. All measurements were made according to the regulations of the Institute for Standardization of Serbia, code of ISO 6439 B: 1997, water quality-determination of phenol index (Institute MOL, Stara Pazova, Serbia), and were performed on UV VIS 1166, Labomed Inc. (LA, California, USA).

Statistical Analysis

Numerical data were analyzed using the computer program *Statgraphics version 4.2* (STScI Inc. and

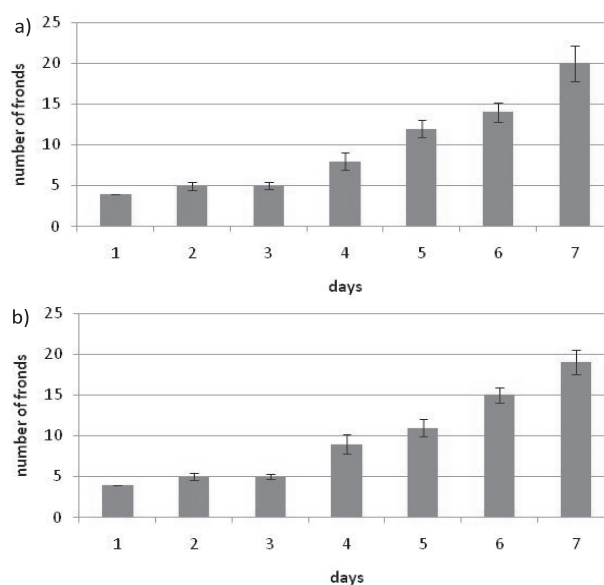


Fig. 1. Multiplication rates of duckweed cultivated on MS – and H – medium.

Statistical Graphics Corporation, 1985-1989, USA). Analysis of variance (ANOVA) and least significant difference (LSD) test at $p \leq 0.05$ were used to determine statistically significant differences between mean values. The graphical representation of the results was done with Microsoft Excel.

Results and Discussion

Effects of the Two Nutrient Media (Murashige-Skoog and Hoagland) with or without Phenol on *L. minor* Multiplication and Morphology

MS and H media have similar effects on *L. minor* multiplication (Fig. 1). After 4 days of cultivation, the effects of MS and H media on multiplication of duckweed were determined as frond duplication (4 new fronds when grown on MS and 5 new fronds on H). After 7 days, the number of newly formed fronds was about four-fold higher than at the beginning of the experiment. Newly formed plants kept their normal morphology on both tested media. Further growth on both media led to regenerating more plants at an almost geometrical rate.

Relative growth rate of *L. minor* declined with increasing phenol concentration on both media (Fig. 2). The trend in RGR decline was constant but slow, and the lowest biomass production was noticed at the highest phenol concentration (100 mg/l). RGR of *L. minor*, growing on medium with 100 mg/l of initial phenol concentration, was diminished and was 8% lower than that on medium without phenol. However, plants remained morphologically unchanged.

Rhizosphere-Associated Bacteria

To assess the diversity of bacteria associated with the roots of the non-sterile plants, we examined the Gram stain reaction and overall morphological

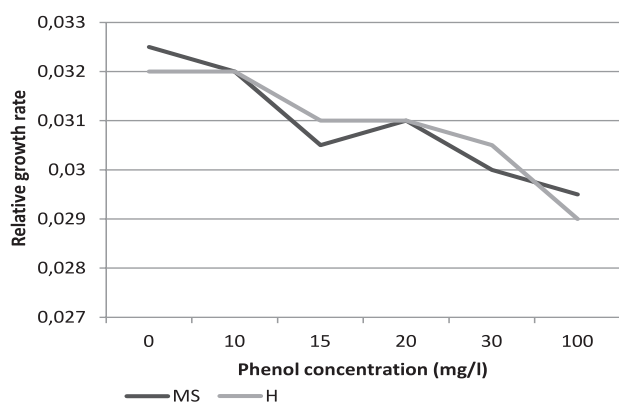


Fig. 2. Relative growth rates of duckweed cultivated on MS – and H – medium supplemented with varying concentrations of phenol (0 – 100 mg/l).

Table 1. Tolerance index (Ti) relative to phenol concentration in the nutrient medium. MS=Murashige and Skoog medium; H=Hoagland medium

Phenol concentration (mg/L)	Ti (MS)	Ti (H)
0	1±0.14	1±0.11
10	0.97±0.12	1.031±0.17
15	0.91±0.11	0.96±0.11
20	0.92±0.10	0.98±0.12
30	0.88±0.14	0.95±0.11
100	0.86±0.15	0.89±0.11

properties of the colonies isolated from the rhizosphere. Morphologically, Gram negative coccoid bacilli were prevalent. Based on MacConkey agar reaction, the majority of isolated cultures were enteric and lactose-fermenting. Based on light microscopy, we identified at least 6 different bacterial genera that are associated with the rhizosphere of *Lemna minor*: enteric bacteria (possibly *Klebsiella* and *Enterobacter*), *Bacillus*, *Pseudomonas*, *Staphylococcus*, and *Micrococcus*.

Phenol – Tolerance of *L. minor*

To investigate the capability of plants to grow in phenol-supplemented medium, the tolerance index (Ti) was calculated. As reported in Table 1, we determined Ti of *L. minor* on both examined media. It is obvious that Ti does not differ significantly between plants grown on MS or H media. Also, Ti indices of plants grown on increment concentrations of phenol did not differ significantly. Actually, Ti index remained surprisingly high even at the highest phenol concentration.

Phenol – Removal Capacities of Aseptic and Non-Aseptic *L. minor*

To determine the possibility of phenol removal from medium by aseptically grown *L. minor* plants and by non-sterile *L. minor* plants, phenol has been added to nutrient media in final concentrations: 5, 10, 15, 20, 30, and 100 mg/l, respectively. Phenol in blank solution was determined after one day. Control plants were grown on medium without phenol. After 4 days, concentration of phenol in the control group (no phenol supplementation) was significantly higher in medium with non-sterile plants (more than two-fold). By day 5, phenol concentration in the control group dropped below 5 mg/l, while it remained two times higher in the experimental group with non-sterile plants (Fig. 3a). Aseptic *L. minor* plants appeared to remove much more phenol from initial concentration of 10 mg/l than the mixed culture of plant and bacteria (Fig. 3b). After 5 days their phenol removal rates were equal. After only 3 days, phenol concentration in the culture of aseptic plants dropped to 50% of initial value

(Fig. 3b). At initial concentration of 15 mg/l of phenol the non-sterile plants removed phenol more efficiently. After five days of cultivation, phenol concentration in the media with aseptic plant cultures dropped to 50% the initial value, while in the mixed cultures it dropped to 33% of the initial concentration (Fig. 3c).

At higher phenol concentrations in growth media (20 and 30 mg/l), non-sterile plants again removed more phenol from the initial concentrations compared to aseptic plants (Figs 3d and 3e). At the highest initial concentration of phenol (100 mg/l), non-sterile plants were significantly more efficient in removing phenol than aseptic plant cultures (Fig. 3f). After five days of cultivation at 100 mg/l, phenol concentration dropped from 100 to 30 mg/l in non-sterile cultures.

Common Duckweed (*L. minor*) is a Promising Phenol – Eliminating the Plant, and the Process is Enhanced in the Presence of the Rhizosphere – Associated Microbial Population

We have examined different media for *L. minor* growth *in vitro* and their impact on plant morphology

and multiplication. MS and H media have been successfully used in agriculture, plant tissue cultivation, and duckweed cultivation [3, 25]. Our results show that the differences in the effect two media have on frond multiplication are negligible, which might not be true for all ecotypes [25]. We have documented a doubling time of 4 days. By comparison, Ziegler et al. tested more than 400 ecotypes of the Lemnaceae family and observed the doubling time ranging from 1.34 to 4.54 days on average [3]. Yamaga et al. [8] noticed that during long-term growth, non-sterile fronds multiplied faster compared to sterile plants, regardless of phenol. We did not observe such a phenomenon. This might be a consequence of specific bacterial community composition, i.e., whether the rhizosphere is dominated by plant-growth-promoting bacteria [8]. As we observed no differences in terms of plant morphology, proliferation, and relative growth between *L. minor* plants grown on Hoagland and MS media *in vitro*, further experiments on phenol removal were carried out exclusively on MS media.

Relative growth rate (RGR) of *L. minor* declined with increasing phenol concentration in medium. The highest RGR reduction was observed when plants were

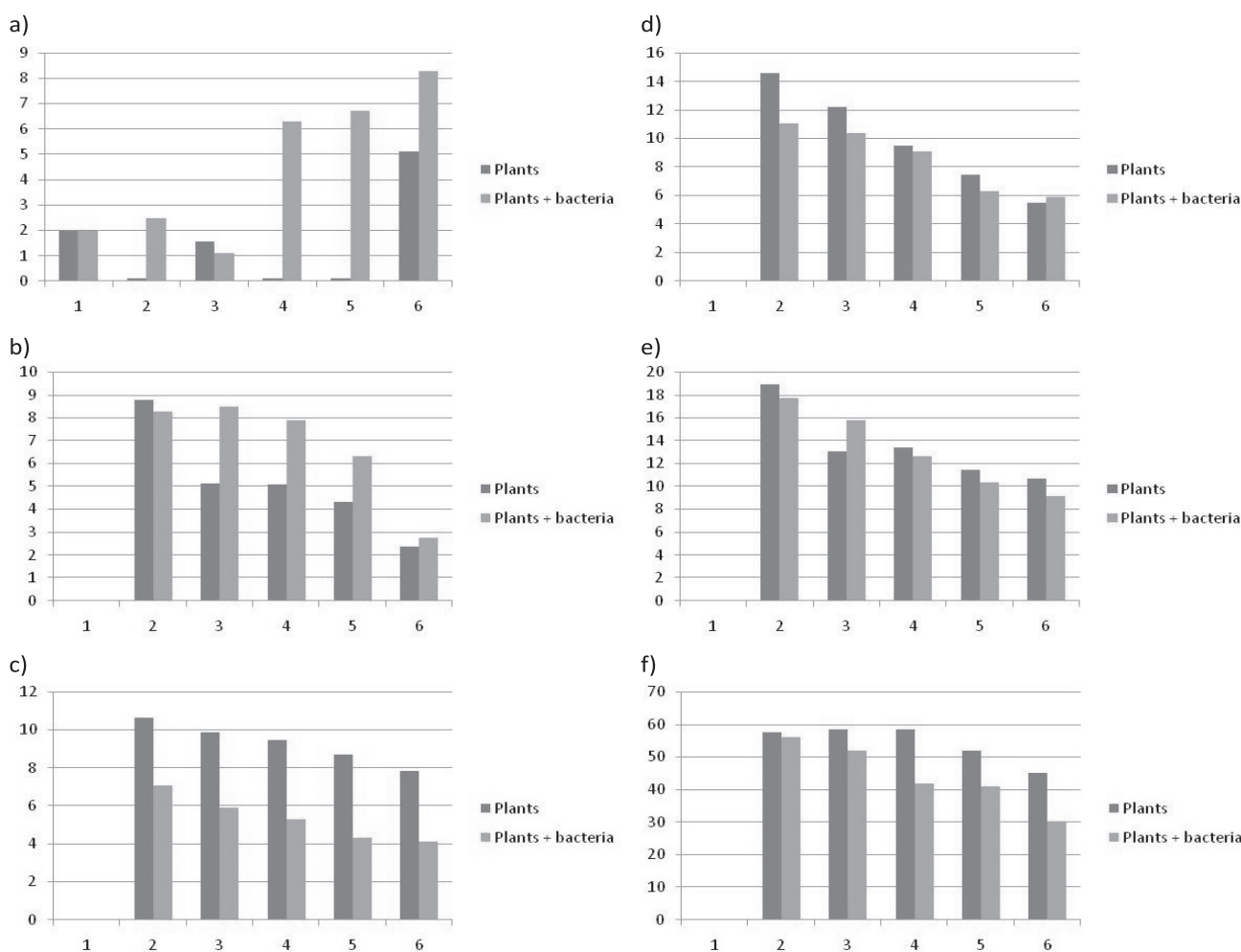


Fig. 3. Removal rate of phenol at different initial concentrations in MS – medium. A – No extrinsic phenol added. Initial concentration of phenol is: B – 10 mg/l; C – 15 mg/l; D – 20 mg/l; E – 30 mg/l; F – 100 mg/l.

exposed to the highest phenol concentration (100 mg/l). Our results are in correlation with Bianconi et al. [26], where a significant decline in RGR was documented when plants of *L. minor* were exposed to high cadmium concentrations. However, *L. minor* population *in vitro* continues to multiply even at elevated phenol concentrations. Enzymatic mechanisms of phenol uptake and transformation by the plants are diverse [27-28]. Although the exact enzymatic status of our experimental plants remains to be examined, all of our plants retained a great survival rate (100%) at all examined phenol concentrations, which suggests that *L. minor* has an inherent and highly active enzymatic system for phenol neutralization. These results are similar to that of Bianconi et al., where *L. minor* was marked as a plant with high bio-concentration factor for Cd and had also a remarkable survival rate [26].

The capability of plants to grow in the presence of a toxic substance is described using tolerance index (Ti). Plants are considered tolerant if their tolerance index (Ti) is higher than 0.6 [23-24, 26]. In our experiments, *L. minor* retained a Ti of more than 0.7 and an unchanged morphology at all tested phenol concentrations, which implies that this plant is indeed a promising bioremediation agent. On top of their simplified anatomy and one of the smallest genomes among higher plants (similar in size and organization to *Arabidopsis thaliana* [2]), Lemnaceae are easy to manipulate genetically *in vitro*, opening yet another way to enhancement of their bioremediation capacity [29].

Due to their efficient metabolism of aromatic compounds and potential of genetic transformation *in vitro*, bacteria are, as a group, generally regarded as efficient bioremediation organisms [8, 12, 17-18]. In our study, we have cultivated in parallel both the aseptic plants and plants associated with naturally occurring microbial populations in the rhizosphere of the duckweed, which are dominated by bacteria. Aseptic cultures of plants at the initial low phenol concentration (10 mg/l) apparently removed more phenol than mixed cultures of plants and bacteria (Fig. 3b). However, these efficiency rates may likely be the consequence of the fact that non-sterile cultures actively produced a comparably high amount of phenolic compounds and of the fact that the 4 AAP method does not discriminate between different phenolic compounds (compare with Fig. 3a). It is safe to assume that both plants and bacteria synthesize certain phenolic compounds in varying ratios (probably caffeic acid, chlorogenic acid, isochlorogenic acid, etc.), leading to falsely positive results.

According to our experiments, non-sterile fronds removed phenol more efficiently than aseptically grown fronds, and the efficiency of removal remained constant at all initial concentrations. Approximately 70% of phenol was removed after 5 days, which may suggest that the rhizosphere bacteria are not only metabolically active when exposed to phenol in the 10-100 mg/l

range, but also highly resistant to phenol. Aseptic plants removed phenol with varying efficiency (approximately 50-70% of the initial phenol eliminated after 5 days). The greatest difference in elimination rates first occurred at 15 mg/l and then at 100 mg/l of initial phenol concentration (Figs 3c and 3f), which may indicate that this is the stress-inducing range of phenol for the common duckweed, while its rhizosphere bacteria remain unaffected. Moreover, bacteria may act to some extent as a protective biological layer, minimizing the oxidative stress and cell wall disruption that occurs with exposure to higher doses of phenol [30]. Finally, all of these observations are consistent with the experiment with giant duckweed (*Spirodela polyrrhiza*), where phenol elimination by aseptically grown plants was negligible, whereas the elimination dependent on mixed culture of plants and rhizobacteria was significantly enhanced [9-10].

Conclusions

We have documented a mutually beneficial relationship between *Lemna minor* (the common duckweed) and the microbial community of its rhizosphere that could be used in bioremediation of phenol-contaminated waters. This relationship apparently allowed the duckweeds to survive 100 mg/l of phenol, which is considered a toxic concentration, and the elimination of 70% of starting 100 mg/l of phenol in only five days. Similarly to other Lemnaceae family members, our specimen of *L. minor* has a typically short doubling time and a high tolerance to phenol as well. The microbial population in its rhizosphere is probably specific to sampling site and the particular duckweed ecotype. The bacterial population of the rhizosphere must be analyzed in more detail (e.g., 16S rRNA sequencing and additional biochemical testing, including phenol tolerance), but the preliminary data presented in this paper are sufficient to propose the duckweed-microbial system (with emphasis on bacteria) as a promising platform for bioremediation of phenol-contaminated waters.

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Conflict of Interest

The authors declare no conflict of interest.

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