

Original Research

# Culture-Dependent Analysis of 16S rRNA Sequences Associated with the Rhizosphere of *Lemna minor* and Assessment of Bacterial Phenol-Resistance: Plant/Bacteria System for Potential Bioremediation – Part II

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## Abstract

In this work, we demonstrate that the rhizosphere of common duckweed (*Lemna minor*) is inhabited with various phenol-resistant bacterial strains. Based on 16S rRNA sequencing, we have identified 60 rhizosphere-associated bacterial isolates belonging to 10 different bacterial genera (*Pseudomonas*, *Hafnia*, *Serratia*, *Enterobacter*, *Micrococcus*, *Stenotrophomonas*, *Xanthomonas*, *Bacillus*, *Staphylococcus* and *Klebsiella*). All isolates have been tested for phenol resistance and ability to utilize phenol as the sole carbon source. 70% of all isolates survived high doses of phenol ( $\geq 200$  mg/L) and at least 27% can be potentially acclimatized by gradual increase of phenol concentration. Finally, based on high phenol resistance, ability to utilize phenol as the sole carbon source and documented low pathogenicity, we propose 5 strains as potentially excellent candidates for bioremediation. These 5 strains taxonomically correspond to *Klebsiella* sp., *Serratia* sp., and *Hafnia* sp., respectively. To the best of our knowledge, this is the first attempt to assess decontamination capacity of *Serratia nematodiphila* and *Hafnia* sp. in the context of bioremediation of phenol-contaminated aqueous media. Although additional analyses are needed, interaction between the common duckweed and the selected bacterial strains may be utilized in future bioremediation strategies.

**Keywords:** bacteria, bioremediation, rhizosphere, phenol resistance

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## Introduction

Phenol contamination is a major environmental and health concern worldwide. Phenol is produced and used in the petrochemical, textile, plastics, and pharmaceutical industries. Therefore it is not surprising that phenol is declared a priority toxic compound in the USA [1]. Although there is no evidence of carcinogenic effects, phenol is a teratogen and an irritant. Phenol concentrations as low as 5 mg/L are considered toxic to all living organisms, while concentrations of 20 mg/L and higher are lethal to water vertebrates. Additionally, phenol is most stable and long-lived in aqueous environments, where it can persist for up to 14 days [1, 2]. A consensus has been reached that the optimal strategy for phenol decontamination of ambient waters includes physical and chemical degradation methods combined with final polishing steps based on the natural ability of some living organisms (primarily bacteria and plants) to neutralize phenol and derived compounds, i.e., bioremediation [2-4]. This is especially true for the aromatic compounds that can still exert adverse effects on the natural environment even years after an incident [5], and aquatic environments are particularly sensitive to this type of pollution due to the fact that phenol is more stable in water than elsewhere, and rapidly dissolves in large volumes of water.

To make the matter even more complex, not all bacterial species are able to degrade phenol or can even survive in a phenol-polluted medium since phenol was not present in significant amounts in the environment until the industrial era. Therefore, bacteria had merely 250 years to adapt, and this evolutionary process is still ongoing [6]. Bacterial species in constant contact with elevated amounts of phenol are under greater selective pressure [7, 8], and therefore it is expected that urbanized areas are the source of many phenol-resistant strains, where resistance is defined as the ability of a living organism to survive adverse conditions. *In vitro*, it is shown that diversity of genes responsible for phenol degradation rises with the phenol increment [8, 9]; furthermore, individual bacterial strains isolated from different microhabitats have been identified as excellent bioremediation agents [10, 11]; however, little is known about the overall distribution of phenol resistance in specific microhabitats. The rhizosphere can be regarded as a microhabitat adjacent to the plant root where microbial communities are aggregating, attracted by the metabolic activity of the plant, and is also a known place of intense horizontal gene transfer, which helps disseminate resistance-related genes [6-9, 11].

Only highly resistant bacterial strains can be used in bioremediation strategies. Moreover, combining water plants and bacterial communities has been proven to enhance the efficiency of bioremediation [12, 13]. Certain species of the Lemnaceae family, such as *Spirrodela polyrhiza* (a cosmopolitan species) and *L. aikoukusa* (Japanese species), are known for their ability to eliminate phenol or derived aromatic

compounds, with their rhizosphere probably harboring a variety of bacterial species of which only a few with phenol-eliminating activity have been isolated and identified thus far [14-16]. However, investigations regarding the decontamination capacity of *Lemna* are incomplete because only a small fraction of the family has been analyzed for the potential of phenol-contaminated water. Furthermore, introducing a new species into an ecosystem is always inherently combined with the risk of disturbing the ecological balance or, in the case of bioremediation, of exacerbating the state of an already contaminated site. By selecting a species indigenous to the area where contamination occurred, this risk would be significantly diminished.

The aim of this work was to isolate and identify bacteria from the rhizosphere of a Lemnaceae representative indigenous to Serbia, the common duckweed (*Lemna minor*, L), grown in a typical urban setting, and to find evidence of bacterial populations with traits that make them good bioremediation candidates.

## Experimental Procedures

### Plant Material Preparation and Isolation of Bacteria

Duckweed was collected from the garden pond of the Siniša Stanković Institute for Biological Research in Belgrade. Pond water is periodically replenished from the communal water supply system. The pond is situated in a typical urban environment, in the centre of the Serbian capital city, and is exposed predominantly to traffic-related pollution. Duckweed was either brushed gently against sterile filter paper (group A), air dried in an aseptic environment (group B), or washed thoroughly in sterilized distilled water to remove larger contaminants (group D). As a control, 1 mL of surrounding water was used to inoculate 5 mL of sterile liquid lysogeny broth (LB) medium (group C) [14]. Cultures were initiated by transferring 100 µL of suspension from each LB media to LB solid agar in Petri dishes. The colonies were subcultivated many times to obtain purity. All rounds of cultivation were done at +27°C. Colonies were stained according to Gram and checked under the microscope for stain reaction and purity of colonies.

### Bacterial DNA Extraction

Total genomic DNA of selected isolates were prepared according to modified protocol by Le Marrec et al. Briefly, overnight cultures were vortexed, briefly centrifuged at 13,000 xg, and washed in TEN buffer. Lysosime for Gram-positive bacteria or proteinase K for Gram-negative bacteria were resuspended in PP buffer and added to cell suspension. Cell lysis was performed at +37°C for 30 minutes. The suspension was subsequently centrifuged and the pellet resuspended in TEN buffer.

Sodium-dodecyl sulphate (10% solution) was added and the suspension thoroughly vortexed until transparent. Standard phenol/chloroform extraction ensued. The upper phase was mixed with isopropanol and centrifuged. DNA in pellet was additionally washed and precipitated in 70% ice-cold ethanol. The samples were left to dry, then resuspended in RNase solution [15].

#### Polymerase Chain Reaction (PCR) of 16S Ribosomal RNA Genes

Deleted “good quality” DNA extracts were used in PCR amplification. To amplify 16S ribosomal RNA genes, two universal primers covering the length between positions 8 and 1492 in 16S rRNA genes were used: UN1-16SF (GAGAGTTTGATCCTGGC) and UN1-16SR (AGGAGGTGATCCAGCCG). For amplification we used KAPA Taq DNA Polymerase (KAPA Biosystems, Boston, USA). PCR conditions were adapted according to manufacturer’s instructions. PCR products (no overlapping bands, no smearing) were then purified with QIAquick Gel Purification Kit (QIAGEN GmbH, Hilden, Germany), according to manufacturer’s instructions. Purified samples were quantified and sent to Macrogen, Netherlands, for sequencing.

#### Sequence and Statistical Analysis

Sequences were analyzed by Nucleotide BLAST (blastn) to find the corresponding sequences in the GenBank database. Ferograms were additionally checked visually in FinchTV, version 1.4.0. Best BLAST matches (91% identity and more) were used as relevant. Phylogenetic trees were constructed in MEGA 7 using the neighbor-joining method. The distances were calculated using the maximum likelihood composite method as the number of base substitutions per site. The topology was evaluated by the bootstrap method with 1000 replicates. Distances were represented as the number of base substitutions per site [16-18]. Bacterial population diversity was quantified through Shannon’s diversity index (H) and Simpson’s diversity index (D):

$$H = - \sum_i^N \left( \frac{n_i}{N} \ln \frac{n_i}{N} \right) \quad (\text{Eq. 1})$$

$$D = \frac{\sum n_i (n_i - 1)}{N(N-1)} \quad (\text{Eq. 2})$$

#### Phenol-Resistance Test

100  $\mu$ L liquid LB overnight culture of each isolate was streaked onto a Petri dish containing Murashige and Skoog (MS) agar with sucrose [19]. The MS agar plates were supplemented with 200, 500, and 1000 mg/L phenol, respectively. Bacteria were grown at +27°C for 5-7 days or until colonies became visible. The colonies were re-streaked from lower to higher concentrations.

Additionally, all isolates were tested immediately from the overnight culture against 1000 mg/L of phenol in MS agar with sucrose.

#### Utilizing Phenol as the Sole Carbon Source

All bacterial strains able to grow on  $\geq 200$  mg/L were transferred to aqueous solution of salts from MS recipe with 20 mg/L of phenol as the sole carbon source. In short, 1 mL of overnight culture in liquid LB was briefly centrifuged at maximum speed (13,000  $\times g$ ). Supernatant was discarded and the cells were resuspended in 1 mL of minimal MS medium. The cultures were grown at +27°C until a significant increase in biomass was observed.

#### Vitek 2 (Biomerieux) Biochemical Characterization of Selected Isolates

Five isolates that have previously been shown to multiply abundantly in minimal MS medium with phenol as the sole carbon source and which possess significant resistance to phenol ( $\geq 500$  mg/L) were biochemically characterized on Vitek 2 (Biomerieux; Mercy, France).

## Results and Discussion

#### Diversity of Rhizosphere-Associated Bacteria, their Phenol Resistance, and Phenol Utilization

Successful cultivation, isolation, and identification of 60 bacterial strains from the rhizosphere of duckweed (Table 1) were performed. Almost all belong to deleted  $\gamma$ -Proteobacteria group, except 3 Terrabacteria species (*Micrococcus luteus*, *Staphylococcus* sp. and *Bacillus* sp.). Isolates represent 8 different bacterial families: Pseudomonadaceae deleted (28 isolates), Xanthomonadaceae (7), Hafniaceae (6), Staphylococcaceae (6), Enterobacteriaceae (6), Yersiniaceae (5), Micrococcaceae (1), and Bacillaceae (1). Based on Shannon diversity indices, group A was slightly more diverse, followed by C, D, and B (Table 2). Of 60 tested isolates, 18 were unable to grow on 200 mg/L of phenol, whereas 6 grew on a maximum of 200 mg/L phenol. Fifteen strains formed colonies at a maximum of 500 mg/L of phenol and ceased to grow on 1000 mg/L. In total, 21 bacterial strains showed varying degree of growth on a maximum of 1000 mg/L of phenol. Among these, 5 isolates grew on 1000 mg/L of phenol after direct inoculation from an overnight culture, i.e., without any prior *in vitro* acclimatization (Fig. 1; Table 1, non-acclimatized isolates are bolded). Within this group, there were pronounced differences in growth speed and biomass increase. Acclimatized isolates showed toxic effects of phenol and needed approximately 9 days to form. Five strains that were grown without prior acclimatization showed less toxic effects. Of these, *Hafnia alvei* C31-106/2,

Table 1. Summary of isolates, their phenol-resistance and ability to grow on phenol as the sole carbon source. (A: duckweeds were brushed against sterile filter paper before transfer to liquid LB medium. B: duckweeds were air-dried in aseptic environment before transfer to LB liquid medium. C: pond water surrounding duckweeds was used to inoculate LB liquid medium. D: duckweeds were washed gently in sterile distilled water and then transferred to LB liquid medium. "No" annotates inability to grow on MS solid agar supplemented with 200, 500 or 1000 mg/L of phenol. Further explanations in text.)

ID	Strain	Closest relative	Access. No. (closest relative)	Ident.	Accession No. ( <i>L. minor</i> rhizosphere isolates)	Phenol resistance				Phenol as the sole C- source
						No	200 mg/L	500 mg/L	1000 mg/L	
1	D 4-1-3-100/1	<i>Staphylococcus haemolyticus</i> 0078	KP236214	99%	MF526904		+			+
2	D 4-1-3-100/2	<i>Staphylococcus haemolyticus</i> CIFRI P-TSB4	JF784022	99%	MF526905			+		
3	D 4-2-100/1	<i>Staphylococcus haemolyticus</i> SS-13	KX964163	99%	MF526906			+		
4	D 4-2-100/2	<i>Staphylococcus haemolyticus</i> LCR 51	FJ976569	99%	MF526907			+		
5	D 4-2-100/3	<i>Staphylococcus haemolyticus</i> strain S1.2.2	KR779793	98%	MF526962			+		
6	D 5-1-102	<i>Enterobacter</i> sp. WJ3	KT318370	100%	MF536889		+			
7	D 6-2-101	<i>Bacillus thuringiensis</i> NBRC3951	AB680181	91%	MF526908	+				
8	A5-106/3	<i>Pseudomonas</i> sp. JL21	JF740045	99%	MF526909				+	
9	<b>A6-104/2</b>	<b><i>Klebsiella oxytoca</i> N7</b>	KM349412	99%	MF526910				+	+
10	B1-105/2	<i>Enterobacter</i> sp. YUST-DW17	HM640295	94%	MF526911		+			+
12	B2-106/5	<i>Staphylococcus</i> sp. EA_L_11	KJ642371	98%	MF526912	+				
14	D1-104/2	<i>Hafnia alvei</i> strain F10	KT767858	99%	MF526958				+	
15	D1-104/3	<i>Pseudomonas oryzihabitans</i> L-1	NR_025881	99%	MF526913		+			+
16	D1-105/4	<i>Lelliottia amnigena</i> strain Lmb019 16S	KT986089	99%	MF526914		+			
17	A3-102/2	<i>Pseudomonas</i> sp. strain X8	KY490127	99%	MF526960			+		+
18	A3-102/3	<i>Serratia fonticola</i> GS2	CP013913	98%	MF526915			+		
19	A3-104/1	<i>Serratia fonticola</i> GS2	CP013913	98%	MF526916				+	
20	A3-104/2	<i>Stenotrophomonas maltophilia</i> Q1	HE862285	99%	MF526917	+				
21	A3-104/3	<i>Pseudomonas putida</i> KAR35	KR054997	99%	MF526918	+				
22	A3-104/4	<i>Serratia fonticola</i> GS2	CP013913	98%	MF526919	+				
23	A3-104/5	<i>Pseudomonas putida</i> L3	T767824	99%	MF526920				+	
24	A5-106/1	<i>Pseudomonas oryzihabitans</i> L-1	NR_025881	99%	MF526921			+		
25	A6-104/1	<i>Pseudomonas oryzihabitans</i> L-1	NR_025881	98%	MF526922				+	
26	A6-104/3	<i>Stenotrophomonas maltophilia</i> Q1	HE862285	98%	MF526923	+				
27	A7-102/1	<i>Serratia</i> sp. I136	JX566540	98%	MF526924				+	+
28	A7-102/2	<i>Pseudomonas</i> sp. E88	KR703537	98%	MF526925	+				
29	B1-104/2	<i>Pseudomonas putida</i> KAR35	KR054997	99%	MF526926			+		+
30	B1-105/1	<i>Pseudomonas brenneri</i> strain NA	KT184488	98%	MF526927	+				

Table 1. Continued.

31	B1-105/3	<i>Pseudomonas putida</i> KAR35	KR054997	99%	MF526928				+	+
32	B2-106/1	<i>Stenotrophomonas maltophilia</i> Q1	HE862285	99%	MF526929	+				
33	B2-106/4	<i>Pseudomonas putida</i> KAR35	KR054997	99%	MF526930				+	+
34	B2-106/6	<i>Pseudomonas oryzihabitans</i> L-1	NR_025881	98%	MF526931	+				
35	B3-106/4	<i>Stenotrophomonas maltophilia</i> Q1	HE862285	99%	MF526932			+		+
36	C31-106/1	<i>Hafnia alvei</i> FDAAR-GOS_158	CP014031	99%	MF526933				+	
37	<b>C31-106/2</b>	<b><i>Hafnia alvei</i> JCM 1666</b>	NR_112985	99%	MF526934				+	+
38	C31-106/3	<i>Pseudomonas gessardii</i> IHBB 9179	KR085817	99%	MF526935	+				
39	C31-106/4	<i>Pseudomonas gessardii</i> IHBB 9179	KR085817	99%	MF526936			+		+
40	C31-106/5	<i>Pseudomonas fluorescens</i> A12	KT767652	100%	MF526959	+				
41	C32-106/1	<i>Hafnia alvei</i> 14	KY849243	98%	MF526937				+	
42	<b>C32-106/2</b>	<b><i>Pseudomonas</i> sp. AceT-1</b>	FJ605388	99%	MF526938				+	+
43	<b>C32-106/3</b>	<b><i>Hafnia paralvei</i> ATCC 29927</b>	NR_116898	99%	MF526939				+	+
44	C32-106/4	<i>Pseudomonas fragi</i> NRRL B-727	LT629783	99%	MF526940				+	
45	C32-106/5	<i>Pseudomonas putida</i> NBRC 14164	KY952686	98%	MF526961			+		
47	C44-104/1	<i>Pseudomonas yamanorum</i> LMG 27247	LT629793	99%	MF526941				+	+
48	C44-104/2	<i>Hafnia paralvei</i> strain ATCC 29927	NR_116898	98%	MF526942			+		
49	C44-106/1	<i>Pseudomonas</i> sp. AMF4009	JQ316310	98%	MF526943			+		
50	C44-106/2	<i>Enterobacter nimipressuralis</i> LMG 10245	JF430421	98%	MF526944				+	
51	<b>D1-104/1</b>	<b><i>Serratia nematodiphila</i> DZ0503SBS1</b>	NR_044385	99%	MF526945				+	+
52	D1-105/1	<i>Pseudomonas panacis</i> D313	KT758723	98%	MF526946	+				
53	D1-105/2	<i>Pseudomonas putida</i> KAR35	KR054997	99%	MF526947				+	+
54	D1-105/3	<i>Pseudomonas oryzihabitans</i> IHB B 13621	KP762549	99%	MF526948			+		
55	D4-106/1	<i>Pseudomonas putida</i> L3	T767824	99%	MF526949			+		
56	D4-106/2	<i>Micrococcus luteus</i> HN-18	KT003262	98%	MF526950	+				
57	5-2-101	<i>Stenotrophomonas maltophilia</i> Q1	HE862285	99%	MF526951		+			
60	A3-102/1	<i>Xanthomonadales bacterium</i> F2V8C04	HG322894	98%	MF526952	+				
62	B2-106/7	<i>Stenotrophomonas</i> sp. LMG 29892	KY973973	98%	MF526953	+				
63	B3-106/1	<i>Pseudomonas putida</i> KAR35	KR054997	99%	MF526954				+	+
64	B3-106/2	<i>Pseudomonas</i> sp. WXBRN2	KJ184953	98%	MF526955	+				
65	B3-106/3	<i>Klebsiella</i> sp. ZA	KP769536	98%	MF526956	+				
66	D12-106	<i>Pseudomonas putida</i> L3	T767824	99%	MF526957				+	+

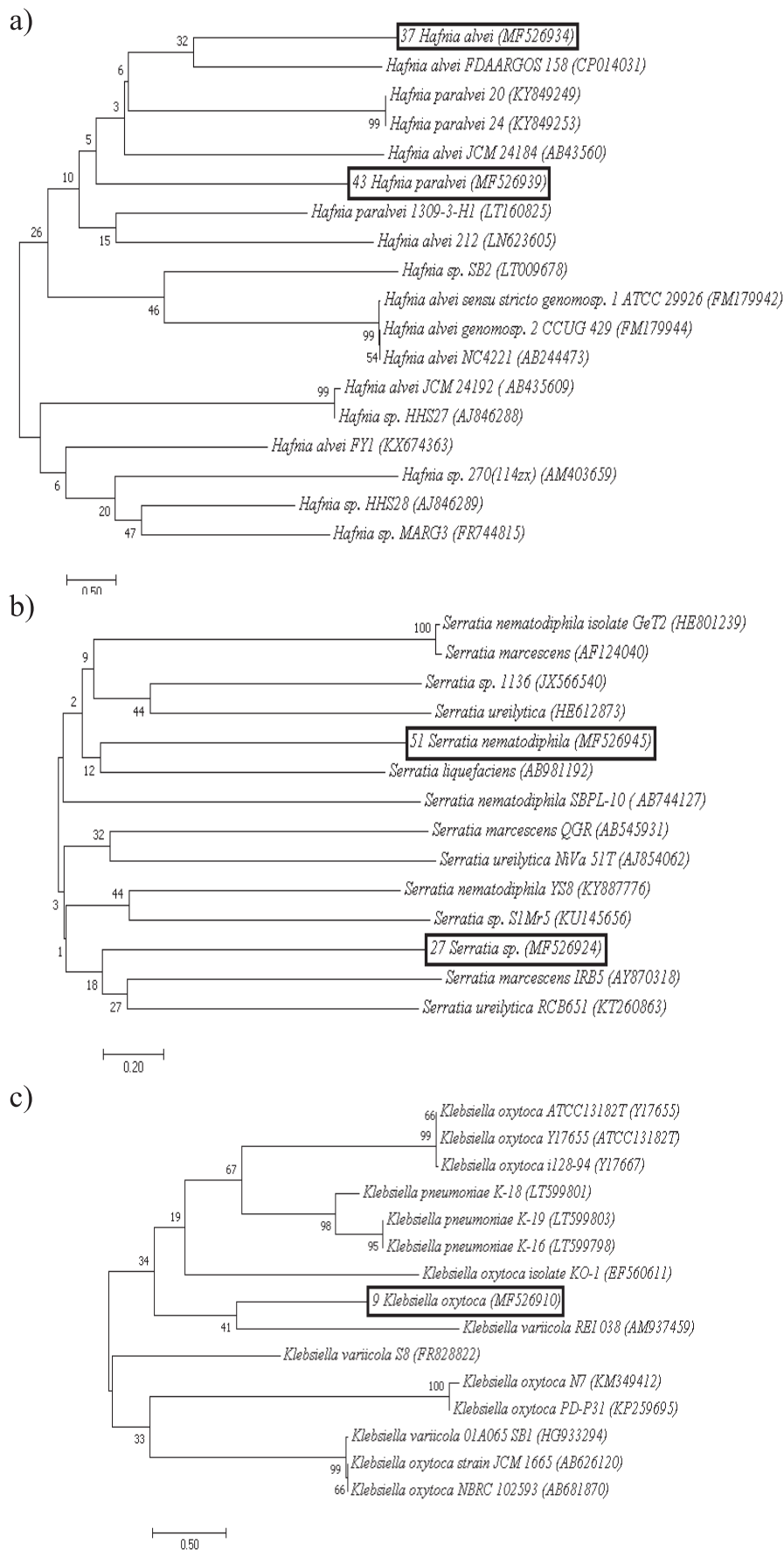


Fig. 1. Evolutionary relationships of 5 strains proposed as the best phenol-eliminating candidates and related species based on partial 16S rRNA sequences (a) – *Hafnia*, b) – *Serratia*, c) – *Klebsiella*; in boxes: 9 *Klebsiella oxytoca*, 27 *Serratia* sp., 37 *Hafnia alvei*, 43 *Hafnia paralvei*, 51 *Serratia nematodiphila*). The scale bar represents number of base substitutions per site.

Table 2. Ecological diversity and phenol-resistance (Shannon's index, H; Simpson's index, D; \*defined as ability to grow on MS agar + sucrose supplemented with 200 mg/L of phenol and more. Additional explanations in text).

Group	Shannon's Diversity Index	Simpson's Diversity Index	Percentage of phenol resistant isolates in the group*	Percentage of isolates able to use phenol as the sole carbon source
A	2.34±0.6	0.103±0.05	60% ±10%	33% ±1%
B	2.03±0.9	0.090±0.04	54% ±16%	21% ±11%
C	2.21±0.7	0.055±0.01	86% ±16%	36% ± 4%
D	2.18±0.7	0.057±0.01	82% ±12%	28% ± 4%
Entire population	2.92	0.054	70%	32%

and *Serratia nematodiphila* D1-104/1, formed visible colonies after 6 days. Colonies of *Klebsiella oxytoca* A6-104/2, deleted, became visible after 7 days. *Pseudomonas* sp. C32-106/2, and *Hafnia paralvei* C32-106/3, became visible after 8 days of cultivation, but exhibited significantly more detrimental effects of phenol (reduced biomass and retarded growth).

Nineteen strains in total were able to grow on minimal medium with 20 mg/L phenol as the sole carbon source. However, the most striking biomass increase after 36 hours of cultivation was observed in 5 isolates: *K. oxytoca* A6-104/2, *S. nematodiphila* D1-104/1, *Serratia* sp. A7-102/1, *H. alvei* C31-106/2, and *H. paralvei* C32-106/3. These strains were characterized by Vitek 2 (Biomerieux, Mercy, France) (Table 3). *Serratia* sp. A7-102/1 and *S. nematodiphila* D1-104/1 have been identified as *Serratia marcescens* (99%). *H. alvei* C31-106/2 and *H. paralvei* C32-106/3 have been identified as *H. alvei* (99%). *K. oxytoca* A6-104/2 has been identified as *K. oxytoca* (94%).

#### Organization of Bacteria Inhabiting the Rhizosphere of *L. minor*

Successful cultivation, isolation, and identification of 60 bacterial strains from the rhizosphere of duckweed (Table 1) were performed. Almost all belong to (deleted)  $\gamma$ -Proteobacteria group, except 3 Terrabacteria species (*Micrococcus luteus*, *Staphylococcus* sp. and *Bacillus* sp.). Isolates represent 8 different bacterial families: Pseudomonadaceae (deleted: family) (28 isolates), Xanthomonadaceae (7), Hafniaceae (6), Staphylococcaceae (6), Enterobacteriaceae (6), Yersiniaceae (5), Micrococcaceae (1), and Bacillaceae (1). Based on Shannon's diversity indices (H), group A was slightly more diverse, followed by C, D, and B (Table 2). Simpson's diversity indices showed more differences in diversity: groups A and B were significantly less diverse than groups C and D. According to Simpson's diversity, the least diverse was group A and the most diverse was group C. Of 60 tested isolates, 18 were unable to grow on 200 mg/L of phenol, whereas 6 grew on a maximum of 200 mg/L phenol. Fifteen strains formed colonies at a maximum of 500 mg/L

of phenol and ceased to grow on 1000 mg/L. In total, 21 bacterial strains showed varying degrees of growth on a maximum of 1000 mg/L of phenol. Among these, 5 isolates grew on 1000 mg/L of phenol after direct inoculation from an overnight culture, i.e., without any prior *in vitro* acclimatization (Fig. 1; Table 1, non-acclimatized isolates are bolded). Within this group, there were pronounced differences in growth speed and biomass increase. Acclimatized isolates showed toxic effects of phenol and needed approximately 9 days to form. Five strains that were grown without prior acclimatization showed less toxic effects. Of these, *Hafnia alvei* C31-106/2, MF526934, and *Serratia nematodiphila* D1-104/1, formed visible colonies after 6 days. Colonies of *Klebsiella oxytoca* A6-104/2, became visible after 7 days. *Pseudomonas* sp. C32-106/2, and *Hafnia paralvei* C32-106/3, became visible after 8 days of cultivation, but exhibited significantly more detrimental effects of phenol (reduced biomass and retarded growth). Nineteen strains in total were able to grow on minimal medium with 20 mg/L phenol as the sole carbon source. However, the most striking biomass increase after 36 hours of cultivation was observed in 5 isolates: *K. oxytoca* A6-104/2, *S. nematodiphila* D1-104/1, *Serratia* sp. A7-102/1, *H. alvei* C31-106/2, and *H. paralvei* C32-106/3. In addition, these strains, proven to thrive in minimal medium with phenol as the sole carbon source and with a significant degree of phenol-resistance ( $\geq 500$  mg/L), were biochemically characterized by Vitek 2 (Biomerieux, Mercy, France) (Table 3). Sequencing results were in good correlation with results provided by Vitek 2: *Serratia* sp. A7-102/1 and *S. nematodiphila* D1-104/1 have been identified as *Serratia marcescens* (99%). *H. alvei* C31-106/2 and *H. paralvei* C32-106/3 have been identified as *H. alvei* (99%). *K. oxytoca* A6-104/2 has been identified as *K. oxytoca* (94%).

#### Organization of Bacteria Inhabiting the Rhizosphere of *L. minor*

Slight differences in plant material preparation resulted in visible genetic differences between the isolates (Tables 1 and 2). Groups A and B possess very

Table 3. Biochemical characterization of bacterial strains that can effectively utilize phenol as the sole carbon source. Characterization was performed on Vitek 2 (Biomerieux; Mercy, France).

Reaction	<i>Klebsiella oxytoca</i> (9)	<i>Serratia nematodiphila</i> (51)	<i>Serratia sp.</i> (27)	<i>Hafnia alvei</i> (37)	<i>Hafnia paralvei</i> (43)
Ala-Phe-Pro-Arylamidase	-	-	-	-	-
H <sub>2</sub> S production	-	-	-	+	-
Beta-glucosidase	+	+	+	-	-
L-Pro-Arylamidase	+	+	+	-	+
Saccharose	+	+	+	-	-
L-Lactate Alcalinisation	+	+	+	+	+
Glycine Arylamidase	(+)	+	+	-	-
O129 Resistance (vibrio comp.)	+	+	+	-	+
Adonitol	+	+	+	+	-
Beta-N-Acetyl-Glucosaminidase	-	+	+	+	+
D-Maltose	+	-	+	-	+
Lipase	-	-	-	-	-
D-tagatose	+	-	-	-	-
Alpha Glucosidase	-	-	-	+	-
Ornithine Decarboxylase	-	+	+	-	+
Glu-Gly-Arg Aminidase	-	+	+	-	-
L-Pyrrolidonil Arylamidase	+	+	+	-	-
Glutamyl arylamidase pNA	-	-	-	+	-
D-Mannitol	+	+	+	-	+
Palatinose	+	-	-	+	-
D-trehalose	+	+	+	+	+
Succinate alcalinisation	+	+	-	+	+
Lysine decarboxylase	+	+	+	-	+
L-malate assimilation	(-)	-	-	-	-
L-arabitol	-	+	+	+	-
D-glucose	+	+	+	+	+
D-mannose	+	+	+	+	+
Tyrosine arylamidase	+	+	+	-	+
Citrate	+	+	+	-	-
Beta-N-Acetyl-Galactosaminidase	-	-	+	-	-
L-Histine Assimilation	-	-	-	+	-
ELLMAN	-	-	-	-	+
D-cellobiose	+	-	-	+	-
Gamma-Glutamyl-Transferase	+	+	-	-	+
Beta-Xylosidase	+	-	-	-	-
Urease	+	-	-	+	-
Malonnate	+	-	-	-	+
Alpha-Galactosidase	+	-	-	-	-



Table 3. Continued.

Coumarate	-	+	+	-	-
L-Lactate assimilation	+	-	-	-	-
Beta-Galactosidase	+	+	+	-	+
Fermentation of glucose	+	+	+	-	+
Beta-Alanine Arylamidase	-	+	-	+	-
D-Sorbitol	+	+	+	-	-
5-ketogluconate	+	-	-	+	-
Phosphatase	+	+	+	-	+
Beta-Glucuronidase	-	-	-	+	-

similar Simpson's diversity indices, with the number of phenol-resistant isolates appearing to be reduced in comparison with C and D (Table 2). It appears that drying out and brushing off of plant material in groups A and B significantly diminishes the diversity of the organisms found in the association with the root, as observed with Simpson's diversity indices and to some extent with Shannon's indices. Interestingly, group C contains predominantly *Pseudomonas* sp. and *Hafnia* sp. strains that are restricted to this group only (Table 1, section C). The complete disjunction in taxonomic composition between group C and groups A, B, and D suggests that group C, obtained by cultivation of bacteria from the pond water, contains bacterial species and strains that are not associated at all with the roots of *L. minor*, while groups A, B, and D contain bacterial strains that might be associated with the rhizosphere of *Lemna* to a varying extent. Moreover, distribution of species and strains across the groups suggests that rhizosphere population is not disorganized, but rather specific, and most probably based on chemotaxis, since almost all bacteria associated with the rhizosphere are known to be actively motile. Based on both Shannon and Simpson's indices, the diversity of each group is diminished compared to the overall ecological diversity of the whole rhizosphere-associated population. This also reinforces further the hypothesis that various bacteria prefer the proximity of the plant root. The rhizosphere attracts bacteria primarily by the phenolic exudates of the plant [24-26], which is an effective explanation of why phenol resistance is so frequent within the rhizosphere-associated bacteria.

#### Finding an Appropriate Bacterial Candidate or Bioremediation of Phenol-Contaminated Waters: Correlation with the Current Scientific Literature

Phenol exerted the expected toxic effect on bacteria, although a remarkably high number of isolates (70%) survived beyond the 200 mg/L point, which is considered a highly toxic and even lethal concentration to the living organisms [1, 2]. Widely distributed phenol resistance is probably attributable to the fact that the sampling site

(the pond) is located in an urban area close to an industrial zone notorious for persistent and high above average values of particulate matter of less than 10 microns in diameter (PM<sub>10</sub>) and soot pollution as the consequence of intensive traffic and coal-tar use during winter [27]. Five isolates were able to grow on 1000 mg/L of phenol without any prior acclimatization, while 16 continued to grow on 1000 mg/L of phenol after streaking and re-streaking from lower concentrations, which led to at least some degree of acclimatization. Among tested isolates, we have selected *H. alvei*, *H. paralvei*, *Serratia* sp., *S. nematodiphila*, and *K. oxytoca* as the best candidates for further analyses based on the exhibited biomass increase in minimal medium and high phenol resistance. *K. oxytoca* probably survived due to its documented phenol biodegradation activity [28, 29]. Additionally, the species is living as an endophyte and a plant growth-promoting bacterium, as has been documented in the case of related strain *K. oxytoca* RS-5 [29]. Plant growth promoting ability is seemingly not uncommon in *K. oxytoca* species [30]. *Serratia nematodiphila* is also a documented endophyte that synthesizes indol-acetic acid (IAA), among other plant growth-promoting hormones [31]. Moreover, closely related species can degrade substituted monocyclic phenols [32]. However, to the best of our knowledge, data concerning phenol decontamination capacity of *S. nematodiphila* are lacking. Regarding *Hafnia* sp., two closely related *H. alvei* and the novel *H. paralvei*, apart from living in symbiosis with the plant, also commonly create biofilm, which can explain the phenol resistance, although the literature data describing phenol elimination is apparently lacking as in the case of *Serratia* sp. [32-35]. Almost all of these bacterial species are opportunistically pathogenic (rare causative agents of infections and/or related to infections of heavily immunocompromised individuals only), and commensal (widespread in the natural environment), which makes them comparatively safe for use in potential bioremediation [36]. The exception is the relatively newfound species of *S. nematodiphila*, which has not yet been associated with any case of infection in humans and invades only insects and their larvae mainly due to a specific mutation of their serralyisin protein [37]. Finally, the predominant genus

*Pseudomonas* has a well-documented biodegradation capacity, and its representatives are generally known as “the cleaners” of the natural environment. One of our isolates, *Pseudomonas* sp. C32-106/2, was able to grow directly on MS agar supplemented with 1000 mg/L phenol, albeit weaker in comparison with the remaining isolates. The most prevalent among our isolates was *Pseudomonas putida* strain KAR35, associated with various rhizobacterial communities [38]. Although *P. putida* has been extensively used as a model organism for studies of phenol degradation kinetics, surprisingly, in our study *Hafnia* sp., *Serratia* sp., and *Klebsiella* sp. showed considerably greater phenol-resistance and an ability to grow on phenol as the sole carbon source [39, 40]. This possibly reflects the specific roles certain strains might acquire over time while occupying a very specific niche within an ecosystem. The vast majority of the efficient phenol degraders has been conventionally isolated from the sites of catastrophic phenol pollution (activated sludge, heavily contaminated coal mine soil, municipal and industrial wastewaters) – however, in this work we demonstrate that considerable phenol-degrading activity and resistance can be found in communal waters of urban surroundings and that the bacterial phenol degraders are diverse and organized within the rhizosphere [41]. The bacteria we identified might be best suited for the lesser but recalcitrant pollution since their resistance to phenol did not exceed 1000 mg/L. On the other hand, the bacterial association with a photosynthesizing, non-invasive macroscopic species might contribute to the quicker biological recovery of the contaminated water. Indeed, the data concerning phenol-degradation and phenol-tolerance of European ecotypes of *L. minor* is largely absent from the scientific literature. Therefore, this research is an early attempt to introduce European (Serbian) ecotype of *L. minor* and its specific rhizosphere bacterial population into the bioremediation of phenolic contamination.

### Conclusions

Rhizosphere of the common duckweed (*L. minor* L.) grown in urban ponds is a natural source of different phenol-resistant bacteria, which reflects the adaptation to urbanization of their natural habitat. Phenol resistance is a *condicio sine qua non* for potential bioremediation candidates. Furthermore, we observed that bacterial rhizosphere-associated communities are not randomly distributed, but visibly organized. This fact is reflected in different population structures relative to plant material preparation and in the striking differences between the free-water populations and rhizosphere populations. It is also worth noting that acclimatization is indeed possible for a significant number of isolated strains. Based on their well-documented low pathogenicity, ability to grow on phenol as the sole carbon source, phenol-resistance and biomass increase in minimal liquid medium, we have selected 5 strains as potentially excellent candidates

for bioremediation. To the best of our knowledge, this is the first mention of *S. nematodiphila*, *H. alvei*, and *H. paralvei* in the context of biological decontamination of phenol-polluted waters. Additional investigations of the dynamics of phenol elimination with a dual plant-bacterial system are needed. However, the utilization of aforementioned bacterial cultures attached with the root of duckweed in the putative bioremediation strategies seems promising.

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### Conflict of Interest

The authors declare no conflict of interest.

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