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Original paper

Physiological profile of microbial communities associated with some plant aquatic species

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Abstract

Freshwater is becoming an increasingly scarce resource in the entire world. An ecologically friendly and cost effective strategy to provide clean water is represented by phytoremediation, which is based on the ability of certain aquatic plants to recycle nutrients. Since the microorganisms that colonize plants are important for growth, the aim of this study was to investigate the plant microbial community functionality as Community-Level Physiological Profiles (CLPP) using EcoPlates™. Duckweed (*Lemna minor, L. trisulca, Spiroldelaarrhiza, W. arrhiza*) and water were sampled from Laboratory Aquariums of Faculty of Biology, University of Bucharest (Bucharest, Romania) and Lake Văcărești (Bucharest, Romania). The Shannon-Wiener and Simpson's indices indicated a high functional diversity in microbial communities attached to duckweed samples. The response from the Ecoplates showed which substrates support the growth of duckweed associated bacteria and free floating aquatic bacteria respectively. All 31 carbon sources were metabolized by duckweed associated bacteria, and only 12 were utilized by water samples: β-methyl-D-glucosidase, D-galactonic acid γ lactone, D-galacturonic acid L-asparagine, Tween 40, Tween 80, D-manitol, L-serine. A total of six carbon sources were preferred by microorganisms associated with duckweed plants: D-xylose, D-galacturonic acid, Tween 80, D-manitol, 4-hydroxy benzoic acid, glycogen In all the aquatic plant samples, while none preferred i-erythritol, 2-hydroxy benzoic acid, L-phenylalanine, α-cyclodextrin, N-acetyl-D-glucosamine, L-threonine, α-ketobutyric acid, D,L-α-glycerol phosphate and putrescein.

Keywords microbial communities, EcoPlates metabolic profile, *Lemnaceae*.

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Introduction

Current technologies for waste water treatment are not able to efficiently reduce high load of organic pollutants produced in the private household and health sector. Recent studies suggest that phytoremediation technologies combined with different conventional are most promising to solve the burning problem of polluting our environment (SCHRÖDER & al [1]). In small settlements, phyto-remediation could be an appropriate and cost-effective approach to remove high amounts of pollutants from heavy metal polluted water and WWTPs (SCHRÖDER & al [2], GATIDOU & al [3], NG & CHAN [4]). The *Lemnaceae* family comprises five genera of *Spirodela*, *Landoltia*, *Lemna*, *Wolffiella*, and *Wolffia*, each species harbouring unique making them suitable for industrial applications such as phytoremediation and bioenergy (AN & al [5]).

Duckweed (*Lemna* species) were studied for their capacity to remove organic compounds (estradiols, toluidine blue, benzotriazols) (NEAG & al [6], SCHRÖDER & al [1]), antimicrobials (IATROU & al [7], DI BACCIO & al [8]), herbicides (CHEN & SCHÄFFERM [9]) from wastewater or heavy metal polluted water (ROMERO-HERNÁNDEZ & al [10]) under different conditions in batch experiments. The result of the studies are indicating that knowledge are needed regarding plant metabolic features and growth characteristics in different abiotic conditions in order to select the most appropriate species (SCHRÖDER & al [2]). Additionally to duckweed source population, its associated microbiome and the contaminant environment need also to be tacking into an account in real-world phytoremediation efforts. In this context we cultivated different populations of duckweed in batch experiments and we investigated their microbial community functionality as Community-Level Physiological Profiles (CLPP).

Materials and Methods

1. Site and sampling

Lake Văcărești is a lake in the south of Bucharest (Romania), with a surface of 140.5 hectares. Two duckweed samples (*L. trisulca* and *S. polyrriza*) were taken in November 2018. A total of four duckweeds and four water samples were collected from aquariums (Aquaterra Laboratory) in sterile plastic bottles and stored in cold (10°C) and in the dark until setting up the experiments (1-2 days).

2. Microbial community metabolic profiles

The substrate utilization pattern by the microbial communities attached to aquatic plants collected from artificial and natural habitats and water microbial communities respectively, was investigated using Biolog Ecoplates. The EcoPlate contains three replicates wells of 31 carbon sources divided in six main groups (CHOI & DOBBS, [11]): carbohydrates, amines, carboxylic acids, phenolic compounds, polymers amino acids. Assessment of substrate catabolism was based on color development from tetrazolium dye, a redox indicator, which changes from colourless to purple.

Water samples were ten-fold diluted in PBS, and then was used to inoculate (150 µl per well) the EcoPlates (PRESTON-MAFHAM & al [12], WINDING [13]). For analysis of microbial communities attached to duckweeds samples, whole plant bodies in each flask were collected and washed with 20 mL PBS. Then, the duckweed samples were transferred into sterile flask containing 20 mL PBS and mixed for 10 minutes at room temperature. The resulted suspension was ten-fold diluted and used to inoculate into each microplate well. The microplates were placed into plastic bags and incubated at 20°C for 7 days, in the dark. Absorbance in the microplates was measured using a microplate reader at 590 nm every 24 h after correction for readings in the control well.

3. Statistical analysis

Average well colour development (AWCD) was calculated as the sum of all blanked substrate absorbance values divided by 31 (GARLAND & MILLS [14]). Functional diversity (S) was assessed by determining the substrate oxidation richness (R) (ZAK & al [15]), which is the number of different carbon sources that were used by microbial communities (i.e. corrected absorbance values>0.25) in the EcoPlate (GARLAND [16]). Also, the final values of each well at 120 h were used to calculate Shannon's diversity index (H), Simpson's diversity index (D) and substrate evenness (E) (SCHUTTER & DICK [17]). $H = -\Sigma(pi \times lnp)$, where pi is the ratio of the parameter for a particular substrate to the sum of parameter values of all substrates. Microbial communities that are able to metabolize more carbon substrates or/and to metabolize them with similar efficiency would have higher values of H. The relative use of a specific carbon source (*pi)* would give 0.032 if all the 31 substrates were equally metabolized, therefore, preferential utilization of a particular source would result in pi values higher than 0.032 (FOLEY & al [18]). The Simpson's diversity index was calculated using the formula: $D = 1-\sum pi\ 2$ (KREBS, [19], MAGURRAN, [20]) and substrate evenness was calculated as follows: E = H/lnS (ZAK & al [15], LUPWAYI & al [21]).

Results and Discussions

BIOLOG Ecoplate has been recognized as a useful tool to study functional diversity and to compare bacterial communities (SMALLA & al [22]). The Biolog EcoPlates provide a metabolic fingerprinting of the culturable microbial community (GARLAND & MILLS [14]; JANNICHE & al [23], GHIMIRE & al [24]). In the present study, Biolog EcoPlates were used to assess physiological profile of water microbial community and attached microbial communities to four duckweed species: *L. trisulca*, *L. minor*, *S. polyrrhiza* and *W. arrhiza* collected from natural and artificial habitats.

The average well color development (AWCD) of all the C sources is linked to the metabolic active cells of microbial communities. The colour development depends on cell density and the functional diversity (JANNICHE & al [23]). Generally lower cell density than the 10^5-10^8 cells/mL are considered to be required for colour development (PRESTON-MAFHAM & al [12]). The AWCD in the Biolog EcoPlate generally followed the same pattern with incubation time but varied for different samples. In case of bacteria detached from plant samples, the AWCD exhibited in the first two days of incubation a lag phase of 48 h, which was then followed by an increase in the number of metabolized C sources that reached a maximum in 96 h (*L. trisulca, L. minor, S. polyrrhiza*) and 120 h (*W. arrhiza*) corresponding to the exponential growth phase and a stationary phase beyond 96h of the incubation period (Figure 1). The AWCD data of the water samples collected from the aquariums of the plants followed also a sigmoidal response but with lag phase of 48 h for (*L. minor*) and longer, of 120 h (*W. arrhiza, L. trisulca*) and of 148 h (*S. polyrrhiza*), an exponential growth phase between 48 and about 144 h (*L. minor*) and 120 and 144 h (*W. arrhiza, L. trisulca*) and 144 and 168 h (*S. polyrrhiza*) and and a stationary phase beyond 144-168 h of the incubation period (Figure 2). CHOI & DOBBS [25] and CHRISTIAN & LIND [26] reported for freshwater environments response curves with a lag phase occurring until about 48 h, an exponential growth phase between 50 and about 150 h. The researchers observed an AWCD of approximately 0.6 after a lag phase of 24 h. Our data indicated for the water samples collected from the aquariums a longer lag and log phases which may be due to a low microbial biomass and to a lower microbial functional diversity. The AWCD for bacteria detached from plant samples averaged 0.61 (0.01-0.8) at 72 h interval, 0.8 (0.38-1.03) and 1.15 (1.01 - 2.15) at 120 h incubation time (Figure 1). The values observed at 72 h are within the range (0.25-1.0) recommended for microbial community classification (GARLAND [16]). In case of water samples, the AWCD was slower. The AWCD reached 0.25 at 120 h only for the water sample collected from the aquarium of *L. minor*, while for the rest of the water samples the AWCD remained under the range of 0.25 beyond 7 days of incubation period (Figure 2).

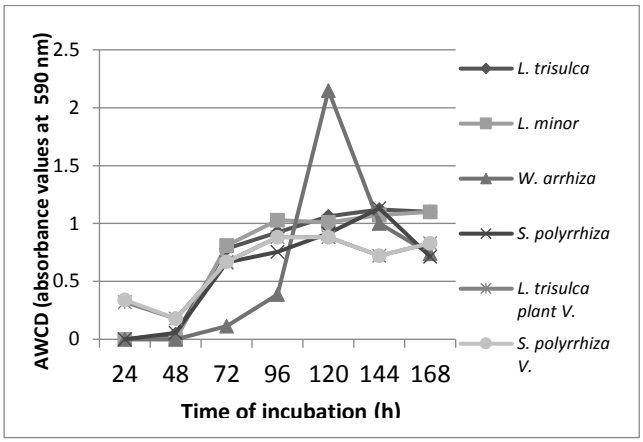

Figure 1. Average well color development (AWCD) obtained for the microbial communities attached to the plant samples *L. trisulca, L. minor, W. arryza,* and *S. polyrrhiza* collected from aquariums and the microbial communities attached to plant samples *L. trisulca* and *S. polyrrhiza* collected from Văcărești.

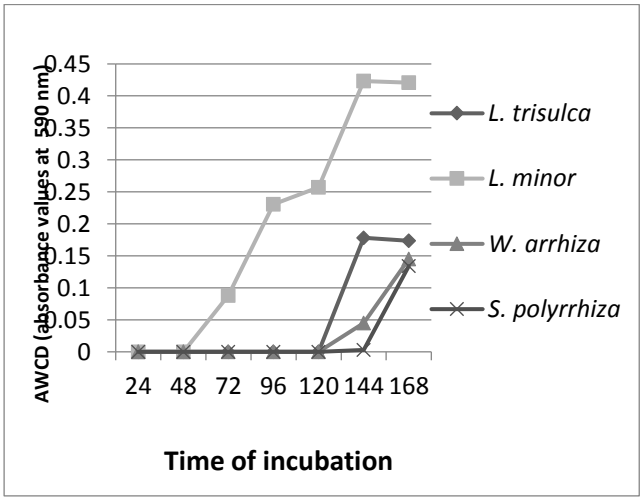

Figure 2. Average well color development (AWCD) obtained for the aquatic microbial community collected from aquariums of plants species: *L. trisulca, L. minor, W. arrhiza* and *S. polyrrhiza*.

The highest functional richness among the analysed microbial communities was detected in *Lemnaceae* plants grown in aquariums. The bacteria attached to *W. arrhiza* were able to metabolize all 31 substrates of the BIOLOG microplate. The biofilms of plant samples collected from a natural ecosystem (Văcărești Lake, Bucharest) metabolized a lower number of C sources. By comparison with the biofilms of *L. trisulca* plant collected from the aquarium,

the microbial communities associated with *L. trisulca* collected from Văcărești Lake did not metabolized, in the analysed time period, a total of seven substrates:β-methyl-D-glucosidase, pyruvic acid methyl ester, glucose-1-phosphate, D-galactonic acid γ lactone, D-malic acid, α-ketobutyric acid, glycyl-L-glutamic acid. These communities greatly metabolized complex C sources (tween 80, tween 40, α-cyclodextrin, and glycogen). It was hypothesized that complex C sources allow biomass accumulation because microorganisms can conserve more energy when catabolizing these compounds (CHAZARENC & al [27]). In contrast, the water samples were the least responsive in the Ecoplate substrate utilization assay, in the analysed time period, being negative for 23 to 28 substrates (Table 2). β-methyl-D-glucosidase, D-galacturonic acid tween 80, L-serine, D-cellobiose, D-galactonic acid γ Lactone, L-asparagine, D-manitol, glycogen, glucose-1-phosphate and tween 40 were the utilized by the free aquatic microorganisms from the aquariums.

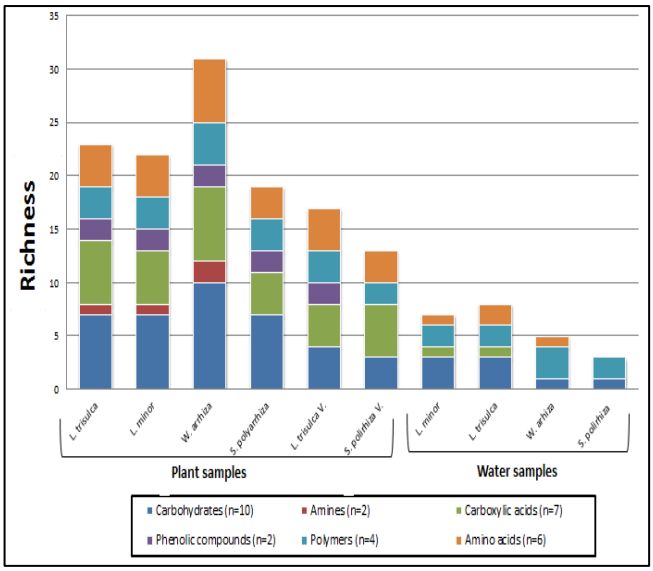

Figure 3. Richness values of the microbial communities associated with aquatic plants and the aquatic microbial communities respectively measured as the summed number of carbon sources oxidezed (O.D. values > 0.25) at day 5 and day 7 in each carbon source group (given in Table 1).

The functional diversity of the microbial communities was analysed using the statistical parameters: Shannon–Wiener index, Simpson's index and substrate evenness (E) in the incubation time of 120 h. The results are shown in Table 1. The diversity of the microbial communities attached to aquatic plant species calculated as the Shannon–Wiener index was 2.57-3.05 and as the Simpson's index was 0.94-0.96, whereas of the aquatic microbial communities was 2.08-2.7 (Shannon–Wiener index) and 0.86-0.93 (Simpson's index). These observations are suggesting a higher functional diversity of the microbial communities that develop biofilms on aquatic plant species by comparison with the free suspension, aquatic microbial communities from the aquariums of the aquatic plant species. A greater functional diversity of microbial communities in freshwater has been linked to greater robustness and functional stability and therefore the ability to adapt to new and fluctuating parameters (LIMA-BITTENCOURT & al [28]).

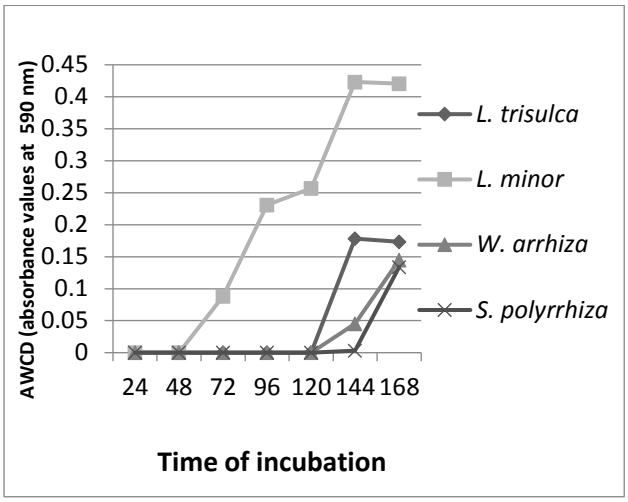

Figure 2. Average well color development (AWCD) obtained for the aquatic microbial community collected from aquariums of plants species: *L. trisulca*, *L. minor*, *W. arrhiza* and *S. polyrrhiza*.

The highest functional richness among the analysed microbial communities was detected in *Lemnaceae* plants grown in aquariums. The bacteria attached to *W. arrhiza* were able to metabolize all 31 substrates of the BIOLOG microplate (Table 1). The biofilms of plant samples collected from a natural ecosystem (Văcărești Lake, Bucharest) metabolized a lower number of C sources. By comparison with the biofilms of *L. trisulca* plant collected from the aquarium, the microbial communities associated with *L. trisulca* collected from Văcărești Lake did not metabolized, in the analysed time period, a total of seven substrates: β-methyl-D-glucosidase, pyruvic acid methyl ester, glucose-1-phosphate, D-galactonic acid γ lactone, D-malic acid,α-ketobutyric acid, glycyl-L-glutamic acid. These communities greatly metabolized complex C sources (tween 80, tween 40, α-cyclodextrin, and glycogen). It was hypothesized that complex C sources allow biomass accumulation because microorganisms can conserve more energy when catabolizing these compounds (CHAZARENC & al [27]). In contrast, the water samples were the least responsive in the Ecoplate substrate utilization assay, in the analysed time period, being negative for 23 to 28 substrates (Table 2). β-methyl-D-glucosidase, D-galacturonic acid

tween 80, L-serine, D-cellobiose, D-galactonic acid γ Lactone, L-asparagine, D-manitol, glycogen, glucose-1-phosphate and tween 40 were the utilized by the free aquatic micro-organisms from the aquariums.

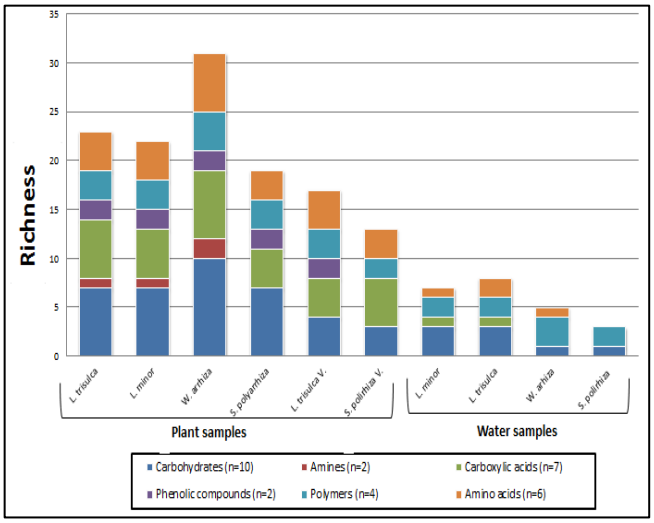

Figure 3. Richness values of the microbial communities associated with aquatic plants and the aquatic microbial communities respectively measured as the summed number of carbon sources oxidized (O.D. values > 0.25) at day 5 and day 7 in each carbon source group (given in Table 1).

The functional diversity of the microbial communities was analysed using the statistical parameters: Shannon–Wiener index, Simpson's index and substrate evenness (E) in the incubation time of 120 h. The results are shown in Table 3. The diversity of the microbial communities attached to aquatic plant species calculated as the Shannon–Wiener index was 2.57-3.05 and as the Simpson's index was 0.94-0.96, whereas of the aquatic microbial communities was 2.08-2.7 (Shannon–Wiener index) and 0.86-0.93 (Simpson's index). These observations are suggesting a higher functional diversity of the microbial communities that develop biofilms on aquatic plant species by comparison with the free suspension, aquatic microbial communities from the aquariums of the aquatic plant species. A greater functional diversity of microbial communities in freshwater has been linked to greater robustness and functional stability and therefore the ability to adapt to new and fluctuating parameters (LIMA-BITTENCOURT & al [28]).

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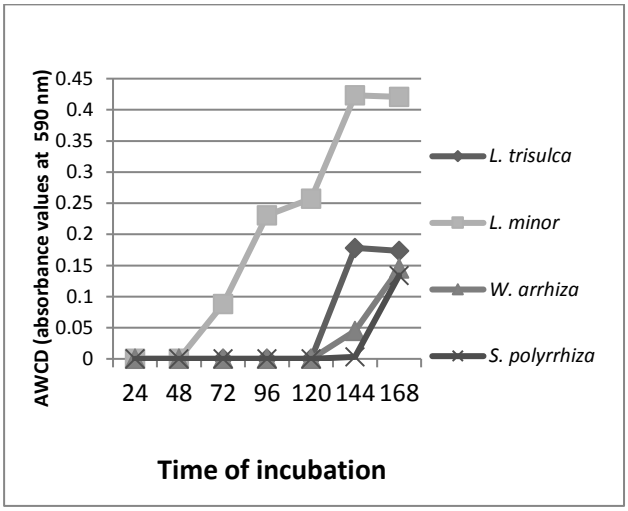

Figure 2. Average well color development (AWCD) obtained for the aquatic microbial community collected from aquariums of plants species: *L. trisulca*, *L. minor*, *W. arrhiza* and *S. polyrrhiza*.

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ISHIZAWA & al [29] reported that bacterial community strongly influences the production speed of duckweed biomass and many of isolates from duckweed-associated bacterial communities have some common characteristics in their taxa and ability to influence plant growth with terrestrial rhizobacteria.

Table 1. Biolog Ecoplates substrates utilization by plant associated microbial communities. An absorption threshold value of 0.25 was used for positive growth response after cultivation for 120 hours at 22°C. (AA): amino acids; (AM): amines/amides; (CAA): carboxilyc & acetic acids; (CH): carbohydrates; (POL): polymers.

C-source	Substrate type	*L. trisulca*	*L. minor*	*W. arryza*	*S. arryza*	*L. trisulca* V.	*S. polyrryza* V.
Water		-	-	-	-	-	-
B-Methyl-D-Glucosidase	CH	+	+	+	+	-	+
D-galactonic Acid γ Lactone	CAA	+	+	+	+	-	+
L-Arginine	AA	+	+	+	+	+	+
Pyruvic Acid Methyl Ester	CH	+	+	+	+	-	+
D-Xylose	CH	+	+	+	+	+	+
D-Galacturonic Acid	CAA	+	+	+	+	+	+
L-Asparagine	AA	+	+	+	+	+	+
Tween 40	POL	+	+	+	+	+	-
i-Erythritol	CH	-	-	+	-	-	-
2-Hydroxy Benzoic Acid	PHE	-	-	+	-	-	-
L-Phenylalanine	AA	-	-	+	-	-	-
Tween 80	POL	+	+	+	+	+	+
D-Manitol	CH	+	+	+	+	+	-
4-Hydroxy Benzoic Acid	PHE	+	+	+	+	+	-
L-Serine	AA	+	+	+	+	+	-
α-Cyclodextrin	POL	-	-	+	-	-	-
N-Acetyl-D-Glucosamine	CH	-	-	+	-	-	-
γ Hydroxybutyric Acid	CAA	-	-	+	-	-	-
L-Threonine	AA	-	-	+	-	-	-
Glycogen	POL	+	+	+	+	+	+
D-Glucosaminic Acid	CAA	+	+	+	+	+	+
Itaconic Acid	CAA	+	+	+	+	+	+
Glycyl-L-Glutamic Acid	AA	+	-	+	-	-	-
D-Cellobiose	CH	+	+	+	+	+	-
Glucose-1-Phosphate	CH	+	+	+	+	-	-
α-Ketobutyric Acid	CAA	+	-	+	-	-	-
Phenylethyil-amine	AM	+	+	+	-	+	-
α-D-Lactose	CH	+	+	+	+	+	-
D,L-α-Glycerol Phosphate	CH	-	-	+	-	-	-
D-Malic Acid	CAA	+	+	+	-	-	+
Putresceine	AM	-	-	+	-	-	-
R (richness)		**22**	**20**	**31**	**18**	**16**	**12**

Table 2. Biolog Ecoplates substrates utilization by aquatic microbial communities. An absorption threshold value of 0.25 was used for positive growth response after cultivation for 120 hours at 22°C. (AA): amino acids; (AM): amines/amides; (CAA): carboxilyc & acetic acids; (CH): carbohydrates; (POL): polymers.

C-source	Substrate type	*L. minor* water sample	*L. trisulca* water sample	*W. arryza* water sample	*S. arryza* water sample
Water		-	-	-	-
B-Methyl-D-Glucosidase	CH	-	+	-	+
D-galactonic Acid γ Lactone	CAA	-	+	-	-
L-Arginine	AA	-	-	-	-
Pyruvic Acid Methyl Ester	CH	-	-	-	-
D-Xylose	CH	-	-	-	-
D-Galacturonic Acid	CAA	+	-	-	-
L-Asparagine	AA	-	+	+	-
Tween 40	POL	-	+	+	+
i-Erythritol	CH	-	-	-	-
2-Hydroxy Benzoic Acid	CAA	-	-	-	-
L-Phenylalanine	AA	-	-	-	-
Tween 80	POL	+	-	+	-
D-Manitol	CH	+	+	-	-
4-Hydroxy Benzoic Acid	CAA	-	-	-	-
L-Serine	AA	+	+	-	-
α-Cyclodextrin	POL	-	-	-	-
N-Acetyl-D-Glucosamine	CH	-	-	-	-
γ Hydroxybutyric Acid	CAA	-	-	-	-
L-Threonine	AA	-	-	-	-
Glycogen	POL	+	+	+	+
D-Glucosaminic Acid	CAA	-	-	-	-
Itaconic Acid	CAA	-	-	-	-
Glycyl-L-Glutamic Acid	AA	-	-	-	-
D-Cellobiose	CH	+	+	+	-
Glucose-1-Phosphate	CH	+	-	-	-
α-Ketobutyric Acid	CAA	-	-	-	-
Phenylethyil-amine	AM	-	-	-	-
α-D-Lactose	CH	-	-	-	-
D,L-α-Glycerol Phosphate	CH	-	-	-	-
D-Malic Acid	CAA	-	-	-	-
Putresceine	AM	-	-	-	-
R (richness)		**7**	**8**	**5**	**3**

Knowledge of the structural and functional biodiversity of microbial communities that colonize *Lemnaceae* plants is important for improving the biomass production of *Lemnaceae* hydrocultures and increasing their wastewater treatment yields.

AWCD values have shown a higher functional diversity of microbial communities associated with aquatic plants compared to the metabolic activity of free, planktonic microbial communities, or fixed by inert support in the analyzed aquatic basins. The metabolic diversity of

Table 3. Microbial diversity calculated as Simpson's Shannon-Wiener indeces.

Plant samples	**Simpson's index (D)**	**Shannon-Wiener index (H)**	**Substrate evenness (E)**
L. trisulca	0.955	2.78	0.899373
L. minor	0.954	2.85	0.951353
W. arrhiza	0.966	2.9	0.844499
S. polyrrhiza	0.946	3.05	1.055228
*L. trisulca*V.	0.946	2.57	0.926932
S.polyrrhiza V.	0.945	2.9	1.209394
Water samples	**Simpson's index (D)**	**Shannon-Wiener index (H)**	**Substrate evenness (E)**
L.trisulca	0.875	2.7	1.298426
L. minor	0.932	2.54	1.221482
W. arrhiza	0.897	2.28	1.416644
S. polyrrhiza	0.865	2.08	1.893298

microbial populations in water samples taken from aquatic basins was lower than that of microbial populations associated with aquatic plants, suggesting a greater diversity of microbial communities adhering to the surface of aquatic plants compared to free, planktonic aquatic communities. The greatest functional diversity has been detected for microbial communities associated with *Lemnaceae* plants grown in aquariums. Bacteria adhering to *W. arrhiza* plants were able to metabolize all 31 substrates of the EcoPlates Bioplates microplate. Biofilms of plant samples collected from the natural ecosystem (Lake Vacaresti, Bucharest) have metabolized a smaller number of carbon sources. Compared to the biofilms of the *L. trisulca* plant collected from the aquarium, microbial communities associated with *L. trisulca* collected from Lake Văcăreşti metabolized a smaller number of substrates, but these communities metabolized a lot of complex C sources (tween 80, tween 40, α-cyclodextrin and glycogen). Complex C sources allow the accumulation of biomass because the microorganisms can conserve more energy when catabolizing these compounds.

Studies on the interactions of lobster plants and colonizing microorganisms can contribute to the development of strategies to modulate these interactions in order to improve bioremediation processes. Also, the use of Biopoly EcoPlates microplates has proven to be a fast and modern method of analysis that can provide useful information about the structural or physiological changes in the microbial communities of aquatic ecosystems caused by various factors of chemical and / or physical factors.

Conflict of interest disclosure

There are no known conflicts of interest in the publication of this article. The manuscript was read and approved by all authors.

Compliance with ethical standards

Any aspect of the work covered in this manuscript has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

Acknowledgments

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