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Gas Chromatography-Mass Spectrometric Study of Low-Molecular-Weight Exogenous Metabolites of Algae-Bacterial Communities in the Laboratory Accumulative Culture

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Abstract: The study of exogenous metabolites of algae-bacterial communities in the laboratory accumulative culture obtained from natural river water was conducted using gas chromatography-mass spectrometry. Exometabolites of the algae-bacterial community (including microalgae and cyanobacteria) in the culture medium were represented by saturated, unsaturated, and aromatic hydrocarbons, carboxylic acids, phenolic, and terpene compounds and their derivatives. Possible biological activities of the discovered exometabolites are considered. The study has demonstrated that an increase in the number of main groups of microorganisms, along with changes in the composition of algae and cyanobacteria, are responsible for the increase in the composition and concentration of metabolites in the microecosystem's culture medium after one month of cultivation. The presence of octacosane in high concentration (0.0603 mg/L; 23.78% of the total content of low molecular weight organic compounds) by the end of exposure accumulative culture is associated with the strong development of the cyanobacterium *Gloeocapsa* sp. in the presence of diatom algae of the genus *Navicula* and green algae of the genera *Chlorella* and *Scenedesmus*. Due to the need to comprehend the ecological and biochemical mechanisms of the formation and functioning of algae-bacterial communities, as well as to predict potential paths of transformation and evolution of aquatic ecosystems, the specificity of exometabolite complexes of algae and microorganisms, as well as their allelopathic and other biochemical interactions in freshwater ecosystems, requires further serious study.

Keywords: algae; cyanobacteria; exometabolites; gas chromatography-mass spectrometry; algae-bacterial communities; accumulative culture

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1. Introduction

Algae and cyanobacteria have a significant role in the formation of the chemical composition and stock of organic compounds in the water basins since they are the primary photosynthetic component of many freshwater ecosystems.

Throughout all stages of growth, organic compounds are released by phytoplankton cells. Algae and cyanobacteria (main representatives of phytoplankton) produce and release in the environment a great multitude of biologically active compounds: hydrocarbons, alcohols, aldehydes, ketones, organic acids (including saturated and polyunsaturated fatty acids), amines, ethers, proteins, carbohydrates, sterols, terpenoids, phytohormones, phenolic compounds, vitamins, etc. [1,2].

Actinobacteria can also be producers of low molecular weight organic compounds (LMWOCs) in aquatic habitats, in addition to plankton algae and cyanobacteria. For example, four volatile substances have so far been found and identified as metabolites of

freshwater actinobacteria [3]: 2-propen-1-amine, n-2-propenyl-; 2-propenal, 3-(1-aziridinyl)-3-(dimethylamino)-; 2-decene, 3-methyl-; and 5-pyrrolidino-2-pyrrolidone.

According to studies [4,5], Cyanoprokaryota can create and excrete a wide range of allelopathic substances that impact other cyanobacteria, eukaryotic algae, bacteria, zooplankton, higher plants, and fish. A study [6] also found that the co-occurring phytoplankton species could be inhibited by the mixotrophic dinoflagellate Akashiwo sanguinea.

It has been demonstrated that some neuro or hepatotoxic compounds and other bioactive metabolites can be released by bloom-forming pelagic cyanobacteria [7]. In this situation, it may be possible to see the synergistic effects of the numerous cyanobacterial metabolites [8].

Furthermore, nitrogen fixation, oxidative response, and toxin production may be affected by allelopathic interactions between phytoplankton species via metabolites [9].

Globally distributed cyanobacterial genera produce toxic peptides called cyanobacterial microcystins. A wide range of other hazardous and/or other bioactive peptides are also produced by cyanobacteria. Some of the metabolites of the cyanobacteria and dinoflagellates can be toxic to zooplankton and fish, as well as livestock that drink water containing these compounds [10]. Numerous cyanobacteria and eukaryotic algae can grow more slowly due to metabolites released by other algae [11].

In algaecenoses, algae and cyanobacteria engage in a variety of interactions, the most crucial of which are allelopathic interactions caused by the synthesis and release of various LMWOCs [12]. In the freshwater ecosystem, cyanobacteria, microalgae, macroalgae, and plants interact allopathically. Allelopathic inhibition is known to be complex and may involve the interaction of several chemical classes, such as phenolic compounds, flavonoids, terpenoids, alkaloids, steroids, carbohydrates, amino acids, etc.

Research on the production of exogenous metabolites (particularly LMWOCs) from freshwater algae-bacterial communities is still rare as compared to marine environments, and little is known about their ecological function in freshwater ecosystems. LMWOCs play an essential role in the exchange of information between aquatic microorganisms, and they can affect ecosystem function as well as the structure and composition of algal-bacterial communities.

Since they affect the flavor and odor of fish, shellfish, drinking water supplies, and recreational waters, low molecular weight metabolites from algae and cyanobacteria are also crucial to the use of aquatic ecosystems.

In addition to the processes of self-purification of water entities from pathogenic microorganisms, understanding the mechanisms of phytoplankton metabolite formation, defining their chemical nature, and transformation in aquatic habitats is of great interest. These processes also involve the acquisition of natural antimicrobial, antifungal, and antialgal agents through controlled biosynthesis using algae. As biotechnological materials for the manufacture of biofuels, commodity chemicals, natural goods, and nutraceuticals, the metabolic products of algae and phototrophic prokaryotes also show significant potential.

There is an increased need for novel naturally occurring biologically active molecules as a result of the rise in diseases that are drug-resistant to multiple treatments [3]. Many of these compounds can be produced by freshwater algae and cyanobacteria.

Since many naturally occurring compounds from aquatic ecosystems have high biological activity and have properties such as immunosuppression, phototoxicity, antitumor, antibacterial, antimicrobial, antifouling, antifungal, and antifungal activity, they are of particular interest [13,14].

With laboratory models of microcosms that have accumulative cultures and imitate natural ecosystems, it is simple to investigate the development and growth of algae-bacterial communities. Studies of algae-bacterial metabolites frequently employ LC-MS (liquid chromatography-mass spectrometry) [15]. Highly effective gas chromatography-mass spectrometry (GC-MS) has also been shown to be one of the most effective ways for identifying as well as for the qualitative and quantitative assessment of natural solute LMWOCs.

In the field of community ecology, experimental microecosystems offer numerous opportunities for understanding the mechanisms of processes that occur in natural aquatic ecosystems. The innovation of this work is the study of the ecological features of the culture of cyanobacteria and microalgae in dynamics using modern gas chromatography-mass spectrometric methods.

The objective of the research is a gas-chromatography-mass spectrometric study of the qualitative and quantitative composition of dissolved LMWOCs produced by cyanobacteria and microalgae in an experimental microecosystem cultivated for 1.5 months at three time points with a fortnight interval.

2. Materials and Methods

The experimental enrichment culture obtained on the basis of sampled river water (the Akhtuba River, the Volga River branch, Astrakhan region, Russia, September 2009) was cultured in the nutrient medium BG_N11 [16] for cyanobacteria. The percentage ratio of the river water and nutrient medium when setting up the experiment was 75% to 25%, respectively. We chose this ratio of the water and medium to intensify the development of cyanobacteria and algae in the presence of the nutrient medium. Cultivation was carried out at 22–25 °C in natural light. To isolate exometabolites, 250 mL of the culture medium together with cells were filtered through a nylon filter with a pore size of 10–50 μm. The resulting culture fluid was then placed in a 250 mL separation funnel, 3 mL of hexane was added and gently shaken for 3 min, inverting the funnel. After 5 min of settling, the fluid was drained, and the hexane that appeared on the surface was drained separately into a glass bottle with a stopper. The extracts were kept in the freezer at −18 °C before conducting GC-MS analysis. When extracted with nonpolar solvents (hexane, petroleum ether, gasoline, etc.), a large group of biologically active substances is released by plants and algae [17]. To replenish the utilized culture medium, 250 mL of BG_N11 medium was added every two weeks.

This study includes data from three ecosystem testing stages: testing stage 1—establishing the accumulative culture by adding river water and nutrient medium; testing stage 2—after two weeks of cultivation; and testing stage 3—after a month of cultivation. We explored a unique ecosystem that had its own unique succession. If a parallel experiment had been set up, it would have been a different ecosystem, which, as a result of the influence of stochastic factors, could have received a different direction of development, possibly very different from another ecosystem. In this case, we would be dealing with a different algobacterial community and possibly a different set of metabolites.

The composition of algae LMWOCs was analyzed in the hexane extracts using a TRACE DSQ II gas chromatography-mass spectrometer (Thermo Electron Corporation) equipped with a quadrupole mass analyzer. TRACE TR_5MS GC Column, 15 m, 0.25 mm ID, film thickness 0.25μ, was used. Helium served as a carrier gas; its ionization voltage was 70 eV. Mass spectra were registered in the scan mode for the whole mass range (30–580 amu) in a programmed temperature regime: oven temperature was held at 35 °C for 3 min and was then programmed to increase to 60 °C at a rate of 2 °C/min. It was kept constant for 3 min and then programmed to increase to 80 °C at a rate of 2 °C/min; it was kept constant for 3 min and then programmed to increase to 120 °C at a rate of 4 °C/min; it was kept constant for 3 min and then programmed to increase to 150 °C at a rate of 5 °C/min; it was kept constant for 3 min and then programmed to increase to 240 °C at a rate of 15 °C/min; and finally, it was then held isothermal for 10 min.

Identification of LMWOCs was carried out using the libraries of mass spectra "NIST-2008" and "Wiley". Linear retention indices [18] obtained using C7–C30 alkane standards were used for more accurate identification. The accuracy of identification of the identified compounds given in the paper was ensured by taking into account the matching factor (Match) and the reverse matching factor (R.Match), which were at least 800–900 (a good match). In many cases, for the most abundant compounds, they were > 900 (an excellent match). Quantitative analysis was performed using benzophenone as an internal stan-

dard. The analysis made it possible to precisely quantify the identified compounds. The diversity of compounds was revealed by qualitative analysis, namely, in the process of the identification of compounds.

Similarity assessment of the LMWOCs complexes, detected at different testing stages, was performed with the use of Jaccard [19] (1) and Sørensen–Czekanowski similarity coefficients [20,21] (2).

$$J = \frac{c}{a + b - c} \quad (1)$$

$$QS = \frac{2c}{a + b} \quad (2)$$

where: c is the number of common LMWOCs for samples A and B; b is the LMWOCs found in sample B; a is the LMWOCs found in sample A.

During the testing period, a microbiological examination of the experiment's accumulative culture was carried out, which included the detection of several bacteria groups and micromycetes, as well as the identification and quantitative assessment of algae and cyanobacteria. The algae and cyanobacteria were identified based on morphological characteristics using the Gollerbakh et al. [22] and Komárek [23] guides—identification cultures were purified and brought to algologically pure strains. Taxonomic diagnostics were carried out taking into account the cultural, physiological, biochemical, and morphological properties of algae and cyanobacteria. These methods are conventional and are based on identification by phenotypic traits. However, at present, for the comparative analysis of algae and cyanobacteria, it is necessary to carry out identification by molecular methods, taking into account the ultrastructure of cells and their morphology. In further research, we are going to taxonomize them in a complex. We conducted a quantitative assessment of the physiological groups of microorganisms using the approach of limiting dilution of nutritional medium [24] to identify autochthonous microbial flora: nutrient agar (NA) (saprotrophs), NA/10, and NA/100 (oligotrophic microorganisms). Gause's medium for actinomycetes [25], Seliber's medium for lipolytic microorganisms [16], Czapek's medium for saccharolytic bacteria [16], and starvation agar [26] for oligotrophic microorganisms were used to discover other microorganisms.

3. Results and Discussion

3.1. Composition of Exogenous Metabolites

In the culture medium of algae and cyanobacteria, GC-MS analysis revealed the presence of saturated, unsaturated, and aromatic hydrocarbons, carboxylic acids, phenolic and terpene chemicals, and their derivatives (Table 1).

Table 1. Composition of exogenous metabolites at the initial stage (Sample 1), after two weeks' cultivation (Sample № 2), and after a month's cultivation (Sample № 3) (RT—retention time, min; RI—linear retention index; %—percent of compound among all LMWOCs; C—compound concentration in water, mg/L); ?—m/z was impossible to determine.

	Compound	Formula	RT	RI	Sample 1		Sample 2		Sample 3	
					%	C	%	C	%	C
1	unidentified m/z 100 [M+], 55 (100)		2.61	799			3.65	0.0085		
2	octane	C_8H_{18}	2.68	800			7.09	0.0165		
3	hexan-3-one	$C_6H_{12}O$	2.73	803	8.06	0.0057			1.86	0.0047
4	hexan-2-one	$C_6H_{12}O$	2.91	809					4.03	0.0102
5	hexan-2-ol	$C_6H_{14}O$	2.94	811	3.21	0.0023				
6	hexan-3-ol	$C_6H_{14}O$	3.02	813					0.61	0.0016
7	unidentified m/z 86 [M+], 86 (100)		3.4	827			3.99	0.0093		
8	unidentified m/z 98 [M+], 56 (100)		3.89	845			1.17	0.0027	0.42	0.0011
9	(E)-hex-2-enal	$C_6H_{10}O$	3.92	846					0.29	0.0007
10	3-methylcyclopentan-1-one	$C_6H_{10}O$	3.93	846	2.75	0.0019				
11	2-(2-methylpentan-2-yl)oxirane	$C_8H_{16}O$	4.06	851					0.79	0.002
12	hexane-2,4-dione	$C_6H_{10}O_2$	5.05	887					0.53	0.0014

Table 1. *Cont.*

	Compound	Formula	RT	RI	Sample 1		Sample 2		Sample 3	
					%	C	%	C	%	C
13	oct-1-en-3-one	$C_8H_{14}O$	5.25	894					0.85	0.0021
14	5-methoxy-2-methylpentan-2-ol	$C_7H_{16}O_2$	6.12	915	5.74	0.004				
15	methyl (E)-5-methoxypent-3-enoate	$C_7H_{12}O_3$	6.25	918					3.03	0.0077
16	3-methylpentane-3-thiol	$C_6H_{14}S$	6.7	928			4.2	0.0098		
17	(5 E)-2-methylhepta-2,5-dien-4-ol	$C_8H_{14}O$	6.73	929					3.47	0.0088
18	(E)-3,7-dimethyloct-2-ene	$C_{10}H_{20}$	7.33	941					7.14	0.0181
19	2,3-dimethyloct-2-ene	$C_{10}H_{20}$	9.39	986					1.71	0.0043
20	5-ethyl-2,4-dimethylhept-2-ene	$C_{11}H_{22}$	9.98	999			2.11	0.0049	1.55	0.0039
21	(E)-hept-2-enal	$C_7H_{12}O$	9.99	999	12.84	0.009				
22	5-methylheptan-1-ol	$C_8H_{18}O$	10.11	1001			2.41	0.0056		
23	3-ethyl-5-methylhept-1-yn-3-ol	$C_{10}H_{18}O$	10.19	1003					1.58	0.004
24	2,2,3,3,4,4-hexamethyloxolane	$C_{10}H_{20}O$	10.61	1010					0.59	0.0015
25	1-methyl-4-prop-1-en-2-ylcyclohexene	$C_{10}H_{16}$	11.29	1021	19.71	0.0139				
26	(E)-3-methyldec-4-ene	$C_{11}H_{22}$	11.36	1022					2.2	0.0056
27	pentylcyclopentane	$C_{10}H_{20}$	11.81	1030					0.6	0.0015
28	(E)-3-methyldec-3-ene	$C_{11}H_{22}$	12.12	1035					0.24	0.0006
29	3,7-dimethylnonane	$C_{11}H_{24}$	12.46	1040					0.78	0.002
30	1-butyl-1-methyl-2-propylcyclopropane	$C_{11}H_{22}$	12.89	1048					0.63	0.0016
31	(Z)-3-methyldec-2-ene	$C_{11}H_{22}$	15.1	1085					0.89	0.0022
32	1-nonylaziridine	$C_{11}H_{23}N$	17.1	1114	4.5	0.0032				
33	(Z)-9-methylundec-2-ene	$C_{12}H_{24}$	17.15	1114					4.16	0.0106
34	1,7,7-trimethylbicyclo[2.2.1]heptan-3-one	$C_{10}H_{16}O$	18.45	1131			0.83	0.0019		
35	1-(1,2,2,3-tetramethylcyclopentyl)ethanone	$C_{11}H_{20}O$	20.9	1162					1.21	0.0031
36	dodec-1-ene	$C_{12}H_{24}$	22.76	1186					0.91	0.0023
37	benzoic acid	$C_7H_6O_2$	23.33	1193			12.05	0.028		
38	dodecane	$C_{12}H_{26}$	23.83	1200	2.68	0.0019	2.2	0.0051		
39	5-ethylnonan-2-ol	$C_{11}H_{24}O$	24.64	1210					1.11	0.0028
40	1,2,4,5-tetraethylcyclohexane	$C_{14}H_{28}$	27.8	1251					0.5	0.0013
41	2-methyltridecane	$C_{14}H_{30}$	37.41	1397			7.32	0.017		
42	3-methyltridecane	$C_{14}H_{30}$	37.5	1398					0.88	0.0022
43	tetradecane	$C_{14}H_{30}$	37.56	1399	2.07	0.0015	1.03	0.0024		
44	tetradec-1-ene	$C_{14}H_{28}$	38.46	1421					1.21	0.0031
45	3-methyltetradecane	$C_{15}H_{32}$	39.37	1444					0.72	0.0018
46	2-methylpentadecane	$C_{16}H_{34}$	45.85	1597					0.31	0.0008
47	undecylcyclopentane	$C_{16}H_{32}$	47.73	1652					0.52	0.0013
48	2-methylheptadecane	$C_{18}H_{38}$	50.57	1741					0.34	0.0009
49	tetradecanoic acid	$C_{14}H_{28}O_2$	51.29	1765			0.57	0.0013		
50	3-methyloctadecane	$C_{19}H_{40}$	52.24	1797					0.38	0.001
51	ethylpentadecylketone	$C_{18}H_{36}O$	54	1860					0.15	0.0004
52	(E)-8-methylheptadec-8-ene	$C_{18}H_{36}$	54.12	1864	0.78	0.0006				
53	5-heptadecenal	$C_{17}H_{32}O$	54.15	1865					0.21	0.0005
54	bis(2-methylpropyl)benzene-1,2-dicarboxylate	$C_{16}H_{22}O_4$	54.3	1871	2.27	0.0016	0.79	0.0018	0.22	0.0006
55	(Z)-nonadec-5-ene	$C_{19}H_{38}$	55.38	1920					0.17	0.0004
56	dibutyl benzene-1,2-dicarboxylate	$C_{16}H_{22}O_4$	55.9	1959	3.93	0.0028	0.68	0.0016	0.37	0.0009
57	hexadecanoic acid	$C_{16}H_{32}O_2$	56.02	1968			0.95	0.0022		
58	3-methylicosane	$C_{21}H_{44}$	56.43	1998	1.98	0.0014	0.16	0.0004	0.88	0.0022
59	ethenyl hexadecanoate	$C_{18}H_{34}O_2$	56.46	2001			0.45	0.0011	0.91	0.0023
60	(3R)-5-[(1S,4as,8as)-5,5,8a-trimethyl-2-methylidene-3,4,4a,6,7,8-hexahydro-1H-naphthalen-1-yl]-3-methylpent-1-en-3-ol	$C_{20}H_{34}O$	56.87	2044			0.64	0.0015		
61	unidentified m/z 290 [M+], 83 (100)		56.98	2056	0.62	0.0004				
62	eicosan-2-ol	$C_{20}H_{42}O$	57.05	2063	1.51	0.0011				
63	(E)-4-methylnonadec-4-ene	$C_{20}H_{40}$	57.05	2063			0.48	0.0011		
64	unidentified m/z 347 [m+], 347 (100)		57.3	2089					0.2	0.0005
65	henicosane	$C_{21}H_{44}$	57.39	2100			0.48	0.0011		
66	docosane	$C_{22}H_{46}$	58.15	2200			1.69	0.0039		
67	unidentified m/z 295 [m+], 71 (100)		58.18	2200					1.05	0.0027
68	unidentified m/z 320 [M+], 95 (100)		58.25	2210	5.75	0.004				
69	unidentified m/z 295 [m+], 83 (100)		58.59	2260					0.49	0.0012
70	unidentified m/z 322 [M+], 69 (100)		58.6	2262	1.72	0.0012				
71	docos-1-ene	$C_{22}H_{44}$	58.63	2266	4.33	0.003				
72	tricosane	$C_{23}H_{48}$	58.82	2300			2.31	0.0054	0.45	0.0012
73	unidentified m/z ? [m+], 347 (100)		58.9	2306			1.22	0.0028		
74	unidentified m/z 347 [M+], 83 (100)		58.91	2308	3.91	0.0028				
75	tetracosane	$C_{24}H_{50}$	59.44	2400			5.33	0.0124		
76	unidentified m/z ? [m+], 81 (100)		59.45	2395					1.31	0.0033
77	unidentified m/z 334 [M+], 44 (100)		59.48	2400	5.45	0.0038				

Table 1. *Cont.*

	Compound	Formula	RT	RI	Sample 1		Sample 2		Sample 3	
					%	C	%	C	%	C
78	2-methyltricosane	$C_{24}H_{50}$	59.5	2403					0.82	0.0021
79	pentacosane	$C_{25}H_{52}$	60.09	2500			6.27	0.0146		
80	unidentified m/z 352 [m+], 71 (100)		60.09	2493					2.61	0.0066
81	unidentified m/z 325 [M+], 44 (100)		60.33	2523	2.54	0.0018				
82	bis(2-ethylhexyl) benzene-1,2-dicarboxylate	$C_{24}H_{38}O_4$	60.44	2537	3.65	0.0026	1.18	0.0027	2.1	0.0053
83	hexacosane	$C_{26}H_{54}$	60.89	2600			6.43	0.015	1.48	0.0038
84	unidentified m/z 426 [m+], 191 (100)		61.46	2647					5.96	0.0151
85	heptacosane	$C_{27}H_{56}$	61.94	2700			7.27	0.0169	4.78	0.0121
86	octacosane	$C_{28}H_{58}$	63.26	2800			5.06	0.0118	23.78	0.0603
87	nonacosane	$C_{29}H_{60}$	65.07	2900			5.52	0.0128		
88	unidentified m/z 441 [m+], 97 (100)	$C_{20}H_{28}O_6$	65.1	2893					6.02	0.0153
89	triacontane	$C_{30}H_{62}$	67.39	3000			4.47	0.0104		
	TOTAL				100	0.071	100	0.233	100	0.254

In total, 22 LMWOCs were detected in the initial sample (native river water, the Akhtuba River), 32 LMWOCs were detected in the second sample, and 53 compounds were detected in the third one (Table 1). Six compounds in the initial sample were not identified. Among the 22 components, limonene (1-methyl-4-prop-1-en-2-ylcyclohexene) (19.71%) and (e)-hept-2-enal (12.84%) account for the largest percentage of total extract. The given compounds are widely distributed in nature and are essential for maintaining the ecological balance in interspecific interactions of bacteria, cyanobacteria, and microalgae, as well as their symbioticates, macrophytes, protozoa, invertebrates, and plants, as well as among different trophic levels [27,28]. These metabolites have a wide range of biological features [29] and participate in a variety of LMWOC activities in water bodies [30].

Limonene is found in a variety of oils, fruits, and plants [31]. It is used to degrease metal before coloring and as an active component in pesticides in household chemistry [32]. Limonene and heptenal, components of juniper and sage extracts, demonstrated antibacterial action against bacteria of the genera *Staphylococcus and Escherichia* and fungi of the genera *Candida* [33].

The summary presented in the book [34] shows that limonene has a wide range of biological activities: anticancer (breast, colon, forestomach, liver, lung, ovarian, prostate, skin), antiasthmatic, antifungal, antibacterial, antihyperglycemic, antiinflammatory, antimutagenic, antiseptic, antispasmodic, antioxidant, antiviral, digestive, appetite suppressant, detoxicant, expectorant, herbicidal, immunomodulatory, muscle relaxant, pesticidal, etc.

Limonene produced by cyanobacteria can inhibit the cell division of green algae, such as *Chlorella vulgaris* [35].

Hexanone found in the sample has been shown to have antibacterial action against seven bacterial species and one micromycete [36]. The studies [37] describe the fungicidal and antibacterial capabilities of the hexanol discovered in the initial sample.

The percentage of phthalates in Sample 1 ranged from 2.27 to 3.93%. Phthalates are contaminants in the environment and are employed in the chemical industry [38]. Bis(2-methylpropyl)benzene-1,2-dicarboxylate (diisobutyl phthalate), dibutyl benzene-1,2-dicarboxylate (dibutyl phthalate), and bis(2-ethylhexyl)benzene-1,2-dicarboxylate (diethylhexyl phthalate) are used in industry as plasticizers (increasing flexibility), solvents for perfume oils, perfume stabilizing agents, in printing paints, in glues, etc. However, there is evidence that in natural conditions, plants, including aquatic ones and algae, also synthesize those compounds, performing as phytotoxins in allelopathic interactions [39,40]. The high percentage of dibutyl phthalate was a characteristic feature of the culture medium of *Oscillatoria neglecta*—12% of total saluted exometabolites [41]. It should be noted that all of the cyanobacteria *Oscillatoria neglecta*, *Anabaena variabilis*, *Anabaena cylindrical*, and green algae *Acutodesmus obliquus* unialgal cultures studied contained phthalates in varying concentrations [41].

It should be emphasized that the existence of phthalates in the analyzed samples is disputed because they can be external contaminants. As to phthalates, further research is required to find out whether their synthesis and decomposition are due to exposure to light and time or are the result of metabolic functions of microbes and algae.

When a culture medium (25% by volume) was added to the initial sample, they could have been introduced. However, the change in their concentration (in some compounds, an increase; in others, a decrease) shows that they were actively involved in the organic matter transformation processes within the microecosystem. At the same time, the total amount of phthalates decreased slightly from the initial sample to the second and third (2 weeks of cultivation and a month of cultivation) from 0.008 mg/L (9.8% of the total concentration of LMWOCs) to 0.007 mg/L (2.7% of the total concentration of LMWOCs).

Dodecane and tetradecane were also discovered in the initial sample. Tetradecane is a naturally occurring and man-made chemical that is employed as a solvent and a synthetic intermediate product. This substance enters the environment via volatile emissions, automobile emissions, sewage waterways, waste disposal sites, and industrial waste. Dodecane is a solvent used in chemical synthesis. This substance is waste from the rubber and paper industries. Furthermore, they are present in the composition of oil products.

It would have been challenging to identify the source of the two alkanes in the initial sample if not for the testing results of the culture medium after two weeks. In the microecosystem, which is linked to the activity of the algae-bacterial community, 13 alkane-type chemicals, including the ones indicated above (Table 1), were found at the second stage of observation.

Alkanes found in aquatic environments are frequently thought to have an anthropogenic origin. However, in natural environments, bacteria, algae, and plants are certain to produce them [39,42]. Compared to the first sample testing, at the second stage, alkane composition has expanded from the initial sample examination, indicating that developing microflora are secreting these substances. Hexacosane and heptacosane appeared in quite high concentrations (6.44% and 7.27%).

The high concentration of alkanes was also defined in the culture medium of monoculture of cyanobacteria *Oscillatoria neglecta* (up to 11.14%) [1]. In the mixing cultures of *Oscillatoria neglecta* and *Anabaena variabilis*, the number of alkanes was reduced. Hexacosane and heptacosane were detected as the basic alkanes in the cultures of green algae *Chlorella kessleri*, *C. vulgaris*, *Chlorella* sp., *Scenedesmus acutus*, *S. acuminatus*, *S. obliquus*, and the cyanobacterium *Spirulina platensis* [43]. As shown, soil-inhabiting cyanobacteria *Microcoleus vaginatus* from the Negev desert produced four linear and more than 60 branched alkanes, the composition of which is extraordinary, and a number of fatty acids, cycling and unsaturated hydrocarbons, aldehydes, alcohols, and ketones [44]. The prevailing compounds were heptadecane (12%), 7-methylheptadecane (7.8%), palmitic acid (6.5%), etc. The essential oil of Anthemis altissima with tricosane as its component demonstrated antimicrobial activity against such bacteria as *Staphylococcus aureus*, *Bacillus subtilis*, *Staphylococcus epidermidis*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* [45]. Tetracosane was detected in the methanolic extract of the mangroves [46]. Nonacosane was a major component of the fractions extracted from the plant Salvia miltiorrhiza [47].

Therefore, it is possible to suggest that the alkanes in the first sample are exometabolites of algae.

The second sample contained 32 LMWOCs, four of which were unidentified (Table 1). Benzoic acid, which was not present in the first sample, was detected in the greatest concentration (12.5%). The antimicrobial and antifungal effects of benzoic acid have been documented [48,49]. Many plants and animals have benzoic acid in their LMWOCs, and it participates in allelopathic interactions in terrestrial and aquatic ecosystems [11,50–52].

Berries, such as cranberry and red cowberry, have a considerable amount of benzoic acid (approximately 0.05%) [53]. Benzoic acid is utilized in the industrial sector to produce valuable compounds such as phenol, benzoyl chloride, and benzoate plasticizers such as

glycol, diethylene glycol, and triethyleneglycol ethers. Benzoic acid and its salts are used to preserve food.

Camphor (1,7,7-trimethylbicyclo[2.2.1]heptan-3-one) was found in sample 2 (Table 1), which is an antibacterial agent with active antagonist characteristics [54]. Camphor's effect on bacteria such as Enterobacter aerogenes and Staphylococcus aureus has been demonstrated [55].

After the experiment was set up, the fatty acids started to show up in the culture media after two weeks (Table 1). Their overall percentage of LMWOCs was 1.52%. According to Jiang et al. [56], Chen et al. [57], Gao et al. [58], and Kurashov et al. [59], fatty acids are known as active allelochemical agents.

Cyanobacteria of the genera *Scytonema* and *Aphanizomenon* secrete fatty-acid components [13,44]. Hexadecanoic acid, with a concentration of 6.54%, was found in soil-inhabiting cyanobacteria [60]. Hexadecanoic acid (palmitic acid) enters the composition of glycerides in most oils: palm oil, black coffee oil, cottonseed oil, cacao oil, etc. Tetradecanoic acid (myristic acid) occurs in nature in the essential oils of many terrestrial and aquatic plants [14,61–63] and takes part in allelopathic interactions [51,64]. This acid was included in a new generation of algaecide to inhibit the development of cyanobacteria and reduce cyanobacterial "blooms" [65]. In addition to being a component of soap and shaving cream, lubricants, and cosmetics, myristic acid is also used to create esters for flavoring and perfumery. At low concentrations, the fatty acids may serve as a possible substitute for traditional antibiotics and biofilm inhibitors [66].

Therefore, the appearance of benzoic acid and fatty acids proves the presence of active allelopathic interactions between certain representatives of the microecosystem.

Manool, which is distinct due to its considerable biochemical, pharmacological, physiological, and toxicological features, should receive special attention. This compound was discovered during the second testing stage. Thus, manool is one of the primary components of blunt-leaved pondweed essential oil (up to 66%) [40]. This article misidentified *Potamogeton obtusifolius* Mert. and W.D.J. Koch as *Potamogeton pusillus* L. (lesser pondweed). According to Kurashov et al. [40], *P. obtusifolius* tissue accumulated manool during the growing season. According to Pratsinis et al. [67], the manool compound found in propolis has an antiproliferative effect. Due to the presence of the diterpene manool in its chemical composition, turpentine from *Copaifera langsdorffii* Desf. has antimicrobial capabilities [68]. Manool exhibits strong selective cytotoxic activity against various tumor cell lines, allowing it to be used to treat cancer without damaging normal cells [69].

The succession in the accumulative culture increased the number of LMWOCs found in the culture medium. It is testified by the results of the third sample examination, where 53 compounds were detected, and 8 of them remained unidentified. The composition of the compounds detected in the third sample differed significantly from both the first and second samples due to its compositional variety, an increase in terpene fraction, saturated hydrocarbons, alcohols, aldehydes, and ketones (Table 1).

3.2. The Similarity Assessment

The similarity assessment of the LMWOCs composition of the microecosystem culture liquids at different stages using the Jaccard and Sorensen similarity coefficients (Table 2) revealed that the composition of exometabolites changed drastically throughout time. For example, after a month of cultivation, the most significant variation was detected between the source water and the culture liquid after a month's cultivation, demonstrating that the composition of the LMWOCs present in the water is predominantly determined by active microflora.

Table 2. Similarity of LMWOCs composition of culture liquid of microsystem at different stages (Sample 1—initial water; Sample 2—after two weeks' cultivation; Sample 3—after a month's cultivation) assessed with Jaccard similarity coefficient (semibold type) and Sørensen–Czekanowski similarity coefficient (*italics*).

	Sample 1	**Sample 2**	**Sample 3**
Sample 1	x	**0.13**	**0.07**
Sample 2	*0.22*	x	**0.15**
Sample 3	*0.13*	*0.26*	x

It is worth noting that when compared to the second testing stage, the third studied stage (a month's microecosystem life) showed a considerable decrease in both the number of alkanes present in the water (from 13 to 4) and their proportion (from 55.16% to 30.48%). Furthermore, the percentage of octacosane increased from 5.06 to 23.78% (Table 1). Alkane absolute content decreased from 0.128 mg/L to 0.076 mg/L. The third testing stage's LMWOC composition likewise contained a high concentration of terpene (E)-3,7-dimethyloct-2-ene (7.14%).

Dodecene and tetradecene, detected in the third testing sample, are referred to as anthropogenic compounds and treated as environmental pollutants [70]. Nevertheless, the fact of their absence in the source water and at the second testing stage (two weeks later) proves that they are synthesized by the microflora being its exometabolites.

3.3. Composition of the Algae-Bacterial Community

Within a month's incubation of the accumulative culture, the development of algae flora was visually and microscopically studied. Two weeks after the experiment setup, the appearance of a green tint in the medium and fouling of the vessel walls in the form of tiny spots were fixed. A thin turbid slick formed on the surface of the medium. At this stage, flocculation at the vessel bottom was also observed.

Cyanobacteria, diatoms, green algae, and euglenophyta were all found in the culture medium of the three samples. Cyanobacteria dominated this assemblage, and diatom algae stood out for their high level of diversity.

The study of the algae flora revealed dominance in the initial sample of the cyanobacterium *Gomphosphaeria naegelina* (Kutz.) as well as the diatom algae *Cocconeis placentula* (Ehr.) and *Navicula* sp. (Bory.). A cyanobacterium *Gloeocapsa* sp. (Kutz.), diatom algae *Stephanodiscus* sp. (Ehr.), Gomphonema constrictum (Ehr.), Melosira sp. (Ag.), Cyclotella sp. (Kutz.), *Cymbella* sp. (Ag.), agreen alga *Chlamidomonas simplex* (Pasch.), and euglenidspecies *Trachelomonas verrucosa* (Stokes) were also found in this sample.

During the microscopic analysis of sample 2, compared to the first sample, an increasing number of cyanobacterial cells of *Gloeocapsa* sp. was detected in the medium strata up to 490 cells/mL. A cyanobacterium *Gomphosphaeria* sp., diatom algae *Navicula* sp., *Cocconeis* sp., *Stephanodiscus* sp., *Cymbella* sp., and a green algae *Chlorella vulgaris* (Beij.) were also present.

The microscopic study of sample 3 has shown the dominance (7.0×10^4 cells/mL) of cyanobacteria of the genus *Gloeocapsa* in the medium strata and diatom algae of the genus *Navicula* in the surface slick. *Cymbella* sp., *C. placentula*, *Nitzschia* sp., *Ankistrodesmus angustus* (Bern), *Actinastrum* sp. (Lagerh.), *Chlorella vulgaris*, *and Scenedesmus* sp. (Turp) were present as well.

As follows from the analysis of the algae flora culture medium, in the first sample, the cyanobacterium *G. naegelina* prevailed, whereas, in the third sample, the cyanobacterium *Gloeocapsa* sp. did; that is, the substitution of the dominants took place. Moreover, these dominants are observed in almost the same proportion: *G. naegelina*—6.8×10^4 cells/mL (initial sample), *Gloeocapsa* sp.—7.0×10^4 cells/mL (after a month's cultivation). In the third sample, we detected clumps of green algae *C. vulgaris* and *Scenedesmus* sp. The presence of octacosane in a high proportion (23.78%) in the third sample concurred with the mass

development of the cyanobacterium *Gloeocapsa* sp. in the presence of the diatom algae of the genus *Navicula* and green algae of the genera *Chlorella* and *Scenedesmus*.

As the algae-bacterial community ran its natural course, a decrease in the total quantitative indicators of the development of microorganisms, algae, and cyanobacteria was not observed. At the same time, their biological activity also remained at a high level as the total concentration of LMWOCs increased. However, the composition of LMWOCs has changed significantly due to the change in dominant species in the community.

The aquatic ecosystem's microorganisms are involved in a complex biocenosis that is characterized by a variety of interactions between them as well as with algae and macrophytes. In water ecosystems, the indicators that respond quickly to environmental changes are microorganisms. Since microbes may break down both naturally occurring and artificially created molecules, the composition of organic and inorganic substances in a water system directly affects how they develop and function.

Studies of the bacterial community in the accumulative culture have revealed its insignificant number and great diversity (Table 3). Among ecologotrophic groups of heterotrophs, microorganisms that may consume high (saprotrophs) and low (oligotrophs) amounts of organic substances, chemotrophs that destroy mineral components, amylo- and saccharolytic, and lipophagic microbes have all been recognized. Microfungi, bacteria, and actinomycetes were found among them. These microorganisms are frequently present in the cyanobacterial communities as satellites [71].

Table 3. Abundance of microflora in the cultural medium of algae and cyanobacteria.

Nutritious Medium	Number of Microorganisms (CUE/mL) and Dominating Morphotypes in the Sample		
	№ 1	№ 2	№ 3
NA	2.0×10^2 Gram-negative bacilli and cocci	2.4×10^2 Gram-positive bacilli	1.5×10^2 Gram-positive bacilli
NA/10	4.6×10^2 Gram-negative bacilli	1.2×10^2 Gram-positive cocci	2.6×10^2 Gram-negative bacilli Gram-positive streptococci
NA/100	0.7×10^2 Gram-negative bacilli	0.9×10^2 Gram-negative bacilli	2.0×10^2 Gram-positive streptococci
Starvation agar	0.7×10^2 Gram-negative bacilli	1.8×10^2 Gram-positive bacilli	0.1×10^2 Gram-positive bacilli
Saliber's	0.6×10^2 Gram-negative bacilli	1.2×10^2 Gram-negative bacilli	2.7×10^2 Gram-negative bacilli
Gause's	0.1×10 *Cladosporium*	0.2×10 Pigmented micromycetes	0.5×10^2 Gram-positive bacilli and cocci Actinomycetes- based
Czapek's	0.1×10 *Fusarium* *Mycelia Sterilia*	0.1×10 *Alternaria*	0.2×10^2 unidentified

The abundance of microorganisms was almost 10^2 cells/mL. The microflora account has shown a general trend of an insignificant rise in the process of cultivating the system (Table 3). However, the third sample's cell count dropped on the "fasting" agar, where only autochthonous microflora grew. Gram-negative rod cells replaced the Gram-positive ones as the major morphotypes.

Gram-negative bacilliform cells predominated in the first sample in all media except Gause's and Czapek's. Gram-positive bacilliform cells and streptococci predominated in the third sample (NA/10 and NA/100). Lipophagic bacteria (Gram-negative cells) were found in all tests in Seliber's medium. Nutrient-agar saprotrophs fluctuated both in abundance and morphotypes in the NA medium. The amylolytic bacteria (Gram-positive

actinomycete-based forms) were detected in Gause's medium. Micromycetes from the genera *Fusarium*, *Alternaria*, and class *Mycelia Sterilia* were found in Czapek's medium.

The microbiological results have shown a negligible abundance of microorganisms in the first sample that used organic compounds (saprotrophs, amylolytic, saccharolytic, and lipophagic) in metabolism, demonstrating the low concentration of organic substances in the initial river water. The results of the GC-MS analysis also showed that the first sample contained the lowest amount of LMWOCs (0.070 mg/L). After that, the concentration of LMWOCs increased up to 0.233 mg/L after two weeks and up to 0.250 mg/L after one month of cultivation (Table 1). This circumstance indicates the active production of organic compounds by microflora in the accumulative culture over time. Their considerable change in composition and quantity indicates that bacteria and algae interact closely in the culture.

4. Conclusions

The experiments have demonstrated that an increase in the number of main groups of microorganisms, along with changes in the composition of algae and cyanobacteria, are responsible for the increase in the composition and concentration of metabolites in the microecosystem's culture medium after one month of cultivation.

The research led to the discovery of various metabolites produced by algae, cyanobacteria, and their bacterial satellites, reflecting their complex ecological and biochemical interactions, including allelopathic ones. The results obtained indicate the importance of ecological and biochemical mechanisms in freshwater ecosystems, which are governed by a variety of exogenous metabolites of populations entering algae-bacterial communities.

Considering that the allelopathic activity of algae, cyanobacteria, and microscopic fungi is, as a rule, caused not by one species-specific compound but by a combination of substances of different natures, a variety of physiologically active compounds are found in exometabolite complexes that have allelochemical potential and influence various aspects of cell metabolism in algae. These compounds seem to be a significant factor in the formation and functioning of algae-bacterial communities. Further research should be aimed, among other things, at finding and identifying groups of compounds that can provide, for example, a synergistic effect in suppressing a particular group or species of microorganisms and algae, e.g., cyanobacteria.

Attention should be paid to the variety of possible functions of phytoplankton LMWOCs, among which the most important appear to be signaling information, allelopathy, and protection from pathogenic microflora and consumers [11]. These functions can be practically significant in the field of environmental management and water quality control. In particular, after identifying the most active allelochemicals against cyanobacteria, they can be further used as natural algicides to control such a hazardous phenomenon as cyanobacterial "flowering".

Due to the need to comprehend the ecological and biochemical mechanisms of the formation and functioning of algal-bacterial communities, as well as to predict potential paths of transformation and evolution of aquatic ecosystems, the specificity of exometabolite complexes of algae and microorganisms, as well as their allelopathic and other biochemical interactions in freshwater ecosystems, requires further serious study.

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