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Communication

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 done 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

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. Various Cyanoprokaryota, Chlorophyceae, Bacillariophyceae,

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Phaeophyceae, Rhodophyceae, and Chrysophyceae representatives have been demonstrated to produce more than 50 phenolic compounds, the majority of which have allelochemical properties.

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 as well as 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.

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The purpose of this paper is to investigate the qualitative and quantitative composition of the solute LMWOCs produced by cyanobacteria and microalgae in the experiment microecosystem using GC-MS.

2. Materials and Methods

On the basis of the nutritional medium BGN11 [16] for cyanobacteria, the experimental accumulative culture was created from river water taken from the Akhtuba River (the Volga river arm) in the area of the Astrakhan region in September 2009. The ratio of river water to the nutritional medium during the experiment was 75% to 25%. Natural light and a temperature range of 22–25 °C were used for cultivation. Exometabolites were isolated using a separating funnel from 500 cm3 of filtered culture medium in 6 ml of hexane. A large range of bioactive compounds are extracted from plants and algae using non-polar solvents (hexane, petroleum ether, benzene, etc.) [17].The extracts were preserved in the refrigerating chamber at -18°C before conducting GC-MS analysis.

The article 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.

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; 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.

By comparing the mass spectra of the LMWOCs found in the water of the accumulative culture with those from the Wiley and NIST_2008 mass spectral libraries, the LMWOCs were identified. By using linear retention indices from a series of straight-chain alkanes (C7-C30), the compounds' identification was verified [18]. With the aid of decafluorobenzophenone and benzophenone (CAS Numbers 119-61-9 and 853-30-4) as internal standards, quantitative analysis was carried out. The St. Petersburg University Resource Center hosted the GC-MS analysis.

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).

$$J = \frac{c}{a+b-c}\ (1)\ ; QS = \frac{2c}{a+b}\ \ (2),$$

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

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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).

					Sample 1		**Sample 2**		**Sample 3**	
	Compound	Formula	RT	RI	%	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
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		

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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

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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		

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58	3-methylicosane	$C_{21}H_{44}$	56.43	1998	1.98	0.0014	0.16	0.0004	0.88	0.002
59	ethenyl hexadecanoate	$C_{18}H_{34}O_2$	56.46	2001			0.45	0.0011	0.91	0.003
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

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77	unidentified m/z 334 [M+], 44 (100)		59.48	2400	5.45	0.0038				
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

The initial sample (Akhtuba native river water) included 22 LMWOCs, 6 of which were unidentified (Table 1). 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 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, 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,

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antispasmodic, antioxidant, antiviral, digestive, appetite suppressant, detoxicant, expectorant, herbicidal, immunomodulatory, muscle relaxant, pesticidal, etc.

Limonene produced by cyanobacteria is able to 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].

To be fair, it should be emphasized that the existence of phthalates in the analyzed samples is disputed because they can be external contaminants. 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

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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%) here. 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 [11,50,51,52].

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 [14,61,62,63] 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. So 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 resulted in an increase in 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).

The similarity assessment of the LMWOCs composition of the microecosystem culture liquids at different stages using Jaccard and Srensen similarity coefficients (Table 2) revealed that the composition of exometabolites changed drastically throughout time. For example, after a month of

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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 quantity 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 LMWOCs 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 are treated as pollutants of the environment [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.

Within a month's incubation of the accumulative culture, the development of algaeflora 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 algaeflora 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.), an euglenidspecies *Trachelomonas verrucosa* (Stokes) were also found in this sample.

During the microscopic analysis of sample 2, as 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 x 104 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 algaeflora culture medium, in the first sample, the cyanobacterium *G. naegelina* prevailed, whereas in the third sample, the cyanobacterium *Gloeocapsa*

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sp.did; that is, the substitution of the dominants took place. What is more, 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, there were 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 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].

The abundance of microorganisms was almost 102 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 grows. Gram-negative rod cells replaced the gram-positive ones as the major morphotypes.

Table 3. Abundance of microflora in 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-positivebacilli	1,5×10^2 Gram-positivebacilli
NA /10	4,6×10^2 Gram-negative bacilli	1,2×10^2 Gram-positivecocci	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-positivestreptococci
Starvation agar	0,7×10^2 Gram-negative bacilli	1,8×10^2 Gram-positivebacilli	0,1×10^2 Gram-positivebacilli
Saliber's	0,6×10^2	1,2×10^2	2,7×10^2

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	Gram-negative bacilli	Gram-negative bacilli	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 MyceliaSterilia*	0,1×10 *Alternaria*	0,2×10^2 unidentified

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, that demonstrates 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. And their considerable change in composition and quantity indicates that bacteria and algae are closely interacting 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 a vast variety of metabolites produced by algae, cyanobacteria, and their bacterial satellites, reflecting their complex ecological and biochemical interactions, including allelopathic ones.

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.

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].

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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Supplementary Materials: The following supporting information can be downloaded at: Preprints.org, Table S1: Composition of exogenous metabolites at the initial stage (Sample 1), after two weeks' cultivation (Sample № 2) and after a month's cultivation (Sample № 3); Table S2: 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*); Table S3:Abundance of microflora in cultural medium of algae and cyanobacteria.

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