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Chapter

The Use of Fluorescence Microscopy to Assess the Suppression of the Development of Cyanobacteria under the Influence of Allelochemicals of Aquatic Macrophytes

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Abstract

The luminescent microscopy (LM) is especially convenient for express analysis of the toxicity of various substances or for detecting the degree of inhibition of the physiological state of cyanobacteria populations as a result of the action of certain chemical compounds on them. In natural water bodies, the suppression of the development of the phytoplankton occurs, in particular, under the influence of low-molecular-weight organic compounds (LMWOCs), metabolites-allelochemicals, of aquatic macrophytes. LM, which allows observing the primary or secondary luminescence of microorganisms, was used by us to study changes in the physiological state of the cyanobacteria cultures of *Synechocystis aquatilis* and *Aphanizomenon flos-aquae* under the influence of allelochemicals of water macrophytes in laboratory experiments. We have shown (including using LM) that selected LMWOCs (linoleic, heptanoic, octanoic, tetradecanoic, hexadecanoic, and gallic acids) possess inhibitory allelopathic activity against cyanobacteria. However, their inhibitory effect was different. The highest values of the suppression index (SI > 10) were recorded (in ascending order) for hexadecanoic, linoleic, tetradecanoic, and gallic acids and a mixture of four allelochemicals (heptanoic, octanoic, tetradecanoic, and gallic acids). The creation of a new generation of algaecides/cyanocides based on LMWOCs of aquatic plants is a very promising strategy for combating “algal blooms.”

Keywords: luminescent microscopy, cyanobacteria, *Synechocystis aquatilis*, *Aphanizomenon flos-aquae*, allelochemicals, allelopathy, carboxylic acids, gallic acid, algal blooms, aquatic macrophytes

1. Introduction

At present, fluorescence methods play an important role in studies of the physiology of photosynthetic organisms. Luminescent microscopy, which makes it

possible to conduct a fairly quick and accurate assessment of the degree of viability of cells of lower and higher plants, manifested in a change in the luminescence of cells, is an integral part of them. This is based on the fact that under microscopy in ultraviolet rays, the cells that differ in their physiological state give different shades of color and brightness of the luminescence.

Luminescent microscopy allows, in particular, to observe the primary fluorescence (autofluorescence) of chlorophyll "a" in the cells of higher and lower plants, as well as in the cells of cyanobacteria, which make it possible to conduct a fairly rapid and accurate assessment of various degrees of cell viability, expressed in changes of their glow [1–3].

In Russia, the basics of applying the method of luminescent microscopy to determine the viability of algae and cyanobacteria cells were developed in the 1960s of the last century [4, 5]. In UV rays, cells with different viabilities were found to yield different colors and brightness of the glow shades. This provides possibilities to differentiate viable and non-viable cells and estimate their number and the degree of degradation of algae and cyanobacteria populations.

The rationale and advantages of this method for fast viability assay for unicellular cyanobacteria, which uses red chlorophyll fluorescence and an unspecific green autofluorescence for the differentiation of viable and non-viable cells without the need of sample preparation, are presented in [6] as well. The method is especially convenient for express analysis of the toxicity of various substances or for detecting the degree of inhibition of the physiological state of cyanobacteria populations as a result of the action of certain chemical compounds on them.

We applied luminescent microscopy to one of the most acute environmental problems of our days, the so-called algal blooms in inland waters, which cause degradation of aquatic ecosystems, deterioration of water quality, and a decrease in the economic potential of reservoirs [7–9]. Among other representatives of photosynthetic plankton, cyanobacteria are the most significant formers of harmful freshwater "algal blooms" (HAB) [10–13]. Inhibiting the growth of undesired cyanobacteria species is crucial for controlling HAB. Moreover, it is most important to prevent the development of HAB, since this will prevent or weaken the tremendous and widespread impact of HAB on human and environmental health, natural and man-made assets, as well as overall ecosystem services [14]. Suppressing of cyanobacteria (and algae of phytoplankton) by allelochemicals released by aquatic macrophytes is reported to be one of the natural mechanisms that maintain a prosperous and clear-water state in aquatic ecosystems (first of all, in shallow lakes and ponds) [15–18].

There are a large number of low-molecular-weight organic compounds (LMWOCs), metabolites of freshwater macrophytes that can perform an allelopathic function in aquatic ecosystems [16, 19–21].

The results of our previous studies using QSAR technology show that carboxylic and gallic acids have a high calculated potential to inhibit the development of cyanobacteria [22].

In order to receive the proofs of the mechanism of suppressing of cyanobacteria by some allelochemicals of aquatic macrophytes, we chose the luminescent microscopy, which allows observing the primary or secondary luminescence of microorganisms. This method was used by us to study changes both in density and in the physiological state of the cyanobacteria cultures of *Synechocystis aquatilis* Sauvageau and *Aphanizomenon flos-aquae* Ralfs ex Bornet and Flahault under the influence of substances-allelochemicals of water macrophytes in a small-scale laboratory microcosm experiments.

2. Material and methods

2.1 Allelochemical identification

In this paper, we use data on the following species of macrophytes: *Ceratophyllum demersum* L., *Potamogeton perfoliatus* L., *Potamogeton natans* L., *Potamogeton obtusifolius* Mert. & W.D.J. Koch, *Potamogeton pectinatus* L., *Nuphar lutea* (L.) Smith., *Nymphaea alba* L., *Myriophyllum spicatum* L., and *Persicaria amphibia* (L.) Gray (**Table 1**). The LMWOCs of these macrophytes were investigated in the plant material, which was collected during the summer of 2013–2017 in the water bodies marked in **Table 1**. The composition and concentrations of LMWOCs in essential oil were determined using a TRACE ISQ gas chromatograph-mass spectrometer (Thermo Electron Corporation) equipped with a quadrupole mass analyzer and Thermo TG-SQC Column (15 m; inner diameter, 0.25 mm; and 0.25 µm film). The technique of sampling macrophytes, sample preparation, and the details of chromatography-mass spectrometric studies are described in detail in [22].

2.2 Assaying allelochemical effects

We used in experiments an axenic strain of *Synechocystis aquatilis* Sauvageau № 1336 from the collection of the living cultures of cyanobacteria, algae and algal parasites (CALU, Collection of Algae of Leningrad University), which was provided by the Centre for Culture Collection of Microorganisms of the Research Park at St. Petersburg University. The strain was isolated from a sample of water taken in the Gulf of Finland near Sosnovy Bor. Another axenic strain of *Aphanizomenon flos-aquae* Ralfs ex Bornet and Flahault was provided by the St. Petersburg Scientific Research Center for Ecological Safety of the Russian Academy of Sciences.

The experiments (three to four replicates) were carried out in microcosms with a volume of 0.5 liters. Cyanobacterial cultures of *S. aquatilis* and *A. flos-aquae* in the exponential phase of growth were introduced into experimental vessels in the form of suspensions.

Cyanobacteria were cultured on medium No. 6 [23]. Cultivation of cyanobacteria and experiments were carried out in a special aquarium using a liquid circulation cryothermostat with cooling and heating {Baths WCR Circulation water bath WCR-MaXircu CR-P8 [Daihan (Witeg)]}. The unit maintained a constant temperature of 25°C during the experiments. The lamp (Lamp Biodesign RIF 80/110/PANORAMA 80/100/DIARAMA 150/200) provided a luminous flux of 1500 lm. The day-night mode (16–8 hours) was set using an adjustable timer (FERON TM50, 3500 W/16A230V).

Due to the promising algicidal effect [24, 25], the following allelochemicals have been used in the present study: linoleic acid, heptanoic acid, octanoic acid, tetradecanoic acid, hexadecanoic acid, and gallic acid. Instead of natural LMWOCs, the purified analogous substances from Acros Organics BVBA and their combination were used in experiments to test their cyanocidal effect on *S. aquatilis* and *A. flos-aquae*.

In experiments, aqueous solutions of these acids were used to test the action of individual compounds. Since some of the allelochemicals used are poorly hydro-soluble, they were dissolved in the organic solvent ethanol in experiments to evaluate their combined allelopathic effects. In experiments, no more than 1 ml of ethanol with the dissolved tested allelochemicals was introduced into the medium in 500 ml vessels to achieve the required concentration in the experimental microcosm. In parallel, experiments with the addition of 1 ml of ethanol without allelochemicals

Species	Water body/coordinates	Number, min-max	(% of total essential oil, min-max)
<i>Ceratophyllum demersum</i> L.	Lakes of Volga-Akhtuba floodplain (Astrakhan Region, Russia) / N 48°29', E 45°34'	3–5	0.27–14.62
<i>C. demersum</i>	Ponds of the Victory Park, (St. Petersburg, Russia)/N 60° 52.025', E 30° 19.91'	4–5	4.9–5.7
<i>Potamogeton perfoliatus</i> L.	Lake Ladoga/N 60° 50', E 31° 33'	1–11	0.10–30.78
<i>P. perfoliatus</i>	Lake Onega/N 61°38', E 35°31'	5–8	1.0–5.28
<i>Potamogeton natans</i> L.	Lakes of Karelian Isthmus, Leningrad Region, Russia/N 61° 07', E 29° 55'	5–7	8.5–56.39
<i>Potamogeton obtusifolius</i> Mert. & W.D.J. Koch	Ponds of the Victory Park, (St. Petersburg, Russia)/N 60° 52.025', E 30° 19.91'	3–5	0.3–4.5
<i>Potamogeton pectinatus</i> L.	Lake Ladoga/N 60° 50', E 31° 33'	2	9.93
<i>P. pectinatus</i>	Lakes of Volga-Akhtuba floodplain (Astrakhan Region, Russia)/N 48°29', E 45°34'	3–5	29.28–40.0
<i>Nuphar lutea</i> (L.) Smith.	River Ild, Yaroslavl Region, Russia/N 58°0.23', E 38°13.53'	4–5	19.05–66.03
<i>N. lutea</i>	Lakes of Karelian Isthmus, Leningrad Region, Russia/N 61° 07', E 29° 55'	4–7	27.43–77.57
<i>N. lutea</i>	The mouth of the Volkhov River, Leningrad Region, Russia/N 60° 07.14', E 32° 19.57'	1	28.74
<i>Nymphaea alba</i> L.	Lakes of Karelian Isthmus, Leningrad Region, Russia/N 61° 07', E 29° 55'	5–10	58.06–61.43
<i>N. alba</i>	Lakes of Volga-Akhtuba floodplain (Astrakhan Region, Russia)/N 48°29', E 45°34'	6–8	49.88–53.05
<i>Myriophyllum spicatum</i> L.	Lakes of Karelian Isthmus, Leningrad Region, Russia/N 61° 07', E 29° 55'	15–17	49.55–52.93
<i>M. spicatum</i>	Lake Ladoga/N 60° 50', E 31°33'	9	26.19
<i>M. spicatum</i>	Lake Naroch (Belarus)/N 54°51', E 26°45'	4	15.41
<i>M. spicatum</i>	Lakes of Volga-Akhtuba floodplain (Astrakhan Region, Russia)/N 48°29', E 45°34'	5	32.09
<i>M. spicatum</i>	Kazachy River, Astrakhan Region/N 46°13.13', E 47°52.57'	8	50.22
<i>M. spicatum</i>	Dniester estuary, Black Sea, Ukraine /N 46°05.11', E 30°27.84'	4–5	43.99–55.39
<i>Persicaria amphibia</i> (L.) Gray	Lake Ladoga/N 60° 50', E 31° 33'	6–7	50.09–60.63

Table 1. The number and relative content (% of total essential oil) of the fatty acids in some species of freshwater macrophytes from different water bodies.

of 500 ml of the medium showed that this does not have an inhibiting effect on the development of cyanobacteria.

The effect of allelochemicals in concentrations that may be characteristic of natural bodies of water was tested. The compounds were added into the cyanobacterial cultures in experimental microcosms to achieve their various final concentrations of 0.001, 0.01, 0.1, 1, 10, and 100 mg/l. Different combinations of these concentrations were used in different versions of the experiments.

Suppression index (SI), defined as the cyanobacterial density in control divided by the cyanobacterial density in an experiment with allelochemicals, was used to assess the degree of suppression of cyanobacterial cultural development.

2.3 Assaying cultural density and physiology state of cyanobacteria

The growth of cyanobacterial cultures and their physiological state were monitored using the MIKMED-26 fluorescence microscope with a $\times 100/1.25$ Plan Achromat oil immersion objective at intervals of 2–7 days in different experiments.

Microscopy was performed using ultraviolet radiation (excitation 330–385 nm/emission 400–420 nm). The physiological state of the culture was estimated by the color of the glow of the cells, and the number of viable and dead cells and their ratio (proportion expressed in %) were calculated.

The change in the fluorescence spectrum of cells containing chlorophyll occurs in the following stages [3, 5, 26]:

1. Bright purple-red glow is typical for chlorophyll-containing cells of the highest viability—the cells are in an active state, intensively dividing or preparing for division (logarithmic growth phase).
2. The red glow of lower brightness (dull maroon or pink-red) is inherent in cells in the stationary phase of growth of algae culture.
3. The orange-red hue of the glow is given by cells that are suppressed under the influence of any factor, and the pale orange glow is given by cells with a very low level of vital activity but still alive.
4. Old cells with weakened vital activity glow dimly red.
5. Bluish-green and olive-green glow is typical for dead cells and detritus.

In some experiments, the density of the cyanobacterial culture was estimated by the characteristic of optical density, which was obtained using a photoelectric concentration colorimeter KFC-2MP. Since the optical characteristics of the aquatic environment change with the growth of cyanobacteria in the experimental vessels, this made it possible to correlate the number of cyanobacteria with the optical characteristics of the medium, which can be recorded using KFC-2MP. The optical density of the culture medium was measured in the red region of the spectrum at a wavelength of 670 nm, which is the maximum absorption of chlorophyll-a [27], available in cyanobacteria.

3. Results

3.1 GC-MS studies

The results of our chromatographic-mass-spectrometric studies of the low-molecular-weight metabolome (LMWM) of various aquatic macrophyte species

from different types of reservoirs [7, 22, 28–30], and unpublished data show that many carboxylic acids, recognized as active allelochemicals, constitute a significant share of the LMWOC composition of the plant essential oil (**Table 1**).

Plants such as *P. natans*, *N. lutea*, *N. alba*, *M. spicatum*, and *P. amphibia* are the most active producers of fatty acids and therefore can have the greatest allelopathic effect on phytoplankton and cyanobacteria. **Figures 1–3** show sections of the chromatograms of essential oils of some aquatic macrophytes, which present the location of the fatty acids found in them, including those that were used in our experiments to study the suppression of the development of cyanobacteria: linoleic, tetradecanoic, hexadecanoic, and octanoic.

According to our data, the following fatty acids have a high content in the essential oil of aquatic macrophytes studied by us, tetradecanoic acid, hexadecanoic acid, linoleic acid, and linolenic acid, and are part of the major LMWM components of many freshwater macrophytes [20, 22, 24, 31]. Some aquatic macrophytes may contain a particularly significant number of carboxylic acids in their LMWM, for example, *Myriophyllum spicatum* L. (**Figure 3**).

3.2 Small-scale laboratory microcosm experiments

3.2.1 Experiment with *S. aquatilis* and linoleic acid

We added the linoleic acid to the *S. aquatilis* culture medium at concentrations of 0.001, 0.01, and 0.04 mg/l. The initial density of cyanobacteria was 460,000 cells/ml. In the experiment with linoleic acid, significant suppression of the development of *S. aquatilis* culture was observed (**Figure 4**). Moreover, the maximum suppression (SI = 12.5) was noted on the fourth day of the experiment at a concentration of the allelochemical of 0.01 mg/l. At the end of the experiment (on day 12), the greatest inhibition of the development of cyanobacteria (SI = 3) was observed at an allelochemical concentration of 0.04 mg/l (**Table 2**).

Figure 5 presents a visualization of the effect of the allelochemical on cyanobacteria during the experiment. The suppression of the development of culture

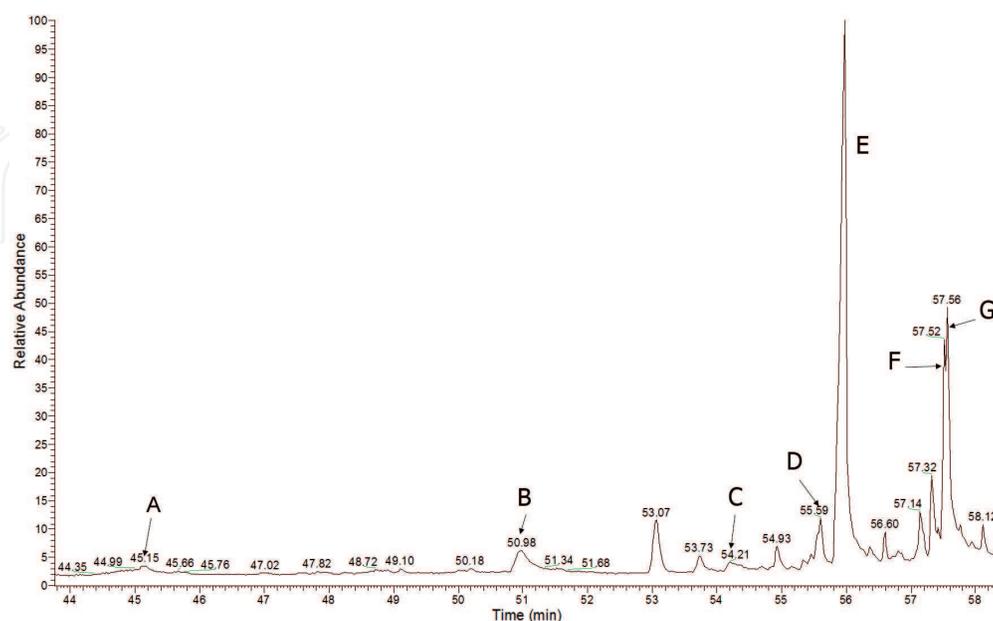


Figure 1. Chromatogram section of *Persicaria amphibia* essential oil (Sortavala skerries, Lake Ladoga) with marked peaks of carboxylic acids: (A) dodecanoic acid, (B) tetradecanoic acid, (C) pentadecanoic acid, (D) palmitoleic acid, (E) hexadecanoic acid, (F) linoleic acid, (G) linolenic acid.

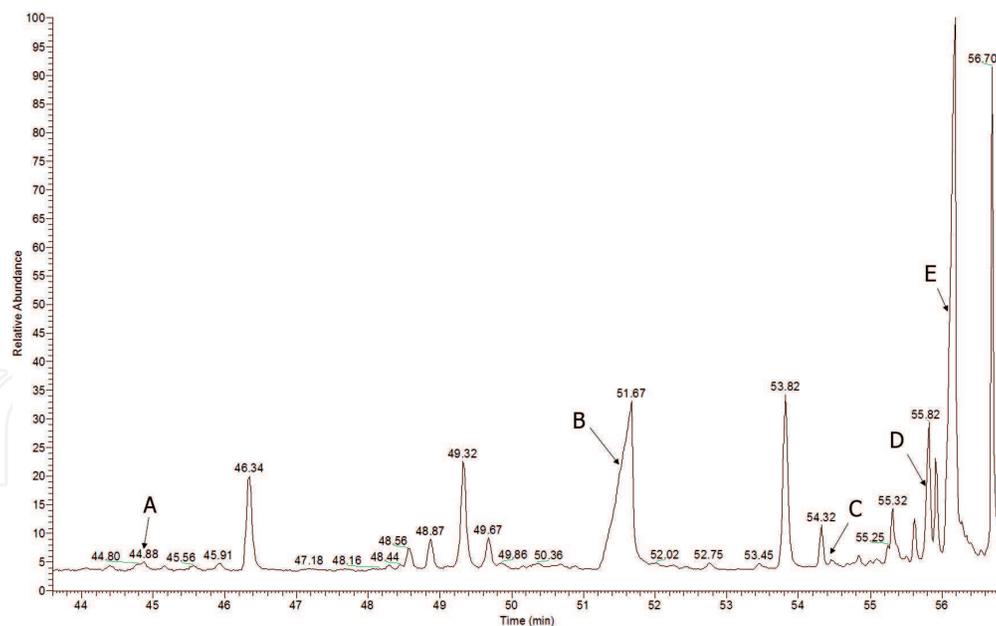


Figure 2.
Chromatogram section of Potamogeton pectinatus essential oil (Lake Obvalovannoe, Volga-Akhtuba floodplain, Astrakhan Region) with marked peaks of carboxylic acids: (A) dodecanoic acid, (B) tetradecanoic acid, (C) pentadecanoic acid, (D) palmitoleic acid, (E) hexadecanoic acid.

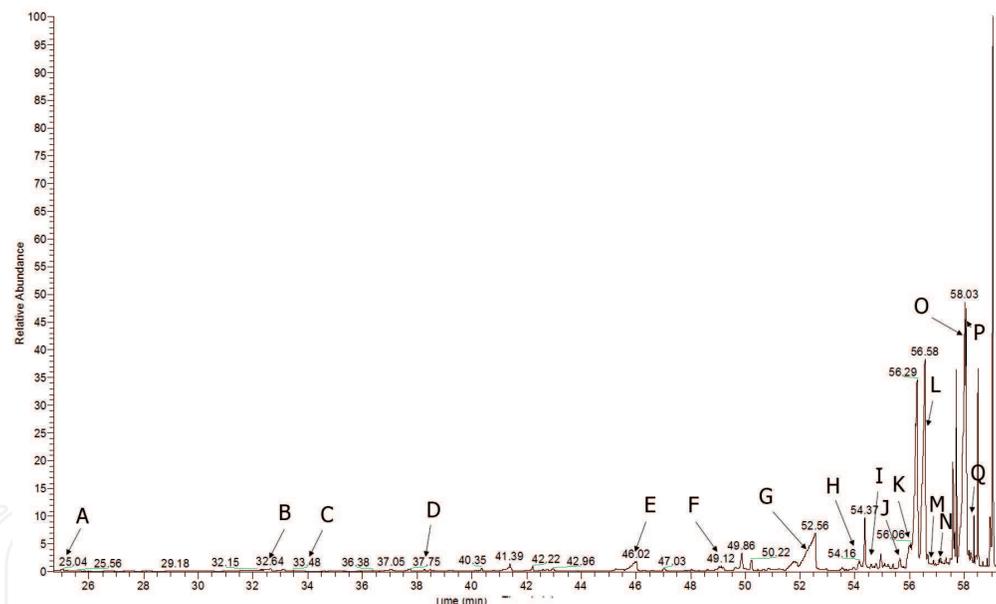


Figure 3.
A chromatogram section of Myriophyllum spicatum essential oil (Lake Uzkoie, Karelian isthmus, Leningrad region) with marked peaks of carboxylic acids: (A) octanoic acid; (B) nonanoic acid; (C) 2-nonenoic acid; (D) decanoic acid; (E) dodecanoic acid; (F) tridecanoic acid; (G) tetradecanoic acid; (H) pentadecanoic acid; (I) 14-pentadecenoic acid; (J) 9-hexadecenoic acid; (K) hexadecanoic acid, Z-11-; (L) hexadecanoic acid; (M) (E)-heptadec-9-enoic acid; (N) cis-10-heptadecenoic acid; (O) linoleic acid; (P) linolenic acid; (Q) octadecanoic acid.

and the suppression of its physiological state, which is expressed in a change in the fluorescent glow of cyanobacteria cells from bright red to orange, pink-green, and bluish-green, is clearly seen.

According to fluorescence microscopy data, changes in the proportion of living and dead cyanobacteria cells were observed when acted upon by linoleic acid (**Figure 4b**). Moreover, the maximum percentage of dead cells (36.5%) was seen on day 5 at the maximum of the studied concentrations of linoleic acid (0.04 mg/l)

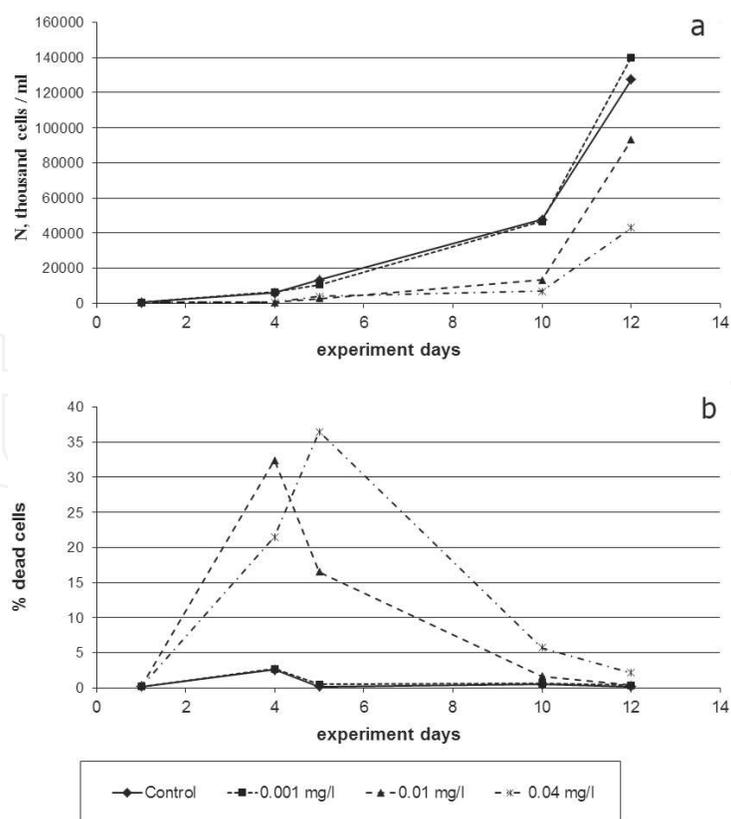


Figure 4. Development of the culture of *S. aquatilis* in the experiment with linoleic acid: (a) the average number (median), (b) % dead cells (median).

Experiment number/ active allelochemical	Object of impact	Maximum SI/ maximum SI by the end of the experiment	Allelochemical concentration (mg/l) at maximum SI/at the greatest SI at the end of the experiment	Day of experiment with maximum SI/total duration of the experiment
1/linoleic acid	<i>S. aquatilis</i>	12.5/3	0.01/0.04	4/12
2/tetradecanoic acid	<i>S. aquatilis</i>	7.5/7.5	0.1/0.1	14/14
3/tetradecanoic acid	<i>S. aquatilis</i>	14.5/11.9	0.1/0.1	8/10
4/hexadecanoic acid	<i>S. aquatilis</i>	10.4/10.4	0.1/0.1	13/13
5/gallic acid	<i>S. aquatilis</i>	30/30	100/100	15/15
6/heptanoic acid	<i>S. aquatilis</i>	1.9/1.9	1/1	17/17
7/octanoic acid	<i>S. aquatilis</i>	3/3	1/1	17/17
8/ mixture of heptanoic, octanoic, tetradecanoic, and gallic acids	<i>S. aquatilis</i>	35.3/8.3	10/10	7/13
9/mixture of heptanoic, octanoic, tetradecanoic, and gallic acids	<i>A. flos-aquae</i>	17,495/17,495	10/10	23/23

Table 2. The maximum suppression of the development of cyanobacteria when exposed to various allelochemicals.

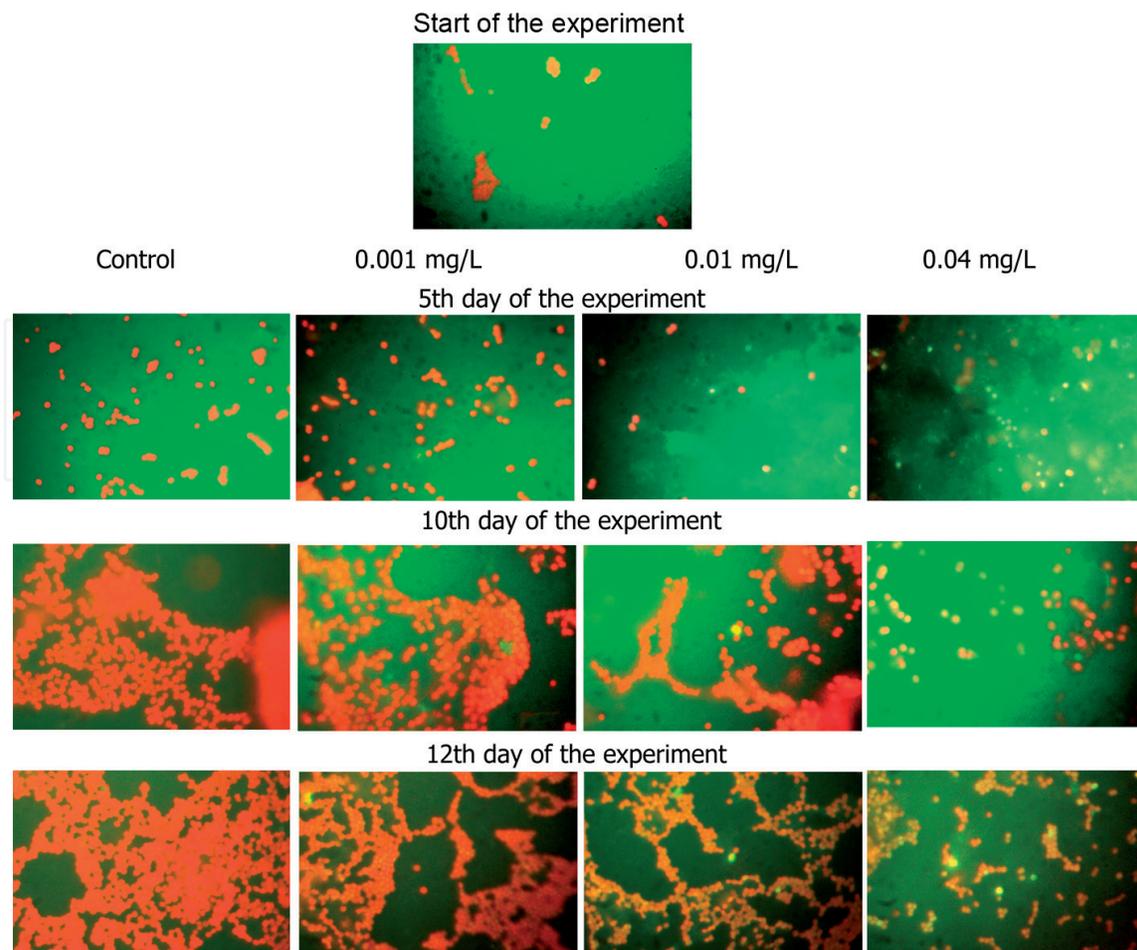


Figure 5. Visualization of changes in the physiological state of *S. aquatilis* culture under the action of linoleic acid.

(**Table 3**). By the end of the experiment (on the 12th day), the proportion of dead cells was insignificant in all variants of the experiments (**Figure 4b**). The observed decrease in the number of *S. aquatilis* by three times compared to the control indicates a physiological slowdown in the growth of the culture.

3.2.2 Experiments with *S. aquatilis* and tetradecanoic acid

According to some researchers [32, 33], tetradecanoic and hexadecanoic acids can be present in natural waters in concentrations from 0.1 to 1000 $\mu\text{g/l}$. In this regard, we added tetradecanoic acid to the *S. aquatilis* culture medium at concentrations of 0.001, 0.01, and 0.1 mg/l. The initial density of cyanobacteria was 440,000 cells/ml in the first series and 210,000 cells/ml in the second series.

The difference between the two series of experiments with tetradecanoic acid, the results of which are shown in **Figures 6–9**, consisted in different initial numbers of cyanobacteria, which in the 1st series was two times higher (440,000 vs. 210,000 cells/ml). Also, as in the case of linoleic acid, in two series of experiments with tetradecanoic acid, as a result of microscopic fluorescence analysis, a distinct inhibition of *S. aquatilis* culture was observed (**Figures 6 and 7**).

In the first series of experiments, the largest proportion of dead cells (19.6%) was recorded on the second day of the experiment, and at the end of the experiment (on day 14), it was 10.4% (**Table 3; Figure 8**). In the second series, with a lower initial concentration of cyanobacteria, the maximum proportion of dead cells was noted at the end of the experiment (on the 10th day) and amounted to 91.7%.

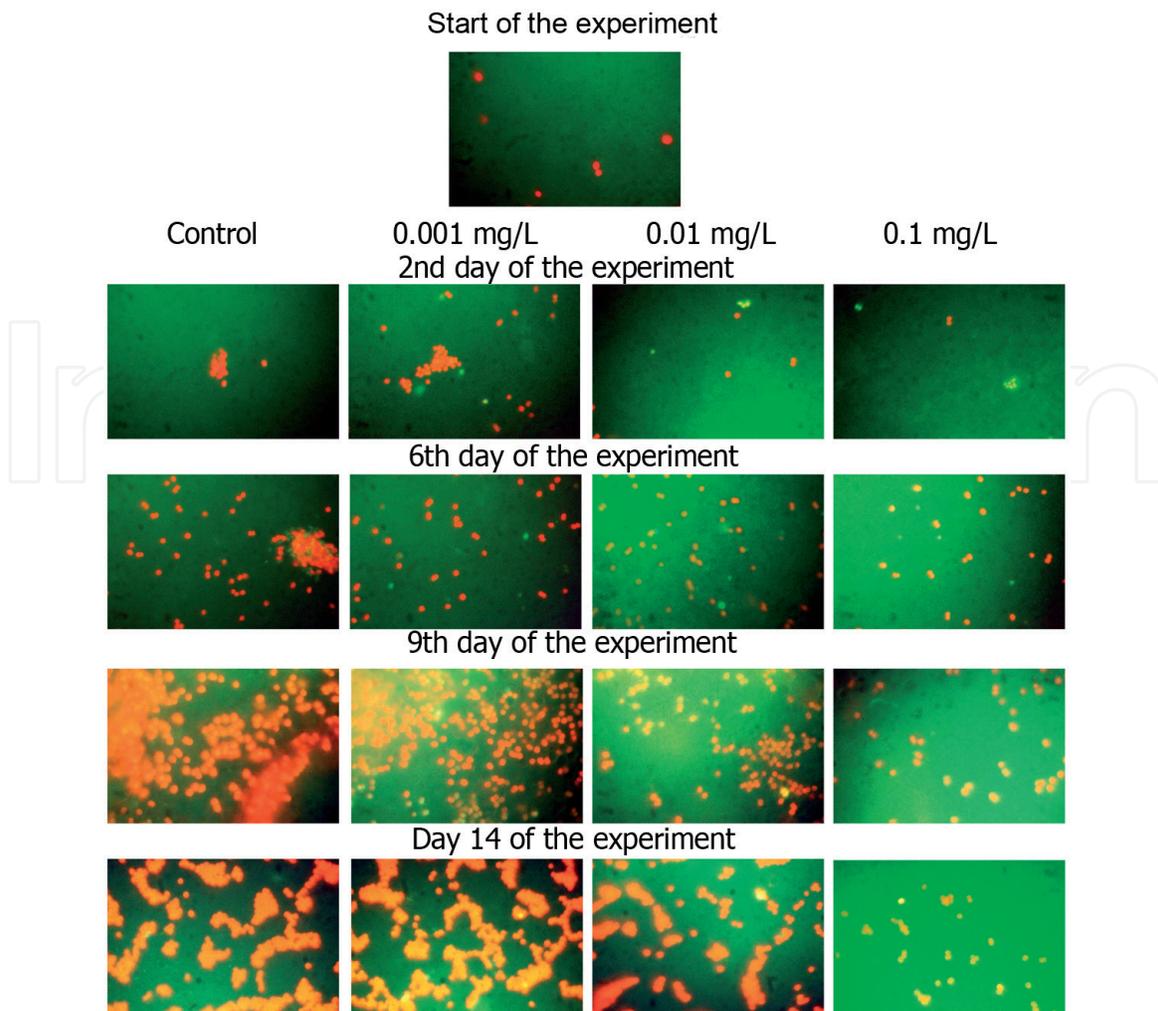


Figure 6. Visualization of changes in the physiological state of *S. aquatilis* culture under the action of tetradecanoic acid (experiment 1).

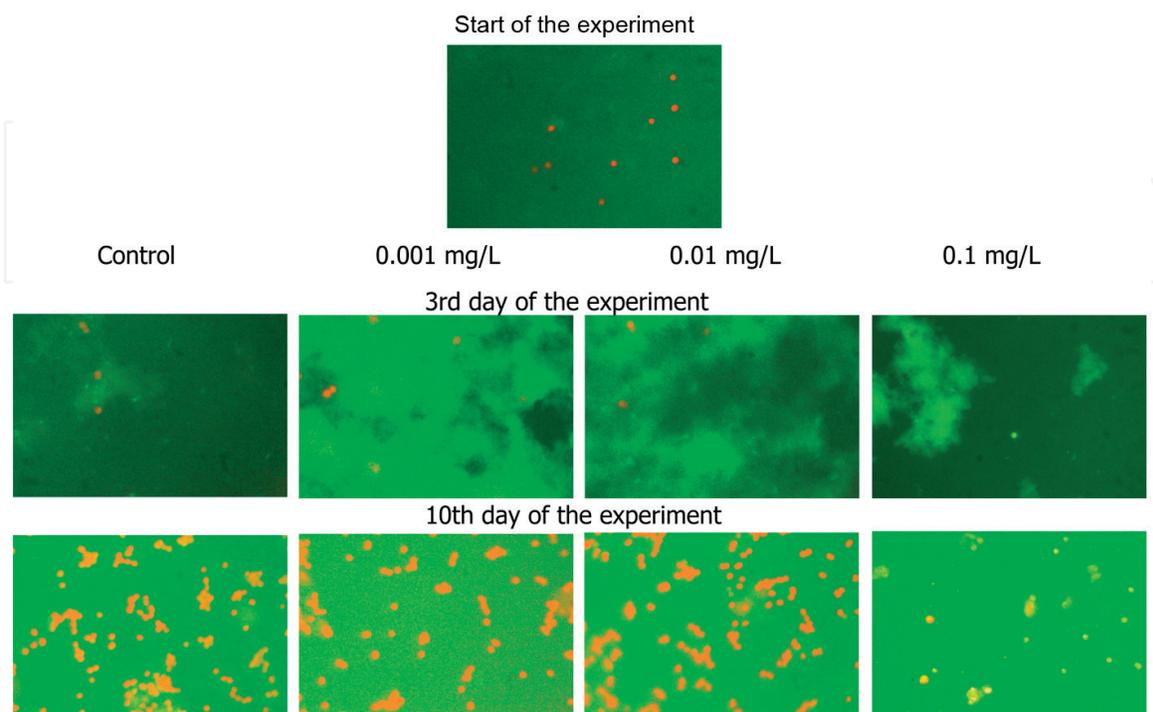


Figure 7. Visualization of changes in the physiological state of *S. aquatilis* culture under the action of tetradecanoic acid (experiment 2).

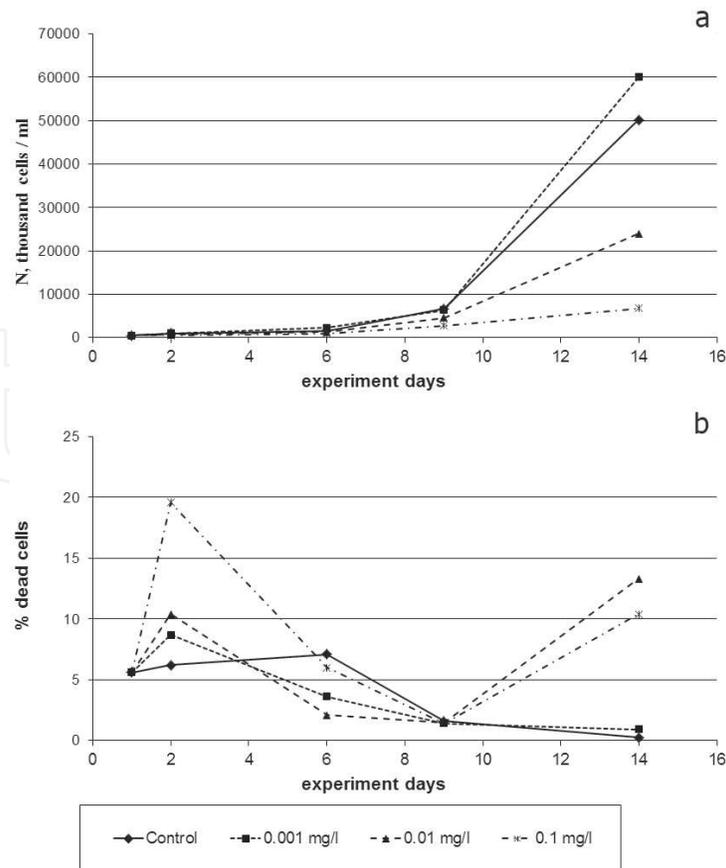


Figure 8.
 Development of the culture of *S. aquatilis* in experiment No. 1 with tetradecanoic acid: (a) the average number (median), (b) % dead cells (median).

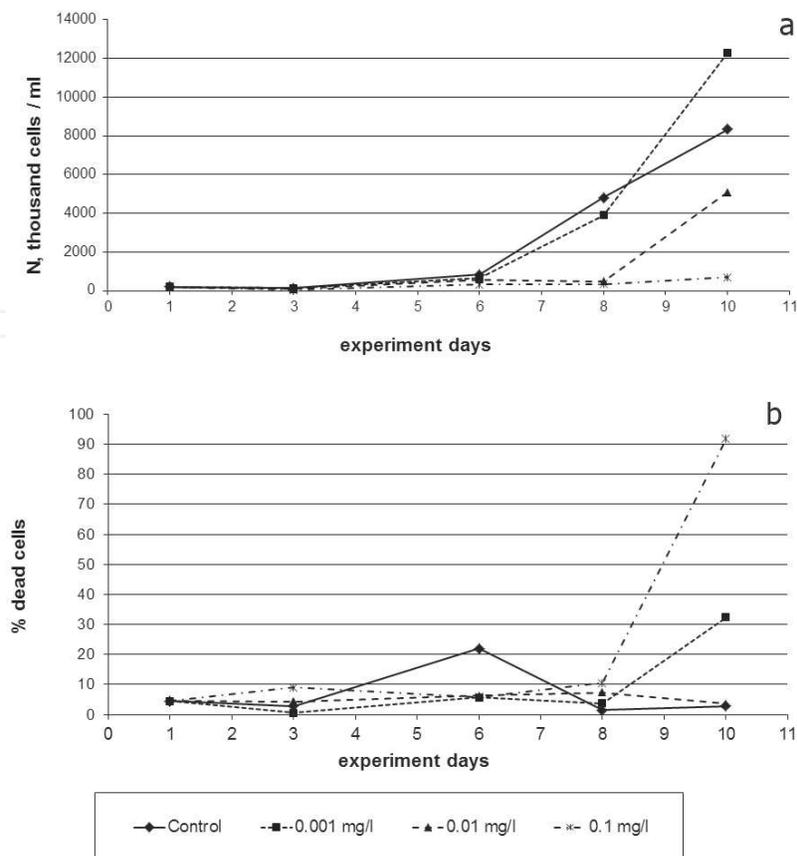


Figure 9.
 Development of the culture of *S. aquatilis* in experiment No. 2 with tetradecanoic acid: (a) the average number (median), (b) % dead cells (median).

Experiment number/ active allelochemical	Object of impact	The maximum proportion of dead cells, %/maximum proportion of dead cells (%) at the end of the experiment	Allelochemical concentration (mg/l)/ the maximum proportion of dead cells	Day of the experiment with the maximum proportion of dead cells
1/linoleic acid	<i>S. aquatilis</i>	36.5/2.4	0.04/0.04	5/12
2/tetradecanoic acid	<i>S. aquatilis</i>	19.6/10.4	0.1/0.1	2/14
3/tetradecanoic acid	<i>S. aquatilis</i>	91.7/91.7	0.1/0.1	10/10
4/hexadecanoic acid	<i>S. aquatilis</i>	23.4/5.9	0.1/0.1	4/13
5/gallic acid	<i>S. aquatilis</i>	22.6/17.6	100/100	13/15
6/heptanoic acid	<i>S. aquatilis</i>	3.6/0.8	1/1	10/17
7/octanoic acid	<i>S. aquatilis</i>	5.4/1.3	1/1	3/17
8/mixture of heptanoic, octanoic, tetradecanoic, and gallic acids	<i>S. aquatilis</i>	4.4/4.4	10/10	13/13
9/mixture of heptanoic, octanoic, tetradecanoic, and gallic acids	<i>A. flos-aquae</i>	>95	10/10	23/23

Table 3.

The maximum proportion of dead cells of cyanobacteria when exposed to various allelochemicals.

The color shade of the cells was very different in the control and in the experiments, where the greatest suppression of cyanobacteria under the influence of tetradecanoic acid was noted. Pink-red clusters of cells were observed in the control, while pink-green and orange-green clusters and single cells were seen in the inhibited culture. With a lower initial density of *S. aquatilis* culture, a higher degree of culture inhibition compared to the control (SI = 11.9) was recorded at the end of the experiment than in the first series of the experiment, where this SI value was 7.5 (**Table 2; Figures 8 and 9**). The greatest inhibition of the development of cyanobacteria was noted at the highest of the studied concentrations (0.1 mg/l) of tetradecanoic acid in two series of experiments.

The lowest of the studied concentrations (0.001 mg/l) did not exert an adverse effect on cyanobacteria. Moreover, in both series of experiments, its stimulating effect was noted, i.e., the number of *S. aquatilis* in experimental microcosms at this concentration was higher than in the control (**Figures 8 and 9**).

3.2.3 Experiment with *S. aquatilis* and hexadecanoic acid

We added the hexadecanoic acid to the *S. aquatilis* culture medium at concentrations of 0.001, 0.01, and 0.1 mg/l. The initial density of cyanobacteria was 31,000 cells/ml. By the end of the experiment (on day 13), differences in the physiological state of *S. aquatilis* culture in the control and at a concentration of the allelochemical of 0.1 mg/l were clearly visible (**Figure 10**). In the experimental microcosms with allelochemical impact, cyanobacteria cells were present in pink-green clusters, while in the control, orange-red clusters of cells were observed in good physiological condition. Even at a concentration of 0.001 mg/l, hexadecanoic acid led to inhibition of the development of *S. aquatilis* culture (**Figure 11**), which was noticeable on the eighth day of the experiment.

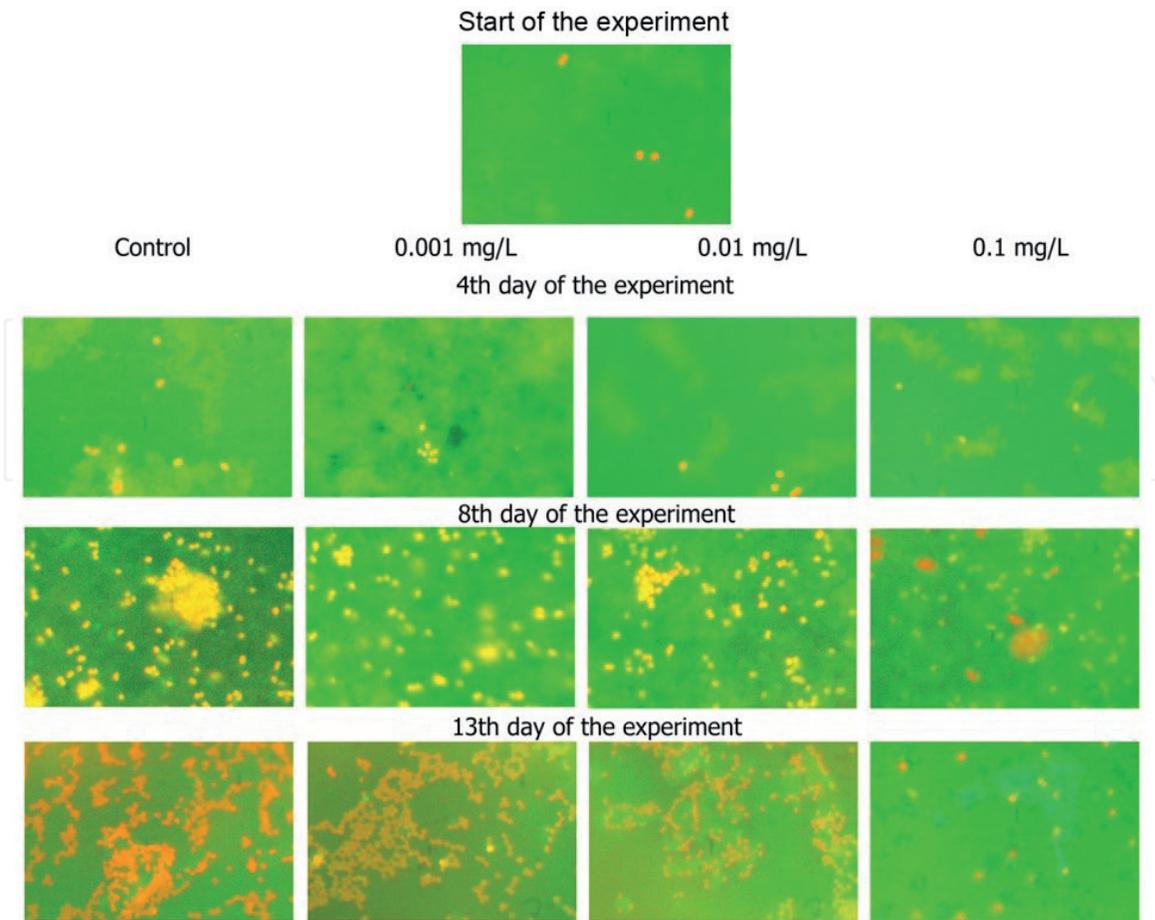


Figure 10. Visualization of changes in the physiological state of *S. aquatilis* culture under the action of hexadecanoic acid.

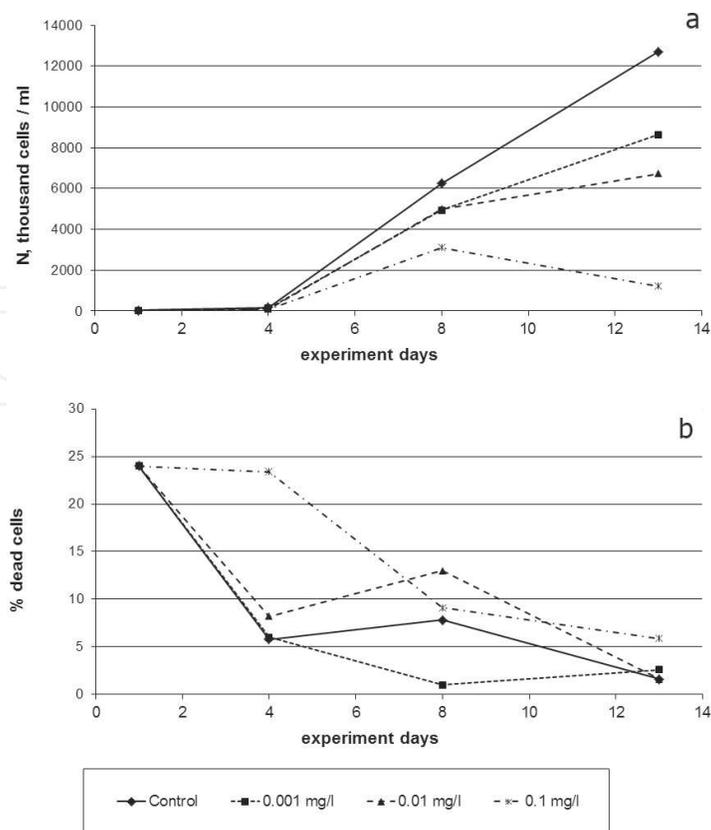


Figure 11. Development of the culture of *S. aquatilis* in the experiment with hexadecanoic acid: (a) the average number (median), (b) % dead cells (median).

The greatest inhibition (SI = 10.4) was observed at the end of the experiment (on day 13) at a concentration of 0.1 mg/l. At the same concentration, the largest number of dead cells was observed, indicating that the culture was suppressed under the influence of the allelochemical. It was evident already on the fourth day of the experiment (23.4%) (**Figure 11; Table 3**). Subsequently, the proportion of dead cells decreased (to 5.9% at the end of the experiment), but a decrease in the number of cyanobacteria was also observed, which indicates the absence of culture growth under the influence of the allelochemical (**Figure 11a**).

3.2.4 Experiment with *S. aquatilis* and gallic acid

In the experiment with *S. aquatilis* and gallic acid, the initial density of cyanobacteria was 40,000 cells/ml. In this experiment, one of the highest values of inhibition of *S. aquatilis* was recorded (**Table 2; Figure 12**).

At the end of the experiment (on the 15th day), the density of the *S. aquatilis* culture in the control was 30 times higher than at the maximal studied concentration (100 mg/l) (**Table 2**). However, even at concentrations of 1 and 10 mg/l, inhibition of cyanobacteria development was observed by 3.3–3.5 times when compared to the control. At the same time, the proportion of dead cells (glowing pink-green or greenish-blue) was especially high at a concentration of 100 mg/l, at which the maximum inhibition of culture development was observed (**Tables 2 and 3; Figure 12**). It should be noted that, as in the case of tetradecanoic acid, the lowest of the studied concentrations of the allelochemical (0.1 mg/l) led to stimulation of the development of cyanobacteria (**Figure 12**).

3.2.5 Experiments with *S. aquatilis* and heptanoic and octanoic acids

In experiments with heptanoic and octanoic acids, the initial density of cyanobacteria was 73,000 cells/ml. Of all the acids tested, heptanoic acid showed the

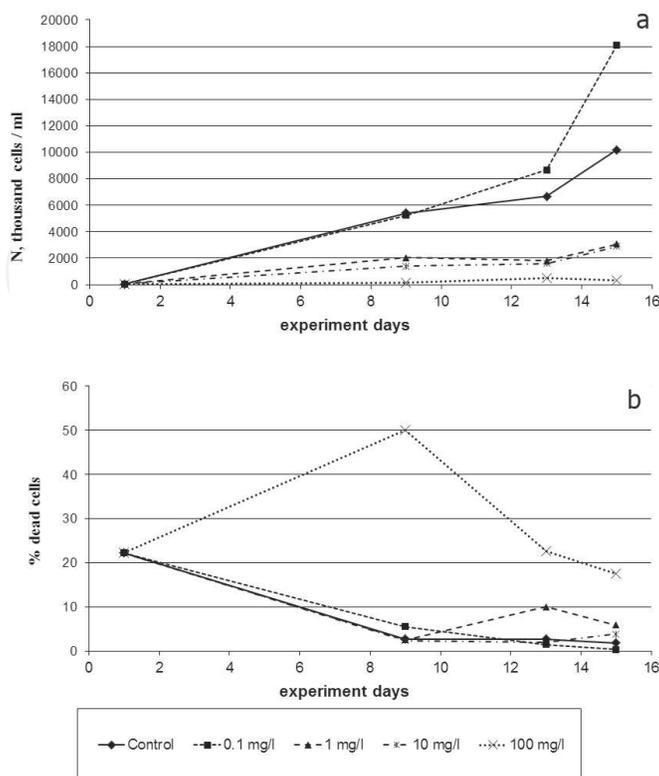


Figure 12.

Development of the culture of *S. aquatilis* in the experiment with gallic acid: (a) the average number (median), (b) % dead cells (median).

least inhibitory effect on *S. aquatilis* culture. So, at the end of the experiment, the density of cyanobacterial cells in the control exceeded that in microcosms with an allelochemical only two times (Table 2; Figure 13a). The differences in the optical density of the culture were even more minor (Figure 13b).

At the same time, it should be noted that the proportion of dead cells in microcosms with the influence of the allelochemical is higher (Figure 13c) than the control, although their absolute values were low. The maximum value was only 3.6% on the 10th day of the experiment (Table 2).

Octanoic acid showed a relatively low but higher than heptanoic acid inhibitory activity against the cyanobacteria *S. aquatilis*. By the end of the experiment (on day 17), the difference in cyanobacterial population density between the control and the allelochemical exposure was three times (Table 2; Figure 14a). Throughout the experiment, the level of development of cyanobacteria in the experimental vessels was lower than in the control. This is especially noticeable in the graphs of changes in the optical density of *S. aquatilis* culture and the proportion of dead cells (Figure 14b and c). The proportion of dead cells when exposed to octanoic acid was always higher than in the control and by the end of the experiment was 1.3%, while the maximum value of this indicator (5.4%) was observed on the third day of the experiment.

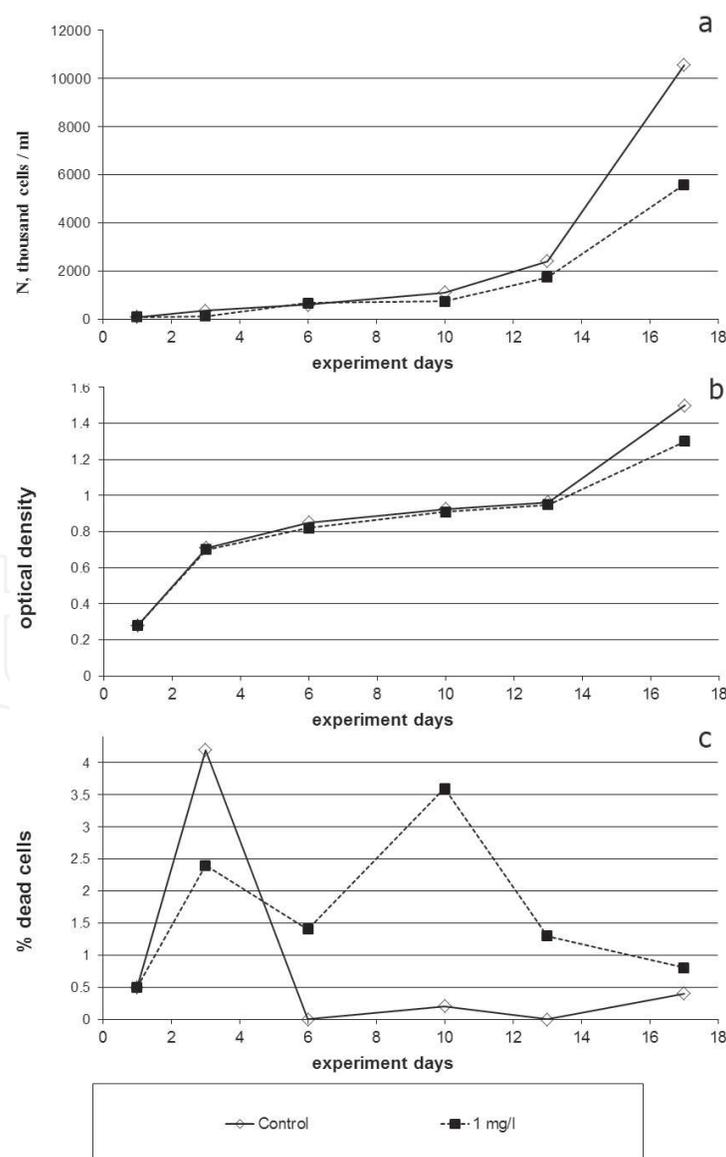


Figure 13. Development of the culture of *S. aquatilis* in the experiment with the effect of heptanoic acid: (a) the average cell density (median), (b) optical density (median), (c) % dead cells (median).

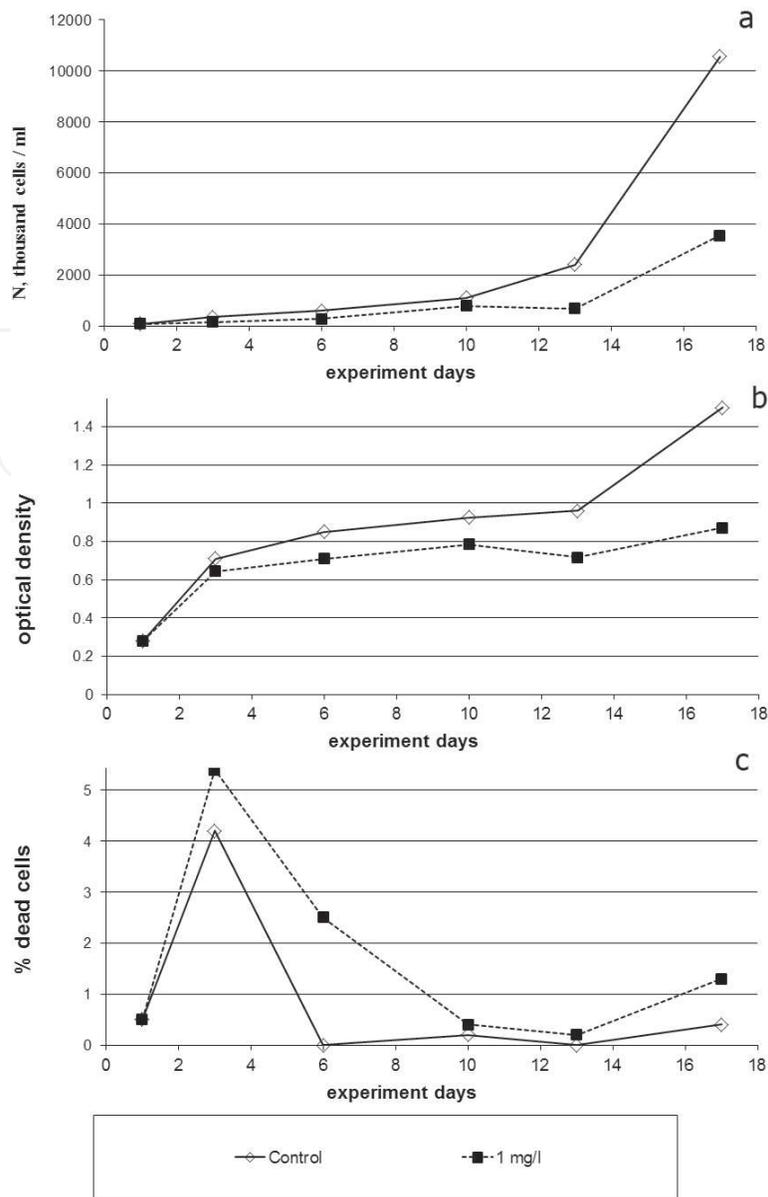


Figure 14. Development of the culture of *S. aquatilis* in the experiment with the effect of octanoic acid: (a) the average cell density (median), (b) optical density (median), (c) % dead cells (median).

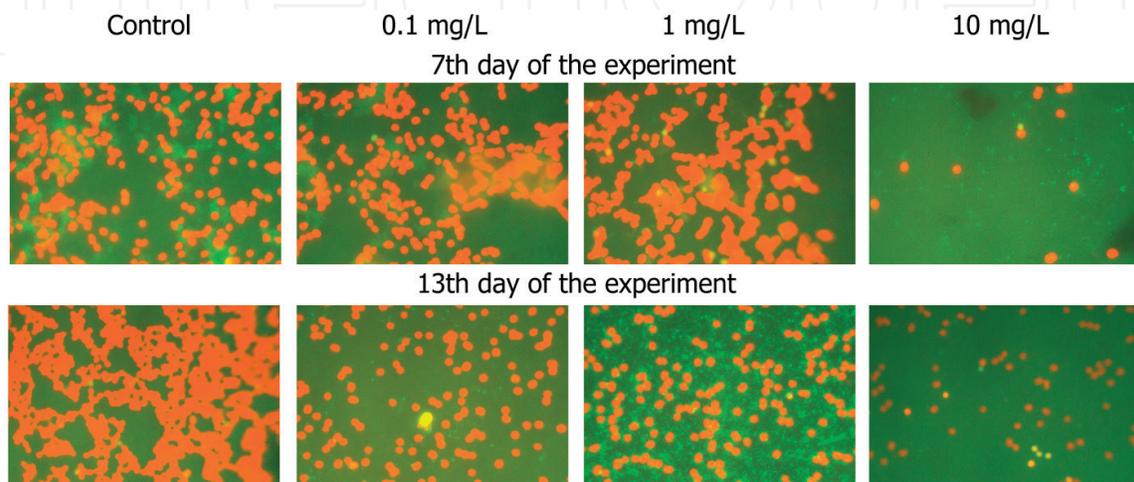


Figure 15. Visualization of changes in the physiological state the culture of *S. aquatilis* in the experiment with the combined effect of heptanoic, octanoic, tetradecanoic, and gallic acids at various concentrations.

3.2.6 Experiment with *S. aquatilis* and the mixture of allelochemicals

Four of the six allelochemicals (heptanoic, octanoic, tetradecanoic, and gallic acids) were combined to obtain their mixture in organic solvent ethanol. The required amount of allelochemicals in equal amounts was dissolved in 1 ml of ethanol and added to experimental vessels to achieve the final concentrations 0.1, 1, and 10 mg/l. No addition of the mixture of allelochemicals was set as the control 1. No addition of the mixture of allelochemicals but with the addition of 1 ml of ethanol was set as the control 2. The initial density of cyanobacteria was 1014,000 cells/ml.

Visual fixation of the state of *S. aquatilis* culture, shown in **Figure 15**, clearly demonstrates a particularly strong inhibition of the development of *S. aquatilis* at a concentration of a mixture of allelochemicals of 10 mg/l when compared to the control. In experimental vessels with a mixture of four allelochemicals, a lower development of cyanobacteria was observed throughout the experiment and at all used concentrations (0.1, 1, 10 mg/l) according to fluorescence microscopy data when compared to the control (**Figure 16a**). More frequently performed the taking

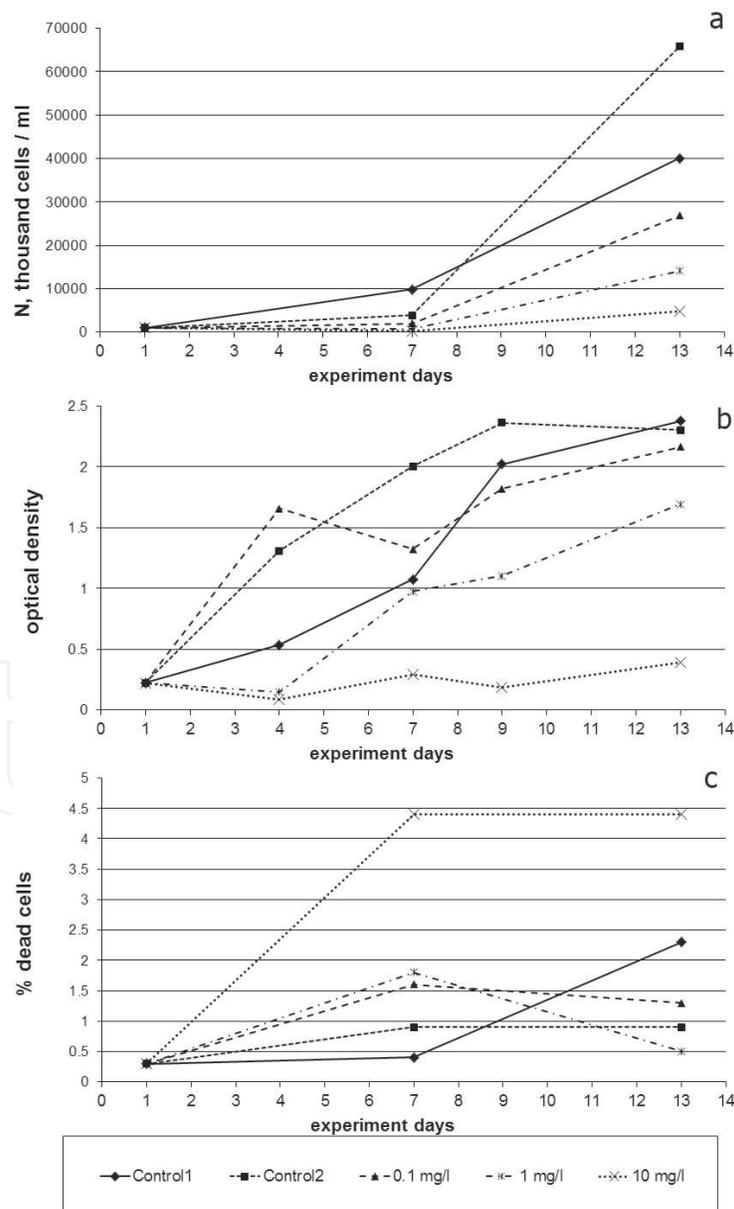


Figure 16. Development of the culture of *S. aquatilis* in the experiment with the combined effect of heptanoic, octanoic, tetradecanoic, and gallic acids at various concentrations: (a) the average number (median), (b) optical density (median), (c) % dead cells (median).

readings of the optical density of culture development showed its increase at a concentration of 0.1 mg/l on day 4 of the experiment more than in both control variants. Later, by the end of the experiment, the optical density at this concentration was lower than in the control. The cyanobacteria abundance and optical density of the culture at allelochemical concentrations of 1 and 10 mg/l throughout the experiment were lower than Controls 1 and 2 (**Figure 16a and b**).

The maximum suppression of the culture of *S. aquatilis* (35.3 times when compared to the Control 1) was noted on the seventh day of the experiment at a concentration of the mixture of allelochemicals of 10 mg/l. By the end of the experiment (on the 13th day) at this concentration, the SI index was 8.3 (**Table 2; Figure 16a**). Also, marked suppression of the development of cyanobacterial culture by the end of the experiment was noted at concentrations of 0.1 and 1 mg/l (1.5 and 2.6 times, respectively) (**Figure 16a**).

In our experiments, we detected no negative influence of ethanol on the culture of *S. aquatilis*. Moreover, in the case of the addition of 1 ml of ethanol to experimental microcosm (Control 2), we have seen more intensive growth of cyanobacteria density in Control 2 toward the end of the experiment in comparison with Control 1 without any additions. Fluorescence analysis also showed that the highest proportion of dead cells of *S. aquatilis* (4.4%) was at a concentration of a mixture of allelochemicals of 10 mg/l (**Figure 16c; Table 3**).

3.2.7 Experiment with *A. flos-aquae* and the mixture of allelochemicals

Experiments with *A. flos-aquae*, a common bloom-forming cyanobacterium producing a hazardous effect in eutrophic freshwater ecosystems, were carried out only with a mixture of four allelochemicals (heptanoic, octanoic, tetradecanoic, and gallic acids). The initial density of cyanobacteria was 1,064,000 cells/ml.

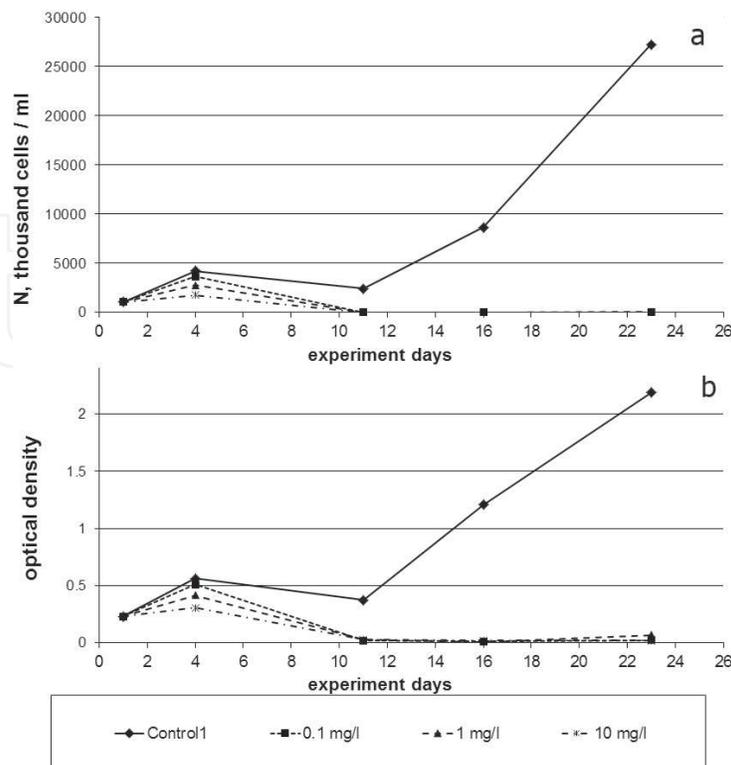


Figure 17. Development of the culture of *A. flos-aquae* in the experiment with the combined effect of heptanoic, octanoic, tetradecanoic, and gallic acids at various concentrations: (a) the average number (median); (b) optical density (median).

This species of cyanobacteria was especially sensitive to the used mixture of allelochemicals. So, at a concentration of 10 mg/l, a complete suppression of the development of cyanobacteria was observed (**Figure 17**). SI at the end of the experiment (on the 23rd day) was 17,495 (**Table 2**). As this takes place, living cells were practically absent.

The proportion of dead cells was more than 95%. At concentrations of 0.1 and 1 mg/l, a strong suppression of the development of cyanobacteria was also observed. By the end of the experiment, SI values amounted to 14,568 and 2130, respectively. However, the proportion of dead cells at these concentrations was small, no more than 2%. This suggests a strong slowdown in the process of reproduction of cyanobacteria.

At the same time, the proportion of dying cells (orange-green shade) in the control on day 23 of culture development averaged 10.5%. For the most part, the culture contained bright red cells in long chains. This effect may be related to the self-inhibition of the culture, whose density in the control reached 27,236,000 cells/ml by the end of the experiment.

4. Discussion

Ultimately, our results showed that organic acids-allelochemicals are widespread in freshwater macrophytes. Of the macrophytes we studied, the largest number of carboxylic acid-allelochemicals was found in *M. spicatum* (**Table 1**), which is one of the aquatic plants with the highest allelopathic potential [34], especially concerning its effect on cyanobacteria [35]. The information available in the literature [36] and our results show that hexadecanoic acid can be attributed to one of the most common allelochemicals in aquatic macrophytes. In [37] it is noted that hexadecanoic acid is part of the major algal inhibiting allelochemicals in planting water of *Hydrilla verticillata*. Already in early studies of the phytotoxic properties of acids, it was shown that hexadecanoic acid possesses phytotoxic properties [38]. For hexadecanoic acid, our study revealed a rather high cyanocidal activity against *S. aquatilis*. It appeared even at concentrations of 0.01 and 0.1 mg/l. At the same time, for other representatives of photosynthetic plankton, for example, red tide algae, hexadecanoic acid displayed relatively weak activity, with LC₅₀ values of 50.6 and 69.5 µg/ml [39]. This may indicate the selectivity of this allelochemical against cyanobacteria.

The studies [33, 38] demonstrated that heptanoic acid also has phytotoxic properties. The work [40] presents data that hexadecanoic acid diminished the seed germination of some epiphytes.

In the samples of aquatic macrophytes we studied, we did not find heptanoic and gallic acids. However, there is ample evidence regarding the presence of heptanoic acid in the composition of metabolites of aquatic macrophytes [41]. In addition, there are results that heptanoic and octanoic acids, being allelochemicals of aquatic macrophytes, can lead to suppressing the development of cyanobacteria [42]. The gallic acid is judged to be the allelochemical compound and phytotoxin [43–45]; it may be found in many aquatic macrophytes [44, 46, 47].

Polyphenols were found to contribute, on average, to 50% of the allelopathic effects of *M. spicatum* on *Microcystis aeruginosa* (Kutzing) Kutzing [48]. As noted earlier [49], the four phenols (pyrogallol, gallic acid, (+)-catechin, and ellagic acid) secreted by *M. spicatum* could contribute to the allelopathic effects of *M. spicatum* at 10–100%. Another part of the allelopathic effect of this macrophyte seems to be due to carboxylic acids, which this species synthesizes especially much (**Figure 3**). Gallic acid and carboxylic acids are reported to be active allelochemicals

[16, 50, 51]. The fact that we did not find this compound in the macrophyte samples we examined may be probably related to the method of obtaining the essential oil of these plants by the method of steam hydrodistillation.

It has been shown that allelochemicals such as heptanoic and octanoic acids can be present in natural waters at concentrations of 100–1000 $\mu\text{g/l}$ [32, 33]. There is evidence that heptanoic and octanoic acids, being allelochemicals of aquatic macrophytes, can lead to a degradation of the development of cyanobacteria [42]. It was also shown that perfluoro modifications of hexanoic, heptanoic, octanoic, and nonanoic acids suppressed the development of cyanobacteria and algae (LC_{50} range: 6.0–24.3 mg/l) [52]. At the same time, cyanobacteria and diatom algae were comparable in sensitivity, but both were more sensitive than green algal species. Fukuda et al. [53] studied the allelopathic potential of heptanoic acid. They reported that a 0.1 M solution of heptanoic acid was able to reduce the viability of crabgrass by 30% compared with the control. Earlier studies with the same compound [54] reported that heptanoic acid was able to significantly reduce the growth of various organelles within the plant *Phragmites* at a concentration of 1.4 mM. It was shown [33] that the EC_{50} (half-maximal effective concentration) is 650 $\mu\text{g/l}$ for heptanoic acid.

Linoleic acid, as an allelochemical, is produced not only by aquatic macrophytes but also, for example, by filamentous green algae (*Uronema confervicolum*, a benthic filamentous green alga) [55]. The effect of linoleic acid on cyanobacteria *M. aeruginosa* has been studied in [56]. The results obtained on the inhibition of *M. aeruginosa* (up to 96%) are in good agreement with our data, showing a high ability of linoleic acid to suppress the development of cyanobacteria. As shown in this work, under the influence of linoleic acid algal cell membrane lipid peroxidation occurred, and the membrane permeability increased, accompanied by the damage of cell membranes. It was concluded that the main mechanism of linoleic acid inhibiting cyanobacterial growth is the destruction of the cyanobacterial cell membrane.

At the same time, our observations using fluorescence microscopy showed the presence of a relatively small number of dead cyanobacterial cells, which differed insignificantly (to the large side) from the control. This does not indicate the death of cells of the cyanobacterial culture but their significantly lower reproduction rate than the control due to the general physiological inhibition of the cyanobacterial population. It is reasonable to hypothesize that there may be another mechanism for inhibiting cyanobacteria in addition to what is indicated in [56].

All of the organic acids tested by us showed inhibitory allelopathic activity against cyanobacteria. However, their inhibitory effect was different in relation to the same species (*S. aquatilis*). The highest values of the suppression index ($\text{SI} > 10$) were recorded (in ascending order) for hexadecanoic, linoleic, tetradecanoic, and gallic acids and a mixture of four allelochemicals (heptanoic, octanoic, tetradecanoic, and gallic acids).

Recently, Zhou et al. [57] reported the inhibitory effects of some allelochemicals on cyanobacteria *M. aeruginosa*. Thus, for tetradecanoic acid, the EC_{50} (mg/l) was 15.5 and, for hexadecanoic acid, 18.23 mg/l [57]. The higher level of inhibition concerning *S. aquatilis* for these allelochemicals, noted in our experiments (**Table 2**), seems to indicate a different sensitivity of different cyanobacteria species to the same allelochemicals. This is supported by our results of evaluating the effect of the same combination of allelochemicals on two different species of cyanobacteria, *S. aquatilis*, and *A. flos-aquae*. For the first species, the resulting SI values for concentrations of 0.1, 1, and 10 mg/l on the 13th day of the experiment were 1.5, 2.8, and 8.3, respectively, and for the second species, on the 11th day, 1606, 1165, and 1063 (at the same concentrations), increasing by the end of the experiment (on day 23) to values of 14,568; 2130; and 17,495, respectively. That is, there was a

particularly strong suppression of the development of the cyanobacterial culture of *A. flos-aquae* up to complete inhibition, whereas in the case of *S. aquatilis*, the inhibitory effect of the mixture of allelochemicals was not so pronounced. This shows the great sensitivity of *A. flos-aquae* to a selected mixture of allelochemicals.

This is also confirmed by the data of Nakai et al. [48], who did not find a significant effect of suppressing another cyanobacterium *M. aeruginosa* by tetradecanoic and hexadecanoic acids. High effective concentrations (68–78 mg/l) were obtained for tetradecanoic acid against red tide microalgae [58]. Cyanobacterial inhibition ability of the fatty acid may be related to the carbon chain length and the degree of their unsaturation. So, it was shown when studying the inhibitory effect of palmitic acid and stearic acid on the growth of *M. aeruginosa* [36].

The data obtained may indicate a higher specific sensitivity of cyanobacteria to long-chain saturated fatty acids than to algae. The cyanobacterial inhibition mechanism of organic acids might be closely related to their chemical structures [59]. This paper shows that fatty acids changed the permeability of cell membranes, which caused the leakage of cell contents, further damaged the membrane structures, and thus affected the level of development of cyanobacterial population including the synthesis of toxins.

The results received to date suggest that among cyanobacteria we will inevitably be faced with the specificity of the action of various acids-allelochemicals, whose anti-cyanobacterial activities will depend on (i) length of carbon chain, (ii) number of unsaturated linkages, and (iii) positions of any double bonds [48]. To this, we need to add the dependence on the concentration of an allelochemical; when the concentration is insufficient to suppress, the reverse stimulating effect of cyanobacteria development can be observed. In some of our experiments, we observed this stimulating effect, for example, in experiments with tetradecanoic, hexadecanoic, and gallic acids.

Until only recently, little experimental evidence has existed to establish that low concentrations of the allelochemicals can stimulate the development of cyanobacteria. Thus, in experiments with *M. aeruginosa* [42], at the lowest concentration of the two organic acids used (benzoic acid and heptanoic acid), higher development of cyanobacteria was observed than in the control. The effect of the allelochemicals mixture in our experiments was higher than in the case of individual components. Thus, under the action of a mixture of allelochemicals (heptanoic, octanoic, tetradecanoic, and gallic acids) on *S. aquatilis*, the maximum SI value (35.3) was obtained (Table 2), while the highest value of SI (30) when on exposure to a single allelochemical was recorded in the case of gallic acid.

A number of studies have shown that a combination of several allelochemicals has a synergistic effect in suppressing the development of cyanobacteria. In [42] it was indicated that benzoic acid and heptanoic acid showed a synergistic effect and that their compound algal inhibition effect was superior to the separate effects of the two organic acids acting independently. It has also been found that when two or three allelochemicals were mixed in specific proportions, the algal inhibition rate increased significantly, thereby indicating allelopathic synergistic interactions [60]. Gniazdowska and Bogatek [61] pointed out that the activity of allelochemicals cannot be explained by just a single mode of action. The synergistic effect of the action of allelochemicals (fatty acids, gallic acids, and pyrogallic acids) in relation to cyanobacteria is indicated in [49, 62–64]. It has also been revealed that allelochemicals from *Chara vulgaris* included three fatty acids: (Z, Z)-9,12-octadecadienoic, tetradecanoic, and hexadecanoic acids. The combined impact of these three fatty acids induced a synergistic inhibitory effect on the growth of toxic cyanobacterium *M. aeruginosa*. Synergistic growth inhibition of these cyanobacteria by a mixture of the polyphenols was reported in [49].

In the experiments with *S. aquatilis*, *A. flos-aquae*, and the mixture of allelochemicals, the situation of a eutrophic reservoir was actually simulated, i.e., they started at a very high initial density of cyanobacteria, which corresponded to the level of hypertrophic reservoirs [65]. It was very interesting to check whether a combination of substances-allelochemicals of aquatic macrophytes could suppress the intensive development of cyanobacteria. The results obtained gave a positive answer. This suggests that new cyanocides made up of a combination of LMWOCs-allelochemicals have the potential to not only inhibit the development of cyanobacteria populations, preventing them from developing to dangerous levels, but also suppress active HAB.

The method of fluorescence microscopy traces changes that are caused in the culture of cyanobacteria by the action of various LMWOCs-allelochemicals of aquatic macrophytes. It was previously shown that the method of fluorescence microscopy is very useful to distinguish producing and non-producing cells of genetically engineered cyanobacteria *Synechocystis* sp. PCC6803 and *Synechococcus* sp. PCC7002 which are used for the production of biofuels [26].

Several studies have attempted to find compounds with pronounced cyanocidal and algicidal activities among natural plant products [60, 66]. In particular, in [67] it is shown for the first time that there are strong inhibition effects ($EC_{50} < 100 \mu\text{g/l}$) of the isoquinoline alkaloids on phytoplankton (including cyanobacteria).

5. Conclusion

Recently, scientific interest in cyanobacteria responsible for the HAB in natural reservoirs has increased significantly. Various ways of controlling the number of these microorganisms in aquatic ecosystems are being investigated, and hence, simple and fast methods of evaluating the viability of their cells are required. The majority of common methods (e.g., plating, fluorescent staining) to determine cell viability are preparation demanding, time-consuming, and rather expensive. The advantages of the fluorescence microscopy method, using red chlorophyll autofluorescence and nonspecific green autofluorescence, are high sensitivity, non-destructive evaluation of the vital state of cells, and differentiation of living and dead cells without sample preparation. The method is time-efficient and accurate, and a large number of samples with small amounts of material can be examined in a short time.

In our study, we have shown (including using fluorescence microscopy) that natural compounds, metabolites of aquatic macrophytes, in particular carboxylic acids and gallic acid, can be used to suppress the development of cyanobacteria populations. Their combined effects are likely to be particularly effective. In other words, the creation of a new generation of algaecides/cyanocides based on LMWOCs of aquatic plants is a very promising strategy for combating HAB. In this case, of course, it will be necessary to take into account that the prices of selected natural products for these goals are usually higher than for conventional products (copper sulfate, aluminum salts, simazine, and many others) used to control cyanobacteria, but the new potential cyanocides likely will have important properties, including (1) selective toxicity toward only undesirable cyanobacteria in the phytoplankton and the absence (or minimal toxicity) toward other hydrobiota and (2) rather fast biological degradation, for an optimal state of the aquatic ecosystem [66].

In fairness, it should be noted that not only LMWOCs-allelochemicals can become the basis for creating new effective methods to combat excessive and undesirable development of cyanobacteria but also such methods as the use of new chlorinated nanoproducts, in particular N-halamine derivatized nanoparticles,

that, as it turned out, enable selective elimination of blooming cyanobacteria while minimizing the effect on cohabiting phytoplankton species [68].

Judging from the results obtained, we can conclude that the method of fluorescence microscopy can be very useful in revealing the inhibitory mechanisms of cyanobacterial populations in experimental studies on the search for natural compounds (in particular, allelochemical metabolites of aquatic macrophytes), on the basis of which a new generation of cyanocides can be created. This “novel weapon” against blooming cyanobacteria seems to be a potentially more interesting product for further development of technologies on HAB control and management.

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Conflict of interest

The authors declare that there is no conflict of interest.

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