Journal of Siberian Federal University. Biology 2023 16(2): 232–251
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EDN: SELYZZ

УДК 577.19+577.29

Using SAR Methodology for Identification of Freshwater Macrophyte Allelochemicals with High Anti-Cyanobacterial Effect against Planktonic Cyanobacteria

Evgeny A. Kurashov[a, c] *, Elena V. Fedorova[b], Julia V. Krylova[a, c], Larisa L. Kapustina[a], Galina G. Mitrukova[a, d] and Elena V. Protopopova[a]

[a] *Institute of Lake Science of the RAS, a separate subdivision of the St. Petersburg Federal Research Center of the RAS Saint Petersburg, Russian Federation*

[b] *Quantori, Global Headquarters Cambridge, USA*

[c] *Papanin Institute for Biology of Inland Waters, RAS Borok, Russian Federation*

[d] *Saint-Petersburg State Chemical and Pharmaceutical University Saint Petersburg, Russian Federation*

Received 17.08.2022, received in revised form 14.02.2023, accepted 12.04.2023

Abstract. Controlling harmful cyanobacterial "blooms" through developing a new generation of algaecides based on allelochemical substances is a challenge facing modern aquatic ecology and biotechnology. The present article is devoted to the use of the SAR (Structure-Activity-Relationship) information technology to identify allelochemicals from aquatic macrophytes (floating-leaved *Nuphar lutea* (L.) Sm. and several species of submerged macrophytes: *Ceratophyllum demersum* L., *Myriophyllum spicatum* L., *Elodea canadensis* Michx, and species of the genus *Potamogeton*) effective against planktonic cyanobacteria. Detection and identification of compounds were performed using gas chromatography-mass spectrometry. The PASS (Prediction of Activity Spectra for Substances) computer program has been applied to predict biological activity spectra of the major components of macrophyte metabolomes and discover their ecological potential against cyanobacteria. A study of the biological activities of major low-molecular-weight organic compounds showed that monocarboxylic acids, gallic acid, cis-6-octadecenoic acid,

* Corresponding author E-mail address: evgeny_kurashov@mail.ru
ORCID: 0000-0002-4486-2804 (Kurashov E.); 0000-0003-1667-5240 (Fedorova E.); 0000-0002-4274-2358 (Krylova J.); 0000-0002-7135-3300 (Kapustina L.); 0000-0002-4128-7840 (Mitrukova G.); 0000-0001-5376-7019 (Protopopova E.)

cis-9-octadecenoic acid, palmitoleic acid, linolenic acid, and 9-cis-12-cis-linoleic acid are the most promising compounds for the experimental verification and creation of nature-like algaecides of a new generation. PASS predictions were successfully compared to the available information on the biological activity of those compounds and confirmed experimentally. The present study shows that some organic acids significantly inhibit the growth of *Synechocystis aquatilis* Sauvageau and *Aphanizomenon flos-aquae* Ralfs ex Bornet and Flahault and can be used as algaecides for suppression of cyanobacteria. The inhibitory effect of the combined mixture of these allelochemicals is stronger than the effect of each individual component, suggesting that there are various mechanisms of cyanobacterial growth inhibition.

Keywords: SAR, PASS, allelopathy, allelochemicals, macrophytes, cyanobacteria, fatty acids, algaecides.

Acknowledgments. The publication of the results of the study was funded by Grant of the Russian Science Foundation No. 22–24–00658, https://rscf.ru/project/22–24–00658. We also express gratitude to the Resource Center «Cultivation of Microorganisms» at St. Petersburg State University for providing the culture of S*ynechocystis aquatilis f*or experiments.

Citation: Kurashov E. A., Fedorova E. V., Krylova J. V., Kapustina L. L., Mitrukova G. G., Protopopova E. V. Using SAR methodology for identification of freshwater macrophyte allelochemicals with high anti-cyanobacterial effect against planktonic cyanobacteria. J. Sib. Fed. Univ. Biol., 2023, 16(2), 232–251. EDN: SELYZZ

Использование методологии SAR для идентификации аллелохемиков пресноводных макрофитов с высоким антицианобактериальным эффектом в отношении планктонных цианобактерий

Е. А. Курашов [а, в], Е. В. Фёдорова [б], Ю. В. Крылова [а, в], Л. Л. Капустина [а], Г. Г. Митрукова [а, г], Е. В. Протопопова [а]

[а]Институт озероведения РАН, обособленное подразделение Санкт-Петербургского федерального исследовательского центра РАН
Российская Федерация, Санкт-Петербург
[б]Квантори, Штаб-квартира
США, Кембридж
[в]Институт биологии внутренних вод им. И. Д. Папанина РАН
Российская Федерация, п. Борок
[г]Санкт-Петербургский государственный химико-фармацевтический университет Минздрава России
Российская Федерация, Санкт-Петербург

Аннотация. Проблема борьбы с опасными цианобактериальными «цветениями» посредством разработки нового поколения альгицидов, основанных на аллелохемиках растений, актуальна

на современной стадии развития водной экологии и биотехнологии. Статья посвящена использованию информационной технологии SAR (связь структура – активность) для выявления эффективных аллелохемиков водных макрофитов (с плавающими листьями – *Nuphar lutea* (L.) Sm., и нескольких видов погруженных макрофитов: *Ceratophyllum demersum* L., *Myriophyllum spicatum* L., *Elodea canadensis* Michx и виды рода *Potamogeton*) против планктонных цианобактерий. Обнаружение и идентификацию соединений проводили с использованием метода газовой хромато-масс-спектрометрии. Компьютерная программа PASS (прогнозирование спектров активности для веществ) была применена для прогнозирования спектров биологической активности мажорных компонентов метаболома макрофитов, чтобы обнаружить их экологический потенциал против цианобактерий. Изучение биологической активности основных низкомолекулярных органических соединений показало, что монокарбоновые кислоты, галловая кислота, цис-6-октадеценовая кислота, цис-9-октадеценовая кислота, пальмитолеиновая кислота, линоленовая кислота и 9-цис-12-цис-линолевая кислота являются наиболее перспективными соединениями для экспериментальной проверки и создания природоподобных альгицидов нового поколения. Прогнозные оценки PASS были успешно сопоставлены с доступной информацией о биологической активности этих соединений, а также подтверждены экспериментально. Было показано, что некоторые жирные кислоты значительно ингибировали рост *Synechocystis aquatilis* Sauvageau и *Aphanizomenon flos-aque* Ralfs ex Bornet and Flahault и могут использоваться в качестве альгицидов для подавления цианобактерий. Поскольку ингибирующий эффект комбинированной смеси аллелохемиков был сильнее, чем у отдельных компонентов, имеются основания предполагать, что существуют различные механизмы ингибирования роста цианобактерий.

Ключевые слова: SAR, PASS, аллелопатия, аллелохемики, макрофиты, цианобактерии, жирные кислоты, альгициды.

Благодарности. Публикация результатов исследования поддержана грантом РНФ No. 22–24–00658, https://rscf.ru/project/22–24–00658. Также выражается благодарность Ресурсному центру «Культивирование микроорганизмов» СПБГУ за предоставление культуры *S. aquatilis* для экспериментов.

Цитирование: Курашов, Е. А. Использование методологии SAR для идентификации аллелохемиков пресноводных макрофитов с высоким антицианобактериальным эффектом в отношении планктонных цианобактерий / Е. А. Курашов, Е. В. Фёдорова, Ю. В. Крылова, Л. Л. Капустина, Г. Г. Митрукова, Е. В. Протопопова // Журн. Сиб. федер. ун-та. Биология, 2023. 16(2). С. 232–251. EDN: SELYZZ

Introduction

Allelopathy is considered a natural phenomenon for both stimulatory and inhibitory effects of one plant upon another, including microorganisms (Mushtaq et al., 2020; Asif et al., 2021; Śliwińska-Wilczewska et al., 2021). Functionally, plants have developed several strategies for interacting with other organisms for self-defense, symbiosis, and sexual attraction (Zhou et al., 2022). The production, accumulation, and release of primary and secondary metabolites (low-molecular-weight organic compounds (LMWOCs)) by aquatic and semiaquatic plants, which inhibit and/or stimulate germination and development

of other plants and microorganisms, is an important mechanism in the interactions between these organisms (Fink, 2007; Li et al., 2020). LMWOCs constitute part of the low-molecular-weight metabolome of aquatic plants and are extremely important for forming hydrobiological communities, thus acting as regulatory agents in aquatic ecosystems (Gurevich, 1978; Fink, 2007; Kurashov et al., 2014; Koksharova, 2020), as well as in terrestrial ecosystems (Allelopathy …, 2013).

Aquatic macrophytes are one of the critical components of inland waters, which fulfill the most important function in formation of the environment. Allelopathic interactions are carried out by synthesising specific compounds – allelochemicals (including volatile substances). In particular, several macrophyte species are active producers of allelochemicals inhibiting the development of phytoplankton in shallow lakes (Hilt, Gross, 2008; Zhou et al., 2019; Zeng et al., 2022).

Despite the importance of this phenomenon in nature, higher plant allelopathy in aquatic habitats has been much less studied than in terrestrial ecosystems, although it should be considered the most important regulatory factor in the dynamics and composition of phytoplankton communities (Cyanobacteria…, 2013; Co-Evolution …, 2020). It has been well reported that allelochemical metabolites can be considered natural algaecides (Hu, Hong, 2008; Balaji-Prasath et al., 2022) and must be the basis of a nature-like convergent technology to suppress the development of cyanobacteria and to prevent "blooms" in water bodies. A number of macrophytes have been reported to contain anti-algal and anti-cyanobacterial compounds (Amorim et al., 2019; Zhang et al., 2019). These results indicate that some macrophytes can be used to control algal and cyanobacterial growth.

To understand the mechanism of the growth inhibition of cyanobacteria by the allelopathic effect of macrophytes, allelochemicals must be identified. At the same time, they can be characterized by many biological effects, which are very difficult to verify experimentally. Moreover, previous studies showed that the number of compounds in aquatic macrophytes can reach more than 200 (Kurashov et al., 2014, 2018). Therefore, it is appropriate to use bio- and cheminformatics approaches.

Developing new biologically active compounds with potential practical applications is one of the most complex and difficult tasks. Experimental testing of the interaction of many chemical compounds with thousands of molecular targets is impossible from an economic and practical perspective. Computer bioinformatic program PASS (Prediction of Activity Spectra for Substances) allows researchers to screen large libraries of chemicals for compounds with desired properties and experimentally test those predicted to be active. So far as there is a relationship between structure and activity, and this principle is called SAR (Structure-Activity-Relationship) in this study, we aim to conduct SAR analysis using a PASS computer program to predict the biological activity spectra of LMWOCs and their mechanisms of action. The PASS computer system can simultaneously predict several hundred types of biological activities (Filimonov et al., 2014, 2018; Poroikov et al., 2019). The prediction is based on the SAR analysis of the training set, which is regularly updated with data on new chemicals and their known biological activities. Once trained, PASS can predict for a test compound all likely biological activities that are included in the training set. In recent years, the use of SAR to identify biological activities for natural compounds has become increasingly widespread (Gillbro et al., 2015; Lagunin et al., 2016; Khameneh et al., 2019; Fattahian et al., 2020). One example is

the identification of natural antioxidants from marine algae that could be developed for further industrial applications (Kelman et al., 2012).

The present study aims to identify allelochemicals released from the floating-leaved *Nuphar lutea* (L.) Sm. and several species of submerged macrophytes – *Ceratophyllum demersum* L., *Myriophyllum spicatum* L., *Elodea canadensis* Michx., and species of genus *Potamogeton* – and to choose among them compounds with the most probable allelopathic potential for suppressing cyanobacteria using SAR methodology.

To achieve these goals, several major LMWOCs (> 1 % of the content of total LMWOCs) predicted by PASS were selected from the metabolome of freshwater macrophytes, and then they were tested in vitro against two species of cyanobacteria: *Synechocystis aquatilis* Sauvageau and *Aphanizomenon flos-aquae* Ralfs ex Bornet and Flahault.

Materials and methods

Plant materials

The LMWOCs of macrophytes *N. lutea*, *C. demersum*, *M. spicatum*, *Potamogeton obtusifolius* Mert. et Koch, *Potamogeton perfoliatus* L., *Potamogeton natans* L., and *E. canadensis* were investigated in the plant material that was collected in the summer seasons of 2015–2017 in the following habitats: (i) oligotrophic Lake Suuri (Karelian Isthmus, Leningrad Oblast, N 61º 07.859′, E 29º 55.076′) with still waters and sticky silt bottom, (ii) an area in the estuary of the Volkhov River (Volkhov Bay, Lake Ladoga, N 60º 07.139′, E 32º 19.566′) with fast waters and silty sand bottom, (iii) ponds of the Victory Park (St. Petersburg, N 60º 52.025′, E 30º 19.91′) with small depths and silt bottom, (iv) different habitats in mesotrophic Lake Ladoga (N 60° 50′, E 31° 33′), and (v) mesotrophic Lake Naroch (N 54° 51′, E 26° 45′). Plant samples were washed to remove debris and air-dried in a closed and darkened room without direct sunlight.

Isolation of oils

Before distillation, the dried plant material was ground to a powder in a Waring BB 25ES blender (Waring, United States). The fraction of organic compounds was obtained from the dry aquatic plant material using the same Clevenger-type apparatus method, commonly used to extract essential oils from terrestrial plants (Clevenger, 1928; Anderson et al., 1993). Hydrodistillation was performed for 8 hours. Then the oils were extracted with hexane (5 mL). The extracts were stored in a freezing chamber (–18 °C) prior to GC–MS analysis.

GC–MS analysis

The concentrations of LMWOCs were determined after hexane extraction 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). Helium served as a carrier gas, the ionization voltage was 70 eV. The mass spectra were registered in scan mode for the whole mass range (30–580 amu) in a programmed temperature regime: the oven temperature was kept at 35 °C for 3 minutes; then the temperature was increased to 60 °C at a rate of 2 °C/minute and kept constant for 3 minutes; after that, the temperature was increased to 80 °C at a rate of 2 °C/minute and kept constant for 3 minutes, increased to 120 °C at a rate of 4 °C/minute and kept constant for 3 minutes, increased to 150 °C at a rate of 5 °C/minute and kept constant for 3 minutes, increased to 240 °C at a rate of 15 °C/minute. Finally, it was held isothermal for 10 minutes.

As an example, Fig. 1 presents chromatogram sections of *Potamogeton perfoliatus* essential oil

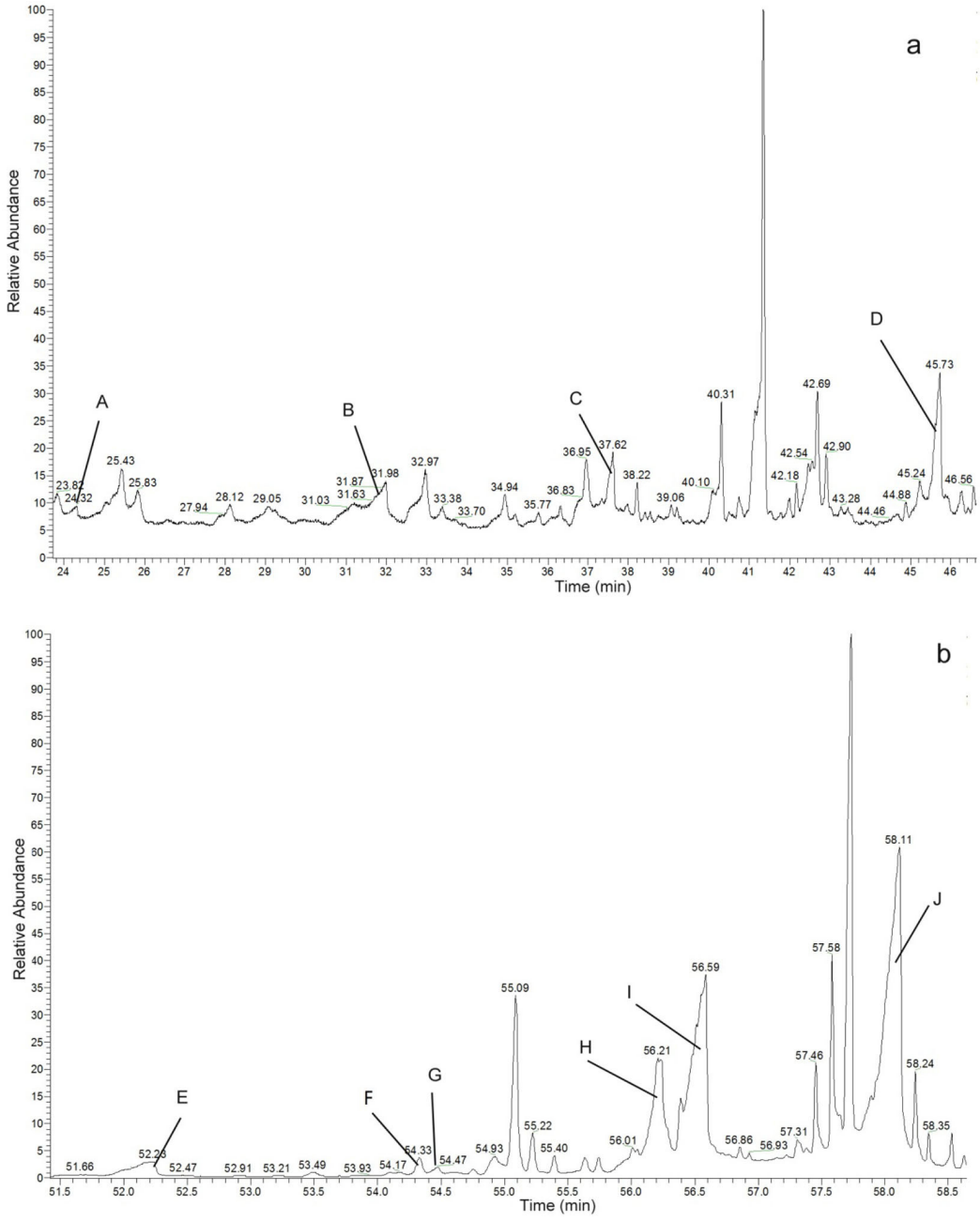

Fig. 1. Chromatogram sections of *Potamogeton perfoliatus* essential oil (Lekhmolahti Bay, Lake Ladoga) with marked peaks of carboxylic acids. a: A – Octanoic acid, B – Nonanoic acid, C – Decanoic acid, D – Dodecanoic acid; b: E – Tetradecanoic acid, F – Pentadecanoic acid, G – 14-Pentadecenoic acid, H – Palmitolinoleic acid, I – Hexadecanoic acid, J – Linolenic acid

(Lekhmolahti Bay, Lake Ladoga) with marked peaks of fatty acids that are of the greatest interest. The LMWOCs determined in the samples of aquatic plants were identified by matching their mass spectra with those from the NIST_2014 and the Wiley mass spectral libraries. The identification of the compounds was confirmed by Linear Retention Indices

obtained from a series of straight-chain alkanes (C 7–C 30) (Zellner et al., 2008). Quantitative analysis was performed using Merck-certified reference materials decafluorobenzophenone and benzophenone (CAS Numbers 119–61–9 and 853–30–4) as internal standards.

PASS analysis

Prediction of Activity Spectra for Substances was carried out with the PASS 2014 Refined version to get biological activity spectra of each compound. PASS 2014 Refined is software for predicting biological activity spectra for organic compounds based on their structural formulas (Filimonov et al., 2014). As input information, PASS uses the information on the structural formula of the molecule represented as a Molfile for a single structure or as an SDfile for a set of structures (Accelrys Inc., http://accelrys.com). PASS output is a list of probable activities with two estimates: P_a – the probability of being active and P_i – the probability of being inactive. Prediction of PASS is based on SAR analysis of the training set containing about one million compounds showing more than 7000 biological activities. Being probabilities, the P_a and P_i values vary from 0.000 to 1.000, however $P_a+P_i \neq 1$, since these probabilities are calculated independently.

Assaying allelochemicals

The experiments were carried out in microcosms with a volume of 0.5 liters. Cyanobacterial cultures of *S. aquatilis* and *A. flos-aquae* were introduced as suspensions. We used in experiments an axenic strain of *Synechocystis aquatilis* Sauvageau No. 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* was provided by the St. Petersburg Scientific Research Center for Ecological Safety of the Russian Academy of Sciences from the culture that is maintained in that Center.

Cyanobacteria were cultured on medium No. 6 of the following composition: KNO_3–1 g / L; K_2HPO_4–0.2 g / L; $MgSO_4 \times 7H_2O$ – 0.2 g / L; $CaCl_2$–0.15 g / L; $NaHCO_3$ 0.2 g / L; trace element solution 1 ml / L. The trace element solution for medium No. 6: $ZnSO_4 \times 7H_2O$ – 0.22 g / L; $MnSO_4$–1.81 g / L; $CuSO_4 \times 5H_2O$ – 0.079 g / L; $NaBO_3 \times 4H_2O$ – 2.63 g / L; $(NH_4)\ 6Mo_7O_{24} \times 4H_2O$ – 1 g / L; $FeSO_4 \times 7H_2O$ – 9.3 g / L; $CaCl_2$–1.2 g / L; $Co(NO_3)_2 \times 4H_2O$ – 0.02 g / L; Na_2EDTA (Trilon B) – 10 g / L (Gromov et al., 1991).

Cultivation of cyanobacteria was 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 was set using an adjustable timer (FERON TM50, 3500W / 16A230V).

Instead of natural LMWOCs, the purified analogous substances (heptanoic acid, octanoic acid, tetradecanoic acid, and gallic acid) from Acros Organics BVBA and their combination were used in experiments to test their algaecidal effects on *S. aquatilis* and *A. flos-aquae*.

The compounds were added to the algal cultures to achieve their final concentrations of 0.001 mg / L, 0.01 mg / L, 0.1 mg / L, 1 mg / L, and 10 mg / L.

The growth of cyanobacterial cultures was monitored using a light microscope (ZEISS Axio Lab.A1) and a luminescent microscope (LOMO

MIKMED-2) at 2–7-day intervals in different experiments.

Results and discussion

In total, 75 major LMWOCs from macrophytes from various water bodies indicated above were identified using the GS-MS method. In order to predict the biological activity profiles, their structural formulas were presented as an SDF-file and then used in the virtual screening based on a commercially available software product, PASS 2014 Refined.

The PASS software product, which predicts biological effects and biochemical mechanisms based on the structural formula of a substance in less than a second with more than 80 % accuracy, can be efficiently used to obtain the predicted biological activity profile for compounds and to find new mechanisms. We previously reported high probabilities of anti-inflammatory, antibacterial, and antifungal activities for some of these compounds (Kurashov et al., 2016).

Despite a large number of biological activities (several thousand) inherent in all identified LMWOCs according to PASS predictions, in this study, only those types of activities were taken into account that could potentially have an inhibitory effect on cyanobacteria.

Although many substances have an inhibitory effect on cyanobacteria, only a few inhibitory mechanisms have been elucidated. A general mechanism for inhibiting cyanobacteria due to the oxidative damage by polyphenol-autoxidized products has been described (Nakai et al., 2001). Other studies suggest that alkaline phosphatase inhibition (Gross et al., 1996; Dziga et al., 2007) and interruption of electron transfer chains of photosystem II (PS II) (Leu et al., 2002; Dziga et al., 2007) can also be inhibitory mechanisms. Superoxide dismutase inactivation and subsequent oxidative damage to algal cells can also be a significant cause of inhibiting the growth of cyanobacteria (Hong et al., 2008a, 2008b; Wu et al., 2019).

However, all explanations are based only on speculation prompted by the chemical characteristics of substances. Direct evidence is missing, and how the substances act against cyanobacteria is now unknown. Thus, further study is needed to explain the inhibitory mechanism. For this purpose, using the literature data, we employ PASS to extract information on specific activities and/or groups of activities of the allelochemicals associated with the biofunctions of cyanobacteria. This is exemplified in Table 1. This table seems to contain the main biological activities of the allelochemicals that we could identify from the PASS analysis and which were supported by experimental evidence found in the literature. However, there may be other mechanisms that we have not considered. Based on the information presented in Table 1, we regard as the most important those specific activities or groups of activities that lead both to inhibition and impairment of the processes of cell membrane formation in cyanobacteria.

The probability P_a reflects, above all, the similarity of the molecular structure of the substance to the molecular structures of the most typical «active» compounds in the corresponding subset in the training set. Therefore, as a rule, there are no direct correlation values P_a with quantitative characteristics of the activity.

The PASS prediction results are usually interpreted and used in a flexible manner: (i) only activities with $P_a > P_i$ are considered possible for a particular compound; (ii) if $P_a > 0.7$, the chance to find the activity experimentally is high; (iii) if $0.5 < P_a < 0.7$, the chance to find the activity experimentally is lower, but the compound is probably not so similar to known biological agents, and (iv) if $P_a < 0.5$, the chance to find the activity experimentally is even lower, but if it

Table 1. Selected biological activities and/or groups of activities and reasons for selecting

1	Peptidoglycan glycosyltransferase inhibitor	Peptidoglycan is an important component of the membrane of bacterial cells, protecting cells from rupture due to turgor and maintaining the shape of the cells. Peptidoglycan is also found in glaucocystophytic algae, as part of the cell walls of cyanells, which are evolutionarily derived from ancient bacteria (Löffelhardt, Bohnert, 1994). Glycosyltransferases are the most attractive target for bacterial inhibition (Mesleh et al., 2016).
2	Groups of activities: UDP-N-acetylglucosamine 4-epimerase inhibitor; N 4-(beta-N-acetylglucosaminyl)-L-asparaginase inhibitor; Acetylgalactosaminyl-O-glycosyl-glycoprotein beta-1,3-N-acetylglucosaminyltransferase inhibitor; Steroid N-acetylglucosaminyltransferase inhibitor; N-acetyllactosaminide beta-1,3-N-acetylglucosaminyltransferase inhibitor; Alpha-N-acetylglucosaminidase inhibitor.	Responsible for the formation of the cell membrane in cyanobacteria, since the supporting skeleton of the bacterial cell wall consists largely of a peptidoglycan polymer (murein). This macromolecule is a heteropolymer built from chains in which the residues of N-acetylglucosamine and N-acetylmuramic acid (N-acetylglucosamine lactate) alternate, and which are interconnected by β-1,4-glycosidic bonds (Schlegel, 1986)
3	Groups of activities: Carboxypeptidase Taq inhibitor; Carboxypeptidase D inhibitor; Gly-X carboxypeptidase inhibitor; Muramoyltetrapeptide carboxypeptidase inhibitor; Metallocarboxypeptidase D inhibitor.	Responsible for inhibiting the growth of the cyanobacterial culture. The presence of activities of the 3rd group indicates the general carboxypeptidase inhibitory activity of the test compound. Thus, a high carboxypeptidase inhibitory activity will help suppress the growth of the cyanobacterial culture.
4	Groups of activities: IgA-specific metalloendopeptidase inhibitor; IgA-specific serine endopeptidase inhibitor; Endopeptidase So inhibitor; Procollagen N-endopeptidase inhibitor; Glutamyl endopeptidase II inhibitor.	Responsible for impairment of the cell membrane formation. It has been shown (Löffelhardt, Bohnert, 1994) that peptidoglycan is sandwiched between the outer and inner membranes in the periplasmic space and that the enzymes are on the outer surface of the inner membrane.
5	Groups of activities: Peptidoglycan beta-N-acetylmuramidase inhibitor; UDP-N-acetylmuramate-L-alanine ligase inhibitor; UDP-N-acetylmuramoylalanine-D-glutamate ligase inhibitor	Responsible for impairment of the cell membrane formation since the biosynthesis of the soluble precursor of UDP-N-acetylmuramyl pentapeptide in the cyanella stroma follows a path similar to that of *E. coli* (Löffelhardt Bohnert, 1994).
6	Groups of activities: Glutamyl endopeptidase II inhibitor; Gamma-D-Glutamyl-meso-diaminopimelate peptidase inhibitor; Pyroglutamyl-peptidase I inhibitor; N-acetyl-gamma-glutamyl-phosphate reductase inhibitor; Gamma-glutamyltransferase inhibitor; Protein-glutamate methylesterase inhibitor.	These activities lead to the inhibition of some green algae (Luque et al., 2006), and it can be expected that they will inhibit the development of cyanobacteria as well.
7	Groups of activities: L-glutamate oxidase inhibitor; D-glutamate oxidase inhibitor; UDP-N-acetylglucosamine 4-epimerase inhibitor; L-3-cyanoalanine synthase inhibitor; D-alanine 2-hydroxymethyltransferase inhibitor; Gamma-D-Glutamyl-meso-diaminopimelate peptidase inhibitor; Diaminopimelate dehydrogenase inhibitor.	Responsible for the degradation of cyanobacteria. All peptidoglycan components were found in cyanells, which explains the lethal effect of antibiotics on these autotrophic organisms (Berenguer et al., 1987).
8	Groups of activities: Cyanoalanine nitrilase inhibitor; 3-Cyanoalanine hydratase inhibitor.	Nitrilases are an important component of the enzyme system of cyanobacteria (Heinemann et al., 2003). In this regard, this can be considered as an important mechanism for limiting the growth of cyanobacteria.

Continuation of the Table. 1

9	Groups of activities: Glycogen (starch) synthase inhibitor; 1,4-Alpha-glucan branching enzyme inhibitor; Phosphorylase inhibitor.	Responsible for suppressing cyanobacterial blooms. Cyanobacteria, as well as most phototrophic microorganisms, use carbon storage mechanisms. The main C-storage of cyanobacteria is glycogen. Synthesis involves four enzymes (Kromkamp, 1987).
10	Groups of activities: 2-Oxoglutarate decarboxylase inhibitor; Oxoglutarate dehydrogenase (lipoamide) inhibitor; 4-Hydroxy-4-methyl-2-oxoglutarate aldolase inhibitor; 4-Hydroxy-2-oxoglutarate aldolase inhibitor.	In cyanobacteria, the main function of the oxidative branch of the TCA cycle (Krebs cycle) is the synthesis of 2-oxoglutarate, which serves as a C-skeleton for the assimilation of ammonia through the GS-GOGAT cycle (glutamate synthesis) (Zhang et al., 2006). Like the previous one, this group of bioactivities will lead to the suppression of the development of cyanobacteria through the inhibition of these processes.
11	Groups of activities: Ferredoxin-NAD+ reductase inhibitor; Aldehyde ferredoxin oxidoreductase inhibitor; Glutamate synthase (ferredoxin) inhibitor; Ferredoxin hydrogenase inhibitor; Ferredoxin-nitrite reductase inhibitor; Ferredoxin-NADP+ reductase inhibitor.	Most studies of ferredoxins were carried out with the model strain of cyanobacteria *Synechocystis* PCC 6803 (Cassier-Chauvat, Chauvat, 2014), which has nine highly conserved ferredoxins. Inhibition of the corresponding functions of the 11th group will lead to inhibition of the development of cyanobacteria.
12	Glucan endo-1,3-beta-D-glucosidase inhibitor.	Leads to disruptions in the glycogen cycle in cyanobacteria (Konishi et al., 1995; Attwater, 2010).
13	Groups of activities: Xylan endo-1,3-beta-xylosidase inhibitor; Xylan 1,4-beta-xylosidase inhibitor.	It was shown in (Attwater, 2010) that the cyanobacteria *Nodularia spumigena* and *Microcystis aeruginosa* produce xylosidase. These activities will inhibit the synthesis of xylosidase in cyanobacteria.
14	Groups of activities: Leucine transaminase inhibitor; Leucine dehydrogenase inhibitor.	Leucine in cyanobacteria, in particular in the genera *Microcystis*, *Nostoc*, *Phormidium*, *Nodularia*, takes part in the biosynthesis of microcystins (Attwater, 2010). In this regard, inhibition of the processes associated with the synthesis of leucine will lead to inhibition of the development of cyanobacteria.
15	Antibacterial	Considered as general antibacterial activity.

is confirmed, the compound might be a new chemical-biological entity.

Thirty major LMWOCs that, according to preliminary data (Macías et al., 2008; Li et al., 2010; Nakai et al., 2012; Kurashov et al., 2014, 2021; Zhu et al., 2021), may be promising algaecidal allelopathic agents against cyanobacteria were selected for the study of biological activities using PASS (Table 2).

In the present study, we applied a stronger criterion, $P_a > 70$ %, to increase the chance of experimental confirmation of the predicted activities. As a result, among 30 LMWOCs, 26 were selected that met the above criterion. The complete list of these compounds with activities and their probabilities is given in Table 3.

Analysis of the results showed that among the selected compounds, 18 contain carboxyl groups and correspond to the highest P_a values. The predicted results are in good agreement with literature data. For instance, it was reported (Jandl et al., 2002; Nakai et al., 2005; Zhai et al., 2022) that saturated and unsaturated carboxylic acids can be active allelochemicals. Moreover, it was shown (Nakai et al., 2000; Lourenção et al., 2021) that gallic acid actively inhibits the

Table 2. P_a values of biological activities predicted by PASS for 30 major LMWOCs of aquatic macrophytes

LMWOCs	Groups of activities as in Table 1							
	1/2	3/4	5/6	7/8	9/10	11/12	13/14	15
Gallic acid	0.568/ 0.543–0.926	0.579–0.779/ 0.475–0.926	0.117–0.169/ 0.679–0.926	0.450–0.926/ 0.723–0.753	0.340–0.458/ 0.445–0.840	0.609–0.878/0.878	0.531–0.862/ 0.522–0.737	0.420
Nonanoic acid	0.814/ 0.561–0.810	0.623–0.954/ 0.854–0.914	0.203–0.216/ 0.697–0.875	0.685–0.810/ 0.605–0.614	0.410–0.620/ 0.745–0.845	0.547–0.714/ 0.945	0.563–0.935/ 0.589–0.665	0.300
Octadecanoic acid	0.814/ 0.561–0.810	0.623–0.954/ 0.854–0.914	0.203–0.216/ 0.697–0.875	0.685–0.810/ 0.605–0.614	0.410–0.620/ 0.745–0.845	0.547–0.714/0.945	0.563–0.935/ 0.589–0.665	0.300
cis-6-Octadecenoic acid	0.743/ 0.440–0.737	0.464–0.933/ 0.788–0.811	0.175/0.533–0.788	0.647–0.737/ 0.533	0.348–0.508/ 0.527–0.764	0.515–0.617/0.909	0.474–0.899/ 0.475–0.638	0.332
cis-9-Octadecenoic acid	0.743/ 0.440–0.737	0.464–0.933/ 0.788–0.811	0.175/0.533–0.788	0.647–0.737/ 0.533	0.348–0.508/ 0.527–0.764	0.515–0.617/0.909	0.474–0.899/ 0.475–0.638	0.332
6,10,14-Trimethylpen-tadecan-2-one	0.498/ 0.306–0.579	0.322–0.726/ 0.613–0.656	0–0.103/ 0.503–0.767	0.496–0.579/ 0.357–0.372	0.250–0.275/ 0.236–0.588	0.281–0.428/0.568	0.118–0.538/ 0.213–0.739	0.297
14-Pentadecenoic acid	0.814/ 0.561–0.810	0.623–0.954/ 0.854–0.914	0.203–0.216/ 0.697–0.875	0.685–0.810/ 0.605–0.614	0.410–0.620/ 0.745–0.845	0.547–0.714/0.945	0.563–0.935/ 0.589–0.665	0.300
Linolenic acid	0.720/ 0.405–0.706	0.419–0.905/ 0.718–0.739	0.151–0.165/ 0.404–0.763	0.611–0.706/ 0.502–0.506	0.285–0.480/ 0.466–0.730	0.474–0.573/0.858	0.384–0.856/ 0.589–0.665	0.300
3,7,11,15-Tetrame-thyl-hexadec-2-en-1-yl acetate	0.406/ 0.187–0.397	0.263–0.637/ 0.286–0.385	0–0.074/ 0.437–0.455	0.220–0.397/ 0.266–0.292	0.192–0.205/ 0.069–0.136	0.162–0.200/0.447	0.071–0.351/ 0.146–0.327	0.418
Dodecanoic acid	0.814/ 0.561–0.810	0.623–0.954/ 0.854–0.914	0.203–0.216/ 0.697–0.875	0.685–0.810/ 0.605–0.614	0.410–0.620/ 0.745–0.845	0.547–0.714/0.945	0.563–0.935/ 0.589–0.665	0.300
Tetradecanal	0.552/ 0.651–0.721	0.453–0.891/ 0.755–0.878	0–0.215/ 0.577–0.794	0.654–0.721/ 0.453–0.454	0.420–0.525/ 0.177–0.803	0.612–0.801/0.738	0.553–0.837/ 0.589–0.665	0.300
5,8,11-Heptadecatrien-1-ol	0.599/ 0.511–0.686	0.235–0.921/ 0.660–0.864	0.113–0.181/ 0.618–0.717	0.532–0.686/ 0.432–0.443	0.391–0.399/ 0.222–0.314	0.404–0.484/0.775	0.515–0.872/ 0.306–0.322	0.317
Hexadeca-7,10,13-trienal	0.439/ 0.496–0.589	0.327–0.779/ 0.591–0.735	0–0.150/ 0.263–0.691	0.503–0.589/ 0.352–0.365	0.300–0.386/ 0.100–0.673	0.449–0.745/0.573	0.370–0.607/ 0.262–0.309	0.410
Benzene, 1-ethenyl-4-methoxy-	0.441/ 0.255–0.599	0.195–0.494/ 0.342–0.371	0–0.063/ 0.269–0.679	0.523–0.599/ 0.467–0.472	0.113–0.180/ 0.076–0.189	0.202–0.444/0.432	0.078–0.280/ 0.145–0.226	0.289
Octanoic acid	0.814/ 0.561–0.810	0.623–0.954/ 0.854–0.914	0.203–0.216/ 0.697–0.875	0.685–0.810/ 0.605–0.614	0.410–0.620/ 0.745–0.845	0.547–0.714/0.945	0.563–0.935/ 0.589–0.665	0.300

Decanal	0.552/ 0.651–0.721	0.453–0.891/ 0.755–0.878	0–0.215/ 0.577–0.794	0.654–0.721/ 0.453–0.454	0.420–0.525/ 0.177–0.803	0.612–0.801/0.738	0.553–0.837/ 0.308–0.416	0.377
Decanoic acid	0.814/ 0.561–0.810	0.623–0.954/ 0.854–0.914	0.203–0.216/ 0.697–0.875	0.685–0.810/ 0.605–0.614	0.410–0.620/ 0.745–0.845	0.547–0.714/0.945	0.563–0.935/ 0.589–0.665	0.300
Pentadecanoic acid	0.814/ 0.561–0.810	0.623–0.954/ 0.854–0.914	0.203–0.216/ 0.697–0.875	0.685–0.810/ 0.605–0.614	0.410–0.620/ 0.745–0.845	0.547–0.714/0.945	0.563–0.935/ 0.589–0.665	0.300
Benzaldehyde	0.540/ 0.575–0.824	0.423–0.796/ 0.700–0.780	0–0.130/ 0.385–0.867	0.798–0.824/ 0.586–0.621	0.282–0.355/ 0.170–0.569	0.598–0.778/0.628	0.334–0.680/ 0.364–0.663	0.334
Hexenal	0.490/ 0.570–0.663	0.386–0.827/ 0.659–0.799	0–0.173/ 0.404–0.747	0.586–0.663/ 0.407	0.353–0.440/ 0.134–0.597	0.543–0.855/0.631	0.447–0.708/ 0.266–0.358	0.331
Benzoic acid, phenylmethyl ester	0.509/ 0.645–0.896	0.292–0.742/ 0.580–0.722	0.096–0.120/ 0.420–0.870	0.803–0.896/ 0.633–0.662	0.327–0.335/ 0.211–0.496	0.503–0.784/0.710	0.383–0.740/ 0.375–0.478	0.262
Heptanoic acid	0.814/ 0.561–0.810	0.623–0.954/ 0.854–0.914	0.203–0.216/ 0.697–0.875	0.685–0.810/ 0.605–0.614	0.410–0.620/ 0.745–0.845	0.547–0.714/0.945	0.563–0.935/ 0.589–0.665	0.300
Tridecanoic acid	0.814/ 0.561–0.810	0.623–0.954/ 0.854–0.914	0.203–0.216/ 0.697–0.875	0.685–0.810/ 0.605–0.614	0.410–0.620/ 0.745–0.845	0.547–0.714/0.945	0.563–0.935/ 0.589–0.665	0.300
Anticopalic acid	0.495/0	0/0	0/0	0/0	0/0	0/0.357	0/0	0.442
1-Butyl-3-(2-chloro-4,6-dimethyl-pyridin-3-yl)-urea	0/0	0/ 0.307–0.714	0/0–0.352	0/0	0/0	0/0	0/0	0
Palmitoleic acid	0.743/ 0.440–0.737	0.464–0.933/ 0.802–0.811	0.175/ 0.533–0.788	0.647–0.737/ 0–0.533	0.348–0.508/ 0.527–0.764	0.515–0.617/0.909	0.474–0.899/ 0.475–0.638	0.332
Tetradecanoic acid	0.814/ 0.561–0.810	0.623–0.954/ 0.854–0.914	0.203–0.216/ 0.697–0.875	0.685–0.810/ 0.605–0.614	0.410–0.620/ 0.745–0.845	0.547–0.714/0.945	0.563–0.935/ 0.589–0.665	0.300
9-cis,12-cis-Linoleic acid	0.720/ 0.405–0.706	0.419–0.925/ 0.781–0.783	0.162–0.165/ 0.490–0.763	0.611–0.737/ 0.502–0.506	0.321–0.480/ 0.466–0.730	0.474–0.573/0.892	0.442–0.886/ 0.442–0.605	0.335
Hexadecanoic acid	0.814/ 0.561–0.810	0.623–0.954/ 0.854–0.914	0.203–0.216/ 0.697–0.875	0.685–0.810/ 0.605–0.614	0.410–0.620/ 0.745–0.845	0.547–0.714/0.945	0.563–0.935/ 0.589–0.665	0.300
Phytol	0.512/ 0.360–0.535	0.319–0.750/ 0.377–0.464	0–0.107/ 0.365–0.598	0.359–0.535/ 0.328–0.347	0.280–0.296/ 0.102–0.237	0.237–0.327/0.529	0.166–0.514/ 0.204–0.416	0.417

Table 3. The number of manifestations of various biological activities (out of 15 groups analyzed) for major LMWOCs of aquatic macrophytes, predicted by PASS

LMWOCs	$0.7<P_a<0.8$	$0.8<P_a<0.9$	$P_a > 0.9$
14-Pentadecenoic acid	1	5	4
Saturated carboxylic acids*	1	5	4
Gallic acid	3	4	4
cis-6-Octadecenoic acid	5	1	3
cis-9-Octadecenoic acid	5	1	3
Palmitoleic acid	5	1	3
Linolenic acid	6	2	1
9-cis,12-cis-Linoleic acid	5	2	1
5,8,11-Heptadecatrien-1-ol	2	2	1
Decanal	4	5	0
Tetradecanal	4	5	0
Benzoic acid, phenylmethyl ester	5	3	0
Benzaldehyde	3	3	0
Hexenal	2	3	0
6,10,14-Trimethylpentadecan-2-one	3	0	0
cis, cis, cis-7,10,13-Hexadecatrienal	3	0	0
1-Butyl-3-(2-chloro-4,6-dimethyl-pyridin-3-yl)-urea	1	0	0

Note: * – Heptanoic acid, Octanoic acid, Nonanoic acid, Decanoic acid, Dodecanoic acid, Tridecanoic acid, Tetradecanoic acid, Pentadecanoic acid, Hexadecanoic acid, Octadecanoic acid

growth of cyanobacterium *M. aeruginosa*. Thus, the study of biological activities in PASS showed that the most promising compounds for further experimental verification are carboxylic acids (saturated and unsaturated) and gallic acid, as substances potentially possessing the largest number of biological activities contributing to the inhibition of development of cyanobacteria (Table 3). The mass spectral patterns of some carboxylic acids are given as an example in the chromatogram sections of *P. perfoliatus* essential oil (Fig. 1).

Four acids were selected for further experimental verification – heptanoic, octanoic, tetradecanoic, and gallic acids – against tested species of cyanobacteria: *S. aquatilis* and *A. flos-aquae*. We did not consider unsaturated carboxylic acids in this study, since they have a rather high market value (https://www.made-in-china.com/). This circumstance, apparently, will limit their use as algaecides.

To test the effects of selected compounds at several concentrations in different cyanobacterial cultures, we applied a classical method performed in microcosms and based on direct cell counts. The experimental results are presented in Fig. 2 and Fig. 3. Our results confirmed that heptanoic, octanoic, tetradecanoic, and gallic acids significantly inhibited the growth of both cyanobacterial species (1.9–7190 times compared to the control) (Fig. 2, 3), especially *A. flos-aquae* (Fig. 3b). Application of even 0.1 mg/L of the combined mixture showed a significant decrease compared to the control (Fig. 3b). It is important that this cyanobacterium is one of the usual species that cause harmful cyanobacterial

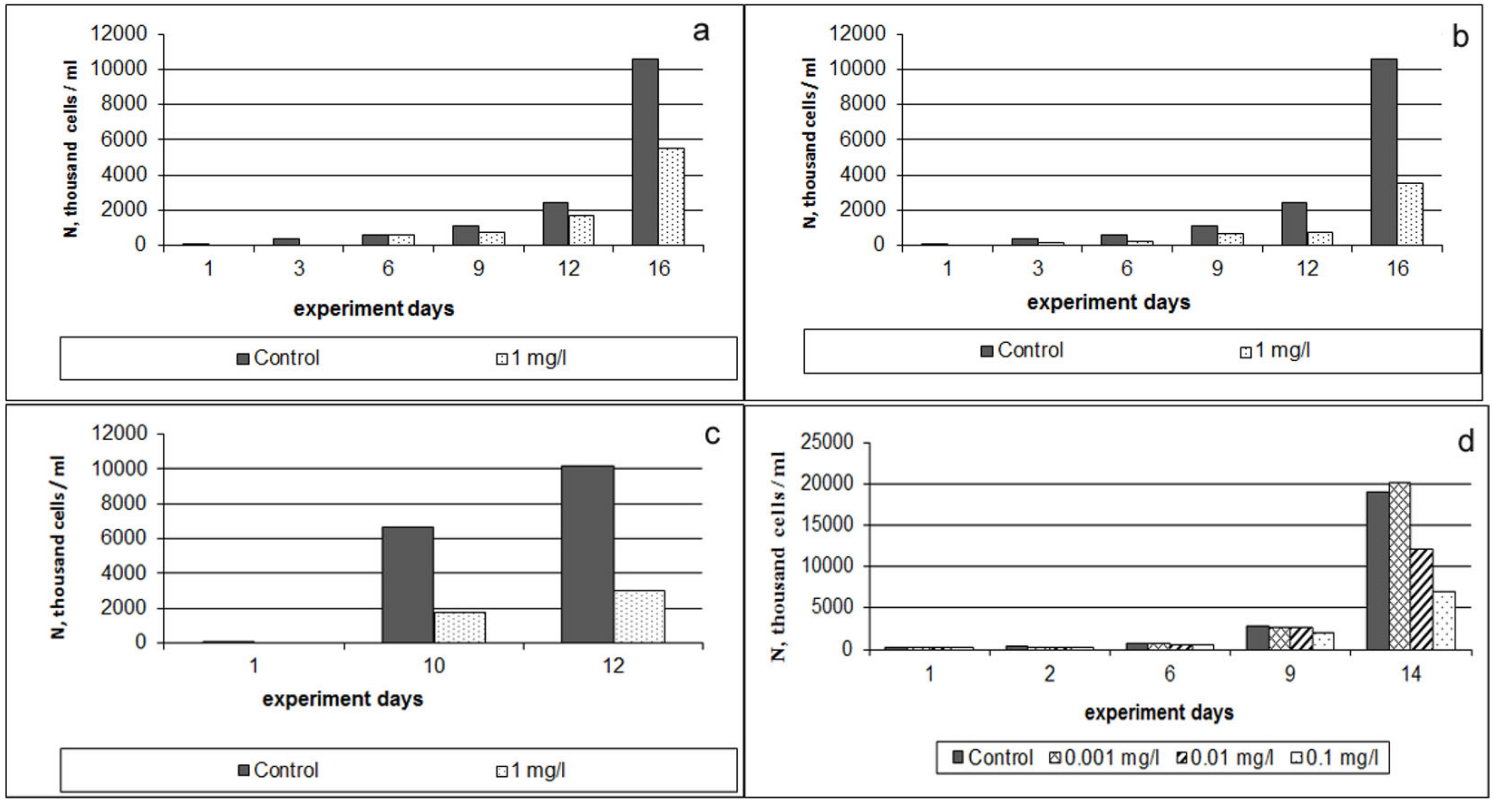

Fig. 2. The average number (median) of the culture of *S. aquatilis* in the experiment with heptanoic acid (a), octanoic acid (b), gallic acid (c), and tetradecanoic acid (d)

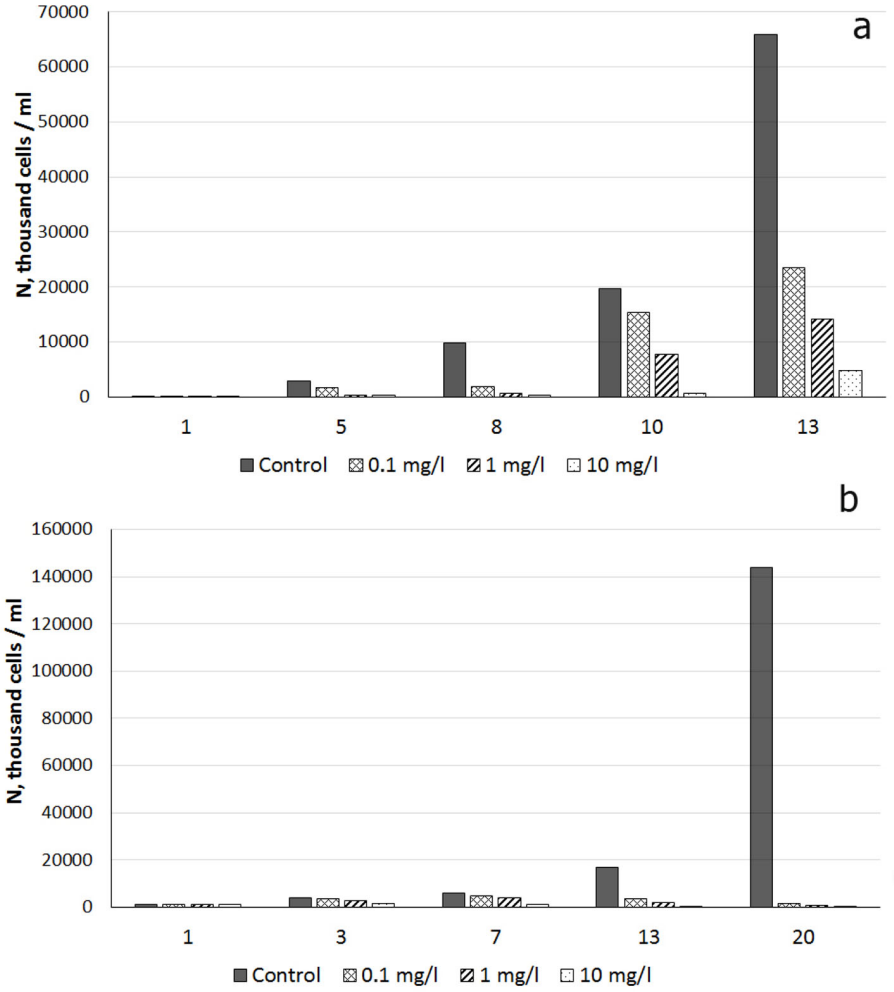

Fig. 3. The average number (median) of the culture of *S. aquatilis* (a) and *A. flos-aquae* (b) in an experiment with the combined effect of heptanoic, octanoic, tetradecanoic, and gallic acids at various concentrations. On the abscissa – the days of the experiment

"blooms" (HCBs) in water bodies (Svirčev et al., 2019; Zerrifi et al., 2021). It forms the most common and noxious type of HCB in freshwater environments, which has potentially serious consequences for environmental and human health.

Because gallic acid, a phenolic compound, and straight-chain saturated fatty acids differ in their chemical properties, their combination may result in different growth inhibition modes. It is reasonable to expect that their common cyanobacterial growth inhibition activities may be additive or synergistic. The inhibitions of cyanobacteria by some substances are known to be synergistic. For example, four polyphenols (pyrogallic acid, gallic acid, ellagic acid, and (þ)-catechin) were identified as allelochemicals antagonistic toward cyanobacteria (Nakai et al., 2000; Zhai et al., 2022). Also, Zhu et al. (2010) studied the combined inhibitory effects of these polyphenols against *M. aeruginosa* and found apparent synergistic effects.

Conclusions

The present study showed that a combined approach can be used to search for effective

allelochemicals against cyanobacteria, including, in the first stage, the SAR methodology followed by experimental verification of the most promising compounds. Our results demonstrated that representatives of saturated and unsaturated fatty acids and gallic acid (possibly other phenolic acids as well) with high activity against cyanobacteria should become components of various combinations for new-generation algaecides to control HCBs.

All compounds tested in this study were toxic for species of cyanobacteria in a dose-dependent manner. Since the inhibitory effect of the combined mixture of these allelochemicals was stronger than the effects of the individual components, it is reasonable to assume that there are various mechanisms of cyanobacterial growth inhibition.

Further research is necessary to investigate the relationship between structural molecular factors and the anti-cyanobacterial activity of different metabolites of freshwater macrophytes. It can also be assumed that various combinations of allelochemicals can be designed to suppress populations of various species of cyanobacteria to achieve the most effective suppression.

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