KLAIPĖDA UNIVERSITY

Donata OVERLINGĖ

CYANOBACTERIA AS A SOURCE OF BIOACTIVE METABOLITES: THEIR POTENTIAL APPLICATION IN BIOTECHNOLOGY AND ENVIRONMENTAL IMPACT

DOCTORAL DISSERTATION

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MELSVABAKTERĖS KAIP BIOAKTYVIŲ METABOLITŲ ŠALTINIS: POTENCIALUS PRITAIKYMAS BIOTECHNOLOGIJOJE IR POVEIKIS APLINKAI

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Abstract

This work presents a comprehensive study on the diversity and occurrence of cyanobacteria and their secondary metabolites in the Curonian Lagoon and coastal Baltic Sea. In the study, the ecological and socioecological significance of toxic cyanobacteria blooms as well as the biotechnological potential of cyanometabolites were explored. Phytoplankton analyses of samples collected in the Curonian Lagoon showed frequent occurrence of *Aphanizomenon*, *Dolichospermum*/*Anabaena*, *Microcystis*, *Planktothrix* and *Woronichinia* genera. Of these, *Dolichospermum*/*Anabaena*, *Microcystis*, and *Planktothrix agardhii* were confirmed by genetic methods as potential MCs producers. The assessment of water quality based on cyanobacteria parameters in the recreational areas of the Lithuanian Baltic Sea coast and Curonian Lagoon indicated a low probability of adverse health effects, with a higher risk in the southernmost part of the Curonian Lagoon. As these two systems are interconnected, the dynamics and structure of cyanobacteria in the Curonian Lagoon have significant impact on diversity and concentrations of cyanotoxins in the coastal areas of the sea. During the comprehensive studies of field samples collected in the Curonian Lagoon, 119 cyanometabolites representing eight different classes of the compounds were detected. Cyanopeptolins and microcystins were found to be most structurally diverse class of cyanopeptides. The observed diversity and considerable variation in rare and potentially new microcystin variants may indicate the presence of different cyanobacteria chemotypes in the lagoon. Bioactivity screening of phytoplankton samples from the Curonian Lagoon confirmed pharmaceutical potential of aquatic microorganisms. The samples were active against antibiotic resistant clinical and environmental bacteria strains, they inhibited serine proteases and reduced the viability of the T47D human breast adenocarcinoma cells.

Key words

Cyanotoxins, risk assessment, bioactivity, structure elucidation, mass spectrometry.

Reziumė

Šiame darbe pateikiamas išsamus melsvabakterių ir jų antrinių metabolitų įvairovės bei paplitimo Kuršių mariose ir Baltijos jūros priekrantėje tyrimas. Jo metu buvo tiriama toksinių melsvabakterių žydėjimo ekologinė ir socioekologinė reikšmė bei melsvabakterių produkuojamų metabolitų biotechnologinis potencialas. Fitoplanktono analizė parodė, kad *Aphanizomenon*, *Dolichospermum* / *Anabaena*, *Microcystis*, *Planktothrix* ir *Woronichinia* gentys yra vienos pagrindinių melsvabakterių genčių, dažniausiai aptinkamų mėginiuose, paimtuose iš Kuršių marių. *Dolichospermum* / *Anabaena*, *Microcystis* ir *Planktothrix agardhii* genetiniais metodais patvirtinti kaip galimi mikrocistinų produkuotojai. Vandens kokybės vertinimas pagal melsvabakterių parametrus Lietuvos Baltijos jūros priekrantės ir Kuršių marių rekreacinėse vietovėse parodė mažą neigiamo poveikio sveikatai tikimybę, didesnę – piečiausioje Kuršių marių dalyje. Kadangi šios abi sistemos yra tarpusavyje susijusios, Kuršių marių melsvabakterių dinamika ir struktūra daro didelę įtaką toksinų įvairovei ir koncentracijai jūros pakrančių zonose. Atlikus išsamius tyrimus Kuršių mariose aptikta 119 melsvabakterių produkuojamų metabolitų, priklausančių aštuonioms skirtingoms klasėms. Nustatyta, kad cianopeptolinai ir mikrocistinai struktūriškai buvo pačios įvairiausios cianometabolitų klasės. Rasta retų ir potencialiai naujų mikrocistinų variantų. Didelė mikrocistinų įvairovė bei jų sezoniniai pokyčiai gali rodyti, jog mariose egzistuoja skirtingi melsvabakterių chemotipai. Fitoplanktono mėginių iš Kuršių marių bioaktyvumo testavimai parodė vandens mikroorganizmų gebėjimą gaminti natūralius produktus, turinčius farmacinį potencialą. Testuojami fitoplanktono biomasės mėginiai buvo aktyvūs prieš antibiotikams atsparias klinikines ir aplinkos bakterijų padermes, taip pat jie slopino serino proteazių aktyvumą ir sumažino T47D žmogaus krūties adenokarcinomos ląstelių gyvybingumą.

Reikšmingi žodžiai

Cianotoksinai, rizikos vertinimas, bioaktyvumas, struktūros nustatymas, masių spektrometrija.

Contents

List of original publications

The material of this study was presented in 4 original publications, published in peer-reviewed scientific journals:

I. Pilkaitytė, R., **Overlingė, D.**, Gaziūnaitė, R. Z., Mazur-Marzec, H., 2021. Spatial and Temporal Diversity of Cyanometabolites in the Eutrophic Curonian Lagoon (SE Baltic Sea). Water, 13, 1760, doi: 10.3390/w13131760.

II. **Overlingė, D.**, Toruńska- Sitarz, A., Kataržytė, M., Gyraitė, G., Pilkaitytė, R., Mazur-Marzec, H., 2021. Characterization and Diversity of Microcystins Produced by Cyanobacteria from the Curonian Lagoon (SE Baltic Sea). Toxins, 13(12), 838, doi: 10.3390/toxins13120838.

III. **Overlingė, D.**, Kataržytė, M., Vaičiūtė, D., Gyraitė, G., Gečaitė, I., Jonikaitė, E., Mazur-Marzec, H., 2020. Are there concerns regarding cHAB in coastal bathing waters affected by freshwater-brackish continuum? Marine pollution Bulletin, 159, doi: 10.1016/j.marpolbul.2020.111500.

IV. **Overlingė, D.**, Toruńska- Sitarz, A., Cegłowska, M., Błaszczyk, A., Szubert, K., Pilkaitytė, R., Mazur-Marzec, H., 2021. Phytoplankton of the Curonian Lagoon as a New Interesting Source for Bioactive Natural Products. Special Impact on Cyanobacterial Metabolites**.** Biomolecules, 11, 1139, doi: 10.3390/biom11081139.

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Author's contributions

I. D. Overlingė performed the data collection *in situ* at the Curonian Lagoon (2015-2017), pre-processing and post-processing the water samples. She contributed to the conceptualization, writing and editing of the manuscript.

II. D. Overlingė performed the data collection *in situ* at the Curonian Lagoon, pre-processing and post-processing the water samples, data analyses. She contributed to the conceptualization of the manuscript, wrote the manuscript draft.

III. D. Overlingė performed the data collection *in situ* at the Curonian Lagoon and coastal Baltic Sea, pre-processing and post-processing the water samples, data analyses. She wrote the manuscript draft.

IV. D. Overlingė performed the data collection *in situ* at the Curonian Lagoon, pre-processing and post-processing the water samples, data analyses. She contributed to the conceptualization of the manuscript, wrote the manuscript draft.

Abbreviations

Abbreviation	Explanation
AEG	Aeruginosamid
AER	Aeruginosin
ANOSIM	Analysis of similarities
ANTX-a	Anatoxin-a
AP	Anabaenopeptin
Asp	Aspartate
BWD	Bathing Water Directive
cHAB	Cyanobacteria harmful algal bloom
Chl *a*	Chlorophyll *a*
CP	Cyanopeptolin
Dha	Dehydroalanine
DNA	Deoxyribonucleic acid
D-Masp	N-methyl-D-aspartate
ELISA	Enzyme-linked immunosorbent assays
EPA	Environmental Protection Agency
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing
Har	Homoarginine
HELCOM	Helsinki Commission Baltic Marine Environment Protection Commission
Hil	Homoisoleucine
Hph	Homophenylalanine
HPLC	High Performance Liquid Chromatography
MC	Microcystin
Mdha	N-methyldehydroalanine
MG	Microginin
m/z	Mass-to-charge ratio
nMDS	Non-parametric multidimensional scaling
NOD	Nodularin
OD	Optical density
PCR	Polymerase chain reaction
PDA	Photodiode array detector
RDA	Redundancy analysis
SE	Southeastern
Ser	Serine
WHO	World Health Organization

1

Introduction

Cyanobacteria are widespread photosynthetic microorganisms that play an important role in the aquatic environment. Under favorable conditions, they form harmful algal blooms in freshwater, estuarine, and marine ecosystems. Due to anthropogenic activities and climate change, the blooms have been increasing in extension and frequency (Buratti et al., 2017; Meriluoto et al., 2017; O'Neil et al., 2012). In temperate regions, cyanobacteria blooms usually occur during the summer and autumn periods, however, in some cases they can also persist during winter and spring (Wejnerowski et al., 2018; Paerl, 2014). The cyanobacteria harmful algal blooms (cHABs) increase the risk of negative impact on water quality, fisheries, tourism, and recreation (Paerl et al., 2019; Wurtsbaugh et al., 2019; Kaloudis et al., 2017).

Estuaries are particularly severely affected by anthropogenic nutrient enrichment from the surrounding watersheds accelerating high biological productivity and the expansion of HABs (Paerl et al., 2018; Preece et al., 2017). Due to hydrologically interconnected systems, e.g., freshwater-marine continuum, waters rich in phytoplankton can be transferred to marine coastal areas and increase the risk associated with cyanobacteria and cyanotoxins. Although many studies on the transfer of cyanobacteria blooms to the coastal areas have been recorded, the presence of cyanotoxins, their concentrations and impact on recreational activities are not well recognized (Preece et al., 2017).

Cyanobacteria species belonging to the orders Oscillatoriales, Nostocales, Chroococcales, and Synechococcales are considered as the main bloom forming species. Amongst them, more than 100 different cyanobacteria taxa (species or genera) produce toxins (cyanotoxins) (Jones et al., 2021; Demay et al., 2019). Although cyanobacteria are not classified as infectious microorganisms, the toxins they synthesize have negative health impacts on living organisms, including humans (Svirčev et al., 2019). Most illnesses caused by cyanotoxins result from ingestion, inhalation, or dermal contact (Kubickova et al., 2019). According to the physiological system that is affected, cyanotoxins are divided into hepatotoxins (microcystins (MCs), nodularin (NOD)), neurotoxins (anatoxin (ANTX-a)), cytotoxins, and dermatotoxins (Buratti et al., 2017; Testai et al., 2016). They can cause various health problems such as stomach cramps, nausea, vomiting, gastrointestinal symptoms, muscle paralysis, respiratory arrest, allergic reactions etc. (Kubickova et al., 2019; Machado et al., 2018; Buratti et al., 2017).

Cyanobacteria blooms are usually composed of toxic and nontoxic species/strains (Svirčev et al., 2019 and references therein). Moreover, the variability of cyanobacteria community can lead to production of multiple types of cyanotoxins (and other secondary metabolites) with numerous variants in each type. For most of the compounds, the toxicological data is insufficient to establish threshold concentrations (Ibelings et al., 2015). In addition, a limited number of available MC variants as quantitative standards (Janssen, 2019; Meriluoto et al., 2017) makes the determination of the total MC concentration difficult. The complexity and high variability of cyanobacteria community and their toxins largely depend on local conditions (Ibelings et al., 2015). In the assessment and management of the risk associated with cyanobacteria blooms, qualitative and quantitative analyses of cyanotoxins are indispensable.

Regulation of Bathing Water Quality based on cyanobacteria. The assessment of bathing water quality in EU countries is carried out in accordance with European Union (EU) Bathing Water Directive (BWD) (EU, 2006). According to this directive, fecal indicator bacteria (*Escherichia coli* and *Enterococci*) are mandatory parameters that should be regularly monitored, while cyanobacteria blooms and the risk associated with their presence are only briefly mentioned. In the absence of clear threshold values for the assessment of water quality based on cyanobacteria, recommendations provided by World Health Organization (WHO) (WHO, 2020) became the basis for the implementation of more specific guidelines for drinking and recreational waters in the EU member states. The main parameters used to set guideline values are cyanobacteria abundance, biomass, chlorophyll *a* (Chl *a*) (during the dominance of cyanobacteria in phytoplankton community), and concentrations of MCs (Chorus and Welker, 2021; WHO, 2020). Visual inspection, cyanobacteria biomass or cell number are among the most frequently used parameters in the EU countries for the assessment of recreational water quality (Ibelings et al., 2015). Lithuania is not an exception. According to the Lithuanian hygiene standard HN 92: 2018 by the Ministry of Health

of the Republic of Lithuania (LRSAM, 2019), the visual inspection should be carried out and, during intensive cyanobacteria proliferation, the cells should be calculated. When the 20,000 cells mL^{-1} threshold is exceeded, the recommendations not to bath should be provided. Although some reports about toxic algal blooms in the Curonian Lagoon were published (Šulčius et al., 2015; Paldavičienė et al., 2009), to date, the monitoring system of cyanotoxins has not been established in Lithuania, and the risk associated with the cyanotoxins transported from the lagoon to the coastal bathing sites has not been assessed.

Detection of cyanotoxins. Among cyanotoxins, MCs are the most widely distributed and structurally diverse compounds (Jones et al., 2021; Janssen, 2019). They belong to cyclic heptapeptides with the general structure of cyclo-(D-Ala^{1}-X^{2}-D-Masp^{3}-Z^{4}-Adda^{5}-D-γ-Glu^{6}-Mdha^{7}), where Adda is 3*S*-amino-9*S*-methoxy-2*S*,6,8*S*-trimethyl-10-phenyldeca-4*E*,6E-dienoic acid, X and Z are variable L-amino acids, D-Masp^{3} is D-erythro-β-methyl-isoaspartic acid, and Mdha is *N*-methyldehydroalanine (Botes et al., 1984; Santikarn et al., 1983). Due to structural modifications in all seven amino acids (mainly in positions 2 and 4), to date more than 280 variants of MCs are listed (Miles and Stirling, 2019).

MCs are synthesized by nonribosomal peptide synthetase and polyketide synthase pathway. The peptides are encoded by *mcyA-J* genes. These genes are frequently used as genetic markers for the detection, differentiation, and identification of MC producers (Sipari et al., 2010; Rantala et al., 2006). Amongst many MC genes, *mcyE* is essential for the synthesis of the Adda D-glutamate part, which is highly conserved in the MC structure (Rantala et al., 2006). It also determines the toxicity of the peptides. These features made the *mcyE* gene a reliable marker for the detection of MC producers (Ngwa et al., 2014; Rantala et a., 2006). Nevertheless, due to the observed *mcy* genes deletions, recombinations, various insertions, the detection of the *mcy* genes, as a single parameter, cannot be directly linked to the synthesis of MCs. Thus, the application of other methods, such as mass spectrometry, must be considered in order to confirm the presence of MCs in the analyzed samples.

Cyanotoxins, usually MCs, are never synthesized alone as one class of compounds. They are always detected with other classes of cyanopeptides, sometimes at similar or even higher concentrations than MCs (Janssen, 2019). Cyanopeptolins (CPs) belong to the most structurally diverse and frequently occurring compounds produced by cyanobacteria. The other groups of cyanopeptides detected in the blooms of cyanobacteria or in cyanobacteria isolates are microginins (MGs), aeruginosins (AERs), and anabaenopeptins (APs) (Janssen, 2019).

Biotechnological potential of secondary metabolites produced by cyanobacteria. Natural products, often called secondary metabolites, are organic molecules of low molecular weight that have diverse and often very potent biological activities. Secondary metabolites are not essential for normal growth, development, or reproduction

of an organism. They increase the potential of the producing organisms to survive interspecies competition, provide defensive mechanisms against stress, and facilitate reproductive processes (Davis and Ryan, 2012). Many secondary metabolites have proved to be invaluable as antibacterial, antifungal, antiviral, anticancer agents. Some of these have found to play a pivotal role in the treatment or prevention of a multitude of biological disorders (Carpine and Sieber, 2021; Mazur-Marzec et al., 2021; Rosales-Mendoza et al., 2020; Swain et al., 2017; Shishido et al., 2015). The bioprospecting of marine and brackish water systems has highly increased during the last few decades. Microalgae and their metabolites became one of the most widely explored resources, mainly in the areas as pharmacy, aquaculture, bioremediation, bioenergy, biorefinery and biopigmentation (Baldisserotto et al., 2019; Singh et al., 2019). Due to the urgent need for more effective and safer medicines for the treatment of cancer, metabolic disorders and infections caused by multidrug- resistant microorganisms, the search for new natural products as lead compounds for drug development has been intensified recently (WHO, 2020; Machowska and Lundborg, 2019; Ventola, 2015).

Most of the bioactive compounds were isolated from the *ex situ* cultures of microorganisms. However, there is still a possibility that the biosynthesis of specific metabolites might not be triggered under laboratory conditions (Lauritano et al., 2018; Welker and von Döhren, 2006). Microalgae are characterized by metabolic plasticity in their natural environment. Under stressed vs. non-stressed conditions they can trigger the synthesis of unique secondary metabolites (Prarthana and Maruthi, 2018). In this case, the analysis of phytoplankton bloom samples can supply valuable information about the metabolic and biotechnological potential of the organisms living in the analyzed ecosystem (Ingebrigtsen et al., 2017).

It is well documented that cyanobacteria are the leaders among the natural sources of bioactive compounds. Nonribosomal oligopeptides belong to the most intensively studied cyanobacterial metabolites. They vary greatly in their chemical structure, biological activity and potential pharmaceutical importance. In many studies, extracts and fractions containing cyanobacterial oligopeptides showed cytotoxic (Nowruzi et al., 2020; Demay et al., 2019), antibacterial (inhibition of the growth of multidrug-resistant pathogens) (Marrez et al., 2019; Swain et al., 2017), anti- inflammatory, antioxidant, antiprotozoal, anticoagulant, and antiviral (Kini et al., 2020). High pharmaceutical potential of cyanobacteria from tropical areas was proven (Hong and Luesch, 2012; Gerwick et al., 2008). Several recent screening studies have shown that the Baltic Sea cyanobacteria are also a rich and attractive source of bioactive peptides of potential therapeutic application (Fidor et al., 2021; Cegłowska et al., 2020; Fidor et al., 2020; Humisto et al., 2016; Spoof et al., 2016; Felczykowska et al., 2015). Previous research in the Curonian Lagoon has only focused on the ecotoxicological assessment of cyanobacteria scums and cyanotoxins from the perspective of the ecosystem and public health (Montvydienė et al., 2020; Šulčius et al., 2017; Šulčius et al., 2015;

Paldavičienė et al., 2009). However, there are no published data on the biological activity of metabolites produced by cyanobacteria and eukaryotic microalgae existing in the Curonian Lagoon.

1.1. Aim and objectives

The aim of the work is to assess the diversity of cyanobacteria from the Curonian Lagoon and the diversity of the secondary metabolites synthesized by these microorganisms. With this respect, the environmental risk and potential biotechnological exploitation of the phytoplankton biomass were delineated.

The following objectives have been raised:

1. To assess the spatial and temporal diversity of cyanobacteria and cyanotoxins, and identify their potential producers in the Curonian Lagoon;
2. To determine the diversity of metabolites produced by the cyanobacteria from the Curonian Lagoon;
3. To assess recreational water quality based on the cyanotoxins synthesized by the cyanobacteria at the fresh-brackish water continuum between the Curonian Lagoon and the Baltic Sea;
4. To assess the biotechnological potential and environmental significance of the secondary metabolites synthesized by the cyanobacteria.

1.2. Novelty of the study

This is the first report on water quality assessment in the Curonian Lagoon and southeastern (SE) coastal Baltic Sea based on the analysis of cyanobacterial community and cyanotoxins. This is also the first study that presents a detailed description of the occurrence and diversity of cyanometabolites (including cyanotoxins) in the Curonian Lagoon and Baltic Sea coast. A list of 119 cyanometabolites was compiled. Among them, the structures of 20 MC variants (among which three might be potentially new variants) were tentatively characterized. The numerous MC variants detected in the Curonian Lagoon provide new information about their geographical distribution and may indicate the presence of different cyanobacteria chemotypes in the lagoon. In the work, pioneered studies on the biological activity of metabolites produced by the cyanobacteria and eukaryotic microalgae existing in the Curonian Lagoon were conducted.

1.3. Scientific and applied significance of the results

The results of this study broaden our knowledge on cyanometabolite diversity, their spatial and temporal distribution in the Curonian Lagoon and coastal Baltic Sea. The occurrence and distribution of cyanotoxins in the coastal Baltic Sea provides a more advanced insight into the Curonian Lagoon impact on water quality in the coastal area. The assessment of water quality based solely on measurements of biomass or abundance of cyanobacteria does not provide a comprehensive evaluation of the environmental risk. In some cases, these measurements could lead to underestimation of risks related to the presence of cyanobactreria in bathing water. Therefore, MCs concentration, as an additional parameter, could provide more accurate assessment of water quality. Data on cyanotoxins structure and dynamics could complement the existing national or regional legislations responsible for monitoring and assessing water quality in recreational areas. The study also indicated that cyanobacteria from the Curonian Lagoon can be explored as a source of biotechnologically important natural products. The obtained results gave a valuable starting point for the further studies into the structure, activity and application of specific metabolites produced by microorganisms from the Curonian Lagoon.

1.4. Scientific approval

Results of this study were presented in 3 international and 2 regional conferences:

1. Regional conference of Marine and Coastal Research (Jūros ir krantų tyrimai), Klaipėda, Lithuania, May 2018;
2. International Conference on Toxic Cyanobacteria (ICTC11), Krakow, Poland, May 2019;
3. International Scientific Conference “The vital nature sign”, Kaunas, Lithuania, May 2019;
4. 1st Ocean4Biotech Conference, Slovenia, Piran, February 2020;
5. Regional conference of Marine and Coastal Research (Jūros ir krantų tyrimai), Klaipėda, Lithuania, October 2020.

2

Materials and Methods

2.1. Study sites

The Curonian Lagoon is the largest lagoon in Europe, with a surface area of 1584 km^2 and a mean depth of 3.5 m (Žaromskis, 1996). The lagoon is a freshwater body divided into two parts (northern and southern) and connected with the sea by the narrow Klaipėda Strait – the only outlet of the lagoon (Ferrarin et al., 2008). Due to irregular brackish water intrusions into the lagoon, the salinity in the northern part fluctuates from 0 to 7 psu (Zemlys et al., 2013). The highest annual average salinities of 3-5.5 psu are determined in the Klaipeda Strait. It gradually decreases towards the south and reaches a freshwater salinity level (<0.5 psu) at approximately 35 km from the sea entrance (Zemlys et al., 2013). The duration and extent of the intrusions of seawater to the Curonian Lagoon depend on the wind-caused rises in water level in the coastal zone (Gasiūnaitė et al., 2008), while freshwater runoff from the Curonian Lagoon to the Baltic Sea is induced by dominating currents due to higher water level in the lagoon than in the Baltic Sea (Čerkasova et al., 2016, Jakimavičius and Kovalenkovienė, 2010). The plume area can vary from 0.3 to 630 km^2, meandering more than 30 km from the outlet of the Curonian Lagoon, mostly northwards (Vaičiūtė, 2012).

In total, nine different sites were investigated for this thesis (Fig. 1). To determine the diversity of cyanometabolites in the Curonian Lagoon during 2013-2017, five sites were selected – Nida Lagoon (located at the Nida city) – represented the central part of the Lagoon, Juodkrantė, Dreverna – transitional areas, representing the western and eastern parts of the lagoon, respectively, Ventė – the area representing

the Nemunas River runoff, and Port (in Paper I named Smiltynė) – a transitional area, which is highly influenced by the Baltic Sea water inflow (Paper I). The diversity of MCs in 2018, 2019 and 2020 was determined based on the comprehensive analysis of the samples collected in the Nida Lagoon (Paper II).

The bathing water quality was assessed based on the concentrations of the cyanotoxins at the Curonian Lagoon and Lithuanian coastal Baltic Sea (Paper III). For this study, six sites were selected – four official bathing sites (Kintai, Melnragė, Palanga and Nida Sea), one potential new bathing site – Nida Lagoon, one site (Port) representing a transitional zone. Sites Nida Sea and Nida Lagoon were named based on the city (Nida) and place (Baltic Sea or Curonian Lagoon).

The biotechnological potential of phytoplankton from the Curonian Lagoon was assessed based on the analyses of samples collected from two sites where the surface accumulation of phytoplankton was the highest: Nida Lagoon and Juodkrantė (Paper IV).

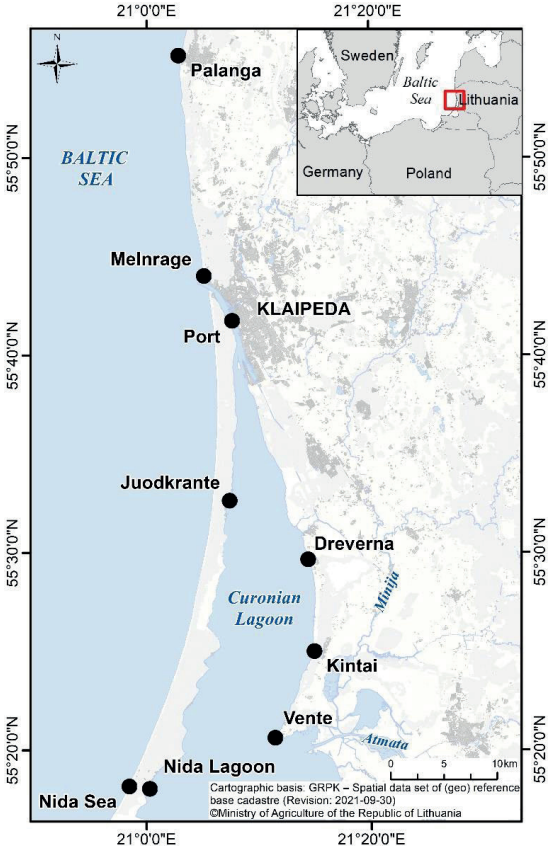

Figure 1. The study area and the locations of the nine study sites: Baltic Sea sites – Palanga, Melnragė and Nida Sea; Curonian Lagoon sites – Port, Dreverna, Kintai, Ventė, Juodkrantė and Nida Lagoon.

2.2. Sampling design

For the Paper I, water samples were collected each season, while for the Papers II-IV only during the official recreational period, i.e., from May 30 till September 19 (except the sample, collected in October 2019) (Table 1). Physical and biological parameters measured or collected for further analyses are provided in Table 1. The detailed description of the environmental measurements (salinity, temperature, pH and chlorophyll *a* (Chl *a*)) are provided in Paper III.

Table 1. Summary of main activities carried out in the Curonian Lagoon and coastal Baltic Sea during the period of 2013-2020.

Paper	Sampling period	Sampling sites	Number of samples	Parameters
Paper I	2013 (August – October)	Nida Lagoon, Juodkrantė, Port, Dreverna, Ventė	127	Salinity, temperature, Chl *a*, phytoplankton composition, diversity of cyanometabolites, concentrations of MC, ANTX-a, NOD
	2014 (July – November)			
	2015 (October – December)			
	2016/2017 (all year round)			
Paper II	2018 (May – September)	Nida Lagoon	10	Phytoplankton composition, diversity and concentrations of MCs, *mcyE*, *mcyE-12R* (*Anabaena*/*Dolichospermum*), *mcyER8* (*Microcystis*), *mcyE-plaR3* (*Planktothrix*)
	2019 (October)		1	
	2020 (July)		1	
Paper III	2018 (May – September)	Nida Lagoon, Port, Kintai, Nida Sea, Melnragė, Palanga	54	Salinity, temperature, pH, Chl *a*, phytoplankton composition, concentrations of MCs, ANTX-a and NOD
Paper IV	2018 (May – September)	Nida Lagoon, Juodkrantė	9	Diversity of cyanometabolites, bioactivity assessment

2.3. Phytoplankton analyses

Microscopical analysis. The quantitative phytoplankton analysis was performed according to the methodology described by Utermöhl, 1958; the phytoplankton abundance and biomass were calculated according to the methodology described by HELCOM (2021) and Olenina et al. (2006). See Paper III for the detailed information about the phytoplankton analysis.

Genetic analysis. DNA was extracted from the environmental samples. Polymerase chain reaction (PCR) cycling conditions were used as in Rantala et al., 2004. For the amplification of *mcy*E gene from all cyanobacteria present in the samples, the primers *mcyE-F2* and *mcyE-R4* (Genomed S.A., Warszawa, Poland) were used. MC-producing *Anabaena*/*Dolichospermum*, *Microcystis*, and *Planktothrix* were targeted with the above-mentioned forward primer and species-specific reverse primers (*mcyE-12R*, *mcyER8*, and *mcyE-plaR3*, respectively) (Genomed S.A., Warszawa, Poland). More detailed description of the genetic analysis is available in Paper II.

2.4. Water quality assessment – guideline values

Cyanobacteria abundance, biomass and MCs concentration are the main guideline values developed by WHO (2003; 1999) for the assessment of water quality affected by cyanobacterial blooms (Table 2). Each of the guideline values corresponds to the guidance level. The Alert Levels for cyanobacteria biomass are developed for drinking water, however, they can also be used for recreational areas to trigger alerts (Chorus and Welker, 2021; WHO, 1999). According to Lithuanian hygiene standard HN 92: 2018 by the Ministry of Health of the Republic of Lithuania (LRSAM, 2019), estimation of cyanobacteria abundance is the only metric for recreational guidelines, related to potential cyanobacterial health impacts. See Paper III for more details.

Recently, the WHO (2021) has updated the guideline values and recommends following the Alert Level system. It focuses mainly on cyanobacteria biomass, concentrations of Chl *a* (with dominance of cyanobacteria) and MCs. The provisional guideline value provided by WHO (2021) for MCs in recreational waters (24 $\mu g\ L^{-1}$) is a bit higher compared to WHO (2003; 1999). During this study (Paper III), the assessment of water quality based on the MCs concentration and cyanobacteria biomass was based on WHO recommendations published in 1999 and 2003 (Table 2). Following the recent guidelines (WHO, 2021), the results of water quality assessment in the field of study did not differ from the assessment based on the previous recommendations.

Table 2. Guidelines and guidance levels of cyanobacteria cells for recreational waters. Redrawn from Paper III.

Parameter	Guideline values	Guidance level
Cyanobacteria abundance	20 000 cyanobacteria cells mL^{-1} (2,4)	Relatively low probability of adverse health effects[2]
	100 000 cyanobacteria cells mL^{-1} (2)	Moderate probability of adverse health effects[2]
	Cyanobacterial scum[2]	High probability of adverse health effects[2]
Cyanobacteria biomass	<0.2 mg L^{-1} *(1)	Alert level 1 (low)[1]
	<10 mg L^{-1} *(1)	Alert level 2 (moderate - high)[1]
	>10 mg L^{-1} *(1)	Very high[1]
MC concentration	2-4 µg MC L^{-1} (2)	Relatively low probability of adverse health effects[2]
	20 µg MC L^{-1} (*Microcystis* dominance)(2)	Moderate probability of adverse health effects[2]
	>1 mg MC L^{-1} (3)	High probability of adverse health effects[2]
	8 µg MC L^{-1} (5)	-

*Guidelines for drinking water, [1]WHO, 1999; [2]WHO, 2003; [3]Codd et al., 2005; [4]LRSAM, 2019; [5]Environmental Protection Agency (EPA), 2019.

2.5. Chemical analyses

Analysis of cyanometabolites. Samples (filtered or freeze-dried) for qualitative and quantitative analysis of cyanometabolites were extracted and then analyzed using the Agilent HPLC system (Agilent Technologies, Waldboronn, Germany) coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer QTRAP5500 (Applied Biosystems, Sciex; Canada) according to the method described by Mazur- Marzec et al. (2015). To determine the content of samples (extracts and fractions), the information-dependent acquisition method (non-target analysis) was used. The detection of CPs in the samples was based on spectral data published by Welker et al. (2006, 2004), Czarnecki et al. (2006), Fuji et al. (2000); MCs – by Bouaïcha et al. (2019); MGs – by Zervou et al. (2020), Carneiro et al. (2012); APs – by Spoof et al. (2016), Welker et al. (2006), Erkhard et al. (1999); AERs – by Nishizawa et al. (2007). For the quantitative analysis of intracellular toxins, the multiple reaction monitoring mode was used. The concentrations of 9 MC variants (dmMC-LR, dmMC-RR, MC-RR, -LR, -YR, -LA, -LF, -LY, -LW), NOD, and ANTX-a were calculated based on the results obtained for

the commercially available standards of the toxins (Alexis Biochemicals, San Diego, USA). For detailed information about the methods, see Papers I-IV.

Fractionation of Phytoplankton Biomass. Extracts for further fractionation were selected based on bioactivity response obtained in four bioassays (see Chapter 2.6). After the extraction of freeze-dried phytoplankton biomass, the supernatants were partially purified and fractionated on Waters Sep- Pak® Vac 20cc C18 cartridges (5 g) (Waters, Milford, MA, USA) (manually) or on flash chromatography Biotage® SNAP KP- C18- HS (120 g) column (Biotage, Uppsala, Sweden) using Shimadzu HPLC system model LC- 20AP (Shimadzu, Canby, OR, USA) equipped with a photodiode array detector (PDA). For detailed information about the methods, see Paper IV.

2.6. Bioassays

In total, 9 different crude extracts of phytoplankton samples were tested. The samples were collected in the Juodkrantė and Nida Lagoon every two weeks from June to August 2018. The extracts showing the highest inhibitions (>50% of the control) were selected for the further fractionation and bioassays.

Antibacterial activity. Broth microdilution assay was performed according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations (http://www.eucast.org). For detailed information about the experimental setup, see Paper IV.

Cytotoxicity Assay. For cytotoxicity assay human breast adenocarcinoma cell line T47D (Merck KGaA, Darmstadt, Germany) was used. The assay was performed according to the colorimetric method described by Felczykowska et al. (2015). More detailed information about the experimental setup can be found in Paper IV.

Enzyme Inhibition Assay. The trypsin inhibition assay was performed according to the methodology described by Pluotno and Carmeli (2005), while the chymotrypsin and thrombin assay followed the procedure by Ocampo Bennet (2007). The detailed experimental setup information is provided in Paper IV.

Acute Toxicity Assay. The toxicity of phytoplankton extracts towards the juvenile freshwater cladoceran *Daphnia magna* was performed according to the procedure described by the producer (MicroBioTests Inc., Gent, Belgium). For detailed information about the experimental setup, see Paper IV.

2.7. Statistical methods

The redundancy analysis (RDA) was applied to assess the relationship between abiotic factors (water temperature, salinity), the biomass of the different cyanobacteria species (used as explanatory variables) and cyanometabolites (used as dependent variables). For detailed information about the statistical methods, refer to Papers I-II.

Non- parametric multidimensional scaling (nMDS) based on the Jaccard and Bray-Curtis similarity coefficients (Bray and Curtis, 1957; Jaccard, 1901) was used to represent the similarities of phytoplankton or dominating cyanobacteria communities among the different samples. One- way ANOSIM tests were used to determine the significances of the degree of separation among the nMDS groups. Detailed information about the statistical methods is provided in Papers II-IV.

3

Results and Discussion

3.1. Cyanobacteria diversity and contribution to phytoplankton biomass

The highest total cyanobacteria biomass of all the Curonian Lagoon sites studied was recorded in the Nida Lagoon site. Mostly, the biomass exceeded 7 mg L^{-1}, with maximum value of 165.8 mg L^{-1} in 2014 September (Paper I – Paper IV). In all other sites, the biomass was mostly lower than 10 mg L^{-1}. The highest cyanobacteria biomass values were usually recorded in late summer and autumn. Cyanobacteria blooms in the Curonian Lagoon are an annual phenomenon. Their biomass varies in a wide range during the year but remains high through all vegetation season (Vaičiūtė et al., 2021). In the scums, cyanobacteria biomass can reach up to 200 mg L^{-1} (Šulčius et al., 2015). In such cases, Curonian Lagoon phytoplankton produces huge amount of biomass which can have negative effects on the ecosystem, but on the other hand, the biomass can provide resources for various applications, such as energy, natural fertilizers of high added value products. Currently, Lithuanian Environmental Protection Agency funded project implemented by Nature Research Centre (Fitobio, 2021-2022), dedicated to assessing the nutrient removal potential from the Curonian Lagoon as a measure for Marine Strategy Water Framework Directive 2008/56/EB (WFD, 2008). However, the presence of toxic compounds in the biomass and the problematics associated with their removal them can limit various applications.

According to this study, which was performed over eight years (2013-2020), the cyanopeptides were most numerous and most abundant in the samples where

Aphanizomenon, *Dolichospermum*/*Anabaena*, *Microcystis*, *Planktothrix* and *Woronichinia* genera dominated (Paper I – Paper IV). The presence of *mcyE* genes amplified with primers specific for *Dolichospermum*/*Anabaena*, *Microcystis*, and *Planktothrix* (*P. agadhii*) genera confirmed the potential producers of MCs in the Curonian Lagoon (Paper II, see Table 2 in Paper II). During the study period, *Aphanizomenon* spp., mainly *Aph. flosaquae*, was the dominating genera in the samples (Paper I). The highest biomass of this species was usually recorded in late summer and early autumn. In almost half of the analyzed samples *Aph. flosaquae* accounted >35% of the total cyanobacteria biomass. The results coincide with the previous studies where *Aph. flosaquae* was represented as the main species responsible for the largest and longest cyanobacteria blooms in the Curonian Lagoon (Vaičiūtė et al., 2021; Olenina, 2012; Gasiūnaitė et al., 2008; Pilkaitytė and Razinkovas, 2006). *Microcystis* and *Planktothrix* genera were also frequently detected in the Curonian Lagoon. During all investigated periods, their biomass did not exceed 4 and 8 mg L^{-1}, respectively. More pronounced dominance of *P. agardhii* was recorded in late spring and during the end of summer and the beginning of autumn, while *Microcystis* spp. – in the middle of summer. Based on the published records, since 1990s, *P. agardhii* has been found more frequently in the Curonian Lagoon and can account for ~50% of the total biomass of cyanobacteria (Bukaveckas et al., 2017; Olenina, 2012; Jaanus et al., 2011). The dominance of *Dolichospermum* was usually recorded in the first half of the summer, with the evident predominance in August 2013 in Ventė and in June 2018 in the Nida Lagoon (Paper I, Paper III). Then, its biomass reached ~7 mg L^{-1}. During the other study period, the biomass of *Dolichospermum* was mostly lower than 1 mg L^{-1}.

As for the coastal Baltic Sea sites, the biomass of cyanobacteria did not exceed 1.5 mg L^{-1} in the Nida Sea and Palanga sites, while in the Melnragė site, located closer to the outlet of the Curonian Lagoon, it was a bit higher but did not exceed 2.5 mg L^{-1} (Paper III). According to the long-term data, the biomass of cyanobacteria rarely exceeds 1 mg L^{-1} in the eastern Baltic Sea (Kownacka et al., 2018). *D. flosaquae*, *Aph. flosaquae*, *W. compacta*, *P. agardhii*, *M. wesenbergii* and *N. spumigena* predominated in the samples collected in the coastal Baltic Sea.

3.2. The diversity and distribution of cyanometabolites

In total, 119 variants of cyanometabolites were detected in the Curonian Lagoon (Paper I – Paper IV). All variants belonged to eight different classes (MC, ANTX-a, NOD, APs, AERs, aeruginosamid (AEG), CPs, MGs). In the coastal Baltic Sea, only MCs, ANTX-a and NOD were analyzed (Paper III).

During the analysis carried out in the Curonian Lagoon in years 2013-2017 (five study sites), 48 variants of cyanometabolites were detected (see Supplementary Material in Paper I) – 10 MCs, 16 APs, 12 AER, 1 AEG, 3 CPs, 4 MGs, NOD and ANTX-a; while during the

research conducted in 2018 (two study sites) – 117 cyanometabolites were identified (see Supplementary Material in Paper II, Paper IV). CPs were found to be the most structurally diverse class of cyanopeptides – in total 53 variants were detected. Peptides from other classes were represented by 3 – 4-fold lower number of structural variants – MCs (20 variants), MGs (18 variants), APs (14 variants) and AERs (13 variants).

The seasonal pattern of MCs diversity and concentrations in the Curonian Lagoon during the 2013-2017 was similar, except in the Nida Lagoon and Port sites. MC-RR accounted for the highest contribution in the total MCs concentration in all investigated sites of the Curonian Lagoon. However, in the Nida Lagoon site a shift from the dominance of MC-RR (2013 and 2014) to the dominance of [Asp^3]MC-RR (2015–2017) was observed. In all investigated sites, the concentrations of MCs during the summer – autumn seasons (June – October) varied within a range 1 – 6 μg L^{-1}, while in the Nida Lagoon, they were mostly higher than 6 μg L^{-1}, with the maximum value 22.8 μg L^{-1} in October 2014. This study also showed that, albeit less frequently, relatively high concentrations of MCs can be detected at the Juodkrantė site as well. In August 2013, it reached 15.8 μg L^{-1}. In addition to the MC variants mentioned before, MC-LR and MC-YR were also frequently detected in the Curonian Lagoon. For more detailed description of the results, see Paper I.

The other two cyanotoxins, NOD and ANTX-a were detected also in the coastal Baltic Sea and Curonian Lagoon (see below Table 4). As for NOD, its concentrations varied from traces to 0.3 μg L^{-1}. The toxin was mainly detected in the coastal Baltic Sea sites and northern part of the Curonian Lagoon (Port, Juodkrantė) (Paper I, Paper III). The concentrations of ANTX-a varied from traces to 2.2 μg L^{-1} and the toxin was mainly detected in the Curonian Lagoon (see below Table 4) (Paper I, Paper III).

The other cyanometabolites most frequently detected during the research conducted in the Curonian Lagoon in years 2013-2017 included APs: AP-A, AP-B, AP-F and Osc-Y. These variants were detected in all investigated sites; however, no pronounced dominance of any specific variant was observed: in almost all samples the mixture of APs was recorded. AERs, AEG A, and MGs belonged to less frequently detected metabolites, but they were detected in all investigated sites, too, except the Port site, where no CPs were recorded. For more detailed description of the results see sections 3.2.1 – 3.2.5 of Paper I.

Based on RDA analysis, APs showed the closest statistical relationship with *P. agardhii*, AER – with *Aph. flosaquae*, AERs – with *Microcystis* spp., CPs – with *Microcystis* spp. and *P. agardhii*, and MGs – with *P. agardhii* (see Paper I, Figure 8). All relationships were statistically significant ($p<0.05$). These results are in line with the previous studies showing the detection of APs in the *Planktothrix* dominated lake and in isolated strains (Grabowska et al., 2014); the correlation between AEG A and *Aph. flosaquae* and *C. issatschenkoi* was recorded by Kust et al., 2020, while *Microcystis* spp. are known as AERs producer (Le Manach et al., 2019); CPs and MGs are mainly linked to the presence of the representatives of *Microcystis* and *Planktothrix* genera (Zervou et al., 2020; Martins et al., 2009; Rohrlack et al., 2009).

3.2.1. A comprehensive study of microcystins

Due to the identified risk related to the presence of MCs and high concentrations of the toxins usually observed in the southern part of the Curonian Lagoon (Nida Lagoon site), more comprehensive research on this class of peptides and their producers was performed in 2018-2020 (Paper II).

In total, 20 MC variants were detected, most commonly with 2 – 5 different variants per sample (Table 3). The enhanced product ion mass spectra of MCs with the suggested structures are provided in Paper II Supplementary material (Figure S3 – S22). Among the numerous MCs detected, one new MC variant with *m/z* 1057 was partially characterized. Moreover, two other MCs with *m/z* 1075 and *m/z* 1068 might belong to the new variants containing serine (Ser) in the position one of the peptides, which is rarely detected. In some cases, the structure elucidation of MCs based on the product ion fragmentation spectrum could lead to misinterpretation of the spectrum. This problem can be encountered when amino acid residues with the same value are present in the peptide, e.g., Glu and Masp, Leu and Ile, Mdha and Dhb, or Tyr and Met(O_2) (see Paper II, Figure 3). MCs with Met(O_2) can be formed during sample processing and are considered post-extraction oxidation artifacts (Miles et al., 2014). Therefore, even if the mass spectrum is rich in fragment ions, the structure elucidation of peptides based on fragmentation pattern should be performed with caution. In these cases, the structure of the compounds should be additionally confirmed by nuclear magnetic resonance spectroscopy, or high-resolution mass spectrometry and accurate mass measurements. However, the application of these analyses is usually restricted by trace amounts of the compounds and difficulties related to their isolation in pure form.

In comparison to other studies, the diversity of MCs observed during this research was relatively high. In German freshwaters, 15 MC variants were detected (Fastner et al., 1999), in Turkish lake – 36 MCs (Yilamaz et al., 2019), in a dam located in South Africa – 41 MC variant (Ballot et al., 2013). In the Curonian Lagoon, the highest diversity of MCs was noted in samples collected on 23 July 2018 (12 variants) and 16 August 2018 (18 variants), when the contribution of cyanobacteria to the total phytoplankton biomass was one of the lowest (see Paper II, Figure 1). The species composition in these samples did not differ evidently from other samples collected during the period from 27 June to 30 August potentially indicating changes at the subpopulation level of the cyanobacteria community. It is known that several cyanobacterial chemotypes can be characterized by different MC patterns and they usually coexist in the same waterbodies (Johansson et al., 2019; Grabowska et al., 2014; Welker et al., 2004). Thus, probably during those two days, when the highest MC structural diversity was recorded in the samples, the environmental conditions were favorable for the proliferation of MC-rich chemotypes and it indirectly influenced the presence of numerous MC variants. However, more detailed research with isolated strains is needed to clarify the diversity of cyanobacteria chemotypes in the Curonian Lagoon.

Table 3. MCs diversity in the field samples collected from the Curonian Lagoon during 2018, 2019, and 2020 ("+": detected; empty cells: not detected, *m/z*–values of MC pseudomolecular ions. In brackets, the value of a doubly charged ion is given).

MC variants	*m/z*	Sampling dates											
		30 May 2018	13 Jun 2018	27 Jun 2018	11 Jul 2018	23 Jul 2018	03 Aug 2018	09 Aug 2018	16 Aug 2018	30 Aug 2018	19 Sep 2018	17 Oct 2019	03 Jul 2020
[Ser^{1}]MC-HtyR or MC-Y(OMe)R	1075					+			+				
MC-WR or [Ser^{1}]MC-HarR	1068								+	+			
MC-X*R	1057					+			+				
MC-?	1054								+				
MC-(H_4)YR	1049					+			+				
MC-YR	1045	+				+			+	+	+		
MC-HphR	1043								+	+			
MC-RR	1038 (519)	+	+	+	+	+	+	+	+	+	+	+	+
[Asp^{3}]MC-YR or [Asp^{3}]MC-M(O_2)R	1031					+		+	+	+			
[Asp^{3}]MC-RY	1031											+	+
MC-FR	1029					+			+				
MC-LW	1025								+				
[Dha^{7}]MC-RR	1024 (512)		+			+			+	+	+	+	+
MC-HilR	1009					+			+				
MC-LY	1002								+				
MC-LR	995	+	+	+	+	+	+		+	+	+	+	+
[Asp^{3}]MC-LY	988								+			+	
MC-LF	986						+		+	+	+		
[Dha^{7}]MC-LR	981		+			+			+	+			
[Asp^{3}]MC-LR	981					+						+	+

*- unknown part of MC.

MC-WR, [Asp^{3}]MC-RY, MC-LW, MC-LY, MC-(H_4)YR, and [Asp^{3}]MC-LY, detected during this study were previously mainly reported from *M. aeruginosa* (Miles et al., 2012; del Campo and Ouahid, 2010; Christiansen et al., 2008; Diehnelt et al., 2006; Bateman et al., 1995), while MC-FR, MC-HilR are characteristic for several *Microcystis* species (Namikoshi et al., 1995; Namikoshi et al., 1992). MC-HphR is

associated with different strains of *Anabaena* (Miles et al., 2014; Fewer et al., 2008; Namikoshi et al., 1992). The diversity of MCs found in phytoplankton samples from the Curonian Lagoon complements information about the geographical distribution of the toxins. The specific MC structures characteristic for different geographical regions are observed worldwide. For instance, Leu[1]-containing MCs were detected only in Canada (LeBlanc et al., 2020), while MC-LA is more frequently detected in the US rather than in European water bodies (Pick, 2016).

3.3. The cHAB in the fresh-brackish water continuum of the Baltic Sea

3.3.1. Dynamics of cyanobacteria biomass and cyanotoxins in the fresh-brackish water continuum

Changes in the salinity values observed during this study indicated the occurrence of two outflow periods from the Curonian Lagoon to the coastal Baltic Sea: in late spring (late May) and in late summer (August) (Paper III). The recreational areas located close to the outlet of the Curonian Lagoon, e.g., Melnragė, were constantly exposed to cyanobacteria and cyanotoxins characteristic to the Curonian Lagoon waters. In addition to the salinity parameter, the water outflow from the Curonian Lagoon was indicated by the observed predominance of various *Dolichospermum* species and the detection of ANTX-a (August 2018). In the Palanga area, which is located approximately 24 km north of the Klaipėda Strait, cyanotoxins characteristic to the Curonian Lagoon were detected only during the second intensive outflow in late summer (August 2018). During this time only a slight increase in the total cyanotoxins concentration was recorded. At the same time, NOD was also detected in Palanga site, which represents typical Baltic Sea water. The presence of NOD potentially indicated that the Curonian Lagoon waters in the Palanga area were already mixed and diluted with the Baltic Sea waters.

To visualize different situations in the coastal Baltic Sea and the Curonian Lagoon, spatial distribution of Chl *a* (due to positive correlation with cyanobacteria biomass, Spearman correlation 0.7, $p<0.05$) and concentrations of cyanotoxins at three investigated dates were presented in Fig. 2 (Paper III). The mid-summer (23 July) and the end of the recreational season (19 September) differed in the contribution of cyanobacteria in phytoplankton biomass, which had an evident impact on the total concentration of cyanotoxins (Fig. 2a, 2c). During the outflow period (2 August), slightly higher biomass of cyanobacteria and total concentration of cyanotoxins were determined in the Curonian Lagoon, while in the plume zone (near the outlet) the total concentration of cyanotoxin increased more than 10-fold (Fig. 2b). If the outflow from the Curonian

Lagoon would be determined having the values of cyanobacteria biomass as shown in the Fig. 2c, significantly higher cyanotoxins concentrations could likely be expected in bathing areas along the coastal Baltic Sea. Independently of the outflow, higher total cyanotoxin concentrations were observed in the Palanga site (0.41 μg L^{-1}) as well (Fig. 2c). During that time, *P. agardhii* was the main dominating species in the sample and potentially contributed to higher concentration of cyanotoxins. *P. agardhii*, especially brackish strains, are known to tolerate higher salinity and show plasticity to their environment (Vergalli et al., 2016). This study also demonstrates that *P. agardhii* transferred through the estuary to the coastal Baltic Sea can persist and survive under higher salinity.

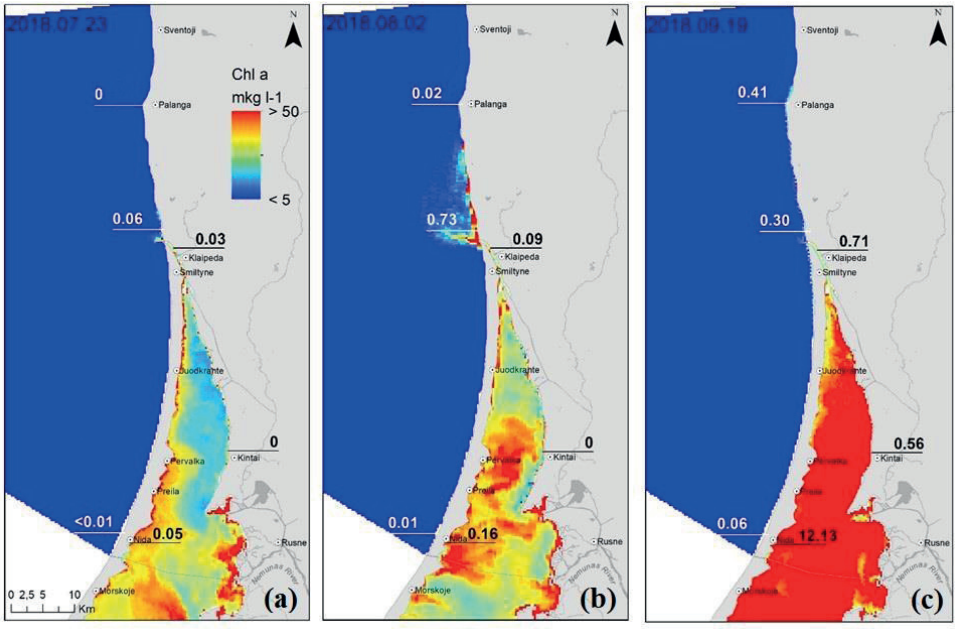

Figure 2. Spatial distribution of Chl *a* (μg L^{-1}) in the Curonian Lagoon and the coastal waters of the Baltic Sea as mapped by Sentinel-3 OLCI satellite data in 300 m spatial resolution during the sampling day: on July 23 (a), August 2 (b, one day before the sampling) and September 19 (c). Numbers indicate the concentration of cyanobacteria toxins (μg L^{-1}) determined *in situ* at the sampling sites (from Paper III).

The opposite phenomenon, i.e., the intrusion of the Baltic Sea waters to the Curonian Lagoon, can also be observed (Paper III). During this study, the salinity changes indicated one intrusion event: brackish waters of the Baltic Sea reached Kintai site (official bathing site) located in the northern part of the Curonian Lagoon. Measurements conducted during the intrusion revealed no evident changes in the biomass of

cyanobacteria and concentrations of cyanotoxins in the Kintai site. Despite the fact that the concentrations of the NOD were relatively low in the Curonian Lagoon during this study (Paper I and Paper III), the previous research by Paldavičienė et al. (2009) in 2007 showed extremely high concentrations of NOD. Therefore, the water transport from the Curonian Lagoon to the Baltic Sea and in the reverse direction raises concerns about bathing water quality.

3.3.2. Assessment of the bathing water quality at the coastal recreational waters

MCs concentrations, abundance (cells mL^{-1}), biomass (measured as biovolume or as concentration of Chl *a*) of cyanobacteria or visual inspection of water color are the main cHAB parameters recommended for the assessment of water quality in recreational areas (Chorus and Welker, 2021; WHO, 2003; 1999). To assess water quality at the coastal Baltic Sea and Curonian Lagoon, two parameters, MC concentrations and cyanobacteria biomass, were used (Paper III). Using these parameters, different results of water quality assessment were obtained. According to the concentrations of MCs measured in the coastal Baltic Sea and Curonian Lagoon, the guideline values did not exceed thresholds recommended by WHO and indicated a low probability of adverse health effects (Table 4). Meanwhile, the biomass of cyanobacteria exceeded the recommended threshold at the Nida Lagoon and Port sites (August – September) (see Paper III, Table 4). The differences between water quality based on the concentrations of MCs and cyanobacteria biomass resulted from the presence of different dominating cyanobacteria species. In the Nida Lagoon site, the dominating cyanobacteria were *P. agardhii* and the representatives of *Dolichospermum* and *Microcystis* genera, which had higher contribution to the total concentration of MCs (Table 4). While in the Port site, when the higher total biomass of cyanobacteria was reached (16 mg L^{-1}), *Aph. flosaquae* accounted for ~90% of the total cyanobacteria biomass (see Paper III, Table 4). In the Curonian Lagoon, as in the Baltic Sea, this species is considered a non-MC producing cyanobacterium (Österholm et al., 2020; Šulčius et al., 2015). In the research done by Paldavičienė et al. (2015), no significant increase in MCs concentrations or their diversity were observed during the bloom of *Aph. flosaquae*. In general, the results showed that cyanobacteria community is very important factor in water quality assessment. With the predominance of non-toxic cyanobacteria, biomass becomes a key indicator of water quality and the analysis of cyanotoxins can be used to confirm or downgrade the risk (WHO, 2021). However, in the presence of toxic cyanobacteria, MCs concentrations should be measured as a mandatory parameter while simultaneously measuring cyanobacteria cell number and/or biomass.

NOD and ANTX-a concentrations in the coastal Baltic Sea and Curonian Lagoon were quite low. However, their detection rises the potential risk for bathers too. NOD

has similar toxicity properties and modes of action as MC-LR (WHO, 2020), while ANTX-a could cause neurotoxic effects and has lethal or sublethal effects on terrestrial organisms (Christensen and Khan, 2020; Weirich and Miller, 2014). However, the official guideline values have not been established for any of these toxins (Chorus and Welker, 2021). Given the potential effects of NOD and ANTX-a on terrestrial organisms, including humans, proper implementation of management and monitoring strategies is required.

The identification of various MC variants and their accurate quantification are very important for the toxicological assessment and monitoring (Bortoli and Volmer, 2014). The following MCs: MC-LR, MC-HilR, MC-LY, MC-YR, presented in Papers I-IV, belong to the highly toxic variants (Bouaïcha et al., 2019). For other MCs detected during this study (Paper II), including potentially new variants, no toxicity data are available (Bouaïcha et al., 2019). In addition, the reliable quantitative assessment of total MCs is impossible due to the lack of reference standards. It should be also noted that MCs, ANTX-a, NOD are not the only harmful compounds produced by cyanobacteria. There are many other classes of peptides, also described in Paper I and Paper IV, which occur as frequently as MCs. They are known as proteases inhibitors or compounds harmful to grazers (Janssen, 2019). Lack of toxicological data and comprehensive knowledge about the activity of cyanobacteria metabolites might lead to the underestimation of the real risk (Paper II).

Table 4. Cyanobacteria biomass (mg L^{-1}), presence and concentrations of cyanotoxins in the samples from the study sites ("+" indicates < 1 µg L^{-1}; "++": indicates > 1 µg L^{-1}; empty cells: not detected; "5–9" indicates the months from May to September) and concentration ranges of the different toxin classes (from the lowest to the highest concentration detected in the samples) (from Paper III).

Cyanotoxin conc. range (µg L^{-1})	SITES	NIDA LAGOON					KINTAI					PORT					NIDA SEA					MELNRAGE					PALANGA				
	Months	5	6	7	8	9	5	6	7	8	9	5	6	7	8	9	5	6	7	8	9	5	6	7	8	9	5	6	7	8	9
$2x10^{-3}$-3.49	dmMC-RR					++						+				+						+									
	MC-LR	+	+	+	++	++						+			+	+						+		+	+	+					+
	MC-RR	+	+	+	++	++				+	+	+	+	+	+	+						+	+	+	+	+				+	+
	MC-YR	+			+	++						+				+										+					
	MC-LA										+											+									
	MC-LF				+	+				+	+	+			+	+					+		+	+	+	+					+
	MC-LY										+											+				+					
	MC-LW										+	+				+					+	+	+	+		+					+
$3x10^{-5}$-0.05	NOD																		+	+				+	+					+	
0.01-2.23	ANTX-a		++	+	++	++								+	+	+									+					+	

3.4. Biotechnological potential of phytoplankton from the Curonian Lagoon

Following a relatively high diversity of cyanopeptides in the Curonian Lagoon detected in 2013-2017 (Paper I), along with the ecological significance, the investigations into the diversity, biological activities of natural products and their specific biotechnological applications were important elements of the study (Paper IV). Therefore, in this section, the biological activities of phytoplankton samples from the Curonian Lagoon were tested using enzymatic, antimicrobial, and cytotoxicity assays as well as acute toxicity assay on *Daphnia*. The results of the tests are presented in Paper IV.

Phytoplankton extracts inhibited the growth of *Enetrococcus faecium* 45 (Table 5), isolated from the Sewage Treatment Plant. The results indicate potential application of phytoplankton from the Curonian Lagoon, especially cyanobacteria, in reducing pathogenic and fecal bacteria present in wastewaters. The growth inhibition of the clinical strain *Staphylococcus aureus* CCNPB/1505 was also recorded. It documents the ability of phytoplankton organisms present in the samples to produce antibacterial compounds (Table 5, see Table 4 in Paper IV). The activity against T47D cancer cells, and high inhibitory activity of the tested extracts and fractions against serine proteases (trypsin, chymotrypsin, and thrombin) also confirmed the pharmaceutical potential of natural phytoplankton products from the Curonian Lagoon (Table 6, see Table 4 in Paper IV).

Table 5. Antibacterial activity of extracts obtained from the Curonian Lagoon phytoplankton. Results are expressed as a percentage of bacterial culture optical density (OD) value compared to untreated control (100% growth). Different colors highlight the differences in OD values of bacterial cultures (the color code is explained below the table) (from Paper IV).

Bacterial strains		Clinical strains												Environmental strains											
		***Staphylococcus aureus* CCNPB/1505**			***Pseudomonas aeruginosa* CCNPB/MBL**			***Acinetobacter baumanii* CCNPB/O**			***Klebsiella pneumoniae* CCNPB/1404**			***Enterococcus faecium* 45**			***Aeromonas salmonicida* 2013**			***Vibrio cholerae* 2329**			***Vibrio diazotrophicus* Cd1**		
Conc., µg mL^{-1}		500	250	125	500	250	125	500	250	125	500	250	125	500	250	125	500	250	125	500	250	125	500	250	125
Extracts*	I																								
	II																								
	III																								
	IV																								
	V																								
	VI																								
	VII																								
	VIII																								
	IX																								

0-20%	20-50%	50-70%	70-100%	100-120%	120-150%

*- the Roman numerals of extracts (I – IX) corresponds to sample ID (I – Sample 1; II – Sample 2 etc.).

Table 6. Enzyme inhibition, cytotoxicity activity, and acute toxicity of phytoplankton extracts. Results are expressed as a percentage value of enzyme inhibition, cell viability (cytotoxicity assay) and cladocerans viability (acute toxicity assay), compared to untreated control. Different colors highlight the differences in values (the color code is explained below the table) (from Paper IV).

Bioassays		Enzyme inhibition assay				Cytotoxicity assay				Acute toxicity assay					
		Trypsin		Thrombin		T47D cells				*Daphnia magna*					
Conc., µg mL^{-1}		45	4.5	45	4.5	200	100	50	25	24 hours			48 hours		
										10	5	2.5	10	5	2.5
Extracts	I														
	II														
	III														
	IV														
	V														
	VI														
	VII														
	VIII														
	IX														

High enzymatic inhibition/ Low cell viability/High toxicity[1] — Low enzymatic inhibition/ High cell viability/Low toxicity[1]

0-20%	20-50%	50-70%	70-100%	100-120%

After the initial testing of the phytoplankton extracts (Table 5, 6), the samples with the highest activity revealed in the assays were chosen for further fractionation and analyses. Extracts IV and V were chosen for antibacterial assays, extract IV for cytotoxicity assays and extracts VIII and IX for enzymatic assays (see Table 4 in Paper IV). In the active fractions obtained from the above mentioned extracts, 117 cyanopeptides were detected (see Table S2 in Paper IV). In the active fractions, CPs and MGs constituted the dominant classes of peptides, while the most intensive ion peaks were observed mainly for AERs (see Table S9, Fig. S2a-j in Paper IV). As complex bloom samples were analyzed, no reliable conclusion about the link between the observed activity and a specific sample component can be established. Cyanobacterial secondary metabolites, such as CPs and AERs are generally considered to be the main classes of cyanopeptides responsible for the inhibition of serine proteases (Janssen, 2019; Mazur-Marzec et al., 2018; Chlipala et al., 2011). MGs are known to be angiotensin- converting enzyme inhibitors (Okino et al., 1993), as well as leucine aminopeptidase, aminopeptidase M, bovine aminopeptidase N and trypsin inhibitors (Bober and Bialczyk, 2017; Ishida et al., 2000). CPs also have cytotoxic activity; microcystilide A, a CP structural variant, showed cell- differentiation- promoting activ-

ity using HL- 60 human leucocytes (Tsukamoto et al., 1993). Moreover, *Microcystis* extracts containing CPs, MGs and other metabolites were active against HepG- 2, colon CaCO- 2 and breast MCF- 7 cancer cell lines (Salem and el Assi, 2020). Cyanotoxins, such as MCs and NOD, also comprise a rich source of natural cytotoxic compounds. They can cause a cellular damage after transport to the cell via organic anion transporting polypeptides which are expressed in cancer cells (Sainis et al., 2010).

In case of tests performed with fractions and extracts, apart from bioactive compounds, the nutritional and defensive effects were observed. In the case of *Vibrio cholerae* 2329, no inhibition but rather growth stimulation was recorded (Table 5). *V. cholerae* is naturally occurring marine and brackish water bacterium. Several species of *Vibrio* are known as pathogenic and can cause severe disease (Kim et al., 2015). Their intensive proliferation mostly correlates with water temperature (>20 °C) (Takemura et al., 2014), which is one of the major causes of cyanobacterial blooms as well (Paerl et al., 2020). The dissolved organic matter resulting from intensive phytoplankton blooms, especially cyanobacteria- derived organic matter, can significantly support the growth of potentially pathogenic *Vibrio* species (Gyraite et al., 2019; Eiler et al., 2007). Such synergy between cyanobacteria and *Vibrio* should be monitored as an element of bathing water quality assessment (WHO, 2003). The opposite results were obtained for environmental, naturally occurring *Aeromonas salmonicida* 2013 and *Vibrio diazotrophicus* Cd1. The tests confirmed the ability of phytoplankton to produce antibacterial compounds (Rojas et al., 2020; Falaise et al., 2016). These compounds might constitute an element of a defense strategy and increase the survival of the producer in extremely competitive environments where a huge variety of bacteria and other microbes co- exists (Ingebrigtsen et al., 2017; Falaise et al., 2016). As the effects of the samples on *D. magna* were rather weak, further tests on this organism were not performed.

The biodiscovery of new compounds in the environment that have dynamic conditions (lagoons, estuaries) provides a relatively high diversity of bioactive metabolites with biotechnological potential (Ingebrigtsen et al, 2017). Promising results obtained during this study coincides with this idea and encourage to continue further studies on the structure, mechanism of action and application of specific metabolites produced by microorganisms from the Curonian Lagoon.

4

Recommendations

Due to the variability of cyanobacteria community composed of different toxic and non-toxic strains, and characterized by different pattern of produced metabolites, the evaluation of water quality based on cyanobacteria parameters becomes complicated. There is no single unique way to provide a scheme of water quality monitoring for cyanobacteria parameters as each waterbody has its own characteristics. The following are the recommendations highlighting the need for more comprehensive monitoring and managing of recreational waters based on cyanobacteria parameters for the coastal and estuarine waters (based on Papers II-IV and adapted according to WHO (2021)). However, every recommendation should be adapted to national and local conditions.

1. *Assessing spatial and temporal patterns of cyanobacteria blooms and scums.* A good understanding of the seasonal dominance of cyanobacteria, species composition and their bloom history might be very useful for planning a monitoring strategy. Although blooms or scums of cyanobacteria are quite difficult to predict, however in some water bodies the annual pattern of blooms may follow quite predictable patterns.

2. *Strengthening competencies.* Due to unique characteristics of different water bodies the documentation (photos or videos) of water color during the bloom and non-bloom periods can be used to strengthen the competencies of responsible authorities carrying out rapid water quality assessment on site. Moreover, a short training courses

in phytoplankton ecology can help to deepen the knowledge and facilitate understanding of cyanobacteria blooms.

3. *Microscopical evaluation of dominant cyanobacteria.* If the water color predicts a possible intense proliferation of cyanobacteria, microscopical evaluation of the dominant cyanobacteria species could assist in providing more precise, rapid information and evaluating the risk for the site users. Due to pronounced differences in the cell sizes of cyanobacteria species, the evaluation of cyanobacteria abundance could lead to the misinterpretation of the potential risk. Therefore, the biomass, Chl *a* or/and phycocyanin concentration (e.g., measured by portable fluorometer for rapid evaluation) can highly support the microscopical assessment.

4. *Determination of cyanotoxins content and concentration.* Because different chemotypes of cyanobacteria can coexist in waterbodies, the concentration of cyanotoxins (MCs, NOD and ANTX-a) should be monitored as a mandatory parameter. Simultaneously the determination of cyanobacteria biomass could also provide a good indication of toxin concentration then cell counts. The analysis of cyanotoxins may depend on the availability of equipment, especially the LC-MS/MS system. If this instrument and experts are not available, the monoclonal or polyclonal enzyme-linked immunosorbent assays (ELISA) or any other portable rapid tests for MCs can be used. It is important to mention, that even if the concentrations of MCs or other toxins are low, but the proliferation of cyanobacteria are quite intensive, other cyanometabolites, which occur as frequently as MCs can cause various allergic reactions or rashes.

5. *Screening of other pathogenic bacteria related to cHABs.* In natural water bodies, rising water temperature, which is one of the major causes of cyanobacterial blooms and proliferation, may also provide an optimal environment for the occurrence of *Vibrio* species. Therefore, it is highly possible that cyanobacteria blooms can support the growth of potentially pathogenic *Vibrio* species. Therefore, such a synergy should be monitored as an element of bathing water quality assessment.

6. *Interconnected systems.* Cyanobacteria blooms usually are observed in the lacustrine environments, however, they can be transferred throughout the hydrologically interconnected systems, like fresh-marine water continuum. Under certain meteorological (e.g., predominant wind direction) and hydrodynamic (e.g., predominant currents) conditions, highly eutrophic water can be transferred to marine coastal areas and increase the risk associated with cyanobacteria blooms. The monitoring and managing cyanobacteria contamination in the systems like fresh-marine water continuum should be strengthened by evaluating it as one interconnected system rather than a series of individual water bodies.

7. *Marine toxins*. Even though cyanobacteria blooms are the most frequent phenomenon observed in fresh and brackish waters, the blooms of other microalgae are also likely to be observed. Among them, dinoflagellates and diatoms are the most relevant microalgae which produce toxic substances that have negative health effects

on humans and other animals. Monitoring of these microalgae species should also be considered, especially in the marine environment, where more intensive recreational activities take place.

8. *Public education and communication.* Sufficient information (posters, various media) about cyanobacteria blooms and related risks must be available for people. Public education about harmful algal and cyanobacteria blooms, their toxins, symptoms experienced after recreational activity could help to increase public awareness.

9. *Future research needs.* Quantitative and qualitative characterization, toxicological assessment of cyanotoxins and other cyanometabolites, more reference standards for the quantitative assessment of cyanotoxins are among the main research needs for recreational waters. Moreover, investigations on symptoms that occur after the exposure to cyanotoxins could contribute to a better understanding of the effects of cyanotoxins on human and animal health.

5

Conclusions

1. *Aphanizomenon*, *Dolichospermum*/*Anabaena*, *Microcystis*, *Planktothrix* and *Woronichinia* genera were most frequently present in samples rich in cyanometabolites. The presence of *mcyE* genes amplified with primers specific for *Dolichospermum*/*Anabaena*, *Microcystis*, and *Planktothrix* genera indicated the cyanobacteria as potential producers of MCs. As the only species among the *Planktothrix* genus found in the Curonian Lagoon was *P. agardhii*, at least a part of the cyanobacteria population belongs to MC producers.

2. Studies on the temporal diversity of cyanobacteria genera revealed the dominance of *Aph. flosaquae* in late summer and early autumn, *Microcystis* spp. – in the middle of summer, *P. agardhii* – in late spring and during the end of summer – beginning of autumn, and *Dolichospermum* spp. – during the first half of the summer. The highest total cyanobacteria biomass and concentrations of cyanotoxins of all the Curonian Lagoon sites studied were recorded in the southernmost part of the Curonian Lagoon which, unlike the transitory areas in the northern part, is confined constantly by water exchange.

3. Within this study, 119 variants of cyanometabolites were detected and grouped to eight different classes (MC, ANTX-a, NOD, APs, AERs, AEG, CPs, MGs). CPs were found to be the dominant and one of the most structurally diverse class of cyanopeptides. In case of spatial distribution, no CPs were recorded at the Port site. Cya-

nometabolites representing other classes, i.e., AERs, APs, MGs were 3 – 4- fold less numerous in different variants and with no dominance of any specific variant among the investigated sites. In total, 20 MC variants were detected. MC-RR accounted for the highest contribution of the total MCs concentration in all investigated sites of the Curonian Lagoon. However, in the Nida site, a shift from the dominance of MC-RR (2013 and 2014) to the dominance of [Asp^3]MC-RR (2015–2017) was observed. One new potential MC variant with *m/z* 1057 was partially characterized, while the MCs with *m/z* 1075 and *m/z* 1068 might belong to new variants as well. Numerous variants and temporal variations in MCs give an assumption that the cyanobacteria community in the Curonian Lagoon is not homogeneous in terms of species or strains.

4. The studies performed in the Lithuanian bathing sites of the Baltic Sea coast showed that water quality based on cyanobacteria parameters corresponded to a low probability of adverse health effects. The bathing site located close to the outlet of the Curonian Lagoon had similar structure of cyanobacterial community and cyanotoxins as the lagoon. During the intensive outflow event, the higher total cyanotoxin concentration was recorded in the northern part of the Lithuanian coast. In contrast, recreational exposure is at a higher risk in the southernmost Lithuanian part of the Curonian Lagoon. The recommended threshold of cyanobacteria biomass (>8 mg L^{-1}) was exceeded in 10% of all analyzed samples.

5. Enzymatic, antimicrobial and cytotoxicity bioassays revealed the high bioactivity of phytoplankton field samples collected from the Curonian Lagoon. In terms of the antibacterial assays, the inhibition of antibiotic- resistant *E. faecium* 45, isolated from the Sewage Treatment Plant, revealed the potential application of cyanobacteria in reducing pathogens and fecal bacteria present in wastewaters. Moreover, extracts and fractions tested during this study revealed the antibacterial activity of cyanobacteria metabolites against clinical, antibiotic- resistant bacterial strains. Inhibitory activity against serine proteases, trypsin, chymotrypsin, and thrombin, also the activity against breast cancer cell line T47D, confirmed the pharmaceutical potential of phytoplankton natural products from the Curonian Lagoon. In the active fractions, CPs and MGs constituted the dominant classes of peptides.

6. The ecological significance of phytoplankton extracts and fractions tested within this study revealed the potential nutritional and defensive effects. Phytoplankton, especially cyanobacteria extracts can support the growth of *V. cholerae* bacteria. Therefore, such a synergy should be monitored as an element of bathing water quality assessment. The inhibition of environmental bacteria (*A. salmonicida* 2013 and *V. diazotrophicus* Cd1) might indicate a defensive strategy of the producer to increase their survival in extremely competitive environments.

6

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7

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8

Summary in Lithuanian

Įvadas

Kenksmingas melsvabakterių žydėjimas pakrančių ir vidaus vandens ekosistemose gali turėti neigiamos įtakos vandens kokybei. Labiausiai pastebimi neigiami padariniai žmonių ir gyvūnų sveikatai, neigiamas poveikis žuvininkystei, turizmui ir rekreacijai. Nors dažniausiai toksiški melsvabakterių žydėjimai stebimi gėlavandenėse sistemose, priekrantės vandenys dėl sąsajos per upes ar estuarijas taip pat yra labai paveikiami. Melsvabakterių gaminami toksinai ir užteršto gėlo vandens pernešimas į jūros pakrančių zonas pasaulyje yra tiriamas jau daugiau nei 20 metų, tačiau toksinų poveikis rekreacinei veiklai nagrinėjamas daug rečiau. Labiausiai paplitę ir struktūriškai gausiausi junginiai tarp toksinų yra mikrocistinai (Jones ir kt., 2021; Janssen, 2019). Ši melsvabakterių produkuojamų metabolitų klasė nėra vienintelė. Mikrocistinai dažniausiai aptinkami ir su kitų klasių metabolitais, kurių koncentracijos labai panašios ar net didesnės lyginant su mikrocistinais (Janssen, 2019). Viena tokių klasių yra cianopeptolinai, kurie dažniausiai pasitaiko mėginiuose, gausiuose melsvabakterių. Kitos metabolitų klasės, aptiktos melsvabakterių žydėjimo metu arba jų izoliuotose padermėse, yra mikrogininai, aeruginozinai ir anabaenopeptinai (Janssen, 2019).

Remiantis 2018 m. atnaujinta Lietuvos higienos norma HN 92:2018 „Paplūdimiai ir jų maudyklų vandens kokybė“, nurodyta atlikti melsvabakterių gausumo tyrimus, tačiau apie saugias toksinų koncentracijas neužsiminta, kaip ir Europos Sąjungos

Maudyklų vandens direktyvoje 2006/7/EB. Pasaulinė sveikatos organizacija (PSO) teikia rekomendacines toksinų bei melsvabakterių biomasės vertes, kuriomis vertinamas pavojus sveikatai, pavyzdžiui, rekreacinėse vietovėse cianotoksinų koncentracija neturi viršyti 24 μg/l, o melsvabakterių biomasė – 8 mg/l (WHO, 2021). Priešingai nei Maudyklų vandens direktyva ir Lietuvos higienos standartas, PSO rekomendacijos oficialiai neįpareigoja, todėl reguliarus cianotoksinų stebėjimas nei pakrančių, nei vidaus gėluose vandenyse Lietuvoje nėra vykdomas.

Melsvabakterės – tai viena pirmaujančių mikroorganizmų grupių, esančių kaip natūralių bioaktyvių junginių šaltinis. Iš jų neribosominiai oligopeptidai yra intensyviausiai tiriami melsvabakterių metabolitai. Jie labai skiriasi chemine struktūra, biologiniu aktyvumu ir galimu biotechnologiniu pritaikomumu. Iš jų dažniausiai nustatomas citotoksinis (Nowruzi ir kt., 2020; Demay ir kt., 2019), antibakterinis (Marrez ir kt., 2019; Swain ir kt., 2017), priešuždegiminis aktyvumas. Keletas naujausių tyrimų parodė, kad Baltijos jūros melsvabakterės taip pat yra gausus bioaktyvių metabolitų šaltinis, turintis potencialų farmacinį pritaikomumą (Fidor ir kt., 2021; Cegłowska ir kt., 2020; Fidor ir kt., 2020; Humisto et al., 2016; Spoof ir kt., 2016; Felczykowska ir kt., 2015). Kuršių mariose, kaip labai eutrofikuotame vandens telkinyje, kasmet pasižyminčiame mesvabakterių žydėjimu, daugiausiai buvo atlikti ekotoksikologiniai melsvabakterių ir jų produkuojamų toksinų tyrimai (Montvydienė ir kt., 2020; Šulčius ir kt., 2017; Šulčius ir kt., 2015; Paldavičienė ir kt., 2009). Vis dėlto publikuotų duomenų apie Kuršių mariose aptinkamų melsvabakterių ir eukariotinių mikrodumblių gaminamų metabolitų biologinį aktyvumą nėra.

Tyrimo tikslas ir pagrindiniai uždaviniai

Šio tyrimo tikslas yra įvertinti Kuršių mariose gyvenančių melsvabakterių ir jų produkuojamų antrinių metabolitų įvairovę, keliamą riziką aplinkai bei biotechnologinį potencialą.

Pagrindiniai uždaviniai:

1. įvertinti Kuršių mariose gyvenančių melsvabakterių ir jų produkuojamų toksinų įvairovę erdvėje ir laike bei nustatyti potencialius jų produkuotojus;
2. nustatyti melsvabakterių produkuojamų metabolitų įvairovę Kuršių mariose;
3. įvertinti Lietuvos Baltijos pajūryje ir Kuršių mariose esančių rekreacinių vietovių vandens kokybę, remiantis melsvabakterių produkuojamų toksinų koncentracijomis;
4. įvertinti melsvabakterių produkuojamų metabolitų biotechnologinį potencialą ir reikšmę aplinkai.

Darbo naujumas

Tai pirmasis Kuršių marių ir pietrytinės Baltijos jūros pakrantės vandens kokybės vertinimo tyrimas, atliktas remiantis melsvabakterių bendrijų ir jų produkuojamų toksinų tyrimais. Taip pat tai pirmasis tyrimas, kuriame išsamiai aprašytas melsvabakterių produkuojamų metabolitų (įskaitant toksinus) paplitimas ir įvairovė Kuršių mariose ir Baltijos jūros pakrantėje. Tyrimo metu sudarytas 119 metabolitų variantų sąrašas. Apibūdinta 20 mikrocistinų variantų, iš kurių 3 gali būti potencialiai nauji variantai. Kuršių mariose aptiktų mikrocistinų įvairovė papildė informaciją apie jų geografinį paplitimą ir atskleidė galimą skirtingų melsvabakterių chemotipų egzistavimą. Atlikti pirmieji Kuršių mariose gyvenančių melsvabakterių ir eukariotinių mikrodumblių gaminamų metabolitų biologinio aktyvumo tyrimai.

Rezultatų mokslinė ir praktinė reikšmė

Tyrimo rezultatai papildė žinias apie melsvabakterių produkuojamų metabolitų įvairovę, jų erdvinį ir sezoninį pasiskirstymą Kuršių mariose ir Baltijos jūros priekrantėje. Toksinų aptikimas ir paplitimas Baltijos jūros priekrantėje suteikė galimybę geriau suprasti Kuršių marių vandens pernašos įtaką priekrantės vandens kokybei. Vertinant vandens kokybę remiantis tik melsvabakterių biomasės arba gausumo matavimais didėja tikimybė, jog rizika, susijusi su melsvabakterių aptikimu, bus nepakankamai įvertinta. Mikrocistinų koncentracijų matavimai, kaip papildomas parametras, galėtų užtikrinti tikslesnį maudyklų vandens kokybės įvertinimą. Tyrimo rezultatai yra svarbūs norint papildyti esamus nacionalinius ar regioninius teisės aktus, atsakingus už vandens kokybės stebėjimą ir vertinimą rekreacinėse zonose. Šis darbas taip pat parodė, kad melsvabakterės, gyvenančios Kuršių mariose, yra svarbus natūralių produktų šaltinis, turintis potencialų biotechnologinį pritaikomumą. Gauti rezultatai skatina tęsti tolesnius tyrimus, susijusius su Kuršių marių mikroorganizmais, jų produkuojamais metabolitais, struktūra bei aktyvumo ir panaudojimo galimybėmis.

REZULTATAI IR DISKUSIJA

Melsvabakterių ir jų produkuojamų metabolitų dinamika

Melsvabakterių įvairovės ir biomasės dinamika. Tiriamu laikotarpiu (2013–2017 m.) didžiausia melsvabakterių biomasė nustatyta Nidos stotyje: 2014 m. rugsėjo mėn. ji siekė 165,8 mg/l; likusiu laikotarpiu biomasė dažniausiai būdavo didesnė nei 7 mg/l

ir retai viršydavo 15 mg/l. Kitose tyrimų vietose melsvabakterių biomasė dažniausiai būdavo mažesnė nei 10 mg/l. Didžiausios melsvabakterių biomasės reikšmės nustatytos vasaros pabaigoje–rudenį.

Mėginiuose, kuriuose buvo gausiai randama melsvabakterių gaminamų metabolitų, dažniausiai aptiktos *Aphanizomenon*, *Dolichospermum* / *Anabaena*, *Microcystis*, *Planktothrix* ir *Woronichinia* gentys. *McyE* genai, pagausinti pradmenimis, būdingais *Dolichospermum* / *Anabaena*, *Microcystis* ir *Planktothrix* gentims, atskleidė, jog šios melsvabakterės yra potencialūs mikrocistinų produkuotojai. Kadangi *P. agardhii* yra vienintelė Kuršių mariose aptinkama *Planktothrix* genties rūšis, remiantis genetiniais tyrimais bent dalis šios populiacijos priklauso mikrocistinų produkuotojams.

Tyrimo laikotarpiu nustatyta, jog *Aph. flosaquae* buvo viena labiausiai vyraujančių rūšių mėginiuose, o jos dominavimas dažniausiai nustatytas vasaros pabaigoje–ankstyvą rudenį. Šie rezultatai sutampa su ankstesniais tyrimais, kuriuose nurodyta, jog *Aph. flosaquae* yra viena pagrindinių melsvabakterių rūšių, atsakingų už didžiausius ir ilgiausiai trunkančius melsvabakterių žydėjimus Kuršių mariose (Vaičiūtė ir kt., 2021; Olenina, 2012; Gasiūnaitė ir kt., 2008; Pilkaitytė ir Razinkovas, 2006). Kitos melsvabakterių gentys, t. y., *Microcystis*, *Planktothrix* ir *Dolichospermum*, taip pat buvo dažnai aptiktos mėginiuose. Nustatyta, kad *Microcystis* spp. dažniausiai dominuoja vasaros viduryje, *P. agardhii* – vėlyvą pavasarį ir vasaros pabaigoje–rudens pradžioje, o *Dolichospermum* spp. – pirmoje vasaros pusėje.

Bendra melsvabakterių biomasė (2018 m.) Baltijos jūros priekrantėje neviršijo 2,5 mg/l. Remiantis ilgo laikotarpio duomenimis, melsvabakterių biomasė rytinėje Baltijos jūros dalyje retai kada viršija 1 mg/l (Kownacka ir kt., 2018). *D. flosaquae*, *Aph. flosaquae*, *W. compacta*, *P. agardhii*, *M. wesenbergii* ir *N. spumigena* rūšys buvo dominuojančios Baltijos jūros priekrantėje.

Melsvabakterių produkuojamų metabolitų įvairovė ir pasiskirstymas. Iš viso Kuršių mariose aptikta 119 melsvabakterių produkuojamų metabolitų, kurie priklausė aštuonioms skirtingoms klasėms (mikrocistinai, anatoksinas, nodularinas, anabaenopeptinai, aeruginozinai, aeruginozamidas, cianopeptolinai, mikrogininai).

2013–2017 m. Kuršių mariose atliktų tyrimų metu aptikti 48 melsvabakterių produkuojami metabolitai: 10 mikrocistinų, 16 anabaenopetinų, 12 aeruginozinų, 1 aeruginozamidas, 3 cianopeptolinai, 4 mikrogininai, nodularinas ir anatoksinas. 2018 m. buvo aptikta 117 metabolitų. Nustatyta, kad cianopeptolinai buvo struktūriškai pati gausiausia peptidų klasė – iš viso aptikti 53 variantai. Kitų klasių metabolitai struktūriškai buvo 3–4 kartus mažiau gausesni: identifikuota 20 mikrocistinų, 18 mikrogininų, 14 anabaenopeptinų ir 13 aeruginozinų variantų. Tarp visų rastų mikrocistinų variantų, trys iš jų – *m/z* 1057, *m/z* 1075 ir *m/z* 1068 – potencialiai priklauso naujiems variantams.

Sezoninė mikrocistinų įvairovės ir koncentracijų kaita 2013–2017 m. buvo panaši, išskyrus Nidos ir Uosto stotis. MC-RR sudarė didžiausią bendrą mikrocistinų

koncentracijos dalį visose tirtose Kuršių marių stotyse. Nidos stotyje buvo stebimas MC-RR ir [Asp^3]MC-RR dominavimo pokytis: 2013 ir 2014 m. mėginiuose dominavo MC-RR variantas, o 2015–2017 m. – [Asp^3]MC-RR. Visose tirtose stotyse mikrocistinų koncentracijos vasaros ir rudens sezonais (birželio–spalio mėn.) svyravo 1–6 µg/l ribose, o Nidos stotyje dažniausiai buvo didesnės nei 6 µg/l. Didžiausia mikrocistinų koncentracija nustatyta 2014 m. spalio mėn. – 22,8 µg/l.

Kiti dažniausiai aptikti melsvabakterių produkuojami metabolitai 2013–2017 m. laikotarpiu buvo anabaenopetinai (AP-A, AP-B, AP-F ir Osc-Y). Šie variantai aptikti visose tirtose vietose, tačiau tarp tiriamų stočių atskiros jų klasės nepasižymėjo aiškiu dominavimu. Aueruginozinai, aeruginozamidas ir mikrogininai priklausė rečiau aptinkamiems metabolitams. Išvardintos metabolitų klasės buvo aptiktos visose tirtose vietose, išskyrus Uosto stotį, kurioje cianopeptolinų nebuvo aptikta. Nemaža mikrocistinų ir kitų metabolitų įvairovė bei sezoniniai pokyčiai rodo, kad melsvabakterių bendrijos rūšių ar padermių lygmenyje nėra homogeniškos. Tai reiškia, jog Kuršių mariose potencialiai gyvuoja kelios ar keliolika skirtingų melsvabakterių chemotipų.

Maudyklų vandens kokybės vertinimas priekrantės rekreaciniuose vandenyse

Melsvabakterių produkuojamų toksinų tyrimai Lietuvos Baltijos pajūrio zonoje atlikti pirmą kartą. Remiantis toksinų įvairove ir koncentracijomis Lietuvos pajūrio maudyklose, nustatyta, jog Kuršių marių vanduo, kuris yra daug produktyvesnis už Baltijos priekrantės vandenis, turi įtakos vandens kokybei priekrantės zonoje. Kuršių marių vanduo dėl tam tikrų susidariusių meteorologinių (t. y., vyraujant pietinių ir rytinių krypčių vėjams) ir hidrodinaminių (t. y., vyraujančių srovių) sąlygų yra išnešamas į pajūrio zoną. Tyrimo metu nustatyta, kad Melnragės stotis, esanti netoli Kuršių marių sąsiaurio, buvo nuolat veikiama melsvabakterių ir jų produkuojamų toksinų, būdingų Kuršių marių vandenims. Pagrindiniais indikatoriais buvo laikomi anatoksino aptikimas mėginiuose, *Dolichospermum* spp. rūšys bei gerokai didesnė mikrocistinų įvairovė. Iki šiol aprašyti tik keli anatoksino atvejai Baltijos jūroje, pvz., Suomijos įlankoje (Chernova ir kt., 2019) ir Gdansko įlankos pakrančių vandenyse (Mazur-Marzec ir kt., 2003). Kuršių marių vandens įtakos zona rugpjūčio mėn. siekė ir Palangos stotį, kurioje toksinų koncentracija šiek tiek išaugo, tačiau ne tiek daug, kiek Melnragėje. Remiantis Pasaulinės sveikatos organizacijos rekomendacijomis, Baltijos jūros ir Kuršių marių maudyklose nustatyta nedidelė neigiamo poveikio sveikatai tikimybė. Visgi pietinėje Kuršių marių (Lietuvos) dalyje aptiktos didesnės toksinų koncentracijos rekreaciniu atžvilgiu gali turėti didesnį potencialiai neigiamą poveikį sveikatai.

Kuršių marių fitoplanktono biomasės biotechnologinis potencialas

Siekiant išsiaiškinti, ar Kuršių marių fitoplanktonas, ypač melsvabakterės, gali būti potencialiai pritaikytos biotechnologijoje, buvo tiriami fitoplanktono ekstraktai naudojant 4 skirtingus biologinius testavimus – fermentinio aktyvumo, antibakterinį, citotoksinį ir ūmaus toksiškumo.

Antibakterinio testavimo rezultatai parodė, jog buvo slopinamas klinikinės bakterijų padermės *Staphylococcus aureus* ir iš nuotekų valymo įrenginių izoliuotos *Enterococcus faecium* augimas. Šie rezultatai atskleidė galimą fitoplanktono, ypač melsvabakterių, gebėjimą gaminti antibakterinius junginius bei jų panaudojimą mažinant patogeninių bakterijų augimą nuotekose. Aplinkos padermių *Aeromonas salmonicida* 2013 ir *Vibrio diazotrophicus* Cd1 augimo slopinimas atskleidė potencialų biologiškai aktyvaus metabolito gamintojo gynybinį mechanizmą, labai svarbų itin konkurencingoje aplinkoje, kurioje egzistuoja didžiulė bakterijų įvairovė. Tyrimo metu nustatyti ir priešingi rezultatai – nedidelis *V. cholerae* augimo stimuliavimas. Tai leidžia daryti prielaidą, kad melsvabakterių žydėjimas gali palaikyti potencialiai patogeniškų *Vibrio* rūšių augimą. Tokią sąveiką reikėtų stebėti kaip maudyklų vandens kokybės vertinimo elementą. Serino proteazių, tokių kaip tripsino, chimotripsino ir trombino aktyvumo slopinimas, taip pat krūties vėžio ląstelių T47D gyvybingumo mažinimas patvirtino Kuršių marių fitoplanktono natūralių produktų farmacinį potencialą. Mėginiuose, kuriuose nustatytas didžiausias slopinantis (inhibicinis) aktyvumas, gausiausiai buvo randami cianopeptolinai ir mikrogininai. Dėl šiame tyrime analizuotų lauko biomasės mėginių sudėtingumo negalima daryti patikimos išvados apie ryšį tarp stebimos veiklos ir konkretaus mėginio komponento.

IŠVADOS

1. Mėginiuose, kuriuose buvo gausiai randama melsvabakterių gaminamų metabolitų, dažniausiai aptiktos *Aphanizomenon*, *Dolichospermum* / *Anabaena*, *Microcystis*, *Planktothrix* ir *Woronichinia* gentys. *McyE* genai, pagausinti pradmenimis, būdingais *Dolichospermum* / *Anabaena*, *Microcystis* ir *Planktothrix* gentims, atskleidė, jog šios melsvabakterės yra potencialūs mikrocistinų produkuotojai. Kadangi *P. agardhii* yra vienintelė Kuršių mariose aptinkama *Planktothrix* genties rūšis, remiantis genetiniais tyrimais bent dalis šios populiacijos priklauso mikrocistinų produkuotojams.

2. Sezoniniai melsvabakterių tyrimai atskleidė, jog *Aph. flosaquae* dominuoja vasaros pabaigoje–ankstyvą rudenį, *Microcystis* spp. – vasaros viduryje, *P. agardhii* – vėlyvą pavasarį ir vasaros pabaigoje–rudens pradžioje, o *Dolichospermum* spp. – pirmoje vasaros pusėje. Iš visų tirtų Kuršių marių vietovių didžiausios melsvabakterių biomasės ir toksinų koncentracijos nustatytos piečiausioje Kuršių marių dalyje, kuri, lyginant su šiaurine tranzitine dalimi, yra nuolat apribota vandens mainų.

3. Tyrimo metu aptikta 119 melsvabakterių produkuojamų metabolitų, kurie buvo priskirti aštuonioms skirtingoms klasėms (MC, ANTX-a, NOD, AP, AER, AEG, CP, MG). Nustatyta, kad CP yra dominuojanti ir viena struktūriškai gausiausių cianopeptidų klasių. Cianometabolitų pasiskirstymo tyrimai Kuršių mariose parodė, kad cianopeptolinai Uosto stotyje nebuvo aptikti. Kitos cianometabolitų klasės, t. y., AER, AP, MG, buvo struktūriškai 3–4 kartus mažiau gausesnės, o tarp tiriamų stočių atskiros jų klasės nepasižymėjo aiškiu dominavimu. Taip pat tyrimo metu aptikta 20 mikrocistinų variantų. MC-RR sudarė didžiausią bendrą mikrocistinų koncentracijos dalį visose tirtose Kuršių marių stotyse. Nidos stotyje buvo stebimas MC-RR ir [Asp^3]MC-RR dominavimo pokytis: 2013 ir 2014 m. mėginiuose dominavo MC-RR variantas, o 2015–2017 m. – [Asp^3]MC-RR. Tarp visų rastų mikrocistinų variantų, trys iš jų – *m/z* 1057, *m/z* 1075 ir *m/z* 1068 – potencialiai priklauso naujiems variantams. Nemaža mikrocistinų įvairovė bei sezoniniai pokyčiai rodo, kad melsvabakterių bendrijos rūšių ar padermių lygmenyje nėra homogeniškos.

4. Baltijos jūros priekrantės maudyklose atlikti tyrimai parodė, kad vandens kokybė, remiantis melsvabakterių parametrais, atitinka mažą neigiamo poveikio sveikatai tikimybę. Visgi toksinų įvairovė ir koncentracijų kaita priekrantės zonoje yra susijusi su Kuršių marių vandenų poveikiu. Intensyvaus Kuršių marių vandenų ištekėjimo metu aukštesnė bendra toksinų koncentracija nustatyta šiaurinėje Lietuvos priekrantės dalyje. Potencialiai didesnė rizika sveikatai nustatyta pietinėje Kuršių marių dalyje. Rekomenduojama melsvabakterių biomasės riba (> 8 mg/l) buvo viršyta 10 % nuo visų tirtų mėginių.

5. Biologinio aktyvumo tyrimai parodė, jog fitoplanktono biomasės mėginiai, surinkti Kuršių mariose, pasižymėjo aukštu fermentiniu, antibakteriniu ir citotoksiškumo aktyvumu. Kalbant apie antibakterinius tyrimus, antibiotikams atsparios *E. faecium* 45, izoliuotos iš nuotekų valymo įrenginių, augimo slopinimas atskleidė galimą melsvabakterių panaudojimą mažinant patogeninių bakterijų augimą nuotekose. Be to, tyrimo metu išbandyti ekstraktai ir frakcijos atskleidė antibakterinį aktyvumą prieš klinikines, antibiotikams atsparias bakterijų padermes. Serino proteazių, tokių kaip tripsino, chimotripsino ir trombino aktyvumo slopinimas, taip pat krūties vėžio ląstelių T47D gyvybingumo mažinimas patvirtino Kuršių marių fitoplanktono natūralių produktų farmacinį potencialą. Aktyviose frakcijose cianopeptolinai ir mikrogininai sudarė dominuojančias peptidų klases.

6. Tyrimo metu tirti fitoplanktono ekstraktai ir frakcijos atskleidė galimą mitybinį ir gynybinį poveikį. Fitoplanktonas, ypač melsvabakterės, gali palaikyti *V. cholerae* bakterijų augimą. Taigi tokią sąveiką (*V. cholerae* ir melsvabakterių) reikėtų stebėti kaip maudyklų vandens kokybės vertinimo elementą. Aplinkos bakterijų (*A. salmonicida* 2013 ir *V. diazotrophicus* Cd1) slopinimas gali rodyti gamintojo gynybinę strategiją, siekiant padidinti jų išgyvenamumą itin konkurencingoje aplinkoje.

9

Publications

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Spatial and Temporal Diversity of Cyanometabolites in the Eutrophic Curonian Lagoon (SE Baltic Sea)

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Abstract: This work aims to determine the profiles of cyanopeptides and anatoxin synthetized by cyanobacteria in the Lithuanian part of the Curonian Lagoon (SE Baltic Sea) and to characterize their spatial and temporal patterns in this ecosystem. Cyanometabolites were analysed by a LC-MS/MS system and were coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer. During the investigation period (2013–2017), 10 microcystins, nodularin, anatoxin-a, 16 anabaenopeptins, including 1 oscillamide, 12 aeruginosins, 1 aeruginosamide, 3 cyanopeptolins and 4 microginins were detected. The most frequently detected metabolites were found at all investigated sites. Demethylated microcystin variants and anabaenopeptins had the strongest relationship with *Planktothrix agardhii*, while non-demethylated microcystin variants and anatoxin had the strongest relationship with *Microcystis* spp. Low concentrations of some microcystins: [Asp^3]MC-RR, MC-RR, MC-LR, as well as a few other cyanopeptides: AP-A and AEG-A were found during the cold period (December–March). Over the study period, *Aphanizomenon*, *Planktothrix* and *Microcystis* were the main dominant cyanobacteria species, while *Planktothrix*, *Microcystis*, and *Dolichospermum* were potentially producers of cyanopeptides and anatoxin detected in samples from the Curonian Lagoon.

Keywords: bioactive cyanometabolites; cyanopeptides; *Aphanizomenon*; *Planktothrix*; *Microcystis*; cyanobacteria; lagoon

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1. Introduction

Cyanobacteria are prokaryotes found in a wide variety of aquatic environments. They grow at high densities, forming 'blooms' that increase in extension and frequency, and follow anthropogenic activities and climate changes [1–3]. In temperate regions, cyanobacteria 'blooms' usually occur during the summer and autumn periods, while some cyanobacteria can also be found in winter and spring [4–7]. Cyanobacteria blooms are composed of toxic and nontoxic organisms representing different taxonomic units. Cyanobacteria species from at least 16 different genera, belonging to the orders *Oscillatoriales*, *Nostocales*, *Chroococcales*, and *Synechococcales* are considered as the main bloom forming species, and more than 100 different cyanobacteria taxa (species or genera) produce toxins [8–10]. The mixtures of various secondary metabolites are detected during naturally occurring cyanobacteria 'blooms'. These include low molecular weight toxins, such as anatoxins, saxitoxins, β-methylamino-L-alanine (BMAA) and cylindrospermopsin, and a wide array of oligopeptides, termed cyanopeptides, including microcystins [11]. To date, 2031 cyanobacterial metabolites have been described, among which approximately 65% are peptides [10]. The majority of these peptides range from 400 to 1900 Da, but most of them are present in the 1000–1100 Da range. Cyanopeptides consist of cyclic and linear compounds [10]. Amongst the bioactive cyanopeptides, microcystins are the most geographically widespread and are not restricted to any climatic zone or geographic range [12]. Over 286 microcystin variants have been identified and structurally characterized so far [13,14]. Other peptide classes

include anabaenopeptins, aerucyclamides, aeruginosins, and microginins [11,12,15]. The vast majority of cyanopeptides studies are related to compounds produced by the isolated strains of cyanobacteria, and the diversity of metabolites naturally present in field samples is less frequently investigated. Moreover, few studies have focused on the presence of cyanobacteria and cyanometabolites in water bodies during the winter period.

Toxic cyanometabolites, especially hepatotoxins, neurotoxins and cytotoxins, might represent health and ecological risks [16]. Hepatotoxins accumulate in liver cells via the organic anion transport system [17]. Microcystins inhibit protein phosphatase and cause oxidative stress, genotoxicity, and the abnormal function of hepatocytes leading to liver damage [17,18]. Neurotoxins are potent toxins that may rapidly disrupt the functioning of the whole organism or its specific organs leading to muscle paralysis, respiratory arrest, and rapid death [17,18]. The naturally occurring cyanobacteria and their toxins are widely used for water quality assessment [19]. However, data on the diversity, occurrence, (eco)toxicology, and effects of many other cyanometabolites on both recreational areas and drinking water supplies are lacking [10,11]. On the other hand, there is a growing interest in the biological activity of these compounds and their biotechnological application. Many of these compounds show cytotoxic, enzyme inhibiting, anti-inflammatory, anti-microbial or anti-fungal activity [20–22]. For example, aeruginosins and cyanopeptolins are serine protease inhibitors, anabaenopeptins inhibit the activity of protein phosphatases, elastase, and carboxy peptidase A, and microginins inhibit amino protease [23]. For this reason, expanded knowledge about the structural diversity of cyanometabolites produced in the Curonian Lagoon is important and can help to direct further studies on their potential application.

Due to the connection with Baltic Sea, the shallow temperate Curonian Lagoon (SE Baltic Sea) experiences a mass occurrence of cyanobacteria representing both brackish and freshwater species [24,25]. In total, about 100 cyanobacteria species have been detected in the Curonian Lagoon [6]. Cyanobacteria blooms typically occur during the summer–autumn period causing a serious threat to aquatic ecosystems and humans using water bodies for recreational purposes [3,4,26]. However, cyanobacteria can also be found during the winter–spring period [6], so also at this time certain effects on aquatic organisms can be exerted [5,27]. To date, studies on cell-bound cyanometabolites in the Curonian Lagoon have been episodic, focusing mainly on microcystins and anatoxin during the summer–autumn periods [28–34], and there are no published data on the diversity of cyanometabolites throughout all seasons. Therefore, the aim of this work was to present a comprehensive long term and all-season inventory of cyanometabolites found in the Curonian Lagoon and to assess the link between the composition of the cyanobacterial community and the cyanometabolites.

2. Materials and Methods

2.1. Study Area

The Curonian Lagoon is a large (surface area 1584 km^2) and shallow (mean depth ~3.8 m) estuarine lagoon in the south-eastern (SE) Baltic Sea. The lagoon is mainly a freshwater body connected with the sea by a narrow Klaipėda Strait; brackish water intrusions enter the lagoon irregularly and the salinity in the northern part fluctuates from 0 to 7 psu [35]. The highest annual average salinities of 3–5.5 psu are determined in the Klaipeda Strait. It gradually decreases towards the south and reaches a freshwater salinity level (0.5 psu) at a distance of approximately 35 km from the sea entrance [35]. The highest water temperature is observed in July–August with a monthly average of 20.2 °C [36].

In the Curonian Lagoon, five stations were selected, representing different hydrological conditions. Station 1 (Nida) is situated in the sheltered zone of the central part of the lagoon (Figure 1); the Juodkrantė (St. 2) and Dreverna (St. 3) stations represent the transitional area of the lagoon and the former is situated in the western part and the latter is situated in the eastern part; the Ventė (St. 4) station is strongly influenced by the runoff

of the Nemunas River; Station 5, situated in the strait, has the largest influence of the Baltic Sea water (Figure 1).

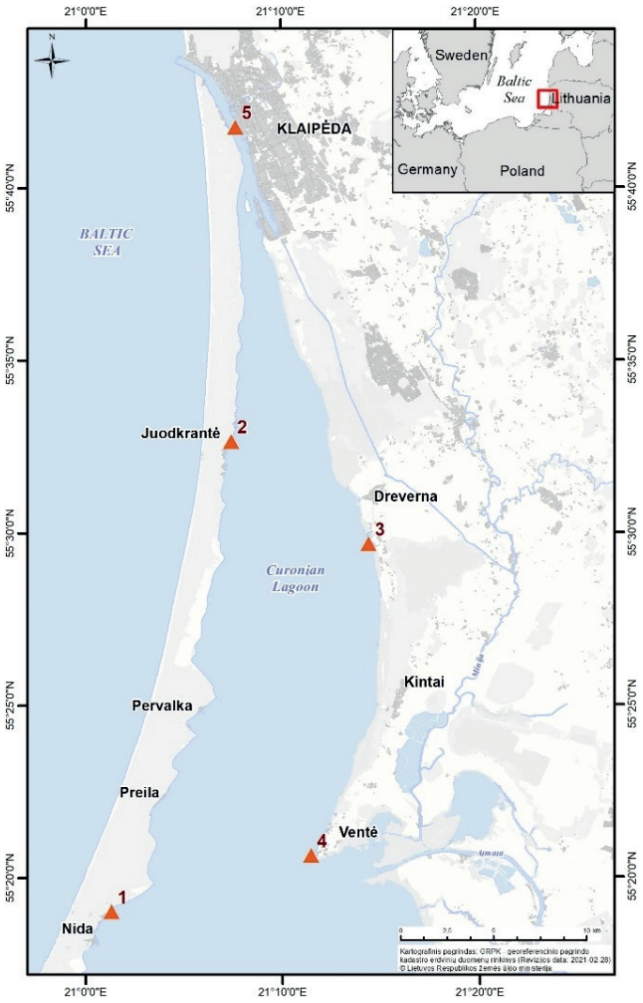

Figure 1. Study sites in the Curonian Lagoon. The numbers indicate the sampling stations: Station 1—Nida, Station 2—Juodkrantė, Station 3—Dreverna, Station 4—Ventė, Station 5—Smiltynė.

2.2. Sampling and Measurement of Environmental Parameters

Sub-surface samples were collected every 1–3 weeks in the Lithuanian part of the Curonian Lagoon. In 2013, samples were collected from August to October, and in 2014 from July to November (63 samples in total) during the cyanobacteria vegetation period

(stations 1–4), while from 2015 (starting from October) to 2017 samples were collected all year round (64 samples in total) at two stations (St. 1 and St. 5) (Figure 1). Phytoplankton samples were fixed with acidic Lugol's iodine solution and analysed using the Utermöhl method up to species or a higher taxonomic level; phytoplankton abundance and biomass (wet weight) according to the closest geometrical shape were calculated based on HELCOM recommendations [37]. Physical water parameters: water temperature (in situ using Eco-Sense® ODO200) and salinity (after transporting samples into the laboratory, using Mettler Toledo, FiveEasy Cond meter F30) were measured during each sampling. Ice presence/absence was recorded as well. Chlorophyll a (Chl a), including all phytoplankton taxa, was measured in situ using a Fluorometer (FluoroProbeII, bbe Moldaenke).

2.3. Analysis of Cyanometabolites

For the analyses of cyanometabolites, water samples of 100–1000 mL (depending on algae density) were filtered on GF/F filters (ø 47 mm). The material on the filter was extracted with 75% methanol (3 mL) and sonicated with an ultrasonic disruptor for 1 min (HD 2070 Sonopuls, Bandeline, Berlin, Germany; 20 kz, 25% duty cycle) [38], followed by a 10 min sonication in a water bath (Sonorex, Bandelin, Berlin, Germany) [39]. After centrifugation at 12,000 *g* for 15 min the supernatant was separated and analysed with a Agilent HPLC system (Agilent technologies, Waldboronn, Germany) coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer QTRAP LC-MS/MS (QTRAP5500, Applied Biosystems, Sciex; Canada) as described in [40]. Peptides were separated on a Zorbax Eclipse XDB-C18 column (4.6 × 150 mm; 5 µm) (Agilent Technologies, Santa Clara, California, USA) by gradient elution with a mixture of 5% acetonitrile in water and 100% acetonitrile, both with 0.1% formic acid. To determine the profile of peptides in the samples, an information dependent acquisition mode (IDA) was used. The structure of the detected peptides was suggested based on collected fragmentation spectra and the spectra published by other authors (e.g., [20,41,42], (Table S1)), while their relative quantities were estimated based on peak areas in the LC-MS chromatogram. For the quantitative analysis of microcystins, nodularin (NOD), and anatoxin-a, for whom standards were available, the multiple reaction monitoring mode (MRM) with the following transitions was used: 1038 → 135q (q-quantifier), 1038 → 103 (dmMC-RR), 1024 → 135q, 1024 → 105 (MC-RR), 1045 → 135q, 1045 → 213 (MC-YR), 981 → 135q, 981 → 213 (dmMC-LR), 995 → 135q, 995 → 213 (MC-LR), 910 → 135q, 910 → 213 (MC-LA), 1002 → 135q, 1002 → 446 (MC-LY), 1025 → 135q, 1025 → 446 (MC-LW), 986 → 135q, 986 → 376 (MC-LF), 825 → 135q, 825 → 389, 825 → 227 (NOD); 166 → 149q, 166 → 131, 166 → 107, 166 → 91 (ANTX-a).

2.4. Data Analyses

For phytoplankton data analysis, potentially toxic cyanobacteria species were pooled into main genera: *Aphanizomenon* (*A. flosaquae*, *A. gracile*, *A. issatschenkoi* (=*Cuspidothrix issatschenkoi*), *Dolichospermum* (*D. affine, D. circinale, D. crassum, D. flosaquae, D. lemmermannii*), *Microcystis* (*M. aeruginosa*, *M. flosaquae*, *M. viridis*, *M. wesenbergii*), and *Woronichinia* (*W. compacta* and *W. naegeliana*).

The redundancy analysis (RDA) was applied using Brodgar (2.7.5) and R (3.3.3) packages to test the relationships between the abiotic factors (water temperature, salinity), the biomass of the different cyanobacteria species (used as explanatory variables) and cyanometabolites produced by cyanobacteria as response variables. All response and biological explanatory variable data were square root-transformed. The Brodgar generated RDA biplots that were interpreted were based on explanatory factor line directions and lengths and response variable lines [43]. Only the most abundant cyanometabolites (found in more than 12% of the samples and NOD) were applied for the statistical analyses.

3. Results

3.1. Abiotic Conditions

The highest water temperature was recorded in July 2014 (26.8 °C, St. One, Figure 2). The average annual water temperature during spring (March–May) was 9 ± 5.8 °C, summer (June–August)—21 ± 2.5 °C, autumn (September–October)—13 ± 4.4 °C, while during winter (December–February) the water temperature was 2 ± 2.5 °C. Ice cover was observed in January–February 2016 (St. One, Figure 2).

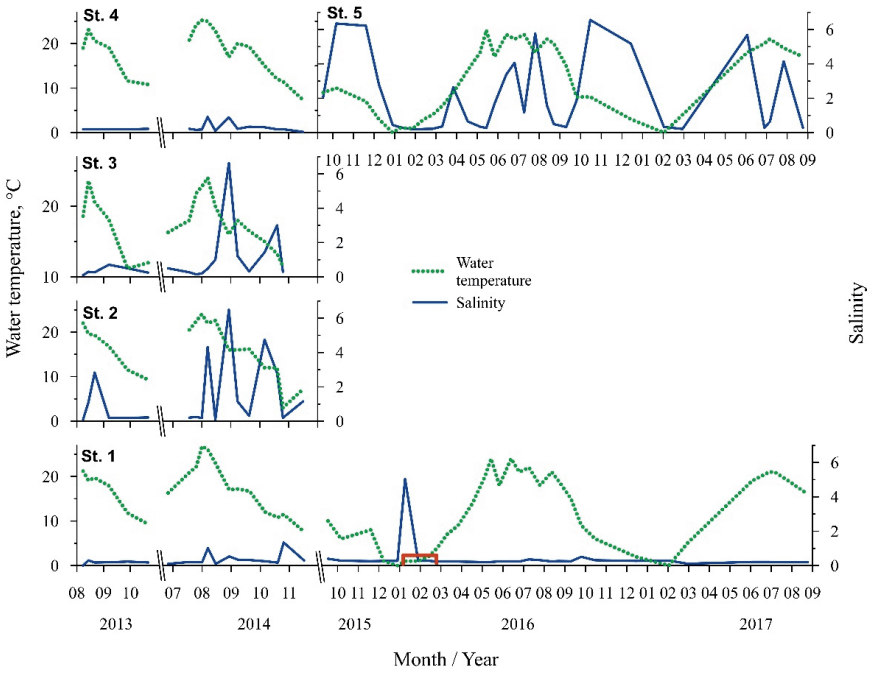

Figure 2. Seasonal fluctuation of water temperature and salinity at different stations. Red line on the axes indicates the presence of ice cover in the Curonian lagoon.

The Baltic Sea water intrusions into the Curonian Lagoon were defined by salinity changes and periodically observed at the stations near the Klaipeda strait. Salinity fluctuations at St. Two and St. Three were dynamic during the summer–autumn period (ranged from 0.1 to 6.6), while at St. Five the fluctuations occurred very often and periodically throughout all the periods of investigation (from 0.2 to 6.6 psu) (Figure 2).

3.2. Diversity of Cyanometabolites at the Study Sites

In total, 48 variants of cyanobacteria metabolites were detected in the Curonian Lagoon during the study period. The compounds belonged to six different peptide classes: microcystins (MC) (including nodularin (NOD), anabaenopeptins (AP), aeruginosines (AER), cyanopeptolins (CP), microginins (Mg), aeruginosamide (AEG), and one alkaloid class—anatoxin (ANTX) (Table S1). Microcystins, anabaenopeptins, and aeruginosins accounted for the highest variety of cyanopeptides, while the lowest variety was observed

for the remaining ones. The lowest numbers of peptides were found in the samples collected from the stations influenced by the Nemunas River (St. Four; 21 compounds) and the Baltic Sea (St. Five; 23 compounds), while the highest numbers were detected at the stations situated along the western coast of the lagoon (St. One and St. Two; 35 and 31 compounds, respectively) (Table S1). The highest diversity of metabolites (40 compounds) was detected in 2014.

3.2.1. Station One (Nida)

In total, 34 metabolites were found at St. One with a maximum of 19 different compounds per sample (Figure 3a, Table S1). The highest diversity of cyanometabolites, including the highest number of aeruginosins (up to 10 variants), was observed in 2014. The most common metabolites throughout the study were AEG-A and AP-A (70% of all samples), Osc-Y (58%), AP-B, AP-F, and AP662 in 43% all samples (Table S1).

Nine microcystins and anatoxin-a were found at Station One during the whole study period (Table S1). Evident of the dominance of microcystin MC-RR was observed in 2013 and July 2014, while [Asp^3]MC-RR dominated the rest of time. MC-RR was found in all the samples, while [Asp^3]MC-RR was found in 67% of the samples (Figure 3b). The highest concentrations of these variants were measured during August–October: MC-RR—15.61 μg L^{-1} in 2013, [Asp^3]MC-RR—22.19 μg L^{-1} in 2014 and 18.77 μg L^{-1} in 2017. The lowest concentrations of MC-RR (up to 0.11 μg L^{-1}) were detected during the January–March period. Microcystins MC-LR and [Asp^3]MC-LR were found in 83% and 35% of the samples, respectively, but at low concentrations (<1.5 μg L^{-1}). Microcystin MC-YR was found in all of the samples except during the cold period (October 2015–April 2016 and December 2016–March 2017), and the highest concentration was detected in 2016 (0.72 μg L^{-1}). Microcystins MC-LY, MC-LF, [Asp^{3}MeSer^7]MC-RR, and [Dha^7]MC-HtyR were found very rarely during the study period (<4% of the samples). Anatoxin ANTX-a was found in July–October in 2013, 2014, and 2016; its concentrations ranged from 0.01 μg L^{-1} to 0.25 μg L^{-1}.

The highest cyanobacteria biomass (166 mg L^{-1}) and Chl *a* (1065 μg L^{-1}) was recorded in September 2014 (Figure 3c). *Microcystis* spp. and *Planktothrix agardhii* were found in all of the samples, including the winter period. *P. agardhii* constituted about a quarter of the total cyanobacteria biomass on average. The highest biomass of *P. agardhii* was recorded in October 2014 (8.3 mg L^{-1}), while the *Microcystis* spp. biomass was only 3.2 mg L^{-1} (July 2014). These cyanobacteria accounted for 26% and 12%, respectively of the total cyanobacteria biomass on average. *Aphanizomenon* spp. was found in 80% of the samples and accounted for more than 90% of the total cyanobacteria biomass in September–October. *Aphanizomenon* was more abundant in 2013–2014 as compared to all the investigated periods. The highest biomass was recorded in September 2014 (160.4 mg L^{-1}). *Woronichinia* was found in 97.9% of the total samples; the largest part of the total cyanobacteria biomass occurred in October–March. The highest biomass of *Woronichinia* was observed in October 2015 (6.9 mg L^{-1}).

3.2.2. Station Two (Juodkrantė)

In total, 31 cyanometabolites were found at St. Two with a maximum of 22 different compounds per sample in 2014 (Figure 4a, Table S1). In terms of toxic cyanometabolites, ten different variants were detected: eight microcystins, anatoxin, and nodularin. Microcystins [Asp^3]MC-RR and MC-RR were predominant and found in all of the samples (Figure 4b). The highest concentration of MC-RR was measured in August 2013 (10.20 μg L^{-1}) while the highest concentration of [Asp^3]MC-RR was measured in August (4.35 μg L^{-1}) and September 2014 (4.85 μg L^{-1}). MC-YR also was found in all of the samples; however, its concentrations did not exceed 1 μg L^{-1}. In comparison to other microcystins, MC-LR and [Asp^3]MC-LR were less abundant (88% and 63% of the samples, respectively) and their concentrations did not exceed 0.70 μg L^{-1}. Microcystins [Asp^3]MC-RY, [Asp^{3}MeSer^7]MC-RR, and [Dha^7]MC-HtyR were found very rarely over the study period (<13% of the samples)

(Table S1). Nodularin was detected in September–November 2014; the concentrations varied from 0.06 to 0.12 μg L^{-1}. ANTX-a was detected in half of the samples, with the highest concentration (0.51 μg L^{-1}) measured in August 2013.

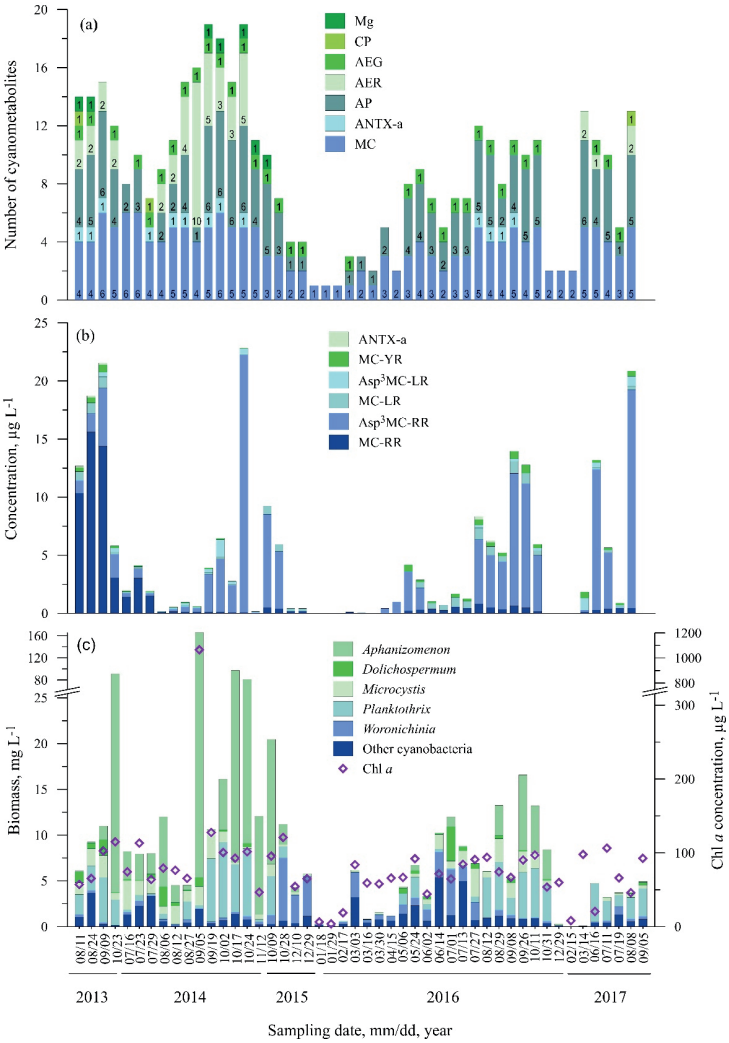

Figure 3. Seasonal changes in the number of cyanometabolites (**a**), concentration of microcystin and anatoxin variants (**b**), cyanobacteria biomass and Chl a concentration (**c**) at St. 1.

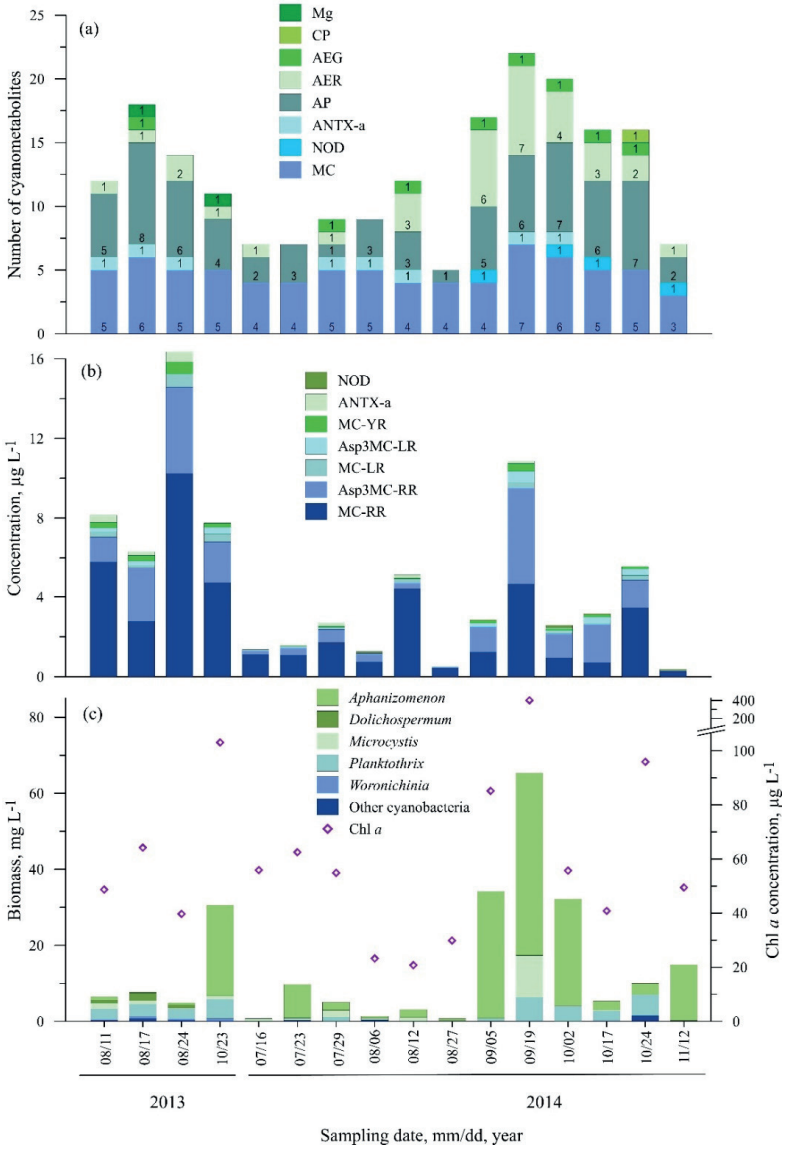

Figure 4. Seasonal changes in the number of cyanometabolites (**a**), concentration of microcystin and anatoxin variants (**b**), cyanobacteria biomass and Chl *a* concentration (**c**) at St. 2.

In terms of other cyanopeptides, 10 anabaenopeptin variants were detected (Table S1). The most common were Osc-Y (found in all of the same samples, except November 2014) and AP-A (found in 81.3% of the samples). Other metabolites were much less common: AP-F, AP-B, and AER 126A were found in 56.3% of the samples, AEG-A and AP676 were found in 50% of the samples (Table S1). Mg 591B was found twice in August and October 2013, while CP978 was found once in October 2014.

The highest phytoplankton biomass and Chl *a* concentrations were measured in September 2014 (65.3 mg L^{-1} and 402.5 μg L^{-1}, respectively), when *Aphanizomenon* dominated (47.7 mg L^{-1}) (Figure 4c). Compared to other cyanobacteria, *Aphanizomenon* constituted the highest contribution to the total cyanobacteria biomass (up to 97% of the total cyanobacteria biomass). *Microcystis* spp. and *P. agardhii* were found in all of the samples. *Microcystis* constituted from 0.2% to 87.4% of the total cyanobacteria biomass, and the highest biomass was measured in September 2014 (10.9 mg L^{-1}). *P. agardhii* constituted up to 63.8% of the total biomass and reached the maximum of 6.3 mg L^{-1} in September 2014 as well. *Woronichinia* was found mainly in 2013, and its highest biomass was observed in October 2013 (0.55 mg L^{-1}).

3.2.3. Station Three (Dreverna)

In total, 27 cyanometabolites were found at St. Three with a maximum diversity of 16 different compounds per sample (Figure 5a, Table S1). Microcystin MC-RR was detected in all of the samples and the highest concentration was measured in October 2014 (5.7 μg L^{-1}) while [Asp^3]MC-RR reached its maximum in September 2013 (1.78 μg L^{-1}) (Figure 5b). Microcystins MC-LR and MC-YR were found in 85.7% and 50% of the samples, respectively; their concentrations did not exceed 0.5 μg L^{-1} (Table S1). Microcystins [Asp^3]MC-LR, [Asp^3]MC-RY and nodularin were rarely found over the investigated period, and the concentrations of each of these variants did not exceed 0.17 μg L^{-1}. ANTX-a was detected in 43% of the samples; the highest concentration was measured in September 2014 (0.18 μg L^{-1}) (Figure 5b).

In terms of other cyanopeptides, nine anabaenopeptins and six aeruginosins were found. The most common anabaenopeptins were AP-A, Osc-Y, and AP-F found in 42.3%, 42.3% and 35.7% of the samples, respectively. The Mg 591B was found in September 2013 and from September to October 2014, while CP978 was found only in August 2014.

Station Three is characterised by the lowest phytoplankton biomass and Chl *a* concentration (Figure 5c). The highest Chl *a* concentration was recorded in October 2014 (76 μg L^{-1}), while the highest cyanobacteria biomass (10.5 mg L^{-1}), dominated by *Aphanizomenon* (9.2 mg L^{-1}), was recorded in early September 2014. During the study period, the total biomass of *P. agardhii* did not exceed 1.8 mg L^{-1}, except September 2013 (4.5 mg L^{-1}).

3.2.4. Station Four (Ventė)

Out of all of the investigated stations, the lowest variety of cyanometabolites was found in samples from Station Four, where only 21 compounds in total and a maximum of 14 different metabolites per sample were detected (Figure 6a). The MC-RR variant constituted the largest contribution to the overall bioactive cyanometabolite concentration (Figure 6b). The highest concentration of this microcystin was measured in October 2014 (7.90 μg L^{-1}). The MC-LR and MC-YR variants were also common. The highest concentrations were measured in October 2014 (0.65 μg L^{-1}) and in September 2013 (0.17 μg L^{-1}), respectively. The predominance of [Asp^3]MC-RR was observed only in September 2013 and its concentration reached 1.40 μg L^{-1}. Microcystin [Dha^7]MC-HtyR, ANTX-a and nodularin were found rarely over the investigated period; the concentrations of ANTX-a and nodularin did not exceed 0.08 μg L^{-1} (Figure 6b).

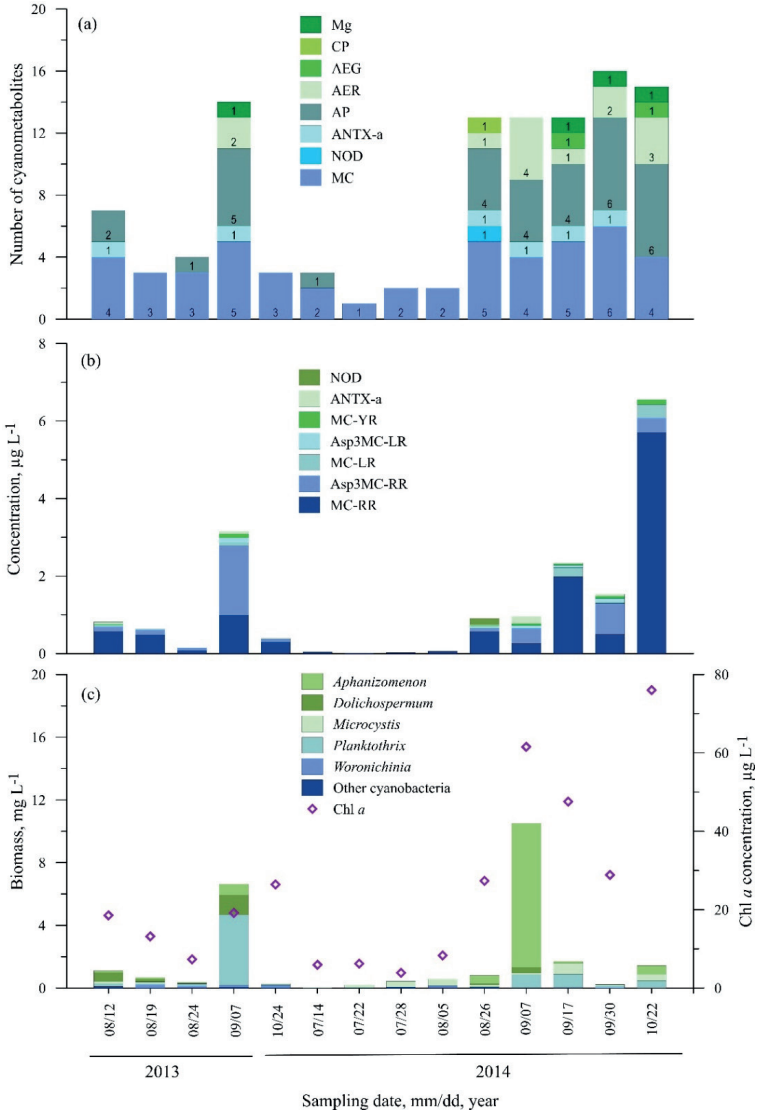

Figure 5. Seasonal changes in the number of cyanometabolites (**a**), concentration of microcystin and anatoxin variants (**b**), cyanobacteria biomass and Chl *a* concentration (**c**) at St. 3.

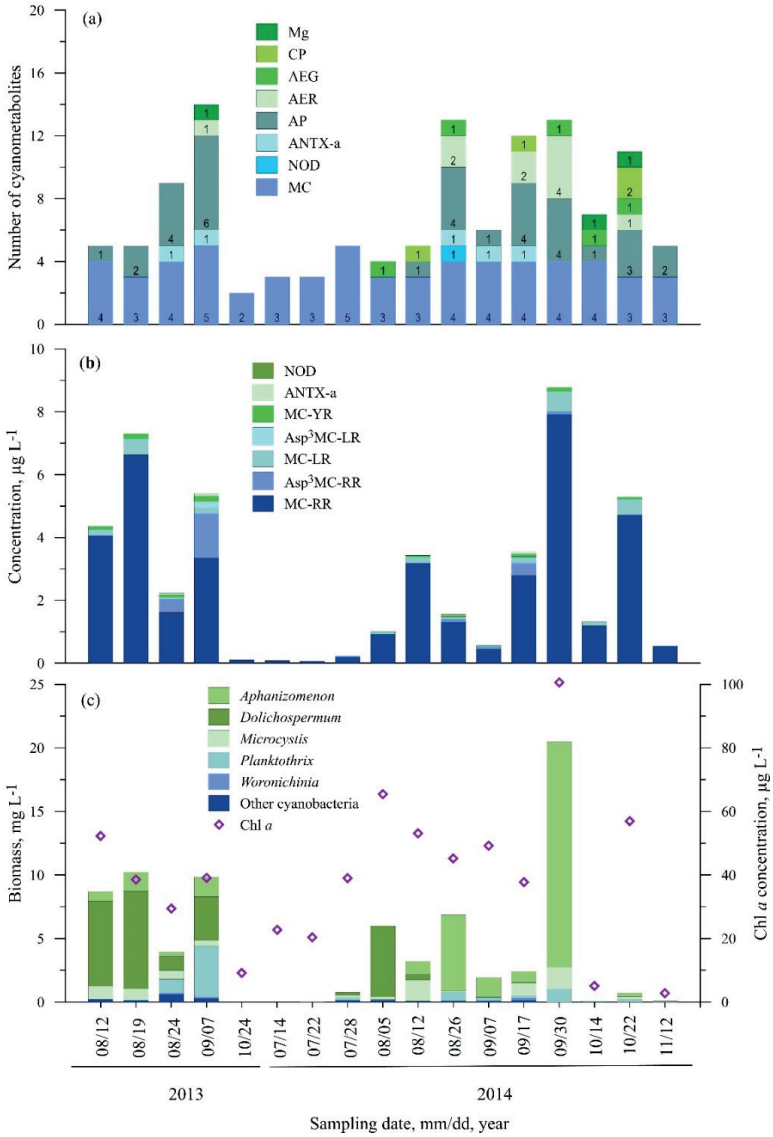

Figure 6. Seasonal changes in the number of cyanometabolites (**a**), concentration of microcystin and anatoxin variants (**b**), cyanobacteria biomass and Chl *a* concentration (**c**) at St. 4.

Among other cyanopeptides, anabaenopeptins were the most dominant compounds—in total, seven anabaenopeptin variants were detected (Figure 6a). The most found anabaenopeptins were AP662, AP676 and Osc-Y (47.1%, 35.3% and 35.3% of the samples, respectively). The aeruginosin and cyanopeptolin variants were found only in 2014.

Aphanizomenon and *Microcystis* were found in 88.2% of the samples (Figure 6c). The highest biomass of *Aphanizomenon* was measured in October 2014 (17.7 mg L^{-1}) and this coincided with the highest Chl *a* concentration (101 μg L^{-1}). The biomass of *Microcystis* was much lower compared to *Aphanizomenon* and did not exceed 2.0 mg L^{-1}. *Dolichospermum* was found in 58.8% of the samples. The highest biomass of this genus was measured in August–September 2013 and early August 2014 (about 7.7 mg L^{-1}). *P. agardhii* was found in 76.5% of the samples, and the highest biomass was measured in September 2013 (4.0 mg L^{-1}).

3.2.5. Station Five (Smiltynė)

A relatively low diversity of cyanometabolites (23) was also characteristic for Station Five; in total six different microcystin variants were detected (Table S1). The microcystin [Asp^3]MC-RR variant dominated and was found in 46.8% of the samples (Figure 7b). The highest concentrations of [Asp^3]MC-RR were recorded in the autumn months: October 2015 (9.04 μg L^{-1}), September 2016 (7.48 μg L^{-1}), and September 2017 (8.13 μg L^{-1}). The MC-RR variant was more common. It was found in 72% of the samples; however, the concentrations did not exceed 0.56 μg L^{-1}. MC-LR and [Asp^3]MC-LR were rarely found (31% and 25% of the samples, respectively); the highest concentrations of these variants were measured accordingly in August 2016 (0.29 μg L^{-1}) and July 2017 (0.49 μg L^{-1}). Nodularin was found only during the short period from July to August 2016, and its concentration ranged from 0.02 to 0.28 μg L^{-1}. ANTX-a was found only in 2016 from August to mid-October with concentrations ranging from 0.02 to 0.19 μg L^{-1}.

In terms of other cyanopeptides, AEG-A, AP-A and Osc-Y were the most common compounds (53.1%, 43.8%, and 40.6% of all samples, respectively). No metabolites were found from late October 2015 to May 2016 except from AEG-A which was detected in December (Figure 7a, Table S1).

Planktothrix and *Woronichinia* were the most common cyanobacteria species at Station Five (100% and 96.9% of the samples, respectively) (Figure 7c). The highest biomasses of *Woronichinia* and *Planktothrix* were recorded accordingly in late December 2015 (5.2 mg L^{-1}) and June 2017 (4.4 mg L^{-1}). *Aphanizomenon* was found in 75% of samples and was the dominant species during the August–October months. The highest biomass was measured in October 2015 (11.6 mg L^{-1}) and in September 2017 (9.7 mg L^{-1}). The highest Chl *a* concentration (92.3 μg L^{-1}) was recorded in October 2015.

3.3. Statistical Analysis

According to the RDA analysis, the environmental factors in combination with the dominant cyanobacteria biomass accounted for 52% of the variance in the concentration of cyanometabolites from the samples taken from the Curonian Lagoon, while two axes explained 83% of the variation (Figure 8). Demethylated variants of MC ([Asp^3]MC-RR and [Asp^3]MC-LR) had a close statistical relationship with *P. agardhii* distribution. The occurrences of AP-A, AP-B, AP662, and Osc-Y were also closely associated with the presence of *P. agardhii*. This species explains 57.33% ($F = 52$, $p = 0.001$) of the variation in the concentrations of the metabolites in the lagoon.

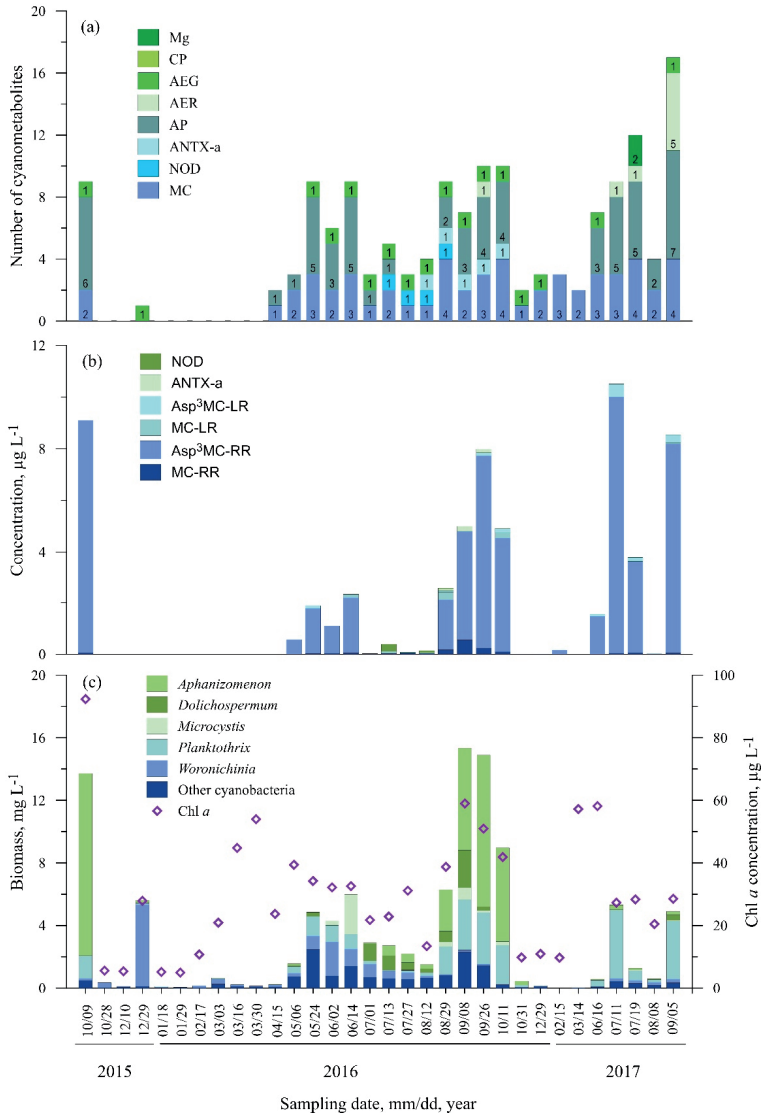

Figure 7. Seasonal changes in the number of cyanometabolites (**a**), concentration of microcystin and anatoxin variants (**b**), cyanobacteria biomass and Chl *a* concentration (**c**) at St. 5.

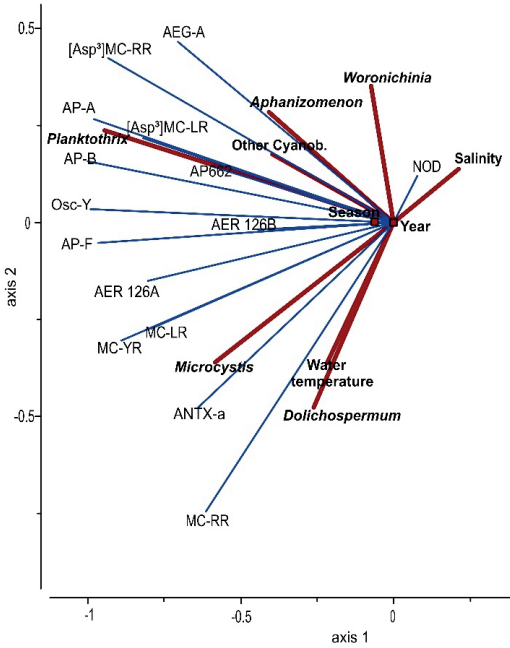

Figure 8. The distribution of the NRPs (response variables, thin blue lines) in relation with the main potentially toxic cyanobacteria and abiotic factors (explanatory variables, solid red lines).

Bioactive cyanopeptides (MC-RR, MC-LR, MC-YR) and anatoxin ANTX-a were closely associated with the occurrence of *Microcystis* spp., *Dolichospermum* spp., and water temperature. All statistical relationships were statistically significant ($p < 0.05$), however, *Microcystis* spp. explains 27.73% (F = 14), *Dolichospermum* spp.—10.02% (F = 3) and water temperature—8.67% (F = 7) of the variation in peptide concentrations. AEG-A had the closest relationship with the genus *Aphanizomenon*, while the contribution of *Aphanizomenon* to the variation was 10.76% (F = 5, $p < 0.05$).

The presence of nodularin in the Curonian Lagoon was very closely connected to the presence of brackish water, which explains 7.17% (F = 6, $p = 0.001$) of the data distribution; and both of them were negatively related with the water temperature. The impact of different years and *Woronichinia* had small though statistically significant contributions to the variation of data (contribution 10.45%, F = 6, $p = 0.001$ and contribution 3.57%, F = 3, $p < 0.05$, accordingly).

4. Discussion

In the present study, we provide the first detailed description of the occurrence and the diversity of cyanopeptides and anatoxin in the Curonian Lagoon (Lithuanian part). Cyanobacteria usually produce toxins and other secondary cyanometabolites as a potential defence mechanism against surrounding aquatic organisms. Cyanometabolites could inhibit the growth of some bacteria and can be toxic to zooplankton ([11,23] and references therein). As many of the compounds are biologically active and frequently toxic, they can pose a risk to the aquatic ecosystem and human health. The problem is global, therefore extended knowledge about cyanobacteria occurrence and metabolic diversity is

needed [44]. Studies on cyanobacteria have been carried out in the Curonian Lagoon for many years, but they have been limited to the structure of the cyanobacterial community and the occurrence of microcystins, nodularin and anatoxin [28,30]. In terms of water quality, the concentrations of microcystins are the parameters for which, according to the WHO and EPA, the recreational values of water bodies are assessed [17,45]. However, other metabolites such as cyanopeptolins, microginins, aeruginosines sometimes have equivalent or even stronger bioactive effects compared to microcystins [11]. Moreover, the water quality in coastal areas can be affected by the water outflows from the eutrophic estuaries/lagoons [32,46]; therefore, an assessment of the diversity and seasonal variation of cyanometabolites can help to improve the estimation of associated risks.

In total, 48 variants of cyanometabolites belonging to seven different classes were identified. Microcystins were the most frequently found cyanopeptides in the Curonian Lagoon, with MC-RR, MC-LR, MC-YR, and [Asp^3]MC-RR being the most common. A similar pattern of microcystin variants has been observed in previous studies conducted in the Curonian Lagoon [28,30–32] and in other freshwater bodies, e.g., in Latvia [47,48], Russia [49], Poland [40,50], Greece [51,52], Bulgaria [53], Estonia [54], France [55], Germany [56], USA [57] or in estuaries and coastal waters ([46,49,58,59] and references therein). According to Mantzouki et al. [60], MC-YR, MC-LR and dmMC-LR variants are the most frequent in European freshwater bodies, while dmMC-RR is less abundant and found in 38% of the investigated lakes. On the other hand, the cell-bound concentration of microcystins falls within the range of concentrations reported from other European water bodies [60], except in some cases (scums) and/or some places, where high microcystin concentrations have been recorded (e.g., 134 μg L^{-1} in the Curonian lagoon [28], >10,000 μg L^{-1} in Lakes Kastoria and Pamvotis (Greece [51]), 1070 μg L^{-1} in Vaya quay (Bulgaria [53]), 25,000 μg L^{-1} in Havel River (Berlin, Germany [61]).

In our study, a shift from the dominance of MC-RR in 2013 and 2014 to the dominance of [Asp^3]MC-RR in 2015–2017 was observed (Figures 3–6). However, the highest microcystin concentration was of [Asp^3]MC-RR in 2014 (St. One). Microcystin [Asp^3]MC-RR is almost by 3-fold more toxic than its methylated variant MC-RR [17]; therefore, this shift can affect the water quality. Microcystin MC-RR was strongly linked with the presence of *Microcystis* spp. and *Dolichopermum* spp., while [Asp^3]MC-RR is strongly linked with the presence of *Planktothrix* (Figure 8). Our results confirm the previous findings that *Microcystis* and *Dolichospermum* species are the main producers of non-demethylated microcystins, while *Planktothrix* is usually linked with demethylated forms of microcystins [40,62]. Moreover, previous studies have shown that *Microcystis* from the Curonian Lagoon contain the *mcy*E gene responsible for microcystins synthesis [63]. Moreover, concentrations of [Asp^3]MC-RR were high in the Curonian Lagoon throughout the study period and are also the highest in other European lakes [60]. The total microcystin concentration recorded in this work exceeded the recommended guideline values for recreational waters provided by US Environmental Protection Agency (US EPA) (8 μg L^{-1}) in 12.6 % of cases. The guideline levels were exceeded at all stations except Station Three. However, these concentrations were within the limits provided by the World Health Organization (24 μg L^{-1}); the highest total microcystin concentration recorded in the lagoon was 22.8 μg L^{-1}. Climatic conditions play an important role in shaping the composition of cyanobacteria species in the water body [64]. Calm and hot weather favour *A. flosaquae* blooms (this study in 2014, [24,64]). On the other hand, during rainy and windy summers, water mixing, and turbidity increases, resulting in *A. flosaquae* replacement by other cyanobacteria species (this study, y. 2017 [24,64]). Despite the low biomass of cyanobacteria in 2017 (summer maximum 5.1 mg L^{-1}), the number of detected metabolites was similar to other years (Table S1). Therefore, it is likely that *A. flosaquae* has no significant contribution to the number of metabolites detected in the samples. Despite the dominance of *A. flosaquae*, other species, particularly *P. agardhii*, *Dolichospermum* spp., and *Microcystis* spp. were considered as the main producers of cyanometabolites.

Cyanobacteria of the main genera recorded in the Curonian Lagoon, *Aphanizomenon*, *Microcystis*, *Planktothrix*, and *Woronichinia*, were also observed during the cold period, and even under the ice cover (this study Figures 3 and 7, [5,6,65–67]. Low concentrations of [Asp^3]MC-RR, MC-RR, and MC-LR, as well as AP-A and AEG-A, indicating the presence of cyanobacteria, were found in the Curonian Lagoon during the December–March period at water temperature 2.9 °C, on average. It is known that microcystins accumulate in the tissues of a wide range of aquatic consumers [68,69]. Therefore, it is not surprising that microcystins have been found in sediments, fish, and mussel tissues in the spring [29,33].

The presence of nodularin at concentrations in the range of 0.01 to 0.28 μg L^{-1}, in the Curonian Lagoon was associated with higher water salinity values. It is known that *N. spumigena*, the only nodularin producer in the Baltic Sea [70,71], could live both in brackish and fresh waters [41,60]. The presence of *N. spumigena* and nodularin in the Curonian Lagoon is associated with Baltic Sea water intrusions to the lagoon [28,32]. According to Zemlys et al. [35], saline water intrusions are more frequent in the northern part of the lagoon, while in extreme cases they could reach the central part. During our study, the farthest station where nodularin was detected was near the Nemunas river avandelta (St. Four). Although Baltic Sea strains of *Nodularia* can grow in culture at 0 g L^{-1} NaCl at least for 70 h [72], it seems that this species has not established a permanent population in the Curonian Lagoon. The detection of the cell-bound nodularin did not always coincide with the detection of *N. spumigena*. Therefore, we can assume that the microscopy method may not reveal the presence of rare species of cyanobacteria.

ANTX-a is a relatively less common bioactive cyanometabolite present in waterbodies worldwide. According to Mantzouki et al. [60], it was found in 39% of the investigated 137 European lakes. In the Baltic Sea region, ANTX-a was found in the Gulf of Finland [49], the Gulf of Gdansk [73], and the south-eastern Baltic coast [32], while it was absent in the Baltic Proper [74,75], the Danish straits [76], and the Vistula Lagoon [77]. In the Curonian Lagoon, anatoxin was not found during the snapshot surveys, even during the blooms of different cyanobacteria (e.g., *M. aeruginosa*, *A. flosaquae*, *P. agardhii*) [30,31], while during long-term surveys, the toxin was reported to be present in 31% (this study) or 47% [32] of the investigated samples. During our study, ANTX-a was found in the lagoon from July to October, but not every year. According to the statistical analysis, the closest relationship of this cyanomethabolite was established with the *Microcystis* genus. However, only few *Microcystis* strains from Japan and Portugal have been found to produce ANTX-a so far [78–80]. It is known that ANTX-a can be also produced by *Aphanizomenon*, *Dolichospermum* (previously known as *Anabaena*), *Gomphosphaeria*, *Limnothrix*, *Lyngbya*, *Planktothrix*, *Planktolyngbia*, *Pseudanabaena*, and *Synechocystis* ([81,82] and references therein). In the Lithuanian lakes, the presence of ANTX-a significantly correlated only with the *C. issatschenkoi*; however, none of the isolated strains of the species showed ANTX-a production [83]. The concentrations of ANTX-a (0–0.515 μg L^{-1}) detected in the Curonian Lagoon are similar to the concentrations determined in Lithuanian lakes [83] and other European lakes [60].

There are numerous references on the composition of cyanopeptides in lakes, while estuaries are poorly studied. Estuaries could be more diverse in species composition, because of the presence of both fresh and fresh-brackish water organisms [6]; therefore, we could expect more potential cyanopeptide producers. In many estuaries and coastal waters, *Microcystis* spp. and *Dolichospermum* (previously known as *Anabaena*) are considered as the main potential microcystin producers ([46] and references therein). Species representing *Aphanizomenon* and *Planktothrix* genera are less common in estuaries, despite the fact that *Aphanizomenon* thrive in brackish waters (e.g., Baltic Sea, [84]), meanwhile *Planktothrix* can grow and produce toxins in waters up to 7.5 g L^{-1} of NaCl [85]. In the Curonian lagoon, depending on the year and season, *Aphanizomenon*, *Planktothrix*, *Microcystis*, *Dolichospermum* or *Woronichinia* can have the largest contribution to the total cyanobacterial biomass.

Until recently, *P. agardhii* was observed only occasionally in the Curonian Lagoon and reached less than 5% of the total phytoplankton abundance ([86] and references

therein). Based on the available literature, from the late 1990s, *P. agardhii* are found all year round (this study, [6,65]) and could account for 13–57% of the total cyanobacteria biomass [25,33,87]. *Planktothrix* tolerates fluctuations in fresh-brackish waters, turbid and or shady conditions [5,64], which are characteristic to the Curonian Lagoon too. Demethylated microcystin variants ([Asp^3]MC-RR and [Asp^3]MC-LR) are usually associated with the presence of *P. agardhii* [40,62]. In previous studies [28,30], only [Asp^3]MC-RR was found in the Curonian Lagoon during the dominance of *Planktothrix*. Microcystins [Asp^3]MC-RR and [Asp^3]MC-LR were the most common toxins in our study. However, we cannot unequivocally attribute the presence of the compounds to a particular cyanobacterium. It has been shown that *P. agardhii* from the Curonian Lagoon has the *mcyE* gene, responsible for toxin production [88].

Usually, more attention is given to widely known bioactive cyanopeptides (e.g., microcystins) and anatoxins, their composition, quantity, and production regulation, etc. Nevertheless, other cyanopeptides could be more harmful to humans and aquatic organisms [11]. In the Curonian Lagoon, anabaenopeptins showed the closest statistical coincidence with *P. agardhii* and had the highest number of variants, of which the most frequent were AP-A, AP-B, AP-F, and Osc-Y. These peptides are also commonly detected in other freshwater bodies dominated by *P. agardhii* [40,89], *Microcystis* spp. [89–91], Coelosphaeriaceae (e.g., *Woronichinia compacta*) [92], in the strain of *Anabaena90* isolated from the fresh environment [93]. The same metabolites AP-A, Osc-Y, AP915, AP807, and AP-D were found in the brackish environment (Gulf of Gdansk, south Baltic Sea), dominated by *N. spumigena* (50% of the cyanobacterial biomass), *A. flosaquae* (40%), and *Dolichospermum* spp. (10%) [94]. According to Janssen ([11] and references therein), in European lakes the concentration of anabaenopeptins usually is higher as compared to microcystins.

A. flosaquae is responsible for the largest and longest cyanobacteria blooms in the Curonian Lagoon (e.g., this study, [6,24,95]). So far, there are no data on its ability to produce microcystins either in the Baltic Sea, or in the Curonian Lagoon [12,30], meanwhile, it is considered as the producer of other classes of bioactive metabolites [12]. Despite the weak relationship between *Aphanizomenon* abundance and concentrations of cyanopeptides, the highest correlation based on the RDA analysis was observed with the aeruginosamide AEG-A. This confirms the findings indicating that *A. flosaquae* and *C. issatschenkoi* are correlated with aeruginosamide [96]. During previous studies in the Curonian Lagoon, AEG-A was found in both samples from *A. flosaquae* and in *M. aeruginosa* dominated communities [31]. However, in that study, AEG-A was found in 52% of the samples collected all year round and in samples dominated by different cyanobacteria: *Aphanizomenon*, *Dolichospermum*, *Microcystis*, *P. agardhii*, *Woronichinia*. Aeruginosamide is also commonly found in waterbodies dominated by *Microcystis* spp. [56,89]. In Poland aeruginosamide was found in several freshwater samples dominated by *P. agardhii* [40]. Recently, 18 different aeruginosamide variants were found in a *Limnoraphis* CCNP1324 culture isolated from the brackish Baltic Sea [97].

Aeruginosins also have a number of structural variants. To date, about 80 variants of the compound from *Microcystis*, *Nodularia*, and *Planktothrix* have been described [10,98]. In phytoplankton samples collected from the Curonian Lagoon, 12 different aeruginosin variants were detected in the summer–autumn period, but they were not common. Typically, the highest number of aeruginosin variants were recorded when *Aphanizomenon* had the highest biomass. Aeruginosins with *m/z* 618 and *m/z* 568 were found in previous research conducted in the Curonian Lagoon during the dominance of *A. flosaquae* and *M. aeruginosa* [31]. Recently, it was found that *A. flosaquae* strains from USA and Finland have aeruginosin biosynthetic gene clusters [99]; therefore, it is possible that in the Curonian Lagoon, *A. flosaquae* is one of the aeruginosin producers as well. The other two species, *P. agardhii* and *Microcystis* spp., which are known to produce aeruginosins [10,98], usually coexist as subdominant species in the Curonian Lagoon. The most common, AER 126A and AER 126B, were previously isolated from *P. agardhii* [100] and were found in a *P. agardhii* dominated freshwater pond as well (Poland, [40]).

Cyanopeptolins and microginins are two other groups with approximately 210 and 72 variants, respectively [12,101]. According to the literature, there are several species in the Curonian Lagoon that can synthesize cyanopeptolins, namely *Microcystis*, *Planktothrix*, and *Dolichospermum* [12], while microginins are synthesized mainly by *Microcystis*, *Planktothrix*, *Synechococcus*, and *Woronichinia naegeliana* [12,101,102]. The amount of data on the occurrence of these two groups of peptides is rapidly increasing in recent years (e.g., [99] and references therein, [40,56,89,103]), however, they are still under-researched compounds [11,101]. During the whole period of our study, we found only three cyanopeptolins of different variants and four microginin variants. Moreover, both cyanopeptolins and microginins were found in the Curonian Lagoon, only occasionally in the summer–autumn period and in less than 4% of all samples, except for microginin Mg 591B, which was identified in 12% of the samples. *M. aeruginosa* strains isolated from Portuguese freshwaters were the source/producers of 11 cyanopeptolins and three microginin variants [104]; from Greek freshwaters, 51 different microginin variants were detected [101]. In the Curonian Lagoon, cyanopeptolins were detected during the dominance of *Microcystis* and *P. agardhii*, while microginins were present during the predominance of *P. agardhii*. The low number of cyanopeptolins and microginins found in the Curonian Lagoon might be attributed to the narrowly specific cyanobacteria species / strain's ability to produce these two compounds, or to unfavourable estuarine conditions for the production of the compounds.

The eutrophic, dynamic, and heterogeneous environment of the Curonian Lagoon is favourable for the growth, diversity, and high abundance of different cyanobacteria species and, consequently, supports the diversity and high concentration of cyanopeptides. Over the past decades, a shorter or absent ice period and increasing water temperature have been observed during the winter in the Curonian Lagoon [105]. Due to this reason, cyanobacteria (especially overwintering species such as *Planktothrix* or *Limnothrix*) and their produced cyanometabolites may be present in water bodies during the winter period [5]. This, in turn, led to the detection of a greater variety and higher concentrations of cyanopeptides, also in winter. Moreover, aeruginosins, anabaenopeptins, cyanopeptolins, and microginins can be as toxic as microcystins [11]. These cyanopeptides may have synergistic effects on aquatic organisms when found together [11,89]. In general, the effects of cyanopeptides on human health have been more frequently investigated than the ecological function of these metabolites in the water ecosystems [106,107].

Despite the fact that *Aphanizomenon* was the dominant cyanobacterium species in our study of the Curonian Lagoon, it was *Planktothrix*, *Microcystis*, and *Dolichospermum* that were the main producers of cyanopeptides and anatoxin in the water body. The cyanometabolites richness shown in our work can form a good starting point for further studies on their role in ecosystem functioning, including their indirect impact on water quality at bathing sites. Knowledge of the effects of environmental conditions on the production and fate of these compounds are important to the general understanding of water ecosystem dynamics.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/w13131760/s1, Table S1: The presence of detected cyanometabolites (*m/z* of their $[M + H]^+$ ion) and cyanobacteria in the Curonian Lagoon during the study period. + – present of the compound or cyanobacteria. AER—aeruginosin, AEG—aeruginosamide, ANTX—anatoxin, AP—anabaenopeptin, CP—cyanopeptolin, MC—microcystin, Mg—microginin, NOD—nodularin, Osc—oscillamide. Stations: 1st—Nida, 2nd—Juodkrantė, 3rd—Dreverna, 4th—Ventė, 5th—Smiltynė. T.s.—this study.

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PAPER II

toxins

MDPI

Article

Characterization and Diversity of Microcystins Produced by Cyanobacteria from the Curonian Lagoon (SE Baltic Sea)

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Abstract: Microcystins (MCs) are the most widely distributed and structurally diverse cyanotoxins that can have significant health impacts on living organisms, including humans. The identification of MC variants and their quantification is very important for toxicological assessment. Within this study, we explored the diversity of MCs and their potential producers from the Curonian Lagoon. MC profiles were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) method, while the potential producers were detected based on the presence of genus-specific *mcyE* gene sequences. Among the numerous MCs detected, one new potential MC variant with *m/z* 1057 was partially characterized. Moreover, two other MCs with *m/z* 1075 and *m/z* 1068 might belong to new variants with serine (Ser), rarely detected in position one of the peptides. They might also represent MC-Y(OMe)R and MC-WR, respectively. However, the application of a low-resolution MS/MS system made the unambiguous identification of the MCs impossible. Based on this example, the problems of peptide structure identification are discussed in the work. Genetic analysis revealed that potential MCs producers include *Dolichospermum*/*Anabaena*, *Microcystis* spp., and *Planktothrix agardhii*. The diversity and temporal variations in MC profiles may indicate the presence of several chemotypes of cyanobacteria in the Curonian Lagoon.

Keywords: microcystin; cyanotoxin; mass spectrometry; structure elucidation

Key Contribution: This study demonstrates the diversity of MCs (20 variants) detected in the Curonian Lagoon. One potentially new MC variant with *m/z* 1057 was characterized based on a partially elucidated structure.

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1. Introduction

Cyanobacteria are widely distributed oxygenic phototrophs that play an important role in the aquatic environment. Under favorable conditions, they form blooms, which have a negative effect on the ecosystem [1]. Although cyanobacteria are not considered infectious microorganisms, the toxins they synthesize have significant health impacts on living organisms, including humans [2].

The species producing cyanotoxins belong mainly to the orders *Oscillatoriales*, *Nostocales*, *Chroococcales*, and *Synechococcales* [3,4]. Among cyanotoxins, microcystins (MCs) are the most frequently studied and structurally diverse compounds [2,4,5]. To date, more than 280 variants of MCs are listed [6]. These toxins cause abnormal hepatocyte functioning leading to liver damage [7,8]. Moreover, there is growing evidence that MCs may also have reproductive and neurological effects [9,10]. Health problems occur after the ingestion of contaminated water or food, or after the absorbance of cyanotoxins through

the skin during recreational activities (e.g., water sport) [7,11]. The World Health Organization (WHO) updated guideline values for MCs in drinking water (specifically for MC-LR variant—1 μg L^{-1}) and water for recreational use (guideline value for MC-LR or total MCs—24 μg L^{-1}) [12]. These values include the sum of all MCs in a sample [7]. However, only a limited number of MC variants as quantitative standards are available [5,13], which makes the determination of the precise value of the total MC concentration difficult. Qualitative analysis of MCs in field samples can also provide valuable data and important benefits for toxicological studies and risk assessment.

MCs are cyclic heptapeptides with the general structure of cyclo-(D-Ala^1-X^2-D-$Masp^3$-Z^4-$Adda^5$-D-γ-Glu^6-$Mdha^7$), where Adda is 3*S*-amino-9*S*-methoxy-2*S*,6,8*S*-trimethyl-10-phenyldeca-4*E*,6*E*-dienoic acid, X and Z are variable L-amino acids, D-$Masp^3$ is D-*erythro*-β-methyl-isoaspartic acid, and Mdha is *N*-methyldehydroalanine [14,15]. The structural modifications can occur in all seven amino acids, but they are mainly recorded in positions 2 and 4 [5,7]. The one-letter amino acid code of two residues is included in the symbols of a specific MC variant (e.g., MC-LR has leucine (L) and arginine (R) in positions 2 and 4, respectively) [5]. MCs are synthesized by the nonribosomal peptide synthetase and polyketide synthase encoded by the *mcyA-J* genes. The organization of the *mcy* cluster was resolved in the representants of the main MCs producing genera: *Dolichospermum* [16], *Microcystis* [17], and *Planktothrix* [18]. *Mcy* genes are frequently used as genetic markers for the detection, differentiation, and identification of MC producers [19,20]. *McyE* is responsible for Adda synthesis, as well as for the addition of D-glutamate into the MC molecule [19]. As this part of the MC structure is crucial for the activity of the compound, *mcyE* has been most widely used to assess the risk associated with cyanobacteria blooms [21]. Apart from general primers used for the amplification of the *mcyE* gene, the presence of toxic *Dolichospermum*, *Microcystis*, and *Planktothrix* genera in the environment has been studied with genus-specific *mcyE* primers [19,22].

Data on the presence of MC-producing cyanobacteria, MCs diversity (including other cyanometabolites), and their concentrations have also been reported from the Curonian Lagoon located in the southeastern part of the Baltic Sea [23–27]. The lagoon is a hypereutrophic, mainly freshwater body, connected to the Baltic Sea by the narrow Klaipėda Strait. In the lagoon, summer cyanobacteria blooms are an annual phenomenon that has been reported for several decades [28]. Most of the cyanobacteria species found in the lagoon belong to fresh–brackish water (42%) and freshwater species (37%) [29]. *Aphanizomenon* spp. is a dominant species in the Curonian Lagoon, co-occurring with *Planktothrix agardhii*, *Microcystis* spp., *Dolichospermum*/*Anabaena* spp., and *Woronichinia* spp. [23,24,28]. Dense cyanobacteria blooms are more frequently observed in the southern and central parts than in the northern part of the water body [28]. MC concentrations in the coastal southern part occasionally reach the guideline values recommended by the WHO, posing a higher risk to humans [24]. In the Russian part of the Curonian Lagoon, the MCs producing cyanobacteria have been detected using molecular methods [30,31]. In both studies, *mcyE* genes and cyanotoxins were associated with the presence of *Microcystis* species.

The aim of this work was to determine and document the diversity of MCs produced by cyanobacteria from the Curonian Lagoon, and identify the source organisms using genus-specific primers. In order to detect and characterize the structure of 20 MC variants present in the field samples, liquid chromatography-tandem mass spectrometry (LC-MS/MS) was applied. Genetic analysis showed that at least part of the cyanobacteria belonging to the *Dolichospermum*/*Anabaena*, *Microcystis*, and *Planktothrix* genera are MC producers.

2. Results

2.1. Cyanobacteria Community

In samples collected from the shore station of the Curonian Lagoon, cyanobacteria biomass varied from 1.17 to 12.39 mg L^{-1}, and accounted for 3–65% of the total phytoplankton biomass (TPB) (Figure 1, Table S2). During most of the summer (27 June 2018–16 August 2018), the composition of cyanobacteria species did not differ (Figure S1),

while late spring–early summer and early autumn were characterized by different communities of cyanobacteria. *Dolichospermum flosaquae* dominated in June (71% of the total cyanobacteria biomass (TCB)), while the contribution of *Planktothrix agardhii* was highest in July (2020), September–October (62–84% of the TCB).

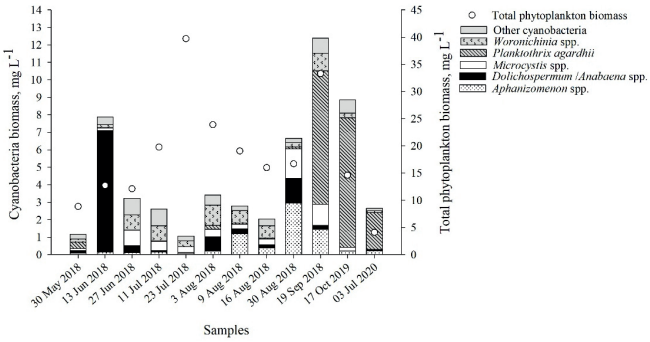

Figure 1. Structure and biomass (mg L^{-1}) of the cyanobacteria community and total phytoplankton biomass (mg L^{-1}) in the collected samples.

The greatest species diversity (eight species) and the differences in relative biomass were found among members of the genus *Dolichospermum* (Figure 2a). Together with *D. flosaquae*, the dominance of *D. crassum* and *D. planctonicum* was recorded (<57% of the TB of the genus *Dolichospermum*) (Figure 2a, Table S2). No evident seasonal differences in the occurrence of *M. flosaquae* and *M. wesenbergii* were observed. The two species were found in all samples, and their contribution to the total biomass was similar in most of the samples (2–88% and 23–51% of the TB of the genus *Microcystis*, respectively) (Figure 2b). A more pronounced dominance of *M. flosaquae* was observed during late spring–early summer (30 May–13 June) (82–88% of the TB of the genus *Microcystis*). *M. viridis* and *M. aeruginosa* represented a slightly different seasonal pattern: the highest contribution of the species biomass was recorded during the middle of the summer (July–August) (26–43% and 28–61% of the TB of the genus *Microcystis*). *Aphanizomenon* genus was mainly represented by *Aph. flosaquae* (0–91% of the TB of the genus *Aphanizomenon*), together with *Aph. gracile* and *Cuspidothrix issatschenkoi* (Figure 2c). *Woronichinia compacta* was the dominant species among representatives of the *Woronichinia* genus (91–100% of the TB of the genus *Woronichinia*) and was present in all samples (Figure 2d).

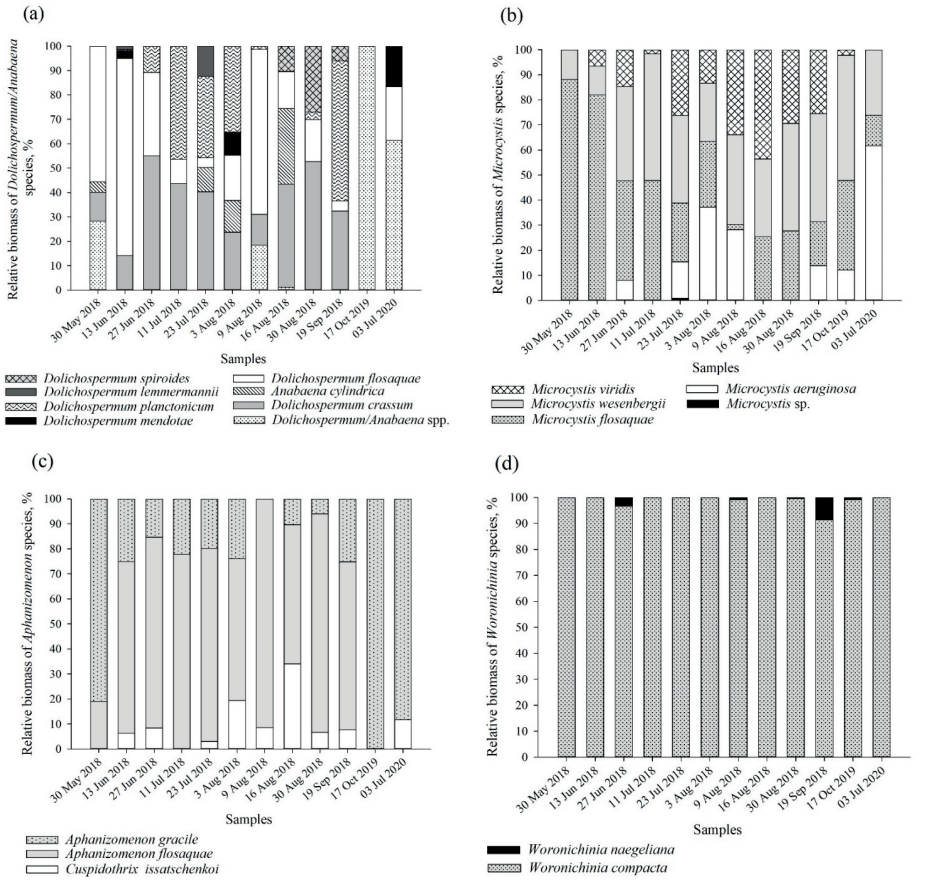

Figure 2. Relative biomass of dominating cyanobacteria species of the genera *Dolichospermum*/*Anabaena* (**a**), *Microcystis* (**b**), *Aphanizomenon* (**c**) and *Woronichinia* (**d**). Relative biomass is calculated from the total biomass of specific genera.

2.2. Microcystins Diversity

In total, 20 MC variants were detected in the samples, most commonly with 2–5 different variants per sample (Table 1, Figures S3–S22). Of these, MC-RR was detected in all samples. Other frequently detected MCs were MC-LR and [Dha^7]MC-RR, which were present in 11 and 7 out of 12 samples, respectively. The following variants belonged to the most rarely recorded: ([Ser^1]MC-HtyR (or MC-Y(OMe)R), MC-WR (or [Ser^1]MC-HarR), MC-(H4)TyrR, MC-HphR, [Asp^3]MC-RY, MC-FR, MC-LW, MC-HilR, MC-LY, and [Asp^3]MC-LY; they were found in only one or two samples. The abbreviations of the amino acids and their full names are provided in Table S1. The highest number of MC variants was detected during July 2018 (12 variants) and in the second half of August 2018 (18 variants).

Table 1. MC diversity in field samples collected from the Curonian Lagoon during 2018, 2019, and 2020 ("+": detected; empty cells: not detected, *m/z*—values of MC pseudomolecular ions. In brackets, the value of a doubly charged ion is given).

MC Variants	*m/z*	Sampling Dates											
		30 May 2018	13 Jun 2018	27 Jun 2018	11 Jul 2018	23 Jul 2018	3 Aug 2018	9 Aug 2018	16 Aug 2018	30 Aug 2018	19 Sep 2018	17 Oct 2019	3 Jul 2020
[Ser^1]MC-HtyR or MC-Y(OMe)R	1075					+			+				
MC-WR or [Ser^1]MC-HarR	1068								+	+			
MC-X^{1}R	1057					+			+				
MC-?	1054								+				
MC-(H_4)YR	1049					+			+				
MC-YR	1045	+				+			+	+	+		
MC-HphR	1043								+	+			
MC-RR	1038 (519)	+	+	+	+	+	+	+	+	+	+	+	+
[Asp^3]MC-YR or [Asp^3]MC-M(O_2)R	1031					+		+	+	+			
[Asp^3]MC-RY	1031											+	+
MC-FR	1029					+			+				
MC-LW	1025								+				
[Dha^7]MC-RR	1024 (512)		+			+			+	+	+	+	+
MC-HilR	1009					+			+				
MC-LY	1002								+				
MC-LR	995	+	+	+	+	+	+		+	+	+	+	+
[Asp^3]MC-LY	988								+			+	
MC-LF	986						+		+	+	+		
[Dha^7]MC-LR	981		+			+			+	+			
[Asp^3]MC-LR	981					+						+	+

Note: Unknown part of MC.

In position 1 of the MCs produced by cyanobacteria from the Curonian Lagoon, D-Ala was mainly present (in 16 of the 19 MCs) (Figure 3). There were also two MC structures, with *m/z* 1075 and *m/z* 1068, where the presence of Ser was suspected. The amino acid residues in position 2 were the most diverse amongst the MCs detected in the Curonian Lagoon samples. In position 2, Leu was found most frequently (7/19), followed by Arg (3/19). The other amino acid residues were present in two (Tyr) or one (Hph, Phe, Hil, Hty, Tyr(OMe), Trp, Har, (H_4)Tyr, M(O2)) MC variant. Position 4 was more conserved and was occupied mainly by Arg (14/19) or optionally by Tyr (3/19), Phe (1/19), and Trp (1/19). In positions 3 and 7, methylated or dimethylated analogues of D-Asp and Dha were present, respectively. No modifications were observed in positions 5 and 6.

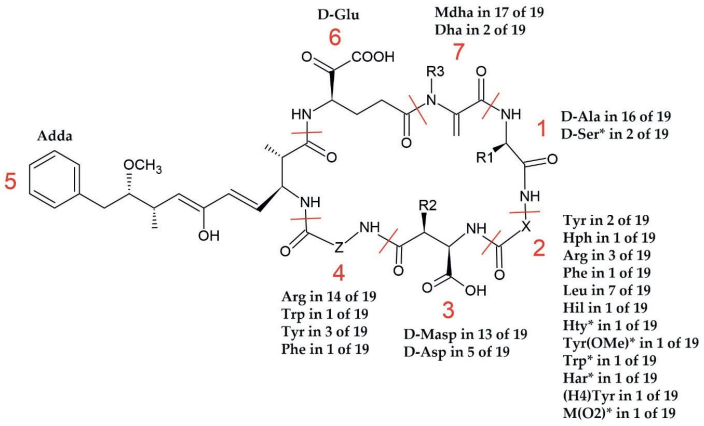

Figure 3. A general structure of MCs and their structural diversity recorded in the samples from the Curonian Lagoon (R1 = CH_3 or CH_2OH; R2 = H or CH_3; R3 = H or CH_3; X and Z—variable L-amino acids). * One of the possible residues. See the text in Section 2.2. The abbreviations of the amino acids and their full names are provided in Table S1.

The collected spectra of three MCs contain fragment ions that can be generated by two different variants: [Ser^1]MC-HtyR/MC-Y(OMe)R (*m/z* 1075) (Figure 4), MC-WR/[Ser^1]MC-HarR (*m/z* 1068) (Figure S4), and [Asp^3]MC-YR/[Asp^3]MC-M(O_2)R (*m/z* 1031) (Figure S12). These peptides differ only in positions 1 and 2. Since the total value of the two residues present in different variants is the same (e.g., Ser^1+Hty^2 and Ala^1+Tyr(OMe)^2; Ala^1+Trp^2 and Ser^1+Har^2; Ala^1+Tyr^2 and Ala^1-M(O2)^2), the fragment ions containing both residues are also the same. The encountered problems are illustrated in Figure 4, Figures S4 and S12.

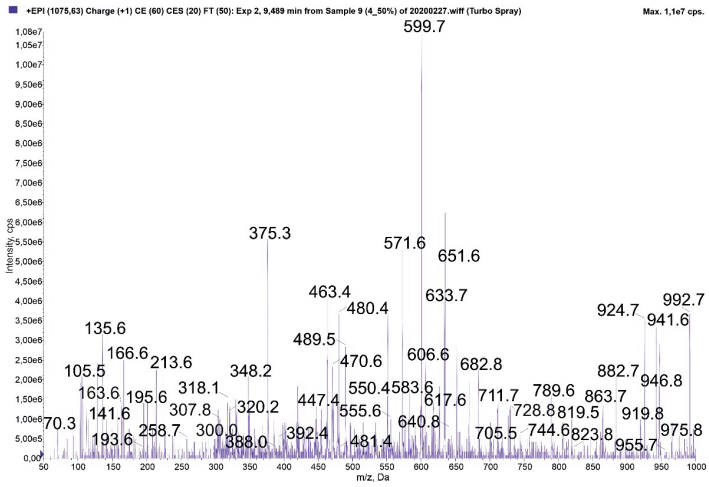

Figure 4. Enhanced product ion mass spectrum of MC with *m/z* 1075. The spectrum can correspond to [Ser^1]MC-HtyR (I) or

MC-Y(OMe)R (II). The structure elucidation of [Ser^1]MC-HtyR (I) was based on the following fragment ions: *m/z* 992 [M + H – Mdha]; 946 [M + H – Glu/Masp]; 941 [M + H – Adda fragment]; 924 [$C_{11}H_{14}O$ + Glu + Mdha + Ser + Hty + Masp + Arg + H]; 919 [M + H – Arg]; 882 [Masp + Arg + Adda + Glu + Mdha + H]; 863 [Ser + Hty + Masp + Arg + Adda + H]; 728 [Masp + Arg + Adda + Glu + H]; 682 [Arg + Adda + Glu + Mdha + H]; 633 [Mdha + Ser + Hty + Masp + Arg + H]; 606 [Glu + Mdha + Ser + Hty + Masp + H]; 599 [Arg + Adda + Glu + H], 571 [Arg + Adda + Glu + H – CO], 550 [Ser + Hty + Masp + Arg + H]; 470 [Arg + Adda + H]; 463 [$C_{11}H_{14}O$ + Glu + Mdha + Ser + H]/[Hty + Masp + Arg + H]; 375 [$C_{11}H_{14}O$ + Glu + Mdha + H]; 348 [$C_{11}H_{14}O$ + Glu + Mdha + H – CO]/[Mdha + Ser + Hty + H]; 307 [Hty + Masp + H]; 300 [Glu + Mdha + Ser + H]; 213 [Glu + Mdha + H]; 163 [$C_{11}H_{14}O$ + H]; 135 Adda fragment.

MC-Y(OMe)R (II) structure elucidation was based on the following fragment ions: *m/z* 992 [M + H – Mdha]; 946 [M + H – Glu/Masp]; 941 [M + H – Adda fragment]; 924 [$C_{11}H_{14}O$ + Glu + Mdha + Ala + Tyr(OMe) + Masp + Arg + H]; 919 [M + H – Arg]; 882 [Masp + Arg + Adda + Glu + Mdha + H]; 863 [Ala + Tyr(OMe) + Masp + Arg + Adda + H]; 728 [Masp + Arg + Adda + Glu + H]; 682 [Arg + Adda + Glu + Mdha + H]; 633 [Mdha + Ala + Tyr(OMe) + Masp + Arg + H]; 606 [Glu + Mdha + Ala + Tyr(OMe) + Masp + H]; 599 [Arg + Adda + Glu + H], 571 [Arg + Adda + Glu + H – CO], 550 [Ala + Tyr(OMe) + Masp + Arg + H]; 480 [Tyr(OMe) + Masp + Arg + H]; 470 [Arg + Adda + H]; 447 [$C_{11}H_{14}O$ + Glu + Mdha + Ala + H]; 375 [$C_{11}H_{14}O$ + Glu + Mdha + H]; 348 [$C_{11}H_{14}O$ + Glu + Mdha + H – CO]/[Mdha + Ala + Tyr(OMe) + H]; 213 [Glu + Mdha + H]; 163 [$C_{11}H_{14}O$ + H]; 135 Adda fragment.

2.3. Genetic Analysis

To identify the possible producers of MCs in the Curonian Lagoon, the polymerase chain reactions (PCRs) were performed with *mcyE* genus-specific primers in addition to *mcyE* general primers. When general primers were used, the PCR products were obtained for all the environmental samples and the two MCs producing cyanobacteria strains *M. aeruginosa* CCNP1102 and *P. aghardii* CCNP1325 (Table 2). The application of *Microcystis*- and *Planktothrix*-specific primers also gave products in all the bloom samples and the relevant cyanobacteria strains. Only for the sample collected on 17 October 2019, no amplification product with *Dolichospermum*-specific *mcyE* primers was detected.

Table 2. The results of PCR amplification with the general *mcy* primers and *Dolichospermum*-, *Microcystis*-, and *Planktothrix*-specific primers ("+"—PCR-positive results, "−"—PCR-negative results, OK500398-OK500432—accession number in GenBank).

Samples	General *mcyE* Primers	*Dolichospermum* Specific Primers	*Microcystis* Specific Primers	*Planktothrix* Specific Primers
30 May 2018	+	OK500398	OK500409	OK500421
13 Jun 2018	+	OK500399	OK500410	OK500422
27 Jun 2018	+	OK500400	OK500411	OK500423
11 Jul 2018	+	OK500401	OK500412	OK500424
27 Jul 2018	+	OK500402	OK500413	OK500425
3 Aug 2018	+	OK500403	OK500414	OK500426
9 Aug 2018	+	OK500404	OK500415	OK500427
16 Aug 2018	+	OK500405	OK500416	OK500428
30 Aug 2018	+	OK500406	OK500417	OK500429
19 Sep 2018	+	OK500407	OK500418	OK500430
17 Oct 2019	+	–	OK500419	OK500431
3 Jul 2020	+	OK500408	OK500420	OK500432
M. aeruginosa CCNP1102	+	–	OK500396	–
P. aghardii CCNP1325	+	–	–	OK500397
Limnoraphis sp. CCNP1324	–	–	–	–
MilliQ water	–	–	–	–

Sequences of the received PCR products were deposited in GenBank under the accession numbers OK500398-OK500432. Sequences obtained in amplification using *Microcystis*-specific primers were 100% similar and grouped with other sequences from potentially toxic *M. flos-aquae*, *M. viridis* of different origin, and one sequence from *Pseudanabaena* sp. CCM-UFV065 (Figure S2). Sequences received from PCRs with *Planktothrix*-specific primers grouped with sequences obtained from *P. aghardii*, *P. rubescens*, and one sequence from *Synechococcus* sp., while those obtained from *Dolichospermum*-specific grouped with other *Dolichospermum*/*Anabaena* sequences.

3. Discussion

In our studies, *Aph. flosaquae*, *M. wesenbergii*, and *W. compacta* form a significant part of the total cyanobacteria biomass [23,32]. The *Aph. flosaquae* population from the Curonian Lagoon, similarly to the one from the Baltic Sea, is considered non-MC producing [33]. During its blooms, no significant increases in MC concentrations and diversity were observed [23,27]. In the recent work by Österholm et al. [34], *mcy* genes were not detected in several analyzed *Aph. flosaquae* genomes. *M. wesenbergii* is rarely considered as an MC producer, and to date, only one work showing *mcyE* gene amplification from this species has been published [35]. As for the *Woronichinia* genus, the presence of MCs in bloom samples dominated by some species, e.g., *W. naegeliana*, has been occasionally recorded [36,37], but no data on toxin production by isolated *W. compacta* strain were published. Based on the reviewed data, we can conclude that although the biomass of *Aph. flosaquae*, *M. wesenbergii*, and *W. compacta* were high, these species do not contribute to the toxicity of blooms in the Curonian Lagoon.

Phytoplankton analyses of samples collected in the Curonian Lagoon showed a frequent occurrence of *Dolichospermum*/*Anabaena*, *Microcystis*, and *Planktothrix* genera among the potential producers of MCs. This was also reflected by the presence of *mcyE* genes amplified with primers specific for these genera. Furthermore, *P. agardhii* is the only species among the *Planktothrix* genus found in the Curonian Lagoon [23], and according to our genetic analyses, at least part of the population belongs to MC producers. *Microcystis*, *Dolichospermum*/*Anabaena*, and *Planktothrix* are frequently found in temperate zones, and usually, during their presence, different variants of MCs are detected [4,35,38–41]. However, assigning a particular MC variant to any of the cyanobacteria genera or species is impossible, especially because the study was performed with the application of field samples with a mixture of cyanobacteria species. This becomes even more complicated by the fact that one cyanobacteria strain can produce a median number of 4–5 MCs simultaneously, with one or two variants being dominant in any single strain [42,43]. However, based on the most identified associations between cyanobacteria and MCs [18,44–46], *M. flosaquae*, *D. flosaquae*, and *P. agardhii*, which were found in almost all samples, potentially can be considered as the main producers of the most frequently detected MC-RR and MC-LR and their demethylated variants ([Asp^3]MC-RY, [Dha^7]MC-RR, [Asp^3]MC-LY, [Asp^3]MC-LR).

Sensitive molecular methods, based on *mcy* genes, were developed to detect and identify MC producers [19,47,48]. Unfortunately, as a result of the observed *mcy* genes deletions, recombinations, and various insertions, the presence of the *mcy* genes cannot be directly linked to the synthesis of MCs. Furthermore, the influence of biotic and abiotic factors on *mcy* gene expression is still poorly recognized, and the knowledge about the changes in the process in given genotypes is limited [19,49]. Studies show that among tested *Dolichospermum* spp. (=*Anabaena*) (126 strains from the Baltic Sea), *Planktothrix* (72 strains from European lakes), and *Microcystis* spp. (18 strains from worldwide), only in 1–13% of the isolates *mcy* genes were not expressed [47,48,50]. Due to a relatively low percentage of *mcy* genes carrying but not expressing genera, the screening of *mcy* genes for the identification of potential MC producers from field samples may give a good qualitative assessment. However, as a single parameter, these genes cannot provide a clear conclusion; therefore, the application of other methods, such as mass spectrometry, must be considered for the detection of MCs.

During our study, 20 different MC variants were characterized based on fragmentation spectra. Among the MCs with a fully elucidated structure, in only two variants the presence of Ser in position 1 was considered, while in others D-Ala was present. This result is in line with previous findings that indicated position 1 as highly conserved and predominantly occupied by Ala. According to Bouaïcha et al. [51], D-Ala^1 was present in 219 of the 279 identified MC variants, while only two D-Ser^1-containing variants were reported. The diversity and frequency of other amino acids in specific positions of MCs from the Curonian Lagoon, as in the case of position 1, correspond to the previous structural studies of the toxins [4,51,52]. Positions 2 and 4 were most variable, while modifications in other positions were minor.

In some cases, structure elucidation based on the product ion fragmentation spectrum was found to be difficult, and could lead to misinterpretation of the spectrum. This problem can be encountered when residues with the same value are present in the peptide, e.g., Glu and Masp, Mdha and Dhb, Leu and Ile, or Tyr and Met(O_2). MCs with Met(O_2) can be formed during sample processing, and are considered post-extraction oxidation artifacts [53]. This fact cannot be excluded in our work, either. In addition, the sequence of two or more residues can produce the products with the same *m/z* value in the spectrum. The structure identification in this study was additionally hampered by the low resolution of the QTRAP5500 system (and *m/z* range limited to 1000). In general, structure elucidation of peptides based on fragmentation pattern, even if the mass spectrum is rich in fragment ions, might be tentative and always should be performed with caution. Moreover, in the case of new variants, the structure elucidation should be confirmed by nuclear magnetic resonance spectroscopy (NMR). However, if sufficient amounts of pure MC variants for NMR analysis cannot be isolated, the application of high-resolution mass spectrometry and accurate mass measurement can provide new data for more reliable structure elucidation.

The highest diversity of MCs was observed in samples collected on 23 July 2018 (12 variants) and 16 August 2018 (18 variants), when the contribution of cyanobacteria to TPB was one of the lowest. Compared to other studies, the diversity of MCs detected during our study is relatively higH—Yilamaz et al. [54] reported more than 36 MCs detected in Turkish lake, Ballot et al. [55] detected 41 MC variants in a dam located in South Africa, and Fastner et al. [46] reported 15 MC variants in German freshwaters. The species composition in the samples collected on 23 July 2018 and 16 August 2018 did not differ evidently from other samples collected during the period from 27 June to 30 August. The significant differences in MC profiles recorded in these samples indicate changes at the sub-population level of the cyanobacteria community. In water bodies, several cyanobacteria chemotypes characterized by different MC patterns usually coexist [56–58]. In the Curonian Lagoon, the environmental conditions on the two days when the highest MC structural diversity was recorded (23 July and 16 August) apparently favored the proliferation of MC-rich chemotypes, and thus, indirectly influenced the presence of numerous, including the more rare, MC variants (e.g., MC-HtyR, [Ser^1]MC-RR, MC-LW, or MC-LY). Environmental conditions have an impact on the structure and dynamics of the cyanobacteria community, but not necessarily on the production of different MC variants [59,60]. Moreover, diversity can increase as a result of relaxation of the adenylation domain [50]. Recombination patterns in the adenylation domains might lead to the synthesis of new MCs [61,62]. More detailed research with isolated strains is needed to clarify the diversity of cyanobacteria chemotypes in the Curonian Lagoon.

The samples from the Curonian Lagoon also displayed diversity and considerable variation in rare and potentially new MC variants throughout the sampling period. MC variants, such as MC-WR, [Asp^3]MC-RY, MC-LW, MC-LY, MC-(H_4)YR, and [Asp^3]MC-LY, detected during our study are mainly produced by representatives of the *Microcystis* genus [63–67]. Furthermore, MC-FR and MC-HilR were detected in the blooms dominated by several *Microcystis* species [68,69], while MC-HphR is associated with different strains of *Anabaena* [53,70]. The list of MCs detected in cyanobacteria bloom samples from the Curonian Lagoon and presented in this study adds new information that can be useful

in establishing the geography of specific MC variants. Geographical differences in the distribution of specific MC variants are observed worldwide. For example, Leu^1-containing MCs were detected only in Canada [71], while MC-LA is more frequently detected in the US rather than in European water bodies [72].

The identification of MC variants and their accurate quantification are very important for toxicological assessment and monitoring [73]. The following MCs: MC-LR, MC-HilR, MC-LY, MC-YR, belong to the highly toxic variants [51]. For other MCs, including the new variants, no toxicity data are available [51]. Moreover, due to the lack of reference standards, the reliable quantitative assessment of total MCs is impossible. It should be also noted that MCs are not the only harmful substances produced by cyanobacteria. There are many other classes of peptides (anabaenopeptins, cyanopeptolins, aeruginosins, etc.), which occur as frequently as MCs, and are mainly known as proteases inhibitors or compounds harmful against grazers [5]. Lack of toxicological data and comprehensive knowledge about the activity of cyanobacteria metabolites might lead to the underestimation of the real risk.

4. Conclusions

This study showed for the first time the diversity of MCs produced by cyanobacteria from the Curonian Lagoons. While genetic analyses indicated the potential of the cyanobacteria to produce MCs, the chemical analyses allowed us to obtain more conclusive results. The detection of 20 different MCs (among which three might be potentially new variants) adds new information about their geographical distribution and may indicate the presence of different cyanobacteria chemotypes in the lagoon.

5. Materials and Methods

5.1. Water Samples

Phytoplankton samples were collected from the shore of the Curonian Lagoon, located in the city of Nida (sampling site coordinates 55.300115, 22.005821) during 2018 (10 samples, every two weeks from May to September), 2019 (1 sample, October), and 2020 (1 sample, July). For DNA analysis, water samples were collected in 2018 from the surface layers (~50 cm) in sterile, darkened plastic bottles. The samples were filtered (0.5–1.0 L; the sample volume depended on the abundance of cyanobacteria) through a 0.22 µm pore size mixed cellulose ester filters (MontaMil® Membrane Filters, Frisenette ApS, Knebel, Denmark) for genetic analysis and GF/F glass fiber filters (Whatman International Ltd., Kent, UK) for MC analysis. Visually, the biomass collected on filters was similar for all samples (intensely green color).

5.2. Phytoplankton Analysis

Phytoplankton samples for microscopic analysis were taken during all sampling events and fixed with acid Lugol's iodine solution. Quantitative analyses of the composition of the phytoplankton community were conducted using a LEICA DMI 3000 inverted microscope (Leica Microsystems CMS, Wetzlar, Germany) at magnifications of ×100 and ×400, according to the method described by Utermöhl [74]. Phytoplankton was identified to the lowest possible taxonomic level using guidelines described in the literature for the freshwater environments [75–78]. According to HELCOM recommendations [79], the phytoplankton abundance (counts L^{-1}) was calculated by multiplying the number of units counted (filamentous cyanobacteria were counted in lengths of 100 µm as one count) with the coefficient C (L), calculated using the following equation:

$$C = \frac{A \times 1000}{N \times a \times V} \quad (1)$$

where A is the cross-section area of the top cylinder of the combined sedimentation chamber (the usual inner diameter is 25.0 mm, giving A = 491 mm^2), N is the number of counted fields or transects, a is the area of a single field or transect, and V is the volume (mL) of sedimented aliquot.

The biomass of phytoplankton (mg L^{-1}) was calculated by the allocation of phytoplankton species (genus) to size classes, according to the scheme of Olenina et al. (2006) and updated appendix available at HELCOM website (https://helcom.fi/helcom-at-work/projects/peg/, accessed on 5 September 2021). The biomass of phytoplankton (mg L^{-1}) was calculated based on the following equation, as recommended by HELCOM [79]:

$$\text{Biomass} = \text{abundance} \times \text{VCU} \times 10^{-6}, \quad (2)$$

where VCU is the volume of the counting unit (μg).

5.3. DNR Extraction and PCR

DNA extraction was performed using the PowerWater® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) and FastDNA™ Spin Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) (samples collected during 2018); DNeasy PowerSoil Pro Kit (QIAGEN, Hilden, Germany) (samples collected during 2019 and 2020). The quality and quantity of extracted DNA were determined with SpectraMax® i3 Platform (Molecular Devices LLC., Sunnyvale, CA, USA) equipped with SpectraDrop Micro-Volume Microplate. PCR products were detected by electrophoresis in 1.5% agarose gel stained with SYBR® Green I (Sigma-Aldrich, St. Louis, MO, USA). DNA isolated from toxic *M. aeruginosa* CCNP1102 and *P. aghardii* CCNP1305 were used as a positive control [57,80]. DNA isolated from *Limnoraphis* sp. CCNP1324, and MilliQ water were used as a negative control [81].

For amplification of the *mcyE* gene from all cyanobacteria present in the samples, the same primers (*mcyE-F2* and *mcyE-R4*) (Genomed S.A., Warszawa, Poland) and PCR cycling conditions were used as in Rantala et al. [82]. MC-producing *Anabaena/Dolichospermum*, *Microcystis*, and *Planktothrix* spp. were targeted with the above-mentioned forward primer and genus-specific reverse primers (*mcyE-12R*, *mcyE-R8*, and *mcyE-plaR3*, respectively) (Genomed S.A., Warszawa, Poland) [19,83]. PCRs were run in 25 μL solution containing approx. 100 ng of DNA, 5 pmol of each specific oligonucleotide primer, 12.5 μL of MyTaq™ Red Mix (Bioline Reagents Ltd., London, UK), in Mastercycler® nexus GSX1 (Eppendorf, Hamburg, Germany).

The PCR products were purified with an ExtractMe DNA clean-up kit (Blirt S.A., Gdańsk, Poland). The PCR fragments were sequenced (Genomed S.A., Warszawa, Poland) using both forward and reverse genus-specific primers used in the amplification. The obtained nucleotide sequences were edited with Chromas Lite 2.1, aligned and assembled, and afterward compared to the sequences in the NCBI GenBank (http://www.ncbi.nlm.nih.gov) using blast algorithm (http://blast.ncbi.nlm.nih.gov). They were deposited under OK500398–OK500432 accession numbers. For phylogenetic analyses, the sequences were aligned using the MEGA version X [84], the alignments were corrected manually. Neighbor-joining (NJ) and maximum likelihood (ML) trees were constructed in MEGA version X. For each tree, a bootstrap analysis of 1000 replications was performed.

5.4. Microcystins Analysis

The collected material was extracted with 75% methanol. Filters were sonicated with an ultrasonic disrupter (1 min) (HD 2070 Sonopuls, Bandelin, Berlin, Germany), then in the water bath (15 min) (Sonorex, Bandelin, Berlin, Germany), and centrifuged (12,000 *g*; 15 min) (Eppendorf 5810R, Hamburg, Germany). The extracts were analyzed using an Agilent HPLC system (Agilent Technologies, Waldboronn, Germany) coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer QTRAP LC-MS/MS (QTRAP5500, Applied Biosystems, Sciex; Toronto, ON, Canada), according to the method described by Mazur-Marzec et al. [85]. The presence of MCs was screened using information dependent acquisition (limit of detection was 0.5–2.0 ng mL^{-1}, depending on the variant). Data were processed with Analyst QS (Version 1.5.1, Applied Biosystems/MDS Analytical Technologies, Concord, ON, Canada, 2008).

5.5. Statistical Analysis

Non-parametric multidimensional scaling (nMDS) based on the Bray–Curtis similarity coefficient [86] was used to represent the similarities of dominating cyanobacteria communities among the different samples. The stress values of the two MDS plots were determined—a stress value of <0.2 indicated an accurate representation of similarity rankings. nMDS was conducted using Primer v6 software (PRIMER-E Ltd., Plymouth, UK).

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/toxins13120838/s1, Table S1: The abbreviations of amino acids and their full names, Table S2: Presence and dominance of cyanobacteria species in the samples from the Curonian Lagoon. Cyanobacteria dominance was expressed as a percentage of total cyanobacteria biomass, Figure S1: nMDS plot based on Bray–Curtis similarity matrix of dominating cyanobacteria species biomass. Only samples for which concentrations of microcystins were calculated in μg L^{-1} were used for RDA analysis, Figure S2: Phylogenetic relationships of sequences retrieved from the studied environmental samples (marked in colors), their closest relatives and representants of other potent microcystin producing genera, based on the alignment of partial (218 b) *mcyE* sequences. Numbers above branches indicate the bootstrap values for NJ and ML method. Accession numbers in NCBI are presented in brackets, Figure S3: Enhanced product ion mass spectrum of microcystin with the suggested structure [Ser^1]MC-HtyR (*m/z* 1075) and the following fragment ions: *m/z* 992 [M + H – Mdha]; 946 [M + H – Glu/Masp]; 941 [M + H – Adda fragment]; 924 [$C_{11}H_{14}O$ + Glu + Mdha + Ser + Hty + Masp + Arg + H]; 919 [M + H – Arg]; 882 [Masp + Arg + Adda + Glu + Mdha + H]; 863 [Ser + Hty + Masp + Arg + Adda + H]; 728 [Masp + Arg + Adda + Glu + H]; 682 [Arg + Adda + Glu + Mdha + H]; 633 [Mdha + Ser + Hty + Masp + Arg + H]; 606 [Glu + Mdha + Ser + Hty + Masp + H]; 599 [Arg + Adda + Glu + H], 571 [Arg + Adda + Glu + H – CO], 550 [Ser + Hty + Masp + Arg + H]; 470 [Arg + Adda + H]; 463 [$C_{11}H_{14}O$ + Glu + Mdha + Ser + H]/[Hty + Masp + Arg + H]; 375 [$C_{11}H_{14}O$ + Glu + Mdha + H]; 348 [$C_{11}H_{14}O$ + Glu + Mdha + H – CO]/[Mdha + Ser + Hty + H]; 307 [Hty + Masp + H]; 300 [Glu + Mdha + Ser + H]; 213 [Glu + Mdha + H]; 163 [$C_{11}H_{14}O$ + H]; 135 Adda fragment; or enhanced product ion mass spectrum of microcystin with the suggested structure MC-Y(OMe)R (*m/z* 1075), and the following fragment ions: *m/z* 992 [M + H – Mdha]; 946 [M + H – Glu/Masp]; 941 [M+H – Adda fragment]; 924 [$C_{11}H_{14}O$ + Glu + Mdha + Ala + Tyr(OMe) + Masp + Arg + H]; 919 [M + H – Arg]; 882 [Masp + Arg + Adda + Glu + Mdha + H]; 863 [Ala + Tyr(OMe) + Masp + Arg + Adda + H]; 728 [Masp + Arg + Adda + Glu + H]; 682 [Arg + Adda + Glu + Mdha + H]; 633 [Mdha + Ala + Tyr(OMe) + Masp + Arg + H]; 606 [Glu + Mdha + Ala + Tyr(OMe) + Masp + H]; 599 [Arg + Adda + Glu + H], 571 [Arg + Adda + Glu + H – CO], 550 [Ala + Tyr(OMe) + Masp + Arg + H]; 480 [Tyr(OMe) + Masp + Arg + H]; 470 [Arg + Adda + H]; 447 [$C_{11}H_{14}O$ + Glu + Mdha + Ala + H]; 375 [$C_{11}H_{14}O$ + Glu + Mdha + H]; 348 [$C_{11}H_{14}O$ + Glu + Mdha + H – CO]/[Mdha + Ala + Tyr(OMe) + H]; 213 [Glu + Mdha + H]; 163 [$C_{11}H_{14}O$ + H]; 135 Adda fragment, Figure S4: Enhanced product ion mass spectrum of microcystin with the suggested structure MC-WR (*m/z* 1068) and the following fragment ions: *m/z* 997 [M + H – Ala]; 985 [M + H – Mdha]; 939 [M + H – Glu/Masp]; 934 [M + H – Adda fragment]; 917 [M + H – Adda fragment – H_2O]; 912 [M + H – Adda]; 856 [M + H – (Glu + Mdha)]; 811 [M + H – (Ala + Trp)]; 728 [Masp + Arg + Adda + Glu + H]; 682 [Arg + Adda + Glu + Mdha + H]; 626 [Mdha + Ala + Trp + Masp + Arg + H]; 599 [Arg + Adda + Glu + H], 582 [Agr + Adda + Glu + H – NH_3]; 571 [Masp + Arg + Adda + H – CO]/[Arg + Adda + Glu + H – CO]; 543 [Ala + Trp + Masp + Arg + H]; 526 [Adda + Glu + Mdha + H]; 472 [Trp + Masp + Arg + H]; 470 [Arg + Adda + H]; 456 [Trp + Masp + Arg + H – NH_3]; 375 [$C_{11}H_{14}O$ + Glu + Mdha + H]; 347 [$C_{11}H_{14}O$ + Glu + Mdha + H – CO]; 341 [Mdha + Ala + Trp + H]; 269 [Masp + Arg + H – NH_3]; 258 [Ala + Trp + H]; 213 [Glu + Mdha + H]; 195 [Glu + Mdha + H – H_2O]; 135 Adda fragment; or enhanced product ion mass spectrum of microcystin with the suggested structure [Ser^1]MC-HarR (*m/z* 1068), and the following fragment ions: *m/z* 997 [M + H – Ala]; 985 [M + H – Mdha]; 939 [M + H – Glu/Masp]; 934 [M + H – Adda fragment]; 917 [M + H – Adda fragment – H_2O]; 912 [M + H – Adda]; 856 [M + H – (Glu + Mdha)]; 811 [M + H – (Ser + Har)]; 728 [Masp + Arg + Adda + Glu + H]; 682 [Arg + Adda + Glu + Mdha + H]; 626 [Mdha + Ser + Har + Masp + Arg + H]; 599 [Arg + Adda + Glu + H], 582 [Agr + Adda + Glu + H – NH_3]; 571 [Masp + Arg + Adda + H – CO]/[Arg + Adda + Glu + H – CO]; 543 [Ser + Har + Masp + Arg + H]; 526 [Adda + Glu + Mdha + H]; 470 [Arg + Adda + H]; 456 [Har + Masp + Arg + H]; 375 [$C_{11}H_{14}O$ + Glu + Mdha + H]; 347 [$C_{11}H_{14}O$ + Glu + Mdha + H – CO]; 341 [Mdha + Ser + Har + H]; 300 [Har + Masp + H]/[Glu + Mdha + Ser + H]; 258 [Ser + Har + H]; 213 [Glu + Mdha + H]; 195 [Glu + Mdha

+ H – H_2O]; 135 Adda fragment, Figure S5: Enhanced product ion mass spectrum of microcystin with the partially elucidated structure MC-XR (*m/z* 1057) and the following fragment ions: 728 [Masp + Arg + Adda + Glu + H]; 682 [Arg + Adda + Glu + Mdha + H]; 599 [Arg + Adda + Glu + H], 571 [Arg + Adda + Glu + H – CO], 470 [Arg + Adda + H]; 375 [$C_{11}H_{14}O$ + Glu + Mdha + H]; 195 [Glu + Mdha + H – H_2O]; 213 [Glu + Mdha + H]; 135 Adda fragment, Figure S6: Enhanced product ion mass spectrum of microcystin *m/z* 1054/528, Figure S7: Enhanced product ion mass spectrum of microcystin with the suggested structure MC-(H_4)YR (*m/z* 1049) and the following fragment ions: *m/z* 978 [M + H – Ala]; 965 [M + H – Mdha]; 920 [M + H – Glu/Masp]; 893 [M + H – Arg]; 753 [Arg + Adda + Glu + Mdha + Ala + H]; 728 [Masp + Arg + Adda + Glu + H]; 725 [Arg + Adda + Glu + Mdha + Ala + H – CO]; 710 [Masp + Arg + Adda + Glu + H – H_2O]; 682 [Arg + Adda + Glu + Mdha + H]; 624 [Mdha + Ala + (H_4)Tyr + Masp + Arg + NH_3 + H]; 607 [Mdha + Ala + (H_4)Tyr + Masp + Arg + H]; 599 [Arg + Adda + Glu + H], 589 [M + H – (Adda + Glu) – H_2O]; 582 [Arg + Adda + Glu + H – NH_3]; 571 [Arg + Adda + Glu + H – CO], 523 [Ala + (H_4)Tyr + Masp + Arg + H]; 495 [Ala + (H_4)Tyr + Masp + Arg + H – CO]; 470 [Arg + Adda + H]; 446 [$C_{11}H_{14}O$ + Glu + Mdha + Ala + H]; 418 [$C_{11}H_{14}O$ + Glu + Mdha + Ala + H – CO]; 375 [$C_{11}H_{14}O$ + Glu + Mdha + H]; 347 [$C_{11}H_{14}O$ + Glu + Mdha + H – CO]; 284 [Glu + Mdha + Ala + H]; 213 [Glu + Mdha + H]; 195 [Glu + Mdha + H – H_2O]; 163 [$C_{11}H_{14}O$ + H]; 155 [Mdha + Ala + H]; 135 Adda fragment; 127 [Mdha + Ala + H – CO], Figure S8: Enhanced product ion mass spectrum of microcystin with the suggested structure MC-YR (*m/z* 1045) and the following fragment ions: *m/z* 974 [M + H – Ala]; 962 [M + H – Mdha]; 916 [M + H – Glu/Masp]; 889 [M + H – Arg]; 834 [M + H – (Glu + Mdha)]; 760 [M + H – (Masp + Arg)]; 683 [Masp + Arg + Adda + Glu + H – NH_3 – CO]; 603 [M + H – (Adda + Glu)]; 599 [Arg + Adda + Glu + H], 576 [Glu + Mdha + Ala + Tyr + Masp + H]; 571 [Arg + Adda + Glu + H – CO]; 520 [Ala + Tyr + Masp + Arg + H]; 470 [Arg + Adda + H]; 448 [Tyr + Masp + Arg + H]; 432 [Tyr + Masp + Arg + H – NH_3]; 375 [$C_{11}H_{14}O$ + Glu + Mdha + H]; 347 [Adda fragmen + Glu + Mdha + H – NH_3 – CO]; 303 [Masp + Arg – NH_3]; 269 [Masp + Arg + H – NH_3]; 213 [Glu + Mdha + H]; 195 [Glu + Mdha + H – H_2O]; 163 [$C_{11}H_{14}O$ + H]; 135 Adda fragment, 127 [Mdha + Ala + H – CO], Figure S9: Enhanced product ion mass spectrum of microcystin with the suggested structure MC-HphR (*m/z* 1043) and the following fragment ions: *m/z* 972 [M + H – Ala]; 909 [M + H – Adda fragment]; 892 [$C_{11}H_{14}O$ + Glu + Mdha + Ala + Hph + MeAsp + Arg + H]; 887 [M + H – Arg]; 882 [M + H – Hph]; 831 [M + H – (Glu + Mdha)]; 728 [Masp + Arg + Adda + Glu + H]; 682 [Arg + Adda + Glu + Mdha + H]; 602 [M + H – (Adda + Glu)]; 599 [Arg + Adda + Glu + H]; 574 [M + H – (Adda + Arg)]; 571 [Arg + Adda + Glu + H – CO]; 518 [Ala + Hph + Masp + Arg + H]; 470 [Arg + Adda + H]; 446 [$C_{11}H_{14}O$ + Glu + Mdha + Ala + H]; 375 [$C_{11}H_{14}O$ + Glu + Mdha + H]; 347 [$C_{11}H_{14}O$ + Glu + Mdha + H – CO]; 213 [Glu + Mdha + H]; 195 [Glu + Mdha + H – H_2O]; 163 [$C_{11}H_{14}O$ + H]; 135 Adda fragment; 127 [Mdha + Ala + H – CO], Figure S10: Enhanced product ion mass spectrum of microcystin with the suggested structure MC-RR (*m/z* 1038/519) and the following fragment ions: *m/z* 910 [M + H – Glu/Masp]; 887 [M + H – Arg]; 884 [M + H – (Mdha + Ala)]; 826 [M + H – (Glu + Mdha)]; 811 [M + H – (Ala + Arg)]; 755 [M + H – (Glu + Mdha + Ala)]; 731 [$C_{11}H_{14}O$ + Glu + Mdha + Ala + Arg + Masp + H]; 702 [$C_{11}H_{14}O$ + Glu + Mdha + Ala + Arg + Masp + H – CO]; 599 [Arg + Adda + Glu + H]; 579 [Adda + Glu + Mdha + Ala + H – H_2O]; 571 [Arg + Adda + Glu + H – CO]; 470 [Arg + Adda + H]; 440 [Glu + Mdha + Ala + Arg + H]; 375 [$C_{11}H_{14}O$ + Glu + Mdha + H]; 311 [Mdha + Ala + Arg + H]; 285 [Glu + Mdha + Ala + H]; 213 [Glu + Mdha + H]; 195 [Glu + Mdha + H – H_2O]; 163 [$C_{11}H_{14}O$ + H]; 135 Adda fragment; 127 [Mdha + Ala + H – CO], Figure S11: Enhanced product ion mass spectrum of microcystin with the suggested structure [Asp^3]MC-RY (*m/z* 1031/516) and the following fragment ions: *m/z* 960 [M + H – Ala]; 916 [M + H – Asp]; 897 [M + H – Adda fragment]; 880 [$C_{11}H_{14}O$ + Glu + Mdha + Ala + Arg + Asp + Tyr + H]; 868 [M + H – Tyr]; 819 [M + H – (Glu + Mdha)]; 760 [M + H – (Arg + Asp)]; 753 [M + H – (Tyr + Asp)]; 735 [M + H – (Tyr + Asp) – H_2O]; 717 [$C_{11}H_{14}O$ + Glu + Mdha + Ala + Arg + Asp + H]; 606 [Tyr + Adda + Glu + H]; 602 [$C_{11}H_{14}O$ + Glu + Mdha + Ala + Arg + H]; 589 [Mdha + Ala + Arg + Asp + Tyr + H]; 555 [M + H – (Adda + Glu)]; 435 [Arg + Asp + Tyr + H]; 426 [Mdha + Ala + Arg + Asp + H]; 375 [$C_{11}H_{14}O$ + Glu + Mdha + H]; 343 [Ala + Arg + Asp + H]; 311 [Mdha + Ala + Arg + H]; 213 [Glu + Mdha + H]; 163 [$C_{11}H_{14}O$ + H]; 135 Adda fragment, Figure S12: Enhanced product ion mass spectrum of microcystin with the suggested structure [Asp^3]MC-YR or [Asp^3]MC-M(O_2)R (*m/z* 1031/516) and the following fragment ions: *m/z* 960 [M + H – Ala]; 902 [M + H – Glu/Masp]; 897 [M + H – Adda fragment]; 880 [$C_{11}H_{14}O$ + Glu + Mdha + Ala + Tyr/M(O_2) + Asp + Arg + H]; 868 [M + H – Tyr/M(O_2)]; 599 [Arg + Adda + Glu + H]; 589 [Mdha + Ala + Tyr/M(O_2) + Asp + Arg + H]; 585 [Asp + Arg + Adda + H]; 562 [Glu + Mdha + Ala + Tyr/M(O_2) + Asp + H]; 506 [Ala + Tyr/M(O_2) + Asp + Arg + H]; 470 [Arg + Adda + H]; 446 [$C_{11}H_{14}O$ + Glu + Mdha + Ala + H]; 435 [Tyr/M(O_2) +

Asp + Arg + H]; 375 [$C_{11}H_{14}O$ + Glu + Mdha + H]; 357 [$C_{11}H_{14}O$ + Glu + Mdha + H – H_2O]; 347 [$C_{11}H_{14}O$ + Glu + Mdha + H – CO]; 318 [Mdha + Ala + Tyr/M(O_2) + H]; 213 [Glu + MeDha + H]; 135 Adda fragment; 127 [Mdha + Ala + H – CO], Figure S13: Enhanced product ion mass spectrum of microcystin with the suggested structure MC-FR (*m/z* 1029) and the following fragment ions: *m/z* 957 [M + H – Ala]; 946 [M + H – Mdha]; 900 [M + H – Glu/Masp]; 895 [M + H – Adda fragment]; 878 [M + H – Glu/Masp – H_2O]; 817 [M + H – (Glu + Mdha)]; 728 [Masp + Arg + Adda + Glu + H]; 682 [Arg + Adda + Glu + Mdha + H]; 604 [M + H – (Adda + Glu) + NH_3]; 599 [Arg + Adda + Glu + H]; 587 [M + H – (Adda + Glu)]; 571 [Arg + Adda + Glu + H – CO]; 560 [M + H – (Arg + Adda)]; 504 [Ala + Phe + Masp + Arg + H]; 470 [Arg + Adda + H]; 433 [Phe + Masp + Arg + H]; 431 [Mdha + Ala + Phe + Masp + H]; 375 [$C_{11}H_{14}O$ + Glu + Mdha + H]; 356 [Ala + Phe + Masp + H]; 348 [Ala + Phe + Masp + H]; 302 [Mdha + Ala + Phe + H]; 284 [Glu + Mdha + Ala + H]; 213 [Glu + Mdha + H]; 195 [Glu + Mdha + H – H_2O]; 163 [$C_{11}H_{14}O$ + H]; 135 Adda fragment; 127 [Mdha + Ala + H – CO]; 120 Phe immonium ion, Figure S14: Enhanced product ion mass spectrum of microcystin with the suggested structure MC-LW (*m/z* 1025) and the following fragment ions: *m/z* 976 [M + H – CH_3OH – NH_3]; 925 [M + H – Mdha – NH_3]; 891 [M + H – Adda fragment]; 873 [$C_{11}H_{14}O$ + Glu + Mdha + Ala + Leu + Masp + Trp + H]; 693 [Adda + Glu + Mdha + Ala + Leu + H – NH_3]; 583 [M + H – (Adda + Glu)]; 580 [Adda + Glu + Mdha + Ala + H – NH_3]; 565 [Mdha + Ala + Leu + Masp + Trp + H – H_2O]; 559 [$C_{11}H_{14}O$ + Glu + Mdha + Ala + Leu + H]; 555 [Mdha + Ala + Leu + Masp + Trp + H – CO]; 509 [Adda + Glu + Mdha + H – NH_3]; 500 [Ala + Leu + Masp + Trp + H]; 472 [Ala + Leu + Masp + Trp + H – CO]; 446 [$C_{11}H_{14}O$ + Glu + Mdha + Ala + H]; 429 [Leu + Masp + Trp + H]; 397 [Mdha + Ala + Leu + Masp + H]; 375 [$C_{11}H_{14}O$ + Glu + Mdha + H]; 347 [$C_{11}H_{14}O$ + Glu + Mdha + H – CO]; 316 [Trp + Masp + H]; 298 [Trp + Masp + H – H_2O]; 288 [Trp + Masp + H – CO]; 213 [Glu + Mdha + H]; 195 [Glu + Mdha + H – H_2O]; 187 [Trp + H]; 163 [$C_{11}H_{14}O$ + H]; 159 Trp immonium ion; 135 Adda fragment; 127 [Mdha + Ala + H – CO], Figure S15: Enhanced product ion mass spectrum of microcystin with the suggested structure [Dha^7]MC-RR (*m/z* 1024/512) and the following fragment ions: *m/z* 890 [M + H – Adda fragment]; 872 [M + H – Adda fragment – H_2O]; 717 [$C_{11}H_{14}O$ + Glu + Dha + Ala + Arg + Masp + H]; 668 [Arg + Adda + Glu + Dha + H]; 588 [$C_{11}H_{14}O$ + Glu + Dha + Ala + Arg + H]; 582 [Dha + Ala + Arg + Masp + Arg + H]; 565 [Dha + Ala + Arg + Masp + Arg + H – NH_3]; 426 [Glu + Dha + Ala + Arg + H]; 297 [Dha + Ala + Arg + H]; 269 [Dha + Ala + Arg + H – CO]; 199 [Glu + Dha + H]; 181 [Glu + Dha + H – H_2O]; 141 [Dha + Ala + H]; 135 Adda fragment; 113 [Dha + Ala + H – CO], Figure S16: Enhanced product ion mass spectrum of microcystin with the suggested structure MC-HilR (*m/z* 1009) and the following fragment ions: *m/z* 992 [M + H – NH_3]; 938 [M + H – Ala]; 926 [M + H – Mdha]; 880 [M + H – Glu/Masp]; 875 [M + H – Adda fragment]; 858 [$C_{11}H_{14}O$ + Glu + Mdha + Ala + Hil + Masp + Arg + H]; 853 [M + H – Arg]; 797 [M + H - (Glu + Mdha)]; 753 [M + H – (Hil + Masp); 728 [Masp + Arg + Adda + Glu + H]; 682 [Arg + Adda + Glu + Mdha + H]; 599 [Arg + Adda + Glu + H]; 584 [Mdha + Ala + Hil + Masp + Arg + H + NH_3]; 573 [$C_{11}H_{14}O$ + Glu + Mdha + Ala + Hil + H]; 567 [M + H – (Adda + Glu)]; 540 [Glu + Mdha + Ala + Hil + Masp + H]; 484 [Ala + Hil + Masp + Arg + H]; 470 [Arg + Adda + H]; 446 [$C_{11}H_{14}O$ + Glu + Mdha + Ala + H]; 412 [Hil + Masp + Arg + H]; 396 [Hil + Masp + Arg + H – NH_3]; 375 [$C_{11}H_{14}O$ + Glu + Mdha + H]; 347 [$C_{11}H_{14}O$ + Glu + Mdha + H – CO]; 213 [Glu + Mdha + H]; 195 [Glu + Mdha + H – H_2O]; 163 [$C_{11}H_{14}O$ + H]; 135 Adda fragment; 127 [Mdha + Ala + H – CO], Figure S17: Enhanced product ion mass spectrum of microcystin with the suggested structure MC-LY (*m/z* 1002) and the following fragment ions: *m/z* 985 [M + H – NH_3]; 902 [M + H – Mdha – NH_3]; 868 [M + H – Adda fragment]; 851 [$C_{11}H_{14}O$ + Glu + Mdha + Ala + Leu + Masp + Tyr + H]; 818 [M + H – (Ala + Leu)]; 693 [Adda + Glu + Mdha + Ala + Leu + H – NH_3]; 580 [Adda + Glu + Mdha + Ala + H – NH_3]; 560 [M + H – (Adda + Glu)]; 559 [$C_{11}H_{14}O$ + Glu + Mdha + Ala + Leu + H]; 509 [Adda + Glu + Mdha + H – NH_3]; 477 [Tyr + Adda + H]; 446 [$C_{11}H_{14}O$ + Glu + Mdha + Ala + H]; 406 [Leu + Masp + Tyr + H]; 397 [Mdha + Ala + Leu + Masp + H]; 375 [$C_{11}H_{14}O$ + Glu + Mdha + H]; 347 [$C_{11}H_{14}O$ + Glu + Mdha + H – CO]; 293 [Masp + Tyr + H]; 213 [Glu + Mdha + H]; 195 [Glu + Mdha + H – H_2O]; 163 [$C_{11}H_{14}O$ + H]; 155 [Mdha + Ala + H]; 136 Tyr immonium ion; 135 Adda fragment; 127 [Mdha + Ala + H – CO]; 86 Ile immonium ion, Figure S18: Enhanced product ion mass spectrum of microcystin with the suggested structure MC-LR (*m/z* 995) and the following fragment ions: *m/z* 977 [M + H – H_2O]; 967 [M + H – CO]; 924 [M + H – Ala]; 912 [M + H – Mdha]; 866 [M + H – Glu/Masp]; 839 [M + H – Arg]; 783 [M + H – (Glu + Mdha)]; 728 [Masp + Arg + Adda + Glu + H]; 712 [M + H – (Glu + Mdha + Ala)]; 682 [M + H – (Ala + Leu + Masp)]; 599 [Arg + Adda + Glu + H]; 571 [Arg + Adda + Glu + H – CO]; 553 [M + H – (Adda + Glu)]; 526 [Adda + Glu + Mdha + H]; 470 [Arg + Adda + H]; 453 [Ala + Leu + Masp + Arg + H – NH_3]; 397 [Glu + Mdha + Ala + Leu + H]; 375 [$C_{11}H_{14}O$ + Glu + Mdha + H]; 347 [$C_{11}H_{14}O$ + Glu

+ Mdha + H – CO]; 269 [Masp + Arg + H – NH_3]; 213 [Glu + Mdha + H]; 195 [Glu + Mdha + H – H_2O]; 163 [$C_{11}H_{14}O$ + H]; 135 Adda fragment, Figure S19: Enhanced product ion mass spectrum of microcystin with the suggested structure [Asp^3]MC-LY (*m/z* 988) and the following fragment ions: *m/z* 854 [M + H – Adda fragment]; 837 [$C_{11}H_{14}O$ + Glu + Mdha + Ala + Leu + Asp + Tyr + H]; 580 [Adda + Glu + Mdha + Ala + H – NH_3]; 546 [M + H – (Adda + Glu)]; 477 [Tyr + Adda + H]; 463 [Ala + Leu + Asp + Tyr + H]; 446 [$C_{11}H_{14}O$ + Glu + Mdha + Ala + H]; 383 [Mdha + Ala + Leu + Asp + H]; 375 [$C_{11}H_{14}O$ + Glu + Mdha + H]; 266 [Glu + Mdha + Ala + H – H_2O]; 251 [Asp + Tyr + H – CO]; 213 [Glu + Mdha + H]; 195 [Glu + Mdha + H – H_2O]; 163 [$C_{11}H_{14}O$ + H]; 135 Adda fragment, Figure S20: Enhanced product ion mass spectrum of microcystin with the suggested structure MC-LF (*m/z* 986) and the following fragment ions: *m/z* 969 [M + H – NH_3]; 857 [M + H – Glu/Masp]; 852 [M + H – Adda fragment]; 802 [M + H – (Ala + Leu)]; 693 [Adda + Glu + Mdha + Ala + Leu + H – NH_3]; 580 [Adda + Glu + Mdha + Ala + H – NH_3]; 559 [$C_{11}H_{14}O$ + Glu + Mdha + Ala + Leu + H]; 544 [M + H – (Adda + Glu)]; 509 [Glu + Mdha + Ala + Leu + Masp + H – NH_3]; 461 [Ala + Leu + Masp + Phe + H]; 446 [$C_{11}H_{14}O$ + Glu + Mdha + Ala + H]; 397 [Glu + Mdha + Ala + Leu + H]; 390 [Leu + Masp + Phe + H]; 375 [$C_{11}H_{14}O$ + Glu + Mdha + H]; 347 [$C_{11}H_{14}O$ + Glu + Mdha + H – CO]; 249 [Masp + Phe + H – CO]; 213 [Glu + Mdha + H]; 195 [Glu + Mdha + H – H_2O]; 163 [$C_{11}H_{14}O$ + H]; 135 Adda fragment; 127 [Mdha + Ala + H – CO], Figure S21: Enhanced product ion mass spectrum of microcystin with the suggested structure [Asp^3]MCLR (*m/z* 981) and the following fragment ions: *m/z* 910 [M + H – Ala]; 847 [M + H – Adda fragment]; 830 [$C_{11}H_{14}O$ + Glu + Mdha + Ala + Leu + Asp + Arg + H]; 753 [Arg + Adda + Glu + Mdha + H]; 668 [M + H – Adda]; 599 [Arg + Adda + Glu + H]; 571 [Arg + Adda + Glu + H – CO]; 539 [M + H – (Adda + Glu)]; 470 [Arg + Adda + H]; 456 [M + H – (Adda + Glu + Mdha)]; 446 [$C_{11}H_{14}O$ + Glu + Mdha + Ala + H]; 383 [Mdha + Ala + Leu + Asp + H]; 375 [$C_{11}H_{14}O$ + Glu + Mdha + H]; 369 [Glu + Mdha + Ala + Leu + H – CO]; 347 [$C_{11}H_{14}O$ + Glu + Mdha + H – CO]; 213 [Glu + Mdha + H]; 195 [Glu + Mdha + H – H_2O]; 163 [$C_{11}H_{14}O$+H]; 135 Adda fragment; 105 Dha, Figure S22: Enhanced product ion mass spectrum of microcystin with the suggested structure [Dha^7]MCLR (*m/z* 981) and the following fragment ions: *m/z* 910 [M + H – Ala]; 852 [M + H – Glu/Mdha]; 830 [$C_{11}H_{14}O$ + Glu + Dha + Ala + Leu + Masp + Arg + H]; 825 [M + H – Arg]; 728 [Masp + Arg + Adda + Glu + H]; 712 [Leu + Masp + Arg + Adda + H]; 599 [Arg + Adda + Glu + H]; 583 [Adda + Glu + Dha + Ala + H]; 571 [Arg + Adda + Glu + H – CO]; 539 [M + H – (Adda + Glu)]; 512 [Adda + Glu + Dha + H]; 470 [Arg + Adda + H]; 432 [$C_{11}H_{14}O$ + Glu + Dha + Ala + H]; 399 [Leu + Masp + Arg + H]; 383 [Glu + Dha + Ala + Leu + H]; 361 [$C_{11}H_{14}O$ + Glu + Dha + H]; 334 [$C_{11}H_{14}O$ + Glu + Dha + H – CO]; 269 [Glu + Dha + Ala + H]; 181 [Glu + Dha + H – H_2O]; 163 [$C_{11}H_{14}O$ + H]; 141 [Dha + Ala + H]; 135 Adda fragment.

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PAPER III

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PAPER IV

biomolecules

MDPI

Article

Phytoplankton of the Curonian Lagoon as a New Interesting Source for Bioactive Natural Products. Special Impact on Cyanobacterial Metabolites

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Abstract: The bioprospecting of marine and brackish water systems has increased during the last decades. In this respect, microalgae, including cyanobacteria, and their metabolites are one of the most widely explored resources. Most of the bioactive compounds are isolated from ex situ cultures of microorganisms; however, analysis of field samples could also supply valuable information about the metabolic and biotechnological potential of microalgae communities. In this work, the activity of phytoplankton samples from the Curonian Lagoon was studied. The samples were active against antibiotic resistant clinical and environmental bacterial strains as well as against serine proteases and T47D human breast adenocarcinoma cells. No significant effect was found on *Daphnia magna*. In addition, using LC-MS/MS, we documented the diversity of metabolites present in field samples. A list of 117 detected cyanopeptides was presented. Cyanopeptolins constituted the largest class of cyanopeptides. As complex bloom samples were analyzed, no link between the observed activity and a specific sample component can be established. However, the results of the study showed a biotechnological potential of natural products from the Curonian Lagoon.

Keywords: phytoplankton; cyanobacteria; antibacterial compounds; enzymatic activity; cytotoxicity; acute toxicity; Baltic Sea

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1. Introduction

The brackish water Curonian Lagoon, located along the south-eastern part of the Baltic Sea, is one of the largest lagoons in Europe. As a highly eutrophic water body, it annually experiences massive blooms of microalgae [1–4]. The spring phytoplankton community is dominated by diatoms, mainly *Stephanodiscus hantzschii*, *Diatoma tenuis*, *Aulacoseira islandica*, *Asterionella formosa* [5,6]. During the summer–autumn seasons, cyanobacteria are the main phytoplankton component. Among them, *Aphanizomenon* spp., *Planktothrix agardhii*, *Dolichospermum* spp., *Microcystis* spp., *Woronichinia compacta*, *Limnothrix redekei* are dominant [1,7,8]. Cyanobacteria and eukaryotic microalgae, mainly diatoms and green algae, generate a high diversity of metabolites, with an important ecological function and/or potential of biotechnological application.

Previous research in the Curonian Lagoon has only focused on the ecotoxicological assessment of cyanobacterial scum and cyanobacterial toxins from the perspective of the ecosystem and public health [7,9–13]. To our knowledge, no published data on the biological activity of metabolites produced by cyanobacteria and eukaryotic microalgae

occurring in this lagoon exist. In terms of cyanobacterial toxins, the presence of various microcystin (MC) analogues, anatoxin-a (ANTX-a) and nodularin (NOD) has been confirmed [7,9,12,13]. Reports on the detection of cyanopeptides in the Curonian Lagoon was also published [10,13].

It is well documented that cyanobacteria are leaders among the natural sources of bioactive compounds. Most frequently, their cytotoxic effect has been described [14,15]. Like other microalgae groups (diatoms, green microalgae), cyanobacteria also show considerable antibacterial potential and can inhibit the growth of multidrug-resistant pathogens [16–18]. In addition, secondary metabolites produced by microalgae and cyanobacteria exhibit anti-inflammatory, antioxidant, anticoagulant, antiprotozoal and antiviral activities [19].

In general, the bioprospecting of marine and brackish water systems has highly increased during the last few decades. In this respect, microalgae and their metabolites are one of the most widely explored resources. Especially in areas such as pharmacy, aquaculture, bioremediation, bioenergy, biorefinery and biopigmentation [20,21]. Due to the urgent need for more effective and safer medicines for the treatment of cancer, metabolic disorders and infections caused by multidrug-resistant microorganisms, the search for new natural products for the pharmaceutical industry has been recently intensified [22–24].

To date, the potential for biotechnological application of more than 10,000 new compounds from these resources has been assessed [21,25]. Most of the bioactive compounds were isolated from ex situ cultures of microorganisms. However, in some cases, the biosynthesis of specific metabolites might not be triggered under laboratory conditions [17,26]. Some microalgae that live naturally in the aquatic environment show metabolic plasticity under stressed vs. non-stressed conditions. Abiotic and biotic stress can provide an extra advantage of triggering the synthesis of secondary metabolites [27]. However, the identification of their producer in field samples can present a challenge and the reproducibility of biological effects is rather unlikely. Therefore, in further studies isolated strains and their metabolites should be explored. On the other hand, the analysis of phytoplankton bloom samples supplies valuable information about the metabolic and biotechnological potential of the organisms living in the analyzed ecosystem [28].

The objectives of this study were: (1) to assess the biotechnological potential of phytoplankton from the Curonian Lagoon using enzymatic, antimicrobial, and cytotoxicity assays as well as acute toxicity assay; (2) to document the diversity of bioactive cyanometabolites from the Curonian Lagoon.

2. Materials and Methods

2.1. Field Samples Collection

The samples (Sample ID 1–9, Table 1) were collected from two stations (Nida and Juodkrante (Figure 1), depending on the highest surface accumulation of the phytoplankton) every second week from June until August in 2018. To collect higher biomass for activity screening, water samples were concentrated using 55 µm Apstein plankton net and centrifuged at 3444× *g*, 8 °C for 10 min (Centrifuge 5810 R, Eppendorf®, Hamburg, Germany). Then, the samples were frozen and freeze-dried. Subsamples for phytoplankton analyzes, both concentrated and non-concentrated, were fixed with Lugol's iodine solution.

Table 1. Testing steps and assays performed with extracts and fractions of the collected samples (N represents Nida site, J—Juodkrante site).

Collected Samples		1st Testing Step		2nd Testing Step		3rd Testing Step	
Sampling dates	Sample ID	Extract ID	Assay	ID [1] of the fractions tested	Assay	ID [2] of the fractions tested	Assay
N2018.06.24	1	I	acute toxicity, antibacterial, enzyme inhibition, cytotoxicity	–	–	–	–
N2018.06.28	2	II		–	–	–	–
J2018.07.11	3	III		–	–	–	–
J2018.07.20	4	IV		IV-[10–100] [3]	antibacterial, cytotoxicity	IV-60-[20; 40; 60; 90; 100] [3]; IV-70-[20; 40; 60; 90; 100] [3]	cytotoxicity
N2018.07.23	5	V		V-[10–100] [3]	antibacterial	–	–
N2018.08.03	6	VI		–	–	–	–
N2018.08.09	7	VII		–	–	–	–
N2018.08.16	8	VIII		VIII-[10–100] [3]	enzyme inhibition	–	–
N2018.08.30	9	IX		IX-[10–100] [3]		–	–

[1]—sample ID indicates: extract number (I–IX); I fractionation, fraction number; [2]—sample ID indicates: extract number; I fractionation, fraction number; II fractionation, fraction number; [3] —numbers in square brackets represent eluent from 10% to 100% methanol (for 2nd Testing Step—every 10% (10 fractions for each extract); for 3rd Testing Step—20%, 40%, 60%, 90% and 100% methanol in MilliQ water); "–"—not tested.

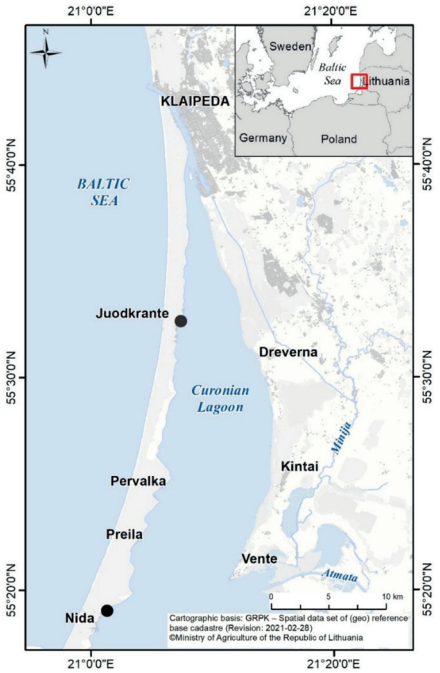

Figure 1. The study area and the locations (black circles) of the two sampling sites in the Curonian Lagoon (Nida and Juodkrante).

2.2. Phytoplankton Analysis

The qualitative and quantitative analyses of the phytoplankton community composition were conducted using a LEICA DMI 3000 (Leica Microsystems CMS, Wetzlar, Germany) inverted microscope at magnifications of ×100 and ×400. The qualitative analysis of concentrated phytoplankton samples was carried out using Nunclon 10 mL 6-well chambers [28]. The quantitative phytoplankton analysis was performed according to the methodology described by Utermöhl [29]; the phytoplankton abundance and biomass was calculated according to the methodology described by HELCOM [30] and Olenina et al. [31]. Detailed description of the methods used for calculation of phytoplankton abundance and biomass can be found in the Supplementary Materials. Phytoplankton was identified to the lowest possible taxonomic level using guidelines described in the literature for the freshwater and brackish environments [32–35].

2.3. Extraction and Fractionation of Phytoplankton Biomass

Freeze-dried phytoplankton biomass was extracted with 75% methanol by vortexing for 15 min and centrifuged (19,837× *g*, 4 °C for 20 min) (Centrifuge 5810 R, Eppendorf®, Hamburg, Germany). The supernatants were diluted with MilliQ water, to lower the concentration of methanol below 10%. Then, the samples were partially purified by passing through preconditioned Waters Sep-Pak® Vac 20cc C18 cartridges (5 g) (Waters, Milford, MA, USA). The extracts were eluted by washing the cartridges with 90% methanol in MilliQ water. The collected extracts were then evaporated using a miVac QUATTRO centrifugal vacuum concentrator (SP Scientific, Ipswich, UK) to the dry residue and depending on the bioassay (antibacterial, enzyme inhibition, cytotoxicity, or acute toxicity) (see methodology below) prepared for the first testing step (1st t.s.) (Table 1).

Based on the bioactivity response obtained in the MTT, antibacterial and enzyme inhibition assays (1st t.s.), extracts IV, V, VIII and IX, were selected for further investigation (Table 1, second testing step (2nd t.s.)). The dried extracts were dissolved in 75% methanol (10 mL) by vortexing for 10 min and diluted in MilliQ water to lower the methanol concentration (<10%), centrifuged (19,837× *g*, 4 °C for 10 min) (Centrifuge 5810 R, Eppendorf®, Hamburg, Germany) and filtered through GF/A filters (Whatman International Ltd., Kent, UK). The supernatants were then loaded onto a preconditioned flash chromatography Biotage® SNAP KP-C18-HS (120 g) column (Biotage, Uppsala, Sweden). Fractionation was performed using Shimadzu HPLC system model LC-20AP (Shimadzu, Canby, OR, USA) equipped with a photodiode array detector (PDA). PDA operated in a range from 190 to 500 nm and during all chromatographic runs, the absorbance at 210 and 280 nm was recorded. Samples were loaded onto a preconditioned column at a flow rate of 12 mL min^{-1}. After washing the resin with MilliQ water the sorbed substances were eluted with methanol: water mixture, gradually increasing the strength of the eluent (by 10% at each step) from 10% to 100% methanol. A volume of 12 mL was collected for each fraction. Based on the results of the MTT assay (2nd t.s.), fractions V-60 and V-70 were further separated (3rd t.s.)). The second (II) fractionation of V-60 and V-70 was performed manually using Waters Sep-Pak® Vac (50 mg) (Waters, Milford, MA, USA). Sorbed metabolites were eluted with 20%, 40%, 60%, 90%, and 100% methanol in MilliQ water. The collected fractions (I and II fractionation) were evaporated as described above.

2.4. Antibacterial Activity

2.4.1. Bacterial Strains

Antibacterial activity was tested against 8 bacterial strains—*Staphylococcus aureus* CCNPB/1505, *Pseudomonas aeruginosa* CCNPB/MBL, *Acinetobacter baumanii* CCNPB/O, *Enterococcus faecium* 45, *Aeromonas salmonicida* 2013, *Vibrio cholerae* 2329, *Vibrio diazotrophicus* Cd1 and *Klebsiella pneumoniae* CCNPB/1404 (Table S1). Four clinical isolates (no. 1–4) were kindly provided by Kamila Korzekwa PhD, Medical Laboratories Center Dialab (Wrocław, Poland) and are now kept in the Culture Collection of Northern Poland at Division of Marine Biotechnology (CCNP), University of Gdańsk. Environmental isolates

were obtained from Ewa Kotlarska PhD, Institute of Oceanology Polish Academy of Sciences (isolates no. 5–7 from IO PAN MB Strain Collection Institute of Oceanology, Polish Academy of Sciences, Molecular Biology Laboratory) and Aneta Łuczkiewicz PhD, Gdańsk University of Technology (isolate no. 8 from Department of Water and Wastewater Technology Strain Collection) [36–38].

2.4.2. Antibacterial Assay

Broth microdilution assay was performed according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations (http://www.eucast.org, accessed on 18 January 2021). Before the experiments, the bacteria were grown overnight on Mueller Hinton (Sigma-Aldrich, Steinheim, Germany) agar at 36 °C. For the experiment, bacterial suspensions (prepared in Ringer's solution and equal to 0.5 of McFarland standard (10^8 CFU mL^{-1})) were diluted to obtain ~1×10^6 CFU mL^{-1}. The assay was performed in 96-well sterile microplates (Eppendorf, Hamburg, Germany). The extracts and fractions were diluted in 2% v/v DMSO and tested in triplicates at concentrations of 500, 250, 100 and 10 μg mL^{-1}. The fractions were prepared using a two-fold serial microdilution method. The concentration of the fractions used in the experiment ranged from 1.95 μg mL^{-1} to 1000 μg mL^{-1}. The 96-well microplates were incubated overnight (Gram− for 16 h, Gram+ for 24 h) at 36 °C. The optical density (OP) of each well was measured at 620 nm using SpectraMax® i3 Platform (Molecular Devices, San Jose, CA, USA). The percentage of growth inhibition was calculated in comparison to the control (bacterial culture without the extract/fraction).

2.5. Cytotoxicity Assay

Cytotoxicity assay was performed on human breast adenocarcinoma cell line T47D (Merck KGaA, Darmstadt, Germany). The assay was performed based on the colorimetric MTT assay method described by Felczykowska [39]. T47D cells were plated at a concentration of 1×10^4 cells per well of 96-well plate containing RPMI1640 (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) medium supplemented with 10% fetal bovine serum (Merck KGaA, Darmstadt, Germany) and penicillin–streptomycin solution (50 U and 0.05 mg mL^{-1}, respectively; Merck KGaA, Darmstadt, Germany) and allowed to attach overnight (24 h of incubation at 37 °C; in CO_2 (5%)). After the incubation, the medium was replaced with a fresh portion of extracts or fractions dissolved in 1% v/v DMSO (Merck KGaA, Darmstadt, Germany) at final concentrations 200, 100, 50 and 25 μg mL^{-1}. The test was performed in three replicates for all the tested extracts, fractions and control samples. Plates were incubated for 24 h (37 °C). Then, 25 μL of MTT solution (4 mg mL^{-1}) was added to each well. After 4 h of incubation, the medium was removed and 100 μL of 100% DMSO was added to dissolve the formazan. The absorbance of the reaction mixtures was measured at 570 nm (with reference wavelength 660 nm) using a microplate reader (Spectramax i3, Molecular Devices, San Jose, CA, USA). Cell survival was calculated as the ratio of the mean absorbance of the tested samples in comparison to the control (mean absorbance of the corresponding solvent) and expressed as a percentage.

2.6. Enzyme Inhibition Assay

Trypsin inhibition assay was performed according to the methodology described by Pluotno and Carmeli [40], chymotrypsin and thrombin assay followed the procedure by Ocampo Bennet [41]. The extracts and fractions were diluted in 1% v/v DMSO at final concentrations of 45 and 4.5 μg mL^{-1}. Standard inhibitors were used as a positive control and 1% v/v DMSO with the addition of buffer, as a negative control. The absorbance of the solutions was measured at 405 nm with the application of a microplate reader (Varioskan Flash Thermo Fisher Scientific OY, Finland).

The final concentrations of enzymes used for the assays were 0.1 mg mL^{-1} for trypsin and chymotrypsin and 0.5 mg mL^{-1} for thrombin. In the case of trypsin and chymotrypsin, the mixtures containing the sample (10 μL) or inhibitor (10 μL), enzyme (10 μL) and

buffer (100 µL) were preincubated for 5 min at 37 °C. Then, the substrate solution (100 µL) was added, and the mixture was incubated for 10 additional min at 37 °C. In the case of thrombin, the sample (10 µL) or inhibitor (10 µL), with the addition of enzyme (10 µL) and buffer (170 µL) were preincubated at 36 °C for 10 min after which 20 µL of the substrate was added. The solution was incubated for another 10 min at 36 °C. The percentage of enzymatic inhibition was calculated in comparison to the positive control.

2.7. Acute Toxicity Assay

The toxicity of phytoplankton extracts towards the juvenile freshwater cladoceran *Daphnia magna* was evaluated in 24-h and 48-h bioassays. The tests were performed according to the procedure described by the producer (MicroBioTests Inc., Gent, Belgium) protocol. The test organisms were prepared for the experiment by incubating their cryptobiotic forms in Standard Medium (SM). The extracts were dissolved in 1% DMSO at final concentrations 10, 5 and 2.5 µg mL^{-1}. Specimens of *D. magna* were exposed to 10 mL of the prepared extracts. The assay plates containing ephippia (5 in each well) were incubated at 20 °C, 6000 lux. The assay was performed in triplicate. The test endpoint was the death of the organisms. The results were presented as the percentage of surviving organisms.

2.8. Analysis of Cyanometabolites

Analyses of cyanometabolites were done using Agilent HPLC system (Agilent Technologies, Waldboronn, Germany) coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer QTRAP LC-MS/MS (QTRAP5500, Applied Biosystems, Sciex; Canada) according to the method described by Mazur-Marzec et al. [42]. Chromatographic separation was performed on a Zorbax Eclipse XDB-C18 column (4.6 µm, 150 mm, 5 µm; Agilent Technologies, Santa Clara, CA, USA). To determine the content of crude extracts and fractions, the information-dependent acquisition method (non-target analysis) was used. Total ion current spectra were used to determine the most intense ion peaks. Data were processed with Analyst QS (Version 1.5.1, Applied Biosystems/MDS Analytical Technologies, Concord, ON, Canada, 2008).

2.9. Statistical Analysis

Non-parametric multidimensional scaling (nMDS) based on the Jaccard similarity coefficient [43] of presence-absence data, was used to represent the similarities of phytoplankton communities among the different samples. Phytoplankton samples were divided into groups with group-average linking [44]. One-way ANOSIM tests were used to determine the significances of the degree of separation among the nMDS groups. The stress values of the two MDS plots were determined, which is considered to adequately represent the similarity between the samples in nMDS plots. A stress value of <0.2 indicates an accurate representation of similarity rankings. nMDS and ANOSIM analyses were conducted using Primer v6 software. All biological experiments were carried out in triplicate, the data presented in this paper is expressed as a mean. The reliability of results was verified through the calculation of standard deviation.

3. Results

3.1. Phytoplankton Community

In total, 178 species were observed: 68 members of Chlorophyta, 35 of Bacillariophyta, 52 of Cyanophyta, 7 of Cryptophyta, 8 of Dinophyta, 3 of Euglenophyta, 2 of Chrysophyta and 1 of Haptophyta (Table S8). The phytoplankton species composition in the concentrated and non-concentrated samples did not differ. Single phytoplankton cell sizes varied from 0.4 to 700 µm, some were chain or colony-forming species.

Phytoplankton biomass and dominating phytoplankton groups differed among the samples (Figure 2, Table S8). Bacillariophyta accounted for the highest biomass in the majority of the samples (Figure 2a,b). The highest total biomass of the Bacillariophyta was measured in Samples 5, 6 and 7 (67–87% of the total phytoplankton biomass (TPB)),

the lowest was in Sample 2 (12% of the TPB). Amongst the diatoms, *Actinocyclus normanii* accounted for the highest contribution of the TPB (30–70% from the TPB), except Sample 2 (4% from the TPB) (Table S8). Chlorophyta dominated only in Sample 2 (61% of the TPB) (Figure 2a,b). Other phytoplankton groups did not exceed the 5% threshold of the TPB.

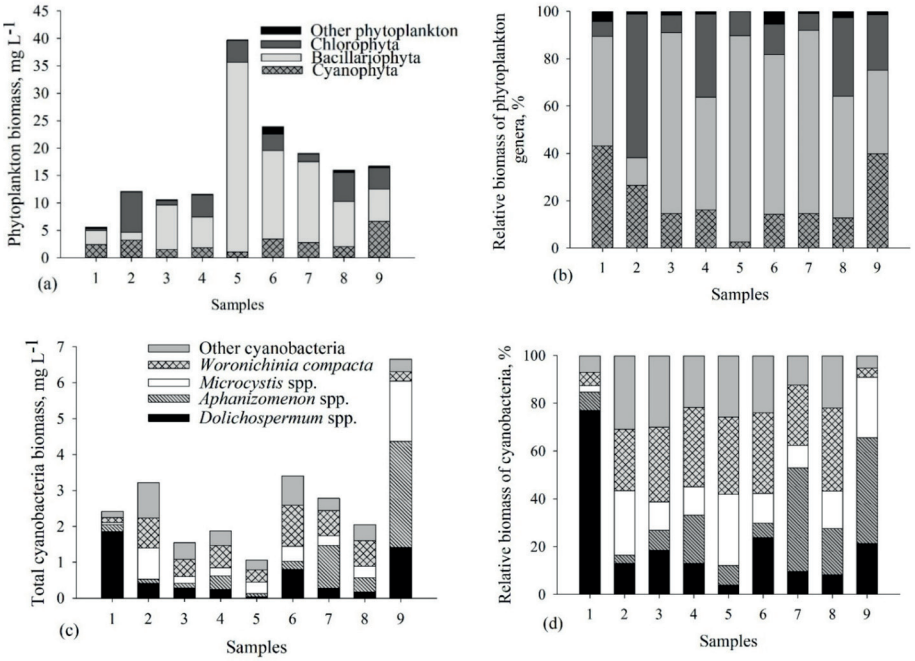

Figure 2. Structure, biomass (mg L^{-1}) and relative biomass of phytoplankton (**a**,**b**) and cyanobacteria (**c**,**d**) community in the collected samples.

The highest contribution of cyanobacteria biomass was measured in Samples 1, 2 and 9 (27–43% of the TPB), the lowest was in Sample 5 (3% of the TPB) (Figure 2c,d, Table S8). *Dolichospermum* spp., *Aphanizomenon* spp., *Microcystis* spp. and *Woronichinia compacta* belonged to the dominant cyanobacterial genera (Figure 2c,d). An evident contribution of *Dolichospermum flosaquae* was observed only in Sample 1 (47% of the total cyanobacterial biomass (TCB)), while in other samples this species accounted for less than 7% of the TCB (Table S8). In other samples, different species of *Dolichospermum* were also present (*D. crassum*, *D. planctonicum*, *D. lemmermanii*) and accounted for not more than 11% of the TCB. In comparison, *Microcystis* genus, *M. wesenbergii* and *M. flosaquae*, had a higher contribution to the biomass of Samples 2, 5 and 9 (~10% of the TCB). The highest contribution of *Aphanizomenon flosaquae* was observed in Samples 7 and 9. *W. compacta* predominated in almost all samples (Samples 2–8) and accounted for 25–35% of the TCB (Figure 2c,d, Table S8).

In order to highlight the importance of different phytoplankton communities leading to the potentially different bioactivity results (Tables 2 and 3, see results below), nMDS analysis of similarity was performed for each of the dominating phytoplankton communities (Bacillariophyta, Chlorophyta and Cyanophyta) separately (Figure S1). According

to the obtained results based on Bacillariophyta and Chlorophyta, six samples out of nine did not differ in species composition and were grouped into one cluster (Group A) (Global R − 1, $p < 0.01$) (Figure S1a,b). Despite the similarity of species composition in Group A, samples showed different bioactivity results in antibacterial, enzymatic, cytotoxic and acute toxicity bioassays (Tables 2 and 3). Considering the Cyanophyta community, nMDS analysis showed that the species composition was highly different among the samples (Global R − 0.765, $p < 0.01$) (Figure S1c) and indicated that the diversity of cyanobacteria influenced the recorded activity.

Table 2. Antibacterial activity of extracts obtained from the Curonian Lagoon phytoplankton. Results are expressed as a percentage of bacterial culture OD value compared to untreated control (100% growth). Different colors highlight the differences in OD values of bacterial cultures (the color code is explained below the table).

Bacterial Strains	Clinical Strains												Environmental Strains											
	Staphylococcus aureus CCNPB/1505			*Pseudomonas aeruginosa* CCNPB/MBL			*Acinetobacter baumanii* CCNPB/O			*Klebsiella pneumoniae* CCNPB/1404			*Enterococcus faecium* 45			*Aeromonas salmonicida* 2013			*Vibrio cholerae* 2329			*Vibrio diazotrophicus* Cd1		
Conc., µg mL^{-1}	500	250	125	500	250	125	500	250	125	500	250	125	500	250	125	500	250	125	500	250	125	500	250	125
Extracts I																								
Extracts II																								
Extracts III																								
Extracts IV																								
Extracts V																								
Extracts VI																								
Extracts VII																								
Extracts VIII																								
Extracts IX																								

0–20% | 20–50% | 50–70% | 70–100% | 100–120% | 120–150%

Table 3. Enzyme inhibition, cytotoxicity activity, and acute toxicity of phytoplankton extracts. Results are expressed as a percentage value of enzyme inhibition, cell viability (cytotoxicity assay) and cladocerans viability (acute toxicity assay), compared to untreated control. Different colors highlight the differences in values (the color code is explained below the table).

Bioassays	Enzyme Inhibition Assay				Cytotoxicity Assay				Acute Toxicity Assay					
	Trypsin		Thrombin		T47D Cells				*Daphnia Magna*					
									24 h			48 h		
Conc., µg mL^{-1}	45	4.5	45	4.5	200	100	50	25	10	5	2.5	10	5	2.5
Extracts I														
Extracts II														
Extracts III														
Extracts IV														
Extracts V														
Extracts VI														
Extracts VII														
Extracts VIII														
Extracts IX														

High enzymatic inhibition/ Low cell viability/High toxicity [1] | Low enzymatic inhibition/ High cell viability/Low toxicity [1]

0–20% | 20–50% | 50–70% | 70–100% | 100–120%

[1]-percentage of live cladocerans.

3.2. Bioactivity Screening of the Phytoplankton Extracts

To evaluate the biological activity of the extracts obtained from the collected phytoplankton, four different assays were applied (Tables 2 and 3, Tables S3 and S4). Considering the antibacterial assay, at least 50% growth inhibition of the clinical strains (compared to the control) was obtained for the extracts II-VI (Table 2 and Table S3). In the case of *S. aureus*, extract IV, at 250 µg mL^{-1}, reduced bacterial growth to less than 20% as compared to the control. Extract V, at 250 and 500 µg mL^{-1}, inhibited two clinical strains, *S. aureus* and *P. aeruginosa* by more than 70% and 50%, respectively. The growth of the environmental strains was inhibited by all tested extracts, except for extract VIII. *V. diazotrophicus* and *A. salmonicida* were found to be most sensitive to phytoplankton extracts I − VII. None of the extracts reduced the growth of *V. cholerae* and even slight growth stimulation was observed for the extracts IV and V. Extract IX only inhibited the growth of *E. faecium*

45 (by 51%). Irrespective of the strain used in the tests, extract VIII did not show any antibacterial activity.

In the enzymatic assay, all tested extracts inhibited trypsin and thrombin at the highest concentration (45 μg mL^{-1}) (Table 3 and Table S4). The strongest effect was exhibited by extracts VIII and IX, which inhibited trypsin and thrombin even at the lowest concentration applied in the assay (4.5 μg mL^{-1}; >50%).

Considering the cytotoxicity assay, extracts II-VII decreased T47D cancer cells viability by more than 80% when applied at the highest concentration (200 μg mL^{-1}). At 50 μg mL^{-1}, extract IV was most active (Table 3 and Table S4).

In the acute toxicity assay, after 24-h exposure, the extracts did not decrease the survivorship of *D. magna* (Table 3 and Table S4). Only the 48-h assay revealed a toxic effect of the samples. The dilutions of the extract I decreased the survivorship of cladocerans by approximately 52% (applied at 2.5 μg mL^{-1}) and 58% (applied at 5 μg mL^{-1}), in comparison with untreated organisms. Other extracts had a lower toxicity effect on *D. magna*; in the tests, 60–90% of the organisms survived.

3.3. Bioactivity Screening of Fractions

The extracts with the highest activity revealed in the assays were chosen for further fractionation and analyses. Extracts IV and V were chosen for antibacterial assays, extract IV for cytotoxicity assays and extracts VIII and IX for enzymatic assays (Table 4). As the effects of the samples on *D. magna* were rather weak and did not exceed 40% of the control, further tests on this organism were not performed.

Table 4. Antibacterial and cytotoxicity activities, and enzyme inhibition of fractions obtained after further separation of the extracts IV, V, VIII and IX.

Antibacterial Assay																				
Extracts	**IV**										**V**									
Fractions [1]	10	20	30	40	50	60	70	80	90	100	10	20	30	40	50	60	70	80	90	100
Staphylococcus aureus CCNPB/1505	–		–	–	–	–	+	++	+	+	–	–	–	–	–	–	+	+	++	+
Pseudomonas aeuruginosa CCNPB/MBL	–		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Enterococcus faecium 45	–		–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Vibrio cholerae 2329	–		–	–	–	–	++	++	–	++	–	–	–	–	–	–	–	+	++	+
Aeromonas salmonicida 2013	–		+	+	+	++	++	++	++	+	+	+	+	++	+	+	+	+	+	++
Vibrio diazotrophicus Cd1	+		+	+	++	+	+	–	+	+	+	++	+	+	+	+	+	+	+	+

Cytotoxicity Assay																				
Extracts	**IV**																			
Fractions [1]	10	20	30	40	50	60	70	80	90	100										
T47D	–		–	–	–	++	++	–	–	–										
Fractions (3rd testing step)	**IV-50**					**IV-60**					**IV-70**									
	20	40	60	90	100	20	40	60	90	100	20	40	60	90	100					
T47D	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–					

Enzyme Inhibition Assay																				
Extracts	**VIII**														**IX**					
Fractions [1]	10	20	30	40	50	60	70	80	90	100	10	20	30	40	50	60	70	80	90	100
Trypsin	–	–	–	+	+	+	++	++	+	+	–	–	+	++	+	+	+	++	+	+
Chymotrypsin	–	–	–	–	+	+	++	+	+	+	–	–	–	+	+	+	+	+	+	+
Thrombin	–	–	–	+	+	+	+	+	+	–	–	–	–	+	++	+	+	+	–	–

[1]—names of fractions (10–100; concentration of MeOH in water (10–100%) used to elute the fractions); "++" indicates bacterial growth/enzymatic/cell viability inhibition by more than 80%; "+" inhibition in the range 50–80%; "−" inhibition lower than 50%. Markings have been applied based on the activity obtained from the lowest tested concentration.

Fractions obtained from extracts IV and V inhibited the growth of four bacterial strains out of six applied in the antibacterial assays (Table 4 and Table S5). The fractions eluted with the solution containing the highest content of organic solvent (i.e., methanol) (IV-70 −

100, V-70 − 100) were highly active against the tested strains, mainly *S. aureus* CCNPB/1505 (bacterial growth reduced to less than 20% of control) and *V. cholerae* 2329 (reduced to less than 10%). The growth of *A. salmonicida* 2013 and *V. diazotrophicus* Cd1 was inhibited by almost all tested fractions (bacterial growth reduced to less than 50% of the control).

In the cytotoxicity assays, the viability of T45D cancer cells was affected by fractions IV-60 and IV-70 (Table 4 and Table S7). The most potent activity was observed for the fraction IV-70, which decreased cell viability to 47% of the control at the lowest concentration used in the assay (25 μg mL^{-1}). This fraction was used for the third testing step and the effect was observed only for the subfraction IV-70-90 (cell viability reduced to 22% at 200 μg mL^{-1}).

In enzyme inhibition assays, fractions 40-100 obtained from the extracts VIII and IX showed inhibitory activity against tested enzymes (Table 4 and Table S6). In the case of trypsin, chymotrypsin and thrombin, the highest inhibitory activity was obtained for fractions 40, 70 and 80 (more than 80% applied at 4.5 μg mL^{-1}), fraction 70 (more than 60% applied at 4.5 μg mL^{-1}) and fraction 50 (more than 60% applied at 4.5 μg mL^{-1}), respectively.

3.4. Analysis of Cyanopeptides

In bioactive fractions obtained from extracts IV, V, VIII and IX, 117 cyanopeptides were detected (Table S2). Although in many cases only partial structure identification was possible, due to the presence of several diagnostic ions in the fragmentation spectra (Figures 3–7, Figures S3–S12), the compounds could be assigned to one of the five cyanopeptide classes: the most numerous cyanopeptolins (CPs; 53 variants), microcystins (MCs; 19 variants), microginins (MGs; 18 variants), anabaenopeptins (APs; 14 variants) and aeruginosins (AERs; 13 variants).

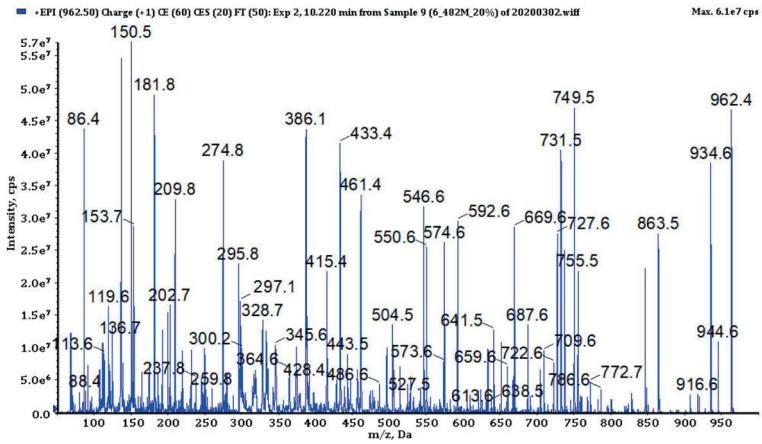

Figure 3. The enhanced product ion mass spectrum of cyanopeptoline CP979 with the suggested structure HA + Asp − [Thr^1 + Tyr^2 + Ahp^3 + Ile^4 + $MeTyr^5$ + Val^6] and the following fragment ions: *m*/*z* 962 $[M + H - H_2O]^+$, 944 $[M + H - 2H_2O]^+$, 934 $[M + H - H_2O - CO]^+$, 916 $[M + H - 2H_2O - CO]^+$, 863 $[M + H - H_2O - HA]^+$, 749 $[M + H - H_2O - (HA + Asp)]^+$, 731 $[M + H - 2H_2O - (HA + Asp)]^+$, 650 $[M + H - H_2O - (HA + Asp) - Val]^+$, 461 $[HA + Asp + Thr + Tyr + H - H_2O]^+$, 386 $[Ahp + Ile + MeTyr + H - H_2O]^+$, 297 $[Asp + Thr + Val + H - H_2O]^+$, 209 $[Ahp + Ile + H - H_2O]^+$, 181 $[Ahp + Ile + H - H_2O - CO]^+$, 150 MeTyr immonium, 136 Tyr immonium, 86 Ile immonium (HA − hexanoic acid, Ahp − 3-amino-6-hydroxy-2-piperidone).

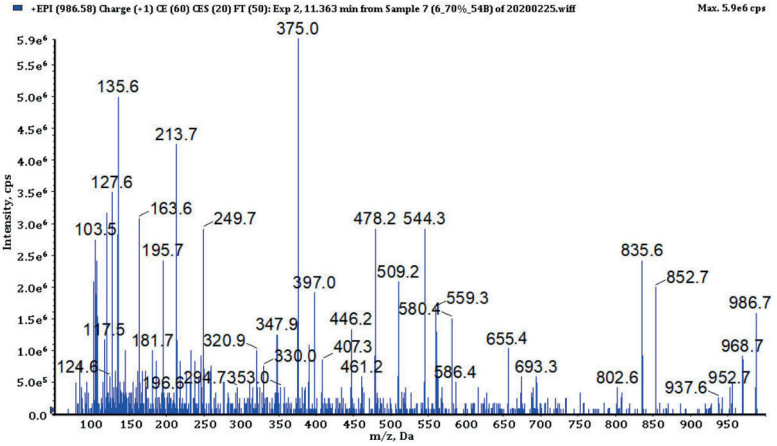

Figure 4. The enhanced product ion mass spectrum of microcystin MC-LF with the following fragment ions: *m*/*z* 986 $[M + H]^+$, 968 $[M + H - H_2O]^+$, 802 [MeAsp + Phe + Adda + Glu + MeDha + H]$^+$, 655 $[M + H -$ Adda $- H_2O]^+$, 580 [Adda + Glu + MeDha + Ala + H $- NH_3]^+$, 559 $[C_{11}H_{14}O$ + Glu + MeDha + Ala + Leu + H]$^+$, 544 [MeDha + Ala + Leu + MeAsp + Phe + H]$^+$, 509 [Adda + Glu + MeDha + H $- NH_3]^+$, 461 [Ala + Leu + MeAsp + Phe + H]$^+$, 446 $[C_{11}H_{14}O$ + Glu + MeDha + Ala + H]$^+$, 407 [Leu + MeAsp + Phe + $NH_4]^+$, 397 [Glu + MeDha + Ala + Leu + H]$^+$, 375 $[C_{11}H_{14}O$ + Glu + MeDha + H]$^+$, 213 [Glu + MeDha + H]$^+$, 127 [MeDha + Ala + H $-$ CO]$^+$, 120 Phe immonium (Adda $-$ (3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) MeDha-methyldehydroalanine).

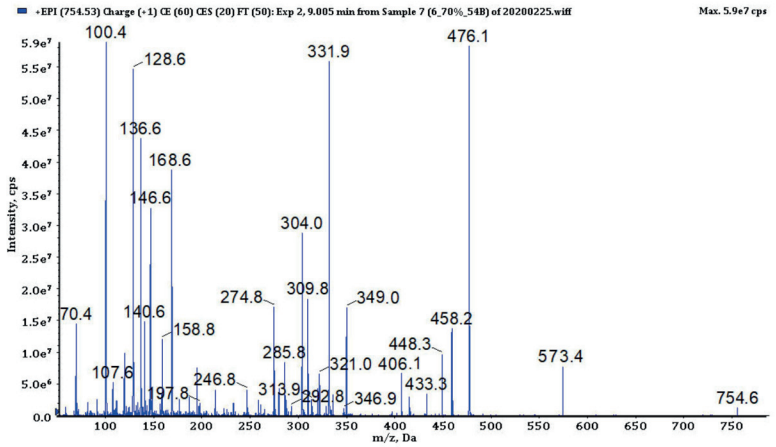

Figure 5. The enhanced product ion mass spectrum of microginin MG753 with the suggested structure Ahda + Tyr + MeIle/MeLeu + Pro + Tyr and the following fragment ions: *m*/*z* 754 $[M + H]^+$, 573 $[M + H -$ Tyr$]^+$, 476 $[M + H -$ (Tyr + Pro)$]^+$, 458 $[M + H -$ (Tyr + Pro) $- H_2O]^+$, 448 $[M + H -$ (Tyr + Pro) $-$ CO$]^+$, 406 [MeLeu + Pro + Tyr + H]$^+$, 349 $[M + H -$ (Tyr + Pro + MeLeu)$]^+$, 331 $[M + H -$ (Tyr + Pro + MeLeu) $- H_2O]^+$, 321 $[M + H -$ (Tyr + Pro + MeLeu) $-$ CO$]^+$, 279 [Pro + Tyr + H]$^+$, 168 [Ahda $- H_2O]^+$, 158 [Ahda $-$ CO]$^+$, 136 Tyr immonium, 128 Ahda fragment, 100 MeLeu immonium, 70 Pro immonium (Ahda $-$ 3-amino-2-hydroxy-decanoic acid).

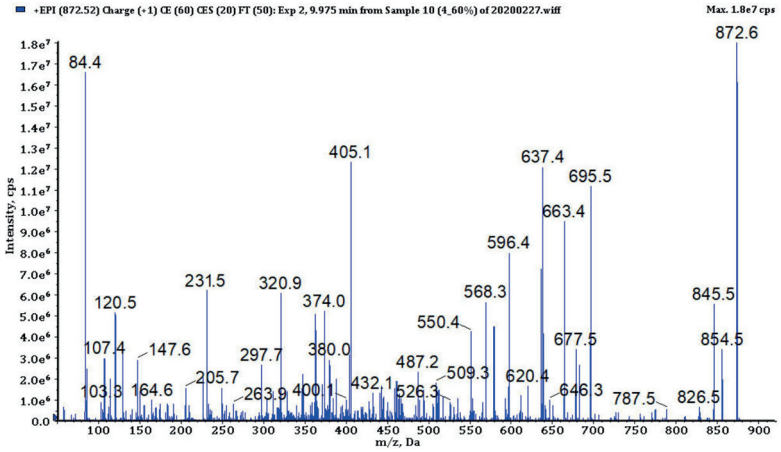

Figure 6. The enhanced product ion mass spectrum of anabaenopeptin AP871 with the suggested structure MeHTyr + CO − [Lys + Val + Hty + MeAla + Phe] and the following fragment ions: m/z 872 $[M + H]^+$, 854 $[M + H - H_2O]^+$, 845 $[M + H - CO]^+$, 826 $[M + H - H_2O - CO]^+$, 787 $[M + H -$ MeAla$]^+$, 773 $[M + H -$ Val$]^+$, 695 $[M + H -$ HTyr$]^+$, 663 $[M + H -$ MeHTyr $- H_2O]^+$, 596 $[M + H -$ (HTyr + Val)$]^+$, 578 $[M + H -$ (HTyr + Val) $- H_2O]^+$, 568 $[M + H -$ (HTyr + Val) $- CO]^+$, 550 $[M + H -$ (HTyr + Val) $- H_2O - CO]^+$, 405 [HTyr + Val + Lys + H$]^+$, 263 [MeAla + HTyr + H$]^+$, 231 [MeAla + Phe − H$]^+$, 164 MeHTyr immonium, 120 Phe immonium, 58 MeAla immonium, 84 Lys.

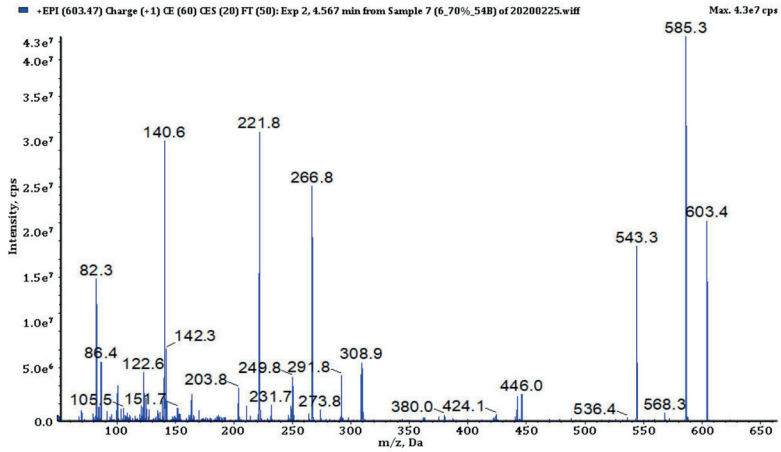

Figure 7. The enhanced product ion mass spectrum of aeruginosin K139 with the structure Hpla + Ile + Choi + Argal and the following fragment ions: m/z 603 $[M + H]^+$, 585 $[M + H - H_2O]^+$, 543 $[M + H - CH_2N_2 - H_2O]^+$, 446 $[M + H -$ Argal$]^+$, 308 [Choi + Argal + H $- NH_3]^+$, 291 [Choi + Argal + H $-NH_2 - H_2O]^+$, 266 [Choi + Argal + H $- CH_3N_2 - H_2O]^+$, 122 and 140 Choi ions, 142 Argal, 86 Leu immonium (Choi − 2-carboxy-6-hydroxyoctahydroindol, Hpla − (4-hydroxy)phenyllactic acid)).

The detection of CPs in the samples was based on spectral data published by Fuji et al. [45], Welker et al. [26,46] and Czarnecki et al. [47]. Structures of the peptides were mainly recognized by the ion peaks at m/z 420, 308, 234, 215, 150 (MeTyr immonium ion) and 120 (Phe immonium ion) that indicate the presence of Ahp + Phe + MeTyr fragment (Figure 3, Figures S3–S5; Ahp-3-amino-6-hydroxy-2-piperidone). In eight CPs (CP1012, CP993, CP986, CP984, CP965, CP951, CP911 and CP886) the substitution of chloride ion at MeTyr was detected by the shift of the peaks at m/z 420, 308 and 150 to m/z 454, 342 and 184. In few CPs spectra, the ion peaks at m/z 386, 274, 209, 181 and 86 that are characteristic for Ahp + Leu + MeTyr were observed. All Tyr^2-containing CPs were detected as dehydrated protonated molecules $[M + H - H_2O]^+$ (Figure 3).

The fragmentation spectra of MCs detected in samples from the Curonian Lagoon were compared with those published by other authors [48–50]. Based on the analysis (Figure 4, Figure S6) and the comparison of the spectra, we concluded that the detected MCs belong to the known MC variants previously described by Bouaïcha et al. [51] and included in CyanoMetDB [52]. Structure elucidation of MGs was mainly based on mass fragmentation spectra of the compounds published by Zervou et al. [53] and Carneiro et al. [54] and on the description of the important diagnostic ions of the peptides included in the work (e.g., m/z 128 for 3-amino-2-hydroxy-decanoic acid Ahda or m/z 142 for MeAhda). The process of structure elucidation of a new MGs variant, MG753, is illustrated in Figure 5 (Figure S7, Figure S8; MG928, MG783, respectively). In structure elucidation of APs, the fragmentation spectra published by Erhard et al. [55], Welker et al. [26] and Spoof et al. [56] were useful. The spectra always contained the peak at m/z 84 derived from the conserved Lys (Figure 6, Figures S9–S11). The kind of residue in a side chain was deduced based on the intensive ion peak at m/z $[M + H - (Arg/Tyr/Ile - CO)]$. The presence of Arg in this position was additionally confirmed by peaks at m/z 201 and 175. The MS/MS spectrum of AP871 and the description of the most characteristic fragment ions are shown in Figure 6. The fragmentation spectra of AERs were compared with those published by other authors [57] and was recognized by the presence of ions at m/z 140 and 122 characteristic for 2-carboxy-6-hydroxyoctahydroindole (Choi), and peaks at m/z 266, 291 and 308 indicating the presence of Choi + Argal fragment (Figure 7, Figure S12; 603, 619, respectively).

In the fractions eluted with the solution containing the highest content of organic solvent (i.e., methanol) (70–100) obtained from extracts IV and V, CPs and MGs constituted the dominant classes of peptides, with the highest number of detected variants, while the most intensive ion peaks in LC-MS chromatograms were observed for AERs and CPs (Table S9, Figures S2a–d). Of these, AER604 had the most intensive ion peak in the chromatograms of fractions IV-70 and V-70. In the fraction IV-80, only variant CP1006 was detected, while in the chromatogram of fraction V-90–CP1014 gave the most intensive ion peak. In the subfraction IV-70-90 the most intensive ion peaks in LC-MS chromatogram were observed for CPs, with CP1015 characterizes by the most intensive peak (Table S9, Figure S2e).

In the fractions (40–100) obtained from extracts VIII and IX, a high number of CPs variants were detected. High diversity of MGs was detected in the fractions VIII-80 and VIII-90, too. However, AERs, and especially AER567, gave the most intensive ion peaks in LC-MS chromatograms of the samples (Table S9, Figure S2f–j).

4. Discussion

Within this study, enzymatic, antimicrobial and cytotoxicity bioassays revealed high bioactivity of phytoplankton field samples collected from the Curonian Lagoon. Our results also showed that species diversity of phytoplankton, especially cyanobacteria, had a significant effect on the different bioactivity results. Bacillariophyta and Chlorophyta, two dominating phytoplankton groups did not differ in species diversity throughout the bioactive samples, while Cyanophyta species composition differed significantly. This fact suggests that secondary metabolites produced by cyanobacteria potentially may have had

a greater influence on the bioactivity results than the compounds produced by eukaryotic microalgae. Unfortunately, as complex bloom samples were analyzed, no reliable conclusion about the link between the observed activity and a specific sample component can be established. As in the study, the LC-MS/MS method optimized for cyanopeptide analysis was used, numerous compounds from this group of natural products could be detected. A list of 117 cyanopeptides is presented in Table S2. CPs were found to be the dominant and one of the most structurally diverse class of cyanopeptides. Peptides representing other classes, i.e., AERs, APs, MGs, MCs, were 3–4-fold less numerous in different variants. The composition of cyanopeptides in the environment highly depends on the diversity of cyanobacteria species and their genetic ability to effectively biosynthesize these metabolites under various biotic and abiotic conditions [52,58–60].

During our study the ecological significance of phytoplankton metabolites was assessed using extracts and fractions obtained from field samples. The samples were tested with the application of different environmental bacterial strains and *D. magna*. The activity of extracts against *D. magna* was rather weak—even after 48 h of incubation more than 50% of the individuals survived (except extract I obtained from Sample 1). *Daphnia* sp. is an important organism in the food chains; it grazes on phytoplankton organisms [61]. In many acute toxicity assays, relatively high concentrations of samples are used, which do not always reflect the real situation in the aquatic environment. These conditions might potentially correspond periods of phytoplankton blooms or their final stages, when cells collapse, and concentrations of the dissolved secondary metabolites increases significantly. Such conditions could have a negative effect on aquatic organisms (including zooplankton) [62,63]. However, during non-bloom periods, zooplankton uses mechanisms that help to maintain persistent coexistence of both groups (cyanobacteria or microalgae and zooplankton). According to the literature, the zooplankton response to microalgae may vary between species or strains. Moreover, zooplankton has detoxification mechanisms to minimize the negative effects of cyanobacteria [64]. In our study the concentrations of extracts used for acute toxicity assay potentially reflected non-bloom conditions and it is possible that the defense mechanisms were effective enough for the organisms to support their survival.

In terms of the antibacterial assays, the growth of almost all environmental bacterial strains, except antibiotic-resistant *V. cholerae* 2329, was inhibited by the tested samples (Table 2). The tests revealed the ability of phytoplankton species to produce antibacterial compounds potently active against environmental, naturally occurring *A. salmonicida* and *V. diazotrophicus*. These compounds might constitute an element of a defense strategy and increase the survivorship of the producer in extremely competitive environments where a huge variety of bacteria and other microbes co-exists [28,65]. In the case of *V. cholerae* 2329, no inhibition or even growth stimulation was observed during our study. *Vibrio* are naturally occurring marine and brackish water bacteria and their intensive proliferation mostly correlates with temperature (>20 °C) and salinity (5–10 ppt) [66]. It is known that several species of *Vibrio* are pathogenic and may cause toxigenic cholera and vibriosis [67]. In natural water bodies, rising water temperature [68], which is one of the major causes of cyanobacterial blooms and proliferation [69,70], may also provide an optimal environment for the occurrence of *Vibrio* species [66,71]. Moreover, the dissolved organic matter resulting from intensive phytoplankton blooms, especially cyanobacteria-derived organic matter, can significantly support the growth of potentially pathogenic *Vibrio* species [71–73]. Such synergy between cyanobacteria and *Vibrio* should be monitored as an element of bathing water quality assessment [74].

Along with the ecological significance, the investigations into the diversity, biological activities of natural products and their specific biotechnological applications were important elements of the study. In the assays, the inhibition of antibiotic-resistant *E. faecium* 45, isolated from the Sewage Treatment Plant, was observed (Table 2). The extracts were more active against *E. faecium* 45 than the tested fractions. It is believed that in some cases the mixtures of bioactive secondary metabolites could act more efficiently, compared with

separated (or pure) metabolites. Such effects can be attributed to additive or synergistic interactions between many different compounds [75]. Moreover, the sample processing may lead to the loss of activity as a consequence of compound degradation or modification. The nutrients removal efficiencies in wastewater treatment plants based on cyanobacteria or microalgae species are well documented [76], while their role and efficiency in pathogen removal are still under investigation [77]. The wastewater treatment systems do not entirely eliminate antibiotic-resistant strains of enterococci in the treated water [77,78], therefore phytoplankton species, or especially cyanobacteria, might assist in reducing pathogens and fecal bacteria present in wastewaters.

Pharmacy is another important field where the application of natural products has great potential [79,80]. Extracts and fractions tested during our study revealed antibacterial activity against clinical, antibiotic-resistant bacterial strains (Tables 2 and 4). Antibacterial activity of eukaryotic microalgae and cyanobacteria extracts, containing different classes of metabolites, is quite often reported in other studies [17,42,81]. During our study, the fractions eluted from solid-phase extraction cartridge with the more hydrophobic solvent (70–100% methanol in MiliQ water) were active against antibiotic-resistant Gram-positive bacteria. The mechanisms of action of cyanobacterial and microalgal metabolites against bacterial cells are not well described [82]. It has been proposed that the resistance of Gram-negative bacteria to metabolites produced by cyanobacteria is due to a hydrophilic outer membrane that blocks the penetration of hydrophobic metabolites through the cell membrane [83].

Our results also revealed high inhibitory activity of the tested extracts and fractions against serine proteases, trypsin, chymotrypsin and thrombin (Tables 3 and 4); activity against T47D cells also confirmed pharmaceutical potential of phytoplankton natural products. Serine proteases have different roles related to human health (digestion, immune response or blood coagulation) [84]. Cyanobacterial secondary metabolites, such as CPs and AERs are generally considered to be the main classes of cyanopeptides responsible for the inhibition of serine proteases [26,58,85,86]. CPs and related depsipeptides also have cytotoxic activities [87]. Amongst CPs, microcystilide A showed cell-differentiation-promoting activity using HL-60 human leucocytes [88]. Research done by Salem et al. [89], showed that *Microcystis* extracts containing CPs, MGs and other metabolites were active against HepG-2, colon CaCO-2 and breast MCF-7 cancer cell lines. Additionally, MGs congeners showed the cytotoxic effect. The compounds were active towards human hepatocellular carcinoma (HepG2) cell line and had genotoxic activity [90]. MGs are also known to be angiotensin-converting enzyme (ACE) inhibitors [91], leucine aminopeptidase, aminopeptidase M, bovine aminopeptidase N and trypsin inhibitors [92,93]. Metabolites produced by other organisms present in bloom samples were also reported to have interesting biological activities. For example, the extracts of *Nitzschia* (diatom) cells exhibited ACE-inhibitory activity [94]; sulfolipids, isolated from several species of *Scenedesmus* (green microalgae) were effective in inhibiting alpha-glucosidase, glutaminyl-peptide cyclotransferase and telomerase [95]. Diatoms are considered to be a source of promising anticancer agents, such as *Synedra acus* produce chrysolaminaran, which exhibits HT-29 and DLD-1 colon cancer cells [96]; the extracts of *Melosira* and *Nitzschia* induce IPC-81 leukemia cell death [97].

Our results support the idea that the biodiscovery of new compounds in the environment that have dynamic conditions (lagoons, estuaries) provides a relatively high diversity of bioactive metabolites with biotechnological potential [28]. The promising results obtained in this work, encourage us for further studies into the structure, activity and application of specific metabolites produced by microorganisms isolated from Curonian Lagoon.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/biom11081139/s1, Figure S1: Phytoplankton abundance and biomass analysis, Figure S1: nMDS plot based on presence/absence Jaccard similarity matrix of Bacillariophyta (a), Chlorophyta (b) and Cyanophyta (c) communities, Figure S2: The total ion current (TIC) spectra of fractions from phytoplankton samples: IV-70 (a), IV-80 (b), V-70 (c), V-90 (d), IV-70-90 (e), VIII-70 (f), VIII-80 (g),

IX-40 (h), IX-50 (i), IX-80 (j), Figure S3: The enhanced product ion mass spectrum of cyanopeptoline CP1055 with the suggested structure OA + Gln − [Thr^1 + Tyr^2 + Ahp^3 + Phe^4 + $MeTyr^5$ + Val^6] and the following fragment ions: m/z 939 $[M + H - H_2O - Val]^+$, 861 $[M + H - H_2O - MeTyr]^+$, 783 $[M + H - H_2O - (OA + Gln)]^+$, 765 $[M + H - 2H_2O - (OA + Gln)]^+$, 666 $[M + H - 2H_2O - (OA + Gln) - Val]^+$, 420 $[Ahp + Phe + MeTyr + H - H_2O]^+$, 150 MeTyr immonium; 136 Tyr immonium; 120 Phe immonium (OA − octanoic acid), Figure S4: The enhanced product ion mass spectrum of cyanopeptoline CP1012 with the suggested structure Ac + Gln − [Thr^1 + Arg^2 + Ahp^3 + Phe^4 + $ClMeTyr^5$ + Ile^6] and the following fragment ions: m/z 1013 $[M + H]^+$, 995 $[M + H - H_2O]^+$, 967 $[M + H - H_2O - CO]^+$, 882 $[M + H - H_2O - Ile]^+$, 864 $[M + H - 2H_2O - Ile]^+$, 842 $[M + H - H_2O - (Ac + Gln)]^+$, 824 $[M + H - 2H_2O - (Ac + Gln)]^+$, 796 $[M + H - 2H_2O - (Ac + Gln) - CO]^+$, 455 $[Ahp + Phe + ClMeTyr + H - H_2O]^+$, 428 $[Ac + Gln + Thr + Arg + H]^+$, 184 ClMeTyr immonium; 120 Phe immonium (Ac − acetyl group), Figure S5: The enhanced product ion mass spectrum of cyanopeptoline CP986 with the suggested structure HA + Gln − [Thr^1 + Arg^2 + Ahp^3 + Leu^4 + $MeTyr^5$ + Val^6] and the following fragment ions: m/z 987 $[M + H]^+$, 969 $[M + H - H_2O]^+$, 941 $[M + H - H_2O - CO]^+$, 870 $[M + H - HA - H_2O]^+$, 760 $[M + H - (HA + Gln)]^+$, 742 $[M + H - (HA + Gln) - H_2O]^+$, 714 $[M + H - (HA + Gln) - H_2O - CO]^+$, 584 $[M + H - (Ahp + Leu + MeTyr)]^+$, 484 $[HA + Gln + Thr + Arg + H]^+$, 386 $[Ahp + Ile + MeTyr + H - H_2O]^+$, 209 $[Ahp + Ile + H - H_2O]^+$, 181 $[Ahp + Ile + H - H_2O - CO]^+$, 150 MeTyr immonium, 136 Tyr immonium, 86 Ile immonium (HA − hexanoic acid), Figure S6: The enhanced product ion mass spectrum of microcystin [Ser^1]MC − HTyrR with the structure Adda − [Glu + Mdha + Ser + HTyr + MeAsp + Arg] and the following fragment ions: m/z 941 $[M + H - Adda]^+$, 923 $[C_{11}H_{14}O + Glu + Mdha + Ser + HTyr + MeAsp + Arg + H]^+$, 918 [Adda + Glu + Mdha + Ser + HTyr + MeAsp + H]$^+$, 863 [Ser + HTyr + MeAsp + Arg + Adda + H]$^+$, 728 [MeAsp + Arg + Adda + Glu + H]$^+$, 682 [Arg + Adda + Glu + Mdha + H]$^+$, 633 [Mdha + Ser + HTyr + MeAsp + Arg + H]$^+$, 599 [Arg + Adda + Glu + H]$^+$, 550 [Ser + HTyr + MeAsp + Arg + H]$^+$, 470 [Arg + Adda + H]$^+$, 375 $[C_{11}H_{14}O + Glu + Mdha + H]^+$, 213 [Glu + Mdha + H]$^+$, 135 Adda fragment, Figure S7: The enhanced product ion mass spectrum of microginin MG928 with the suggested structure MeAhda + Phe + MeLeu + HTyr + Pro + Tyr and the following fragment ions: m/z 929 $[M + H]^+$, 748 $[M + H - Tyr]^+$, 651 $[M + H - (Tyr + Pro)]^+$, 633 $[M + H - (Pro + Tyr) - H_2O]^+$, 474 $[M + H - (HTyr + Pro + Tyr)]^+$, 456 $[M + H - (HTyr + Pro + Tyr) - H_2O]^+$, 348 [MeAhda + Phe + H]$^+$, 330 $[MeAhda + Phe + H - H_2O]^+$, 182 $[MeAhda - H_2O]^+$, 172 $[MeAhda - CO]^+$, 150 Hty immonium ion, 142 MeAhda fragment, 120 Phe immonium, 100 MeLeu immonium, Figure S8: The enhanced product ion mass spectrum of microginin MG783 with the suggested structure MeAhda + Val + MeLeu + HTyr + Tyr and the following fragment ions: m/z 784 $[M + H]^+$, 603 $[M + H - Tyr]^+$, 426 $[M + H - (HTyr + Tyr)]^+$, 408 $[M + H - (HTyr + Tyr) - H_2O]^+$, 299 $[M + H - (MeLeu + HTyr + Tyr)]^+$, 281 $[M + H - (MeLeu + Htyr + Tyr) - H_2O]^+$, 182 $[MeAhda - H_2O]^+$, 172 $[MeAhda - CO]^+$, 142 MeAhda fragment, 100 MeLeu, Figure S9: The enhanced product ion mass spectrum of anabaenopeptin AP885CL with the suggested structure MeHTyr + CO − [Lys + Ile + Hty + MeAla + Phe] and the following fragment ions: m/z 886 $[M + H]^+$, 868 $[M + H - H_2O]^+$, 858 $[M + H - CO]^+$, 773 $[M + H - Ile]^+$, 709 $[M + H - HTyr]^+$, 691 $[M + H - HTyr - H_2O]^+$, 651 $[M + H - (CO + MeHTyr)]^+$, 633 $[M + H - (CO + MeHTyr) - H_2O]^+$, 405 $[M + H - MeHTyr - (Hty + Ile)]^+$, 320 $[M + H - MeHTyr - (MeAla + Hty + Ile)]^+$, 263 [MeAla + Hty + H]$^+$, 120 Phe immonium, 107 Tyr/HTyr, 84 Lys, Figure S10: The enhanced product ion mass spectrum of anabaenopeptin AP841CL with the suggested structure Tyr + CO − [Lys + Ile + Hph + MeAla + Phe] and the following fragment ions: m/z 842 $[M + H]^+$, 824 $[M + H - H_2O]^+$, 814 $[M + H - CO]^+$, 729 $[M + H - Ile]^+$, 681 $[M + H - Hph]^+$, 661 $[M + H - Tyr - H_2O]^+$, 635 $[M + H - (CO + Tyr)]^+$, 405 $[M + H - Tyr - (Hph + Ile)]^+$, 387 $[M + H - Tyr - (Hph + Ile) - H_2O]^+$, 320 $[M + H - Tyr - (MeAla + Hph + Ile)]^+$, 247 [MeAla + Hph + H]$^+$, 120 Phe immonium, 84 Lys, Figure S11: The enhanced product ion mass spectrum of anabaenopeptin AP915 with the suggested structure Tyr + CO − [Lys + Val + HTyr + MeHTyr + Ile] and the following fragment ions: m/z 916 $[M + H]^+$, 898 $[M + H - H_2O]^+$, 888 $[M + H - CO]^+$, 817 $[M + H - Val]^+$, 803 $[M + H - Ile]^+$, 739 $[M + H - HTyr]^+$, 721 $[M + H - HTyr - H_2O]^+$, 725 $[M + H - MeHTyr]^+$, 709 $[M + H - (CO + Tyr)]^+$, 612 $[M + H - (MeHTyr + Ile)]^+$, 477 $[M + H - Tyr - (HTyr + Val)]^+$, 369 [MeHTyr + HTyr + H]$^+$, 305 [MeHTyr + Ile + H]$^+$, 164 MeHTyr immonium, 84 Lys, Figure S12: The enhanced product ion mass spectrum of aeruginosin AER618 with the suggested structure Hpla + Leu/Ile + Choi + Arg and the following fragment ions: m/z 619 $[M + H]^+$, 601 $[M + H - H_2O]^+$, 559 $[M + H - H_2O - CH_2N_2]^+$, 455 $[M + H - Hpla]^+$, 325 $[Choi + Arg + H - NH_3]^+$, 307 $[Choi + Arg + H - NH_3 - H_2O]^+$, 282 $[Choi + Arg + H - CH_2N_2 - H_2O]^+$, 70 and 175 Arg, 122 and 140 Choi ions, 86 Leu/Ile immonium, Table S1: Bacterial strains used in the antibacterial activity assay,

Table S2: Microcystins and other oligopeptides detected in the fractions obtained from the Curonian Lagoon. Red color indicates active fractions; X indicates non-determined amino acids; empty cells—microcystins and other oligopeptides not detected, Table S3: Antibacterial activity of extracts obtained from Curonian Lagoon phytoplankton. Results are expressed as a percentage of bacterial culture OD value compared to untreated control (100% growth). Different colors highlight the differences in OD values of bacterial cultures (the color code is explained below the table), Table S4: Enzyme inhibition, cytotoxicity activity, and acute toxicity of phytoplankton extracts. Results are expressed as a percentage value of enzyme inhibition, cell viability (cytotoxicity assay) and cladocerans viability (acute toxicity assay), compared to untreated control. Different colors highlight the differences in values (the color code is explained below the table), Table S5: Antibacterial activity of fractions obtained after further separation of the extracts IV and V. Results are expressed as a percentage of bacterial culture OD value compared to untreated control (100% growth). Different colors highlight the differences in OD values of bacterial cultures (the color code is explained below the table), Table S6: Enzyme inhibition of fractions obtained after further separation of the extracts VIII and IX. Results are expressed as a percentage value of enzyme inhibition compared to untreated control. Different colors highlight the differences in values (the color code is explained below the table), Table S7: T47D cancer cells viability of fractions obtained after further separation of the extracts IV, and fractions IV-50, IV-60 and IV-70. Results are expressed as a range of percentage effect compared to untreated control. Different colors have been applied to highlight the differences in values (the color code is explained below the table), Table S8: Presence and dominance of phytoplankton species in the samples (1–9) from the Curonian lagoon. Cyanobacteria dominance was expressed as a percentage of total cyanobacteria biomass. The dominance of other species of phytoplankton was expressed as a percentage of total phytoplankton biomass. The color code is explained below the table. Table S9. Microcystins (MCs) and other oligopeptides detected in the most active fractions obtained from the phytoplankton samples collected in the Curonian Lagoon (the numbers in parentheses are given to distinguish compounds with the same molar mass but different amino acid sequences).

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