

Special Issue Reprint

Harmful Algae in a Changing World

Where Did We Come from and Where Are We Going?

Edited by
Katia Comte

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Harmful Algae in a Changing World: Where Did We Come from and Where Are We Going?

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Guest Editor

Katia Comte



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About the Editor

Katia Comte

Katia Comte received her PhD degree in Hydrobiology–Ecology (with a specialization in Phycology) from the University of Aix-Marseille, France (IMBE former IMEP Unit), and continued as a post-doctoral researcher in the period 2001–2007 at the Pasteur Institute (Paris, France), the Institute of Phycology (Trebou, Czech Republic) and the University of Biological Sciences (Bristol, UK) where she acquired molecular and taxonomic expertise on freshwater bloom-forming and toxic cyanobacteria. Building on this experience, in 2008 she joined the MCAM research unit (UMR7245) within the AVIV department of the National Museum of Natural History (Paris) to continue to pursue her main interests in ecotoxicology and environmental health, particularly the study of abiotic and biotic factors that may promote toxigenic cyanobacteria genotypes, as well as the impact of cyanotoxins on organisms and their fate in the trophic web. Since 2021, her research has focused on the interactions between toxic cyanobacteria and their potential predators, particularly ciliates (unicellular protists). Due to her recent appointment as curator of the MNHN’s free-living protist collection (i.e. “CEU” within RBCell collection) in 2025, she plans to expand her observations to other predator–prey pairs in marine environments on photosynthetic protists’ prey (a.k.a. microalgae) and atypical heterotrophic protists’ predators, absent from other international collections.

Preface

Freshwater and marine ecosystems are increasingly disturbed by the intensification of harmful algae blooms (HABs) including photosynthetic prokaryotes (i.e. cyanobacteria) and protists (i.e. dinoflagellates, diatoms, etc..) which seriously threaten food webs and human health worldwide.

Although eutrophication and climate warming contribute significantly to the actual frequency of HABs, the extent to which these rapid changes will affect their widespread distribution, potential dominance and toxicity will constitute a crucial global issue for years to come.

This Special Issue provides an overview of current research on the early and large-scale detection of HAB species in some understudied settings, and highlights innovative approaches to the fluorescent detection of multiple toxins that are potentially applicable to all water systems. As such, it aims to consider new environmental management strategies to mitigate future HABs.

Katia Comte

Guest Editor

Editorial

Harmful Algae in a Changing World: Where Did You Come from and Where Are We Going

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Aquatic environments, whether freshwater, brackish, or marine, are increasingly disrupted, in terms of frequency, extent, geographic distribution, and duration, by the massive, worldwide proliferation of harmful and/or nuisance algae, the so-called Harmful algal blooms (HABs), which are a global phenomenon that poses a major threat to human and animal health and ecosystems. While the majority of freshwater HABs are caused by cyanobacteria (cHAB), the marine bloom-forming species (approximately 300 species) are microalgae with detrimental effects, 80 of which are toxin-producing species belonging to eukaryotic flagellates such as Dinoflagellates and Raphidophytes (responsible for red-tides), as well as other groups including Diatoms, Haptophytes, Dictyophytes, and prokaryotic Cyanobacteria.

Not all species responsible for HABs act in the same way, nor do they all pose the same hazards to ecosystems and their biocenoses. A wide variety of secondary metabolites and modes of action (most of which are still poorly understood) exist within harmful species, leading to the classification of HABs into three types: the first includes “true” toxic species that produce various toxins that affect the entire food web (from protists to higher trophic levels, including humans) and cause various episodes of poisoning. The second type is defined by “non-toxic” species, whose large biomass alone is sufficient to induce serious damage and mass mortality of aquatic organisms through a drastic drop in dissolved oxygen concentration (transition from hypoxia to anoxia). The third type is represented by “non-toxin-producing” species exhibiting cytotoxic or ichthyotoxic properties, *via* multiple and not yet fully elucidated biochemical and physical mechanisms, such as the lytic properties of the membranes of other organisms (for example, the gills of fish), the production of reactive oxygen species (ROS), the harmful effects of certain polyunsaturated fatty acids (PUFAs), and the production of dense mucus. These “ichthyotoxic” microalgae represent approximately 70 to 80 species of phytoplankton, including dinoflagellates, raphidophytes, dictyophytes, and marine haptophytes from around the world.

There is a current consensus regarding the main causes of the global expansion of HABs worldwide, which is the excessive and accelerating eutrophication process by anthropogenic pressure in all water bodies. The ever-increasing input of nutrients (primarily phosphorus and nitrogen) from agricultural land, livestock farming, and their discharges into coastal waters poses a major global challenge that is difficult to contain due to its persistent storage resulting from massive accumulation in sediments over decades in aquatic environments.

Another major issue is climate change, with global warming projected at 1.7 to 4.9 °C between 1900 and 2100. In addition to the temperature rise in all aquatic systems, other direct and indirect consequences will appear, such as sea level rise (from 0.1 to 0.9 m in

2100–IPCC, 2001), ocean acidification due to increased CO₂, thermohaline, and/or water column stratification on a global scale, as well as extreme weather events (floods and droughts).

The extent to which these abiotic changes, identified as key stressors for environments, will affect the biodiversity, the expansion of the HABs species (and therefore their toxicity), and the functioning of water systems, including ecosystem services and economic resources (aquaculture, fisheries, tourism, etc.), constitutes a crucial issue for centuries to come on a global scale.

This Special Issue was open to the scientific community working on harmful algal blooms in freshwater and marine environments. It addresses the differences between past and present approaches to studying these HABs species (“where do we come from?”), and innovative approaches related to new methods of investigation that enable the early detection of species responsible for HABs or the measurement of their toxin concentrations in water samples (“where are we going?”).

In the first article, Wejnerowski et al. [1] analyzed the cyanobacterial community structure in an eutrophic lake in Poland, and compared the diversity of species between summer and winter seasons with a multiphasic approach, including classical microscopy observations, molecular analysis (genes implying in the toxin synthesis), and analytical techniques (HPLC-DAD, LC/MS, and ELISA) for two cyanotoxins detection (microcystin: MC and cylindrospermopsin: CYN) from water samples and isolated strains from the field. They found a large dominance of cyanobacteria on the water surface (around 94% in summer and 70% in winter), mainly represented by Oscillatoriales species, including *Planktothrix* and *Limnothrix* genera in summer, and dominated by *Limnothrix* in winter. They confirmed that the *Planktothrix* strains were MC-producing species, while no other toxins were detected by analytical methods, with the exception of the *cyrJ* gene in the water samples. Interestingly, they observed a shift within the cyanobacterial community: the year-round dominance of *P. agardhii*, noted in previous studies (2006–2008) in this lake, gave way to a permanent bloom of *Limnothrix redekei*, recently observed in many European lakes. This study provided a new confirmation that eutrophic temperate lakes are “bloom-friendly” for potentially toxic cyanobacteria throughout the year, thus highlighting the need for regular eco-phyco-toxicological monitoring of freshwater systems.

The following article, by Agasild et al. [2], also used the *mcyE* reference gene to detect microcystin-producing cyanobacterial strains, thus exploring a new avenue of research based on the transfer of these MCs within zooplankton communities and their impact on the food web. To date, few studies have examined the ingestion of these microcystins by different zooplankton taxa or the differences in ingestion capacity according to their feeding modes (grazers, filter feeders), using samples collected in situ during toxic blooms. This study provided original data from a eutrophic lake in Estonia, where *Microcystis* dominated the water body. They detected and quantified the *mcyE* gene by qPCR in the gut contents of crustaceans collected in situ, and they found that all guts contained *mcyE*, with significant variations between grazers, including a higher abundance in cyclopoid copepods than in cladocerans. The *mcyE* gene proved to be the preferred tool here, because it is present in a single copy per genome, allowing for an accurate assessment of *Microcystis* cell abundance in each grazing crustacean. They demonstrated that the transfer of toxic cells into the food web is primarily determined by the dynamics of specific and top grazers (omnivorous copepods being the predominant consumers compared to the generalist feeders like daphnia), rather than by the abundance of toxic cyanobacterial strains in natural aquatic environments.

The interactions between toxic species and the food web, in terms of detection and/or bioaccumulation, were also addressed in the article by Litaker et al. [3] using a “hybrid”

socio-toxicological approach. This approach combined the analysis of historical data dating back to 1948 concerning a traditional belief among Native Alaskan populations (that consuming a butter clam was safe if it had been prepared beforehand and its siphon removed), with a rigorous evaluation of different protocols and their level of safety, based on the detection of the phycotoxin STX (saxitoxin) in each tissue (using HPLC coupled with FLD). This marine toxin was produced by the dinoflagellate *Alexandrium catenella*, which frequently bloomed in Alaskan waters during the summer months, while tending to disappear during the winter.

By analyzing the STX content in all supposedly "edible" tissues, they unequivocally refuted the traditional belief that simple cleaning was sufficient to reliably reduce STX concentration and render the clam "safe" for consumption, particularly during the summer when values reaching 1000 µg STX eq. 100 g of tissue (capable of causing severe symptoms) were recorded. The presence of STX was also observed in winter, well after the disappearance of the *A. catenella* bloom, highlighting the ongoing risk of shellfish contamination by this neurotoxin and underscoring the lack of any means to predict when and where highly toxic shellfish will be harvested, which can have moderate-to-severe public health consequences.

While this study examined the STX concentrations from the common dinoflagellate *Alexandrium* sp and assessed the actual toxicity of ingesting shellfish contaminated with PSP, the other two articles [4,5] focused on the detection of potentially toxic marine species in two anthropized locations, a port in Tahiti [4] and an aquaculture area (in New Zealand) [5] by the use of a fairly recent technique: environmental DNA in situ (eDNA).

Fernandez et al. [4] used an alternative molecular method, environmental DNA (eDNA) metabarcoding of water samples, to assess the diversity of potentially toxic marine species in the port of Papeete (Tahiti), which could be a hotspot for a new large-scale distribution of species responsible for HABs. They identified a total of 21 potentially toxic species, including three directly associated with mass fish mortality events in this area and 18 other species known to produce a wide range of phycotoxins, including ciguater toxins (PSPs), other CFP toxins, and domoic acid (ASPs type, produced by diatoms). They found that the eDNA of *Gambierdiscus* sp. coincided with several ciguatera outbreaks in Tahiti between 2018 and 2024 and demonstrated that the majority of HABs detected by metabarcoding were currently present throughout the port area, from shallow to deep waters, potentially posing significant risks to seafood consumption and, consequently, to human health. Although this study did not confirm the actual presence of toxins in port waters (due to a lack of additional toxicological data), and therefore could not formally demonstrate a direct causal relationship, these results suggest the usefulness of eDNA analysis for the early detection of HABs and its potential as a promising tool for the routine monitoring and prevention of human risks associated with new alien contamination by various non-native species in these transit environments.

The study by Biessy et al. [5] was conducted in a New Zealand aquaculture area where shellfish farms are highly exposed to blooms of *Alexandrium pacificum*, a PSP-producing species that could impair biological resources and lead to economic losses for the region. The authors investigated biological interactions during and after *Alexandrium* blooms using high-throughput sequencing on prokaryotic and eukaryotic communities over two consecutive summers. They found significant changes in the composition of microbial communities, dominated by Rhodobacterales and Flavobacteriales during the *Alexandrium* bloom, and a high abundance of Pelagibacteriales during post-bloom. A shift towards the dominance of Syndiniales and Gymnodiniales eukaryotes was noted during the decline of the bloom, suggesting their possible ecological role in regulating and terminating this phase, as Syndiniales are known for their parasitic interactions (leading to the death

of their hosts). Concurrently, they found that copepods, particularly the *Paracalanus*, dominated the water column during the bloom period, indicating their high capacity to feed on toxic dinoflagellates and tolerate their toxicity levels. They emphasized the need to investigate parasite groups as well as grazing zooplankton as potential bioagents for mitigating harmful algal blooms (HABs) in aquaculture and marine environments. Their study makes an innovative contribution by combining metabarcoding and bloom ecology to reveal biotic interactions and key ecological factors, with a view to developing targeted management strategies to mitigate future HABs.

The last three articles [6–8] address *in vivo* experimental studies focused on the development of new molecular tools for the specific detection of toxins directly in producing cells or in water samples. They exploit the chemical nature of DNA structure to develop fluorescence-based methods enabling the specific detection of NRPS (non-ribosomal peptide synthetases) involved in the synthesis of microcystins (MCs) and anabaenopeptides APS [7,8] or of oligonucleotides specific to the marine phycotoxins [6].

The study by Al Tabban et al. [6] described a fluorescent approach for the simultaneous detection of six marine toxins (including MCs, anatoxin-a, STX, OA, brevetoxin (BTX), and cylindrospermopsin (CYN)) *in vivo*, on strains, and in water samples. This original method relies on a duplex-to-complex structure-switching approach using an aptamer sequence (used as a bioreceptor) specific to each targeted toxin, which is labeled with a fluorescent dye and combined with their corresponding cDNA, conjugated with a fluorescent quencher. In the absence of toxin in the water sample, the aptamer–cDNA complex is formed, and the fluorescence is quenched (=inhibited). If the toxin is present in the sample, the aptamer binds to its specific target (in this case, the toxin) and releases the cDNA (the aptamer's affinity for its target being higher than its affinity for the cDNA used). An aptamer–toxin complex then forms, and fluorescence is restored, its intensity being correlated with the toxin concentration. High sensitivity was observed for the detection of all six toxins, with good selectivity for each aptamer and no cross-reactivity. These results demonstrate that the aptamer-based technique could be an excellent alternative to traditional methods (immunoassays and analytical techniques such as LC/MS) for the *in situ* and simultaneous detection of various toxins, which is crucial for ensuring water safety.

The last two articles by Kurmayer et al. [7,8] described an innovative method based on the incorporation (i.e., “clickable”) of non-natural amino acids into the adenylation domain of the NRPS of two common freshwater cyanotoxins: microcystins (MCs) in *Microcystis* cells and anabaenopeptides (APS) in *Planktothrix agardhii*. The juxtaposition of a fluorochrome to these amino acids provided chemo-selective labeling, allowing the clickable cyanopeptide (i.e., chemically modified cyanopeptide) to be localized in the cells and to track the intracellular dynamics by analytical chemistry and high-resolution imaging (laser-scanning confocal microscopy). The first study [7] confirmed a quantitative relationship between clickable modified cyanopeptide content and the signal intensity resulting from chemo-selective labeling (during maximum growth) throughout time-lapse build-up or decline experiments. The second study [8] focused more specifically on the time-lapse experiment, using the pulse feeding of three non-natural amino acids to observe the synthesis and/or decay of chemically modified MC and APS in *Microcystis* and *Planktothrix* cells under maximum growth rate conditions. The authors demonstrated a rapid increase in clickable MC and APS content, linked to the growth rate, and a steady decay of clickable MC and APS through dilution due to the growth, rather than a regulated or induced release during the synthesis process. This highlights a new method for tracking toxin synthesis and its intracellular dynamics, and paves the way for promising management strategies for preventing risks to water safety and public health through the early detection of toxin-producing cells in water bodies.

To conclude, this Special Issue presents new data and innovative approaches to better understand the distribution and presence of harmful algal blooms in all aquatic systems, as well as their potential expansion in the face of global surface water warming and the resulting unforeseen environmental changes.

While these articles offer new perspectives on the early and large-scale detection of HAB species (including the presence of their respective toxins) in marine and freshwater ecosystems, using both classical and exploratory molecular tools, they all focus on toxin-producing species. This underscores the need to complement broader research efforts on other classes of HABs, including “non-toxic” species associated with animal mortality or health impairments, for which these latest innovative fluorescent-toxin-complex techniques will not be applicable. This need is all the more fundamental as an acceleration in the widespread distribution and frequency of these “nuisance” algae is predicted through flash blooms due to global warming and a rapidly changing world.

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Article

Prokaryotic and Eukaryotic Community Succession and Potential Parasitic Interactions During Two *Alexandrium pacificum* Blooms in Aotearoa New Zealand

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Abstract

Harmful algal blooms (HABs), caused by the dinoflagellate *Alexandrium pacificum*, are increasingly frequent in the Marlborough Sounds, an important aquaculture region in Aotearoa New Zealand. *Alexandrium pacificum* produces paralytic shellfish toxins and blooms cause significant economic and ecological disruptions through contamination of edible shellfish. High-throughput sequencing of prokaryotic and eukaryotic communities was used to investigate community dynamics during bloom events across two consecutive summers. Distinct successional shifts were observed, with prokaryotic communities dominated by Rhodobacterales and Flavobacteriales during blooms, and increased abundance of the SAR11 clade (Pelagibacterales) post-bloom. Eukaryotic diversity was dominated by *Alexandrium* species (Gonyaulacales) during the bloom, and subsequently shifted towards Syndiniales, Gymnodiniales, and Peridiniales as blooms collapsed. Significant correlations indicated potential ecological roles for these taxa in bloom regulation, particularly Syndiniales, which could indicate parasitic interactions. Depth profiles revealed consistent microbial composition throughout the water column, validating depth-integrated sampling strategies for community studies. This research describes changes in the composition of microbial communities during two *A. pacificum* blooms, suggesting that species interactions (e.g., via parasitism) may play a role shaping bloom dynamics. Further studies incorporating environmental parameters, especially nutrient dynamics linked to anthropogenic activities, are necessary to better understand the drivers of blooms in this important aquaculture region.

Keywords: dinoflagellates; metabarcoding; depth sampling; Syndiniales; community composition

Key Contribution: The abundance of Pelagibacterales ASVs increased post-bloom period and the Syndiniales ASVs correlated with *Alexandrium* bloom collapse. Depth-integrated sampling represents full water column communities and microbial interactions may regulate *Alexandrium* bloom dynamics.

1. Introduction

Over the past decades, reports of marine harmful algal blooms (HABs) have increased in frequency, duration, and geographical distribution. This has been attributed to global climate changes and to anthropogenic impacts, such as eutrophication [1,2]. HABs can cause significant impacts on human and animal health due to the production of toxic or

bioactive compounds [3]. HABs can also have major economic [4,5] and environmental repercussions [6], for example, by restricting the harvest of shellfish until toxins depurate or by directly impacting on the health of fish and shellfish species [7].

In the marine environment, paralytic shellfish toxins (PSTs) are produced by HAB species from the genera *Alexandrium*, *Gymnodinium*, and *Pyrodinium*. PSTs negatively impact the shellfish aquaculture industry, causing harvest closures worldwide, with species from the genus *Alexandrium* the most commonly implicated [8]. *Alexandrium pacificum* (previously known as *A. catenella* in Aotearoa New Zealand) can develop long-lasting bloom events as well as extensive cyst beds, which can drive reoccurring bloom events. *Alexandrium pacificum* commonly occurs along the east coast of the North Island of Aotearoa New Zealand, but it was only detected for the first time in the top of the South Island in 2010. It now causes regular HAB events in one of the main shellfish production regions of Aotearoa New Zealand—the Marlborough Sounds [9]. Since 2010, it has resulted in widespread PST contamination of shellfish with associated closures of harvests for several months each year [9].

Conditions leading to the recurrence of HAB species have attracted a great deal of interest in the last two decades and previous studies have investigated the ecological niche of *A. pacificum* [10,11]. *Alexandrium pacificum* blooms were found to be associated with water surface temperature between 21 and 25 °C, salinities of around 34 psu or higher than 37 psu, and these blooms dominated when both NO₃ and NH₄ levels were high [12]. Increasingly, biotic interactions between microbial taxa, including competition, parasitism, and predation, have been recognized as critical factors regulating HABs dynamics. Previous research suggests certain bacterial groups may directly influence the growth and toxicity of dinoflagellate blooms, either by enhancing bloom initiation through nutrient cycling or by contributing to bloom termination via competition for essential nutrients [13–15]. Interspecific competition could also play a role in the promotion or decline of phytoplankton blooms via allelopathy [16], with some toxic *Alexandrium* species shown to have allelopathic activity [17]. It has also only been suggested that pressure from parasites can have a great impact on HAB dynamics, particularly their elimination [18].

High-throughput DNA sequencing techniques provide a powerful approach for capturing the diversity, composition, and succession patterns of microbial communities at high resolution, significantly expanding upon traditional microscopy and culture-based methodologies [19–21]. These methods enable a more comprehensive understanding of microbial interactions and their potential roles in influencing harmful algal bloom dynamics. Previous research utilizing high throughput sequencing for examining HAB interactions has largely focused on bacterial taxa and in freshwater environments [22,23]. However, a study on associations amongst bacteria, archaea, and microeukaryotes during a spring bloom of the dinoflagellate *A. pacificum* showed that Gammaproteobacteria and Bacteroidetes were predominant during the initial bloom stage, while Alphaproteobacteria, Cyanobacteria, and Actinobacteria were the most abundant taxa present during bloom onset and termination [24]. For eukaryotes, as expected, the bloom was dominated by *A. pacificum*, followed by a mixed assemblage of diatoms, green algae, rotifers, and other microzooplankton during bloom termination [24]. Interactions and response of microbial communities during marine HAB events are still not well understood.

The aim of this study was to investigate successional changes in both prokaryotic and eukaryotic communities during two distinct *Alexandrium pacificum* blooms in the Marlborough Sounds region of Aotearoa New Zealand, across two different summer periods, using high-throughput sequencing methodologies. The study site, Opuia Bay, experiences regular blooms of *A. pacificum* (previously *A. catenella*). This species has been well characterized at this location and all strains are known to produce paralytic shellfish

toxins [9,25]. During blooms, bivalve species exceed the regulatory level of 0.8 mg/kg shellfish meat as saxitoxin equivalents for extended periods of time [25]. Plankton dynamics in this region are critically understudied despite the recurrent economic and ecological impacts of HABs. By comparing microbial community structure in two distinct *A. pacificum* bloom events, the study provides insights into why certain microbial species dominate and identifies factors influencing bloom development and termination, thereby enhancing our ability to predict and manage these harmful events in this commercially important region of Aotearoa New Zealand.

2. Results

2.1. Microscopic Observations

The microscopic observations showed three distinct bloom peaks of the dinoflagellate *A. pacificum* during the summer of 2016. The first and largest peak occurred on 7–24th of January, the second peak was from the 24th of February to 9th of March, and the smallest and last peak started on the 31st of March and finished on the 11th of April (Figure 1A). The total dinoflagellates number peaked on the 11th of February at 1062×10^3 cells L^{-1} , due to a mixed bloom of *Karenia longicanalis* (reaching 934×10^3 cells L^{-1}) and *Polykrikos geminatum* (reaching 126×10^3 cells L^{-1} ; Figure 1B). During the 2017 summer, only one peak of *A. pacificum* occurred (9 February–1 March, Figure 1A), with extremely high cell concentrations (241×10^3 cells L^{-1}). This species dominated the total dinoflagellate counts for that date. This was followed by a mixed bloom of *Karenia longicanalis* and *Pentapharsodinium* sp.; both species do not produce PSTs (Figure 1B).

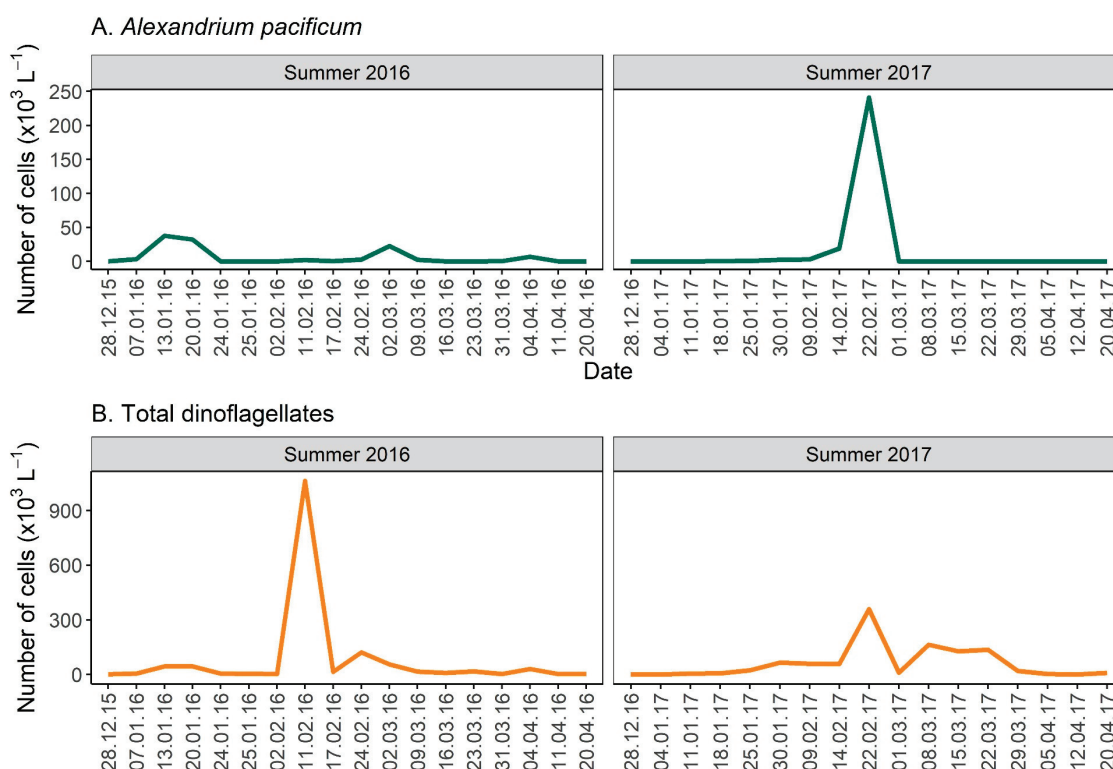


Figure 1. *Alexandrium pacificum* counts ((A); $\times 10^3$ cells L^{-1}) and total dinoflagellates counts ((B); $\times 10^3$ cells L^{-1}) using microscopic observations during algal blooms in Opua Bay, Aotearoa New Zealand in the summers of 2016 and 2017.

2.2. Community Composition Using Metabarcoding

After quality filtering the 65 water samples, the 16S rRNA gene barcode generated 3971 unique ASVs and the 18S rRNA barcode generated 2962 ASVs. There were no

noticeable changes in the observed bacterial diversity during the *A. pacificum* blooms for both years with the observed number of ASVs varying between 100 and 330 but the eukaryotic diversity decreased during the blooms (from 200 to 300 ASVs to <50 ASVs during the blooms). Very few Metazoan ASVs were present in the samples (less than 20 at all times) and numbers did not fluctuate during the different blooms.

The two dominant orders of prokaryotes were Rhodobacterales (13–72%) and Flavobacteriales (4–51%) in all samples for both summers (Figure 2A). Alteromonadales were also present in most samples but at lower abundance (0–46%). There were no discernible changes in the prokaryotic composition during the blooms, except for an increase in the relative abundance of the SAR11 clade order following the *A. pacificum* bloom. Regarding the eukaryotic abundance, there were three distinct blooms of Gonyaulacales (with the genus *Alexandrium* representing 95–100% of the abundance) during the summer of 2016 and one single longer bloom during the summer of 2017. For both years, as the *Alexandrium* bloom disappeared, Syndiniales and Gymnodiniales abundances increased, as well as Peridinales to a smaller extent (Figure 2B). Most of the families present in the Syndiniales order were either Amoebohyphaceae or unclassified. Regarding Metazoan community composition, Crustacea was the dominant order (49–100%) in the water column for most samples and there were no changes observed during the blooms, with the dominant genera being from copepods (*Paracalanus* (27–90% of the total relative abundance), *Oithona*, and *Centropages*).

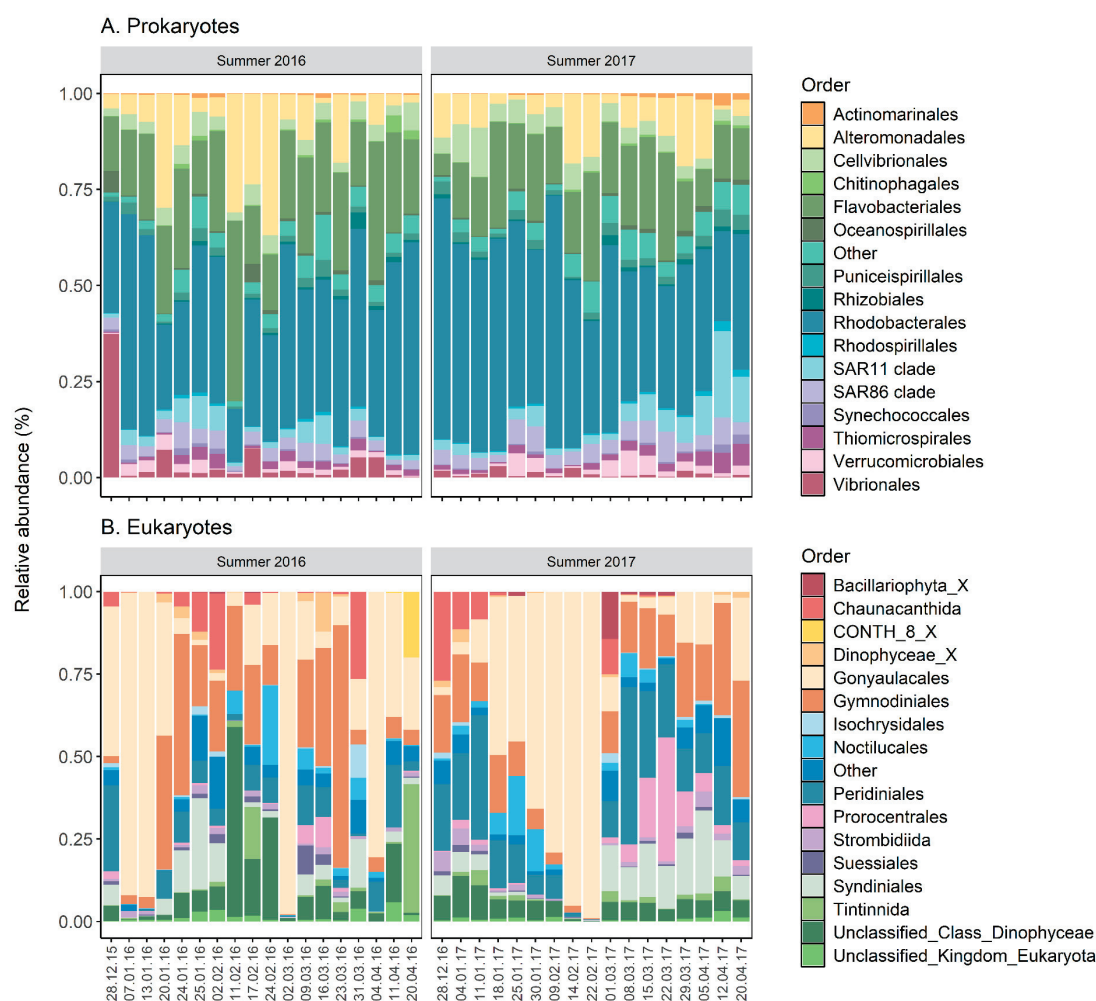


Figure 2. Stacked bar plots showing the relative abundance of prokaryotic (A) and eukaryotic (B) community composition at the order levels from water samples collected during blooms of *Alexandrium pacificum* in Opua Bay, Aotearoa New Zealand.

Multivariate analysis (PERMANOVA) showed significant differences in community composition amongst the two summers for prokaryotes (Bray–Curtis, $p = 0.006$, $F = 2.41$) but no significant difference in eukaryotes (Bray–Curtis, $p = 0.37$, $F = 1.04$) communities. After combining the communities from the two summers, the PERMANOVA analysis showed significant differences in community composition amongst the two stages of the blooms (during the bloom: ‘Bloom’, and pre- and post-bloom combined: ‘No bloom’; Figure 3) for prokaryotes (Bray–Curtis, $p < 0.001$, $F = 2.90$) and eukaryotes (Bray–Curtis, $p < 0.001$, $F = 7.65$).

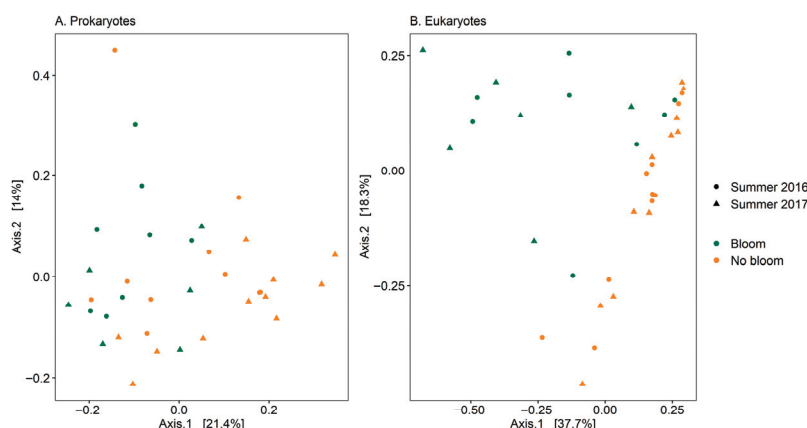


Figure 3. Principal Coordinates Analysis (PCoA) based on Bray–Curtis dissimilarities with 999 permutations of prokaryotic (A) and eukaryotic (B); including metazoans) community composition of water samples collected during *Alexandrium pacificum* blooms and pre- and post-blooms (‘no bloom’) at the Amplicon Sequence Variant level from Opuia Bay, Aotearoa New Zealand. For each axis, in square brackets, the percentage of variation explained was reported.

Three eukaryotic orders had significant relationships with changes in the abundance of Gonyaulacales (composed mainly by the genus *Alexandrium*). The strongest relationship was identified for Syndiniales ($r^2 = 0.443$, $p < 0.001$, Figure 4A). There were also significant but weak linear relationships with Gymnodiniales ($r^2 = 0.135$, $p = 0.03$; Figure 4B) and Peridiniales ($r^2 = 0.113$, $p = 0.048$; Figure 4C).

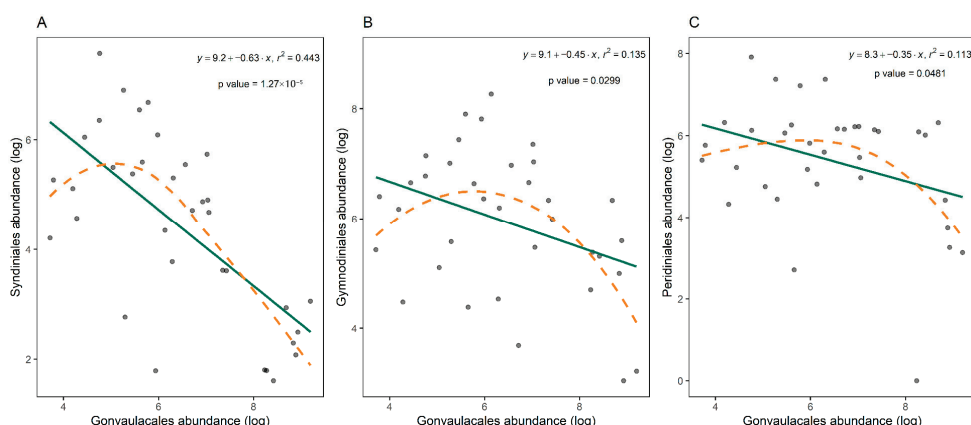


Figure 4. Linear regressions between (x) log-transformed Gonyaulacales abundance and (y) log-transformed abundances of Syndiniales (A), Gymnodiniales (B), and Peridiniales (C) from water samples collected during *Alexandrium pacificum* blooms. Correlation coefficients (r^2), significance of the relationship ($p < 0.05$ taken as significant), and linear equations are presented. The solid line represents the linear regression, and the dashed line represents the smooth curve of a locally estimated scatterplot smoothing (loess) regression.

2.3. Depth Profile and Community Composition

No noticeable changes were observed in the prokaryotic composition for each day at different depths, at the order level, with Rhodobacterales, Flavobacteriales, and Alteromonadales dominating the water column (Figure 5A). An increase in the relative abundance of bacteria from the SAR11 clade was observed once the bloom has terminated. There were also very little changes in the eukaryotic composition at different depths with Gonyaulacales dominating the entire water column during the bloom periods and Gymnodiniales being the dominant eukaryotic order outside of the bloom periods (Figure 5B). The order Strombidiida was only present in the 0 and 3 m samples and the Syndiniales abundance increased in the deeper samples (from 9 to 15 m). There were no changes in the metazoan composition for the different depths, with Crustacea almost exclusively dominating the water column with the copepod genus *Paracalanus*.

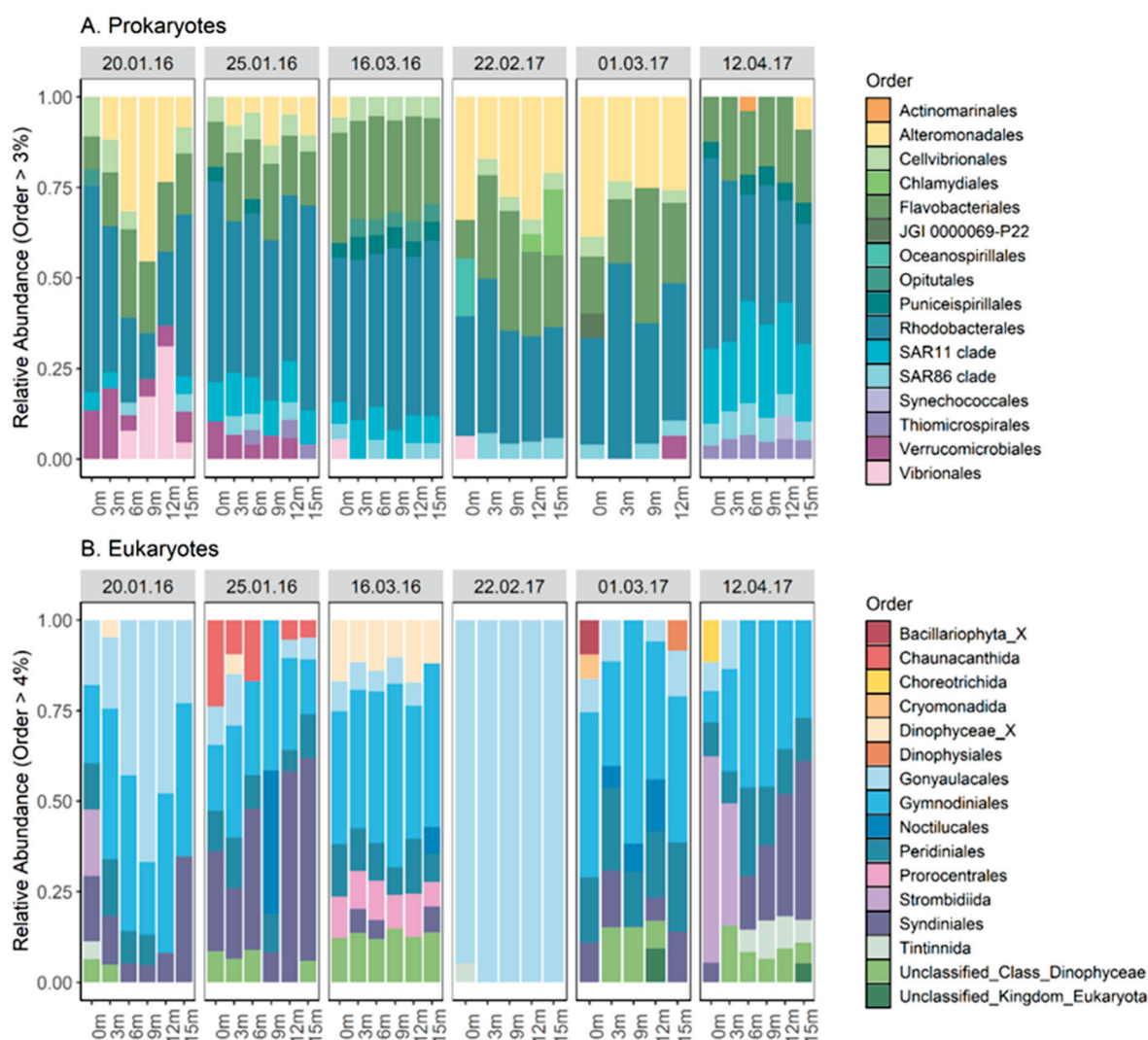


Figure 5. Stacked bar plots showing the relative abundance of prokaryotic (A) and eukaryotic (B) compositions at the order level in water samples collected at different depths in the water column in 2016 and 2017.

3. Discussion

The increasing frequency, duration, and geographic spread of HABs are of growing ecological and economic concern, including in Aotearoa New Zealand [5]. This study showed the recurrence of *A. pacificum* bloom events over two summers in the Marlborough

Sounds region of Aotearoa New Zealand, a commercially important aquaculture area. This study focused on summer blooms from 2016 and 2017 but blooms of PST-producing *A. pacificum* still occur every year, or several times per year in Opuia Bay, the study site. This region was and still is regularly affected by shellfish farm closures due to PST contamination [5,9]. The structure of prokaryotic and eukaryotic communities and bloom dynamics between the two sampled periods highlight the complexity of ecological factors that drive bloom initiation and collapse.

The shifts in prokaryotic and eukaryotic communities during the bloom succession underline the importance of microbial interactions as potential biological drivers. For instance, blooms of *Alexandrium pacificum* coincided with reduced microbial diversity and dominance of specific bacterial taxa such as Rhodobacterales and Flavobacteriales, and the succession towards Syndiniales and Gymnodiniales during bloom decline suggests these groups may play critical roles in the termination phase. Thus, understanding these microbial community dynamics and their interactions with environmental factors is vital to predict and potentially mitigate the detrimental impacts of recurrent *A. pacificum* blooms in this region.

One important shift in the prokaryotic community was the increase in the relative abundance of bacteria from the SAR11 clade order, now known Pelagibacterales, an order of the Alphaproteobacteria class [26]. This is one of the most abundant bacterial orders in ocean surface waters and Pelagibacterales are usually considered oligotrophs that can feed on dissolved organic carbon and nitrogen [27–29]. This result suggests the potential roles of Pelagibacterales in nutrient cycling during bloom collapse and should be investigated further.

Regarding the changes in the relative abundance of the order Gonyaulacales, identified as *Alexandrium pacificum* using microscopy, increases in the relative abundance of Syndiniales, Gymnodiniales, and Peridiniales taxa were observed after the collapse of the blooms. These findings indicate that some of these groups could have roles in the ecology of the *Alexandrium* blooms, such as parasitism, competition, allelopathy, or grazing pressure. This was particularly noticeable, with a strong significant and positive relationship observed for the group Syndiniales, with a low relative abundance before the bloom and an increase after the bloom. Syndiniales (Dinophyceae) are a common and diverse parasitic group, but their ecology remains poorly understood [30]. All described Syndiniales automatically kill their host, including other eukaryotes (e.g., dinoflagellates) and metazoans [31]. This finding suggests a parasitic relationship potentially influencing bloom termination between Gonyaulacales (*A. pacificum* in this case) and Syndiniales. A previous study hypothesized that the decline in toxic *Alexandrium minutum* bloom in a French estuary may have been due to an introduced dinoflagellate parasite (e.g., *Amoebophyra* sp.) [18].

Our study also showed that copepods (*Paracalanus* spp.) dominated the water column both during the blooming period and outside of blooming events. Previous studies have looked at the importance of mesozooplankton (i.e., copepods) grazing as a potential factor of HAB collapse. Results showed that copepods can graze on toxic microalgae, regardless of the toxicity levels [32]; the ingestion of toxic dinoflagellate cells is species-specific [33] and could be an important factor affecting the bloom development of the dinoflagellates [34]. This highlights the potential of copepods, particularly species of *Paracalanus*, as an avenue for exploring HAB mitigation in Aotearoa New Zealand.

Metabarcoding techniques have enabled the study of fine-scale HAB dynamics, with the ability to undertake a detailed, culture-free analysis of microbial communities throughout the water column during a bloom event, characterizing taxa from multiple trophic levels. But there are also limitations to this method. One crucial step in metabarcoding studies is the choice of a targeted gene region, as it could affect the community compo-

sition obtained [35–37]. For the eukaryotic 18S rRNA gene, the short V9 region or the longer V4 region is the most commonly targeted and thus well represented in reference databases [38,39]. However, dinoflagellates have unusually large genomes with associated high rDNA copy number and lots of intra-specific variation [40,41]. It is worth noting that metabarcoding studies of phytoplankton species can be biased towards dinoflagellates and this may influence the levels of diversity and relative abundance of these taxa in studies that target rRNA genes [42]. Additionally, when studying community dynamics, the taxonomic identification of species based on DNA sequence data relies heavily on accurate and well-curated reference databases. Regarding the prokaryotic communities, articles assigned to cyanobacteria were low in this study, but it is worth noting that the primers used can be biased towards detection of general bacterial and might not be appropriate for the detection of cyanobacterial species [43]. Also, the reference databases for all eukaryotes, particularly for dinoflagellates, vary in completeness and availability, depending on the taxonomic group targeted [44]. Relevant to this study, very little is known about the majority of Syndiniales species [45], and this could limit their identification from eukaryotic communities.

The differences in both prokaryotic and eukaryotic community structure at different depths in the water column were also studied. For each sampling date, only some small changes in the relative abundance of the communities within the water column were observed in the prokaryotic communities. It was similar for the eukaryotic communities, apart from the order Strombidiida which was only present on the top layer (0–3 m), and the order Noctilucales, only present in the middle layer (3–12 m). This highlights that a composite sample of the whole water column, when possible, is enough to have a representative community composition for the entire water column for environmental DNA studies, as long as replication is adequate.

Environmental conditions and parameters were not recorded in this study, limiting the understanding of abiotic drivers of *A. pacificum* blooms in the Marlborough Sounds region and this should be the focus of future studies. This information would help understand if *A. pacificum* blooms in this region are also associated with a specific range of water surface temperatures (21–25 °C), salinities (34–37 psu), and if these blooms dominate when both NO₃ and NH₄ levels were high, as demonstrated by Bravo, Vila, Masó, Figueroa, and Ramilo [12]. Blooms of *A. pacificum* have been a recurring issue in the Marlborough Sounds since 2010, particularly in Opuia Bay, the focus of this study [9,46]. The Tory channel, where the study site is located, is naturally high in nitrate due to upwelling from the nearby Cook Strait, a strait that separates the North and South Islands of Aotearoa New Zealand [47], and it is likely that these conditions promote the formation of HABs. Prior to European settlement, the shoreline of Opuia Bay was covered in lowland forest and dominated by native forest in the higher elevations [48]. Native forests were cleared in the mid-1800s and today, the majority of the catchment is colonized by introduced pine trees *Pinus radiata* [49]. Impacts of forestry on the aquatic ecosystems can be significant (e.g., increase in fine sediments and nutrients after logging activities [49–52]) and the impacts of forestry practices on the ecology of algal communities and water quality has been demonstrated in freshwater systems [53–55]. Potentially, changes in the catchment from the pine forestry in Opuia Bay could be associated with the exacerbation of HAB species in this region. Environmental conditions preceding bloom events should be prioritized for any future studies to determine abiotic drivers of HABs.

4. Conclusions

To conclude, this study provided insights into microbial community dynamics associated with *A. pacificum* blooms in an important aquaculture region. We showed that

prokaryotic communities were dominated by Rhodobacterales and Flavobacteriales during blooms, with Pelagibacterales (SAR11 clade) increasing post-bloom, suggesting a role in nutrient cycling during bloom decline. Eukaryotic succession was characterized by *Alexandrium* dominance followed by rises in Syndiniales, Gymnodiniales, and Peridinales, with Syndiniales strongly correlated with bloom collapse, pointing to potential parasitic interactions. Depth-integrated sampling was validated as representative of whole water column dynamics, strengthening its use for future HAB studies. These findings highlight the novel contribution of combining high-throughput sequencing with bloom ecology to reveal biotic interactions that may regulate bloom termination. They will help focus efforts for future investigations into microbial interactions and environmental and ecological drivers, critical for developing predictive tools and targeted management strategies to mitigate HABs in Aotearoa New Zealand. Future research should prioritize characterizing environmental factors, including nutrient dynamics influenced by nearby forestry and aquaculture practices, to further understand the conditions triggering HAB events.

5. Materials and Methods

5.1. Study Site

Samples were collected from a boat moored at a sampling station in Opua Bay (Marlborough Sounds, Aotearoa New Zealand; $41^{\circ}16'23.2''$ S $174^{\circ}12'04.1''$ E; Figure 6A). This site was chosen as it has recurring dense blooms of PST-producing *A. pacificum* every year since 2010 [9] and is known to contain particularly high numbers of *A. pacificum* cysts in the sediments [46]. Sampling was carried out regularly over two summer periods (December 2015 to April 2016 and December 2017 to April 2018). Water samples were collected using van Dorn water bottle casts, at three-meter intervals from surface to 15 m (i.e., at 0 m, 3 m, 6 m, 9 m, 12 m, and 15 m). Depth samples were then pooled and subsampled for analysis, as described below. To understand the differences in communities at different depths, during the two summers, some sampling points were randomly selected and also analyzed as discrete depth profiles. A CTD/fluorometer cast to measure chlorophyll-*a* fluorescence was also made at each sampling occurrence.

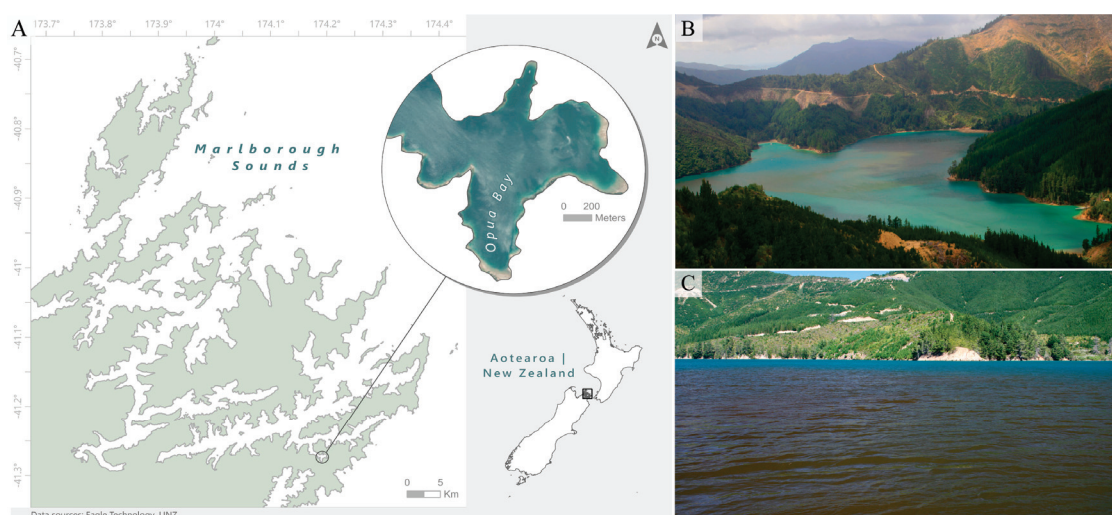


Figure 6. (A) Map of the study location, Opua Bay (Marlborough Sounds, Aotearoa New Zealand), and photographs from January 2016 of *Alexandrium pacificum* blooms within Opua Bay, showing (B) aerial view of the Bay when a bloom is occurring, and (C) water discoloration.

5.2. Microscopy Analysis

From each sample, a 100 mL subsample was preserved in Lugol's solution for microscopic analyses. Subsamples (10 mL) were taken and examined using inverted light microscope (LM) and Utermöhl chambers. The number of dinoflagellate cells per liter, including *A. pacificum*, was calculated.

5.3. DNA Extraction, Polymerase Chain Reaction, High Throughput Sequencing, and Bioinformatics

From each water sample, two liters were filtered on a sterile 0.45 µm filter and DNA was extracted from the filter using the DNeasy PowerSoil Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions, and negative extraction controls were included every 24th sample. The V3-V4 regions of the bacterial 16S ribosomal RNA (rRNA) gene (Bact341F- 5'- CCT ACG GGN GGC WGC AG-3' and Bact785R- 5'-GAC TAC HVG GGT ATC TAA TCC-3') [56,57] and the V4 region of the eukaryote nuclear 18S rRNA gene (Uni18SF: 5'-AGG GCA AKY CTG GTG CCA GC-3' and Uni18SR: 5'-GRC GGT ATC TRA TCG YCT T-3') [58] were amplified by Polymerase Chain Reaction (PCR). PCRs, purification, and library preparation were undertaken, as described in Biessy et al. [59], and using a Nextera Index kit (Illumina, San Diego, CA, USA).

Bioinformatic pipelines for both the 16S and 18S rRNA genes were identical unless otherwise stated. Primers were removed from the raw reads using 'cutadapt' with 1 mismatch allowed [60] and subsequently processed using the DADA2 package [61] within R [62]. Reads were truncated (230 and 228 bp for forward and reverse reads, respectively) and filtered with a maximum number of 'expected errors' (maxEE) threshold of two (forward reads) and four (reverse reads). Those reads not matching this criterion were discarded from further analysis. A parametric error matrix was constructed based on the first 108 bp of the sequences. The processes for dereplication, singletons, and chimera removal were undertaken using the protocol described in Biessy et al. [63].

The resulting chimera-checked Amplicon Sequence Variants (ASVs) were used for taxonomic classification against the SILVA 138 database for the 16rRNA [64] and the PR2 database [65]. The sequences were classified based on the rdp classifier [66] with a bootstrap of 50 so as to be able to obtain classifications at higher taxonomic levels. The results were combined into a phyloseq object [67] and for the 16S rRNA dataset sequences assigned as microeukaryotes, chloroplasts and mitochondria were removed. The sums of the reads from the negative controls were evaluated and contamination ASVs were subtracted from all samples.

For comparisons between samples, subsampling to an even depth was undertaken for each sample at a depth of 9000 and 10,000 reads for the prokaryotic and eukaryotic datasets, respectively. The Metazoan were a subset out of the eukaryotic sequences to be analyzed on their own. Alpha diversity numbers were calculated using the estimate_richness function from the phyloseq package in R [67] at any given day; however, statistical differences in alpha diversity could not be assessed due to lack of replication. Phylogenetically annotated 16S rRNA and 18S rRNA sequences were used to characterize prokaryotic and eukaryotic community compositions of each island type at order, family, and genus levels. Stacked bar plots, and abundance bubble plots were generated using the package ggplot2 [68] in R, based on the average relative abundance of sequence reads attributed to a given order, family, or genus within each summer. The proportion of explained variance between each year on the ASV composition dissimilarities was evaluated using a permutational analysis of variance (PERMANOVA) test implemented by the adonis function in the vegan package [69]. The PERMANOVA test was also used to determine the dissimilarities between the community composition during the bloom (>25% relative abundance of the

genus *Alexandrium* at any given day) and pre- or post-bloom and visualization of the observed patterns was obtained by means of a principal coordinates analysis (PCoA) using a Bray–Curtis similarity matrix. Relationships between some eukaryotic and prokaryotic orders and the Gonyaulacales order were determined using linear regressions.

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Conflicts of Interest: The authors declare no conflicts of interest.

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Article

Metabarcoding Reveals Diversity of Potentially Toxic Algae in Papeete Port (Tahiti)

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Abstract

Harmful algae are transported in various compartments of maritime vessels, making ports with heavy maritime traffic potential hotspots for their introduction and spread. In this study, we investigate the port of Papeete (Tahiti, French Polynesia), a key hub for numerous South Pacific shipping routes. Using metabarcoding on DNA extracted from water samples (environmental DNA, eDNA) we identified 21 species of harmful algae comprising to Bacillariophyceae (4), Dinophyceae (14), and Haptophyta (3 species). Three of those species are directly associated with fish mortality events without recognized toxigenic capacity. The remaining harmful algae species are known to produce a wide range of toxins, like the ciguatera toxin produced by endemic *Gambierdiscus* sp., domoic acid, haemolysins, yessotoxins, and others. Health risks such as ciguatera and paralytic shellfish poisoning were identified. An increase in *Gambierdiscus* frequency in Papeete port waters was parallel to an increase in ciguatera fish poisoning events in Tahiti, which suggests the value of eDNA analysis for early warning of harmful algae presence. Management measures, including banning fishing near the ports, could prevent public health risks associated with harmful algae blooms.

Keywords: harmful algae; health risks; seafood resource risks; metabarcoding; French Polynesia

Key Contribution: Environmental DNA (eDNA) reveals a high number of harmful algae in Papeete port (Tahiti). The prevalence of *Gambierdiscus* eDNA is in accordance with the number of ciguatera poisoning outbreaks in Tahiti in different years; eDNA could be used for early detection of harmful algae.

1. Introduction

Harmful algae are toxin-producing algae capable of excessive proliferation in aquatic environments, a phenomenon known as harmful algae blooms (HABs, thereafter). Inadvertent consumption of these algae, typically through contaminated seafood, can cause severe health effects, and may even be lethal in some cases, depending on the type and amount of toxin ingested [1]. Given the increasing global occurrence of HABs, their detection and prediction are especially important. Therefore, monitoring harmful algae has been enhanced in recent years [2].

In the central and south Pacific Ocean, most reported HAB events are linked to seafood toxins, principally due to ciguatera [2]. Ciguatera fish poisoning (CFP) is caused by the dinoflagellate species of the genus *Gambierdiscus* and has been documented in Hawaii and

other Pacific islands, with incidence rates reaching up to 100,000 cases annually [3]. In French Polynesia, consumption of seafood contaminated with ciguatoxins represents a major public health issue [4]. CFP incidence rates are dangerously high in some archipelagos [5], for example, on Rapa Island in the Australes [6], with a hotspot of *Gambierdiscus* species reported in Tahiti [5,7].

Harmful algae can be transported in ballast water [8], in bilge water [9], or even via biofouling on ship hulls [10], making ports potential entry points and reservoirs that should be surveyed as possible sources of these organisms. Conventional harmful algae surveys are based on collecting water samples and scanning them for microalgae using a light microscope; in addition, or as an alternative, molecular tools are also employed, providing a high sample throughput rate and sensitivity [11]. A method of harmful algae detection that is increasingly employed involves metabarcoding on environmental DNA (eDNA), that is, DNA extracted directly from water or sediment filter samples. Metabarcoding consists of amplifying from that DNA a sequence that serves to identify species, i.e., DNA barcode, followed by massive sequencing (Next Generation Sequencing, NGS) and bioinformatic analysis to compare sequences against reference databases for taxonomic identification [12,13]. Metabarcoding of water and sediment samples has been used to detect harmful algae in multiple studies [8,14].

In this study, we applied eDNA metabarcoding on water samples from Papeete port (Tahiti). Papeete, the capital of French Polynesia, hosts the major port of the whole region ($-17.5333^{\circ}/-149.5666^{\circ}$ coordinates; Figure 1) with facilities for general, bulk, and oil cargo, handling approximately 1,900,000 t of international cargo and 2,940,000 passengers annually [15]. Given this high volume of maritime traffic, we expected to find a relatively high number of toxic algae, particularly *Gambierdiscus* species. The objectives of this study were therefore (1) to inventory algae classified as harmful and (2) to assess the potential health risks of these species based on the toxins they are known to produce. To examine the relationship between the prevalence of harmful algae detected from metabarcoding and the reported toxicity outbreaks reported in the region, we compared the results obtained in 2018 [16] and 2023 (present study) for *Gambierdiscus* and CFP.

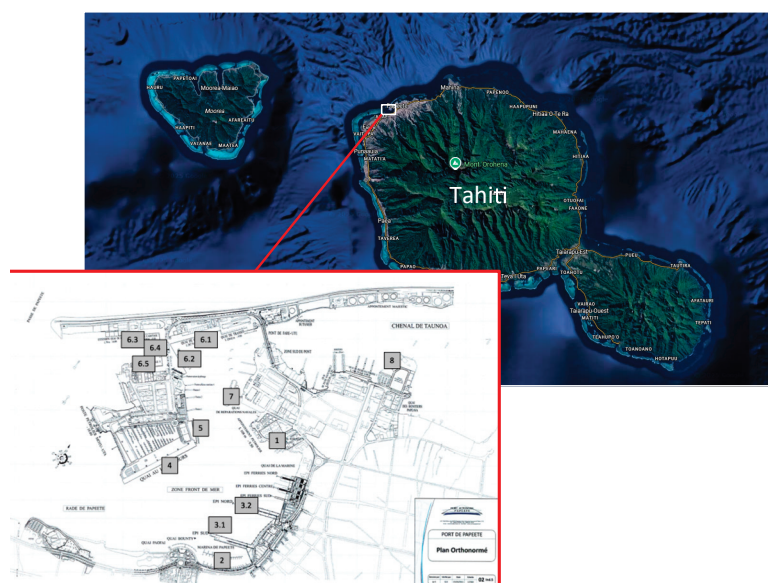


Figure 1. Map of Tahiti Island showing Papeete port. Modified from Google (n.d.). Retrieved on 28 June 2025. The location of the sampling points is shown in the map of the port (shown above on the left). The numbers (1, 2, 3.1, 3.2, 4, 5, 6.1 to 6.5, 7, 8) are the codes of the sampling points.

2. Results

The raw metabarcoding data (high-throughput sequencing, HTS) are available in NCBI database with the Bioproject number PRJNA1282046. A total of 1,738,896, 2,464,840, and 3,664,168 quality reads of the 18S rRNA, RBCL, and COI genes, respectively, were obtained from water samples taken from 13 sampling locations evenly distributed across Papeete port to have a good coverage of its area (Supplementary Table S1, where the letter S in the sample codes refers to samples taken from the surface and the letter F to deeper samples). Shallow (surface) samples provided 48.8%, 50.4%, and 51.6% of the quality sequences, respectively, for 18S rRNA, RBCL, and COI genes. Of the total quality reads, 1,501,192 were taxonomically assigned to 191 phytoplankton species (Supplementary Table S2), while 3697 (0.25%) corresponding to 65 phytoplankton taxa could not be assigned down to a species level. The taxonomic composition (Table 1) shows a higher proportion of diatoms (Bacillariophyta), followed by dinoflagellates (Myzozoa), both in terms of the number of species and the quality sequences assigned to them.

Table 1. Taxonomic summary of the phytoplankton found in this study from Papeete port using metabarcoding. Number of species and number of quality sequences per phylum are presented, combining the three markers.

Phylum	Species	Sequences
Bacillariophyta	115	881,323
Chlorophyta	5	18,852
Cryptophyta	3	133
Crysophyta	1	73
Haptophyta	4	1581
Heterokontophyta	1	3393
Myzozoa	52	125,937
Ochrophyta	10	69,793

In total, 21 species known to be associated with HABs [16] were found, with the majority being dinoflagellates (14 species), followed by diatoms (4 species), and 3 Haptophyta species (Table 2). The proportion of species associated with HABs over the total number of phytoplankton species ranged between 0.147 and 0.207 across the sampling sites. The sites were homogeneous for this distribution of potentially harmful algae (contingency chi-square $\chi^2 = 1.119$, 12 degrees of freedom, $p = 0.99$).

Many species were detected across all the sampling locations, whereas *Amphidinium carterae* and *Gambierdiscus carpenteri* were less frequent, each found only from at only a single location. Three species (*Margalefidinium fulvescens*, *Margalefidinium polykrikoides*, and *Tripos fusus*) are known to be associated with fish mortality events but not with specific toxicogenic activity [17–19]. The remaining species are well-documented toxin producers [17]. Those of the genus *Pseudo-nitzschia* produce domoic acid; *Akashiwo sanguinea*, *Amphidinium carterae*, and *Pheocystis globosa*, hemolytic toxins; *Alexandrium affine*, paralytic shellfish poisoning toxins; *Amphidoma* and *Azadinium* species, azaspiracids; *Coolia canariensis* and *Protoceratium reticulatum*, yessotoxins; *Gambierdiscus* species, maitotoxins and ciguatoxins (CFP toxins); *Ostreopsis ovata*, ovatoxins; *Chrysochromulina leadbeateri*, sterolysins; and *Prymnesium polylepis*, prymnesins (Table 2).

Table 2. Total number of quality sequences (Reads) obtained from Papeete port for each potential toxin producer species [17]. The number of port sampling locations from which each species was found is indicated.

Class	Species	Main Toxins	Reads	Sampling Locations
Bacillariophyceae	<i>Pseudo-nitzschia caciantha</i>	Domoic acid	12	4
Bacillariophyceae	<i>Pseudo-nitzschia cuspidata</i>	Domoic acid	3395	13
Bacillariophyceae	<i>Pseudo-nitzschia delicatissima</i>	Domoic acid	62	12
Bacillariophyceae	<i>Pseudo-nitzschia galaxiae</i>	Domoic acid	694,147	13
Dinophyceae	<i>Akashiwo sanguinea</i>	Hemolysin-like	313	13
Dinophyceae	<i>Alexandrium affine</i>	PSP toxins	252	10
Dinophyceae	<i>Amphidinium carterae</i>	Hemolysins	13	1
Dinophyceae	<i>Amphidoma languida</i>	Azaspiracids	3056	13
Dinophyceae	<i>Azadinium poporum</i>	Azaspiracid	71	11
Dinophyceae	<i>Azadinium spinosum</i>	Azaspiracids	476	13
Dinophyceae	<i>Coolia canariensis</i>	Yessotoxin analogue	10	2
Dinophyceae	<i>Gambierdiscus carpenteri</i>	Maitotoxin	11	1
Dinophyceae	<i>Gambierdiscus pacificus</i>	Ciguatoxin- and MTX-like toxins	33	4
Dinophyceae	<i>Margalefidinium fulvescens</i>	Associated with fish kills without confirmed toxin production	130	12
Dinophyceae	<i>Margalefidinium polykrikoides</i>	Associated with fish kills without confirmed toxin production	2884	13
Dinophyceae	<i>Ostreopsis ovata</i>	Ovatoxins	683	13
Dinophyceae	<i>Protoceratium reticulatum</i>	Yessotoxin	48	11
Dinophyceae	<i>Tripes fusus</i>	Associated with fish kills, without confirmed toxin production	513	13
Haptophyta	<i>Chrysochromulina leadbeateri</i>	Sterolysin-like	254	13
Haptophyta	<i>Phaeocystis globosa</i>	Hemolysins	288	13
Haptophyta	<i>Prymnesium polylepis</i>	Prymnesins	119	13

Based on the predominant toxin produced by each species and the frequency of the species in the port, our results indicate that producers of the majority of toxins were potentially present throughout the entire port area, with 53.2% of reads from shallow water and the remainder from deep water samples, showing a fairly balanced distribution across the water column (Figure 2). However, it was not equal for all the toxin producers. Notable exceptions included CFP toxins producers, only found in five sampling locations and mainly in shallow-water samples (61.4% of the reads); PSP toxins producers, present in 10 locations (46.8% reads from shallow samples); and yessotoxin and prymnesin producers, found at most or all sampling locations in deeper waters (39.7% and 35.3% reads, respectively, from shallow samples).

The threats of the harmful algae found from Papeete port in Tahiti are summarized in Table 3, based on the effects of the toxins they produce [20–33], the species affected, and known carriers of toxin producers. The effects range from neurotoxicity and inflammation to increased mortality, affecting from shellfish, fish resources, and humans. Carriers of toxin-producing algae are primarily shellfish and fish, but also crustaceans (Table 3).

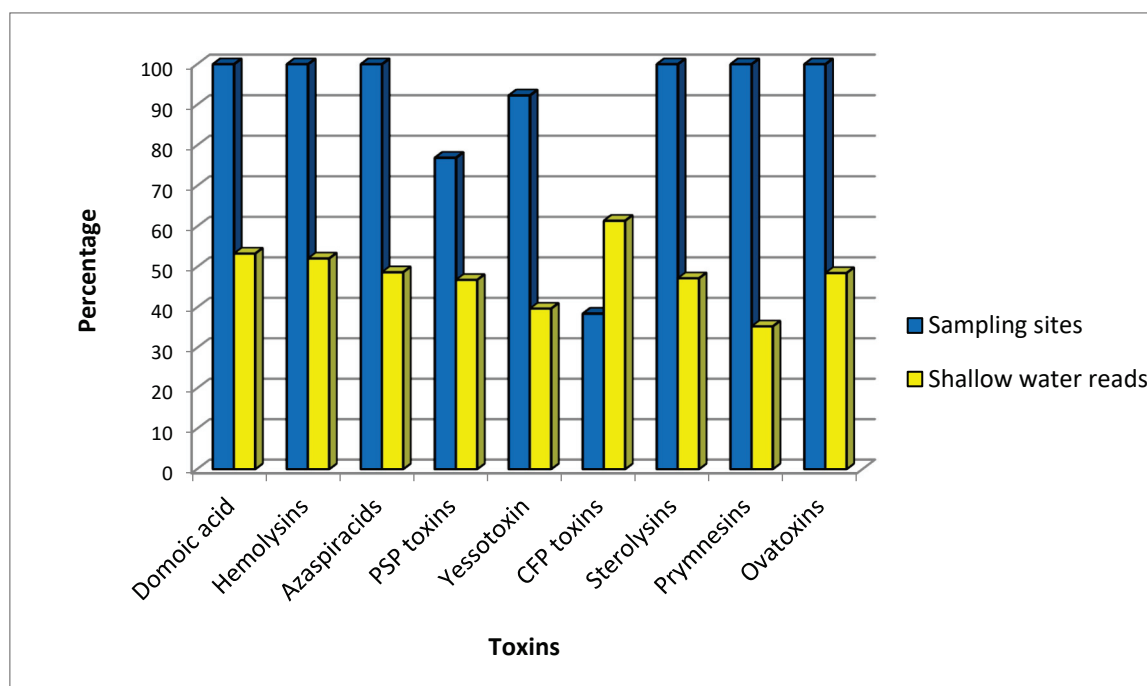


Figure 2. Frequency and depth of toxin producer species in Papeete port, measured, respectively, from the percent of sampling locations with the presence of species producing a toxin, and the proportion of reads found from surface water samples. PSP, paralytic shellfish poisoning; CP, ciguatera poisoning.

Table 3. Summary of harmful effects of the toxins potentially produced by HABs found from Papeete port using metabarcoding. References are given for each toxin. AZP, azaspiracid poisoning; ASP, amnesic shellfish poisoning; PSP, paralytic shellfish poisoning; CP, ciguatera poisoning.

Toxin	Main Harmful Effects	Affected Species	Carriers of Producing Algae
Azaspiracids [20]	AZP	Humans	Shellfish, crabs
CFP toxins [21]	CFP, reduced recruitment	Humans, fish	Fish
Domoic acid [22]	ASP, neurotoxicity	Humans, birds, mammals	Shellfish, finfish
Hemolysins, hemolysin-like [23,24]	Increased mortality	Shellfish; finfish	Shellfish
Ovatoxins [25]	Inflammatory response	Humans	Direct contact
Prymnesins [26,27]	Increased mortality	Fish, shellfish, seabirds	Direct contact/shellfish
PSP toxins [28,29]	PSP	Humans	Shellfish, fish, crustaceans
Sterolysins, sterolysin-like [30,31]	Increased mortality	Fish	Direct contact
Yessotoxin and analogues [32,33]	Increased mortality	Fish, shellfish	Shellfish

Several harmful algae found in this study had been found from metabarcoding in Papeete port in 2018 [16]: *Coolia canariensis*, *Protoceratium reticulatum*, *Margalefidinium polykrikoides*, and *Gambierdiscus*. Five out of the 13 Papeete port samples contained *Gambierdiscus* eDNA in our study in 2023 (Figure 3). In contrast, only one of the 16 samples analyzed from Papeete port in 2018 contained eDNA of *Gambierdiscus* [16]. In the two studies, *Gambierdiscus* eDNA was detected with the same 18S rDNA marker, thus a bias due to the marker is unlikely. The risk difference between 2018 and 2023 was 0.406 (95% confidence 0.129–0.842), being statistically significant ($z = 0.13$ with $p = 0.033$), suggesting an increase in the prevalence of species causative of CFP in 2023.

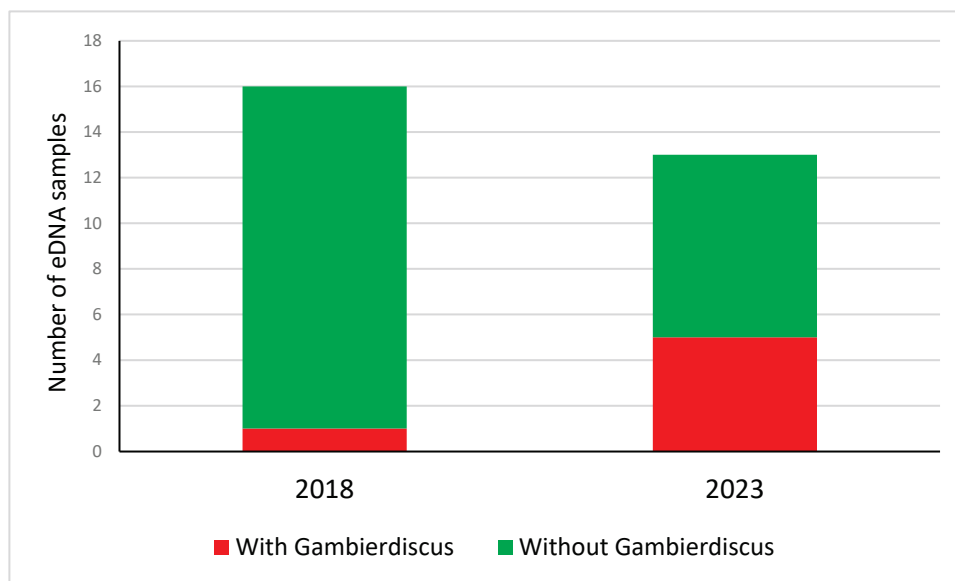


Figure 3. Number of eDNA samples from Papeete port with or without detected *Gambierdiscus* eDNA in 2018 [16] and 2023 (this study).

In French Polynesia, the official data of harmful algae events are recorded from the Ciguatera Fish Poisoning Monitoring Program. In the period 2018–2024, a total of 655 events were recorded in all the islands of the whole region, of which 278 occurred in Tahiti Island. The evolution of the events along the studied period is presented in Figure 4. The difference between Tahiti and the rest of islands (excluding Tahiti) in the distribution of CFP events along the seven years considered was highly significant ($\chi^2 = 20.17$, 6 d.f., $p = 0.003$). It can be observed that, unlike in the rest of islands in the region, in Tahiti, the events increased noticeably in 2023 and even more in 2024 (5 events in 2018, followed by 8 in 2019 versus 20 events in 2023, followed by 22 in 2024). This coincides with the respective prevalence of *Gambierdiscus* species in Papeete port in 2018 and 2023 (see Figure 3).

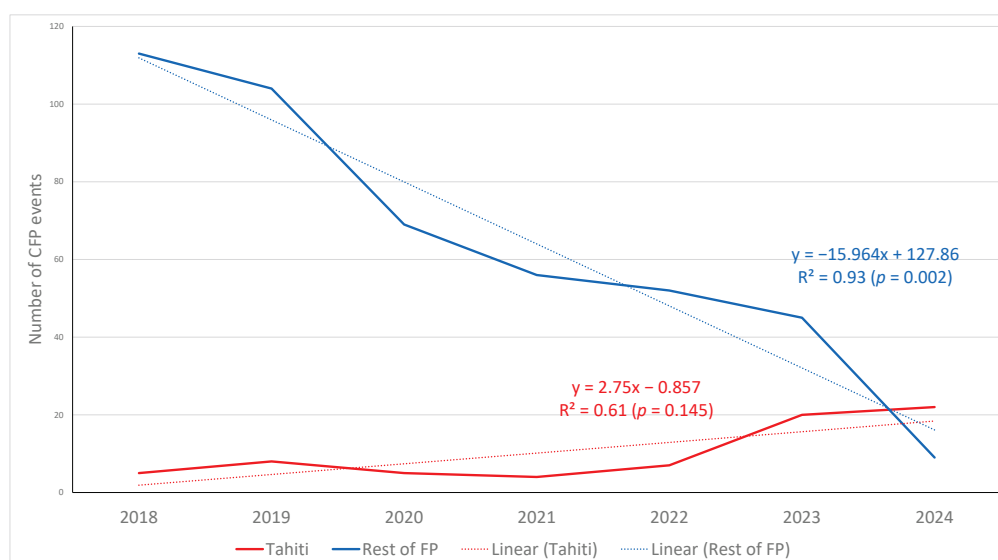


Figure 4. Number of ciguatera fish poisoning (CFP) events in Tahiti Island and in the rest of French Polynesia (FP) islands from 1 January 2018 to 31 December 2024. Graph generated by the authors using the data downloaded from the HAEDAT database for French Polynesia in the indicated period, filtering by Tahiti reports (red line) or without Tahiti (blue line).

3. Discussion

The detection of 21 potential HAB producers within Papeete port supports the hypothesis that ports act as hubs of harmful microalgae that are transported in different boat compartments [8–10]. Several species that pose serious toxicity risks for human health were present at all sampling sites, like the domoic acid producers *Pseudo-nitzschia cuspidata* and *P. galaxiae* [22], the azaspiracids producers *Azadinium* and *Amphidoma* [20], or *Ostreopsis ovata*, which produces ovatoxins [25]. Other locations outside the port should be sampled to confirm this hypothesis.

Two *Gambierdiscus* species associated with CFP were identified at five locations. This relatively low diversity could be unexpected given that Tahiti islands are considered hotspots of *Gambierdiscus* with seven known species of this genus [5], but these two species were the only detected in Papeete port in previous studies using a similar metabarcoding methodology [34], thus they would be stable in the port. The metabarcoding methodologies employed in previous studies on Papeete port [16,34] are not entirely comparable with the present study, principally due to different water volumes oscillating between 3 L [16] and 700 L [34] per replicate. Nevertheless, finding the same two species (28.6% of the *Gambierdiscus* described in all the island) in a single port using different water volumes in different years is already noticeable and points at the reproducibility of the results over time. Although the two species found in this study, *G. carpenteri* and *G. pacificus*, were not highly toxic in previous Tahitian reports [5], the risk of CFP remains if fish around the port are consumed.

The coincidence between the prevalence of *Gambierdiscus* in Papeete port measured from eDNA and the number of CFP events in Tahiti in 2018 and 2023, although cannot be taken as a direct cause-effect in absence of toxin studies, could be an indirect indicator of the validity of the eDNA as an early alert system of harmful algae presence, supporting other studies [8,14]. These results suggest an increase in CFP agents in Papeete port, perhaps due to the introduction of new species and strains of harmful algae via maritime traffic over the years, something that should be studied more carefully including toxin analysis in the *Gambierdiscus* found in the port for comparison with toxins found in CFP outbreaks in Tahiti.

With respect to the potential threats of the harmful algae found in Papeete port in Tahiti (Table 3), their potential toxins indicate threats to both human health and seafood resources [20–32]. Azaspiracids, produced by *Azadinium* and other dinoflagellates [20], are known to cause azaspiracid poisoning (AZA), which manifests as nausea, vomiting, diarrhea, and other gastrointestinal symptoms. CFP toxins (ciguatoxin, maitotoxin) cause a range of symptoms that include neurological, gastrointestinal, and cardiovascular problems in humans, and reduced recruitment in fish [21]. The domoic acid is a neurotoxin that affects humans and other vertebrates like seabirds and marine mammals, causing amnesic shellfish poisoning characterized by short-term memory loss and brain damage [22]. Hemolysins reduce survival of affected fish [23] and shellfish resources, including pearl oysters [24]. Ovatoxins [25] present in marine aerosols produce inflammatory response by inhalation, causing diverse symptoms like rhinorrhea, eye and nose irritation, and general malaise in humans. Prymnesins are toxic metabolites that deter grazing in herbivore fish and have cytotoxic effects in fish and shellfish, reducing their survival; they also harm bivalve predators like seabirds [26,27]. Paralytic shellfish poisoning (PSP) in humans is caused by neurotoxins, principally saxitoxin, produced by *Alexandrium* species that are carried by shellfish but also by fish and crustaceans [28,29]. Sterolysins [30,31] produced by different microalgae cause recurrent fish kills. Finally, yessotoxins and their analogues are another type of toxins that causes mortality in different fish and shellfish species [32,33].

Some harmful algae that are ubiquitously detected in the port can cause mortality to fisheries resources, impacting both finfish and shellfish (Table 2). Those that reduce bivalve survival, like *Akashiwo sanguinea* [24], may be of special concern in French Polynesia for the risk that may pose to pearl oysters that are a key resource in the region [35]. Moreover, the five species detected in 2018 [16,34] and again in 2023 are probably established in Papeete port, as opposed to brief introductions. *Coolia canariensis*, *Protoceratium reticulatum*, *Margalefidinium polykrikoides*, and the two *Gambierdiscus* species commented on above would also be of special concern, because they could spread from the port that may act as a reservoir for them.

This study contributes to the growing body of work employing metabarcoding for harmful algae detection [8,14,36]. The method is reliable and efficient for the description of plankton communities when based on high-quality data [37], and using three metabarcodes, we are capturing the majority of phytoplankton diversity [38]. However, there is a limitation: with the metabarcodes here employed, we are identifying species but not the strains that produce toxins. The method could be improved, amplifying genes that code for the toxins, or that are able to recognize toxin-producing strains. This way the toxic load could be estimated in water samples. Another way of improving the accuracy of health danger evaluation in port ecosystems could be to combine metabarcoding surveys with direct measurements of toxins potentially produced by the species found from eDNA. More generally, routine checking of harmful algae in locally consumed seafood as well as in seafood exports would ensure safe and healthy consumption of French Polynesian fishing and aquaculture resources.

Future research directions should explore the diversity and the frequency of HAB producers in other maritime ports, and explore for methods to prevent the transport of microalgae by ships. Potential HAB mitigation methods include physical aeration to prevent blooms [39], clays to flocculate algal cells and their ichthyotoxins [40], or biological agents like bacteria and viruses to control the microalgae [41]. However, the methods developed so far remain partially efficient; more methods to inhibit the proliferation of HABs would be useful [42,43]. Promising methods based on cultivation of seaweeds in sites of interest that may reduce the lethal effects of some microalgae [44] could be explored. More importantly, methods to treat hulls and ballast water to avoid shipping transport of microalgae, like biocides, heat treatment, and others [45], should be further developed.

These findings strongly suggest that consuming fish and shellfish collected within or nearby the port may be a risk to human health. In French Polynesia, fishing is prohibited inside port areas, with angling being permitted only nearby [46]. In other regions illegal angling fishing within ports is reported as relatively frequent [47], it being not rare to see people angling from some piers within port areas, especially in small ports. Although this study focused on the presence of toxin-producer species rather than direct toxin measurements and recognizing that not all the strains are equally toxic [48], applying the precautionary principle, a ban of fishing nearby this and other ports, and a control of illegal angling within ports would be recommended.

Finally, here we have focused on port areas that are impacted by maritime transport. Harmful algae can also be naturally introduced in the South Pacific by oceanographic driving. An example is the expansion of *Pseudochattonella verruculosa* to the south due to changes in water column stratification associated with global climate change; its convergence with *A. catenella* caused a catastrophic HAB event in Chilean waters in 2016 [49]. It is difficult to estimate the relative weight of maritime transport and natural oceanographic shipping on the expansion of harmful algae, but from our results it seems that controlling port areas will help to control HABs. Regional early warning systems could combine molecular methods like metabarcoding, remote sensing, and oceanographic records.

4. Materials and Methods

4.1. Sampling and eDNA Extraction

Sampling was conducted between 22 May and 5 June of 2023. At each of 13 sampling sites within Papeete port, three replicates of 20 L water were collected from two depths (surface and 2 m above the substrate, ranging from 1 to 15 m depth) and using two filter pore sizes (1.2 and 0.2 μm) to capture as much diversity as possible. DNA was extracted from the filters using the Quick-DNA Fecal/Soil Miniprep Kit (Zymo Research, Irvine, CA, USA), with modifications optimized for marine samples. The protocol combined mechanical and chemical cell lysis followed by column-based purification and a final inhibitor removal step, ensuring DNA quality for downstream PCR amplification. The replicates were joined by sampling point and depth for obtaining a higher eDNA quantity.

DNA samples were purified using the Mag-Bind RXNPure Plus magnetic beads (Omega Biotek, Norcross, GA, USA), following the instructions provided by the manufacturer. Magnetic beads were added in 1.5 times the volume of the original DNA extract to retrieve the whole spectrum of DNA fragment sizes in the samples. The purified DNA was resuspended in 60 μL . We quantified the DNA concentration in each extract using the Qubit High Sensitivity dsDNA Assay (Thermo Fisher Scientific; BIOGEN Científica, Madrid, Spain).

4.2. DNA Metabarcoding Library Preparation and Sequencing

DNA metabarcoding library preparation and sequencing were performed by AllGenetics and Biology SL www.allgenetics.eu (accessed on 16 July 2025). DNA metabarcoding procedures followed Douard et al. [50], based on three libraries that are summarized next. The first one was constructed by amplifying a 365 bp fragment of the chloroplastic *rbcl* gene using a cocktail of primers [51]:

Forward

- Diat_rbcL_708F_1 (5' AGGTGAAGTAAAAGGTTTCWTACTTAAA 3')
- Diat_rbcL_708F_2 (5' AGGTGAAGTTAAAGGTTTCWTAYTTAAA 3')
- Diat_rbcL_708F_3 (5' AGGTGAAACTAAAGGTTTCWTACTTAAA 3')

Reverse

- R3_1 (5' CCTTCTAATTTACWACWACTG 3')
- R3_2 (5' CCTTCTAATTTACWACAACAG 3')

In the second library, a fragment of the mitochondrial COI gene of around 365 bp (including primer sequences) was amplified using the primers mICOLintF [52] as forward and jgHCO2198 [53] as reverse. In the third, the V4 domain of the nuclear 18S rRNA gene (460 bp) was amplified with the primers Uni18SF and Uni18SR [54].

All the primers had 5' Illumina sequencing primer sequences attached. In the first amplification step, PCRs were carried out in triplicate in a final volume of 12.5 μL , containing 1.25 μL of template DNA, 0.5 μM of the primers, 3.13 μL of Supreme NZYTaq 2x Green Master Mix (NZYTech), and ultrapure water up to 12.5 μL . The reaction mixture was incubated with an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, annealing temperature for 45 s, 45 s, 72 °C for 45 s, and a final extension step at 72 °C for 7 min. Annealing temperatures were 47 °C, 51.4 °C, and 47.4 °C for the first, second, and third library, respectively. The products from the three PCR replicates were pooled together for the subsequent steps.

A second amplification step of 5 cycles and annealing at 60 °C was carried out, attaching oligonucleotide indices for multiplexing different libraries in the same sequencing pool [55]. Negative PCR controls without DNA were included in all the rounds. These blanks were also pooled together (BPCR) for library preparation.

The libraries were run on GreenSafe (NZYTech)-stained agarose gels (2%) to check the library size under UV light. Then, they were purified with Mag-Bind RXNPure Plus magnetic beads (Omega Bio-tek) and pooled in equimolar quantities using Qubit dsDNA HS Assay (Thermo Fisher Scientific) for quantification. A NovaSeq PE250 flow cell (Illumina) was employed for sequencing, aiming at two Gb of output for each marker.

4.3. Bioinformatics Analysis

Standard bioinformatics analysis was performed [50]. First, traces of adapter dimers were removed using Cutadapt v3.5 [56], and sequencing indices were trimmed after a demultiplexing step. FASTQ file quality was checked using FastQC [57]. The output was summarized using MultiQC [58].

Qiime2 2021.4 [59] was employed for further analyses. Primers were trimmed with the q2-cutadapt plugin [56]. DADA2 [60] was used for denoising via q2-dada2. To construct a local database, nucleotide sequences were downloaded from NCBI using the e-search queries “COI NOT environmental NOT unclassified sequences NOT bacteria”, “18S NOT uncultured NOT fungi NOT land plants NOT unclassified NOT unidentified NOT archaea NOT bacteria NOT viruses NOT genome”, and “rbcL NOT uncultured NOT algae NOT unclassified NOT genome” using Rescript within qiime2 [23].

The quality-filtered sequences were taxonomically assigned using the q2-feature-classifier [61] with an exigent assignation (97% as percent of identity and e-value below 10×10^{-50}), for high confidence on the species status considering the metabarcodes employed.

An ASV (Amplicon Sequence Variant) table was constructed with the reads of each assigned species by sample, retaining those assigned at least to a genus level and eliminating DNA from humans and other terrestrial taxa. For downstream analysis, only those samples with >200 reads in the curated ASV table were considered. Taxonomic references and distribution of the species assigned from DNA were validated and confirmed using WoRMS [62] and the French National Inventory of Natural Heritage [63] databases.

4.4. Harmful Algae Events in French Polynesia

The harmful algae events recorded in French Polynesia for the period 2018–2024 were downloaded from the Harmful Algae Events Database HAEDAT [64]. The following search terms were employed: Country, French Polynesia; period years, 2018–2025; nature of the harmful event: any; organism affected: any; associated syndrome: any; species implicated in toxin transmission (transvector): any.

In the French Polynesian network for ciguatera surveillance, CFP cases are reported on a volunteer basis. The reporting forms [65] include the archipelago, island, and city/village as geographical information, but not the exact location within a city with coordinates. Thus, it is not possible to know if CFP events occurred within Papeete port.

4.5. Statistical Analysis

Statistics was carried out with the free PAST software v. 4.03 [66]. Distributions like the number of CFP events in the years considered in Tahiti and the rest of the French Polynesian islands were compared using contingency chi-square analysis. The prevalence of *Gambierdiscus* (detected from eDNA) in Papeete port was compared between 2018 and the current dataset based on frequency differences and z to estimate their significance. The standard $p < 0.05$ significance threshold was adopted.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/toxins17080424/s1>. Table S1: DADA analysis of the Next Generation sequences obtained from Papeete port water samples; Table S2: List of phytoplankton species and the total number of sequences obtained from each sampling location within Papeete port.

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Article

Time-Resolved Visualization of Cyanotoxin Synthesis via Labeling by the Click Reaction in the Bloom-Forming Cyanobacteria *Microcystis aeruginosa* and *Planktothrix agardhii*

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Abstract: In non-ribosomal peptide synthesis of cyanobacteria, promiscuous adenylation domains allow the incorporation of clickable non-natural amino acids into peptide products—namely into microcystins (MCs) or into anabaenopeptins (APs): 4-azidophenylalanine (Phe-Az), *N*-propargyloxy-carbonyl-L-lysine (Prop-Lys), or *O*-propargyl-L-tyrosine (Prop-Tyr). Subsequently, chemo-selective labeling is used to visualize the clickable cyanopeptides using Alexa Fluor 488 (A488). In this study, the time-lapse build up or decline of azide- or alkyne-modified MCs or APs was visualized during maximum growth, specifically MC biosynthesis in *Microcystis aeruginosa* and AP biosynthesis in *Planktothrix agardhii*. Throughout the time-lapse build up or decline, the A488 signal occurred with heterogeneous intracellular distribution. There was a fast increase or decrease in the A488 signal for either Prop-Tyr or Prop-Lys, while a delayed or unobservable A488 signal for Phe-Az was related to increased cell size as well as a reduction in growth and autofluorescence. The proportion of clickable MC/AP in peptide extracts as recorded by a chemical-analytical technique correlated positively with A488 labeling intensity quantified via laser-scanning confocal microscopy for individual cells or via flow cytometry at the population level. It is concluded that chemical modification of MC/AP can be used to track intracellular dynamics in biosynthesis using both analytical chemistry and high-resolution imaging.

Keywords: microcystin; anabaenopeptin; time-lapse experiments; pulse-feeding; chemo-selective labeling; high-resolution microscopy; flow cytometry

Key Contribution: The quantitative relationships between the clickable microcystin/anabaenopeptin content and the resulting signal intensity from chemo-selective labeling using Alexa Fluor 488 are reported through time-lapse signal build up/decline experiments.

1. Introduction

Cyanobacteria frequently dominate aquatic ecosystems and have received much attention due to their ability to produce numerous toxic secondary metabolites, so-called cyanotoxins, for example, microcystins (MCs), anatoxins, and cylindrospermopsins. Cyanotoxin production is a global problem as it negatively affects freshwater quality. It is generally agreed that cyanobacterial harmful algal blooms (cHABs) are increasing in frequency, a phenomenon that is to some extent attributable to global warming and eutrophication [1,2]. The negative ecological and health impacts of cHABs have been documented, although

there are still significant gaps in our understanding of cyanotoxin function. One remaining challenge is to understand the synthesis and regulation of cyanotoxins in the cell, which is also linked to the function of these toxins in nature.

Aside from ecosystem relevance, cyanotoxins comprise pharmacologically active compounds with potential antibacterial, fungal, or cytostatic effects. MCs, as well as many other toxic/bioactive metabolites, are synthesized by large multifunctional enzyme complexes via the thio-template mechanism, known as non-ribosomal peptide synthesis (NRPS) [3]. Through this pathway, both proteinogenic and non-proteinogenic amino acids (AAs) are sequentially incorporated into the growing peptide chain [4]. For example, it has been estimated that 48 steps are needed for the biosynthesis of MCs, of which 45 have been assigned to catalytic NRPS domains [5].

Common bloom-forming cyanobacteria include the genera *Dolichospermum*, *Microcystis*, and *Planktothrix*, which produce MCs and/or anabaenopeptins (APs) among other non-ribosomally synthesized peptides, such as aeruginosins, cyanopeptolins, and microginins. Moreover, they synthesize ribosomally and post-translationally modified peptides such as aerucyclamides, microviridins, and prenylagaramides [6]. For MCs, inhibition of protein phosphatase 1 and 2A in the nanomolar concentration range has been observed repeatedly [1–3]. For other peptides, such as APs, inhibition of serine proteases and the resulting toxicity to aquatic crustaceans has been reported [6–8]. It has been postulated that intercellular cocktails of various toxins or peptides may provide better protection due to synergistic interactions but also make co-evolutionary responses more difficult [9]. This hypothesis is supported by the observation that, for certain peptides synthesized via NRPS, there is a trend in evolution toward increased structural diversity, either via domain duplication [10] or point mutation and recombination within NRPS domains [11,12].

The same genotypes produce MC structural variants with variable AAs—that is, MC-RR vs. MC-LR and MC-HtyR—implying co-production of MC variants carrying Arg or Leu and Hty in position 2 of the MC-molecule [13–15]. Similarly, research has documented the co-occurrence of AP B or AP F and AP A or OscY [11,12,16], implying co-production of AP variants carrying Arg and Tyr in exocyclic position 1 of the AP molecule. For AP synthetase, we have genetically characterized and heterologously expressed the adenylation (A-)domain of the initiation module (ApnAA1) of the NRPS [11]. It was used in vitro as a model system to biochemically characterize the genetic variation observed within these A-domains. This endeavor provided the first evidence that particular A-domain genotypes are capable of activation of chemically divergent AAs—that is, Arg vs. Tyr—with comparable efficiency. The findings also demonstrated promiscuity for other proteinogenic AAs. In another study, the authors crystallized such an A-domain and elucidated the structural basis of substrate activation; they described a so-called bispecific A-domain [17]. From this crystallographic analysis, it could be inferred that ApnAA1-domain genotypes carrying substitutions defining the so-called specificity conferring code [18] led to a conformational change that allows for Arg and Tyr activation in parallel. Thus, only a few critical substitutions that cause a conformational change during substrate activation are responsible for the observed promiscuity. Subsequently, research has identified more genotypes from strains co-producing APs with three or more different AAs (Arg or Tyr or Lys) in position 1 [12].

Understanding the molecular basis for such promiscuity has opened the possibility of introducing unnatural AAs (non-AAs) with azide or alkyne groups into MCs or APs, enabling visualization via a subsequent click chemistry reaction [17,19]. In general, click chemistry involves the introduction of a functional group that is covalently attached to a substrate and introduced into a biomolecule of interest (i.e., introducing a non-AA into a peptide). The functional group reacts chemo-selectively, in the presence of all

the functional groups found in a living cell, with an added chemical probe (i.e., Alexa Fluor 488 [A488]), through a chemical reaction such as the classical copper-catalyzed azide-alkyne cycloaddition (CuAAC) [20,21]. For this aim, non-AAs have been tested (i.e., 4-azidophenylalanine [Phe-Az], *N*-propargyl-lysine [Prop-Lys], and H-L-propargyl-tyrosine [Prop-Tyr]) to enable subsequent biomolecular labeling of the resulting modified peptide using A488 fluorophore azide or alkyne [21–23] (Figure 1).

Although MC/AP fluorescent labeling by chemo-selective reaction has been documented [21,22], questions on the labeling sensitivity and background fluorescence through autofluorescence (AF) remain. Surface bloom-forming cyanobacteria, such as *Microcystis aeruginosa*, have lower photosynthetic efficiency than cyanobacteria stratifying in the metalimnion, such as *Planktothrix* spp. (e.g., see Figures 5.4 and 5.5 in [24]). For example, *M. aeruginosa* has a lower chlorophyll *a* specific absorption efficiency compared with *Phormidium* and *Trichodesmium* and other taxa of the Phormidiaceae (Microcoleaceae) sensu [25,26]. In contrast, *Planktothrix* spp. has higher phycobilin contents, particularly phycoerythrin [27], enabling relatively higher growth rate under shade conditions [28].

The higher AF in *Planktothrix agardhii* may also lead to an increased signal-to-noise ratio. We recently tested a second fluorophore, Alexa Fluor 405 (A405), in the blue light spectrum, in parallel to A488 for *P. agardhii* and *M. aeruginosa* [22]. A405 also resulted in peptide labeling, but the labeling intensity was generally lower and the distinction from natural background AF was reduced, limiting its use in *Planktothrix* spp. Hence, the selection of the excitation/emission spectrum of the fluorophore is of particular significance for cyanobacteria with stronger AF, such as *Planktothrix* spp. Currently, it is not known how labeling signal intensity quantitatively relates to the clickable MC/AP content.

Time-lapse build up and decline experiments have been performed to better understand the synthesis of MC/AP through tracing via clickable non-AA incorporation [23]. The build up of A488 labeling signal in cells was observed by growing them in the presence of non-AAs under maximum growth conditions. Samples were taken every 12 h for the first 48 h, and a final sample was taken after 168 h (7 days). In addition, the decline of labeling signal was also determined by growing the cells under maximum growth conditions. At T0, following a 48 h incubation with non-AAs, the cells were transferred to fresh medium and harvested directly after washing and sampled every 24 h. In the present study, the results for A488 signal intensity were compared directly to the clickable AP/MC content as revealed by chemical-analytical methods reported earlier [23]. The A488 signal intensity was determined using two independent methods: direct examination of individual cells with high-resolution microscopy and examination of the total population with flow cytometry (FCM). This study reports a quantitative correlation between the amount of clickable MC/AP determined through chemical analysis and A488 signal intensity through the click chemical reaction.

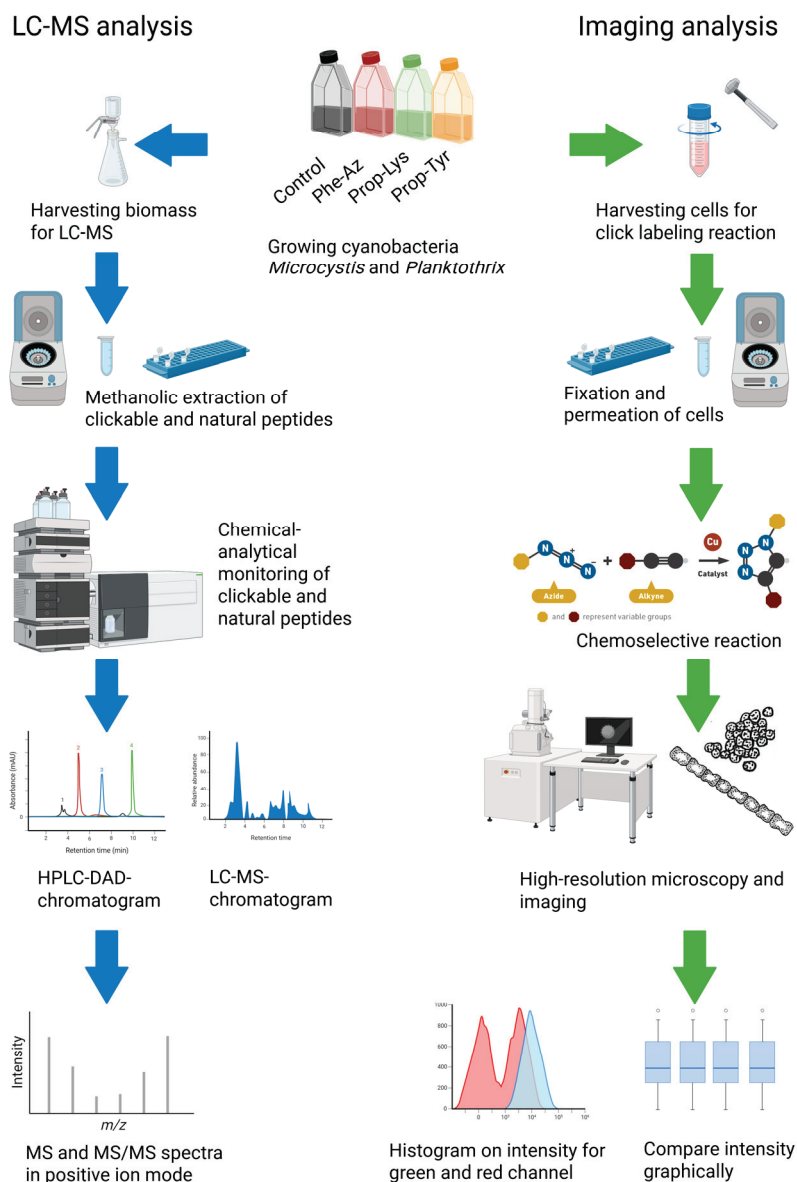


Figure 1. General overview of the protocol steps for the two tasks related to the click-chemical labeling of cyano-peptide synthesis in the cyanobacteria *Microcystis aeruginosa* or *Planktothrix agardhii*. The blue arrows indicate the protocol steps in the chemical-analytical analysis workflow [21–23], while the green arrows indicate the steps in chemo-selective labeling and microscopic analysis. Created in BioRender. Deng, L. (2025) <https://BioRender.com/f6m1hhc>.

2. Results

2.1. Qualitative Observation of A488 Signal Generation During Time-Lapse Build up via High-Resolution Microscopy

The A488 signal intensity was monitored qualitatively and quantitatively via laser-scanning confocal microscopy during time-lapse experiments for the cyanobacteria *M. aeruginosa* and *P. agardhii*. For *M. aeruginosa*, A488 signal build up resulting from clickable MC synthesis occurred quickly (within 12–24 h) for cells fed Prop-Tyr or Prop-Lys, while control cells (i.e., grown in the absence of non-AAs and processed under identical conditions) showed negligible A488 signal. For Phe-Az-fed cells, A488 signal build up was delayed, occurring after 24–36 h (Figure 2). Notably, the same heterogeneous distribution for the A488 signal reported in a previous study [21] was observed from the first time point after pulse feeding (i.e., at T1 [12 h]). In contrast, AF appeared to be distributed more

homogeneously within the cell during the entire experimental period. For *P. agardhii*, A488 signal build up resulting from clickable AP synthesis also occurred quickly for cells fed Prop-Tyr or Prop-Lys (12–24 h). In contrast, there was no signal build up for Phe-Az-fed cells when compared with control cells (Figure 3). The delayed or unobservable A488 signal generation for Phe-Az-fed cells was related to the general reduction in growth, as reported previously [23]. Accordingly, for the Phe-Az-fed cells, the cell diameter and volume increased in both cyanobacteria compared with the control cells or Prop-Tyr- or Prop-Lys-fed cells (Tables 1 and S1). There was a rather heterogeneous distribution of A488 signal for the Prop-Tyr -and Prop-Tyr-fed cells from the beginning (T1 = 12 h), which will be investigated in detail in a forthcoming article. Similarly to *M. aeruginosa*, AF of *P. agardhii* was found to be distributed more evenly within the cells throughout the entire experiment.

Table 1. The mean ($\pm$ standard error (SE) of the mean) cell diameter and cell volume during time-lapse signal build up and decline in *M. aeruginosa* strain Hofbauer and *P. agardhii* strain no371/1. Control = cells grown and processed under identical conditions but without non-AA substrate. Graphical data are shown in Figures S1 and S2. Measurements for each time point are shown in Table S1.

Strain	Experiment	Control	Phe-Az	Prop-Lys	Prop-Tyr	<i>n</i>	<i>p</i> -Value ^{1,2} (Treatment)	<i>p</i> -Value ³ (Time)
Cell diameter (μm)								
<i>M. aeruginosa</i>	build up	4.3 $\pm$ 0.04 ^a	4.59 $\pm$ 0.08 ^b	4.42 $\pm$ 0.04 ^c	4.47 $\pm$ 0.04 ^c	72	0.001 ***	<0.001 ***
	decline	4.05 $\pm$ 0.03 ^a	4.59 $\pm$ 0.1 ^b	4.23 $\pm$ 0.03 ^c	4.14 $\pm$ 0.03 ^{ac}	60	<0.001 ***	0.008 **
<i>P. agardhii</i>	build up	3.52 $\pm$ 0.02	3.56 $\pm$ 0.02	3.53 $\pm$ 0.02	3.52 $\pm$ 0.02	72	0.084	<0.001 ***
	decline	3.58 $\pm$ 0.05 ^a	3.74 $\pm$ 0.03 ^b	3.58 $\pm$ 0.04 ^a	3.49 $\pm$ 0.06 ^a	60	0.001 **	0.197
Cell volume (μm^3)								
<i>M. aeruginosa</i>	build up	42.4 $\pm$ 1.1 ^a	52.4 $\pm$ 2.9 ^b	46.2 $\pm$ 1.3 ^c	47.8 $\pm$ 1.4 ^c	72	<0.001 ***	<0.001 ***
	decline	35.3 $\pm$ 0.8 ^a	53.1 $\pm$ 3.9 ^b	40.3 $\pm$ 0.9 ^c	38 $\pm$ 0.7 ^{ac}	60	<0.001 ***	0.008 **
<i>P. agardhii</i>	build up	133.3 $\pm$ 1.6	137.2 $\pm$ 2	134 $\pm$ 1.3	134 $\pm$ 1.4	72	0.083	0.01 *
	decline	140.5 $\pm$ 3.8 ^a	154 $\pm$ 2.8 ^b	140.5 $\pm$ 3.5 ^a	133 $\pm$ 4.2 ^a	60	0.002 **	0.398

¹ Two-way repeated measures analysis of variance (RM ANOVA) (grouping factor). *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$; ² Superscript letters (^{a,b,c}) indicate subgroups of non-AA treatments that were not significantly different based on post hoc pairwise comparison (Bonferroni test, $p > 0.05$); ³ Two-way RM ANOVA (time factor).

2.2. Qualitative Observation of A488 Signal Reduction During Time-Lapse Decline via High-Resolution Microscopy

In addition to the increase in the A488 signal intensity, its decline was monitored during time-lapse experiments. Specifically, *M. aeruginosa* and *P. agardhii* were grown in the presence of non-AAs for 48 h and subsequently transferred to the new medium under maximum growth conditions. Similarly to the A488 signal intensity build up, the A488 signal intensity decline was rather fast: within 24 h for *M. aeruginosa* fed Prop-Tyr or Prop-Lys (Figure 4) and within 48 h for *P. agardhii* fed Prop-Tyr or Prop-Lys (Figure 5). There was a slower decline for Phe-Az-fed *M. aeruginosa*, while *P. agardhii* fed Phe-Az showed no difference compared with the control cells. Corresponding to the reduced growth rate, the Phe-Az fed cells also had an increased cell diameter and increased cell volume compared with the control cells or the Prop-Tyr or Prop-Lys fed cells (Tables 1 and S1 and Figures S1 and S2).

In general, in *M. aeruginosa* the heterogeneous distribution of the A488 signal remained visible until the end of the experiment. In contrast, for *P. agardhii*, there was a temporal sequence leading to the disappearance of heterogeneous structures in the cell: Heterogeneous structures that occurred as distinct entities were replaced gradually and partially by

ring-like formations in the peripheral area of the cell. The formation of these structures will be explored in more detail in a subsequent article.

2.3. Quantification of A488 Signal Intensity and AF via High-Resolution Microscopy During Time-Lapse Build up and Decline

Next, the labeling signal intensity was quantified by recording both the A488 signal intensity and AF per cell volume or per cell from 20 randomly selected cells per technical replicate and time point. Corresponding to the analysis of micrographs (Figure 2), A488 signal intensity increased quantitatively by 4.4- and 4.2-fold within 12 h for Prop-Tyr- and Prop-Lys-fed *M. aeruginosa*. In comparison, Phe-Az-fed *M. aeruginosa* showed a slower increase in labeling signal: 2.8-fold after 24 h, and a plateau phase after 36 h (Figures 6a and S3).

The A488 signal intensity increased by 3.5- and 2.4-fold within 12 h for Prop-Lys- and Prop-Tyr-fed *P. agardhii*, respectively. In contrast, the A488 signal intensity from Phe-Az-fed cells remained low and did not differ from the control cells during the entire time-lapse signal build up experiment (Figure 6b). For both cyanobacteria, the Phe-Az-fed cells consistently showed the lowest AF per cell volume or cell (Figures 7 and S4). Thus, Phe-Az feeding reduced cell division and growth [23], increased cell size, and significantly reduced AF during the entire experiment (Tables 2 and S2). Somewhat unexpectedly, the ratio of A488 intensity to AF intensity was high for Phe-Az-fed *M. aeruginosa* and even *P. agardhii* (Figure S5).

During the time-lapse signal decline experiment, both cyanobacteria showed a fast (24 h) decrease in A488 labeling intensity—by 4.4- and 3.2-fold for Prop-Tyr- and Prop-Lys-fed cells, respectively—while AF remained rather constant (Figure 6c,d vs. Figure 7c,d and Table S2). Only Phe-Az-fed *M. aeruginosa* showed a delayed decline in A488 signal intensity (i.e., 2-fold at 48 h), which was attributed to the reduced growth rate. Accordingly, AF became significantly reduced in Phe-Az-fed cells during the entire experiment. As apparent in the micrographs (Figures 4 and 5), Phe-Az-fed *P. agardhii* did not differ from the control cells at any time during the entire time-lapse decline experiment.

Signal co-localization analysis was used to quantify the heterogeneous distribution of A488. Specifically, the correlation of the voxels (three-dimensional pixels) between the A488 signal and the AF per cell was determined. In general, for *M. aeruginosa*, there was less difference in the co-localization indices between the treatments (Tables 3 and S3 and Figures S6 and S7). In contrast, Prop-Lys- and Prop-Tyr-fed *P. agardhii* showed a significantly reduced correlation compared with the control cells. Note that, even when excluding Phe-Az-fed cells because of toxic side effects, the other two treatments differed significantly from the control cells. In summary, qualitative description of high-resolution micrographs was supported by quantitative evaluation of the A488 signal and AF.

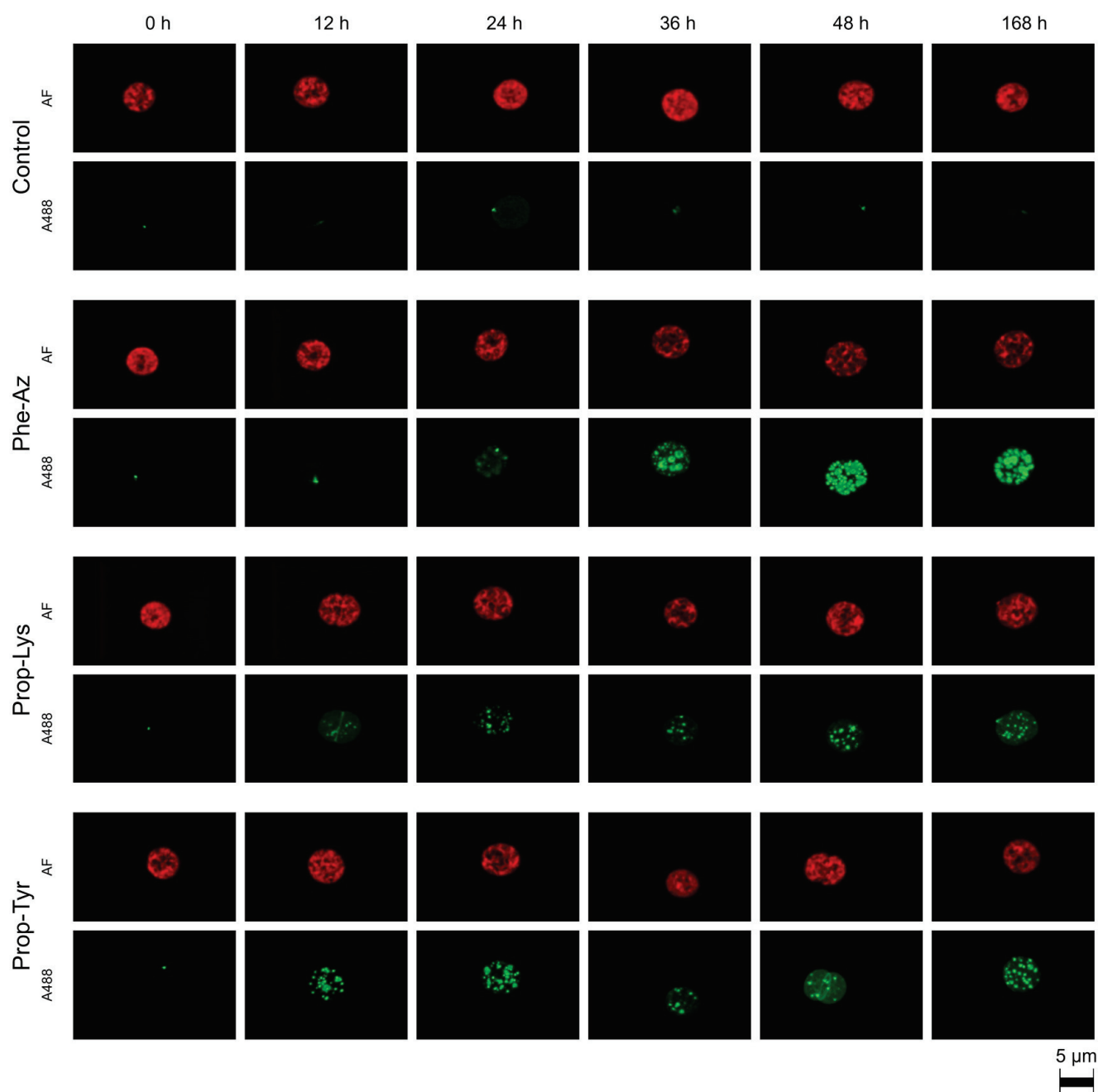


Figure 2. High-resolution micrographs of *M. aeruginosa* strain Hofbauer showing the increase in Alexa Fluor 488 (A488) labeling signal related to clickable MC synthesis via time-lapse build up during feeding of non-AAAs (4-azidophenylalanine [Phe-Az], *N*-propargyl-lysine [Prop-Lys], and H-L-propargyl-tyrosine [Prop-Tyr]). Control cells were grown in the absence of non-AAAs and processed under identical conditions. The natural autofluorescence (AF) is shown in red, while the A488 labeling signal is shown in green.

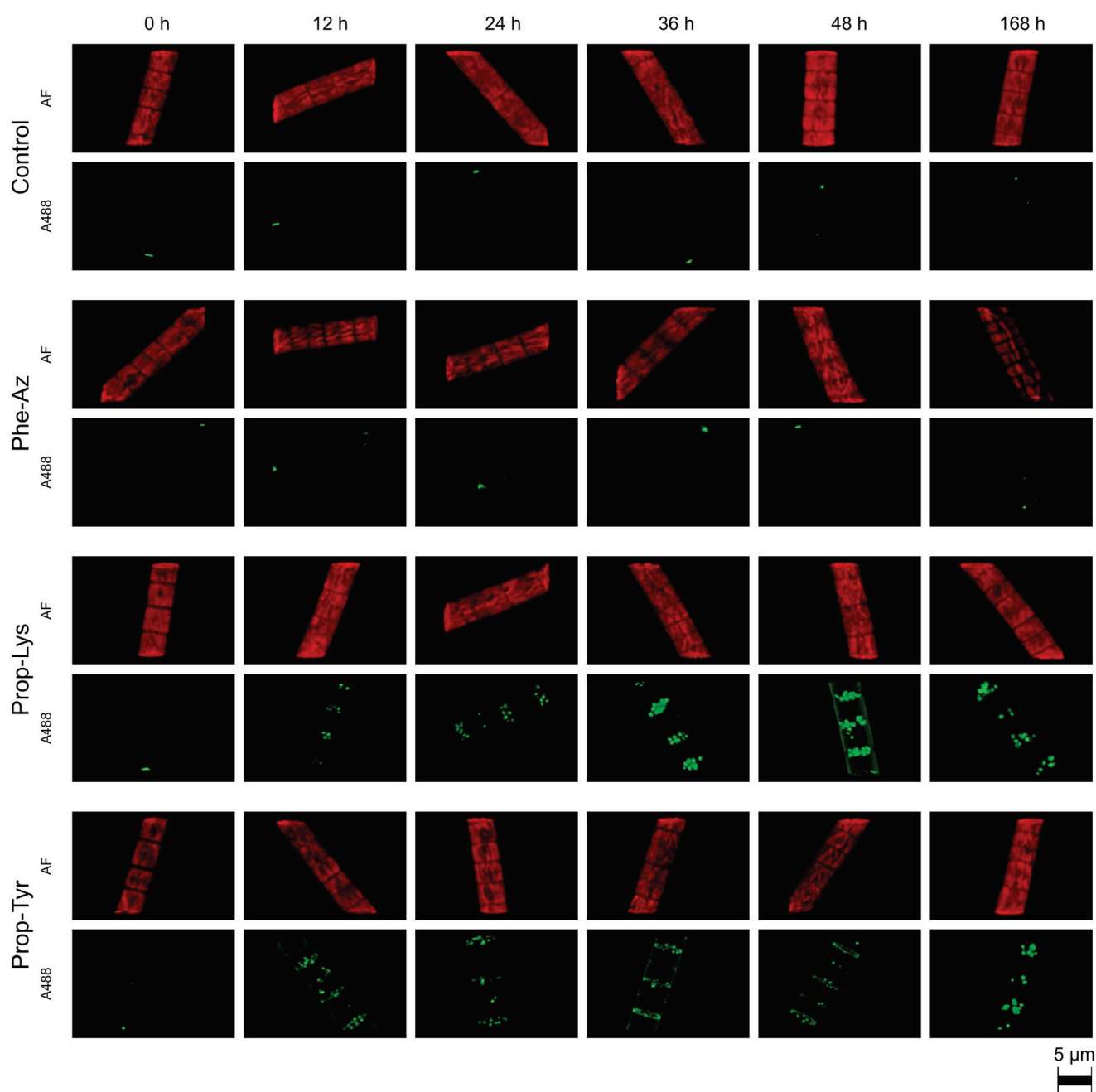


Figure 3. High-resolution micrographs of *P. agardhii* strain no371/1 showing the increase in Alexa Fluor 488 (A488) labeling signal related to clickable AP synthesis via time-lapse build up during feeding of non-AAAs (Phe-Az, Prop-Lys, Prop-Tyr). Phe-Az-modified AP synthesis was not related to A488 labeling compared with control cells. The natural autofluorescence (AF) is shown in red, while the A488 labeling signal is shown in green.

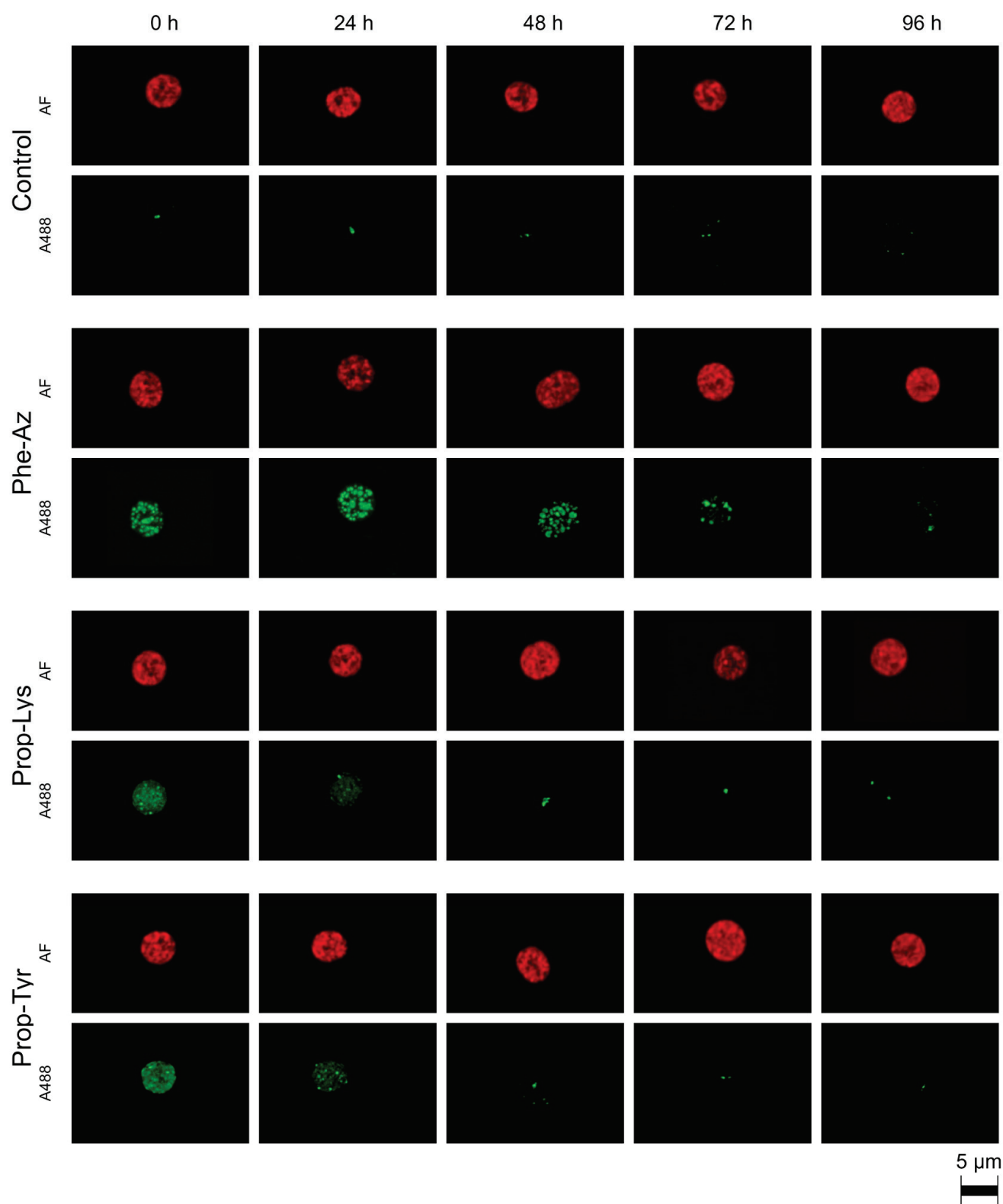


Figure 4. High-resolution micrographs of *M. aeruginosa* showing the decrease in Alexa Fluor 488 (A488) labeling related to the decline in clickable MC synthesis via pulsed feeding of non-AAAs (Phe-Az, Prop-Lys, Prop-Tyr). The natural autofluorescence (AF) is shown in red, while the A488 labeling signal is shown in green.

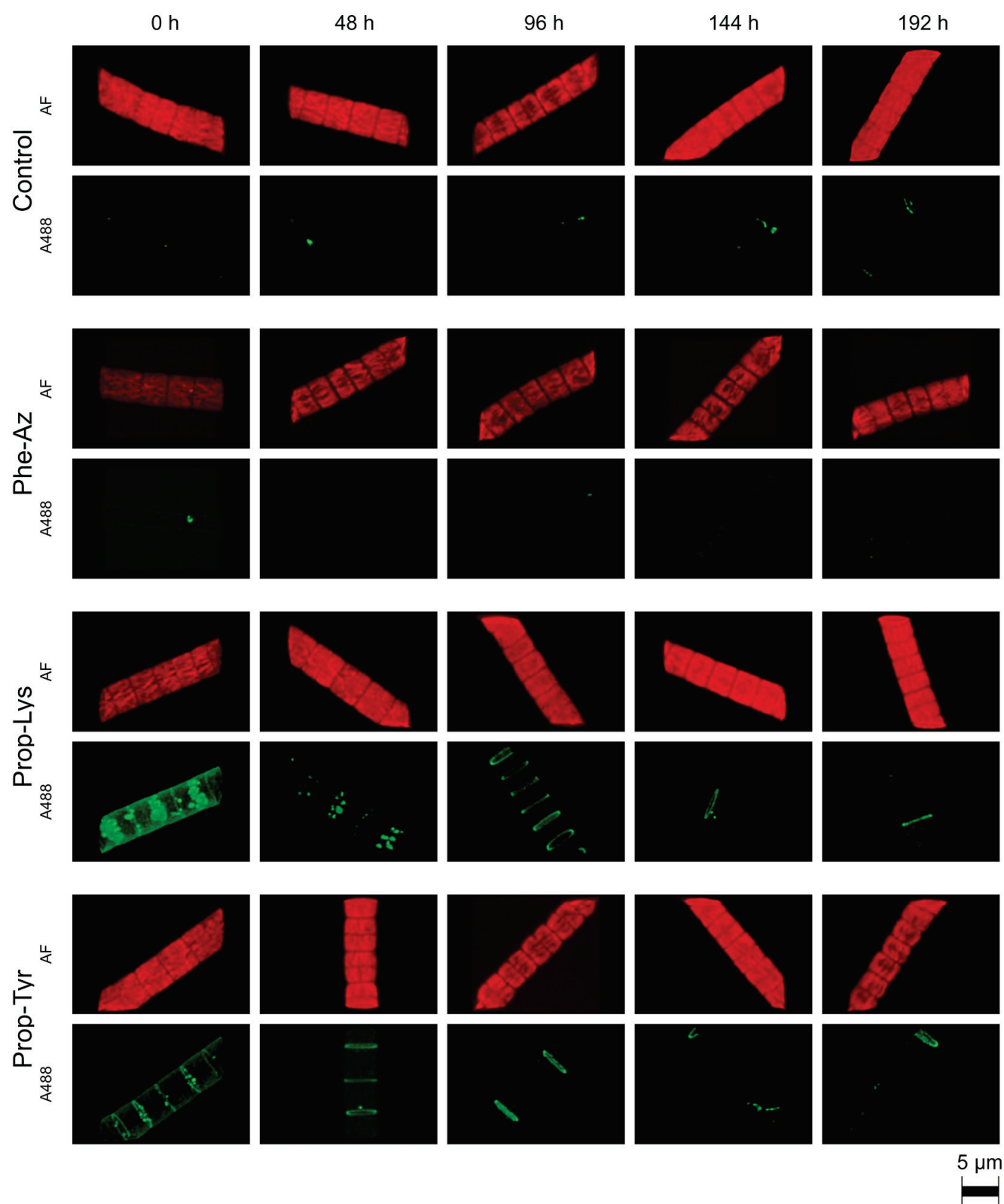


Figure 5. High-resolution micrographs of *P. agardhii* showing the decrease in A488 labeling signal related to the decline in clickable AP synthesis via pulsed feeding of non-AAs (Phe-Az, Prop-Lys, Prop-Tyr). Phe-Az-modified AP synthesis was not related to A488 labeling compared with control cells. The natural autofluorescence (AF) is shown in red, while the A488 labeling signal is shown in green.

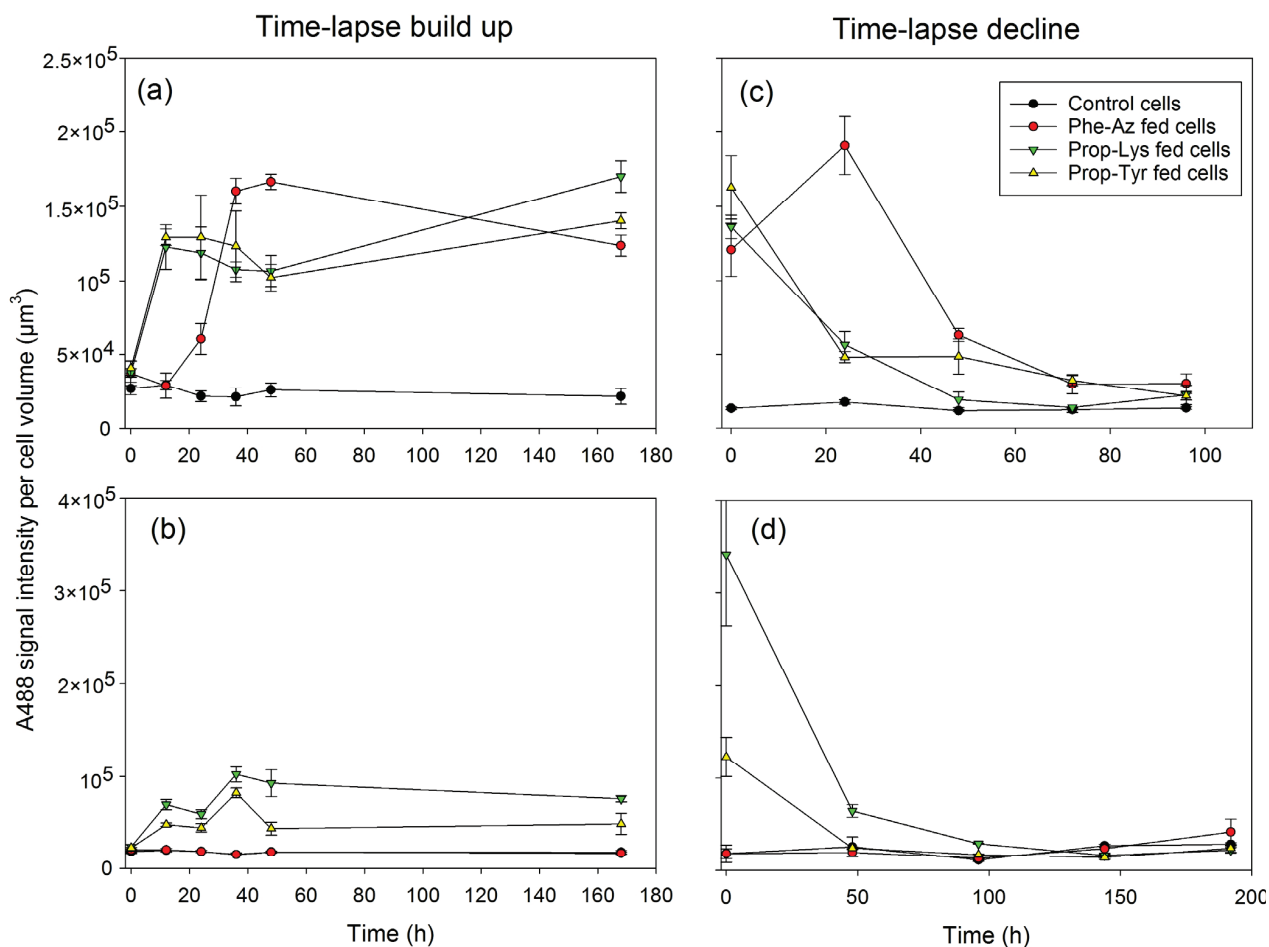


Figure 6. The mean ($\pm$ SE) A488 signal intensities per cell volume (in μm^3) of *M. aeruginosa* (a,c) or *P. agardhii* (b,d) during time-lapse experiments related to the build up (a,b) or decline (c,d) of clickable MC (a,c) or clickable AP (b,d). A488 signal intensities were quantified from images as shown in Figures 2–5.

Table 2. The mean ($\pm$ SE) A488 signal intensity ($\times 10^4$) and autofluorescence (AF) ($\times 10^5$) per cell volume (in μm^3) and A488/AF ratio during time-lapse signal build up and decline in *M. aeruginosa* and *P. agardhii*. The mean ($\pm$ SE) fold changes compared with the control cells are given in parentheses. Graphical data are shown in Figures 6, 7 and S5. Measurements for each time point are shown in Table S2.

Strain	Experiment	Control	Phe-Az	Prop-Lys	Prop-Tyr	<i>n</i>	<i>p</i> -Value ^{1,2} (Treatment)	<i>p</i> -Value ³ (Time)
A488 signal intensity ($\times 10^4$)								
<i>M. aeruginosa</i>	build up	2.4 ± 0.18^a	9.6 ± 1.4^b (4.2 ± 0.6)	11.1 ± 1^{bc} (4.7 ± 0.5)	11.1 ± 1^{bc} (4.7 ± 0.5)	72	<0.001 ***	<0.001 ***
	decline	1.4 ± 0.07^a	8.7 ± 1.7^b (6.0 ± 1)	5 ± 1.1^c (3.6 ± 0.9)	6.3 ± 1.3^d (4.6 ± 1.1)	60	<0.001 ***	<0.001 ***
<i>P. agardhii</i>	build up	1.8 ± 0.07^a	1.8 ± 0.07^a (1.0 ± 0.03)	7 ± 0.67^b (4.0 ± 0.4)	4.8 ± 0.48^{ab} (2.8 ± 0.3)	72	<0.001 ***	0.002 **
	decline	2.2 ± 0.25^a	2.3 ± 0.4^b (1.1 ± 0.2)	9.5 ± 3.3^a (5.1 ± 2)	4.0 ± 1.2^a (2.1 ± 0.66)	60	0.003 **	0.002 **
AF intensity ($\times 10^5$)								
<i>M. aeruginosa</i>	build up	0.8 ± 0.03^a	0.6 ± 0.03^b (0.8 ± 0.04)	0.78 ± 0.03^a (1.0 ± 0.03)	0.76 ± 0.03^a (1.0 ± 0.03)	72	<0.001 ***	0.001 ***

Table 2. Cont.

Strain	Experiment	Control	Phe-Az	Prop-Lys	Prop-Tyr	<i>n</i>	<i>p</i> -Value ^{1,2} (Treatment)	<i>p</i> -Value ³ (Time)
<i>P. agardhii</i>	decline	3.5 ± 0.24 ^a	2.4 ± 0.23 ^b (0.67 ± 0.06)	3.5 ± 0.2 ^a (1.0 ± 0.3)	3.5 ± 0.22 ^a (1.0 ± 0.3)	60	0.003 **	0.234
	build up	1.7 ± 0.09 ^a	1.2 ± 0.08 ^b (0.8 ± 0.06)	1.5 ± 0.04 ^a (0.9 ± 0.03)	1.5 ± 0.06 ^a (0.9 ± 0.04)	72	0.003 **	0.003 **
	decline	6.1 ± 0.25 ^a	1.9 ± 0.44 ^b (0.3 ± 0.08)	5.5 ± 0.21 (0.9 ± 0.03)	5.3 ± 0.22 ^a (0.9 ± 0.05)	60	<0.001 ***	0.011 *
A488/AF ratio								
<i>M. aeruginosa</i>	build up	0.32 ± 0.02 ^a	1.98 ± 0.34 ^b	1.48 ± 0.15 ^{bc}	1.57 ± 0.16 ^{bc}	72	<0.001 ***	<0.001 **
	decline	0.05 ± 0.01 ^a	0.44 ± 0.08 ^b	0.16 ± 0.04 ^{bc}	0.2 ± 0.05 ^{bc}	60	<0.001 ***	0.078
<i>P. agardhii</i>	build up	0.12 ± 0.01 ^a	0.16 ± 0.01 ^a	0.51 ± 0.06 ^{bc}	0.39 ± 0.06 ^{bd}	72	<0.001 ***	<0.001 ***
	decline	0.04 ± 0.004 ^a	0.25 ± 0.08 ^b	0.19 ± 0.07 ^a	0.08 ± 0.02 ^a	60	0.017 *	0.076

¹ Two-way repeated measures analysis of variance (RM ANOVA) (grouping factor). *** *p* < 0.001, ** *p* < 0.01, * *p* < 0.05; ² Superscript letters (^{a,b,c,d}) indicate subgroups of non-AA treatments that were not significantly different based on post hoc pairwise comparison (Bonferroni test, *p* > 0.05); ³ Two-way RM ANOVA (time factor).

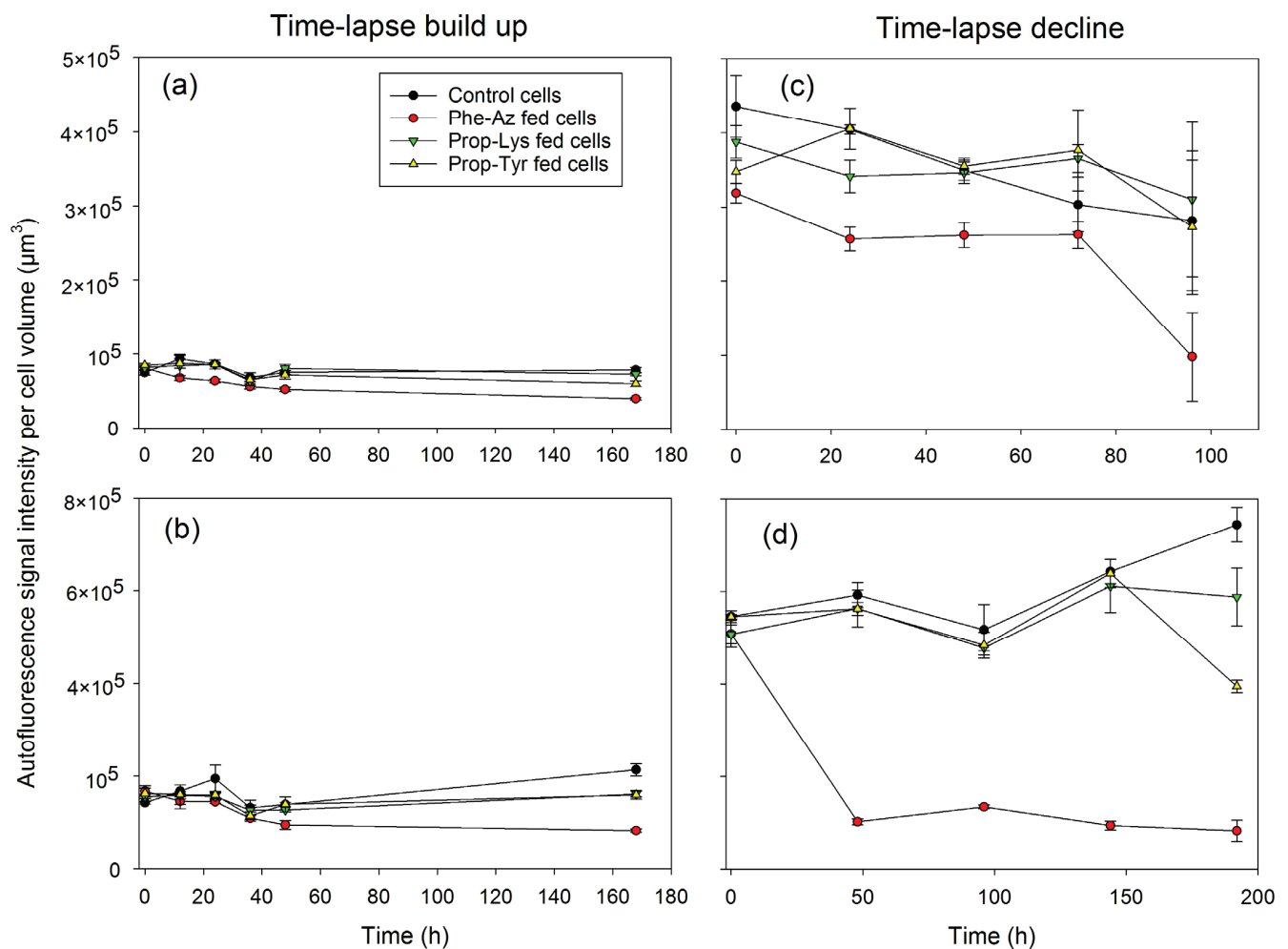


Figure 7. The mean ($\pm$ SE) autofluorescence intensity per cell volume (in μm^3) of *M. aeruginosa* (a,c) or *P. agardhii* (b,d) during time-lapse experiments related to the build up (a,b) or decline (c,d) of clickable MC (a,c) or clickable AP (b,d). AF signal intensities were quantified from images, as shown in Figures 2–5.

2.4. Relationship Between Clickable Cyanotoxin Content and Labeling by the Click Reaction as Quantified from High-Resolution Microscopy

Because aliquots of cells were analyzed for their AP/MC content, as described in a previous study [23], the A488 signal intensity results were compared directly. Based on the original data, there were significant linear relationships between the A488 signal intensity and the percentage of clickable MC/AP based on the total MC/AP content or μg of clickable MC/AP per mg of dry weight (DW). Overall, the $\log(x + 1)$ -transformed data showed higher coefficients of determination (R^2), i.e., the proportion of variation in the A488 signal intensity explained by the clickable MC/AP content in the linear regression model. All reported regression curves were statistically significant ($p < 0.001$ for both intercept and slope, sample size [n] = 72 time-lapse build up, $n = 60$ for time-lapse decline; Figures 8 and S8).

In general, the A488 labeling intensity data for the three non-AA treatments consistently correlated to the observed clickable MC/AP content. The control cells—grown and processed under identical conditions but without non-AA substrate—always showed minimum A488 intensity. This minimum intensity probably resulted from background fluorescence given that a previous study provided no evidence for non-specific binding of the A488 fluorophore for the same *M. aeruginosa* strain [22]. For *M. aeruginosa*, Prop-Tyr feeding led to the highest clickable MC-Prop-Tyr content along with the highest A488 signal intensity (Figures 8a,c and S8a,c). On the contrary, the Prop-Lys-fed cells showed lower A488 labeling intensity, consistent with the lowest overall proportion of clickable MC-Prop-Lys in total MC. The Phe-Az-fed cells showed a more variable and intermediate A488 labeling intensity and proportion of MC-Phe-Az in total MC.

For *P. agardhii*, the highest A488 labeling intensity correlated to the highest percentage of AP-Prop-Lys in the peptide extract (Figures 8b,d and S8b,d). For Prop-Tyr-fed cells, there was a lower proportion of clickable AP-Prop-Tyr that corresponded to a minimum or lower A488 signal intensity. In contrast, synthesis of clickable AP-Phe-Az was observed, but it did not result in any A488 labeling intensity. Because the growth of Phe-Az-fed cells was reduced dramatically, the clickable AP-Phe-Az content in μg per DW also was also low (Figure S8b,d). Thus, toxic side effects caused by Phe-Az feeding in *P. agardhii* not only reduced cell division and growth [23], but also dramatically reduced AF and A488 labeling intensity.

Table 3. The mean ($\pm$ SE) of co-localization coefficients between A488 signal intensity vs. autofluorescence (AF) signal intensity of *M. aeruginosa* or *P. agardhii* during time-lapse experiments using pulsed feeding of non-AAs to observe the build up or decline of clickable MC or AP. Graphical data are shown in Figures S6 and S7. The measurements for each time point are shown in Table S3.

Strain	Experiment	Control	Phe-Az	Prop-Lys	Prop-Tyr	<i>n</i>	<i>p</i> -Value ^{1,2} (Treatment)	<i>p</i> -Value ³ (Time)
Object Pearson's co-localization coefficient								
<i>M. aeruginosa</i>	build up	0.22 $\pm$ 0.01	0.18 $\pm$ 0.01	0.21 $\pm$ 0.02	0.2 $\pm$ 0.01	72	0.288	0.03 **
	decline	0.36 $\pm$ 0.01	0.32 $\pm$ 0.01	0.35 $\pm$ 0.01	0.34 $\pm$ 0.01	60	0.105	0.006 **
<i>P. agardhii</i>	build up	0.23 $\pm$ 0.02 ^a	0.2 $\pm$ 0.02 ^{ab}	0.19 $\pm$ 0.02 ^{ab}	0.17 $\pm$ 0.02 ^b	72	0.022 *	0.003 **
	decline	0.47 $\pm$ 0.03 ^a	0.14 $\pm$ 0.09 ^b	0.25 $\pm$ 0.03 ^{ab}	0.18 $\pm$ 0.03 ^b	60	0.006 **	0.229
Object Spearman's co-localization coefficient								
<i>M. aeruginosa</i>	build up	0.41 $\pm$ 0.02	0.37 $\pm$ 0.02	0.39 $\pm$ 0.03	0.39 $\pm$ 0.02	72	0.727	0.006 **
	decline	0.64 $\pm$ 0.01 ^a	0.61 $\pm$ 0.01 ^b	0.63 $\pm$ 0.01 ^{ab}	0.63 $\pm$ 0.01 ^{ab}	60	0.035 *	0.532

Table 3. Cont.

Strain	Experiment	Control	Phe-Az	Prop-Lys	Prop-Tyr	n	p-Value ^{1,2} (Treatment)	p-Value ³ (Time)
<i>P. agardhii</i>	build up	0.68 ± 0.02 ^a	0.67 ± 0.02 ^a	0.53 ± 0.03 ^b	0.46 ± 0.04 ^b	72	0.001 ***	<0.001 ***
	decline	0.54 ± 0.04 ^a	0.22 ± 0.11 ^b	0.56 ± 0.03 ^a	0.44 ± 0.05 ^{ab}	60	0.013 *	0.258

¹ Two-way repeated measures analysis of variance (RM ANOVA) (grouping factor). *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$; ² Superscript letters (^{a,b}) indicate subgroups of non-AA treatments that were not significantly different based on post hoc pairwise comparison (Bonferroni test, $p > 0.05$); ³ Two-way RM ANOVA (time factor).

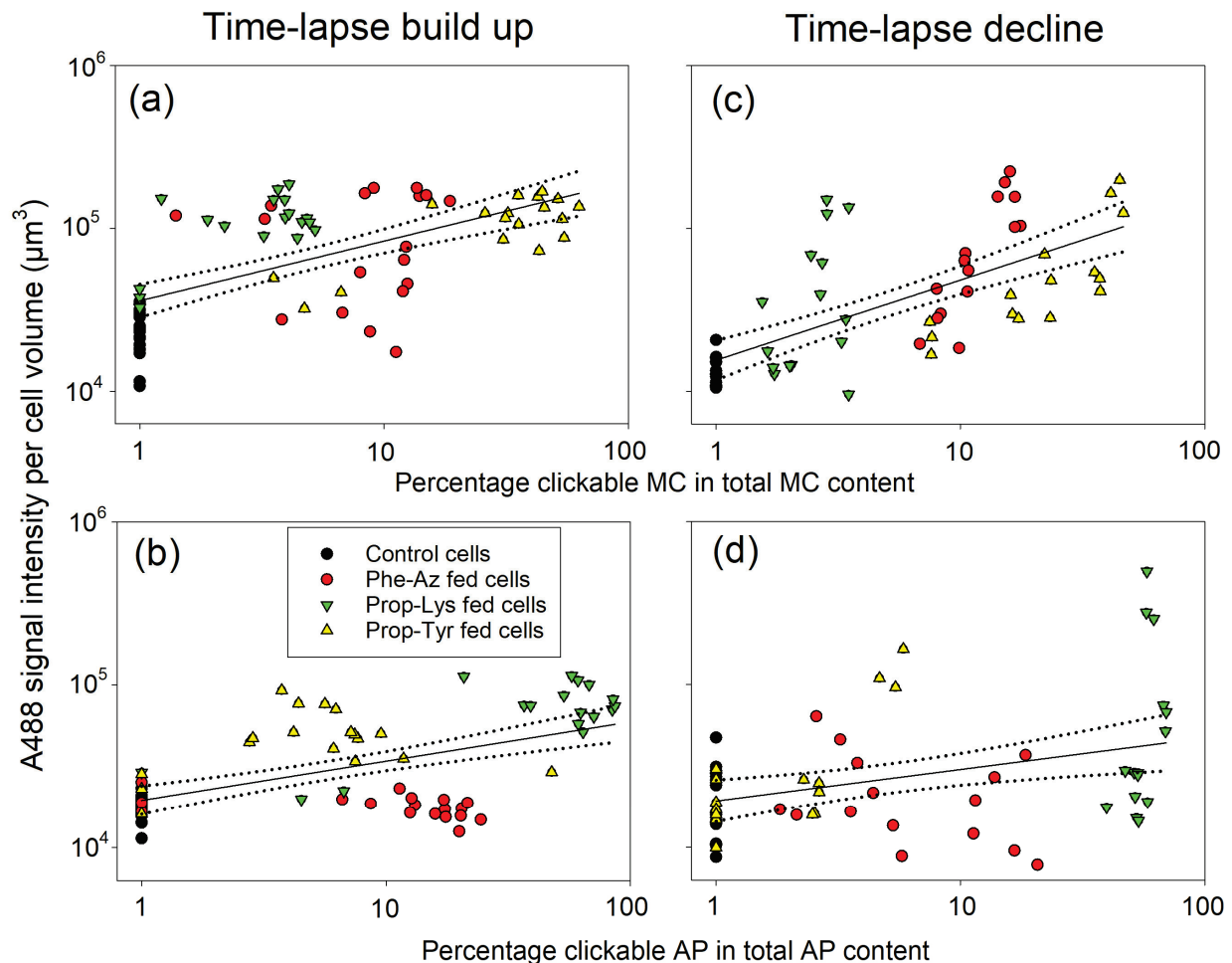


Figure 8. Alexa Fluor 488 (A488) signal intensity per cell volume (in μm^3) of *M. aeruginosa* (a,c) or *P. agardhii* (b,d) during time-lapse experiments using pulsed feeding of non-AAAs vs. the percentage of clickable MC (a,c) or clickable AP (b,d), as recorded by chemical-analytical methods reported previously [23]. Details of linear regression lines: (a) $Y = a + bx$, where y is $\log(x + 1)$ A488 signal intensity and x is $\log(x + 1)$ percentage of clickable MC/AP in total MC/AP as determined by liquid chromatography–mass spectrometry [23]. The dotted lines indicate 95% confidence intervals. Regression curves: (a) $y = 4.524 + 0.374x$ ($R^2 = 0.46$, $p < 0.001$), (c) $y = 4.193 + 0.493x$ ($R^2 = 0.48$, $p < 0.001$), (b) $y = 4.289 + 0.243x$ ($R^2 = 0.33$, $p < 0.001$), (d) $y = 4.281 + 0.198x$ ($R^2 = 0.14$, $p = 0.004$), without Phe-Az-fed cells (b) $y = 4.321 + 0.329x$ ($R^2 = 0.68$, $p < 0.001$), (d) $y = 4.308 + 0.233x$ ($R^2 = 0.22$, $p = 0.001$). MC/AP contents for each time point are shown in Tables S4 and S5.

2.5. Relationship Between the Clickable Cyanotoxin Content and Labeling by the Click Reaction as Quantified by FCM

In addition to high-resolution microscopy, FCM was used to estimate the number of A488-labeled cells/filaments resulting from chemo-selective labeling. Because FCM easily enumerates thousands of particles (cells) to discriminate labeled from non-labeled subpop-

ulations, it was applied as a complementary and more quantitative approach compared with microscopic observation. During the time-lapse build up experiment, labeled subpopulations appeared at T0, showing an increase in BL1-A fluorescence (Figures S9 and S10, Table S6). During the time-lapse signal build up, labeled subpopulations of Prop-Lys- and Prop-Tyr-fed cells differentiated gradually from control cells. In other words, the overlap in the frequency distribution curves for individual subpopulations decreased over time. From T1 onwards, the maximum particle frequency of the BL1-A (A488) signal varied by 1–2 orders of magnitude from the maximum BL1-A frequency for the control cells. In contrast, the unlabeled control cells always showed a narrow range of BL1-A fluorescence intensity. During the time-lapse decline experiment, the BL1-A fluorescent subpopulation also declined and became undetectable compared with the rather sharp fraction of unlabeled control cells (Figures S11 and S12).

Corresponding to A488 intensity per cell volume, the BL1-A percentage of cells correlated positively to the proportion of clickable MC/AP in the total MC/AP content (Figure 9) or μg of clickable MC/AP content per mg of DW (Figure S13). For *M. aeruginosa*, Prop-Tyr-fed cells presented the highest clickable MC content, which correlated with a high proportion of BL1-A particles. Similarly, Phe-Az-fed cells showed a positive relationship between clickable MC synthesis and the percentage of BL1-A fluorescent particles (Figure 9a,c). In contrast, for *P. agardhii*, Phe-Az fed cells incorporated Phe-Az into modified AP but did not show increased fluorescence compared with controls (Figure 9b,d). Given that AF was also dramatically reduced in Phe-Az-fed cells (Figure 7), it is concluded that Phe-Az had the strongest toxic side effects in *P. agardhii*.

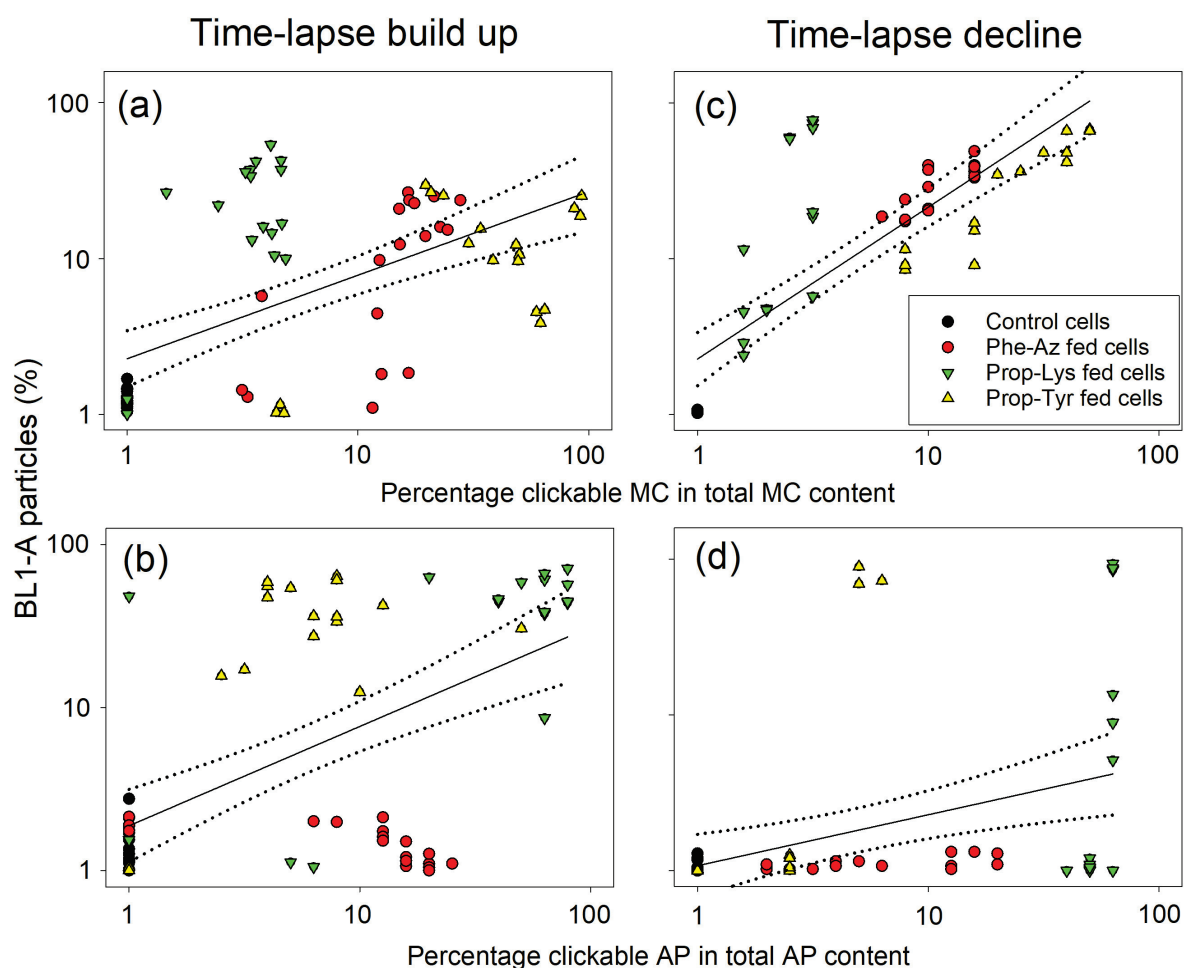


Figure 9. BL1-A percentage of cells (A488-labeled particles) of *M. aeruginosa* (a,c) or *P. agardhii* (b,d) during time-lapse experiments vs. the percentage of clickable MC (a,c) or AP (b,d) as recorded by flow

cytometry. Details of linear regression lines: (a) $Y = a + bx$, where y is $\log(x + 1)$ A488 particles and x is $\log(x + 1)$ percentage of clickable MC/AP in total MC/AP as determined by liquid chromatography–mass spectrometry [23]. The dotted lines indicate 95% confidence intervals. Regression curves: (a) $y = 0.36 + 0.54x$ ($R^2 = 0.33$, $p < 0.001$), (c) $y = 0.36 + 0.97x$ ($R^2 = 0.64$, $p < 0.001$), (b) $y = 0.23 + 0.64x$ ($R^2 = 0.35$, $p < 0.001$), (d) $y = 0.03 + 0.33x$ ($R^2 = 0.15$, $p = 0.002$), without Phe-Az-fed cells (b) $y = 0.365 + 0.825x$ ($R^2 = 0.62$, $p < 0.001$), (d) $y = 0.091 + 0.36x$ ($R^2 = 0.18$, $p = 0.004$). MC/AP contents for each time point are shown in Tables S4 and S5.

3. Discussion

3.1. Sensitivity of Chemo-Selective Labeling and Visualization via A488

In general, cyanobacterial AF can disturb fluorescence signal detection because of spectral overlap with commonly used fluorophores, including Cy3, Cy5, and FITC [29]. While methods to improve the signal-to-noise ratio have been developed continuously [30,31], the choice of the appropriate fluorophore (i.e., A488) is of the utmost importance [32]. In the present study, the A488 labeling signal responded sensitively, which is consistent with the early synthesis of clickable MC/AP as observed in peptide extracts reported earlier [23]. During the time-lapse increase experiment, the A488 signal could be reliably differentiated from background fluorescence (i.e., AF) as early as T1 (after 12 h). For both *M. aeruginosa* and *P. agardhii*, the AF signal intensity (i.e., emission spectrum 680–730 nm) was generally higher than the signal for A488 (i.e., 500–550 nm), (Tables 2 and S2).

Both *M. aeruginosa* and *P. agardhii* contained low amounts of clickable MC/AP as early as T0: *M. aeruginosa*, $5\% \pm 1\%$ for Phe-Az and $4\% \pm 1\%$ for Prop-Tyr; *P. agardhii*, $3\% \pm 2\%$ for Prop-Lys (see Tables S4 and S5, and Figures 1 and 2 in [23]). The early incorporation of non-AA at T0 can be explained by the manipulation time (approximately 1 h) including centrifugation and washing in phosphate-buffered saline (PBS) until fixation in 2% paraformaldehyde (PFA). These low percentages, however, did not result in a significant increase in A488 signal intensity between treatments based on two-way repeated measures analysis of variance (RM ANOVA) and post hoc pairwise multiple comparisons (Bonferroni t -test, $p > 0.95$, Figure 6). Consequently, to determine the detectability vs. the A488 signal-to-noise ratio more precisely, another time-lapse build up experiment in hourly time intervals would be needed. There was no difference in the timing of clickable MC vs. AP synthesis between the two genera *M. aeruginosa* and *P. agardhii*, which can be explained at least partly by the comparable growth rates of these strains, that is, $r = 0.35$ – 0.6 and 0.2 – 0.4 day^{-1} , respectively (see Tables 1 and 2 in [23]).

FCM was more sensitive than high-resolution microscopy, as already at T0 during the time-lapse build up experiment labeled subpopulations were detected in the Prop-Tyr and Prop-Lys fed cells (Figures S9 and S11). In FCM, the rectangular gating tool allows one to discriminate relatively low numbers of particles compared with control cells, supporting the early discrimination of labeled from non-labeled subpopulations. Under the specified conditions, during time-lapse build up, only 27–149 “false” positive particles for *M. aeruginosa* and 0–33 “false” positive particles for *P. agardhii* were differentiated, resulting in 0.04–0.69% and 0–1.75% of the total population, respectively ($n = 18$). For FCM, the observed noise implies a limit of detection of around 1%. In contrast, the natural A488 signal intensity per cell volume recorded from the control cells in the microscope showed greater variation: During time-lapse build up, it was 50–150% for *M. aeruginosa* and 75–118% for *P. agardhii* ($n = 18$). Thus, the potential limit of detection for A488 labeling is a 1.5-fold and 1.2-fold excess of the natural A488 intensity in *M. aeruginosa* and *P. agardhii*, respectively.

So far, only cells treated chemically (i.e., fixed in 2% PFA and permeabilized using Triton X-100, Bio-Rad, Vienna, Austria) have been used [21]. While this protocol has the advantage of processing aliquots for different instrumentation simultaneously (e.g., cell

numbers, chemical analysis, microscopy, and FCM), in the future live cell observation would be advisable in combination with FCM. In this study, the BL1-A fluorescent *M. aeruginosa* cells with clickable MC-Prop-Tyr fluctuated from T1 to T5, implying that clickable MC-Prop-Tyr was not produced constantly in the cells from one time point to the next (Figure 9, Table S6). In addition, the distribution curve form of histograms showing the relative frequency (%) of BL1-A fluorescent particles changed during the time-lapse increase (Figures S9 and S11). Catalyst-free cycloadditions that avoid toxic copper side effects, such as the strain-promoted azide–alkyne cycloaddition (SPAAC) [33,34], have been developed but applied less frequently. Such protocols could make it possible to monitor the same cells of an experimental population repeatedly.

3.2. Quantitative Relationship Between the Clickable Cyanotoxin Content and A488 Labeling Signal by the Click Reaction

Overall, the early visibility of clickable MC/AP is in accordance with other studies on the NRPS synthesis rate. Moschny et al. [35] reported that, when using precursor-directed biosynthesis of MC in *M. aeruginosa*, the amount of clickable MC extracted from the biomass most likely increased in an exponential manner during the first 36 h, reaching a maximum at 48 h. Accordingly, for heterologously expressed NRPS domains, researchers have observed significant amounts of clickable peptide products within a few hours [36]. In penicillin biosynthesis, for the NRPS enzyme (ACV synthetase) forming the tripeptide ACV δ -(L-R-aminoadipyl)-L-cysteinyl-D-valine, under optimal conditions in vitro, a linear rate of ACV formation was observed for at least 120 min [37]. Together with our previous report [23], a chemical rationale has been provided by (i) analyzing aliquots via high-performance liquid chromatography–mass spectrometry and protonated mass (MS^2/MS^3) fragmentation analysis for the clickable MC/AP content and (ii) the subsequently created A488 signal in the cells using the click reaction (Tables S4 and S5).

For *M. aeruginosa*, the proportion of clickable MC obtained through all three non-AAs correlated to the A488 signal intensity per cell (Figure 8a,c) and BL1-A fluorescent particles as recorded by FCM (Figure 9a,c). When testing Phe-Az or Prop-Lys individually, there were significant linear relationships between the percentage of clickable MC vs. the A488 signal intensity (T0–T5, $n = 18$). In contrast, for Prop-Tyr, the highest A488 signal intensity was recorded from T1 onwards, consistent with the fact that clickable MC-Prop-TyrR showed the most efficient synthesis. The variable but consistent differences in clickable MC/AP synthesis can be explained by the variable catalytic promiscuity among different NRPS genotypes [23].

For *P. agardhii*, the A488 signal intensity per cell volume also correlated to the proportion of clickable AP-Prop-Lys and AP-Prop-Tyr in total AP (Figure 8). For the entire population, the proportion of the labeled subpopulation also correlated with the clickable AP-Prop-Lys or AP-Prop-Tyr proportion (Figure 9). In contrast, there was no such relationship for AP-Phe-Az. Recently, toxic side effects have been reported for *Pseudomonas aeruginosa* when using 4-azido-L-homoalanine (1.5 mM concentration in the medium) to modify the siderophore pyoverdine via NRPS [38]. The possible reasons for azide-based toxicity have been discussed [23], including the well-known, exceptional reactivity of the azide group [39]. Despite evidence of incorporation into MC/AP, Phe-Az also decreased AF significantly in both cyanobacteria (Figure 7). Thus, one possible reason for reduced AF is intracellular decomposition of Phe-Az resulting in reactive nitrenes causing the overall toxic side effects. Nevertheless, for *P. agardhii*, even under toxic conditions, the modified peptide AP-Phe-Az was still synthesized at an intermediate proportion ($12.5 \pm 1.7\%$, ranging from 5.6–20.5%) relative to AP-Prop-Lys ($50 \pm 6.8\%$, ranging from 3.5–85.4%) and AP-Prop-Tyr ($6.4 \pm 2.5\%$, ranging from 1.8–10.8%) (Tables S4 and S5 and Table 3 in [23]). In summary, although Phe-Az is incorporated into MC/AP to some extent, most of the

non-incorporated Phe-Az in the cell might have resulted in the formation of nitrenes via uncontrolled decomposition processes counteracting the expected A488 fluorescence signal. Obviously, *M. aeruginosa* was less sensitive to Phe-Az than *P. agardhii* despite showing high intracellular Phe-Az content (Figure S9 in [23]), implying genus-specific differences in detoxification mechanisms.

3.3. Spatiotemporal Changes in the A488 Signal in Cyanobacteria During the Time-Lapse Decline Experiment

As reported previously, there was a rather heterogeneous distribution of the A488 signal for clickable MC/AP products; this signal was quantified in relation to AF using standard co-localization indices, namely Object Pearson's and Object Spearman's co-localization coefficients (Tables 3 and S3 and Figures S6 and S7). For *M. aeruginosa*, the co-localization of A488 signal vs. AF on average tended to decrease (although the decrease was not significant), indicating the presence of distinct A488 signal entities in the cell. In contrast, for *P. agardhii* both co-localization indices of A488 signal vs. AF decreased significantly as a result of A488 signal build up and decline. This heterogeneity could be explained by (i) structures facilitating MC/AP accumulation in the cell and/or (ii) MC/AP biosynthesis. For the former, it has been argued that the intracellular MC/AP content sometimes exceeds the theoretical solubility threshold [40]. A dual labeling protocol has been used to test whether lipids might be related to MC solubility and thus spatially related to clickable MC accumulation occurring as distinct entities [22]. However, pairwise co-localization indices between both lipids (visualized using Bodipy staining) and clickable MC (A405) were not increased compared with analogous indices for AF. The two hypotheses, accumulation vs. biosynthesis of MC/AP in *M. aeruginosa* and *P. agardhii*, will be tested in another forthcoming study via advanced image analysis.

Interestingly, in Prop-Lys- and Prop-Tyr-fed *P. agardhii*, the distinct entities located towards the cell septa were replaced gradually by ring-like formations in the peripheral area of the cell (Figure 5). It is tempting to speculate that the ring-like formations originated from distinct entities (i.e., representing AP-Prop-Lys or AP-Prop-Tyr) as leftovers from diffusion because the synthesis of AP-Prop-Lys/Tyr stopped 48 h earlier. In contrast, during time-lapse decline, there was no such fading signal for *M. aeruginosa*. Given that *M. aeruginosa* showed distinct entities distributed all over the cell, it might be more difficult to see the diffusing clickable MC because of a lower spatial concentration. Notably, using a fluorescence in situ hybridization probe specific for the mRNA transcribed from the *mcyA* gene part of the *mcy* gene cluster in *M. aeruginosa* PCC7806, a so-called “nonuniform labelling, concentrated in spots” has been reported [41,42]. The authors concluded that their fluorescence technique allowed the visualization of the variation in the expression of the *mcy* synthesis genes in *M. aeruginosa*. Using the same fluorescent probe, non-uniform labeling results have been reproduced using *M. aeruginosa* strain PCC7806 [43]. It has been suggested repeatedly that MC carrying *N*-methyl-dehydroalanine (Mdha) at position 7 of the MC molecule binds covalently to abundant proteins, possibly protecting them from photooxidation, and undergoes several other molecular interactions in the *M. aeruginosa* cell [44]. Initial attempts have been made to test co-localization of clickable MC-Prop-Tyr and abundant proteins (e.g., using RbcL and FtsZ [cytosolic proteins] and PsbA [a thylakoid protein]) in *M. aeruginosa*. A double labeling protocol was applied by immunolabeling of proteins (green A488) with chemo-selective labeling of clickable MC (blue A405) and revealed increased co-localization coefficients between PsbA and AF, but not between clickable MC and immunolabeled proteins [22]. In the future, predictions about various cell structures/organelles interacting with cyanotoxins or not could be tested via chemo-selective labeling.

3.4. The Potential of Visualizing Cyanotoxin Synthesis via Chemo-Selective Labeling in Gene or Peptide Function Studies

The application of fluorescence-based technology for MC detection in the environment, cells, or other biota has been intensively tested [43,45]. In general, the feeding of clickable non-AAs or precursor-directed synthesis of clickable MC/AP in the environment is considered to be less applicable, and one might think, rather, of functional research applications—for example, via heterologous expression systems [46]. For example, heterologous expression of MC-LR in *Escherichia coli* was first reported in 2017 [47], and then more recently in a model cyanobacterium *Synechococcus elongatus* PCC7942 [48]. For *E. coli*, the large MC biosynthesis (NRPS) gene cluster (55,000 base pairs) was cloned using fosmids and heterologously expressed in *E. coli*. Notably, the natural bidirectional promoter was not sufficient for any detectable MC product. Only the introduction of a strong bidirectional tetracycline-inducible promoter led to the ability to detect the corresponding MC product [49]. Through chemo-selective labeling and cyanotoxin synthesis on the cellular level in the host organism, such as *E. coli*, the MC production process could be observed directly. Such a heterologous expression system has also been used to investigate the biosynthetic flexibility of the MC biosynthesis pathway [50] or to test the functions of accessory genes [51]. A flexible MC biosynthesis pathway can help to produce clickable MC structural variants with specific moieties to compare moiety-dependent biological properties, such as eukaryotic cellular uptake rates [52]. Another application might involve an investigation of the role of environmental factors, such as macronutrients and light conditions under standardized heterologous expression conditions. For example, soon after the elucidation of the MC biosynthesis pathway [5], binding boxes of the ubiquitous transcription factors NtcA (a transcriptional regulator of genes involved in nitrogen assimilation) and Fur (a ferric uptake regulator) were identified within the bidirectional promoter region and could be tested using chemo-selective labeling more directly under standardized in vivo conditions. In addition, the direct effects of irradiance (i.e., high vs. low light) regulated the transcription of the MC biosynthesis genes in *Microcystis* [44,53]. In summary, chemo-selective labeling should allow for the ability to monitor the molecular basis of potential factors in regulation more directly, in real time, and with high temporal resolution.

4. Conclusions

In conclusion, time-resolved analysis of the A488 signal intensity points to a rather specific and sensitive CuAAC reaction between clickable modified toxins/peptides and the A488 signal based on qualitative and quantitative correlation. On a spatial scale during the time-lapse increase experiment, the heterogeneous A488 signal structures, described as distinct entities previously, became visible from the beginning and varied in their location on a subcellular level. On a temporal scale during the time-lapse decline experiment, the heterogeneous structures in the cell either faded or were gradually replaced by a ring-like patch formation in the peripheral area of the cell. Thus, real-time observation of cyanotoxin synthesis by means of click chemistry has the potential to track intracellular synthesis using both chemical-analytical analysis and high-resolution imaging and can be used to study regulation and potential intracellular function.

5. Materials and Methods

5.1. Study Organisms and Experimental Design

As described previously, clonal strain cultures of *M. aeruginosa* strain Hofbauer and *P. agardhii* strain no371/1 were grown semi-continuously under maximum growth conditions in BG11 medium [54] at 20 °C using illumination conditions of 50 µmol photons

$\text{m}^{-2} \text{s}^{-1}$ (16:8 h light/dark cycle) [23]. Details of strain origin and identification were reported [21].

For time-lapse signal build up, at T0 the treatments were supplemented once with Phe-Az or Prop-Lys or Prop-Tyr, resulting in a final concentration of 0.05 mM [23] (Figure 10). Control cell cultures were grown under identical conditions but in the absence of non-AAs. At T0, the cells were harvested directly after adding the non-AA to the medium within 1 h. To observe the change in the A488 signal intensity, culture flasks were harvested every 12 h for the first 48 h (T1–T4), with a final harvesting at T5 (168 h).

For time-lapse signal decline, cultures were grown for 48 h in the presence of one of the three non-AAs. At T0, following 48 h of non-AA feeding incubation, the cultures were washed by centrifugation (4000 rpm for 5 min) and transferred to fresh (non-AA free) BG11 medium. Analogously to the build up experiment, at T0, the cells were harvested after transfer into new medium within 1 h. The time points T1–T4 represented every 24 h for *M. aeruginosa* and every 48 h for *P. agardhii*. All harvest steps were performed at room temperature.

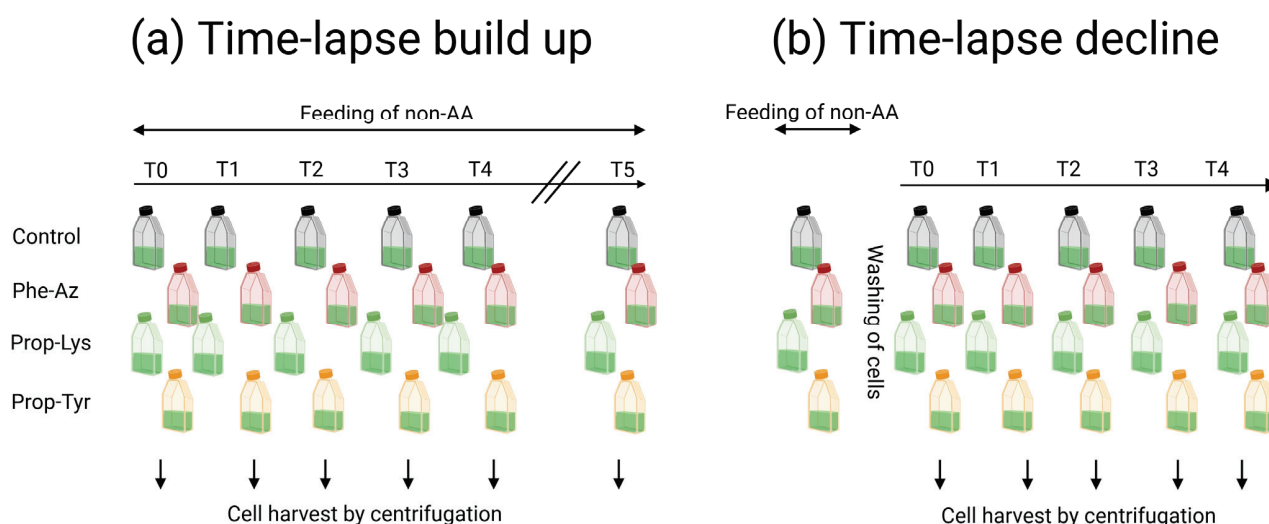


Figure 10. Experimental design for time-lapse experiments to allow for real-time observation of clickable MC/AP synthesis in bloom-forming cyanobacteria: (a) time-lapse build up experiment and (b) time-lapse decline experiment. Three technical replicates were measured for each time point and non-AA treatment. Note that the corresponding clickable MC/AP contents as recorded from aliquots, as well as the growth rates, have been reported previously [23]. Created in BioRender. Deng, L. (2025) <https://BioRender.com/yvkfgrd>.

5.2. Cell Harvesting and Fixation

Cells from 40-mL culture flasks were harvested by centrifugation ($4000\times g$, 15 min) after breaking the gas vesicles using the hammer, cork, and bottle method. The detailed protocol has been published elsewhere [21]. In brief, the cells were washed with freshly prepared $1\times$ PBS, fixed with 2% PFA for 15 min, washed, incubated in Triton X-100 (0.1% in PBS, v/v) for 10 min, washed with PBS, and stored in $50\ \mu\text{L}$ PBS at $4\ ^\circ\text{C}$ for the click reaction (see Section 5.3) the following day.

In parallel, cells were filtered on glass fiber filters by vacuum filtration and extracted for natural and clickable peptides as described previously [23]. The quantitative data on clickable cyanopeptide synthesis as recorded via liquid chromatography–mass spectrometry from aliquots obtained during the same experiments have been reported [23]. In this study the same data were used to explore the relationship between the clickable peptide content and labeling by the click reaction.

5.3. Chemo-Selective Labeling of MC/AP Synthesis via the Click Reaction

The cells were fixed the day before and stored in $1 \times$ PBS at 4°C in the dark until use for the CuAAC reaction using a commercial chemical reaction buffer (C10269, Fisher Scientific, Vienna, Austria) containing the reaction buffer, a buffer additive, 100 mM copper sulfate solution, Milli Q water, and the fluorophore, namely A488 azide (A10266, Fisher Scientific) for non-AA treatments Prop-Lys and Prop-Tyr or A488 alkyne (A10267, Fisher Scientific). A488 azide/alkyne is a photostable and bright green fluorescent dye with excitation/emission maxima of 495/519 nm and with a molecular weight of 861.04/773.91 Da. The reaction volumes and components of the click chemical reaction were as follows: 200 μL of MQ water, 22 μL of $(10\times)$ reaction buffer (containing Tris-buffered saline), 5 μL of 100 mM CuSO_4 (reduced state), 25 μL of $(10\times)$ buffer additive, and 1 μL of A488 azide/alkyne (1 mM aliquots stored at -20°C).

Cyanobacterial pellets were incubated in PBS containing 2% bovine serum albumin (BSA) for 30 min at room temperature, and subsequently transferred into the CuAAC chemical reaction buffer for 60 min in the dark at room temperature, vortexing every 10 min. Then, the samples were centrifuged at 8000 rpm for 10 min, and the supernatant was discarded. Finally, the cells were washed twice, the first time with PBS containing 2% BSA washing solution and the second time with PBS only. The cells were dispersed on coverslips (24 mm $\times$ 24 mm, 0.17 ± 0.01 mm, Hecht Assistant, Sondheim vor der Rhön, Germany) and briefly air-dried before they were embedded in ProLong Diamond Antifade Mountant (Fisher Scientific) for 48 h in the dark.

5.4. Microscopic Recording of Signal Labeling

Imaging was performed as described earlier [21,22] with a high-resolution laser scanning microscope DMI 6000 SP8 (Leica Microsystems, Wetzlar, Germany) with a $100\times$ HCX Plan Apo 1.4 oil objective. The emission spectral range in channel 0 (A488) was narrowed down from 504 to 580 nm to 500–550 nm to enable the visualization of another fluorophore (i.e., to allow for dual labeling). Images were acquired at an XY resolution of 50 nm and Z resolution of 150 nm (resulting in 30–40 scans in depth per cell). In general, during image acquisition care was taken to prevent oversaturation conditions: the power of the white light laser (WLL) was adjusted prior to scanning the cell. Additionally, the Leica motion correction collar was adjusted independently for each microscopic slide to increase image quality and to reduce refractive index mismatch caused by the cover slip. Cells for imaging were selected randomly. For each time point and treatment, 20 randomly selected cells were viewed for each of three technical replicates.

All images were deconvolved with Huygens Essential version 23.10 (Scientific Volume Imaging, Hilversum, The Netherlands, <http://svi.nl>), using the default conditional maximum likelihood estimation algorithm. The total intensity per cell was compared for two channels (channel 0, A 488, 498 nm excitation; channel 1, AF, 620 nm).

5.5. Calculation of Co-Localization Indices

Two co-localization indices were used as a statistical measure of the spatial relationship between the two channels: Pearson's linear correlation coefficient and Spearman's coefficient based on the intensity ranks instead of intensity values. Both coefficients indicate how strongly two variables correlate with each other: A value of +1 indicates a perfect positive correlation, while a value of -1 means a perfect negative correlation. To minimize the background influence, Object Pearson's co-localization coefficient and Object Spearman's co-localization coefficient were used [55,56]. All co-localization parameters were estimated in Huygens Essential version 23.10.

5.6. FCM Analysis

Standard FCM was used to quantify the number of stained cells/filaments resulting from the chemo-selective click reaction. The particles were recorded using the Attune Acoustic Focusing Cytometer (Life Technologies, Thermo Fisher Scientific, Darmstadt, Germany) via the blue (BL, 488 nm, 50 mW) solid state laser. Forward scatter and BL1-A fluorescence (band pass filter at 530 ± 15 nm) were used for all measurements to discriminate stained and non-stained populations of cells at a flow rate of 0.1 mL min^{-1} . The acquisition volume was 0.5 mL. The target populations were quantified by rectangular gating on forward scatter vs. BL-1A fluorescence as compared with control cells.

5.7. Statistical Analysis

Three technical replicates were measured for each time point and non-AA treatment. The cell diameter and cell volume (Table 1) and A488 signal intensity, AF, and the A488/AF ratio (Tables 1–3 and S1–S6) were compared with two-way RM ANOVA (consisting of the grouping factor: three non-AA treatments and one control; and the time factor: T0–T4 (T5), three replicates). The time factor was always significant ($p < 0.001$). The assumption of normality (based on the Shapiro–Wilk) was not met in part for the A488 intensity data and not met for the A488/AF ratio data, while the equality of variance (based on the Brown–Forsythe test) was always met ($p < 0.05$). Because ANOVA results are generally considered robust if the sample sizes between treatments are equal, it was still used for the analysis. The Bonferroni *t*-test was used for the post hoc pairwise multiple comparison procedure, with $p < 0.05$ considered to indicate a significant difference.

For linear regression analysis, the data on clickable MC/AP in the total MC/AP content and the A488 signal intensity as recorded by both microscopy and FCM were $\log(x + 1)$ transformed to make the data linear and to meet the assumptions of normality (based on the Shapiro–Wilk test) and a constant variance. The linear regression curves between A488 signal intensity (or % BL1-A particles as recorded by FCM) and the percentage of clickable MC/AP in the total MC/AP content (Figures 8 and 9) or μg of clickable MC/AP per mg of DW (Figures S8 and S9) were calculated. The sample size was 72 for time-lapse signal build up experiments and 60 for time-lapse signal decline experiments. For data that did not meet the assumptions of a normal distribution or equal variance, the Spearman rank-order correlation was calculated, with a significant positive correlation in each case. All tests were performed using Sigma Plot for Windows version 14.0.

5.8. Chemical Analytical Analysis

As reported previously [21–23], the original MC/AP structural variants and the new (clickable) MC/AP structural variants were differentiated and quantified from aliquots and are summarized in Tables S4 and S5. In *M. aeruginosa* strain Hofbauer, the original MC was composed of demethylated MC variants DAsp-MC-YR $[\text{M} + \text{H}]^+ 1031.5$ and D-Asp-MC-LR $[\text{M} + \text{H}]^+ 981.5$ and methylated MC variants MC-YR $[\text{M} + \text{H}]^+ 1045.5$ and MC-LR $[\text{M} + \text{H}]^+ 995.5$, which were partly transformed into clickable MC. For Phe-Az, the clickable variants DAsp-MC-Phe-AzR $[\text{M} + \text{H}]^+ 1056.5$ and MC-Phe-AzR $[\text{M} + \text{H}]^+ 1070.5$, for Prop-Lys the variants DAsp-MC-Prop-LysR $[\text{M} + \text{H}]^+ 1078.5$ and MC-Prop-LysR $[\text{M} + \text{H}]^+ 1092.5$, and for Prop-Tyr the variants DAsp-MC-Prop-TyrR $[\text{M} + \text{H}]^+ 1069.5$ and MC-Prop-TyrR $[\text{M} + \text{H}]^+ 1083.5$ were reported [21–23]. In *P. agardhii* strain no371/1, the original AP structural variants included AP C $[\text{M} + \text{H}]^+ 809.5$, AP B $[\text{M} + \text{H}]^+ 837.5$, and AP A $[\text{M} + \text{H}]^+ 844.5$. The clickable APs included AP-Phe-Az $[\text{M} + \text{H}]^+ 843.3$, AP-Prop-Lys $[\text{M} + \text{H}]^+ 891.5$, or AP-Prop-Tyr $[\text{M} + \text{H}]^+ 882.5$.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/toxins17060278/s1>, Figure S1: Mean ($\pm$ SE) cell diameter of *M. aeruginosa* strain Hofbauer (a, c) or *P. agardhii* strain no371/1 (b, d) during time-lapse experiments related to the build up (a, b) or decline (c, d) of clickable MC (a, c) or clickable AP (b, d). Control cells were grown in the absence of non-AAAs and processed under identical conditions. Figure S2: Mean ($\pm$ SE) cell volume of *M. aeruginosa* (a, c) or *P. agardhii* (b, d) during time-lapse experiments related to the build up (a, b) or decline (c, d) of clickable MC (a, c) or AP (b, d). Figure S3: Mean ($\pm$ SE) A488 signal intensity of *M. aeruginosa* (a, c) or *P. agardhii* (b, d) per cell during time-lapse experiments related to the build up (a, b) or decline (c, d) of clickable MC (a, c) or AP (b, d). Figure S4: Mean ($\pm$ SE) autofluorescence intensity of *M. aeruginosa* (a, c) or *P. agardhii* (b, d) per cell during time-lapse experiments related to the build up (a, b) or decline (c, d) of clickable MC (a, c) or AP (b, d). Figure S5: Mean ($\pm$ SE) ratio of A488 intensity vs. autofluorescence intensity of *M. aeruginosa* (a, c) or *P. agardhii* (b, d) during time-lapse experiments related to the build up (a, b) or decline (c, d) of clickable MC (a, c) or AP (b, d). Figure S6: Mean ($\pm$ SE) of Object Pearson's co-localization coefficient between A488 signal intensity vs. autofluorescence signal intensity of *M. aeruginosa* (a, c) or *P. agardhii* (b, d) during time-lapse experiments related to the build up (a, b) or decline (c, d) of clickable MC (a, c) or AP (b, d). Figure S7: Mean ($\pm$ SE) of Object Spearman's co-localization coefficient between A488 signal intensity vs. autofluorescence signal intensity of *M. aeruginosa* (a, c) or *P. agardhii* (b, d) during time-lapse experiments related to the build up (a, b) or decline (c, d) of clickable MC (a, c) or AP (b, d). Figure S8: A488 signal intensity per cell volume (in μm^3) of *M. aeruginosa* (a, c) or *P. agardhii* (b, d) during time-lapse experiments vs. (a, c) μg of clickable MC or (b, d) μg of clickable AP per mg of dry weight (DW). Details of linear regression lines: $y = a + bx$, where y is $\log(x + 1)$ A488 signal intensity and x is $\log(x + 1)$ of clickable MC/AP per mg of DW as determined by LC-MS previously [23]. Dotted lines indicate 95% confidence intervals. (a) $y = 4.57 + 4.89x$ ($R^2 = 0.33$, $p < 0.001$), (c) $y = 4.29 + 6.81x$ ($R^2 = 0.38$, $p < 0.001$), (b) $y = 4.31 + 10.97x$ ($R^2 = 0.38$, $p < 0.001$), (d) $y = 4.28 + 8.55x$ ($R^2 = 0.29$, $p < 0.001$). Figure S9: Histograms showing relative frequency (%) of BL1-A fluorescent particles in logarithmic scale (blue laser (BL, 488 nm, 50 mW), (band pass filter at 530 ± 15 nm) as recorded by flow cytometry for *M. aeruginosa* during time-lapse build up experiment in order to quantify labeled subpopulations (three technical replicates are shown). Figure S10: Histograms showing relative frequency (%) of BL1-A fluorescent particles in logarithmic scale (blue laser (BL, 488 nm, 50 mW), (band pass filter at 530 ± 15 nm) as recorded by flow cytometry for *P. agardhii* during time-lapse build up experiment in order to quantify labeled subpopulations (three technical replicates are shown). Figure S11: Histograms showing relative frequency (%) of BL1-A fluorescent particles in logarithmic scale (blue laser (BL, 488 nm, 50 mW), (band pass filter at 530 ± 15 nm) as recorded by flow cytometry for *M. aeruginosa* during time-lapse decline experiment in order to quantify labeled subpopulations (three technical replicates are shown). Figure S12: Histograms showing relative frequency (%) of BL1-A fluorescent particles in logarithmic scale (blue laser (BL, 488 nm, 50 mW), (band pass filter at 530 ± 15 nm) as recorded by flow cytometry for *P. agardhii* during time-lapse decline experiment in order to quantify labeled subpopulations (three technical replicates are shown). Figure S13: A488 labeled particles (percentage of total particles) of *M. aeruginosa* (a, c) or *P. agardhii* (b, d) during time-lapse experiments vs. percentage of clickable MC (a, c) or AP (b, d). Details of linear regression lines: (a) $Y = a + bx$, where y is $\log(x + 1)$ A488 particles and x is $\log(x + 1)$ μg of clickable MC/AP per mg dry weight (DW) as determined by LC-MS [23]. Dotted lines indicate 95% confidence intervals. Regression curves: (a) $y = 0.48 + 6.52x$ ($R^2 = 0.21$, $p < 0.001$), (c) $y = 0.56 + 13.05x$ ($R^2 = 0.49$, $p < 0.001$), (b) $y = 0.29 + 28.6x$ ($R^2 = 0.39$, $p < 0.001$), (d) $y = 0.03 + 14x$ ($R^2 = 0.31$, $p < 0.001$), without Phe-Az fed cells (b) $y = 0.46 + 30.2x$ ($R^2 = 0.52$, $p < 0.001$), (d) $y = 0.08 + 13.8x$ ($R^2 = 0.3$, $p < 0.001$). Table S1. Mean $\pm$ SE cell diameter and cell volume for each time point as recorded during time-lapse signal build up and decline experiments in cyanobacteria *M. aeruginosa* strain Hofbauer and *P. agardhii* strain no371/1. Two-way RM ANOVA was used to test for differences between treatments: Grouping factor (three non-AA treatments and control), time factor (T0–T4, T5), three replicates (graphical data are shown in Figures S1 and S2). Table S2: Mean $\pm$ SE A488 signal intensity ($\times 10^4$) and autofluorescence ($\times 10^5$) per cell volume (in μm^3) and A488/Autofluorescence ratio for each time point as

recorded during time-lapse signal build up and decline experiments in cyanobacteria *M. aeruginosa* and *P. agardhii*. Two-way RM ANOVA was used to test for differences between treatments: Grouping factor (three non-AA treatments and control), time factor (T0–T4, T5), three replicates (graphical data are shown in Figures 6, 7 and S5). Table S3: Mean $\pm$ SE of co-localization coefficients between A488 signal intensity vs. autofluorescence signal intensity for each time point as recorded during time-lapse experiments using pulsed feeding of non-AAs in order to observe the build up or decline of clickable MC or AP. Two-way RM ANOVA was used to test for differences between treatments: Grouping factor (three non-AA treatments and control), time factor (T0–T4, T5), three replicates (graphical data are shown in Figures S6 and S7). Table S4: Mean ($\pm$ SE) proportion of clickable MC in total MC (clickable and natural MC variants) or of clickable AP in total AP (clickable and natural AP variants) for each time point as recorded during time-lapse experiments using pulsed feeding of non-AAs in order to observe the build up or decline of clickable MC or of clickable AP. Two-way RM ANOVA was used to test for differences between treatments: Grouping factor (three non-AA treatments and control), time factor (T0–T4, T5), three replicates (graphical data are shown in Kurmayer and Moron [23], Figures 1 and 2). Table S5: Mean $\pm$ SE proportion of clickable MC in μg per mg of DW or of clickable AP in μg per mg of DW for each time point as recorded during time-lapse experiments using pulsed feeding of non-AAs in order to observe the build up or decline of clickable MC or AP. Two-way RM ANOVA was used to test for differences between treatments: Grouping factor (three non-AA treatments and control), time factor (T0–T4, T5), three replicates (graphical data are shown in Kurmayer and Moron [23], Supplementary Figures S5 and S6). Table S6: Mean $\pm$ SE percentage (%) of BL1-A fluorescent particles and cell (particle) counts (blue laser (BL, 488 nm, 50 mW), (band pass filter at 530 ± 15 nm) as recorded by flow cytometry during time-lapse experiments using pulsed feeding of non-AAs in order to observe the build up or decline of clickable MC or AP. Two-way RM ANOVA was used to test for differences between treatments: Grouping factor (three non-AA treatments and control), time factor (T0–T4, T5), three replicates).

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Abbreviations

The following abbreviations are used in this manuscript:

A488	Alexa Fluor 488 (fluorophore)
A405	Alexa Fluor 405 (fluorophore)
AF	Autofluorescence
AP	Anabaenopeptin
cHAB	cyanobacterial harmful algal bloom
DW	Dry weight of cells extracted for AP/MC peptides
CuAAC	Copper catalyzed azide-alkyne cycloaddition
FCM	Flow cytometry
MC	Microcystin
n	Sample size (used in statistical analysis)
non-AA	non-natural (clickable) L-amino acid
NRPS	Non-ribosomal peptide synthesis
Phe-Az	4-azidophenylalanine
Prop-Lys	N-propargyloxy-carbonyl-L-lysine
Prop-Tyr	O-propargyl-L-Tyrosine
RM-ANOVA	two-way repeated measures analysis of variance

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Article

Paralytic Shellfish Toxins in Alaskan Butter Clams: Does Cleaning Make Them Safe to Eat?

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Abstract: Butter clams (*Saxidomus gigantea*) are a staple in the subsistence diets of Alaskan Native communities and are also harvested recreationally. This filter-feeding species can accumulate saxitoxins (STXs), potent neurotoxins produced by late spring and summer blooms of the microalga *Alexandrium catenella*. The consumption of tainted clams can cause paralytic shellfish poisoning (PSP). Traditional beliefs and early reports on the efficacy of removing clam siphons have created the impression that cleaning butter clams by removing certain tissues makes them safe to eat. However, the toxin distribution within clams can vary over time, making the practice of cleaning butter clams unreliable. This study tested the effectiveness of the cleaning methods practiced by harvesters on Kodiak Island, Alaska. Specifically, butter clams were cleaned by removing different tissues to produce samples of “edible” tissues that were tested for STX content. The results were compared to historical data from a study conducted in Southeast Alaska from 1948 to 1949. Using these data, the risk for an average-sized man and woman consuming 200 g of edible tissue was calculated. The results showed that for clams containing $>200 \mu\text{g STX-equivalents } 100 \text{ g edible tissue}^{-1}$, no cleaning method reduced the concentration of STXs in the remaining tissue below the regulatory limit. Meals containing $>900 \mu\text{g STX-equivalents } 100 \text{ g edible tissue}^{-1}$ posed a substantial risk of moderate or severe symptoms. No cleaning method assured that untested butter clams are safe to eat.

Keywords: paralytic shellfish poisoning (PSP); toxicity risk; safe consumption; *Saxidomus gigantea*; subsistence harvesting

Key Contribution: The removal of certain tissues from Alaskan butter clams is often assumed to make those clams that have accumulated neurotoxic saxitoxins safer to eat. This study confirms that no cleaning method ensures sufficient toxin removal to ensure untested butter clams are safe to eat.

1. Introduction

The butter clam (*Saxidomus gigantea*), found from the Aleutian Islands (Alaska, AK) to mid California, has been a staple in the subsistence diets of Native communities of Alaska and the temperate, northwest coast of the United States and Canada for thousands

of years [1]. It is an abundant and valued species with cultural significance. The Southeast Alaskan cultures and Inuit of Qikiqtarjuaq (formerly Broughton Island), among many others in the Pacific Northwest, are reported to have relied on clams as an essential part of their traditional diets [2]. Shell middens, some as old as 9000 years, document that clams, especially butter clams, were abundant food items [3,4]. Clam digging was often much more than a harvesting activity. It was a social event, often associated with potlatches or seasonal ceremonies.

Some communities gathered clams throughout the year, while others focused on clamming during the winter when hunting and fishing were difficult due to intemperate weather. Another reason for collecting clams primarily in winter may have been concern about PSP during the summer. This illness is caused by STXs, potent neurotoxins produced by phytoplankton species, such as *Alexandrium catenella*, that can bloom seasonally in cool temperate waters. STXs accumulate in shellfish, with over a third (35%) of reported paralytic shellfish poisoning (PSP) illnesses in Alaska caused by consuming contaminated butter clams, likely because they may retain STXs for two years or more [5,6]. From 2011 to 2021, the frequency of annual reported PSP cases ranged from 4 to 17, with most instances of less severe cases not being reported. Symptoms typically occur within 30 to 60 min after consuming tainted clams and include numbness spreading from the lips and mouth to the face, neck, and extremities; dizziness; headache; nausea; vomiting; arm and leg weakness; paralysis; respiratory failure; and, in severe cases, death [7].

In the mid-1940s, the vast shellfish resources along the Alaskan coast were recognized and significant efforts were directed toward shellfish utilization. This included the establishment of a Southeast Alaska clam canning industry in 1943 [8]. In 1946, the peak harvest year, STXs were found in shipments of butter clams by the United States Food and Drug Administration. This led to the discontinuation of clam processing in Southeast Alaska. Following this event, extensive sampling was conducted to determine the STX concentrations in butter clams harvested from Southeast Alaskan beaches [9]. These studies revealed that butter clams harvested in any season could be toxic, that toxins were not evenly distributed among clam tissues, and that removing the siphon reduced, but did not eliminate, PSP risk.

This information regarding the varying toxin content in different butter clam tissues was shared with many coastal communities. In some of these communities, it has become common practice to remove the intestinal tract, gills, and black tip of the siphon before consuming the rest of the clam. Over time, a common misconception has arisen among many subsistence and recreational harvesters that by removing these tissues, the remaining parts of the clam are safe to eat [10]. In other cases, clams are not cleaned at all, particularly when they are small, resulting in no reduction in toxin content. Since the State of Alaska does not test recreationally or subsistence-harvested shellfish, some Native communities have established their own testing procedures [11,12]. Only commercially harvested shellfish intended for interstate commerce are regularly tested for STXs [10].

In this study, three different current or historical datasets regarding STX-eq. concentrations in butter clams were examined to more fully understand (1) how removing different clam tissues impacts STX concentrations, (2) the likelihood of consuming a clam capable of causing mild to serious illness, and (3) how meal size and the weight of the consumer may potentially impact the risk of illness. The first dataset consisted of a reanalysis of the tissue-specific STX-eq. concentrations measured in butter clams collected on Kodiak Island, Alaska, from 2015 to 2018 by Kibler et al. [10]. We know that STXs are not evenly distributed throughout each tissue type [10] and these data made it possible to calculate, for the first time, how all the practical ways of removing specific tissues, or combinations of tissues, would impact the STX-eq. concentrations in the remaining portions of the

clam to be consumed. For simplicity, we will hereafter refer to any combination of tissues retained for consumption as the “edible” portion of the clam and the discarded tissues as the “non-edible” portion.

The second dataset was obtained by analyzing the concentrations of STX-eq. in butter clams that were prepared using cleaning methods commonly practiced in communities in Kodiak, AK. The clams were dissected into what was considered edible (body and part of the lower siphon) and non-edible (viscera, gills, black tip of the siphon) portions. Traditionally, it has been assumed that the edible portion of the clam was less toxic than the discarded non-edible tissues. The resulting data provided valuable information on methods used by local harvesters that can be shared with other communities.

The third combined dataset consisted of unpublished, historical STX-eq. data from butter clam samples collected in Southeast Alaska from 1948 to 1949 by Chambers and Magnusson [9] and from 1963–1965 by Neal [13]. Both studies collected time series data on STX concentrations in whole clams, while the Chambers and Magnusson study additionally included STX concentration in whole clams minus the siphon. These data provided valuable information including (a) comparative data on the toxin content of butter clams collected in Southeast Alaska compared to the Kodiak region. This comparison is important due to preliminary evidence of toxin content differences in *A. catenella* cells between Kodiak and Southeast AK [14]; (b) changes in toxin concentration from siphon removal that can be directly compared to one of the cleaning methods evaluated using the Kibler et al. dataset [10]; (c) publication of the original data that underpin our understanding of how cleaning may impact toxicity; and (d) information on the extent to which toxin concentrations may decrease in winter, which is relevant for understanding whether cleaning clams may reduce toxicity more in the winter compared to the summer months when toxic *A. catenella* blooms occur.

It should also be noted that the impact of removing various tissues on toxin content in the studies above is based on the concentration of STX-eq. in edible tissue relative to the regulatory safety limit of 80 µg STX 100 g tissue^{−1} for shellfish [15,16]. While the specificity of this limit has been questioned, it is generally considered sufficient for protecting public health [17]. Because this safety limit is quite conservative, it can be difficult for individuals who consume butter clams regularly, due to their nutritional and cultural requirements, to understand intuitively. This is particularly true in cases where private testing has shown that the STX-eq. concentrations are above regulatory levels in butter clams that have been consumed with no apparent ill effects. A more intuitive approach may be to demonstrate the potential risk of PSP toxicity from eating an average sized meal of untested butter clams. To address this issue, we used a recent modeling study by Arnich and Thébault [18] to estimate how the STX-eq. concentrations in the edible tissue and the size of the meal can impact toxicity, as well as how often harvested clams would likely cause significant illness. The resulting findings on the impacts of cleaning butter clams, from the perspectives of both concentration and amount of toxin ingested, are intended for use in developing more effective educational materials for communicating the risk of PSP to Alaskan communities.

2. Results

2.1. Effects of Removing Various Tissues, or Combination of Tissues, on Toxin Concentrations in Alaskan Butter Clams

The study conducted on butter clams from Kodiak Island between 2015 and 2018 (Kibler et al. [10]) showed that the concentration of STX-eq. 100 g tissue^{−1} varied among different tissue types (viscera, siphon black tip, siphon neck, and body) was variable (see Appendix A). The average weight of whole shucked butter clams was 15.6 ± 2.2 g. Higher STX concentrations were observed in viscera during the month of June, which is consistent

with the time when *A. catenella* blooms often occur (Appendix A, panel A). Blooms can vary in intensity among years and locations, resulting in large variations in the amount of STX accumulated by butter clams. The black tip of the siphon consistently retained $>100 \mu\text{g STX-eq. } 100 \text{ g tissue}^{-1}$, except for batches in May 2017 (Appendix A, panel B). Toxin concentrations in the neck tissue followed a similar pattern to that observed in the black tip (Appendix A, panel C). The body tissues exhibited the highest STX concentrations in June and July, with the exception of 2017 (Appendix A, panel D). Overall, whole-clam STX concentrations mirrored those of the body tissues alone, consistent with the body contributing the largest amount of tissue (Appendix A, panels D, E).

The extent to which the removal of tissues reduced the concentration of STX-eq. in edible tissues differed. This variability was associated with changes in the tissue STX-eq. concentrations in the different tissues over time. When clams actively feed on *A. catenella* blooms, the STX concentration in the gut contents can be very high ($>500 \mu\text{g STX-eq. } 100 \text{ g tissue}^{-1}$) (Appendix A Figure A1). During and following a bloom, toxins differentially migrate to other tissues. Because of this variable toxin distribution, the overall trend showed that removing tissues generally reduced the overall toxin concentration in the edible tissues relative to the whole clam (e.g., April and May samples; Figure 1), but not always. For instance, in the July samples, more of the STX-eq. load was present in the body tissue instead of the gut and siphon tip. In this case, the average toxin content in the whole clam on a 100 g basis was less than that found in the body alone (Figure 1). A notable finding was that, in the June and July samples where whole-clam toxicities exceeded $200 \mu\text{g STX-eq. } 100 \text{ g tissue}^{-1}$, every type of cleaning method failed to reduce STX concentrations below the recommended regulatory level. The only relatively low toxicities seen during a June collection was in 2016, when the butter clams had toxicities similar to those observed in the April and May samples (Figure 1; Supplementary Table S1). This may be due to the clams in the June 2016 collection not having been exposed to an *A. catenella* bloom. The overall percent reduction in STX concentration relative to whole clams for the various edible tissues was as follows: (a) whole clam minus gut, $6.8 \pm 20.1\%$; (b) whole clam minus gut and siphon black tip, $7.9 \pm 8.5\%$; (c) whole clam minus entire siphon, $9.6 \pm 17.3\%$; and (d) clam body only, $15.4 \pm 17.8\%$. The data on variations in STX congener composition in individual tissues and how these differences can affect the STX-eq. estimates are discussed in Kibler et al. [10].

2.2. Impact of Siphon Removal on Toxin Concentration in Southeast Alaska Butter Clams

The data from Chambers and Magnusson [9] showed that the siphons of butter clams collected from May 1948 to January 1949 in Southeast Alaska had high but variable concentrations of STX-eq. (Figure 2; Supplementary Table S2). Although this tissue made up less than 20% of the overall body weight, it substantially contributed to the total toxin content in the clam. Removing this tissue led to a substantial decrease in overall STX content in the remaining tissues (Table 1). Despite this, removing the siphon from butter clams containing $>200 \mu\text{g STX-eq. } 100 \text{ g tissue}^{-1}$, which was the case for a majority of the clams tested, only rarely reduced the toxin content in the edible tissues below the regulatory level of $80 \mu\text{g STX-eq. } 100 \text{ g tissue}^{-1}$ (Figure 2).

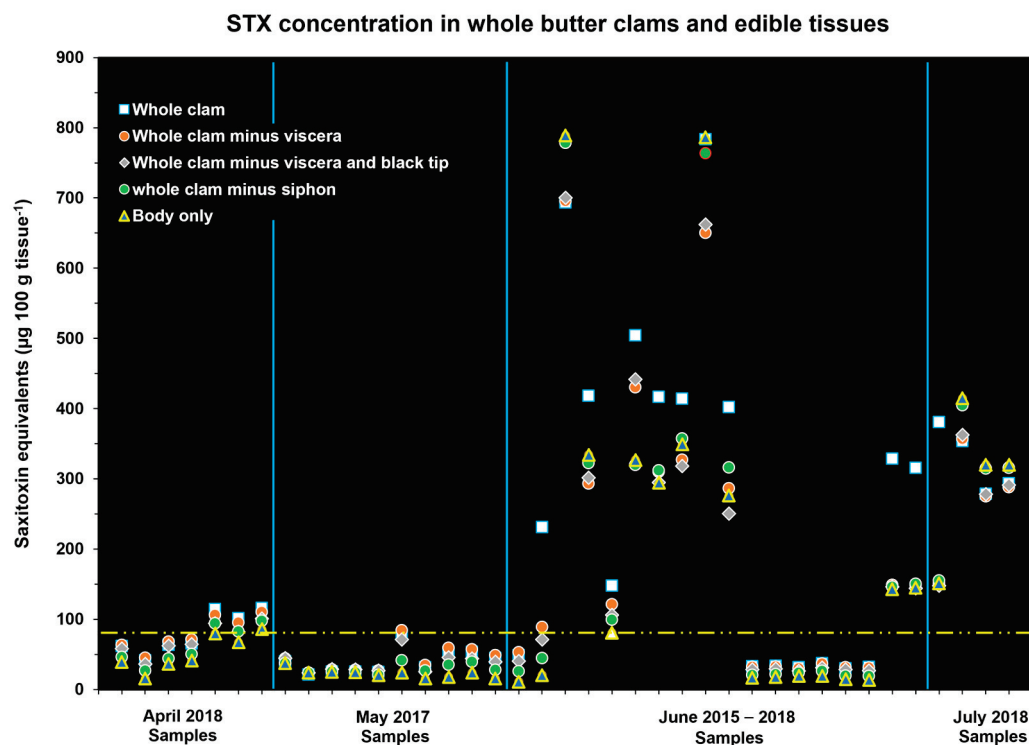


Figure 1. The $\mu\text{g STX-eq. } 100 \text{ g tissue}^{-1}$ for whole butter clams and the different types of edible tissues. The yellow dashed line represents the regulatory limit ($80 \mu\text{g STX-eq. } 100 \text{ g tissue}^{-1}$, $N = 39$). The blue lines indicate the month of the year in which samples were collected.

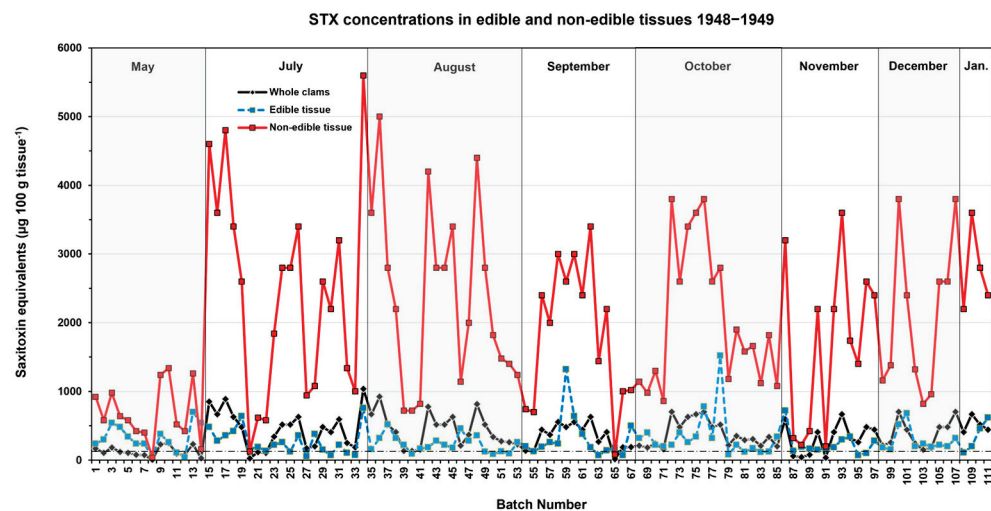


Figure 2. Time series of STX-eq. concentrations in whole butter clams, as well as edible (body and gut) and non-edible (entire siphon) tissues collected in Southeast Alaska from 1948–1949 [9]. The black dashed line represents the regulatory limit ($80 \mu\text{g STX-eq. } 100 \text{ g tissue}^{-1}$).

Table 1. The percentage reduction in overall STX-eq. concentrations in whole clams compared to the edible tissues calculated from the information presented in Figure 2.

Month	Mean	SD	n
May	22	20	15
July	57	23	19
August	60	18	19
September	48	27	14

Table 1. *Cont.*

Month	Mean	SD	n
October	53	18	17
November	45	28	13
December	52	14	10
January	59	21	4
Overall	49	24	111

2.3. Seasonal Changes in Butter Clam STX Concentrations

The Kodiak study conducted by Kibler et al. [10] from 2015 to 2018 did not involve the collection of samples during colder months from November to April. Consequently, the study was unable to assess the effectiveness of different cleaning methods during this time of year. The time series of whole-clam STX concentrations obtained from Chambers and Magnusson [9] and Neal [13] (Figure 3; Supplementary Tables S2 and S3) showed that STX-eq. concentrations declined in some winters but not others. The December and January data (Figure 2 also) showed that high STX-eq. concentrations can persist into the winter months. None of the cleaning methods tested using the Kibler et al. [10] data would reduce STX-eq. concentrations in the higher-toxicity butter clams below the regulatory level for safe consumption.

2.4. Comparison of Two Cleaning Methods Practiced by Alaskan Native Communities

Both Method 1 (where the visceral contents are gently squeezed out and the gills and siphon black tip are removed), and Method 2 (where the visceral mass, gills, and siphon black tip are removed) reduced STX-eq. concentrations in the edible tissue on average, but the amount of that reduction varied (Figure 4). When toxin levels in the whole clam exceeded $200 \mu\text{g STX-eq. } 100 \text{ g tissue}^{-1}$, neither cleaning method consistently lowered STX concentrations below the regulatory limit of $80 \mu\text{g STX-eq. } 100 \text{ g edible tissue}^{-1}$. On average, the reduction in STX-eq. concentrations for Method 1 was $61.4 \pm 20.4\%$ ($N = 13$) relative to whole clams and for Method 2 it was $45.2 \pm 23.3\%$ ($N = 21$) (Supplementary Table S4).

2.5. Risk Associated with Consuming Meal Contaminated by Saxitoxins

The probability of developing PSP symptoms when a certain amount of STX-eq. is consumed by an average sized man or woman in the United States was calculated based on the data presented by Arnich and Thébault [18], and is shown in Table 2. Using the concentration data from Figure 1, the amount of STX that would be ingested if 200 g of whole or variously cleaned (edible) butter clam tissues were consumed in a single meal was calculated (Figure 5). A comparison of the toxin consumed in a 200 g meal with the amounts of toxin that cause illness showed that most of the butter clams assayed in the 2015–2018 Kodiak contained a sufficient toxin concentration to be of concern (Table 2).

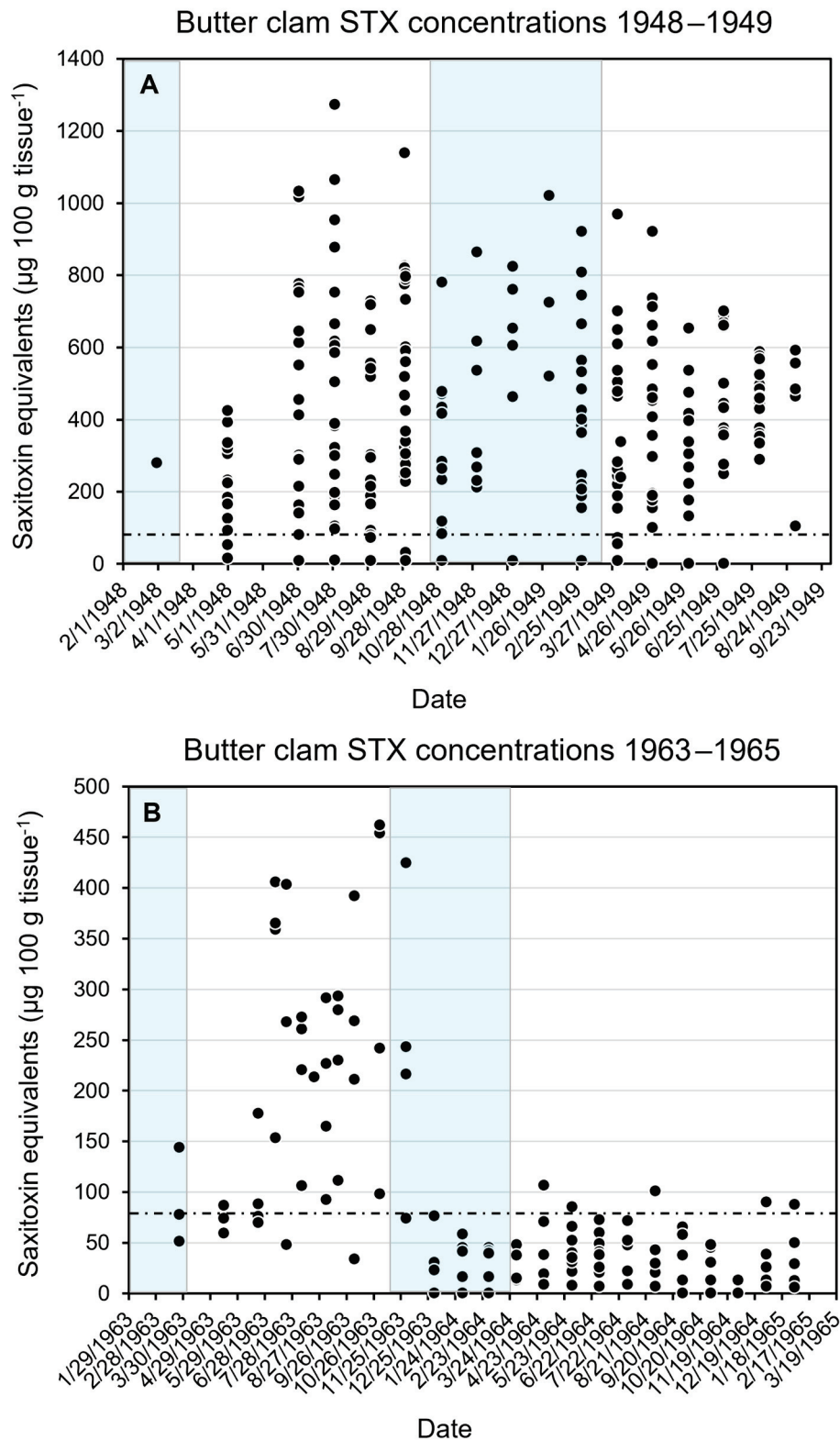


Figure 3. Time series of total clam STX concentration data reported by (A) Chambers and Magnusson (1948–1949; N = 250) [9] and (B) by Neal (1963–1965; N = 123) [13]. The blue shading on the graphs indicates the period from November to March when water temperatures are cooler. The black dashed line indicates the regulatory limit ($80 \mu\text{g STX-eq. } 100 \text{ g tissue}^{-1}$).

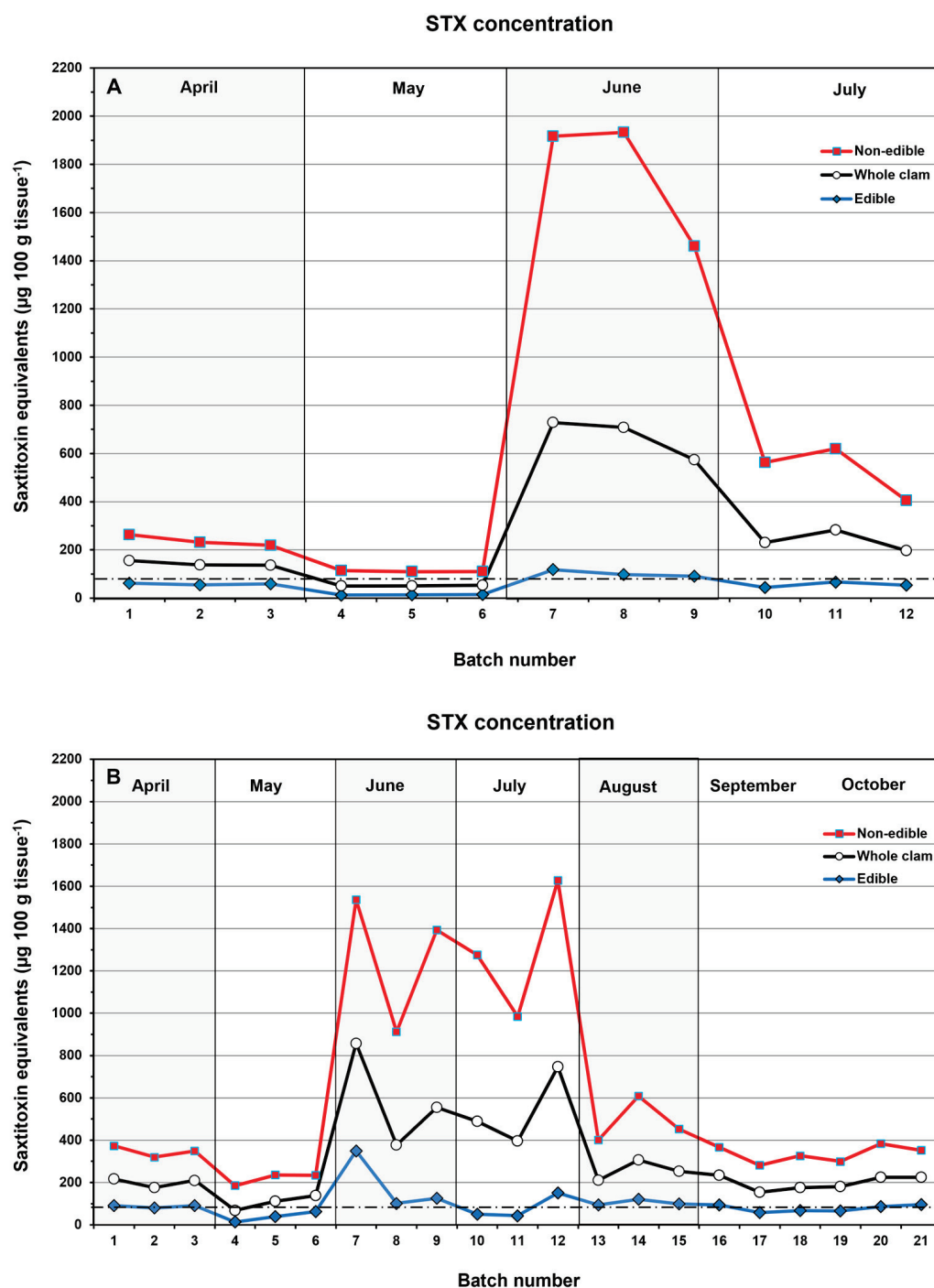


Figure 4. Time series of the STX-eq. concentrations in the edible and non-edible butter clam tissues prepared following (A) Method 1 and (B) Method 2. Each month, three replicate batches were collected on the same day of the month (April to July 2018 for Method 1, April to October 2018 for Method 2). The three individual replicates collected each month are plotted separately to show inter-batch variability. The black dashed line indicates the regulatory limit ($80 \mu\text{g STX-eq. } 100 \text{ g tissue}^{-1}$; $N = 21$).

Table 2. The probabilities of an average-sized man or woman experiencing no symptoms, mild symptoms, severe symptoms, or death after ingesting different amounts of STX-eq. are derived from the toxin kg^{-1} data presented in Arnich and Thébault [18].

Average-Sized Man (90.6 kg) (μg STX-eq. Ingested)	Average-Sized Woman (78 kg) (μg STX-eq. Ingested)	Probability of No Symptoms	Probability of Mild Symptoms	Probability of Moderate Symptoms	Probability of Severe Symptoms	Probability of Death
91	78	88.67%	9.22%	1.57%	0.53%	0.00%
906	775	45.49%	30.66%	12.99%	10.59%	0.27%
9060	7750	7.55%	19.50%	19.43%	46.37%	7.15%
90,600	77,500	0.30%	2.40%	5.30%	47.70%	44.30%
906,000	775,000	0.00%	0.05%	0.26%	11.59%	88.10%

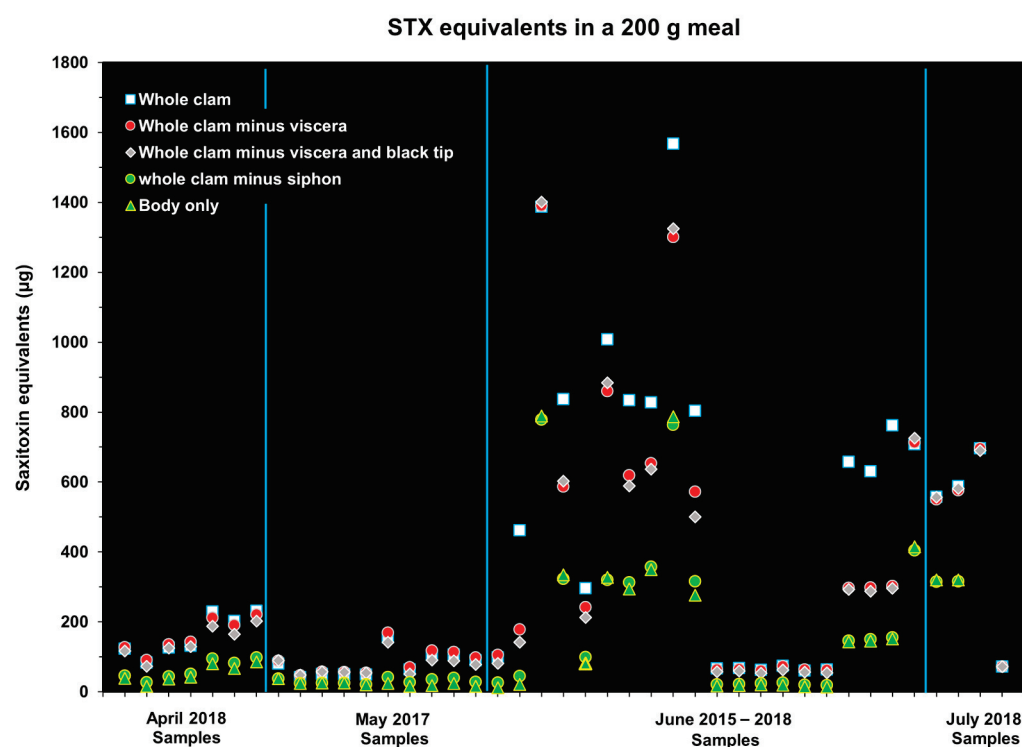


Figure 5. STX-eq. concentrations that would be ingested if 200 g of edible tissue(s) were consumed. The concentration data used to determine the quantity of STX ingested were from the 2015–2018 Kodiak study (see Figure 1, $N = 39$). The blue lines indicate the month of the year in which samples were collected.

3. Discussion

This study demonstrated that no cleaning method for Alaskan butter clams reliably reduced STX concentrations enough to make them safe for consumption (Figures 1 and 2). Whole clams routinely contain between 200 and 500 μg STX-eq. 100 g tissue^{-1} during the summer months, with concentrations between 500 and 1000 μg STX-eq. 100 g tissue^{-1} not being uncommon (Figures 1 and 2). In fact, 63% ($N = 447$) of the whole-clam batch samples in this study had STX-eq. concentrations exceeding the regulatory limit of 80 μg STX-eq. 100 g tissue^{-1} . The combined data, including the cleaning methods practiced in local Kodiak AK communities, also showed that when clams contained $>200\text{ }\mu\text{g}$ STX-eq. 100 g tissue^{-1} , the cleaning methods rarely produced concentrations in edible tissues below the 80 μg STX-eq. 100 g tissue^{-1} regulatory level (Figures 1, 2 and 4). In some instances where the STX contamination had time to migrate into the body of the clam, the removal

of other tissues containing lower STX-eq. concentrations actually resulted in increased toxin concentrations in the ingested tissues. Chambers and Magnusson [9] also reported no correlation between the STX content in butter clams and beach terrain, tidal magnitude, water temperature, or amount of daylight. This reinforces the reality that without testing, it is impossible to predict when a highly toxic clam will be harvested.

Another common practice in Alaska is harvesting butter clams in the winter, which is logical given that *A. catenella* blooms do not occur during that time. The results presented here indicate that STX concentrations in butter clams fluctuate seasonally, but not always to the same extent. In the 1964 study, the concentrations increased in summer, and then declined and remained relatively low throughout the rest of the year (Figure 3B). In contrast, during the 1948–1949 study, the STX concentrations were much higher overall and only dropped slightly after the summer months (Figure 3A). Elevated wintertime concentrations are also evident in Figure 2. It is possible that some of the seasonal differences in STX-eq. concentrations observed were due to whether the local clam populations had converted the STX congeners they retained to lower or higher toxicity forms [10]. The data presented do not allow this possibility to be assessed. Cumulatively, these data are consistent with potentially lower STX concentrations during the winter in some years and not others. Consequently, harvesting in the winter does not reliably reduce the probability of consuming toxic butter clams. This is supported by reports from Canada documenting the occurrence of PSP cases in every month of the year [19].

The data also indicated that, based on Arnich and Thébault [18], an average-sized man or woman consuming even 100 µg STX-eq. would have a ~10% chance of developing mild symptoms and a ~2% chance of developing moderate symptoms (Figure 5; Table 2; [18]). This represented 61.5% of the butter clam batches tested in the Kibler et al. [10] study (N = 39; Figure 5). Mild symptoms include headache, abnormal sensations such as tingling, pricking, numbness, dizziness, vertigo, nausea, and vomiting. Moderate symptoms encompass incoherent speech, involuntary eye movement, rapid pulse, lack of voluntary coordination of muscle movements, shortness of breath, and backache. The modeled response to consuming 900 µg STX-eq. was a ~31% chance of mild symptoms, a 13% chance of moderate symptoms, an ~11% chance of severe symptoms, and a ~0.27% chance of death. Severe symptoms include motor speech disorders, difficulty swallowing, suspension of breathing, weakness of arms and legs, pronounced respiratory difficulties, muscular paralysis, and respiratory arrest (without death). The modeled response based on the reanalyzed data from Kibler et al. [10] indicated that 7.7% of the samples contained >900 µg STX-eq. (N = 39; Figure 5). The findings of this study on the risk of consuming butter clams containing different amounts STX-eq. in an average meal are consistent with the study by Gibbard and Naubert [19], which predicted that consuming 200, 1000, and 2500 µg STX-eq. is needed to cause mild, severe, or extreme symptoms. McFarren et al. [20] also estimated that the ingestion of between 2000 and 4000 µg STX-eq. constitutes a lethal dose.

It is important to note that there is a much higher risk of death from severe symptoms in Alaska because many of the communities consuming clams for subsistence are located in remote areas without adequate medical care. Transport times to medical facilities are long, which can exacerbate the severity of symptoms and substantially increase health risks for Alaska residents.

4. Conclusions

Given that shellfish are the third most commonly consumed traditional food among Natives in Alaska [21] and that a disproportional number of PSP victims are Alaska Natives (53%) [6], with butter clams accounting for 35% of reported PSP incidents, dissuading recreational and subsistence harvesters from consuming untested shellfish, especially

butter clams, is imperative. Our results unequivocally show that no cleaning method reliably makes untested butter clams safe to eat. Furthermore, there is no means of reliably predicting when and where untested, highly toxic clams will be harvested. The findings also indicate that while the probability of severe illness from consuming cleaned clams is not common, it can occur and that unreported mild to moderate illness from consuming cleaned clams is likely a common occurrence.

Involving community members in testing the effectiveness of their traditional shellfish cleaning methods, as was carried out in this study, represents an important step in providing credible information to the population most vulnerable to PSP. The results from this study on cleaning efficacy, the potential risk of consuming a highly toxic clam, and the amount of toxin potentially consumed in an average-sized meal can also be used to develop other outreach and education materials regarding the risks of consuming untested butter clams.

5. Materials and Methods

5.1. Butter Clam Collection

Batches of 10–15 butter clams were collected for processing. Where possible, three separate batches of butter clams were collected within a two-meter radius. Small specimens (<4 cm in length) were avoided as they were considered too small for subsistence harvesting. The clams were then scrubbed, rinsed with tap water to remove sediment and debris, shucked, and drained [22]. These clams were dissected in various ways to produce different edible and non-edible portions as described in Section 5.3. The $\mu\text{g STX-eq. } 100 \text{ g tissue}^{-1}$ for each portion was then determined using High-Performance Liquid Chromatography coupled with fluorescence detection (HPLC-FLD).

5.2. Saxitoxin Analysis

Because of the high degree of variability in STX-eq. concentrations in individual clams, determinations regarding the safety of butter clams are based on assaying batches of 12 homogenized clams. Concentrations exceeding $80 \mu\text{g STX-eq. } 100 \text{ g tissue}^{-1}$ measured via mouse bioassay or HPLC-FLD are considered to exceed the regulatory limit. The mouse bioassay represents a functional assay where time to death is determined and converted to mouse units (MU). One MU is defined as the minimum quantity of toxin needed to kill a mouse within 24 h. HPLC methods measure the structural variants of STX (congeners) found in a sample. Because these congeners vary significantly in toxicity, the total STX-eq. is calculated by summing each measured congener concentration multiplied by its Toxicity Equivalency Factor (TEF) [23]. STX is assigned a TEF of 1.0, with higher toxicity congeners having a TEF > 1.0 and lower ones assigned TEF values < 1.0. One MU is equal to 0.2 STX-eq. [15]. Samples taken as part of this study were analyzed by HPLC and any historical data containing concentrations in MU $100 \text{ g tissue}^{-1}$ were converted to $\mu\text{g STX-eq. } 100 \text{ g tissue}^{-1}$ as described above.

All tissue samples for HPLC-FLD analysis were immediately frozen at -20°C and held pending shipment to the National Oceanic and Atmospheric Administration (NOAA) Laboratory in Beaufort, North Carolina, for analysis. These samples were analyzed via HPLC with precolumn oxidation using the standard methods of Lawrence et al. [24], refined by Ben-Gigirey et al. [25] and Harwood et al. [26]. Briefly, the samples were processed using a Kinematica Polytron model PT-MR 2500E homogenizer fitted with a 12 mm dispersing head (Kinematica, Inc., New York, NY, USA). A 5 g subsample of homogenized tissue was extracted with 3 mL 1% acetic acid in a 100°C water bath for 5 min. After cooling at 4°C , the sample was centrifuged at 4500 rpm ($2940\times g$) for 10 min, and the supernatant was collected. The remaining pellet was re-extracted and the supernatants combined. Then, 1 mL of the combined extract was passed through a conditioned SPE C18 cartridge, pH-adjusted to 6.5, and diluted to 4 mL for oxidation with periodate and peroxide. PSP

toxins were quantified using Agilent 1100 (Santa Clara, CA, USA) or Waters Aquity Arc (Milford, MA, USA) HPLC systems coupled to FLD and 5 μ m C18 columns (150 $\times$ 4.6 mm, Phenomenex, Inc., Torrance, CA, USA). The Agilent fluorescence detector was an Agilent 1100 G1321A FLD and the Waters detector was a 2475 FLR detector. Fluorescence excitation was set to 340 nm and emission to 395 nm. The mobile phase (A) consisted of 0.1 M ammonium formate, adjusted to pH 6 $\pm$ 0.1 with 0.1 M acetic acid, while mobile phase (B) consisted of 0.1 M ammonium formate with 5% acetonitrile, also adjusted to pH 6 $\pm$ 0.1 with 0.1 M acetic acid. The mobile phase was delivered by an Agilent 1200 series LC. The flow rate was set at 2 mL min^{−1}. The LC gradient was as follows: 0–5% mobile phase B in the first 5 min, 5–70% B for the next 4 min, held at 70% B for 1 min, and back to 100% A over the next 2 min. The 100% A was held for an additional 2 min to allow for column equilibration before subsequent sample injections [27]. Concentrations of STX, neosaxitoxin (neoSTX), decarbamoyl saxitoxin (dcSTX), gonyautoxins 2 and 3 (GTX2,3), decarbamoyl gonyautoxins 2 and 3 (dcGTX2,3), gonyautoxins 1 and 4 (GTX1,4), gonyautoxin 5 (GTX5), and the di-sulfated toxins C1 and C2 were quantified using standards purchased from the National Research Council Canada (Halifax, NS, Canada). Isomers GTX 1,4, GTX 2,3, and C1,C2 were not separated using precolumn oxidation and were measured simultaneously. In keeping with the Alaska Department of Environmental Conservation’s protocols, toxicity equivalency factors (TEFs) from the European Food Safety Authority 2009 [16] were used to convert congener concentrations to STX–eq., with the higher TEF used for unresolved congener pairs. Throughout this study, toxin concentrations in clams and tissue components are reported in total STX–eq. The contribution of individual congeners to the clam toxin pool was calculated by weight based on STX–eq. The fraction of clam toxin concentrations associated with specific tissues (% toxin) was calculated as the STX–eq. in each tissue relative to the total toxin pool in that tissue component. The LOQ data for this method are reported in Turner et al. [27].

5.3. Determining STX–eq Concentrations in Edible and Non-Edible Tissues

The effects of cleaning on STX–eq. concentrations in edible butter clam tissues were determined in three separate studies, as noted in the Introduction. The various tissue types (viscera, siphon black tip, neck of the siphon, and the main body of the clam) analyzed in the Kibler et al. study [10] are shown Figure 6 for reference. The weights of each tissue type and the corresponding STX–eq. concentrations were used to calculate the concentration in the edible and non-edible portions shown in Table 3. Specifically, the resulting μ g STX–eq. per weight of edible tissues was converted to a 100 g tissue basis by multiplying by 100 and dividing by the weight of the edible tissue. The concentrations of STX–eq. in each tissue type for each batch of clams analyzed were then plotted in order of the month of the year in which they were collected. The percent reduction in STX–eq. in edible tissue versus whole clams was calculated as the concentration in whole clams minus that in edible tissue divided by the whole clam concentration and multiplied by 100.

Table 3. Different ways in which tissues can reasonably be partitioned into retained edible tissue(s) and discarded non-edible tissue(s).

Tissues Retained as Edible	Tissues Discarded as Non-Edible
Whole clams (includes viscera, black tip at the end of the siphon, siphon neck, and body of the clam)	
Black tip, neck, body	Viscera

Table 3. Cont.

Tissues Retained as Edible	Tissues Discarded as Non-Edible
Siphon neck, clam body	Viscera, black tip
Clam body	Viscera, black tip, siphon neck
Viscera, clam body	Black tip, siphon neck



Figure 6. The various dissected clam tissues analyzed for STX concentration. (A) Shucked butter clam, (B) excised siphons without the black tip, (C) body with siphon neck, (D) black tips, (E) body tissue, (F) dissected visceral tissue (viscera shown in dish), and the location of the viscera in the body of the butter clam.

The STX concentration data presented in a 1954 report by Chambers and Magnusson [9] for whole clams and clams without the siphon were assessed using mouse bioassay and converted to STX-eq. as previously described [15,28]. The original data sheets did not specify the weights of the siphon compared to the rest of the body, but the final report indicated that siphons accounted for 14.3 to 19.9% of the body weight, with values of around 18 to 19% being more common. Based on this information, it was assumed that the weight of the non-edible siphons was 18.5% of the total weight and that the remaining 81.5% was edible tissue. Using the $\mu\text{g STX-eq. } 100 \text{ g tissue}^{-1}$ concentrations in the edible and non-edible tissues, and assuming 100 g of tissue was used for the analysis of STX, the concentration in whole butter clams was calculated. The concentrations of STX-eq. in the whole clams, edible, and non-edible tissues were then plotted by the month of the year in which the batch samples were collected. The percent reduction in STX-eq. in edible tissue compared to whole clams was calculated as described above.

The study of butter clam cleaning practices in communities on Kodiak Island, AK, was conducted as follows. In both Methods 1 and 2, the clams were opened by cutting the adductor muscles, removed whole from the shell, and strained. In Method 1, performed by Alaska Sea Grant personnel, the visceral contents were gently squeezed out, and the gills and black tip of the siphon were removed. The remaining light-colored meat, including the buttons, neck, mantle, and visceral mass with the foot, represented the edible tissue. In Method 2, carried out by a local harvester, the visceral mass (including the gills) was entirely cut out along with the black tip (Figure 7). The remaining light-colored meat, including the buttons, neck, mantle, and foot, represented the edible tissues. For the analysis, three replicate batches containing 12 butter clams each were harvested each month from either Mission Beach (57.7922, -152.3863) between April and July of 2018 or Shipwreck Beach (57.2106, -153.2961) between April and October 2018. The edible and non-edible tissues were then frozen, shipped, and analyzed as described above. The concentrations for whole clams, edible, and non-edible tissues from each batch were again plotted by the month in which the samples were collected, and the percent reduction in STX-eq. concentrations was calculated.

5.4. Seasonal Changes in Saxitoxin Concentrations in Butter Clams

The studies examining how different cleaning techniques could affect butter clam STX-eq. concentrations were not conducted during the colder months. If STX-eq. concentrations remained high in clams during the winter, the impacts of cleaning should be similar to those observed during the warmer months. To address this issue, time series data on whole butter clam STX-eq. collected in Southeast Alaska from February 1948 to September 1949 [9] and from January 1963 to March 1965 [13] were utilized. If toxin levels remain comparable to those in summer, then the cleaning results would likely be comparable. The data from the two different time series were plotted separately to illustrate the seasonal variation in toxin concentrations.

5.5. Estimates of PSP Risk Based on the Amount of Toxin Consumed in an Average-Sized Meal

The risk associated with an average-weight man (90.8 kg) or woman (77.6 kg) consuming a typical meal containing 200 g of butter clam tissue [18,29] was determined based on the STX-eq. in the edible tissues obtained from the 2015 to 2018 Kodiak study and the modeled relationship between the amount of toxin consumed per body weight and the likelihood of developing different severity symptoms. To calculate this risk, the average body weight for a man and a woman was multiplied by the modeled $\mu\text{g STX-eq. kg.b.w.}^{-1}$ for different risk levels [18,29]. This provided an estimate of the total STX-equivalents that would produce no symptoms, mild symptoms, medium symptoms, severe symptoms, or death. Next, we

determined the average serving size for butter clams, which is approximately 240 g [30], and erred on the conservative side by assuming a portion size of 200g of edible butter clam tissues per meal. The average portion size was then multiplied by the STX-eq. for edible tissues obtained from the analysis of the Kibler et al. data [10] (Figure 1) to estimate the amount of toxin consumed. The toxicity risk was then assessed by comparing the STX-eq. consumed versus the total STX needed to produce symptoms of different severity.

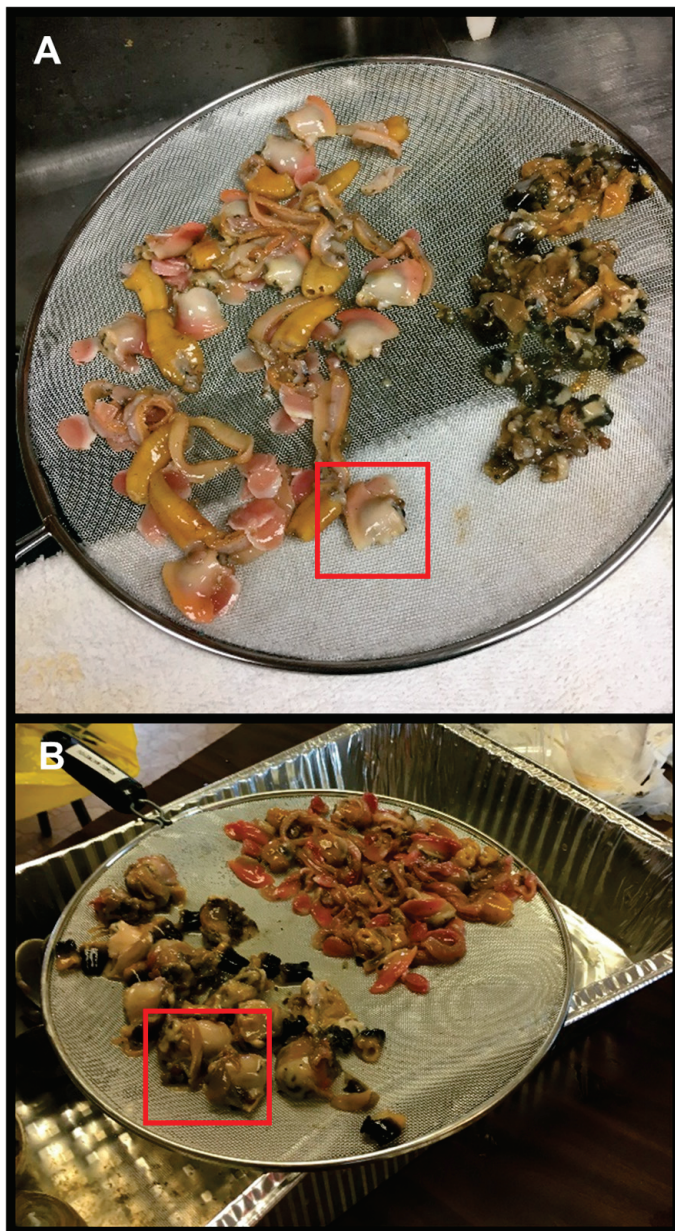


Figure 7. Edible tissues (lighter) versus non-edible tissues (darker) prepared using two methods. (A) In Method 1, the visceral content was expressed while keeping the overall visceral mass intact (red square) and the gills and black tip of the siphon was excised. (B) In Method 2, both the whole visceral mass including gills (red square) and black tip were removed resulting in less remaining tissue compared to Method 1.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/toxins17060271/s1>: Table S1: Data on how different cleaning methods impact concentration of STX-eq. in edible tissues based on data from the 2015 to 2018 study in Kodiak, Alaska ¹¹; Table S2: Sampling locations for determining toxicity in whole clams from Chambers and Magnusson (1950) ⁹ and Neal (1967) ¹⁰; Table S3: The time series of toxicity in whole clams obtained from 1948–1949 ⁹ and 1963–1965 ¹⁰; Table S4: Butter clam toxicity data for whole clams, edible tissue, and non-edible tissue obtained using methods practiced in native communities on Kodiak Island, AK. Edible tissue consists of the body and various amounts of the siphon.

Author Contributions: Conceptualization, J.A.M., S.R.K., R.W.L. and P.A.T.; methodology, J.A.M., S.R.K., R.W.L., D.R.H. and W.C.H.; formal analysis, J.A.M., R.W.L., P.A.T., D.R.H. and W.C.H.; resources, J.A.M., S.R.K., R.W.L. and P.A.T.; data curation, J.A.M., S.R.K., R.W.L., P.A.T., D.R.H. and W.C.H.; writing—original draft preparation, J.A.M., R.W.L. and P.A.T.; writing—review and editing, J.A.M., S.R.K., R.W.L., P.A.T., D.R.H. and W.C.H.; visualization, R.W.L.; supervision, J.A.M., S.R.K., R.W.L. and P.A.T.; project administration, J.A.M., S.R.K. and R.W.L.; funding acquisition, J.A.M., S.R.K., R.W.L. and P.A.T. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: Author P.A. Tester was employed by the company OceanTester. The remaining authors declare that their research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Appendix A

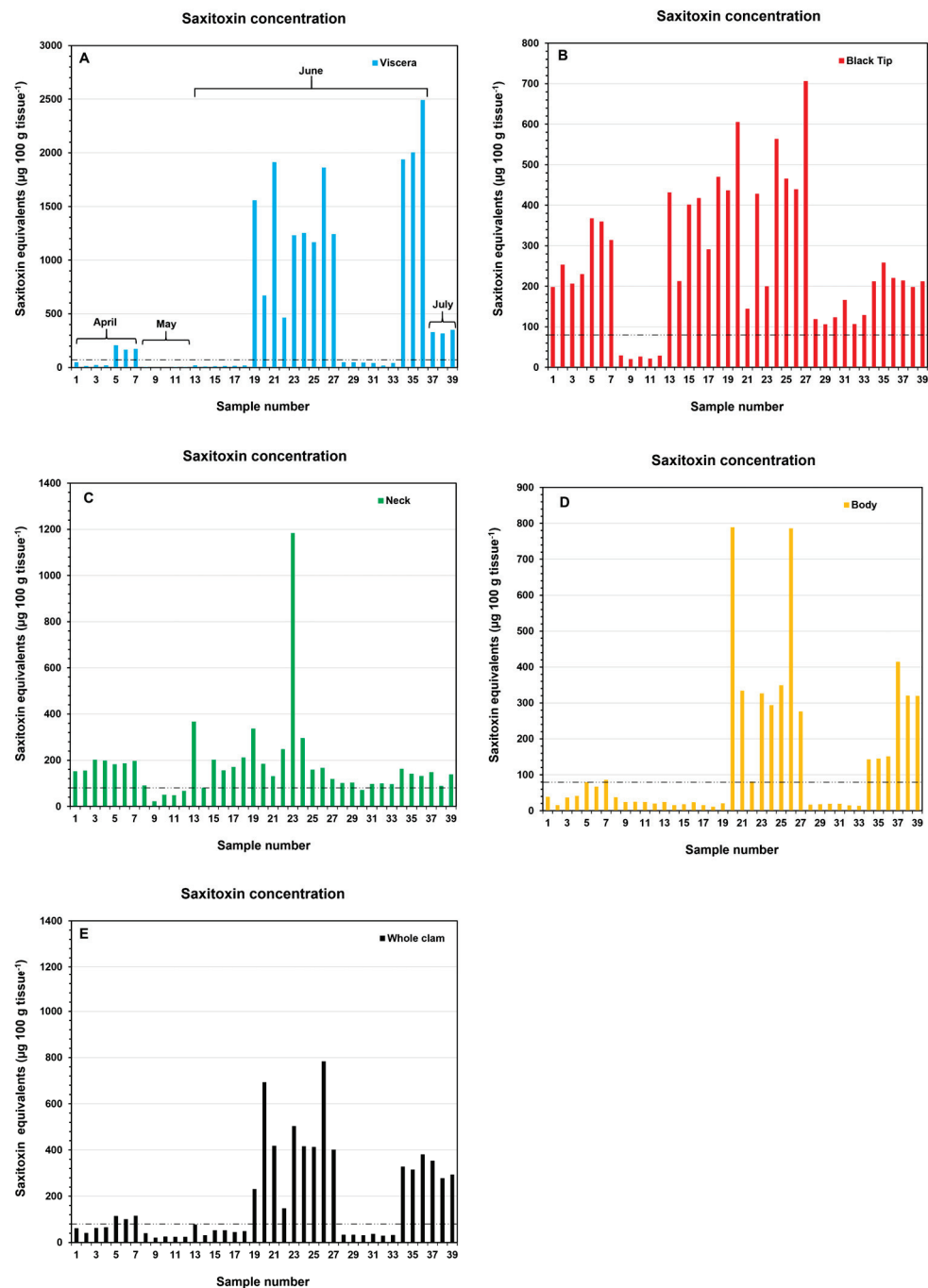


Figure A1. STX-eq. 100 g tissue⁻¹ levels from whole clams and various tissue types dissected from 39 batches of 11 to 12 butter clams each. The panels display the concentrations found in (A) the viscera, (B) siphon black tip, (C) neck (the remainder of the siphon without the black tip), (D) main body of the clam, and (E) whole clam. The samples were collected between 2015 and 2018 from either Mission Beach (MB) or Shipwreck Beach (SB) located on Kodiak Island, AK. The batches were organized by the month they were collected, but do not represent a time series (see Figure 1; Kibler et al. [10]).

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Article

Crustacean Zooplankton Ingestion of Potentially Toxic *Microcystis*: In Situ Estimation Using *mcyE* Gene Gut Content Detection in a Large Temperate Eutrophic Lake

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Abstract: Grazing by zooplankton can regulate bloom-forming cyanobacteria but can also transfer toxin-producing cells, as well as toxic metabolites, to the food web. While laboratory investigations have provided extensive knowledge on zooplankton and toxic cyanobacteria interactions, information on zooplankton feeding on toxin-producing cyanobacteria in natural water bodies remains scarce. In this study, we quantified *Microcystis*-specific *mcyE* synthase genes from the gut contents of various cladoceran and copepod taxa to assess the in situ crustacean community and taxon-specific ingestion of potentially toxic *Microcystis* in Lake Peipsi, a large eutrophic lake in Estonia, Northern Europe. *Microcystis* cells with *mcyE* genes were found in all crustaceans examined. However, some species, such as the cyclopoid copepod *Mesocyclops leuckarti*, were more efficient in ingesting potentially toxic *Microcystis* than other co-occurring cladocerans (*Daphnia* spp., *Bosmina* spp., *Chydorus sphaericus*) and copepods (*Eudiaptomus gracilis*). The amount of toxigenic *Microcystis* cells grazed by crustacean population changed temporarily, and copepods were the predominant consumers of toxigenic *Microcystis* during several months of the 5-month study period. Crustacean ingestion of toxigenic *Microcystis* was not related to *Microcystis* biomass or *mcyE* gene copy numbers in the environment but was instead related to the abundance of major crustacean grazers. Our findings emphasize the close interaction between crustacean zooplankton and toxigenic *Microcystis*, indicating that some species may play a more significant role in linking toxic cells within the food web than others.

Keywords: *Microcystis*; toxic cyanobacteria; cladocerans; copepods; aquatic food web; qPCR

Key Contribution: This study determined that toxigenic *Microcystis* cells can be ingested by various naturally occurring cladoceran and copepod species among the zooplankton community in a eutrophic lake. The dynamics of crustacean zooplankton grazing on toxigenic *Microcystis* is influenced by the presence of efficient grazers that can collect toxic cells from the environment more effectively than other co-occurring species.

1. Introduction

Cyanobacterial blooms and their metabolites are a significant global concern, impacting freshwater ecosystems, fisheries, and tourism [1–3]. In aquatic food webs, zooplankton play a crucial role in regulating algal biomass, also feeding on potentially toxic

cyanobacteria such as *Microcystis*. Since most microcystins are intracellularly bound within cyanobacterial cells [4], zooplankton feeding on these toxigenic cells becomes a significant mechanism for removing toxic algae from the water. Zooplankton collect the toxigenic cells in their guts and store the assimilated toxins in their tissues [5,6]. This, however, highlights the contradictory role of zooplankton grazing in the aquatic ecosystem. While the consumption of toxigenic cyanobacterial cells reduces harmful blooms and lowers cyanotoxin risk in the environment, zooplankton also serve as food for juvenile and planktivorous fish. Thus, they form a key link in transferring ingested toxic cells and accumulating intracellular microcystins up the food chain [7].

The highest risk from toxic cyanobacteria in ecosystems, impacting food webs and fish, is typically associated with the peak occurrence of toxin-producing strains or toxic metabolites in the environment [8]. Cyanotoxins, such as microcystins produced by *Microcystis* spp., can accumulate in aquatic organisms, leading to bioaccumulation through the food web. This can result in significant health risks for higher trophic levels, including fish, birds, and mammals, as reviewed in Chen et al. [9,10]. However, when considering zooplankton as the primary vector between cyanotoxins and fish in water bodies, the dynamics of major grazing zooplankton and the production of toxic strains may not always align. The consumption of toxigenic cells by zooplankton and the subsequent accumulation of cyanotoxins depend not only on the abundance of microcystin-producing strains in the environment but also on the presence of grazers capable of feeding on toxic strains [5]. For instance, general grazing zooplankton, such as *Daphnia*, may ingest a large number of potentially toxic cells [11] before being preyed upon and digested by predator organisms [7,12]. Although some metazoan zooplankters, such as small bacterivorous rotifers and cyclopoid copepods, may be abundant during bloom periods, larger-sized crustacean species (e.g., daphniids, calanoids) tend to decline in many eutrophic lakes during periods of massive development of potentially toxic cyanobacteria [13,14].

Several factors may contribute to this phenomenon, such as the adverse effects of cyanotoxins directly impacting zooplankton grazing rates, abundance, and behavior [15–17]. Poor food conditions due to low content levels of essential biomolecules affecting reproduction, including interference from filamentous cyanobacteria during feeding, also play a role [18,19]. Additionally, intensive predation by newly hatched 0+ fish can alter grazers' abundance and community structure during the summer bloom [20].

In temperate lakes, the onset of blooms typically coincides with favorable light and temperature conditions, leading to peak biomass during the warmest months [21,22]. In freshwater ecosystems, microcystin production is most frequently documented from the genera *Microcystis*, *Dolichospermum*, and *Planktothrix*, and species from *Microcystis* are the predominant producers of microcystins in eutrophic waters worldwide [23,24]. The growth rate of the genus *Microcystis* is highly sensitive to water temperature, accelerating at temperatures above 10 °C [25,26]. Similarly, zooplankton abundance and biomass increase when temperature and food availability support active production and population growth [27]. Toxic and non-toxic strains of the same cyanobacterial species often coexist in the environment [24,28]. Environmental factors, including nutrient concentrations, temperature, and pH, can influence their relative abundance [29–32] and chemical interactions with grazers [33]. This highlights the complexity of interactions affecting the timing of the flow of toxic cyanobacteria into the food web. There remains an open question of whether the transfer of toxic cells to pelagic food web is primarily driven by the dynamics of major grazers or by the abundance of toxigenic strains in the water. To our knowledge, no studies have directly addressed this in natural water bodies yet.

PCR methods have emerged as promising tools for the in situ estimation and quantification of zooplankton ingestion of toxic cyanobacteria. For instance, research demonstrated

that the copepod species *Pseudodiaptomus forbesi* can ingest toxic *Microcystis* strains during blooms in the San Francisco Estuary [5]. Both microzooplankton (20–200 µm) and mesozooplankton (200–2000 µm) communities have been observed to feed on both toxic and non-toxic strains of *Microcystis* [34]. Furthermore, Sotton et al. [7] demonstrated the transfer of toxic *Planktothrix rubescens* via grazing zooplankton to predatory zooplankton, which mediated the microcystin contamination in zooplanktivorous whitefish. Although qPCR detection of toxin-producing genes from consumers is rarely applied in field investigations, it could provide valuable information regarding toxic cyanobacteria–food web interactions. Specifically, it could shed light on major trophic links involved in the movement of cyanotoxins and contribute to understanding the regulation of toxigenic cyanobacteria in natural environments.

To address the knowledge gap, we studied the taxon-specific ingestion of potentially toxic *Microcystis* cells by major cladoceran and copepod taxa among crustacean zooplankton in the large, shallow Lake Peipsi (Figure 1). We focused on toxigenic *Microcystis* as the major bloom-forming cyanobacteria in this lake. In Peipsi, *Dolichospermum* spp. and *Planktothrix* spp. can also produce microcystins, but the respective *mcyE*-producing cells are present in considerably lower abundances compared to the concurrent *Microcystis* spp. [35]. Previous analyses of the ingestion of potentially microcystin-producing *Dolichospermum* and *Planktothrix* by different zooplankton species have not detected the ingestion of these cyanobacteria above the rate of the reliable quantification level [11]. Therefore, we presume that *Microcystis* is the major microcystin-producing cyanobacteria moving through the pelagic food web in Peipsi.

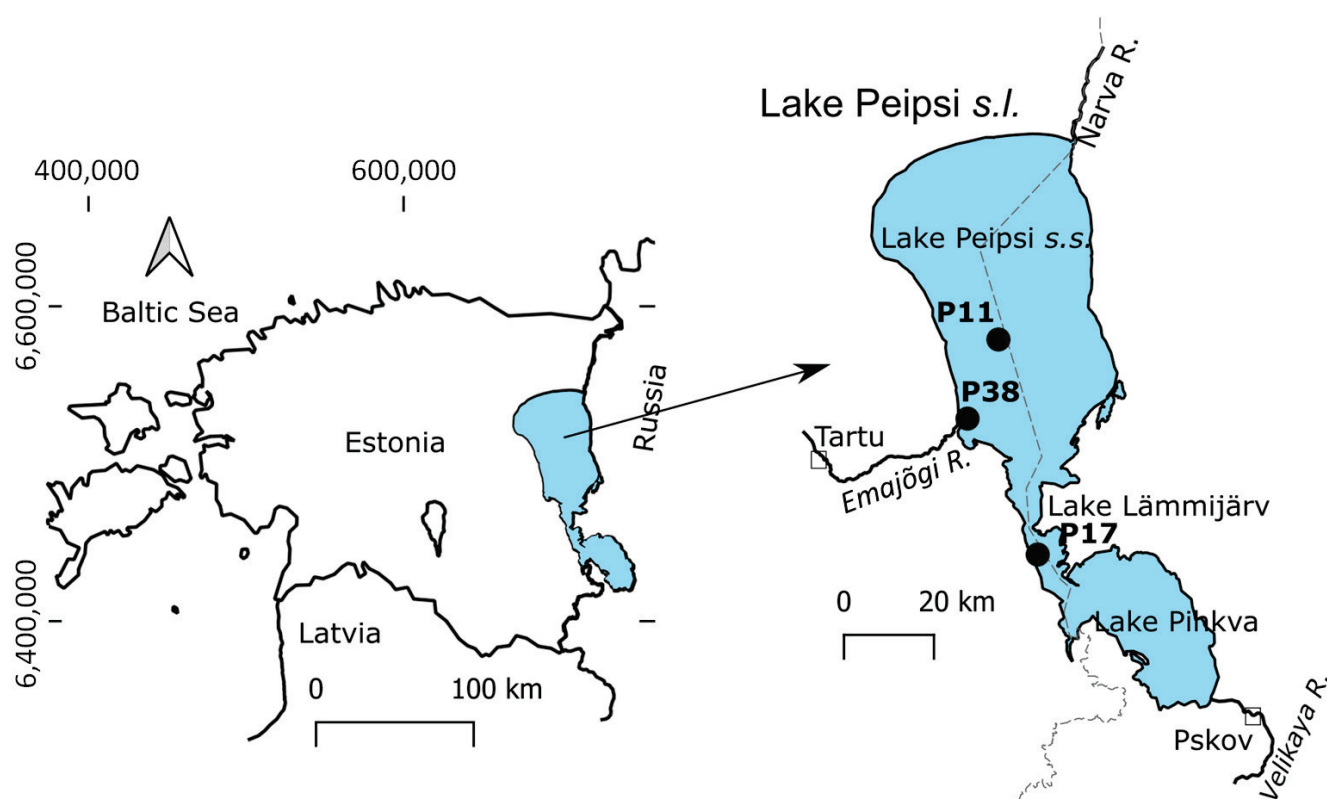


Figure 1. Location of sampling sites in Peipsi *sensu lato* (s.l.): P11 and P38 in Peipsi *sensu stricto* (s.s.) and P17 in Lämmijärv.

Our previous research revealed that the major crustaceans—*Daphnia* spp., *Bosmina* spp., *Eudiaptomus gracilis*, and the predatory cladoceran *Bythotrephes longimanus*—can ingest cyanobacteria, including potentially toxic *Microcystis*, either directly or indirectly [11].

However, the research indicated that some species might be more efficient at this process and thus could potentially be more significant mediators of toxic cyanobacteria to the aquatic food web.

Our working hypotheses were that (1) despite different feeding modes, various taxa in the crustacean community are able to ingest toxic *Microcystis* cells; (2) cladocerans, specifically the generalist feeder *Daphnia*, form the central link connecting toxigenic *Microcystis* to the food web in a eutrophic lake; and (3) the ingestion of toxigenic *Microcystis* by the crustacean community depends more on the dynamics of efficient consumers of toxic cells than on the seasonal availability of toxin-producing *Microcystis* in the environment.

2. Results

2.1. Environmental Variables

During the study period, the total phosphorus (TP), total nitrogen (TN), and chlorophyll-*a* (Chl-*a*) values increased from the northern basin towards the central basin (Lämmijärv) (Table 1). Water temperature was relatively similar at all three study sites, peaking in July at a maximum of 25.7 °C. Based on water quality parameters and the OECD classification [36], Lake Peipsi *sensu stricto* (s.s.) is classified as eutrophic, while Lämmijärv is considered a hypertrophic part of the lake.

Table 1. Water quality parameters in sampling sites in Lake Peipsi s.s. and Lake Lämmijärv in 2021 and trophic state classification according to Caspers [36].

	P11 Peipsi s.s.		P38 Peipsi s.s.		P17 Lämmijärv	
	Mean	Range	Mean	Range	Mean	Range
TP, µg/L	37.83	23–58	41.17	26–78	101	39–160
TN, µg/L	706.67	380–1000	901.67	530–1300	1091.67	660–1600
NO ₃ [−] , µg/L	37.17	20–100	143.83	20–610	58.83	20–220
NO ₂ [−] , µg/L	3.50	3–6	4.33	3–7	3.83	3–7
NH ₄ ⁺ , µg/L	27.17	10–53	23	10–74	24	10–44
Water Temp, °C	15.8	7–25	16.0	6.9–25.4	15.9	6.7–25.7
Secchi depth, m	1.7	0.8–2.4	1.2	0.6–1.3	0.7	0.5–1.1
pH	8.52	8.2–8.8	8.47	7.9–8.8	8.51	8.2–9.2
O ₂ , mg/L	10.38	8–13.2	10.25	8.4–12.1	9.78	7.2–11.8
Chl- <i>a</i> , µg/L	17.95	6.1–30.5	22.65	6.3–44.8	57.77	20.9–109
OECD clasif.	Eutrophic		Eutrophic		Eutrophic/hypertrophic	

2.2. Seasonal Dynamics of Phytoplankton, *Microcystis* spp. And *mcyE* Copy Numbers

Cyanobacteria and diatoms dominated the phytoplankton biomass (Figure 2A). Cyanobacterial biomass varied between 0.04 and 11.14 mgWW/L across the sampled areas and periods. *Microcystis* formed 4 to 89% of the cyanobacterial biomass and was presented by species *M. viridis*, *M. aeruginosa*, *M. botrys*, and *M. wesenbergii*. The biomass of *M. viridis* was particularly notable, forming the highest biomass in all sampling sites, 12.8–26.8% of total cyanobacteria, followed by *M. wesenbergii* (5.1–16.7%), *M. botrys* (14.5–12.9%), and *M. aeruginosa* (2–6.7%). *Microcystis* peaked in August (P17) or in September (P11, P38), with approximately 2- to 4-times higher biomasses in the hypertrophic part of the lake (P17) compared to P11 and P38 in Peipsi s.s. (Figure 2B). Despite these biomass differences, the abundances of *Microcystis* with *mcyE* synthetase genes were generally similar in all sites, with peak occurrences in September (Figure 2B).

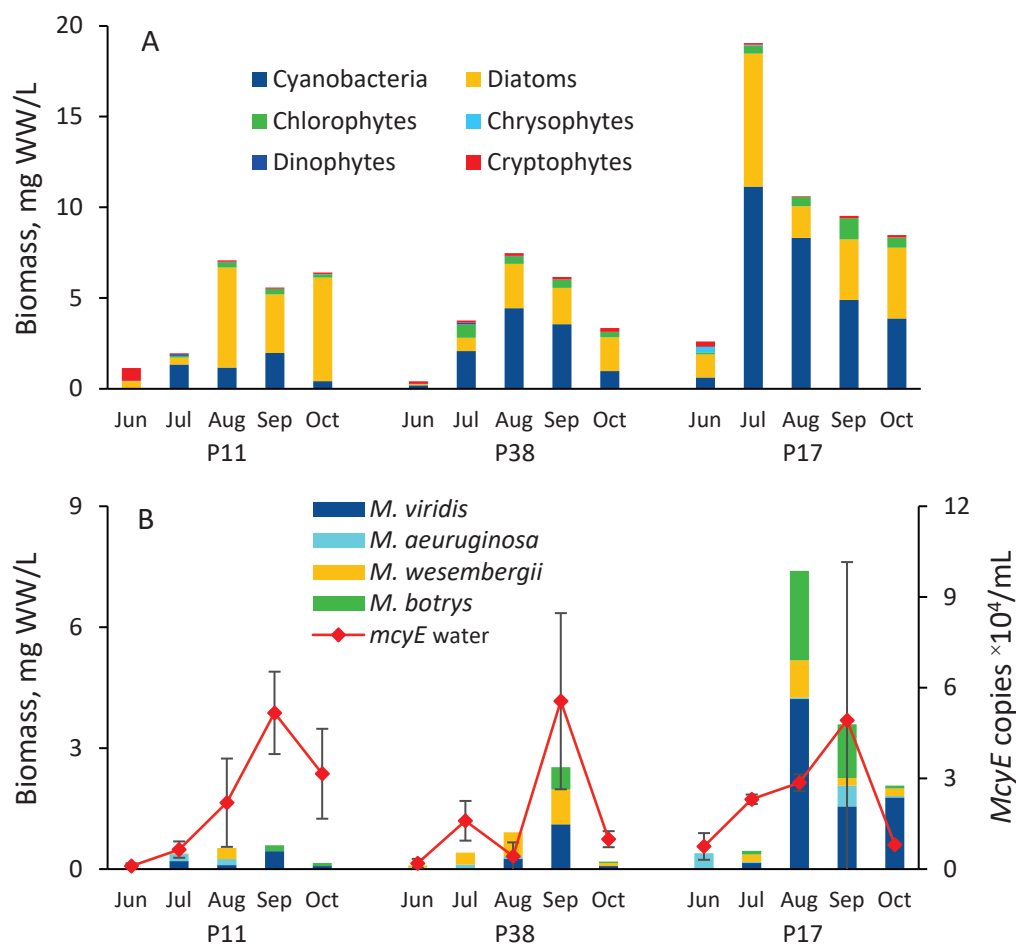


Figure 2. Phytoplankton composition and biomass (A); seasonal dynamics of biomasses of *Microcystis* species and *Microcystis mcyE* copy numbers ($\pm$ SD) (B) in sampling sites P11, P38, and P17 in Lake Peipsi in 2021.

2.3. Seasonal Dynamics of Crustacean Zooplankton Communities

The abundance and biomass of the studied crustaceans (cladocerans, adults copepods, and copepodites) ranged from 20 to 803 individuals/L and 0.27–3.98 mgWW/L, respectively (Figure 3), comprising an average of 90% of the whole crustacean biomass (composition, abundance, and biomass shown in Supplementary Figure S1). *Daphnia* spp. (*D. galeata*, *D. longispina*, *D. cristata*, and *D. cucullata*) among filtering cladocerans, and *Eudiaptomus gracilis* and *Mesocyclops leuckarti* among copepods were the most abundant taxa in the studied lake areas. Crustacean abundance, biomass, and composition varied across the different lake areas. Larger-sized species, such as *D. galeata* and *E. gracilis*, were generally most abundant in the eutrophic Peipsi s.s. (P11). By contrast, smaller-sized species like *M. leuckarti* and *Chydorus sphaericus* dominated the population in the hypertrophic part of the lake (Lämmijärv, P17). Predatory cladocerans *Leptodora kindtii* and *Bhytothrephes longimanus*, as well as the large current-feeding copepod *Heterocope appendiculata*, were presented in limited numbers.

2.4. Crustacean Ingestion of Potentially Toxic *Microcystis*

Microcystis mcyE gene copies were detected in the guts of all analyzed crustacean taxa. Taxon-specific ingestion of *Microcystis mcyE*-containing cells varied across dates and sites (Supplementary Figure S2). The highest taxon-specific ingestion was measured for the current-feeding copepod *H. appendiculata* and the ambush-feeding copepod *M. leuckarti* (Table 2). Among filter-feeding cladocerans, *Daphnia* spp. exhibited higher values than

Bosmina spp. and *C. sphaericus*. Throughout the analyzed period, *Microcystis* cells with *mcyE* genes were most frequently detected in the guts of *M. leuckarti* and *Daphnia* spp., while in predatory cladocerans (*L. kindtii*, *B. longimanus*), this was detected only sporadically.

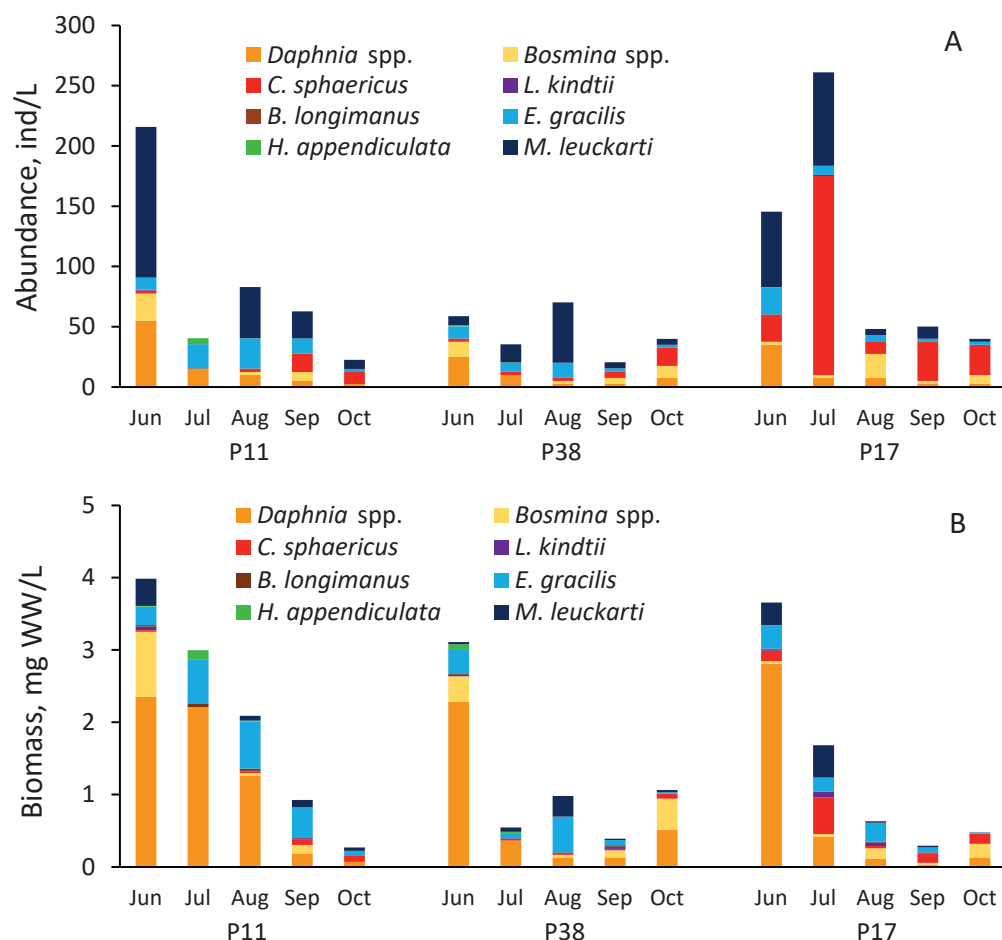


Figure 3. Seasonal dynamics of abundance (A) and biomass (B) of major crustacean taxa in sampling sites P11, P38, and P17 in Lake Peipsi in 2021.

Table 2. Mean and maximum taxon-specific ingestion and mean weight-specific ingestion of potentially toxic *Microcystis* cells (based on *mcyE* synthetase gene detection in consumer's guts) of the most abundant cladoceran and copepod taxa in Peipsi in 2021.

Taxon	Average ($\pm$ SD) Length, mm	Maximum Taxon-Specific Ingestion, <i>mcyE</i> Cells/ind	Mean ($\pm$ SD) Taxon-Specific Ingestion, <i>mcyE</i> Cells/ind	Mean ($\pm$ SD) Weight-Specific Ingestion, <i>mcyE</i> Cells/100 mg
Cladocerans				
<i>Daphnia</i> spp.	1.34 $\pm$ 0.21	303	20 $\pm$ 21	30 $\pm$ 25
<i>Bosmina</i> spp.	0.66 $\pm$ 0.13	61	16 $\pm$ 17	50 $\pm$ 65
<i>Chydorus sphaericus</i>	0.31 $\pm$ 0.03	22	3 $\pm$ 5	216 $\pm$ 150
Copepods				
<i>Eudiaptomus gracilis</i>	1.16 $\pm$ 0.09	74	12 $\pm$ 23	60 $\pm$ 60
<i>Heterocope appendiculata</i>	1.93 $\pm$ 0.12	6428	1203 $\pm$ 2192	611 $\pm$ 1004
<i>Mesocyclops leuckarti</i>	0.85 $\pm$ 0.07	610	92 $\pm$ 138	494 $\pm$ 562

Maximum ingestion rates indicated that *Daphnia* spp. was capable of ingesting hundreds of *Microcystis* toxigenic cells (303 *mcyE*-containing cells/ind), whereas smaller-sized cladocerans, *Bosmina* spp. and *C. sphaericus*, had fewer toxigenic cells in their guts. Among

copepods, *H. appendiculata* had the highest number of *mcyE*-containing cells (6429 cells/ind), followed by *M. leuckarti*.

Weight-specific ingestion of potential microcystin-producing cells was, on average, highest in *H. appendiculata* (611 *mcyE*-containing cells/100 mg of grazer body mass) and *M. leuckarti* (494 *mcyE*-containing cells/100 mg) (Table 2). Cladocerans had much lower weight-specific ingestion, with the highest average values estimated for the smallest cladoceran, *C. sphaericus* (216 *mcyE*-containing cells/100 mg) (Table 2).

Total crustacean population feeding (sum of cladoceran and copepod ingestion) on *Microcystis* toxigenic cells varied between 199 and 27,736 *mcyE*-containing cells/population L, with most dates showing values below 1000 *mcyE* cells/population L. Crustacean ingestion levels were similar in P11 and P38 but remarkably higher in June and July in P17 (Figure 4A; Supplementary Figure S3). On average, across the sampling areas and period, the copepod community (*M. leuckarti*, *E. gracilis*, *H. appendiculata*) was slightly more efficient in ingesting the microcystin-producing cells, accounting for 54% compared to 46% for cladocerans (*Daphnia* spp., *Bosmina* spp., *C. sphaericus*, *L. kindtii*, *B. longimanus*) (Figure 4B). When present in plankton, the cyclopoid copepod *M. leuckarti* often constituted the majority of crustacean ingestion of *Microcystis* microcystin-producing cells, particularly notably in P17 during June and July. Additionally, *Daphnia* spp., *C. sphaericus*, and *E. gracilis* made significant contributions on certain dates and sites (Figure 4A).

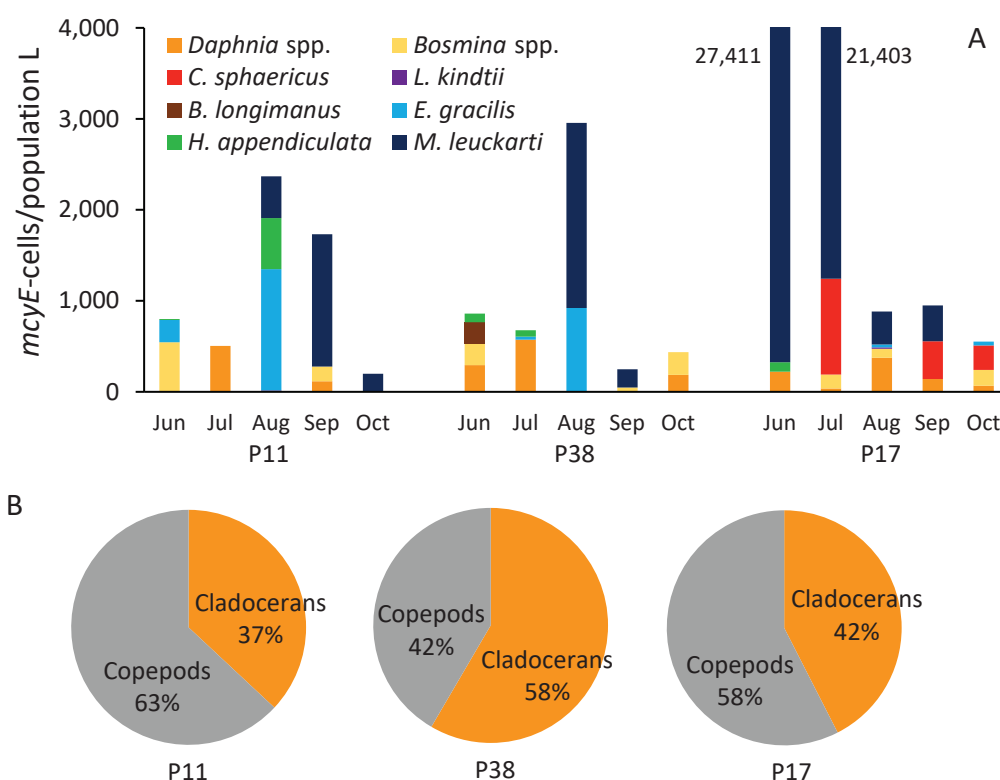


Figure 4. Seasonal dynamics of crustacean population feeding on potentially toxic *Microcystis* cells (based on the detection of *mcyE*-containing cells in consumer' guts) in sampling sites P11, P38, and P17 in Lake Peipsi in 2021; ingestion by various cladoceran and copepod taxa (A); proportional contribution (%) of cladoceran and copepod ingestion (B).

2.5. Relationships of Crustacean Ingestion of *mcyE*-Containing Cells with Crustacean Assemblage Composition, *Microcystis* Biomass, and Environmental Parameters

First, we tested the sampling site difference in *mcyE* abundances and crustacean populations' ingestion (cladocerans, copepods, total crustaceans). As there were no statistical differences in *mcyE* abundance and grazer population ingestion of potentially

toxic *Microcystis* cells between the lake sites (Mann–Whitney U-test, multiple pairwise comparisons, $p > 0.05$), we pooled the data from all three sites and analyzed lake-wide relationships of crustacean ingestion of *mcyE*-containing cells between crustacean assemblage and environmental parameters.

Secondly, in analyzing the relationship between crustacean ingestion and variable grazers' abundances and biomasses, we found a significant positive correlation between the ingestion of potentially toxic *Microcystis* cells by the total crustacean population and copepod abundances ($r = 0.68$, $p < 0.01$) (Table 3). Among the copepod species analyzed, the strongest relationship was found with the abundances and biomasses of *M. leuckarti* and *E. gracilis*. Respective correlations were not observed for cladocerans. The biomass of *Daphnia* spp. and the biomasses of cladocerans and crustaceans were significantly negatively correlated to the biomass of *Microcystis* and *mcyE* gene copy numbers in lake water. The ingestion of *mcyE*-containing cells (by cladocerans, copepods, total crustaceans, or individual crustacean taxa) was not significantly correlated with *Microcystis* biomass or the *Microcystis mcyE* copy numbers in lake water (Spearman correlations, $p > 0.05$).

Table 3. Spearman correlation coefficients (r_s) between the total crustacean population ingestion of toxigenic *Microcystis* (based on *mcyE* synthetase gene detection in grazer gut content) and various grazers' abundance, biomass; *Microcystis* biomass; *mcyE* gene copy numbers in Lake Peipsi water in 2021.

Grazers	Total Crustacean Ingestion (<i>mcyE</i> Cells/L) vs. Grazer Abundance	Total Crustacean Ingestion (<i>mcyE</i> Cells/L) vs. Grazer Biomass	<i>Microcystis</i> Biomass vs. Grazer Biomass	<i>McyE</i> water (Copies/L) vs. Grazer Biomass
Cladocerans	0.398	0.311	−0.607 **	−0.718 **
Copepods	0.684 **	0.425	−0.318	−0.346
Crustaceans	0.684 **	0.368	−0.571 *	−0.732 **
<i>Daphnia</i> spp.	0.23	0.204	−0.674 **	−0.666 **
<i>Bosmina</i> spp.	0.01	−0.032	−0.108	−0.2803
<i>C. sphaericus</i>	0.251	0.322	0.118	0.2795
<i>E. gracilis</i>	0.610 *	0.538 *	−0.084	−0.3067
<i>M. leuckarti</i>	0.705 **	0.620 *	−0.329	−0.1787
<i>H. appendiculata</i>	−0.698	0.030	0.151	−0.2125
<i>L. kindtii</i>	−0.243	0.099	−0.361	0.0311
<i>B. longimanus</i>	−0.57	−0.059	−0.479	0.0035

Correlation is significant at * $p < 0.05$; ** $p < 0.01$

Finally, we tested the relationships between cladoceran, copepod, and total crustacean ingestion of toxigenic *Microcystis* and environmental physio-chemical parameters. Spearman correlation analyses showed clear positive relationships with nutrients, *Microcystis* biomass, and *mcyE* gene copy numbers in lake water but no significant relationships with crustacean population (cladoceran, copepod, or total crustacean) ingestion of potentially toxic *Microcystis* (Table 4). Similarly, no association between environmental parameters and cladoceran, copepod, and total crustacean population ingestion of toxigenic *Microcystis* cells was observed from the principal component analysis (PCA) across the studied months (Figure 5). The PCA shows cladoceran, copepod, and total crustacean population ingestion, *Microcystis* species biomasses, and *Microcystis mcyE* copy numbers as variables, linearly fitting the environmental variables of nutrients (TP, PO_4^{+} , TN, NO_3^{-} , NO_2^{-} , NO_4^{+}), temperature, pH, Secchi depth, and chl-*a* with the PCA ordination space. The first component (PCA1) and second component (PCA2) combined accounted for 57.1% of the variation (PCA1 = 37%; PCA2 = 20.1%).

Table 4. Spearman correlation coefficients (r_s) between environmental variables and cladoceran, copepod, and total crustacean population ingestion (*mcyE*-containing cells/L), *Microcystis* biomass, and *McyE* copy numbers in Lake Peipsi water in 2021.

Variables	Cladoceran, Ingestion (<i>mcyE</i> Cells/L)	Copepod, Ingestion (<i>mcyE</i> cells/L)	Total Crustacean Ingestion (<i>mcyE</i> Cells/L)	<i>Microcystis</i> (mg/L)	<i>McyE</i> Water (Copies/L)
Water temp, °C	0.443	0.288	0.461	−0.237	−0.296
NO ₃ [−] , µg/L	0.436	−0.346	−0.142	−0.467	−0.399
NH ₄ ⁺ , µg/L	0.393	0.161	0.014	−0.244	−0.335
NO ₂ [−] , µg/L	0.288	−0.026	0.159	−0.479	−0.467
TN, µg/L	0.412	−0.163	−0.225	0.321	0.607 *
TP, µg/L	−0.036	0.431	0.256	0.726 **	0.637 *
PO ₄ ⁺ , µg/L	−0.246	0.358	0.211	0.888 ***	0.632 *
Secchi depth, m	0.075	−0.222	−0.052	−0.758 **	−0.571 *
pH	−0.045	0.189	0.119	0.097	−0.03
Chl- <i>a</i> , µg/L	0.016	0.463	0.293	0.814 ***	0.664 **

Correlation is significant at * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, respectively.

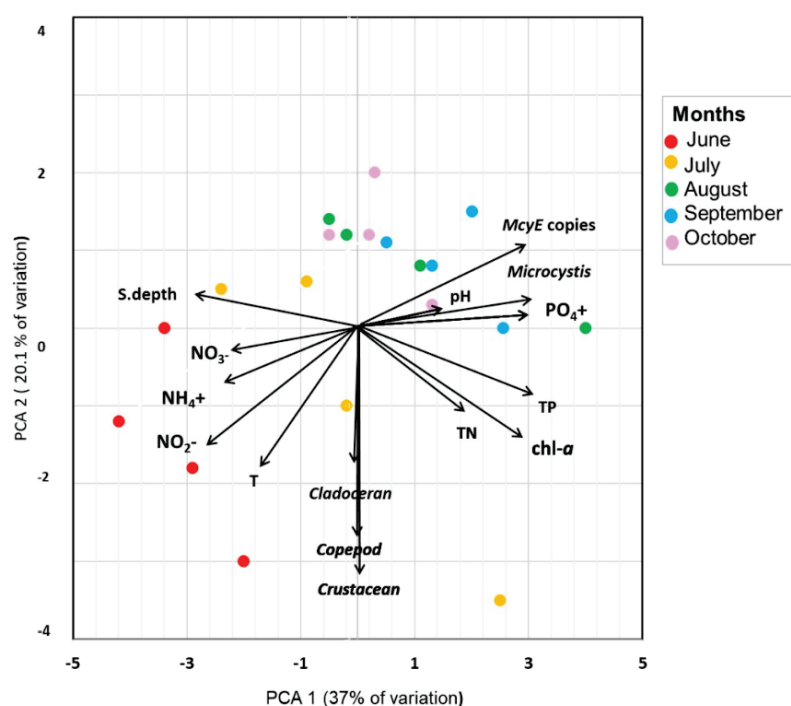


Figure 5. Principal component analysis plot displaying the association between cladoceran, copepod, and total crustacean population ingestion (*mcyE* cell/L), *Microcystis* biomass, *McyE* copy numbers, and environmental variables in Lake Peipsi in 2021 grouped by month.

3. Discussion

Laboratory analyses have demonstrated that various zooplankton taxa are capable of consuming toxigenic *Microcystis* [37]. We confirmed this in situ in a eutrophic lake, showing that despite different feeding modes, all analyzed cladoceran and copepod taxa (*Daphnia* spp., *Bosmina* spp., *C. sphaericus*, *M. leuckarti*, *E. gracilis*, *H. appendiculata*, *L. kindtii*, *B. longimanus*) contained toxin-producing *Microcystis* cells in their guts. Most of these taxa (e.g., *Daphnia* spp., *Bosmina* spp., *C. sphaericus*, *M. leuckarti*, and *E. gracilis*) are commonly occurring among zooplankton communities in eutrophic waters [38]. They could thus impact *Microcystis* populations in these waterbodies. In Peipsi, these taxa constitute a major part of zooplankton biomass [39].

In this study, the feeding assessment of various taxa provided an interesting insight into crustacean zooplankton interactions with toxigenic *Microcystis*.

We expected that the generalist-feeding *Daphnia* that is able to feed on variable types and sizes of algae, including toxic cyanobacteria [37], has the highest capability to collect *Microcystis* toxigenic cells among the studied crustaceans. However, this expectation was not confirmed. Although *Daphnia* spp. had the highest taxon-specific ingestion rate among filtering cladocerans, and it showed the capability to ingest microcystin-producing cells throughout the season (as seen in hypertrophic P17), surprisingly, copepods were generally more efficient in collecting the toxigenic *Microcystis*. This made copepods the predominant feeders of toxigenic *Microcystis* for several months in Peipsi, while cladocerans contributed earlier in the season when daphniids were abundant in the plankton.

We found significant interspecific differences in the consumption of the toxigenic *Microcystis*, especially among copepods. Although occurring in low numbers in Peipsi, the large-sized calanoid *H. appendiculata* was able to consume toxigenic cells with high taxon-specific ingestion. A relatively high number of toxigenic cells was also detected in the guts of the small cyclopoid copepod *M. leuckarti*. It is important to note that, due to their generally omnivorous nature of feeding [40–42], copepods may obtain toxic cyanobacterial cells either by feeding directly on algae or through their animal prey, such as rotifers and protozoa [34]. This may explain the substantial numbers of potentially toxic *Microcystis* cells in the guts of *M. leuckarti* and *H. appendiculata* compared to cladocerans.

However, the opposite results were obtained for another calanoid copepod, *E. gracilis*. Laboratory experiments revealed that this species can successfully avoid consuming toxic strains through cue-based selective avoidance triggered by cellular microcystin and can efficiently graze on alternative, non-toxic prey [15,43]. Such behavior could also explain the limited ingestion of toxigenic *Microcystis* by *E. gracilis* in Peipsi. Analyzing all sites and dates of this study, *E. gracilis* had generally fewer samples, indicating ingestion of toxic *Microcystis* than other co-occurring crustaceans. An earlier in situ zooplankton feeding study from Peipsi, which assessed gut phytoplankton marker pigment composition [11], indicated an active selection against colonial and filamentous cyanobacteria but a high preference for cryptophytes in this copepod. Collectively, these results indicate that *E. gracilis* has a modest effect on toxic *Microcystis* in Peipsi and probably in other lakes with high *Microcystis* biomass.

Analyzing of crustacean weight-specific ingestion revealed that the small-sized crustaceans *C. sphaericus* and *M. leuckarti*, which are characteristic species of highly eutrophic water bodies [27,44,45], are capable of high weight-specific ingestion of potentially toxic *Microcystis*. Based on crustacean population ingestion, we can presume that, with abundant populations, as observed in hypertrophic P17 in this study, *C. sphaericus* and *M. leuckarti* are capable of removing a substantial amount of toxigenic cells from the lake water. The observed pattern may indicate a high tolerance or effective detoxification of microcystins [46], which may explain their prevalence in bloom-dominated lakes. However, future research is necessary to confirm this. Research from hypertrophic Lake Ringsjön (Sweden) has shown that a natural community dominated by cyclopoid copepods and small cladocerans could suppress the blooms of potentially toxic *Dolichospermum*, *Microcystis*, and *Planktothrix* species [47]. This study did not assess the grazing impact; still, as toxic and non-toxic strains of *Microcystis* co-occur in the environment [28], and the tested zooplankters are likely able to ingest both [34,48,49], it is assumed that the total crustacean feeding on *Microcystis* was much higher than the measured ingestion of only toxigenic strains in this study.

Although this was not the scope of our study, we presumed that *Microcystis* colonies were ingested either directly, as smaller and medium-sized (<60 µm) colonies can be consumed by various crustaceans [50–52], or alternatively via scraping the surficial cells of

larger colonies [53,54]. The colony size of *Microcystis* typically increases with the development of cyanobacterial blooms [55], making them easier for grazers to handle earlier in the season [56]. In Peipsi in July 2014, the colony sizes of *Microcystis* spp. ranged from 48 to 96 µm in diameter, with a predominance of smaller colonies; by September, the share of larger colonies had increased (Kersti Kangro, Estonian University of Life Sciences, unpublished data). The use of colonial cyanobacteria, such as *Microcystis*, as a food along with other algae aligns with our earlier crustacean zooplankton feeding study in Peipsi [11]. Gut phytoplankton marker pigment analyses indicated that colonial cyanobacteria (characterized by the carotenoids zeaxanthin and canthaxanthin) often constituted significant proportions and were preferred algae in the diets of *Daphnia* spp. and *Bosmina* spp. At the same time, diatoms, chlorophytes, and cryptophytes were less prevalent. Other crustaceans, such as *E. gracilis*, which preferentially feed on cryptophytes, likely cannot completely avoid ingesting *Microcystis*.

A major result of our study is that the potential risk of toxic cyanobacteria to the aquatic food web and ecosystem cannot be solely assessed based on the dynamics of the abundance of toxigenic strains in the environment despite the common presumptions [8]. As zooplankton represent the primary link transferring the toxic *Microcystis* into the food web, their role in this process must be thoroughly assessed. In Peipsi, the crustacean community feeding on toxigenic strains was inversely related to *Microcystis* biomass and the *mcyE* gene abundance in lake water. Instead, feeding was positively correlated with the abundance of copepods, primarily of the small cyclopoid *M. leuckarti*. This supports the view that copepods might play a significant role in consuming the toxic *Microcystis* in Peipsi. Thus, the occurrence and abundance of major consumers seem to be the main factor determining the grazing dynamics of toxic cells. This is further illustrated by the fact that in all studied areas of Peipsi, the dynamics of the biomasses of crustaceans, particularly cladocerans, and *Microcystis*, with its toxin-producing strains, had opposite patterns. These results highlight the predominant role of grazers, rather than toxin-producing strains, in controlling the flow of toxic *Microcystis* into the pelagic food web.

The widespread presence of toxic *Microcystis* cells in the diet of various crustacean zooplankton observed in this study provides further insights into the functioning of food webs in lakes dominated by toxic bloom-forming *Microcystis*. Based on analyses in 2021 in Peipsi, our results suggest that during the growing period, the toxic cyanobacteria were actively incorporated into the pelagic food web as the lake water consistently contained toxigenic *Microcystis*-contaminated zooplankton. This also poses a potential toxicity threat to young-of-the-year fish, as well as zooplanktivorous smelt (*Osmerus eperlanus* morfa *spirinchus*) and vendace (*Coregonus albula*), which primarily rely on crustacean zooplankton [57,58]. Microcystin concentrations, however, have not yet been measured in fish or zooplankton from Lake Peipsi.

Microcystis species common in our lake are all documented for their ability to produce microcystins, although their toxicity levels vary. While *M. aeruginosa* is a well-known microcystin producer, other species, such as *M. wesenbergii*, *M. viridis*, and *M. botrys*, also contribute to the microcystin burden in the environment [1,59]. Based on a previous study in Peipsi, LC-MS/MS analysis identified a total of 14 microcystin variants in the samples, with MC-RR being the most abundant, found in 93% of analyzed samples, followed by MC-LR and its methylated variants in 92% of the samples. These microcystin variants were closely associated with *M. wesenbergii* and *M. aeruginosa* [35]. Available data of microcystin concentrations in the lake have indicated relatively low concentrations (<1 mg/L) of microcystins in the depth-integrated water samples in open water areas but extremely high concentrations (>2000 µg/L) in inshore scum areas [35,60].

4. Conclusions

Our research provided a unique example from a natural lake, indicating that various crustacean zooplankters are capable of consuming and transferring toxigenic *Microcystis* to the pelagic food web. We also showed that some crustacean species (e.g., *M. leuckarti* in Lake Peipsi) are more efficient at collecting toxic cells from the environment than other co-occurring species (e.g., *E. gracilis*). Our findings further emphasize that relying solely on *mcyE* gene dynamics in water may introduce bias when assessing the temporal *Microcystis* toxicity risk to the food web. Understanding the dynamics of major grazers may be beneficial for predicting the temporal flow of toxigenic cells to zooplankton and zooplanktivorous fish. However, it must be noted that this current study is based on only one-year analyses of grazer feeding activity on potentially toxic *Microcystis*. The dynamics of toxin-producing strains and zooplankton communities may substantially vary between the years within the same waterbody [61,62] and alter the grazing patterns. We, therefore, encourage further research to verify the results of this study to obtain better predictions of food web contamination with zooplankton-grazed toxic cells and assimilated cyanotoxins in lakes with *Microcystis* bloom occurrences.

5. Materials and Methods

5.1. Study Site

Lake Peipsi *s.l.* (*sensu lato*) (57°51′–59°01′ N, 26°57′–28°10′ E, 30 m a.s.l.) is a large non-stratified eutrophic lake between Estonia and Russia with a surface area of 3555 km² and mean and maximum depth of 7.1 m and 15.3 m, respectively (Figure 1). Lake Peipsi *s.l.* consists of three basins from north to south: Peipsi *s.s.*, Lämmijärv, and Pihkva. Due to the large area, variable hydrology, morphometry, and bottom topography, the lake varies in its trophic state, with increasing total phosphorus (TP), total nitrogen (TN), and chlorophyll-*a* (chl-*a*) values from the northern basin (Peipsi *s.s.*) towards the central basin (Lämmijärv) and southern basin (Pihkva) [35,63]. During the growing season (May–October), diatoms and cyanobacteria prevail in the phytoplankton biomass. *Microcystis* is the major toxin-producing cyanobacterial genus, and, along with other potentially toxic genera such as *Gloeotrichia*, *Dolichospermium*, *Aphanizomenon*, and *Planktothrix*, forms the highest cyanobacterial biomass during the summer months (July–August) or early autumn (September) [35]. Cyanobacterial biomass, including *Microcystis*, increases towards the southern basins. Conversely, metazoan zooplankton have higher biomass values in the northern, moderately eutrophic Peipsi *s.s.* (mainly formed by *Daphnia galeata* and *Eudiaptomus gracilis*) compared to the hypertrophic Lämmijärv, where generally smaller species prevail (*Daphnia cucullata*, *Chydorus sphaericus*, *Mesocyclops leuckarti*) [64]. The lake is typically ice-covered from December to April [65]. The water is well mixed by wind and well aerated by waves and currents, with no permanent stratification of temperature, dissolved oxygen, or hydrochemical parameters during the ice-free period.

5.2. Sampling

In this study, samples were collected monthly from June to October 2021 from the routine state monitoring stations in Lake Peipsi (*s.s.*) (P11, P38) and Lämmijärv (P17) (Figure 1). Water chemistry analyses were performed as part of the state monitoring program by the Estonian Environmental Research Centre following international and Estonian quality standards.

Depth-integrated water at 1 m intervals was collected with a Limnos water sampler (with a free flow design for vertical sampling) from the entire water column and mixed in a tank. Subsamples were taken from this water for analyses of phytoplankton community

composition and biomass, molecular detection of *mcyE* genes of potentially toxic *Microcystis*, and zooplankton community composition, abundance, and biomass.

To identify zooplankton composition and biomass, 20 L of the depth-integrated water was filtered through a 48- μ m mesh plankton net and concentrated into a 200 mL sample jar. Phytoplankton and zooplankton samples were fixed with acidified Lugol's solution at a final concentration of 1% and kept in the dark until further analysis.

To assess the abundance of *mcyE* genes in water samples, 90 to 1000 mL of the depth-integrated water was filtered on-site using Sterivex filter capsules (pore size 0.2 μ m) (Merck Millipore Sterivex™, Darmstadt, Germany). Samples were filtered in triplicate. Filters were stored in 96% ethanol at -80°C until further analysis.

For analyzing potentially toxic *Microcystis* in crustacean gut content, depth-integrated samples were collected with vertical tows of a plankton net (300 μ m mesh) until a sufficient amount of material was obtained. The collected bulk zooplankton was instantly rinsed with deionized water to clean the sample from phytoplankton as much as possible, concentrated in a small volume, and immediately frozen in liquid nitrogen. The samples were preserved at -80°C in the laboratory until further analyses.

5.3. Phyto- and Zooplankton Biomass, Crustacean Preparation for Molecular Analyses

Phytoplankton cells were enumerated with an inverted microscope (Nikon Eclipse Ti-S, Nikon Instruments INC, Melville, NY, USA) at $\times 400$ magnification, using the Utermöhl technique [66]. Phytoplankton taxa were identified to the lowest possible taxonomic level, and each counted taxon was converted to biovolumes by measuring cell, trichome, or colony dimensions. Phytoplankton biomass was expressed as mg WW/L (milligrams of wet weight per liter of lake water).

Zooplankton biomass and community composition were analyzed under a stereomicroscope (Nikon SMZ1500, Nikon Instruments INC, Melville, NY, USA, up to $\times 120$ magnification) in a Bogorov chamber. Crustacean length was converted to wet weight as described by Studenikina and Cherepakhina [67] and Balushkina and Winberg [68]. Zooplankton taxa accounting for 20% or more of the biomass were considered abundance and biomass dominants, respectively [69].

To prepare zooplankton samples for molecular analyses, the frozen bulk zooplankton samples were thawed to separate the most abundant taxa. These consisted of three dominant grazing cladocerans, *Daphnia* spp. (*D. galeata*, *D. longispina*, *D. cristata*, *D. cucullata*), *Bosmina* spp. (*B. berolinensis*, *B. gibbera*, *B. thersites*), and *Chydorus sphaericus*; two current-feeding calanoid copepods, *E. gracilis* and *Heterocope appendiculata*; one ambush-feeding cyclopoid copepod, *M. leuckarti*; and two predatory cladoceran species, *Leptodora kindtii* and *Bhytotrephes longimanus*. Whenever possible, three replicates were made per sample. For samples of *Daphnia* spp. and *E. gracilis*, 50 individuals were collected per replicate whenever possible. For smaller taxa (*Bosmina* spp., *C. sphaericus*, *M. leuckarti*), 50–200 individuals were separated. For larger-sized crustaceans (*H. appendiculata*, *L. kindtii*, and *B. longimanus*), generally fewer than 50 individuals per replicate were separated. For copepod samples, only adult and copepodite stages were used. Lengths of approximately 30 individuals per sample were measured to calculate the weight-specific ingestion of various crustacean grazers. The collected specimens were repeatedly rinsed with deionized water to minimize contamination by non-ingested algae, visually inspected to ensure no external algal cells were stuck on the animals, and then placed into 1.5 mL microtubes for DNA extraction.

5.4. DNA Extraction and Molecular Analyses

Genomic DNA from zooplankton was extracted using the NucleoSpin® Tissue Kit (MACHEREY-NAGEL, Düren, Germany) following the manufacturer's instructions. DNA

from integrated water samples was extracted using a modified NucleoSpin® Tissue Kit for Sterivex filter capsules. All extractions were made under the laminar flow hood to protect samples and avoid contamination. The initial steps of the extraction protocol were modified as follows: (1) Ethanol was removed from the SX filter capsule using a 3 mL syringe and centrifuged at 5000 RCF for 30 min. (2) Meanwhile, the SX filter capsules were cut open, and the filter was removed using a sterile scalpel and forceps and placed into 5 mL safe-lock tubes. (3) After centrifugation, the remaining ethanol was carefully removed from the pellet; the pellet was therefore resuspended in 630 µL of warm T1 buffer and 70 µL of Proteinase K. (4) The resuspended pellet in T1 buffer and Proteinase K mixture was pipetted onto the SX filter. (5) Garnet and glass beads (Qiagen, Hilden, Germany) were added to the filters, and the mixture was vortexed at high speed for 10 min. (6) The samples were then incubated at 56 °C overnight to lyse. (7) After the incubation step, the rest of the protocol was followed as instructed in the protocol, but the volume for buffer B3 and ethanol was 700 µL.

The quality and quantity of the extracted DNA were assessed using a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). The DNA was stored at −80 °C until further analysis. To quantify potential microcystin producers among the genera *Microcystis*, in the samples, *Microcystis*-specific *mcyE* qPCR was performed using an absolute quantification method with an internal standard curve. MC-producing *Microcystis* sp. 205 (HAMBI/UHCC Culture Collection, University of Helsinki) was used to construct standard curves in the qPCR analysis. Information about the approximate genome size of *Microcystis* was taken into account for calculations. A more detailed description about the standard curve construction and calculations can be found in Koskeniemi et al. [70]. Each 10 µL qPCR reaction mixture included the following components: 1× HOT FIREPol® Probe Universal qPCR Mix (Solis BioDyne, Tartu, Estonia), 0.3 µM of both forward and reverse primers, 0.3 µM of a fluorescently labeled TaqMan probe, 5 µL of 10-fold diluted template DNA, and molecular-grade dH₂O to make up the remaining volume. Each environmental sample was tested in triplicate. Additionally, each qPCR analysis plate contained negative control samples and positive standard DNA dilutions. All qPCR reactions were conducted on a LightCycler® 480 System (Roche Life Science, Indianapolis, IN, USA) using a 384-well platform using the following protocol: 95 °C for 12 min for initial denaturation, 40 cycles of 95 °C for 15 s, and 62 °C. Results were analyzed using LightCycler® Software 1.5. The *mcyE* gene was chosen to detect and quantify potential microcystin-producing *Microcystis* because of its established role in microcystin production and its reliability as a molecular marker. Since *mcyE* is typically found as a single copy per genome, it is ideal for assessing the abundance of potentially toxic *Microcystis* cells in both environmental samples and grazers [31,71,72].

5.5. Data Analyses

The abundance of potentially toxic cells in zooplankton guts represents their last feeding activity before sampling. Based on the abundances of *Microcystis* cells with the *mcyE* gene in zooplankton gut contents, we calculated the following indices: taxon-specific ingestion, maximum ingestion, weight-specific ingestion, and population ingestion (cladoceran, copepod, total crustaceans). Taxon-specific ingestion was calculated as the mean value of results of replicated qPCR analysis for each date when the taxon's sample was prepared; the results are given as ingestion of *mcyE*-containing cells per individual. Maximum ingestion is the highest measured abundance of cells with the *mcyE* gene in consumer gut content and refers to the taxon's highest capability to ingest potentially toxic *Microcystis* cells under in situ conditions. Weight-specific ingestion was calculated using the taxon's average weight and ingestion of *Microcystis* cells with the *mcyE* gene, provided as *mcyE*-containing cells

per 100 µg of grazer body mass. Population ingestion (cladoceran, copepod, or total crustacean) of *Microcystis* cells with the *mcyE* gene was calculated by summing taxon-specific ingestions, considering the respective consumer numbers in one liter of lake water.

For statistical analyses, the principal component analysis was performed to analyze the ingestion of cells with *mcyE* synthetase genes and the biomass of *Microcystis* and to compare the consumption (population ingestion) by different zooplankton in various months. We also analyzed the effects of environmental variables on zooplankton ingestion and *Microcystis* biomass. PCA was performed with the function “prcomp”. Spearman’s rank correlation (r_s) with the function “cor.test” was used to determine the relationship between the abundance and biomass of zooplankton, *Microcystis* biomass, *mcyE* abundance, and physiochemical indices versus crustacean ingestion on *mcyE*-containing cells. PCA and Spearman’s rank correlation analyses were performed with the RStudio 4.1.2 package and its extensions. Additionally, the nonparametric Mann–Whitney U-test was used to test the significance of differences in *mcyE* abundances and crustacean ingestion of *mcyE*-containing cells between sampling sites. The workflow was performed using STATISTICA 13.2 software.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/toxins17010042/s1>, Figure S1: Composition, abundance, and biomass of crustacean zooplankton communities in Lake Peipsi in 2021 in sampling sites P11, P38, and P17. Cladocerans (A, B); copepods and predatory cladocerans (C, D). Figure S2: Temporal variation in the individual ingestion of potentially toxic *Microcystis* cells based on *mcyE* synthetase gene detection in the gut contents of the studied cladoceran and copepods taxa in Peipsi (*Daphnia* spp., *Bosmina* spp., *Chydorus sphaericus*, *Eudiaptomus gracilis*, *Mesocyclops leuckarti*, *Heterocope appendiculata*, *Leptodora kindtii*, *Bhythotrephes longimanus*) in 2021. Figure S3: Temporal variation in most abundant grazer population (*Daphnia* spp., *Bosmina* spp., *Chydorus sphaericus*, *Mesocyclops leuckarti*, *Heterocope appendiculata*, *Eudiaptomus gracilis*, and cladocerans, copepods, and total crustaceans) ingestion of potentially toxic *Microcystis* cells in liters of lake water in Peipsi in 2021 based on *mcyE* synthetase gene detection in grazer gut contents.

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Article

Real-Time Observation of Clickable Cyanotoxin Synthesis in Bloom-Forming Cyanobacteria *Microcystis aeruginosa* and *Planktothrix agardhii*

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Abstract: Recently, the use of click chemistry for localization of chemically modified cyanopeptides has been introduced, i.e., taking advantage of promiscuous adenylation (A) domains in non-ribosomal peptide synthesis (NRPS), allowing for the incorporation of clickable non-natural amino acids (non-AAAs) into their peptide products. In this study, time-lapse experiments have been performed using pulsed feeding of three different non-AAAs in order to observe the synthesis or decline of azide- or alkyne-modified microcystins (MCs) or anabaenopeptins (APs). The cyanobacteria *Microcystis aeruginosa* and *Planktothrix agardhii* were grown under maximum growth rate conditions ($r = 0.35\text{--}0.6$ and $0.2\text{--}0.4$ (day^{-1}), respectively) in the presence of non-AAAs for 12–168 h. The decline of the azide- or alkyne-modified MC or AP was observed via pulse-feeding. In general, the increase in clickable MC/AP in peptide content reached a plateau after 24–48 h and was related to growth rate, i.e., faster-growing cells also produced more clickable MC/AP. Overall, the proportion of clickable MC/AP in the intracellular fraction correlated with the proportion observed in the dissolved fraction. Conversely, the overall linear decrease in clickable MC/AP points to a rather constant decline via dilution by growth instead of a regulated or induced release in the course of the synthesis process.

Keywords: microcystin synthesis; anabaenopeptin synthesis; time-lapse experiments; molecule tracing; pulse-feeding; unnatural amino acids; click-chemistry; fluorescent labeling; growth rate

Key Contribution: Tracking of MC/AP synthesis by using promiscuous A domains in NRPS, allowing for the incorporation of clickable non-AAAs into peptide products. During time-lapse experiments, a fast increase in clickable MC/AP content has been related to the growth rate.

1. Introduction

In addition to the visually repulsive effect of cyanobacterial blooms in freshwater and brackish water, considerable amounts of toxins are produced and released into the water, with dangerous effects for the entire food web, at the end of which humans are. Within the cyanotoxins, the microcystins (MCs) and related nodularins are globally considered the most abundant [1]. To understand cyanotoxin synthesis within cyanobacterial cells, the intracellular storage mechanism of cyanotoxins, and the factors regulating cyanotoxin occurrence outside the cell are considered of outermost importance to develop exposure scenarios that are at the basis of risk management [1].

Typically, MCs are produced inside the cells while high extracellular concentrations have been observed less frequently, i.e., after cell lysis [2]. Using individual cell culture studies under laboratory conditions, both intracellular and extracellular MCs have been measured, and the general finding was that in exponentially growing cell cultures, <10% of the total MC pool is found to be extracellular. Such experiments have been performed using a few selected strains only (i.e., *Microcystis aeruginosa* PCC 7806), somewhat limiting the possibility of testing variability in ecophysiological responses via comparing a larger

number of genotypes or strains [3]. For example, comparing the extracellular peptide content among 56 strains of *Planktothrix agardhii* and *P. rubescens*, the percentage of extracellular peptides (from total peptide concentration) varied from 0 to $62 \pm 23\%$ (SE) for MC and 0 to $58 \pm 22\%$ for anabaenopeptin (AP) [3,4]. While the majority of the samples (77%) were found to contain less than 10% of total MC dissolved, a few strains showed a higher proportion of dissolved MC. Nevertheless, it is generally understood that MC release into the medium is primarily related to cell aging and resulting cell lysis [5], while MC production has been traditionally linked to cell growth [6,7].

Typically, those analyses are performed in parallel to strain growth under defined culture conditions, and the intra- vs. extracellular MC or other peptide concentrations are determined by fractionation, i.e., from cells filtered on glass fiber filters, while corresponding extracellular MCs are determined from the filtrate [8]. A more direct approach to monitor MC/peptide synthesis is to create a traceable MC peptide pool and to follow the fate of both intra- and extracellular MCs during time-lapse experiments. This has been attempted in the past, i.e., by supplying radioactive inorganic carbon to *M. aeruginosa* in cultures for 6 h and subsequent incubation in the dark [5]. The fate of this radioactive MC pool was studied by following its radioactivity in time-course experiments. Notably, no evidence for losses from the intracellular MC pool could be found either under high light or under low light conditions.

In the last years, the use of click chemistry to assist in subcellular localization of chemically modified MC/APs has been applied. In particular, visualizing the synthesis of MCs and APs directly in the cell of cyanobacteria was established by using chemo-selective reaction [9], i.e., following the principle of the copper catalyzed azide-alkyne cycloaddition (CuAAC) reaction [10]. Accordingly, using chemical-analytical methods the targeted modification of MC/AP peptides through the incorporation of an alkyne or azide side chain could be shown, i.e., for MC structure in pos. 2 in *M. aeruginosa* or AP structure in exocyclic pos. 1 in *P. agardhii* [9]. The targeted peptides then become available as CuAAC reaction sites for specific fluorophores visualized using high-resolution microscopy.

The chemo-selective labeling process requires the incorporation of amino acids carrying azide or alkyne moieties into the modified MC or AP peptide to allow its targeting. The incorporation of such non-natural amino acids (non-AAs) is possible since cyanobacteria assimilate various diverse amino acids from the media [11], and certain promiscuous adenylation (A) domains integrate the non-AAs into the growing peptide product during non-ribosomal peptide synthesis (NRPS). For the first time, those promiscuous A domains were described in *M. aeruginosa* some years ago [12,13]. The encoding genes have possibly evolved in order to increase the structural diversity of the resulting bioactive peptide products, i.e., for MC in *M. aeruginosa* [13] or for AP in *P. agardhii* [14]. In particular, for MC synthesis, the first A domain encoded via the *mcyB* gene was related to the co-production of MC molecules carrying Arg, Tyr, and Leu at pos. 2 [12,13]; while for AP synthesis, the first A domain of the *apnA* gene was related to Arg, Tyr, and Lys at pos. 1 of the AP molecule [14]. However, not all genotypes have evolved towards promiscuity in relevant A domains, but rather, certain phylogenetic clades have been differentiated either by producing only AP molecules with Arg at pos. 1, or with both Arg and Tyr at pos. 1, or with three or more AA at pos. 1 (e.g., Figure 6 in Entfellner et al., 2017 [15]). Consequently, if A domains of NRPS retained their substrate specificity towards a corresponding substrate, such incorporation of non-AA into the growing peptide product will not be possible. During previous studies, two cyanobacteria have been used for click-chemistry, i.e., *M. aeruginosa* strain Hofbauer carrying natural MC variants with Tyr and Leu at pos. 2 of the MC molecule and *P. agardhii* strain no371/1 carrying Arg, Tyr, and Lys at pos. 1 of the AP molecule. While no AP synthesis could be detected for *M. aeruginosa* strain Hofbauer, *P. agardhii* strain no371/1 has lost the genetic basis for MC synthesis and is considered nontoxic [4].

The CuAAC reaction with targeted peptides has been tested for its specificity through the use of genetically modified *P. agardhii* strain NIVA-CYA126/8, for which a specific toxin or peptide such as MC or AP has been eliminated previously [14]. In particular,

P. agardhii strain NIVA-CYA126/8 wild type as well as its peptide knock-out mutants, which have been inactivated either in MC or in cyanopeptolin or in microviridin synthesis, all showed modified clickable AP and labeling using Alexa 488 [16]. In contrast, for the NIVA-CYA126/8 mutant inactivated in AP synthesis, no labeling was observed compared with the controls [16].

In this study, we applied pulse feeding of three different non-natural L-amino acids (non-AAs), i.e., 4-azidophenylalanine (Phe-Az), N-propargyloxy-carbonyl-L-lysine (Prop-Lys), or O-propargyl-L-Tyrosine (Prop-Tyr) to track and trace the intra- and extracellular dynamics of clickable MC/AP synthesis in a time series to better understand the synthesis process in a temporal manner. Using standard peptide analysis and fragmentation via LC-MSⁿ, the build up of a traceable MC/AP pool was observed. Conversely, the decline of traceable MC/AP was followed subsequent to pulse feeding and the transfer of cells to a new medium without non-AAs. The relationships between clickable MC/AP content and resulting signal intensity from subsequent labeling using Alexa Fluor488 will be reported in a forthcoming article. Most importantly, we observed a rapid increase in clickable MC/AP content by chemical-analytical analysis (maximum after 12–24 h). Conversely, after pulsed feeding, a linear decrease in the modified peptides with time was observed (0–96 h or 0–192 h). Thus, both the build up and decline point to a rather constant production of targeted MC/AP, mostly related to growth, which is in line with the current understanding of MC or other bioactive peptide synthesis in cyanobacteria.

2. Results

2.1. Growth of Cyanobacteria in Strain Cultures During Time-Lapse Experiments Using Pulsed Feeding of Non-AAs

In general both strains, *M. aeruginosa* and *P. agardhii*, grew linearly in the presence of all three non-AAs; however, one particular non-AA (Phe-Az) was frequently found to reduce growth when compared to controls (i.e., cells grown and processed under identical conditions but without substrate) or in the presence of the other two non-AAs (Prop-Lys, Prop-Tyr), as shown in Tables 1 and 2 (Supplementary Figures S1 and S2). Based on optical density (OD), the average growth rates varied from 0.58 ± 0.13 (SE) (day^{-1}) during build up to 0.35 ± 0.05 during decline in the controls of *M. aeruginosa*. In contrast, during addition of Phe-Az, the growth decreased to 0.38 ± 0.15 and -0.04 ± 0.07 , respectively. For *P. agardhii*, growth rates varied from 0.47 ± 0.1 to 0.22 ± 0.07 in controls during build up and decline experiments, respectively, and similarly to *M. aeruginosa*, the cell (filament) numbers as indicated by OD were found to be reduced in the presence of Phe-Az (Table 1). Analogously, based on dry weight (DW), reduced biomass was observed during Phe-Az addition, while Prop-Lys and Prop-Tyr did not affect the production of biomass (Table 2).

Table 1. Mean $\pm$ SE optical density and growth rates as recorded during time-lapse signal build up and decline experiments in cyanobacteria *M. aeruginosa* strain Hofbauer and *P. agardhii* strain no371/1. Control = cells grown and processed under identical conditions but without non-AA substrate.

Strain	Experiment	Control	Phe-Az	Prop-Lys	Prop-Tyr	<i>p</i> -Value ¹
Optical density						
<i>M. aeruginosa</i>	build up	0.06 ± 0.02^a	0.02 ± 0.002^b	0.06 ± 0.02^a	0.06 ± 0.02^a	<0.001
	decline	0.09 ± 0.01^a	0.04 ± 0.002^b	0.1 ± 0.02^a	0.1 ± 0.02^a	<0.001
<i>P. agardhii</i>	build up	0.09 ± 0.03^a	0.06 ± 0.01^b	0.09 ± 0.03^a	0.09 ± 0.03^a	<0.001
	decline	0.34 ± 0.05^a	0.29 ± 0.05^b	0.37 ± 0.06^a	0.36 ± 0.06^a	<0.001

Table 1. Cont.

Strain	Experiment	Control	Phe-Az	Prop-Lys	Prop-Tyr	<i>p</i> -Value ¹
Growth rates (day ^{−1})						
<i>M. aeruginosa</i>	build up	0.58 ± 0.13 ^{ab}	0.38 ± 0.15 ^a	0.62 ± 0.12 ^b	0.59 ± 0.13 ^{ab}	0.023
	decline	0.35 ± 0.05 ^{ab}	−0.04 ± 0.07 ^b	0.34 ± 0.06 ^{ab}	0.36 ± 0.1 ^a	0.021
<i>P. agardhii</i>	build up	0.47 ± 0.1	0.52 ± 0.27	0.53 ± 0.15	0.53 ± 0.1	0.93
	decline	0.22 ± 0.07	0.2 ± 0.04	0.2 ± 0.03	0.21 ± 0.03	0.19

¹ Two-Way Repeated Measures ANOVA (grouping factor). Superscripts indicate homogeneous groups as revealed by post hoc pairwise comparison (Bonferroni test).

Table 2. Mean ± SE dry weight and growth rates as recorded during time-lapse signal build up and decline experiments in cyanobacteria *M. aeruginosa* strain Hofbauer and *P. agardhii* strain no371/1. Control = cells grown and processed under identical conditions but without non-AA substrate.

Strain	Experiment	Control	Phe-Az	Prop-Lys	Prop-Tyr	<i>p</i> -Value ¹
Dry weight (mg/mL)						
<i>M. aeruginosa</i>	build up	0.03 ± 0.005 ^a	0.02 ± 0.001 ^b	0.03 ± 0.006 ^a	0.03 ± 0.005 ^a	0.001
	decline	0.06 ± 0.007	0.05 ± 0.004	0.05 ± 0.005	0.06 ± 0.007	0.11
<i>P. agardhii</i>	build up	0.04 ± 0.008 ^a	0.03 ± 0.004 ^b	0.04 ± 0.007 ^a	0.04 ± 0.008 ^a	<0.001
	decline	0.15 ± 0.03	0.11 ± 0.02	0.14 ± 0.02	0.16 ± 0.03	0.099
Growth rates (day ^{−1})						
<i>M. aeruginosa</i>	build up	0.17 ± 0.15	0.28 ± 0.3	0.52 ± 0.3	0.21 ± 0.21	0.19
	decline	0.26 ± 0.09	0.18 ± 0.1	0.21 ± 0.13	0.28 ± 0.12	0.85
<i>P. agardhii</i>	build up	0.37 ± 0.06	0.22 ± 0.09	0.28 ± 0.17	0.31 ± 0.12	0.63
	decline	0.21 ± 0.09	0.18 ± 0.06	0.2 ± 0.05	0.24 ± 0.07	0.60

¹ Two-Way Repeated Measures ANOVA (grouping factor). Superscripts indicate homogeneous groups as revealed by post hoc pairwise comparison (Bonferroni test).

2.2. Observation of Clickable Peptide Synthesis in Cyanobacteria Strains During Time-Lapse Build Up and Decline Experiments

As reported previously, both original MC/AP structural variants, as well as new (clickable) MC/AP structural variants, were observed in the presence of all three non-AAs, i.e., demethylated MC variants DAsp-MC-YR and D-Asp-MC-LR and methylated MC variants MC-YR and MC-LR were partly transformed into modified MC, i.e., either DAsp-MC-Phe-AzR [M + H]⁺ 1056.5 and MC-Phe-AzR [M + H]⁺ 1070.5, or DAsp-MC-Prop-LysR [M + H]⁺ 1078.5 and MC-Prop-LysR [M + H]⁺ 1092.5, or DAsp-MC-Prop-TyrR [M + H]⁺ 1069.5 and MC-Prop-TyrR [M + H]⁺ 1083.5. Analogously, for AP, the original AP structural variants AP C [M + H]⁺ 809.5, AP B [M + H]⁺ 837.5, and AP-A [M + H]⁺ 844.5 were transformed into clickable AP, i.e., either AP-Phe-Az [M + H]⁺ 843.3 or AP-Prop-Lys [M + H]⁺ 891.5 or AP-Prop-Tyr [M + H]⁺ 882.5. All of these structural variants have been identified previously using MS² and MS³ fragmentation analysis [9,16].

In general, MC/AP peptide production was maintained in the presence of all three non-AAs (Phe-Az, Prop-Lys, Prop-Tyr), i.e., using two-way repeated measures (RM)-ANOVA, the time factor was always found to be highly significant (*p* < 0.001).

Except for Phe-Az in *M. aeruginosa*, the total MC or AP contents (intracellular MC-LR equivalents in ng/mL or AP-B equivalents in ng/mL) were constantly increasing over time (Supplementary Figures S3 and S4). This principal relationship between growth and peptide production resulted in total MC content ranging from 0.2 to 2.2 µg MC/mg DW during the time-lapse build up and 0.2–2.3 µg MC/mg DW during time-lapse decline.

Analogously, the total AP content ranged from 0.15 to 0.7 µg AP/mg DW and 0.04–0.56 µg AP/mg DW during the time-lapse build up and time-lapse decline, respectively.

Nevertheless, when comparing MC or AP contents between non-AA treatments, significant differences in total intracellular peptide content vs. moderate differences in percentage of extracellular peptide were found (Table 3). When compared with controls, often the Phe-Az treatment changed in total intracellular peptide content, which was related to the lower growth rates (i.e., Tables 1 and 2). In contrast, the percentage of total extracellular MC contents were found to be less altered in the presence of Prop-Tyr, Prop-Lys, or Phe-Az, while corresponding AP contents did not change significantly.

Table 3. Mean ± SE total MC/AP contents (ng/mg DW), percentage extracellular peptide from total peptide and percentage of clickable MC/AP intra-and extracellular peptides recorded during time-lapse signal build up and decline experiments in cyanobacteria *M. aeruginosa* strain Hofbauer and *P. agardhii* strain no371/1. MC or AP were quantified as equivalents of MC-LR [M + H]⁺ 995.5 or AP-B [M + H]⁺ 837.5. Control = cells grown and processed under identical conditions but without non-AA substrate.

Strain	Experiment	Control	Phe-Az	Prop-Lys	Prop-Tyr	p-Value ¹
Intracellular total peptide content (ng/mg DW)						
<i>M. aeruginosa</i>	build up (MC)	558 ± 49 ^a	823 ± 120 ^b	766 ± 117 ^{ab}	677 ± 64 ^{ab}	0.023
	decline (MC)	530 ± 39 ^{ab}	367 ± 38 ^b	757 ± 67 ^{ab}	811 ± 119 ^a	0.026
<i>P. agardhii</i>	build up (AP)	337 ± 39 ^{ab}	267 ± 20 ^a	299 ± 38 ^a	418 ± 59 ^b	0.003
	decline (AP)	258 ± 35	245 ± 25	247 ± 26	249 ± 34	0.965
Percentage of extracellular peptide from total						
<i>M. aeruginosa</i>	build up (MC)	22 ± 1.7 ^a	21 ± 1.6 ^a	20 ± 1.3 ^a	26 ± 2.5 ^b	<0.001
	decline (MC)	7.2 ± 1.2 ^a	12.8 ± 2.2 ^b	11.6 ± 1.4 ^{ab}	10.5 ± 1.2 ^{ab}	0.009
<i>P. agardhii</i>	build up (AP)	7.6 ± 0.9	6.6 ± 0.3	9.3 ± 1.6	6.2 ± 0.8	0.055
	decline (AP)	3.9 ± 0.3	5 ± 0.7	4.8 ± 0.9	4.4 ± 0.5	0.482
Percentage of clickable intracellular peptide						
<i>M. aeruginosa</i>	build up (MC)	0 ± 0 ^a	14.1 ± 1.6 ^b	2.2 ± 0.3 ^c	43.2 ± 7 ^d	<0.001
	decline (MC)	0 ± 0 ^a	11 ± 1 ^b	1.5 ± 0.2 ^c	24.6 ± 3.6 ^d	<0.001
<i>P. agardhii</i>	build up (AP)	0 ± 0 ^{ad}	12.5 ± 1.7 ^{ac}	50 ± 6.8 ^b	6.4 ± 2.5 ^{ac}	<0.001
	decline (AP)	0 ± 0 ^a	7.3 ± 1.7 ^c	55.4 ± 2.1 ^d	1.4 ± 0.4 ^{ab}	<0.001
Percentage of clickable extracellular peptide						
<i>M. aeruginosa</i>	build up (MC)	0 ± 0 ^a	19.6 ± 3.1 ^b	1.5 ± 0.6 ^a	67.1 ± 9 ^c	<0.001
	decline (MC)	0 ± 0 ^a	10.7 ± 2.3 ^{ab}	0.2 ± 0.1 ^a	28.2 ± 7.5 ^b	0.01
<i>P. agardhii</i>	build up (AP)	0 ± 0 ^a	9.7 ± 2.3 ^b	38.9 ± 6.6 ^b	10.9 ± 3.2 ^b	<0.001
	decline (AP)	0 ± 0 ^a	8.7 ± 4.9 ^a	56.2 ± 8.8 ^b	10.4 ± 5.9 ^{ab}	0.006

¹ Two-Way Repeated Measures ANOVA (grouping factor). Superscripts indicate homogeneous groups as revealed by post hoc pairwise comparison (Bonferroni test).

In addition, the percentages of clickable MC/AP peptides differed significantly between non-AA treatments, i.e., for MC in *M. aeruginosa*, the non-AA Prop-Tyr revealed the highest percentage of modified MC during both build up and decline experiments in both intracellular and extracellular fractions, i.e., MC-Prop-TyrR [M + H]⁺ 1069.5 and [M + H]⁺ 1083.5 followed by MC-Phe-AzR resulting in [M + H]⁺ 1056.5 and [M + H]⁺ 1070.5 and MC-Prop-LysR resulting in [M + H]⁺ 1078.5 and [M + H]⁺ 1092.5 (Table 3). In contrast, for AP in *P. agardhii*, the non-AA Prop-Lys revealed the highest percentage of modified AP,

i.e., AP-Prop-Lys $[M + H]^+$ 891.5 followed by AP-Phe-Az $[M + H]^+$ 843.3 and AP-Prop-Tyr $[M + H]^+$ 882.5 (Table 3).

Regarding the time-lapse build up, MC-Prop-TyrR showed a steep proportional increase until 24–48 h (>60% of total MC), followed by a plateau phase, but continued to increase until day 7 (168 h), resulting in > 80% of the total MC content. The less abundant MC-Phe-AzR and MC-Prop-LysR are already saturated after 24 h at >20% or >3% of total MC content, respectively (Figure 1, Supplementary Figure S5). Conversely, during time-lapse decline, MC-Prop-TyrR decreased linearly from >40% down to 10% of total MC, while MC-Phe-AzR and MC-Prop-LysR showed only minor, albeit significant, decreases. Analogously to *M. aeruginosa*, AP-Prop-Lys in *P. agardhii* showed a steep increase during the first 24 h, reaching a plateau phase at 60% of the total AP content (Figure 2, Supplementary Figure S6), while AP-Phe-Az and AP-Prop-Tyr saturated after 24 h at >10% or >5%, respectively. During time-lapse decline, the most abundant AP-Prop-Lys decreased linearly in proportion, while in comparison to control cells, less decrease in the percentage of μ g AP-Prop-Lys per mg DW could be observed (Supplementary Figure S6).

Thus, intracellular synthesis of modified MC/AP was observed, reaching a plateau rather fast (24–48 h). The efficiency of incorporation of various non-AAs into the MC/AP molecule varied between cyanobacteria strains and/or synthesis pathways. Conversely, for time-lapse decline, the proportion of modified MC/AP decreased linearly. However, clickable MC/AP peptides were removed from the cells at a slower rate than their appearance during build up.

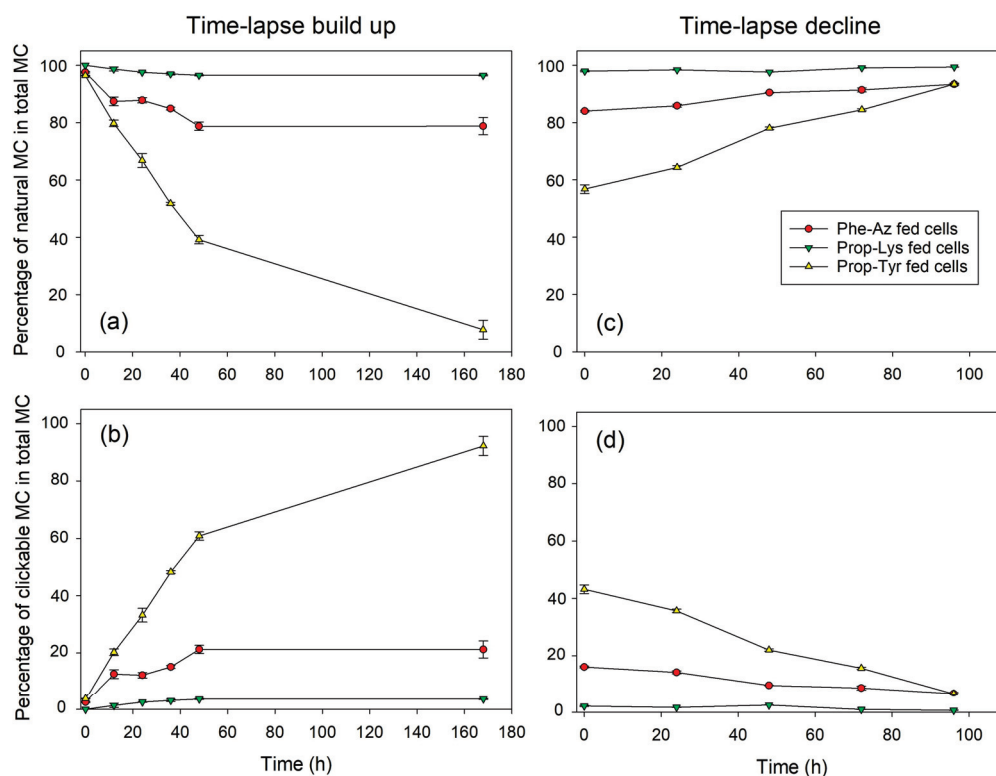


Figure 1. Mean ($\pm$ SE) proportion of natural and clickable MC in total MC (cellular fraction, composed of four MC structural variants: DAsp-MC-YR, MC-YR, DAsp-MC-LR, MC-LR) during time-lapse experiments using pulsed feeding of non-natural amino acids (non-AAs) in order to observe the build up (a,b) or decline (c,d) of azide- or alkyne-modified MC in *M. aeruginosa* strain Hofbauer. Control cells were grown and processed under identical conditions but without non-AA substrate and could not show any clickable MC synthesis.

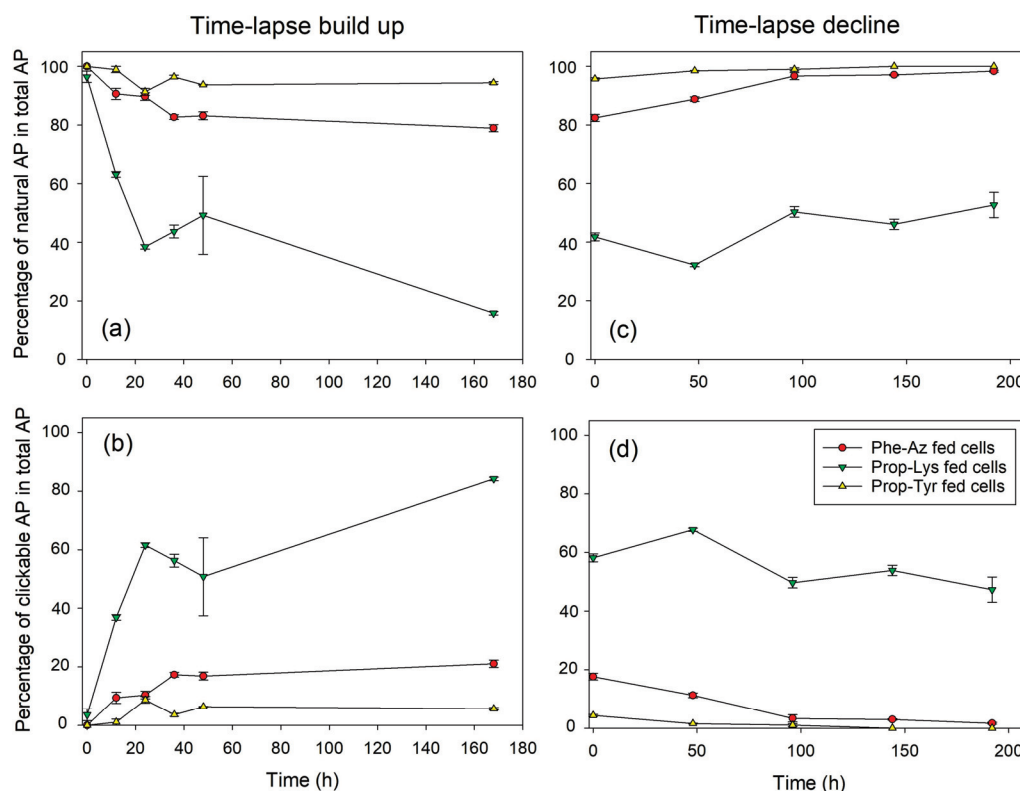


Figure 2. Mean ($\pm$ SE) proportion of natural and clickable AP in total AP (cellular fraction, composed of four AP structural variants: unknown AP, AP-C, AP-B, AP-A) during time-lapse experiments using pulsed feeding of non-natural amino acids (non-AAs) in order to observe the build up (a,b) or decline (c,d) of azide- or alkyne modified AP in *P. agardhii* strain no371/1. Control cells were grown and processed under identical conditions but without non-AA substrate and could not show any clickable MC synthesis.

2.3. Dissolved Fraction of Clickable and Natural MC/AP Peptides

In general, the ranking orders observed in the proportion of clickable MC/AP in the intracellular fraction, i.e., MC-Prop-TyrR > MC-Phe-AzR > MC-Prop-LysR in *M. aeruginosa* or AP-Prop-Lys > AP-Phe-Az > AP-Prop-Tyr in *P. agardhii* was the same as those found in the extracellular fraction (Table 3). The percentages of clickable and natural MC peptides in dissolved vs. intracellular fraction were compared for both time-lapse build up and decline experiments, and linear regression curves were calculated (Supplementary Figures S7 and S8). While the slope of linear curves ranged between $y = 1.52x$, $y = 0.52x$, and $y = 1.19x$, the coefficient of variation of regression curves varied between $R^2 = 0.94, 0.39, 0.8$ for MC-Prop-TyrR, MC-Prop-LysR, and MC-Phe-AzR, respectively. Thus, clickable MC proportion in the medium was found to be linearly related to the respective intracellular proportion and linear relationships did not differ significantly from natural MC-LR in slope (i.e., ANCOVA, interaction between grouping and covariate $p = 0.08, 0.64, 0.86$ for MC-Prop-TyrR, MC-Prop-LysR, and MC-Phe-AzR, respectively). Using the equal slope model in a one-way analysis of covariance (ANCOVA), a significant difference in intercept was found when comparing the adjusted group means of MC-Prop-Tyr vs. MC-LR ($p < 0.001$).

In contrast to MC variants in *M. aeruginosa*, for clickable AP variants AP-Prop-Tyr and AP-Phe-Az in *P. agardhii*, the slope of linear regression curves was dissimilar when comparing with regression curves for natural AP-A ($p = 0.58, p = 0.15$ for AP-Prop-Tyr and AP-Phe-Az, respectively), i.e., AP-Prop-Tyr ($R^2 = 0.48$), $y = 2.41x$ and AP-Phe-Az ($R^2 = 0.51$), $y = 0.84x$. For the most abundant AP-Prop-Lys, a significantly reduced slope of the regression line between intracellular vs. extracellular fraction was found ($R^2 = 0.83$, $y = 0.91x$) when compared with natural AP-A ($R^2 = 0.85$, $y = 2.13x$), as recorded from the

same peptide extract (ANCOVA, $p = 0.008$). In other words, the proportion of AP-Prop-Lys in the medium was found to be underrepresented by a factor of 2 when compared to the proportion of natural AP-A (Supplementary Figure S8). In summary, all modified MC/AP peptides could be quantified in the extracellular fraction, suggesting a more constant release procedure similar to their natural congeners.

2.4. Relationship of Clickable MC/AP Peptide Synthesis to Growth in Cyanobacteria Strains During Time-Lapse Experiments

Because of the variation in growth rate between non-AA treatments, it was important to find out whether growth or cell division rate could be related to clickable MC/AP net production rate. Thus, clickable MC or AP net production rates were calculated from intracellular MC-LR equivalents in ng/mL or AP-B equivalents in ng/mL by $\ln(x + 1)$ transformation and were plotted against the growth rate (day^{-1}) calculated from OD (Figure 3).

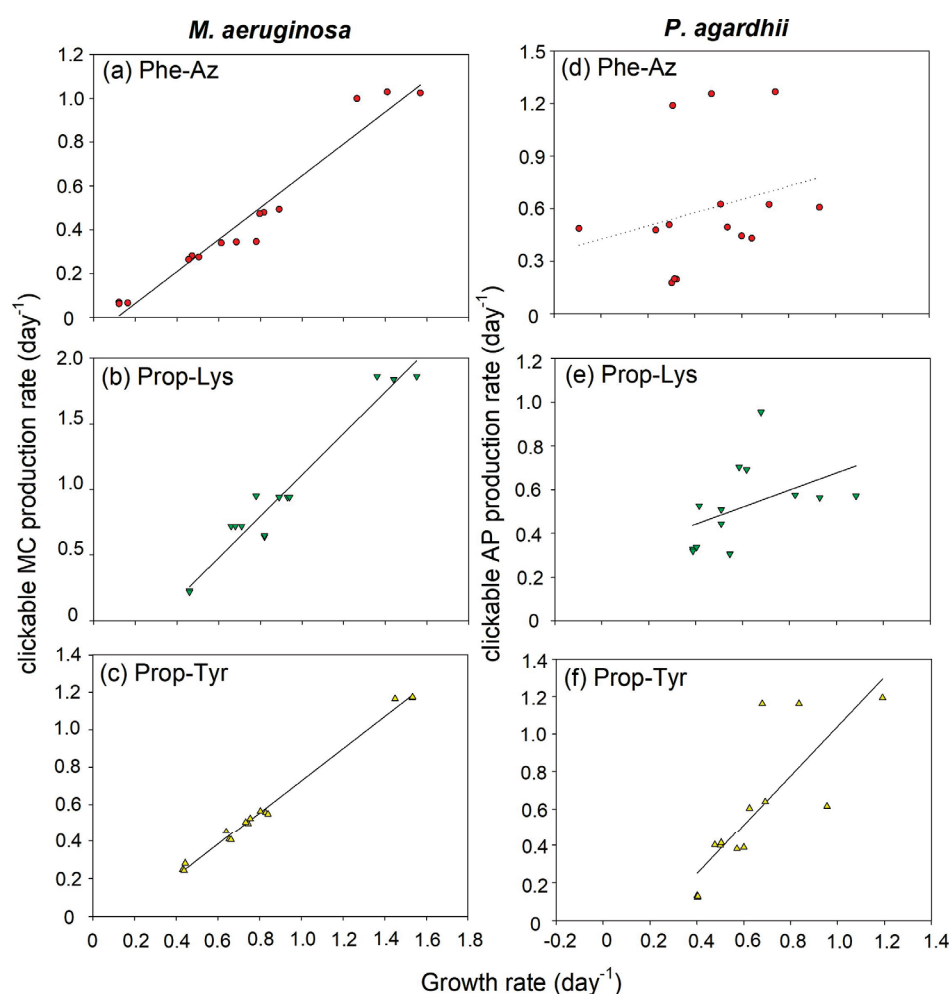


Figure 3. Relationship between growth rate (day^{-1}) and (a–c) the clickable MC net production rate (day^{-1}) in *M. aeruginosa* strain Hofbauer (calculated from $\ln(x + 1)$ MC-LR equivalents in ng/mL) and (d–f) the clickable AP net production rate (day^{-1}) in *P. agardhii* strain no371/1 (calculated from $\ln(x + 1)$ AP-B equivalents in ng/mL) during time-lapse experiments using feeding of non-natural amino acids (non-AA) in order to observe the build up of azide- or alkyne-modified MC/AP. Details of linear regression curves are as follows: (a) MC-Phe-AzR ($y = -0.08 + 0.73x$, $R^2 = 0.95$, $p < 0.0001$), (b) MC-Prop-LysR ($y = -0.47 + 1.58x$, $R^2 = 0.95$, $p < 0.0001$), (c) MC-Prop-TyrR ($y = -0.13 + 0.86x$, $R^2 = 0.99$, $p < 0.0001$), (d) AP-Phe-Az (not significant, $p = 0.33$), (e) AP-Prop-Lys ($y = 0.29 + 0.39x$, $R^2 = 0.21$, $p = 0.099$), (f) AP-Prop-Tyr ($y = -0.28 + 1.32x$, $R^2 = 0.65$, $p = 0.0005$), where y is MC/AP production rate (day^{-1}) and x is growth rate (day^{-1}).

Overall, the highest clickable MC/AP production rates were observed in the most rapidly growing cultures. In *M. aeruginosa*, for all non-AA treatments, a significant linear relationship between cell growth and the daily production of MC-Phe-AzR, MC-Prop-LysR and, MC-Prop-TyrR was found (Figure 3a–c). In *P. agardhii*, for AP-Phe-Az, the coefficient of determination R^2 was lowest and no significant slope in regression was found ($p = 0.33$), while for AP-Prop-Lys and AP-Prop-Tyr, marginally or highly significant linear relationships between cell growth and the daily production were obtained (Figure 3d–f). Thus, for Phe-Az in *P. agardhii*, the synthesis of AP-Phe-Az was not coupled to growth, implying that cells stopped growing after the synthesis of AP-Phe-Az. Notably, for *M. aeruginosa*, the integration of Phe-Az into MC reduced the growth, but had less impact on the principal relationship between clickable MC synthesis rate and the (reduced) daily cellular growth. In summary, an overall influence of cell growth on clickable MC/AP peptide net production via non-AA integration was observed, implying that the principal relationship between cell growth and MC/AP production is still applicable.

2.5. Relationship of Clickable MC/AP Peptide Content Decline and Growth in Cyanobacteria Strains During Time-Lapse Experiments

During the time-lapse decline experiments, overall, a linear decrease in clickable (intracellular) MC/AP in proportion to total MC/AP was observed over time. Using growth rates calculated from OD (see above), the theoretical decline of modified MC/AP recorded at T0 was calculated and compared to the observed decline as reported above (Figures 1d and 2d). If the decline in intracellular new MC/AP peptide is significantly faster than the theoretical decline through dilution by cellular growth, one may conclude on clickable MC/AP peptide breakdown or active vs. passive transport processes.

Two-Way Repeated Measures ANOVA was used to compare the observed and the theoretical estimates in the proportion of intracellular MC (Figure 4a–c) and AP (Figure 4d–f). The time factor was always found to be significant ($p < 0.001$) (i.e., 24–96 h for MC in *M. aeruginosa*). For MC-Phe-AzR, a much faster decline than predicted from the lowest cell division rate (Supplementary Figure S1) was observed, indicating potential cell lysis. In contrast, for MC-Prop-LysR and the most abundant MC-Prop-TyrR no significant difference between theoretical and observed proportions (the grouping factor) was detected ($p > 0.05$). Thus, even for the most abundant MC-Prop-TyrR, a linear decline during time course, mostly via cell division rate or cell growth, was concluded.

Analogously, for clickable APs, the time factor showed a significant decrease (i.e., 48–196 h in *P. agardhii*). For AP-Phe-Az and AP-Prop-Tyr, the corresponding theoretical AP proportions tended to be slightly increased, which was found to be marginally significant ($p = 0.02$ – 0.03). For AP-Prop-Lys, the intracellular proportion appeared to decline less rapidly when compared with other clickable MC/AP peptides (Figure 4e). The slower decline could be related to the fact that AP-Prop-Lys in the medium was found to be underrepresented 2-fold when compared to the proportion of natural AP-A (Supplementary Figure S8). In summary, the overall linear decrease in clickable MC/AP points to a rather constant decline via dilution by growth instead of a regulated or induced release during the synthesis process.

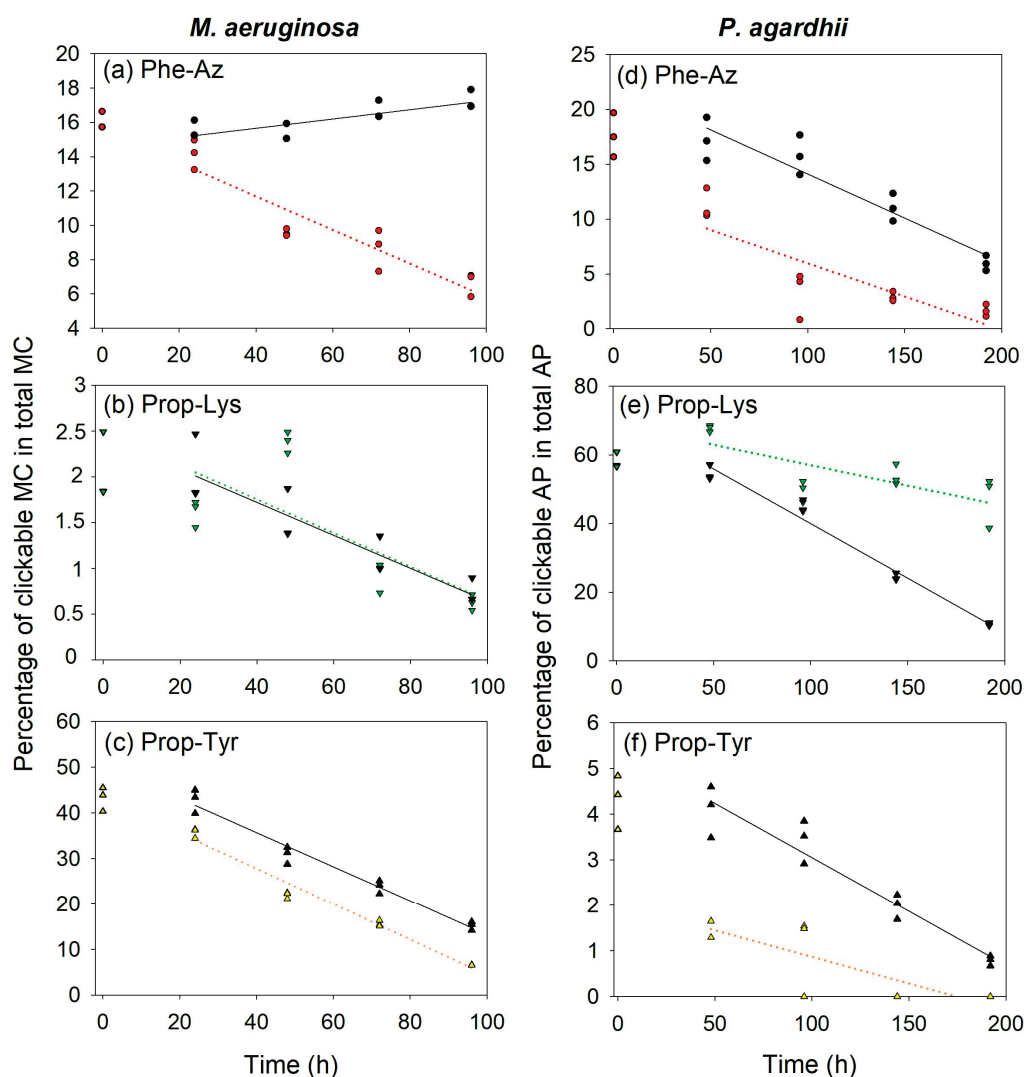


Figure 4. Proportion of individual clickable MC in *M. aeruginosa* (a–c) or clickable AP in *P. agardhii* (d–e) in total MC/AP (cellular fraction) during time-lapse experiments using pulsed feeding of non-natural amino acids (non-AAs) in order to observe the decline of (a) MC-Phe-AzR, (b) MC-Prop-LysR, (c) MC-Prop-TyrR in *M. aeruginosa*, or (d) AP-Phe-Az, (e) AP-Prop-Lys, (f) AP-Prop-Tyr in *P. agardhii* strain no371/1. Using growth rates, the theoretical decline of clickable MC/AP was calculated (black symbols, straight line) and compared to the observed decline (colored symbols, dotted line). Note that the scale at the y-axis is different, as production efficiency differs between non-AAs (Figures 1 and 2).

2.6. Fate of Non-Natural Amino Acids (Non-AAs) During Build Up and Decline

During all time-lapse build up and decline experiments, the quantity of the different non-AAs in the cellular and dissolved fraction was recorded in parallel to LC-MS peptide analysis. Using the same chromatographic separation conditions, the non-AAs, Phe-Az, Prop-Lys, and Prop-Tyr eluted at 5.4 min $[M + H]^+$ 207.5, 3.4 min $[M + H]^+$ 229.5, and 5.2 min $[M + H]^+$ 220.5, respectively. Typically, during the entire build up experiments, non-AAs were detected in the cellular fractions (Supplementary Figure S9). For *M. aeruginosa*, Prop-Tyr and Phe-Az decreased towards the end but remained available in cells, while Prop-Lys increased three-fold till the end of the experiment (168 h) (Supplementary Figure S9a). Similarly, for *P. agardhii*, Prop-Lys was increasing linearly by up to three-fold till the end of the experiment (168 h), while Prop-Tyr and Phe-Az were not detected.

Dissolved quantities of non-AA were, in general, one to two magnitudes higher than cellular contents and tended to decrease during build up experiments (Supplementary Figure S10). In contrast, during signal decline, dissolved non-AAs were not detected

in *M. aeruginosa*. In *P. agardhii*, dissolved non-AAs occurred at T0 but were reduced to undetectable from 48 h onwards (Supplementary Figure S10). Thus, at T0, non-AAs were removed by washing through centrifugation and resuspension of the cells in BG11 medium. In contrast, during build up experiments, non-AAs remained dissolved in the medium and were available in unlimited amounts.

3. Discussion

This study is the first to track MC/AP synthesis by using promiscuous adenylation (A) domains in non-ribosomal peptide synthesis (NRPS), allowing for the incorporation of clickable non-AAs into the peptide products. While the results of visualization using the clickable amino acids as fluorescent tags will be reported in a forthcoming paper, the monitoring of modified MC/AP synthesis alone can lead to a better understanding of natural product synthesis in bloom-forming cyanobacteria. The following insights were obtained and will be discussed:

3.1. Variable Efficiency of Clickable MC/AP Production Using Different Non-AAs

In this study, a rapid increase in most clickable MC/AP peptides was observed, reaching a plateau after 24–48 h (Supplementary Figures S5 and S6). Not all non-AAs were integrated into the new modified MC/AP peptide with the same efficiency. For *M. aeruginosa*, MC-Prop-TyrR was produced much more efficiently than MC-Prop-LysR or MC-Phe-AzR. For *P. agardhii*, AP-Prop-Lys was produced most efficiently, while both AP-Prop-Tyr and AP-Phe-Az were produced in lower amounts. In general, it is well-known that cyanobacteria uptake amino acids from the media [11]. Accordingly, during build up experiments, non-AAs have been detected in the cellular fraction consistently (Supplementary Figure S9) and it is unlikely that non-AA intracellular availability caused the variable efficiency of integration into the peptide product.

Instead, it is known that A domains in NRPS are the key enzymes enabling the integration of unnatural AA into the growing peptide [17,18]. However, activation of non-AA must be compatible with the essential subsequent catalytic reactions, such as the step of peptide bond formation, i.e., condensation (C) domains are also known to exhibit moderate to high substrate specificity [19]. For example, an erroneously loaded non-AA by an A domain, which is not accepted by the responsible C domain, would prevent peptide bond formation, resulting in the build up of intermediate peptide products [20]. Thus, it is likely that for *M. aeruginosa* strain Hofbauer, the most efficient integration of Prop-Tyr into pos. 2 of MC-YR or MC-LR can explain its highest share in total MC pool (>80%). In a similar vein, it can be assumed that for *P. agardhii* strain no371/1, the most efficient integration of Prop-Lys into AP peptide product is likely related to the highest share of AP-Prop-Lys (>80%) in the total AP pool.

3.2. Effects of Non-AAs on Cell Division or Growth in Study Organisms

In this study, three different non-AAs were tested in order to better describe the influence on MC/AP production and growth. It is generally understood that non-AAs with alkyne or azide moieties react very specifically with their reaction partner, i.e., without side reactions or negative influence on native biochemical processes and growth. The specificity of the reaction is one criterion to be considered as a bio-orthogonal reaction [21]. In this study, Phe-Az addition distinctly reduced growth for *M. aeruginosa* as well as *P. agardhii* from the beginning (Tables 1 and 2, Supplementary Figures S1 and S2). It should be noted that Phe-Az (4-azido-L-phenylalanine) is one of the most versatile non-AAs in common use, i.e., it is widely applied for protein labeling through bio-orthogonal reactions [22]. On the other hand, Phe-Az has also been described as reactive via decomposition that may cause intracellular damage by reactive nitrenes, causing structural rearrangement of proteins or even crosslinking [23]. Nevertheless, despite this inherent reactivity, a significant positive relationship between MC-Phe-AzR production and cell division rate was observed for *M. aeruginosa* (Figure 3a). In contrast, as previously observed [16], *P. agardhii* cells appeared

more negatively affected, filaments were shorter and looked disintegrated and only a weak relationship between AP-Phe-Az production and cell division could be observed (Figure 3d). In general, a positive relationship between the production of MC (and other toxins) and cell division rate in individual strains has been postulated by authors Orr and Jones [6], suggesting that although MCs and other toxins (such as anatoxin a) are secondary metabolites the MC net production depends primarily on the cellular growth rate. Thus, environmental conditions influence MC production rather indirectly via the cellular growth rate. Although this significant interdependence is not understood on a cellular level, it fits to the overall consistent MC production in strain cultures observed regularly under laboratory conditions, even when severe physiological stress conditions have been investigated [24]. In summary, a high degree of interdependence between non-AA containing clickable MC/AP production and cellular growth has been observed, implying that the addition of non-AAs (even if available in excess amounts) did not overrule the tight coupling between primary and secondary metabolism during maximum growth rate conditions.

3.3. Decrease in Targeted MC/AP Content During Time-Lapse Experiment

Overall, a rather linear decrease in the proportion of clickable MC/AP was observed over time. Cells were grown as clonal strain cultures at maximum growth rate under the specified semi-continuous culture conditions, following the turbidostatic principle [25]. Thus, the influence of physiological stress or cell lysis was considered of minor importance [26]. At the onset of this study, it was hypothesized that if the decline in clickable MC/AP labels would occur faster than expected from the theoretical decline through dilution by cellular growth, one may infer intracellular processing or active/passive release processes into the medium. This was observed for MC-Phe-AzR, implying that the decline of clickable MC occurred actively/passively under conditions when cell division was very much reduced. However, the linearity of the decrease implies a constant release process instead of a regulated or induced release in the course of the synthesis pathway (Figure 4).

On the other hand, the intracellular proportion of clickable AP-Prop-Lys appeared to decline less rapidly than observed for the MC-Prop-LysR peptides (Figure 4). Accordingly, the clickable AP-Prop-Lys peptides occurred with a lower proportion in the medium when compared to its natural congener AP-A (Supplementary Figures S7 and S8). These results would imply that clickable APs were retained in the cell to some extent and not readily released into the medium as was observed for natural AP-A. Whether such a delay in the release of clickable APs into the medium can be confirmed by subcellular localization via peptide labeling remains to be proved. Preliminary results on peptide labeling using the above-mentioned chemo-selective reaction revealed an increased concentration of signal intensity near the membrane region of the cells (author's unpublished data). If such a mechanism of intracellular compartmentalization can be confirmed via imaging analysis it would constitute an interesting observation on the intracellular enrichment of AP peptide before its release into the medium.

4. Conclusions

In summary, chemical-analytical methods quantitatively show the targeted synthesis of MC/AP peptides by incorporating an alkyne or azide side chain, which then enables visualization via chemo-selective reaction for linking fluorophores. The variable efficiency in clickable MC/AP production probably can be linked to the varying degree of promiscuity in accepting non-AA substrates by corresponding NRPS. The tight coupling between primary metabolism, i.e., cell division and growth with secondary metabolism, i.e., MC synthesis, can explain why clickable MC production is still related to growth even if the growth rate is significantly reduced (i.e., by Phe-Az). Conversely, the rather linear decrease in clickable MC/AP proportion in total MC/AP content with growth implies that dilution by growth is still the major responsible force in intra- and extracellular dynamics of clickable MC/AP synthesis.

5. Materials and Methods

5.1. Study Organisms and Growth Conditions

Precultures of clonal *Microcystis aeruginosa* strain Hofbauer (isolated from Neusiedlersee, Austria in 1982) and *Planktothrix agardhii* no371/1 (isolated from Moose Lake, Alberta, Canada in 2005) were maintained in a semi-continuous culture condition in BG11 medium [27] at 20 °C and photosynthetically active radiation of 50 $\mu\text{E m}^{-2} \text{s}^{-1}$ (using fluorescent lamps Master TL-D 90 De Luxe 36W/965, Philips, Vienna, Austria) following the turbidostatic principle [3]. Under our culture conditions, *M. aeruginosa* strain Hofbauer grew in single cells only, while *P. agardhii* strain no371/1 continued to grow in long filaments (e.g., hundreds of micrometers in length). All experiments were performed in the same dedicated climate chamber under the specified conditions. During the experiments (i.e., years 2021–2022), the temperature ranged between 18.4 and 20.6 °C (mean 19.9 ± 0.1 , $n = 31$), while irradiation varied on the shelf between 36 and 58 $\mu\text{E m}^{-2} \text{s}^{-1}$ (mean 47 ± 2 , $n = 31$). In general, precultures (40 mL culture volume in 250 mL transparent cell culture flasks, CytoOne T-75, Starlab, Vienna, Austria) were started from cultures maintained at maximum growth rate conditions until they reached an optical density ($\text{OD}_{600\text{nm}}$) = 0.1 (1 cm cuvette, BioSpectrometer basic, Eppendorf AG, Vienna, Austria), then diluted to OD = 0.01 and 0.025 for *M. aeruginosa* and *P. agardhii*, respectively. As described, OD = 0.01 was equal to 662 ± 265 filaments/mL of *P. agardhii* strain no371/1 or $62,866 \pm 29,059$ cells/mL of *M. aeruginosa* strain Hofbauer [9]. Growth rates were calculated from OD or dry weight (DW) using the following formula: $\mu (\text{day}^{-1}) = (\ln \text{OD}_{t+1} - \ln \text{OD}_t) / \Delta t$, where $\ln$ is the natural logarithmic function and Δt is the time in days.

The overall workflow is presented in Figure 5. For both time-lapse signal build up experiments, three technical replicates were inoculated for each time point (T0–T5) under identical conditions. At T0, treatments were supplemented once with L-isomeric non-AAAs, i.e., either 4-azidophenylalanine (Phe-Az), (Carl Roth, Karlsruhe, Germany), N-propargyloxy-carbonyl-L-lysine (Prop-Lys), (Sichem, Bremen, Germany), or O-propargyl-L-Tyrosine (Prop-Tyr), (Iris Biotech GmbH, Marktredwitz, Germany) (Figure 6), diluted in 20 mM NaOH, resulting in 0.05 mM final non-AA concentration (adding 100 μL of stock solution of 20 mM non-AA solution into 40 mL of culture volume). Control cell cultures were grown in the absence of non-AAAs but supplemented with 100 μL of 20 mM NaOH only. For T0, the cells were harvested directly after adding the non-AA to the medium within 1 h. In order to observe the incorporation of the non-AA into the cyanobacterial peptides, culture flasks were harvested every 12 h for the first 48 h, with a final harvesting at 168 h.

Analogously, to observe the time-lapse decline of the peptide signal, the precultures were diluted to an OD = 0.01 and 0.05 for *M. aeruginosa* and *P. agardhii*, respectively. Three technical replicates were inoculated per strain and grown for 48 h in the presence or absence of the three non-AAAs, as described above (Phe-Az, Prop-Lys, Prop-Tyr). At T0, following the 48 h incubation, cultures were centrifuged at 4000 rpm for 5 min and washed with sterile BG11 medium under sterile conditions. Cultures were centrifuged again at 4000 rpm for 5 min, resuspended in fresh non-AA free sterile BG11 medium, and transferred to fresh culture flasks. For T0, cells were harvested directly after washing and transferring into fresh BG11 medium within 1 h. Time points T1–T4 were every 24 h for *M. aeruginosa* and every 48 h for *P. agardhii*. The manipulation of cells was carried out at room temperature.

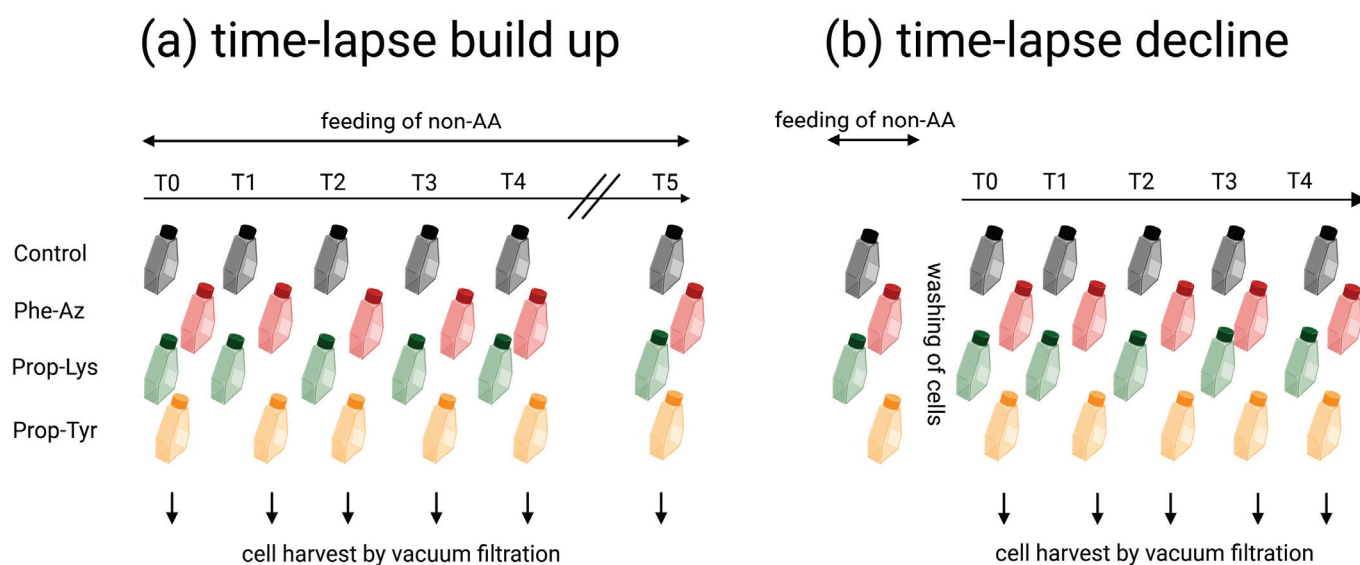


Figure 5. Workflow of time-lapse experiments using pulsed feeding of non-AAs for real-time observation of clickable MC/AP synthesis in bloom-forming cyanobacteria (the workflow was the same for both *M. aeruginosa* and *P. agardhii*): (a) time-lapse build up experiments; (b) time-lapse decline experiments. Created with BioRender.com. Note that the labeling of clickable peptides via chemo-selective reaction with fluorophore and high-resolution microscopy and flow-cytometry analysis using Alexa Fluor488 will be reported in a follow-up article.

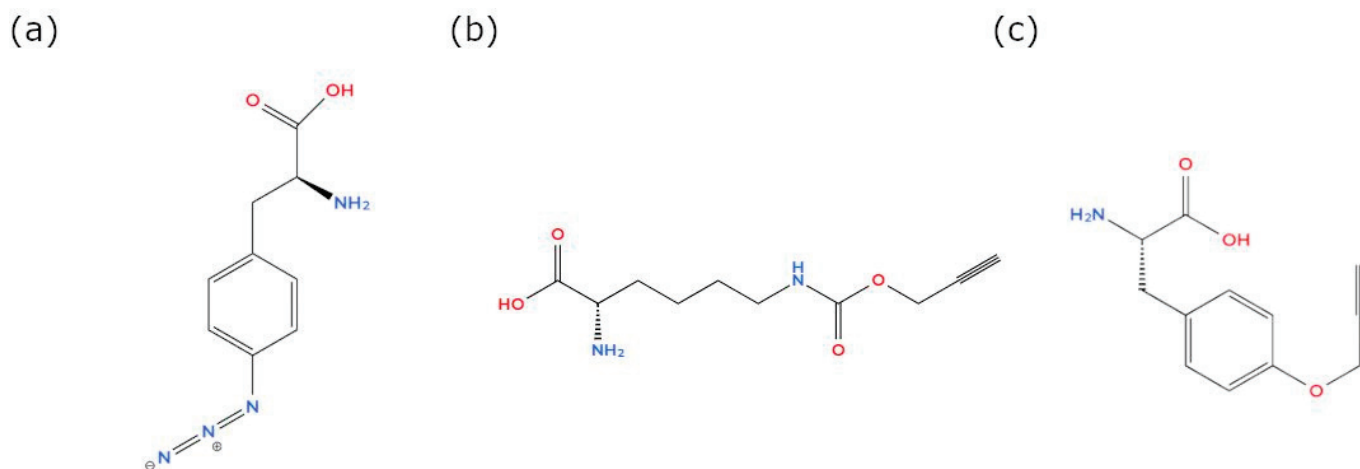


Figure 6. Chemical structures of non-AA molecules used for clickable microcystin (MC) synthesis in *M. aeruginosa* and for clickable anabaenopeptin (AP) synthesis in *P. agardhii*: (a) 4-Azido-L-phenylalanine (Phe-Az, MW 206.20 g/mol), (b) N-Propargyl-L-Lysine (Prop-Lys, MW 228.25 g/mol), (c) O-Propargyl-L-tyrosine (Prop-Tyr, MW 219.24 g/mol).

5.2. Cell Harvesting and Fixation

At T0–T4 (T5), cells from culture flasks (40 mL culture volume) were harvested via low-vacuum filtration using pre-weighed glass fiber filters (GF/C, Ø = 2.5 cm, ROTILABO® Typ CR261, Carl Roth, Karlsruhe, Germany). The filters with the collected biomass were dried using a vacuum centrifuge at RT for 4 h and dry weight was determined. Filters were stored frozen (−20 °C) until peptide extraction.

For each sample, the extracellular MC and AP peptide concentrations were determined from GF/C filtrate using standard solid phase extraction (SPE) [28]. Briefly, C18 columns (Sep-Pak® Vac tC18 cartridge, 1 cc/100 mg, 37–55 µm, Waters Corporation, Vienna, Austria) were conditioned with 3 mL of 100% methanol and equilibrated using 3 mL of BG11

medium according to the manufacturer's instructions. Subsequently, the GF/C filtrate was allowed to flow through the column at one drop per second and the dry column was stored frozen ($-20\text{ }^{\circ}\text{C}$) until eluting with 1 mL of 80% (*v/v*) methanol for HPLC-MS analysis.

5.3. Peptide Extraction and HPLC-MS Analysis

The intracellular peptide content was extracted from the dried biomass using aqueous methanol on ice, as described previously [3]. In brief, the filters carrying the algal biomass were dispersed with a pistil in the reaction tube and incubated in aqueous 50% (*v/v*) methanol (1.5 mL volume). The extracts were shaken on ice (30 min) and centrifuged, and the clear supernatant was transferred into new 2 mL reaction tubes, which were evaporated to dryness in a vacuum concentrator (no heating). This procedure was repeated two times to ensure efficient peptide extraction. After complete evaporation of the solvent (4.5 mL), the extract was stored frozen.

For Liquid Chromatography—Mass Spectrometry (LC-MS), the dried extracts were resuspended using 150 μL of 100% methanol. After complete resuspension of the pellet, 150 μL of MilliQ (MQ) water was added. Extracts were centrifuged and from the clear supernatants, 100 μL was injected into the HPLC-DAD (HP1100, Agilent, Vienna, Austria). Peptides were chromatographically separated using a LiChrospher[®] 100, octyldecyl silane (ODS), 5 μm particle size, LiChroCART[®] 250-4 HPLC cartridge system (Merck, Darmstadt, Germany) using a linear water/acetonitrile (0.05% trifluoroacetic acid) gradient from 80:20 to 50:50 in 45 min at a flow rate of 1 mL/min and at $30\text{ }^{\circ}\text{C}$ oven temperature.

In order to quantify the extracellular peptide composition, the C18 columns were eluted with 1 mL of 80% (*v/v*) methanol, evaporated to dryness using the vacuum concentrator, and re-dissolved in 110 μL of methanol. Finally, 100 μL was injected under identical chromatographic conditions as for the intracellular peptide extract. Recovery tests using 50 ng of MC-RR $[\text{M} + \text{H}]^{+}$ 1038.5, MC-YR $[\text{M} + \text{H}]^{+}$ 1045.5, and MC-LR $[\text{M} + \text{H}]^{+}$ 995.5 analytical standards (Simris Biologics, Berlin, Germany) revealed recoveries of $83 \pm 3\%$ (SE), $64 \pm 11\%$, $73 \pm 8\%$ using UV DAD at 240 nm, respectively. For MS peak area (base peak chromatograms, BPC), the recovery results obtained in parallel were $48 \pm 11\%$, $160 \pm 36\%$, and $61 \pm 13\%$ for MC-RR, MC-YR, and MC-LR, respectively.

The HPLC system was coupled to an ESI-MS (Electrospray Ionization Mass Spectrometer) ion trap (amaZon SL Ion Trap MS, Bruker Daltonik, Bremen, Germany) and operated in positive ionization mode. Nitrogen and helium were used as sheath gas and collision gas, respectively (43 psi, 9 L/min, $250\text{ }^{\circ}\text{C}$), with 5 kV capillary voltage. MC and AP variants were assigned according to their retention time, protonated mass $[\text{M} + \text{H}]^{+}$, and automatically recorded fragmentation patterns for MCs [29,30] or APs [31,32]. Under these conditions, the limit of detection for analytical standards MC-RR $[\text{M} + \text{H}]^{+}$ 1038.5, MC-YR $[\text{M} + \text{H}]^{+}$ 1045.5, and MC-LR $[\text{M} + \text{H}]^{+}$ 995.5 was 10 ng per injection (Simris Biologics, Berlin, Germany). To compare the total MC/AP content, all peptides were quantified in equivalents of either AP-B $[\text{M} + \text{H}]^{+}$ 837.5 or MC-LR. According to the linear regression curves $y = 1 \times 10^7x + 1 \times 10^8$ and $y = 2 \times 10^7x - 4 \times 10^8$, y was the integrated area in the base peak chromatogram (BPC), and x was ng of AP-B (1–270 ng) or MC-LR (1–180 ng) injected [9]. In order to test peptide quantification, both UV peak area (at 240 nm for MC or 210 nm for AP) and MS peak area (base peak chromatograms, BPC or extracted ion chromatograms, EIC) were compared: For MC total BPC (EIC) area vs. total UV area correlated by $R^2 = 0.90$ ($y = 6 \times 10^{-7}x$) and $R^2 = 0.98$ ($y = 8 \times 10^{-7}x$), signal build up, and signal decline, respectively, where y is UV_{240} peak area and x is BPC peak area. Similarly, for AP total BPC (EIC) area vs. total UV area correlated by $R^2 = 0.95$ ($y = 6 \times 10^{-7}x$) and $R^2 = 0.96$ ($y = 4 \times 10^{-7}x$) for signal build up and signal decline, respectively (Supplementary Figure S11). To quantify MC/AP modification, the BPC (EIC) peaks were manually integrated to express the individual peak area in the percentage of the total manually integrated peak area. For LC-MS the Bruker Compass DataAnalysis version 4.2 was used.

5.4. Statistical Analysis

For comparing growth data and clickable MC/AP contents, the two-way repeated measures analysis of variance (two-way RM ANOVA) was used (grouping factor: three non-AA treatments and one control; time factor: T0–T4 (T5), three replicates). The time factor was always found to be significant ($p < 0.001$). Assumptions on normality (Shapiro-Wilk) or equality of variance (Brown-Forsythe) were sometimes not met ($p < 0.05$). Since ANOVA test results are generally considered to be robust if sample sizes between treatments are equal, the statistical test results were still reported. For the pairwise multiple comparison procedure, the Bonferroni t -test ($p < 0.05$) was used.

For comparing growth with peptide production, cellular growth rates (calculated from OD at T0) were calculated using the formula $y = (\ln(x)_{t+1} - \ln(x)_{t0})/\Delta t$ where y is the growth rate (day^{-1}), x is the OD, and Δt is the time in days ($t + 1 - t_0$). Analogously, the clickable MC or AP net production rates were calculated from intracellular MC/AP using the following formula: clickable MC/AP production rate (day^{-1}) = $(\ln(x + 1)_{t+1} - \ln(x + 1)_{t0})/\Delta t$, where x is either MC-LR equivalents in ng/mL or AP-B equivalents in ng/mL. The linear regression curves (Figures 3 and 4) were calculated and tested according to standard linear regression analysis.

The linear regression curves between the proportion of intracellular (independent variable) vs. extracellular (dependent) MC/AP peptides were compared in terms of slope using one-way analysis of covariance (ANCOVA) via the equality of slopes model, which tested the assumption that there is no interaction between the grouping factor (i.e., modified MC/AP vs. natural MC/AP) and the covariate (intracellular proportion of corresponding MC/AP peptide). If the equal slope model was passed, the equality of the intercepts was tested by comparing the adjusted group means via pairwise multiple comparison (Holm-Sidak, $p < 0.05$). All tests were performed using Sigma plot version 14.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/toxins16120526/s1>. Supplementary Figure S1: Mean ($\pm$ SE) optical density of strain cultures of (a,c) *M. aeruginosa* strain Hofbauer and (b,d) *P. agardhii* strain no371/1 during time-lapse experiments using pulsed feeding of non-natural amino acids (non-AAs) under maximum growth rate conditions in order to observe the build up (a,b) or decline (c,d) of azide- or alkyne-modified MC or AP peptides; Supplementary Figure S2: Mean ($\pm$ SE) dry weight (mg/mL) of strain cultures of (a,c) *M. aeruginosa* Hofbauer and (b,d) *P. agardhii* no371/1 during time-lapse experiments using pulsed feeding of non-natural amino acids (non-AAs) under maximum growth rate conditions in order to observe the build up (a,b) or decline (c,d) of azide- or alkyne-modified MC or AP peptides; Supplementary Figure S3: Mean ($\pm$ SE) cellular concentration of (a,d) total, (b,e) natural, and (c,f) clickable MC in ng of MC-LR equivalents/mL (composed of four MC structural variants: DAsp-MC-YR, MC-YR, DAsp-MC-LR, MC-LR) during time-lapse experiments using pulsed feeding of non-natural amino acids (non-AAs) in order to observe the increase (c) or decline (f) in azide- or alkyne-modified MC in *M. aeruginosa* strain Hofbauer; Supplementary Figure S4: Mean ($\pm$ SE) cellular concentration of (a,d) total, (b,e) natural, and (c,f) clickable AP in ng of AP-B equivalents/mL (composed of four AP structural variants: AP (unknown), AP-C, AP-B, AP-A) during time-lapse experiments using pulsed feeding of non-natural amino acids (non-AAs) in order to observe the increase (c) or decline (f) in azide- or alkyne-modified AP in *P. agardhii* strain no371/1; Supplementary Figure S5: Mean ($\pm$ SE) cellular content of natural and clickable MC in percentage of control in *M. aeruginosa* strain Hofbauer (ng MC-LR equiv./mg DW, composed of four MC structural variants: DAsp-MC-YR, MC-YR, DAsp-MC-LR, MC-LR) during time-lapse experiments using pulsed feeding of non-natural amino acids (non-AAs) in order to observe the build up (a,b) or decline (c,d) of azide- or alkyne-modified MC. (Control = cells grown and processed under identical conditions but without non-AA substrate); Supplementary Figure S6: Mean ($\pm$ SE) cellular proportion of natural and clickable AP in total AP in percentage of control in *P. agardhii* strain no371/1 (ng AP B equiv./mg DW, composed of four AP structural variants: AP unknown, AP-C, AP-B, AP-A) during time-lapse experiments using pulsed feeding of non-natural amino acids (non-AAs) in order to observe the build up (a,b) or decline (c,d) of azide- or alkyne-modified AP. (Control = cells grown and processed under identical conditions but without non-AA substrate); Supplementary Figure S7: Percentage of clickable and natural MC peptides in dissolved vs. intracellular fraction in *M.*

aeruginosa strain Hofbauer during time-lapse experiments using pulsed feeding of non-natural amino acids (non-AAAs) in order to observe the build up (open symbols) or decline (closed symbols) of azide- or alkyne-modified MC. Details of linear regression curves: (a) MC-Prop-TyrR ($R^2 = 0.94$), $y = 1.5186x$; (b) MC-LR (Prop-Tyr fed cells), ($R^2 = 0.87$), $y = 0.856x$; (c) MC-Prop-LysR ($R^2 = 0.39$), $y = 0.523x$; (d) MC-LR (Prop-Lys fed cells), ($R^2 = 0.99$), $y = 0.9467x$; (e) MC-Phe-AzR ($R^2 = 0.8$), $y = 1.1864x$; (f) MC-LR (Phe-Az fed cells), ($R^2 = 0.91$), $y = 0.9185x$, where y was percentage of extracellular MC in total extracellular MC and x was percentage of intracellular MC in total intracellular MC. The dotted line represents the 1:1 reference line; Supplementary Figure S8: Percentage of clickable and natural AP peptides in dissolved vs. intracellular fraction in *P. agardhii* strain no371/1 during time-lapse experiments using pulsed feeding of non-natural amino acids (non-AAAs) in order to observe the build up (open symbols) or decline (closed symbols) of azide- or alkyne-modified AP. Details of linear regression curves: (a) AP-Prop-Tyr ($R^2 = 0.48$), $y = 2.4073x$; (b) AP-A (Prop-Tyr fed cells), ($R^2 = 0.9$), $y = 1.78x$; (c) AP-Prop-Lys ($R^2 = 0.83$), $y = 0.913x$; (d) AP-A (Prop-Lys fed cells), ($R^2 = 0.85$), $y = 2.133x$; (e) AP-Phe-Az ($R^2 = 0.51$), $y = 0.842x$; (f) AP-A (Phe-Az fed cells), ($R^2 = 0.91$), $y = 2.542x$, where y was percentage of extracellular AP in total extracellular AP and x was percentage of intracellular AP in total intracellular AP. The dotted line represents the 1:1 reference line; Supplementary Figure S9: Mean $\pm$ SE cellular content of non-AAAs (Phe-Az, Prop-Lys, Prop-Tyr) eluting at 3.4 min (Prop-Lys), 5.2 min (Prop-Tyr), 5.4 min (Phe-Az) quantified in μg of non-AA per mg of DW in peptide extract during time-lapse experiments using pulsed feeding of non-AAAs in order to observe the build up (a,c) or decline (b,d) of azide- or alkyne-modified MC/AP peptide in *M. aeruginosa* (a,b) and *P. agardhii* (c,d); Supplementary Figure S10: Mean $\pm$ SE dissolved quantity of non-AAAs (Phe-Az, Prop-Lys, Prop-Tyr) eluting at 3.4 min (Prop-Lys), 5.2 min (Prop-Tyr), 5.4 min (Phe-Az) quantified in μg of non-AA per mg of DW during time-lapse experiments using pulsed feeding of non-AAAs in order to observe the build up (a,c) or decline (b, d) of azide- or alkyne-modified MC/AP peptide in *M. aeruginosa* (a,b) and *P. agardhii* (c,d). Explanation for missing data in (a): No SPE measurements were performed at T1 (12 h); Supplementary Figure S11: Comparison of total MC (a) or (b) AP peptide quantification using either MS peak area (base peak chromatograms, BPC or extracted ion chromatograms, EIC) or UV peak area (at 240 nm for MC or 210 nm for AP) during all time-lapse experiments. In (b), three outliers (white triangles) were omitted from calculating the regression line for build up experiments.

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Article

Design of a Duplex-to-Complex Structure-Switching Approach for the Homogeneous Determination of Marine Biotoxins in Water

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Abstract: In this report, we describe a fluorescent assay for the detection of six marine toxins in water. The mechanism of detection is based on a duplex-to-complex structure-switching approach. The six aptamers specific to the targeted cyanotoxins were conjugated to a fluorescent dye, carboxyfluorescein (FAM). In parallel, complementary DNA (cDNA) sequences specific to each aptamer were conjugated to a fluorescence quencher BHQ1. In the absence of the target, an aptamer–cDNA duplex structure is formed, and the fluorescence is quenched. By adding the toxin, the aptamer tends to bind to its target and releases the cDNA. The fluorescence intensity is consequently restored after the formation of the complex aptamer–toxin, where the fluorescence recovery is directly correlated with the analyte concentration. Based on this principle, a highly sensitive detection of the six marine toxins was achieved, with the limits of detection of 0.15, 0.06, 0.075, 0.027, 0.041, and 0.026 nM for microcystin-LR, anatoxin- α , saxitoxin, cylindrospermopsin, okadaic acid, and brevetoxin, respectively. Moreover, each aptameric assay showed a very good selectivity towards the other five marine toxins. Finally, the developed technique was applied for the detection of the six toxins in spiked water samples with excellent recoveries.

Keywords: marine toxins; aptamers; fluorescence; quenching; water

Key Contribution: In this report, we describe an aptameric assay for the homogeneous detection of six marine toxins in water. The principle of the assay is based on a FAM-labelled aptamer and its complementary DNA conjugated to the fluorescence quencher BHQ1. In the absence of the toxin, the aptamer hybridizes to the cDNA and the fluorescence is blocked. By adding the toxin, the aptamer binds to its target and releases the cDNA, restoring thus the fluorescence intensity. excellent analytical performance was achieved for the six marine toxins. The breakthroughs or highlights of the manuscript. Authors can write one or two sentences to describe the most important part of the paper.

1. Introduction

Cyanobacteria, also called blue-green algae, are photosynthetic microorganisms found in water bodies worldwide [1]. While they are essential to many aquatic ecosystems, some cyanobacteria species produce potent toxins, known as cyanotoxins, which threaten human and animal health [2]. Eutrophication, driven by nutrient pollution, particularly

phosphorus and nitrogen, is a major factor contributing to cyanobacterial blooms [3]. These blooms can lead to hypoxia, loss of biodiversity, and the production of large quantities of toxins [4]. Cyanotoxins can be found in drinking water, recreational activities, and contaminated food, including fish and seafood [5]. Humans and animals exposed to these toxins can suffer from various forms of damage, including hepatotoxicity, nephrotoxicity, and neurotoxicity [6].

Cyanotoxins are classified according to their chemical structure and biological effects. The most well-studied cyanotoxins include microcystins: cyclic heptapeptides produced by various cyanobacterial species such as *Microcystis*, *Anabaena*, and *Planktothrix* [7]. Microcystins are potent inhibitors of protein phosphatases, leading to liver damage and promoting tumor growth even at low concentrations [8]. Microcystin-Leucine-Arginine (MC-LR) is the most toxic and commonly studied variant [9]. Cylindrospermopsins (CYN), also known for their hepatotoxicity, are commonly produced by *Cylindrospermopsis raciborskii* and *Aphanizomenon ovalisporum* [10]. In parallel, it has been demonstrated that CYN has cytotoxic and genotoxic effects [11]. Besides hepatotoxicity, some cyanotoxins classes have neurotoxic effects [12]. Anatoxins (ANTXs) act as potent agonists at nicotinic acetylcholine receptors, leading to paralysis and potentially fatal respiratory failure [13]. Anatoxins, including anatoxin-a, anatoxin-a (S), and homoanatoxin-a are produced by *Anabaena*, *Oscillatoria*, and *Aphanizomenon* species [14]. Known as paralytic shellfish compounds, saxitoxins (STXs) are also neurotoxic, able to block sodium channels in nerve cells [15]. Finally, brevetoxins (BTXs), lipophylic polyether toxins, have been also reported as neurotoxic cyanotoxins. They are produced by the marine dinoflagellate *Gymnodinium breve* [16,17]. In addition to cyanotoxins, okadaic acids (OAs) constitute a very important class of marine biotoxins worldwide. OAs are causative agents of diarrhetic seafood poisoning, produced by microalgae, *dinoflagellates* *Dinophysis* spp. and *Prorocentrum* spp. [18]. Okadaic acid was also considered a potential inhibitor of phosphatases 1 and 2A, causing the hyperphosphorylation of tau protein, leading to Alzheimer's disease [19].

Detecting these toxins is crucial for ensuring the safety of drinking water, recreational water, and seafood. Various techniques have been developed to detect cyanobacteria toxins, each with its own strengths and limitations [20]. Toxin bioassays involve the use of living organisms, such as fish, mice, or cultured cells, to detect the presence of cyanotoxins based on their toxic effects [21,22]. While bioassays can provide information on the overall toxicity of a sample, they are not specific to individual toxins and raise ethical concerns due to the use of live animals [23]. Assays based on whole cells, which monitor the cytotoxic effects of toxins on cultured cells, offer a more ethical and specific alternative, though they still lack the precision of other methods such as immunoassays or chromatography and mass spectrometry techniques [24]. Immunochemical techniques, such as enzyme-linked immunosorbent assay (ELISA), are widely used for the detection of cyanotoxins. These assays are based on the specific binding of antibodies to cyanotoxins, which can be quantified to determine toxin concentrations. ELISA is relatively simple, cost-effective, and can be used for high-throughput screening [25]. However, immunoassays may sometimes lack specificity due to cross-reactivity with similar compounds, which can lead to false positives or negatives [26]. Chromatographic techniques such as HPLC (high-throughput liquid chromatography) and LC-MS (liquid chromatography coupled to mass spectrometry) are also used to separate and quantify marine toxins. LC-MS is a highly sensitive and specific technique used for detecting and quantifying multiple cyanotoxins simultaneously. Toxins are separated according to their chemical properties by liquid chromatography, then identified and quantified based on their mass-to-charge ratio using mass spectrometry [27]. Despite its sensitivity, LC-MS is also expensive, requires specialized equipment and expertise, and is not well suited for rapid field testing. Polymerase chain reaction (PCR) techniques have been used for detecting cyanobacteria by targeting their genomes or specific genes involved in toxin production, such as the *mcy* gene cluster responsible for microcystin biosynthesis [28,29]. PCR is valuable for early warning and monitoring purposes; however, it requires specialized equipment and technical expertise.

In addition to PCR-based detection methods, nucleic acids have attracted great interest in the field of biosensing. They can be used in DNA hybridization biosensors targeting a complementary DNA or RNA. In parallel, they can be used as bioreceptors called ‘aptamers’ in affinity-based biosensors to target different classes of molecules, cells, or microorganisms [30]. Aptamers are synthetic oligonucleotides generated by the SELEX process, and are characterized by their high affinity, specificity, and stability [31]. Their easy synthesis and outstanding binding properties make them a good alternative for antibodies in modern bioanalysis [32]. Various aptamers have been generated for a wide range of molecules, including marine toxins [33]. Given the chemical nature of aptamers, the latter can be labeled with different probes and chemical groups, allowing their use with electrochemical and optical detection methods [34]. Recently, we reported an electrochemical aptasensing platform for the simultaneous determination of five cyanotoxins in water [35]. It is worth noting that many cyanotoxins are usually present in the same sample, and multiplex detection is of great importance for ensuring water safety. Herein, we developed a fluorescent aptamer-based assay for the detection of six important marine toxins present in water (MC-LR, ANTX, SXT, CYN, OA, and BTX). The developed assay takes advantage of the capability of nucleic acids to form duplex structures based on the Watson–Crick helix. For this purpose, the aptamer sequences labeled with a fluorescent probe were used, and combined with their corresponding cDNA labeled with a fluorescent quencher. Following the FRET (fluorescence resonance energy transfer) principle, the duplex formation leads to fluorescence extinction. However, since aptamers’ affinity for their targets is much higher than their affinity for the short cDNA used, the complex fluorescent aptamer–toxin is formed, releasing the quencher-labeled cDNA and thus restoring the fluorescence intensity. Based on this fluorescent duplex-to-complex structure-switching approach, we achieved a highly sensitive detection of the six marine toxins within the wide linear range of 0.05 to 100 nM.

2. Results and Discussion

2.1. Principle of the Aptamer-Based Assay

Figure 1 depicts the sensing mechanism of the fluorescent aptameric assay. The detection strategy is based on a duplex-to-complex structure-switching approach. Before the addition of the toxin, the fluorescent ssDNA aptamer binds to its complementary sequence to form a duplex structure. Therefore, BHQ1 conjugated to the 5′ end of the cDNA quenches the fluorescence of the aptamer by a transfer of energy phenomenon. In the presence of the target, the aptamer changes its conformation to form a complex with its target. Consequently, it releases the BHQ1-labeled cDNA, thus inducing the recovery of the FAM fluorescence. Therefore, the toxin amount can be measured by monitoring the increasing fluorescence intensity. Herein, we explore this sensing strategy for the detection of six cyanotoxins: microcystin-LR, anatoxin-α, saxitoxin, cylindrospermopsin, okadaic acid, and brevetoxin.

To study the mechanism of detection, a proof-of-concept experiment was performed. Each FAM-labeled aptamer was subjected to a fluorescence measurement before and after formation of the duplex. Further, the fluorescence intensity was assessed after incubation of the duplex with a fixed concentration of the targeted toxin (5 nM). Figure 2a–f show the fluorescence spectra recorded at 518 nm, for the six toxins. The figures show a high fluorescence intensity in the red lines corresponding to the free FAM–aptamers. A significant drop in the fluorescence intensity was noted after the addition of the BHQ1-labeled cDNA (blue line). This decrease returns to the quenching efficiency of BHQ1 on the fluorophore and confirms the formation of the aptamer–cDNA duplex. Subsequently, the fluorescence was restored (black line), validating the binding between the aptamer and the target. The quenching efficiency was calculated for each duplex as $((F_0 - F)/F_0)\%$, where F_0 is the fluorescence intensity of the aptamer and F is the fluorescence intensity of the aptamer–cDNA duplex. The quenching percentages were over 67%, except for CYN and OA, where the quenching efficiency was 54% and 50%, respectively.

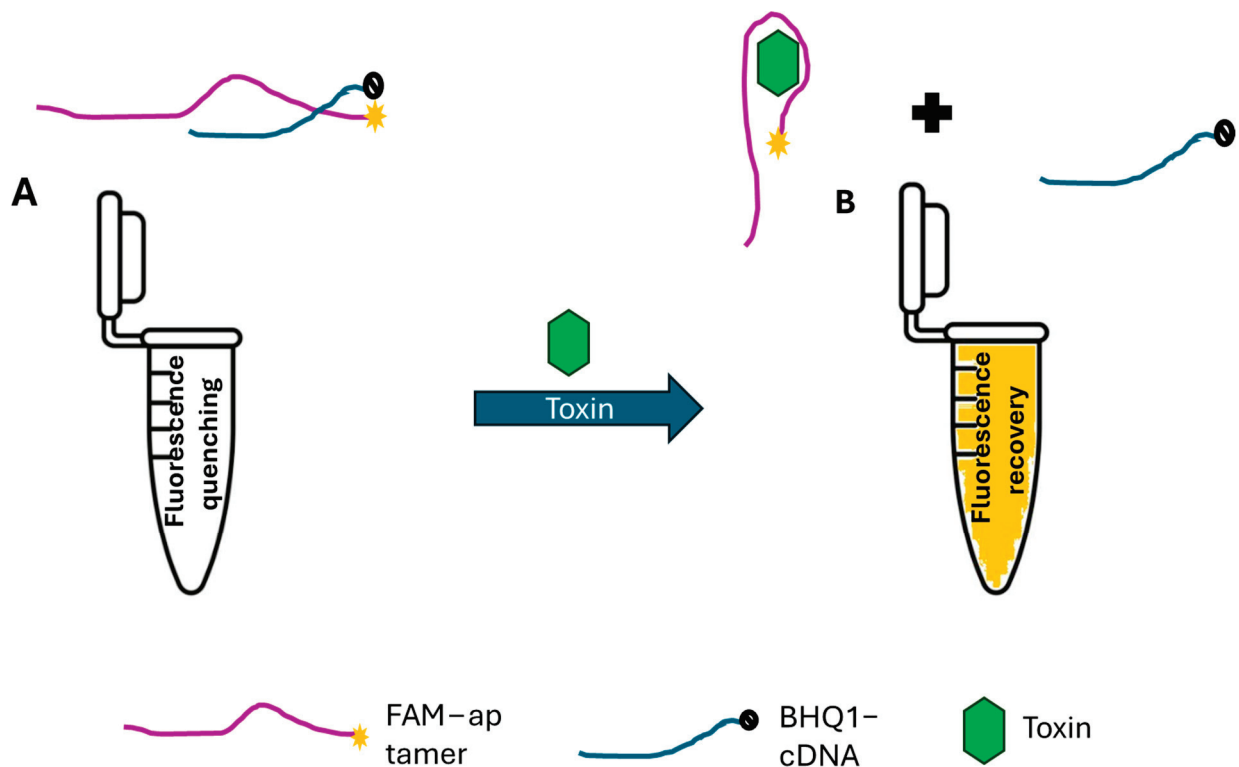


Figure 1. Sensing principle of the fluorescence aptameric assay. (A) Fluorescence quenching induced by the BHQ1-labeled cDNA sequence after the formation of the aptamer–cDNA duplex. (B) Fluorescence recovery after formation of the aptamer–toxin complex.

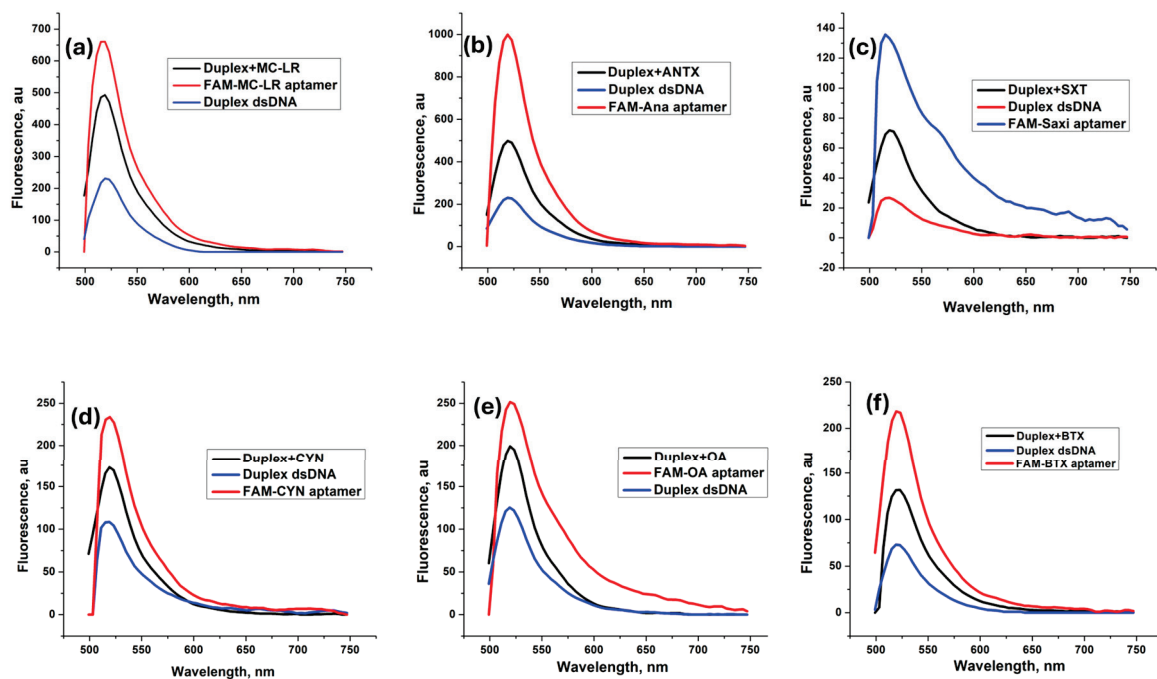


Figure 2. Fluorescence spectra of the FAM–aptamers, the aptamer–cDNA duplex, and the aptamer–target complex: microcystin-LR (a), anatoxin (b), saxitoxin (c), cylindrospermopsin (d), okadaic acid (e), and brevetoxin (f). Fluorescence measurements were performed at an emission wavelength of 518 nm.

2.2. Analytical Performance

Under the optimized conditions, the duplex-to-complex structure-switching approach was applied for the six toxins by designing the corresponding duplexes based on their aptamers and cDNA sequences labeled with FAM and BHQ1, respectively. For each toxin, the aptameric assay was performed in the absence and presence of different concentrations of the target. Figure 3a–f display the fluorescence spectra recorded for microcystin-LR, anatoxin- α , saxitoxin, cylindrospermopsin, okadaic acid, and brevetoxin, respectively. As expected, we observed that the fluorescence intensity increases by increasing the toxin concentration from 0.05 to 100 nM. For the six toxins, we noted a very low fluorescence intensity in the absence of the target. This is mainly due to the strong quenching effect of BHQ1 on the fluorophore resulting from the formation of the aptamer–cDNA duplex. By increasing the toxins' concentrations, the fluorescence intensities increased gradually at the emission wavelength of 518 nm. This increase confirms the specific binding of the different toxins to their aptamers, leading to the dissociation of the duplex and the recovery of the FAM fluorescence. This returns to the fact that the aptamer's affinity toward the target is much higher than its affinity for its cDNA sequence.

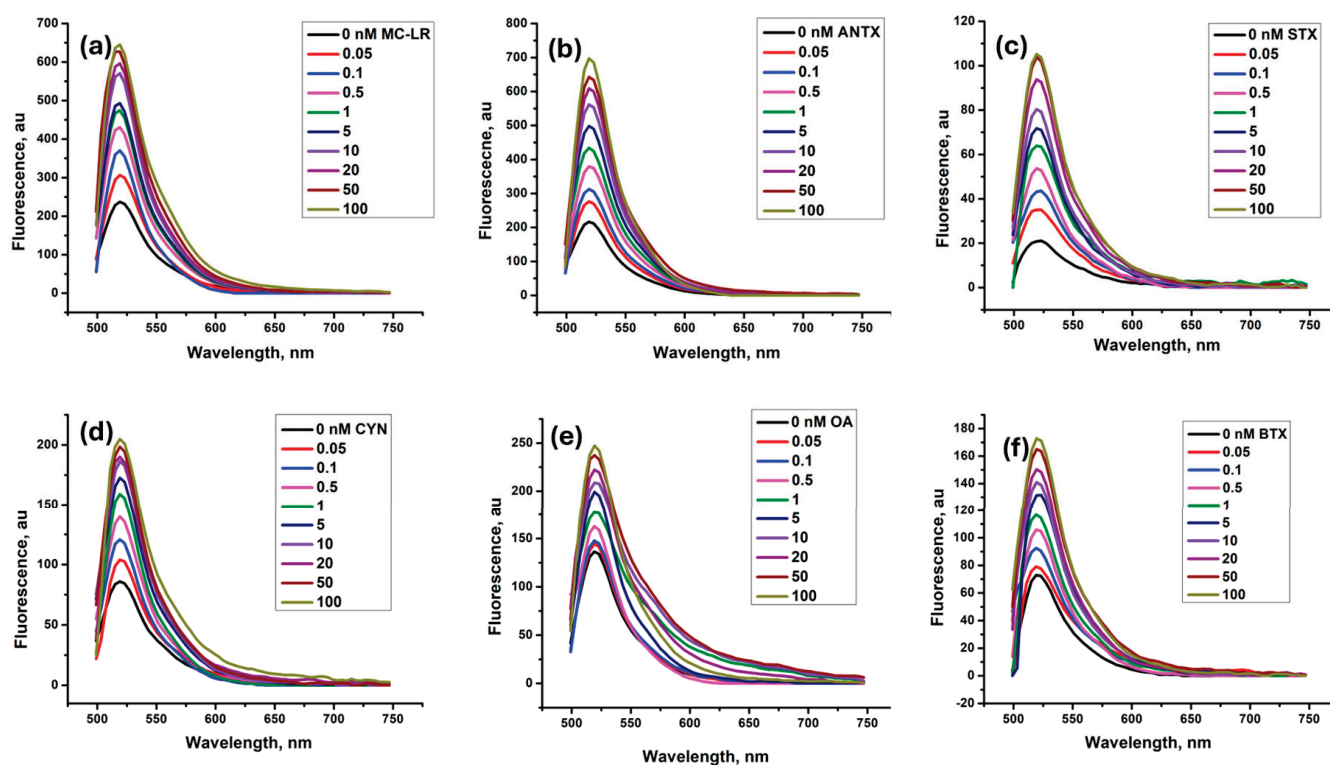


Figure 3. Fluorescence spectra of the six FAM–aptamers after incubation with increasing concentrations of their specific targets: microcystin-LR (a), anatoxin (b), saxitoxin (c), cylindrospermopsin (d), okadaic acid (e), and brevetoxin (f). Fluorescence measurements were performed at an emission wavelength of 518 nm.

The fluorescence spectra obtained in Figure 3 were used to plot the calibration curves for each toxin. For this, the fluorescence intensity (I) was plotted as a function of the logarithm of toxin concentrations. Figure 4a–f show the calibration curves for microcystin-LR, anatoxin- α , saxitoxin, cylindrospermopsin, okadaic acid, and brevetoxin, respectively.

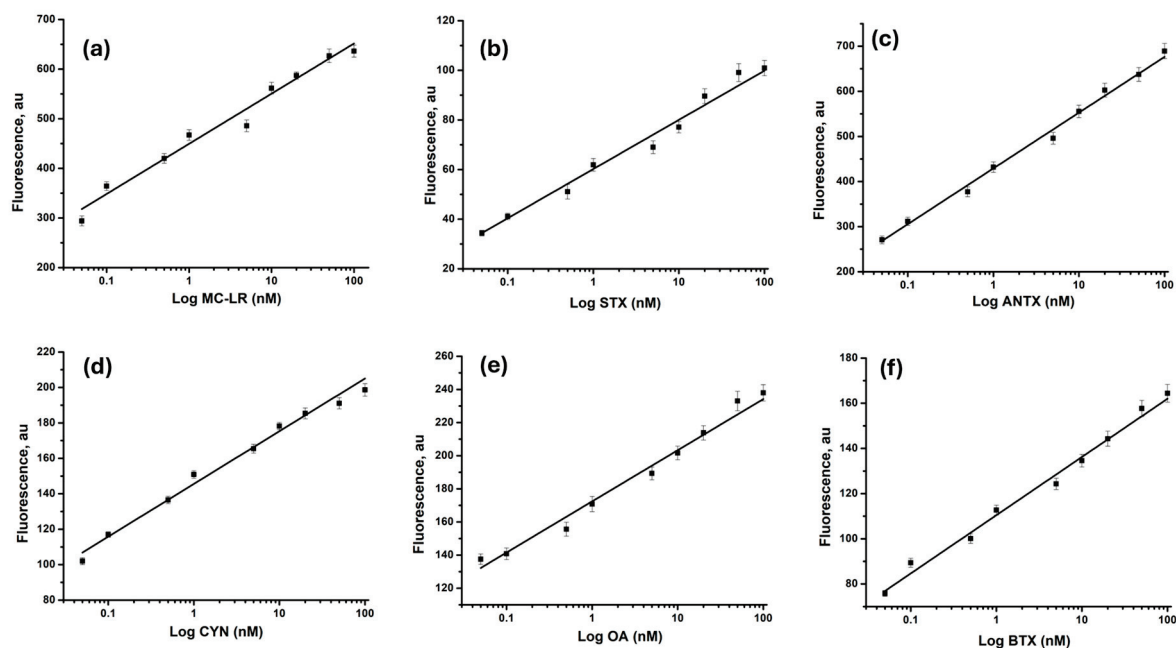


Figure 4. Calibration plots of the recorded fluorescence intensities as a function of the logarithm of cyanotoxins' concentrations (nM): microcystin-LR (a), anatoxin (b), saxitoxin (c), cylindrospermopsin (d), okadaic acid (e), and brevetoxin (f).

The calibration curves show good linearity, with an excellent coefficient of determination (R^2) and low detection limits (LODs), for the different toxins (Table 1). The fluorescent aptameric assay was applicable in the range of 0.05 to 100 nM for the six analytes.

Table 1. Analytical performance of the fluorescent aptameric assay for MC-LR, ANTX, STX, CYN, OA, and BTX.

Toxin	Linear Regression	R^2	LOD ng/mL
MC-LR	$I = 449.43 + 101.09 \text{ Log MC-LR concentration (nM)}$	0.97	0.15
ANTX	$I = 429.11 + 123.65 \text{ Log ANTX concentration (nM)}$	0.99	0.06
STX	$I = 60.19 + 19.83 \text{ Log STX concentration (nM)}$	0.99	0.075
CYN	$I = 145.56 + 29.76 \text{ Log CYN concentration (nM)}$	0.99	0.027
OA	$I = 172.4 + 30.88 \text{ Log OA concentration (nM)}$	0.98	0.041
BTX	$I = 110.44 + 25.75 \text{ Log BTX concentration (nM)}$	0.99	0.026

Based on the regression equations, the limit of detection (LOD) was calculated for each toxin as $3\sigma/\text{slope}$, where σ is the standard deviation of the blank samples. The obtained LODs, shown in Table 1, are much lower than those obtained in our previous work reporting the electrochemical detection of the same cyanotoxins [35].

2.3. Selectivity and Real Sample Applicability

Aiming to confirm the specificity of each aptameric assay for its targeted toxin, a cross-reactivity study was carried out. In brief, each duplex composed of FAM–aptamer/BHQ1–cDNA was incubated with the same concentration (5 nM) of its specific target and the five non-specific toxins, separately. The fluorescence intensity of the duplex was measured at 518 nm, before and after incubation with each toxin. Then, the percentage of fluorescence recovery was calculated as $F = I/I_0\%$, where I_0 and I are the fluorescence intensities of the free duplex and the complex aptamer–cDNA–toxin, shown in Figure 5a–f. We observed a negligible fluorescence recovery of the duplexes for the non-specific toxins, in contrast

to a significant recovery with the specific analyte. Therefore, we confirmed the absence of cross-reactivity of the different aptameric duplexes with the non-specific toxins.

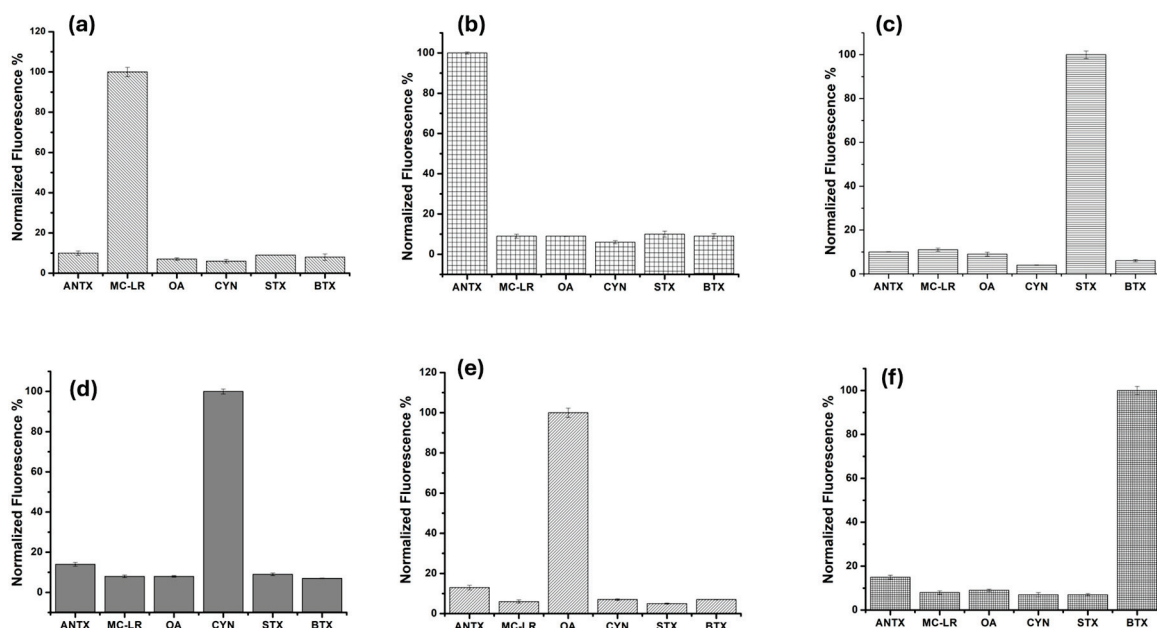


Figure 5. Cross-reactivity study of the aptameric assays for MC-LR (a), ANTX (b), STX (c), CYN (d) OA (e), and BTX (f) aptasensors towards the non-specific cyanotoxins (concentration of 5 nM).

Finally, we tested the application of our methodology in the detection of different toxins in real water samples. As shown in Table 2, excellent recoveries were obtained, with RSDs (relative standard deviations) lower than 5%.

Table 2. Application of the aptamer-based assay in the determination of microcystin-LR, anatoxin- α , saxitoxin, cylindrospermopsin, brevetoxin, and okadaic acid in spiked water samples.

Cyanotoxin	Spiking Concentration (nM)	Measured Concentration (nM)	Recovery Percentage (%)	RSD (%)
MC-LR	10	9.87	98.7	4.25
	25	25.02	102	2.21
	100	99.87	99.87	1.8
CYN	10	10.08	108	4.65
	25	24.92	99.68	4.9
	100	99.73	99.73	1.5
ANTX	10	10.06	106	3.8
	25	24.89	99.56	4.56
	100	98.97	98.97	3.78
STX	10	9.48	94.8	2.23
	25	24.91	99.64	4.27
	100	99.78	99.78	1.99
BVTX	10	10.1	110	4.89
	25	24.87	99.48	3.32
	100	99.23	99.23	4.81
OA	10	9.73	97.3	3.99
	25	25.08	108	1.76
	100	99.96	99.96	2.93

3. Conclusions

Cyanotoxin testing is of great importance for ensuring water and food safety. Aptamer-based assays constitute an excellent alternative to the traditional techniques used for toxin detection, including chromatography and immunoassays. In this work, a fluorescent aptameric assay based on a duplex-to-complex structure-switching approach was successfully applied to quantify six marine toxins: microcystin-LR, anatoxin- α , saxitoxin, cylindrospermopsin, okadaic acid, and brevetoxin. We first investigated the quenching effect of the BHQ1-labeled cDNA sequences on the FAM-labeled aptamers, followed by fluorescence recovery analysis after binding of the six aptamers to their specific toxins. Under the optimized conditions, the homogeneous assay showed a wide linear range of quantification from 0.05 to 100 nM. In addition, high sensitivity was achieved, with LODs ranging from 0.026 to 0.15 nM. Moreover, no significant cross-reactivity was observed for the six aptameric assays, showing the high selectivity of the aptamers. Finally, we demonstrated the applicability of our method in water samples, achieving good agreement with the calibration curve established in buffer. Our approach constitutes a very good alternative for immunoassays and aptamer-based assays targeting a single analyte, particularly for toxins commonly present in the same environment.

4. Experimental

4.1. Chemicals and Instrumentation

The chemicals and instrumentation used in this work are described in Supplementary Materials.

4.2. Design of the Aptamer–cDNA Duplex

First, the duplex DNA was prepared by binding each aptamer sequence to its complementary DNA. For this, 350 μ L of the FAM-labeled aptamer was incubated with the same volume of the BHQ1-labeled cDNA. A concentration of 500 nM of each sequence was used. The mixture was kept in a 90 °C water bath, followed by slow cooling to room temperature for 3 h in dark. This process decreases the distance between the fluorophore–quencher pair, thus enhancing the quenching of fluorescence. In parallel, a control mixture was prepared using the same protocol by incubating the FAM-labeled aptamers with binding buffer. Finally, the prepared duplexes were stored at 4 °C until further use.

4.3. Aptamer-Based Assay

Each duplex of the hybridized aptamer–cDNA pairs was used in the detection of its specific toxin. For this, the aptamer–cDNA (100 nM) was incubated with different concentrations of the targeted analyte ranging from 0.05 to 100 nM for 30 min. The fluorescence measurements were conducted before and after incubation with the increasing concentrations of the six toxins. To study the specificity of the proposed aptamer assay, the different duplexes were cross-reacted with the non-specific toxins at a fixed concentration of 5 nM, separately. Then, the corresponding fluorescence intensities were compared to those obtained with the specific toxin. Finally, the fluorescent assay was applied for the detection of the six toxins in real samples. Water samples, collected in Riyadh city, were spiked with different concentrations (10, 25, and 100 nM) of microcystin-LR, anatoxin- α , saxitoxin, cylindrospermopsin, okadaic acid, and brevetoxin. Fluorescence determination was carried out under the same conditions described above.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/toxins16110476/s1>, Table S1. Aptamers and cDNAs used in the fluorescent assay.

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Article

Community Structure and Toxicity Potential of Cyanobacteria during Summer and Winter in a Temperate-Zone Lake Susceptible to Phytoplankton Blooms

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Abstract: Cyanobacterial blooms are increasingly common during winters, especially when they are mild. The goal of this study was to determine the summer and winter phytoplankton community structure, cyanotoxin presence, and toxigenicity in a eutrophic lake susceptible to cyanobacterial blooms throughout the year, using classical microscopy, an analysis of toxic cyanometabolites, and an analysis of genes involved in biosynthesis of cyanotoxins. We also assessed whether cyanobacterial diversity in the studied lake has changed compared to what was reported in previous reports conducted several years ago. Moreover, the bloom-forming cyanobacterial strains were isolated from the lake and screened for cyanotoxin presence and toxigenicity. Cyanobacteria were the main component of the phytoplankton community in both sampling times, and, in particular, Oscillatoriales were predominant in both summer (*Planktothrix/Limnothrix*) and winter (*Limnothrix*) sampling. Compared to the winter community, the summer community was denser; richer in species; and contained alien and invasive Nostocales, including *Sphaerospermopsis aphanizomenoides*, *Raphidiopsis raciborskii*, and *Raphidiopsis mediterranea*. In both sampling times, the blooms contained toxigenic species with genetic determinants for the production of cylindrospermopsin and microcystins. Toxicological screening revealed the presence of microcystins in the lake in summer but no cyanotoxins in the winter period of sampling. However, several cyanobacterial strains isolated from the lake during winter and summer produced anabaenopeptins and microcystins. This study indicates that summer and winter blooms of cyanobacteria in the temperate zone can differ in biomass, structure, and toxicity, and that the toxic hazards associated with cyanobacterial blooms may potentially exist during winter.

Keywords: cyanotoxins; cyanobacterial blooms; cyanotoxin immunoassays; high-performance liquid chromatography–mass spectrometry; toxigenicity

Key Contribution: Toxic cyanobacteria genotypes are present in summer and winter lake phytoplankton. Health hazards related to MCs and CYN can occur throughout the year.

1. Introduction

Excessive nutrient input into aquatic ecosystems, together with climate change, accelerates their eutrophication and makes them more susceptible to cyanobacterial blooms—it is a phenomenon that constitutes a global problem. A recent estimate [1] showed that eutrophication, just in lakes, will increase by 25–200% by the middle of the 21st century and double or quadruple by the end of the century as a result of the changing climate. The more eutrophic the lake ecosystems, the more incidences of bloom-forming cyanobacteria in lakes and, consequently, the greater the challenge from the aquatic environment-management perspective.

The blooming of cyanobacteria carries numerous negative consequences for the ecosystem, from light limitation [2] and alterations of food-web structure [3] to biodiversity loss [4]. Among the characteristics of cyanobacteria that determine their harmfulness (e.g., ability to form surface scums [5] and low nutritional value to consumers [6]), the ability of these prokaryotes to synthesize and secrete a variety of toxic metabolites [7,8] is probably the most important. According to the National Rivers Authority [9], 25%–75% of cyanobacterial blooms can be toxic, and they may cause the mortality of aquatic organisms and pose a threat to other users of water resources [10,11]. Moreover, toxic cyanobacterial blooms, as well as the release of toxic cyanometabolites in water, e.g., during bloom collapse, can impair the recreational and economic functions of water bodies and disqualify the lake/reservoir as a drinking water resource (e.g., see [12]). At the same time, preventing toxic cyanobacterial blooms and mitigating existing problems can come at a high cost [13,14].

Considering the response of bloom-forming cyanobacteria to climate change, one can conclude that aquatic ecosystems face a future with more toxic cyanobacterial biomass if projections regarding increases in temperature and concentrations of greenhouse gases in the atmosphere are realized by the end of the 21st century [15]. For example, an increase in temperature, apart from intensifying the growth of cyanobacteria [16], was found to increase the production of toxic metabolites for some cyanobacteria (e.g., nodularin for *Nodularia*, [17], saxitoxin for *Aphanizomenon*, [18], or microcystins for *Planktothrix*, [19]). Other toxic cyanobacteria, e.g., *Microcystis aeruginosa*, can also exhibit similar responses when a temperature rise is accompanied by extra nutrient input [20]. Apart from increasing toxin production, temperature elevation can favor toxic genotypes and, consequently, promote their dominance in the cyanobacterial community [21].

Blooms also like it cold—there are increasing reports of cyanobacterial bloom-forming taxa and even active blooms in wintertime, even under the ice cover, and they are often formed by the same bloom-forming and potentially toxic species as in summertime [22–28]. The presence of toxic cyanometabolites in winter blooms (anatoxin-a [29] and microcystins [25,30,31]) shows that cyanobacterial blooms can be “cold and toxic”. Synthesis of cyanotoxins can also aid cyanobacteria in acclimation to a lower water temperature, as shown by Stark et al. [32] for microcystin-producing *Microcystis aeruginosa* and its non-toxigenic mutant strain. Moreover, toxic cyanometabolites, i.e., microcystins, were found even in cyanobacteria living in extremely cold glacial ecosystems [33,34]. In addition, winter populations of cyanobacteria can exert toxic effects on other species in the aquatic environment [31,35].

The main objective of the present study was to determine the community structure, toxicity, and toxigenicity of the summer and winter blooming of cyanobacteria and to compare whether cyanobacterial diversity in the studied lake has changed in relation to previous reports conducted several years ago. Therefore, we sampled a shallow, eutrophic lake, Lubosińskie Lake, in summer and winter, analyzed the structure of cyanobacterial communities, and studied the presence of cyanotoxins in the lake and in the isolated strains of cyanobacteria. The strain toxigenicity and real capability to produce toxigenic cyanometabolites were not previously investigated in cyanobacteria from this lake. Our study provides special insight into the toxicity of summer and winter cyanobacterial blooms and the potential threats associated with ongoing climate change.

2. Results

2.1. Physicochemical Parameters and Phytoplankton Structure in Lubosińskie Lake

The surface-water temperature of Lubosińskie Lake was 22.6 °C on 2 September 2019 and 4.2 °C on 11 February 2020, while water transparency was 0.2 m and 0.4 m, respectively. There was no ice cover on the lake during winter sampling. Values of the remaining measured parameters of surface water during summer and winter sampling, respectively, were as follows: 8.8 and 8.5 (pH), 616 and 711 $\mu\text{S}/\text{cm}$ (conductivity), 2.23 and 1.6 mg N/L (total nitrogen), 0.126 and 0.435 mg/L (N- NO_3), 0.004 and 0.012 mg/L (N- NO_2), 2.1 and 1.15 mg/L (Kjeldahl nitrogen), 0.215 and 0.24 mg P/L (total phosphorus), and 0.081 and 0.137 mg P/L (total reactive phosphorus).

The phytoplankton biomass was 29.4 mg/L on 2 September 2019 and 9.4 mg/L on 11 February 2020. Cyanobacteria constituted ~94% of summer and ~70% of winter phytoplankton (Figure 1a). Apart from cyanobacteria, summer phytoplankton contained members of Bacillariophyceae (two taxa), Chlorophyta (five taxa), Cryptophyceae (four taxa), Dinophyceae (one taxon), and Haptophyceae (one taxon). In winter, Bacillariophyceae were represented by two taxa, Chlorophytes by one taxon, Cryptophyceae by three taxa, Dinophyceae by two taxa, and there were no representatives of Haptophyceae. Numerical values of phytoplankton taxa biomass and species or genus authority are outlined in Supplementary Materials S1.

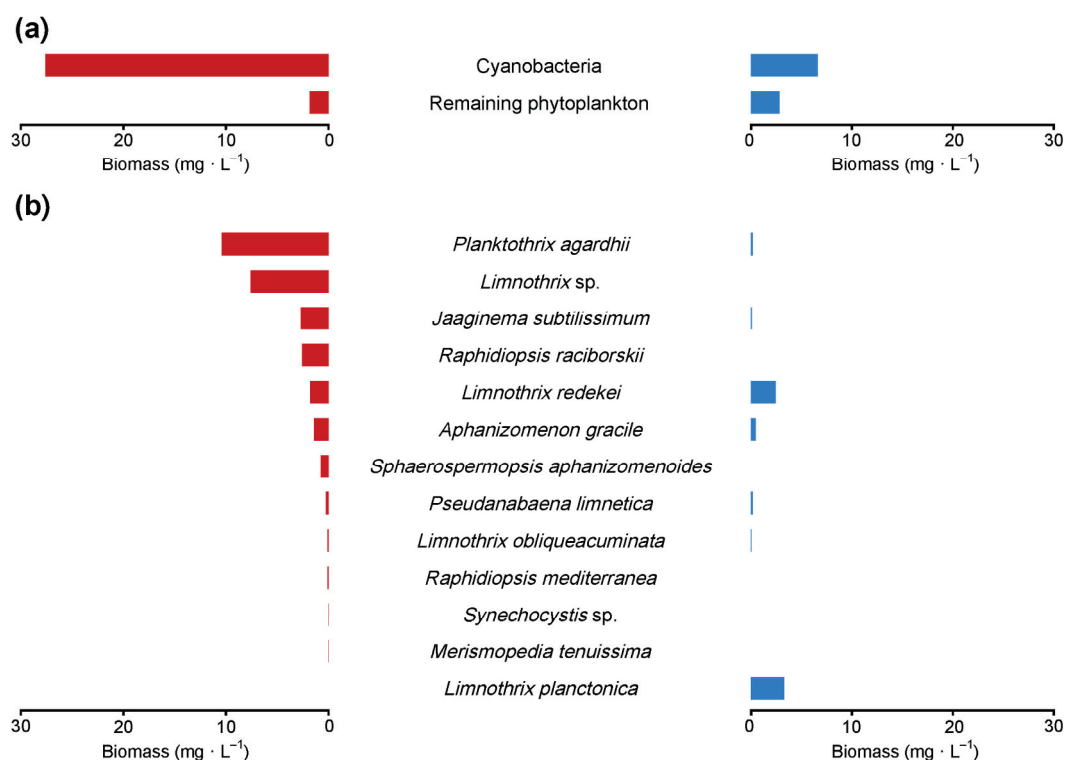


Figure 1. The proportion of cyanobacteria in the phytoplankton (a) and quantitative structure of cyanobacterial communities (b) of the Lubosińskie Lake during summer (left panel) and winter (right panel). No bar means that a given taxon was absent.

The summer cyanobacterial bloom was dominated (~61% of total biomass) by *Planktothrix agardhii* and *Limnothrix* sp. (10.39 and 7.58 mg/L, respectively); there were also four subdominants (*Jaaginema subtilissimum*, *Raphidiopsis raciborskii*, *Limnothrix redekei*, and *Aphanizomenon gracile*) of biomass ranging from 2.69 to 1.39 mg/L; and six taxa (*Sphaerospermopsis aphanizomenoides*, *Pseudanabaena limnetica*, *Limnothrix obliqueacuminata*, *Raphidiopsis mediterranea*, *Synechocystis* sp., and *Merismopedia tenuissima*) of biomass less than 0.8 mg/L (Figure 1b, left panel). Two *Limnothrix* species dominated the cyanobacterial biomass

in winter (*L. planktonica*, 3.27 mg/L; and *L. redekei*, 2.41 mg/L) (Figure 1b, right panel) (Figure 2), while the biomass of other taxa (*A. gracile*, *P. limnetica*, *P. agardhii*, *J. subtilissimum*, and *L. obliqueacuminata*) was lower than 0.5 mg/L.

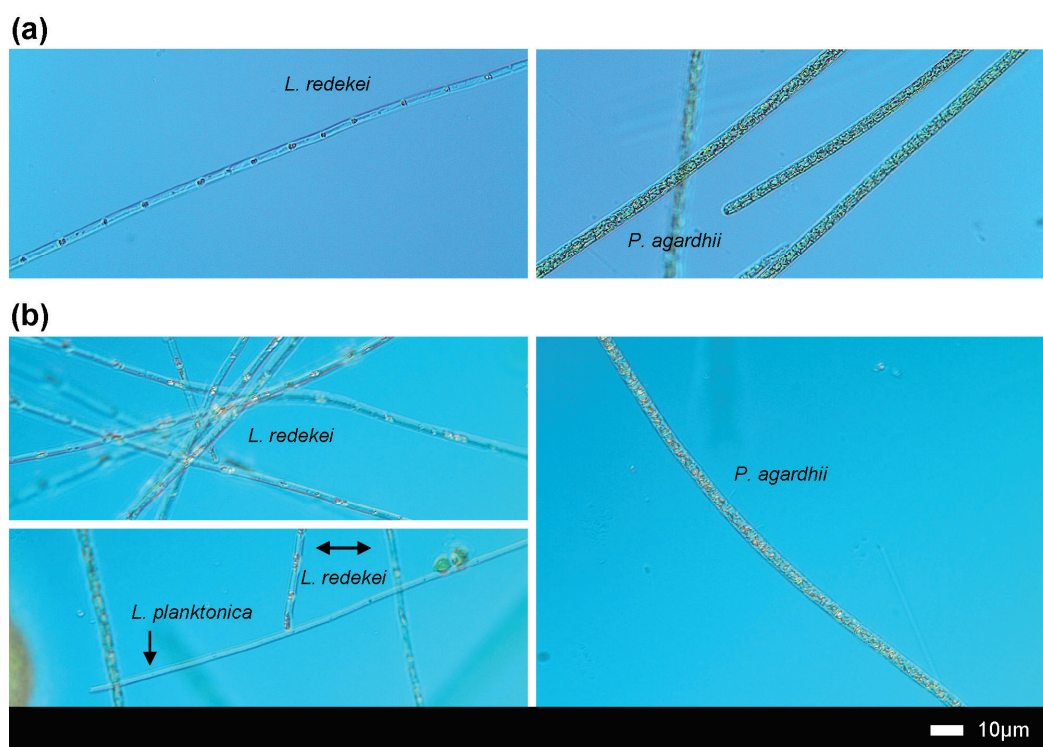


Figure 2. Micrographs of *Limnothrix* and *Planktothrix* specimens from summer (a) and winter (b) samples, conducted using light microscope with Nomarski interference contrast.

2.2. Overview of Cyanobacteria Strains Isolated from the Lake during Summer and Winter

Microscopic inspection of successfully growing strains showed that the summer set of strains comprised *P. agardhii* (W49 and W67 strains) and *R. raciborskii* (W73 and W88 strains), whereas the winter set included four isolates of *A. gracile* (W4, W58, W71, and W89) and one *P. agardhii* (W70) (Figure 3). Specimens of each strain possessed key characteristic morphological features of the species to which they were classified: (1) *A. gracile*: trichomes solitary and free-floating, vegetative cells 2.4–3.4 μm wide and 3.5–7.4 μm long in the middle of trichomes, apical cells non-hyaline and capitate at the end, barrel-shaped and intercalary heterocytes (3.4–4.7 μm wide and 5.7–8.9 μm long), akinetes (3.6–7.2 μm wide and 16.9–26.1 μm long), cylindrical, possessing at poles often cup-shaped sheath formations opened towards the neighboring vegetative cells; (2) *P. agardhii*: trichomes mostly solitary and free-floating sometimes with fine sheaths, vegetative cells 3.7–5.2 μm wide and 1.6–3.8 μm long, apical cells bluntly conical or sometimes capitate; (3) *R. raciborskii*: free-floating trichomes, vegetative cells 1.9–3.4 μm wide and 7.1–15.9 μm long, heterocytes 2.5–3.8 μm wide and 5.7–11.4 μm long, drop-like, terminal and present at one or two ends of a trichome, akinetes of 3.5–5.7 μm width and 11.8–24.1 μm length, cylindrical-oval and adjacent to the heterocytes or slightly distant from these cells.

Phylogenetic analyses based on *rpoB* gene sequencing showed that sequences of W4, W58, W71, and W89 strains clustered together and exhibited a short phylogenetic distance to reference sequences of *A. gracile* and *A. flos-aquae* from GenBank (Figure 4). Considering that specimens in each of these four strains had neither hyaline terminal/subterminal cells nor the tendency to aggregate into fascicles, these strains were finally determined to be *A. gracile*. Sequences of W49 and W67 strains from summer phytoplankton and the sequence of W70 strain from winter clustered together with the reference sequences of *P. agardhii*. The remaining two sequences (W73 and W88 strains) clustered together

with sequences of *Raphidiopsis*, and they had slightly shorter phylogenetic distance to *R. raciborskii* than to *R. curvata*, *R. curvispora*, or *R. brookii*. Pairwise distance matrix between cyanobacterial strains isolated from summer and winter phytoplankton of Lubosińskie Lake and comparative sequences from GenBank using the Kimura2-parameter model is outlined in Supplementary Materials S2.

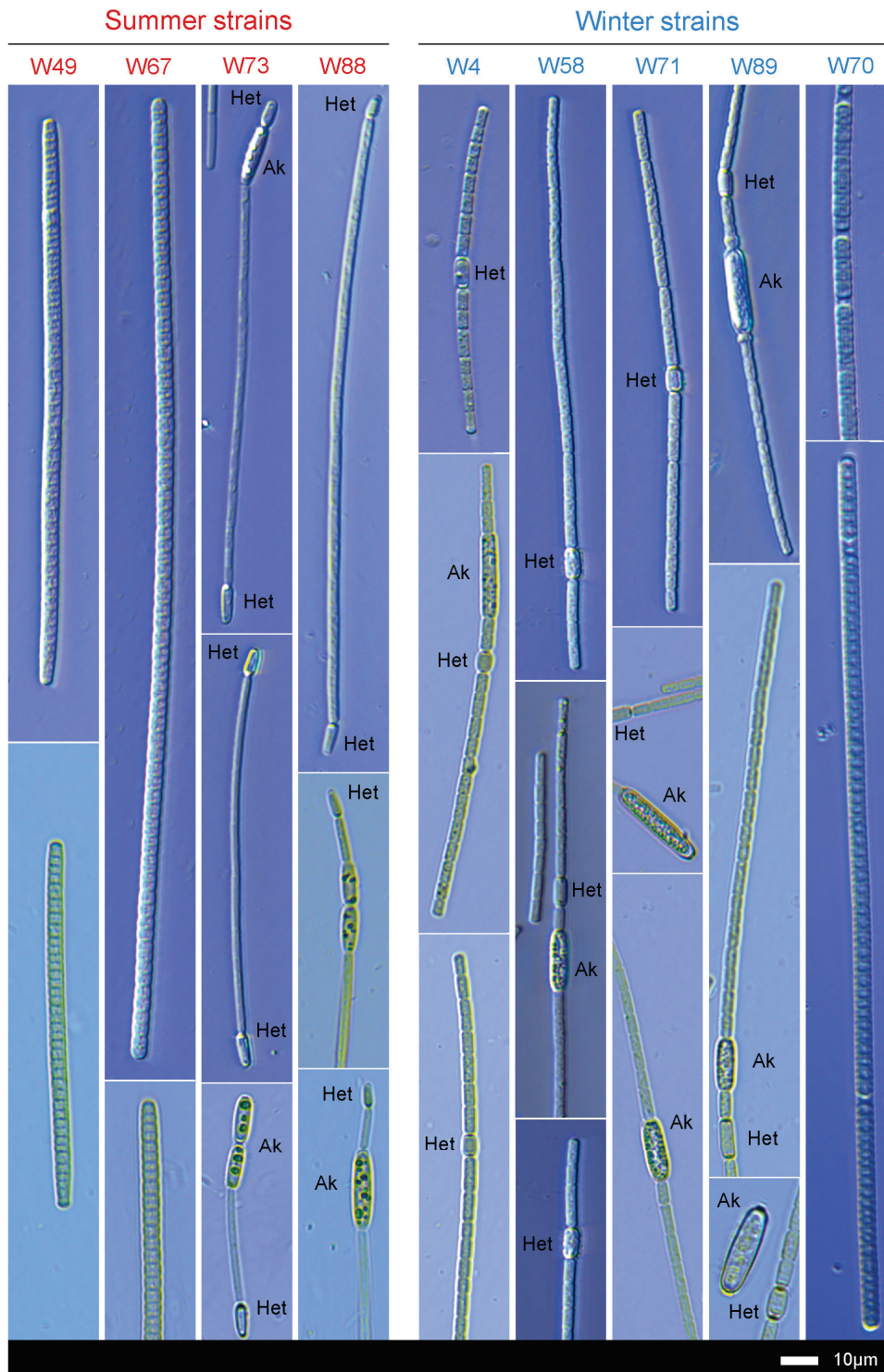


Figure 3. Micrographs of cyanobacterial strains isolated from summer and winter phytoplankton of Lubosińskie Lake, conducted using light microscope with integrated modulation contrast.

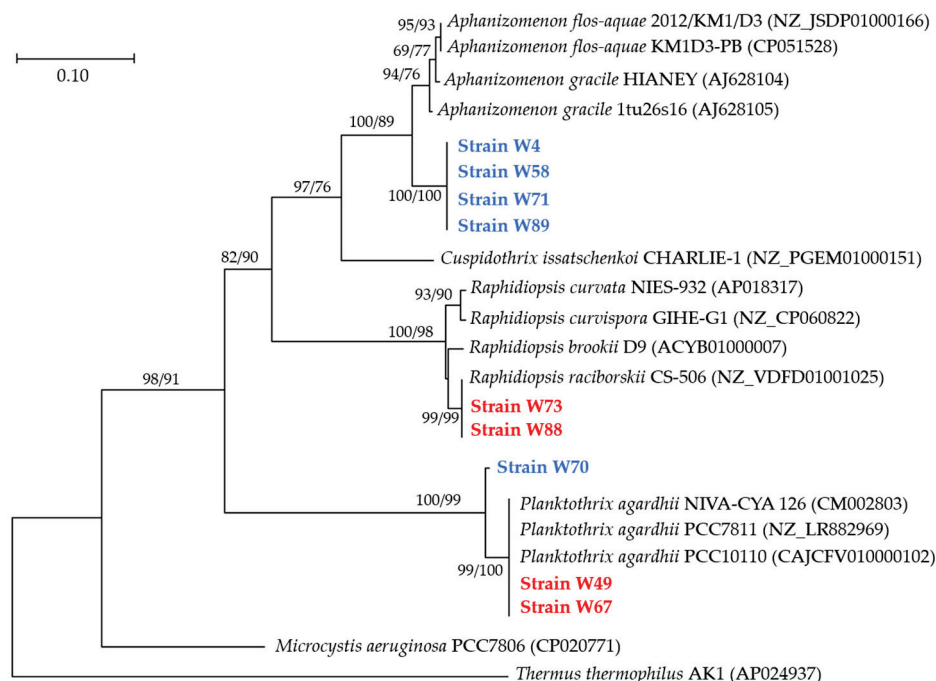


Figure 4. Phylogenetic reconstruction based on the *rpoB* gene for the isolated cyanobacterial strains from the Lubosińskie Lake. The tree was constructed using the NJ topology and the numbers associated with the nodes represent the bootstrap support values of NJ and ML analyses, respectively. Only bootstrap supports $\geq 50\%$ were reported. The bar above the tree represents nucleotide substitutions per position. Accession numbers were given for *rpoB* sequences obtained from GenBank. The sequences of *Microcystis aeruginosa* PCC7806 and *Thermus thermophilus* AK1 were used as outgroups to cluster the representative strains in the phylum Cyanobacteria. Red and blue colors indicate summer and winter strains, respectively.

2.3. Cyanometabolites and Toxigenicity Genes

The toxicological screening of water from Lubosińskie Lake using HPLC-DAD revealed the presence of dmMC-RR (10.58 $\mu\text{g/L}$), MC-YR (1.14 $\mu\text{g/L}$), and dmMC-LR (1.94 $\mu\text{g/L}$) in the summer, whereas none of the examined microcystin forms was detected in winter. Summer and winter lake water did not contain APs and STXs, according to the ELISA immunoassay. The results from the HPLC-DAD chromatography and ELISA immunoassay for lake-water samples are presented in Supplementary Materials S3. The toxigenicity assessment showed the presence of *mcyE* and *cyrJ* genes both in summer and winter samples, while *anaF* was found in neither (Figure 5).

The results of the toxicological screening of strains are summarized in Table 1. APs were detected in all examined strains of *A. gracile* and *P. agardhii*—the concentration of APs was above the detection limit, 2 $\mu\text{g/L}$, in each case (Supplementary Materials S4). Strains of *R. raciborskii* were free of APs. The ELISA immunoassay also showed that *P. agardhii* W67 and *A. gracile* W4 and W89 strains contained anatoxin-a, and the concentrations were very close to the lower detection limit. Saxitoxins were not detected by ELISA in the summer or in the winter set of strains. The noncompetitive immunoassay (TRFIA) revealed *P. agardhii* from summer (W67) and winter (W70) to be positive for MCs/NOD, while the test gave negative results in the case of other examined strains (Supplementary Materials S5). The HPLC-DAD showed the presence of dmMC-RR and dmMC-LR in summer and winter isolates of *P. agardhii* (W67 and W70, respectively), and MC-YR in summer strain W67 (Figure 6). Other forms of microcystins were not detected in these strains by this chromatographic method (Supplementary Materials S6). The remaining strains were free of all examined MC forms based on HPLC-DAD. The LC-MS technique confirmed the presence of demethylated forms of MC-RR in the W67 and W70 strains, and MC-YR in W67 (Figure 7), and the absence of other investigated MC

forms in these strains (Supplementary Materials S7). Cylindrospermopsin, anatoxin-a, and nodularin were not detected in any of the screened strains by HPLC-DAD and LC-MS (Supplementary Materials S6 and S7, respectively). Screening of strains for genes involved in production of the selected cyanotoxins revealed the presence of *mcyE* gene (potential for synthesis of microcystins) in *P. agardhii* W67 and W70 strains (Supplementary Materials S8). The *cyrJ* (gene marker for cylindrospermopsin synthesis) and *anaF* (gene marker for anatoxin-a synthesis) were not detected in any of the examined cyanobacterial strains isolated from the lake.

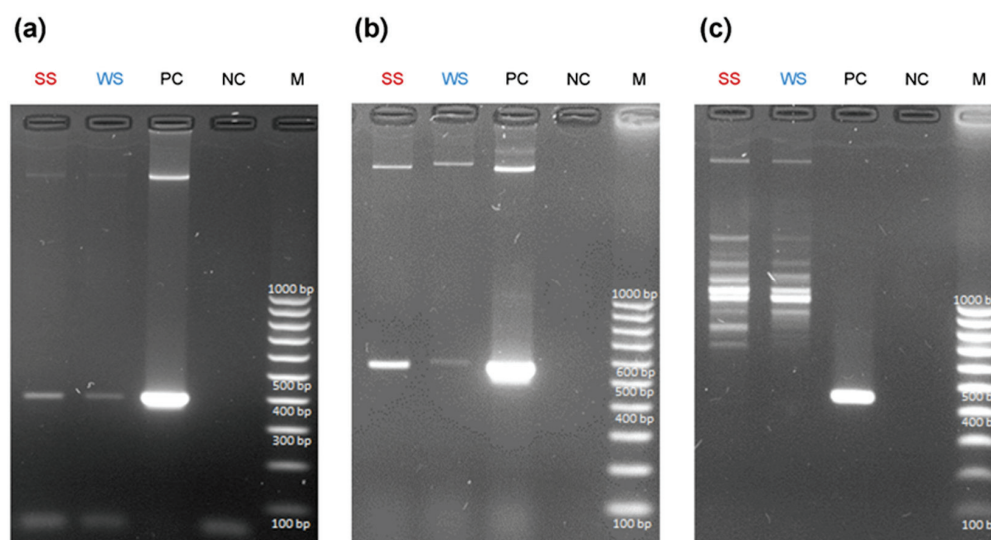


Figure 5. Qualitative amplification of toxigenic genes in summer and winter samples from Lubosińskie Lake: (a) *mcyE* (405 bp)—potential for production of MCs; (b) *cyrJ* (578 bp)—potential for production of CYN; and (c) *anaF* (467 bp)—potential for production of ATX-a. SS in red color—summer lake-water sample, WS in blue color—winter lake-water sample, PC—positive control (*mcyE*, *Microcystis aeruginosa* PCC7806; *cyrJ*, *Raphidiopsis raciborskii* CS505 Australia; *anaF*, *Cuspidothrix issatschenkoi* NIVA-CYA 711); NC—negative control; M—DNA size marker.

Table 1. Summary of the toxicological screening of summer and winter lake-water samples and cyanobacterial strains isolated from summer and winter phytoplankton of Lubosińskie Lake. Note: “+” means a given cyanometabolite or gene involved in its synthesis was detected; “(f+)” means false positive result; “NA”—missing data. Lack of symbol means the absence of a given cyanometabolite or gene responsible for its synthesis.

Cyanometabolite	Method	Summer Sampling				Winter Sampling			
		<i>P. agardhii</i> W49	<i>P. agardhii</i> W67	<i>R. raciborskii</i> W73	<i>R. raciborskii</i> W88	<i>A. gracile</i> W4	<i>A. gracile</i> W58	<i>A. gracile</i> W71	<i>A. gracile</i> W89
APs	ELISA	+	+			+	+	+	+
ATX-a	ELISA		(f+)			(f+)			(f+)
	HPLC-DAD						NA		
	LC-MS <i>anaF</i> gene						NA		

Table 1. Cont.

Cyanometabolite	Method	Summer Sampling				Winter Sampling			
		<i>P. agardhii</i> W49	<i>P. agardhii</i> W67	<i>R. raciborskii</i> W73	<i>R. raciborskii</i> W88	<i>A. gracile</i> W4	<i>A. gracile</i> W58	<i>A. gracile</i> W71	<i>A. gracile</i> W89
CYN	HPLC-DAD LC-MS <i>cyrJ</i> gene						NA NA		
MCs	HPLC-DAD LC-MS <i>mcyE</i> gene		+				NA NA		+
NOD	HPLC-DAD LC-MS						NA NA		
MCs/NOD	TRFIA		+				NA		+
STXs	ELISA								

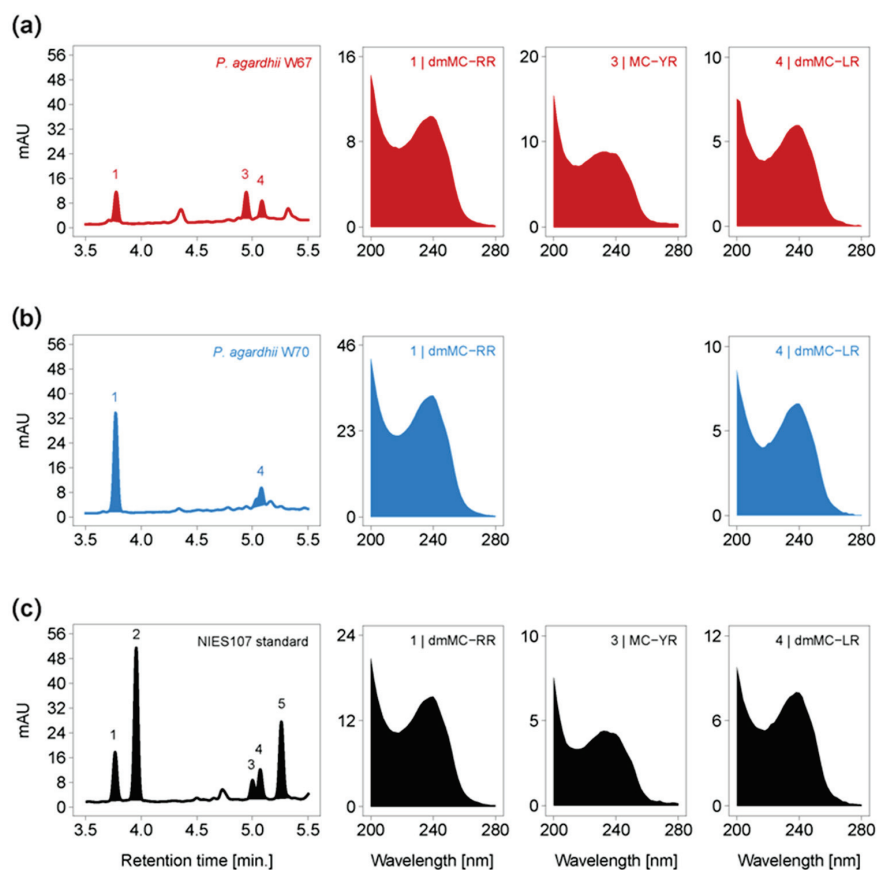


Figure 6. Visualized HPLC-DAD chromatograms and compound spectra of dmMC-RR (1, RT = 3.776 min), MC-YR (3, RT = 4.945 min), and dmMC-LR (4, RT = 5.086 min) in summer *P. agardhii* strain W67 (a); dmMC-RR (1, RT = 3.771 min) and dmMC-LR (4, RT = 5.081 min) in winter *P. agardhii* strain W70 (b); and dmMC-RR (1, RT = 3.766 min), MC-RR (2, RT = 3.955 min), MC-YR (3, RT = 5.001 min), dmMC-LR (4, RT = 5.071 min), and MC-LR (5, RT = 5.260 min) in the standard NIES107 (c). Summer and winter MCs-producing strains are marked, respectively, in red and blue.

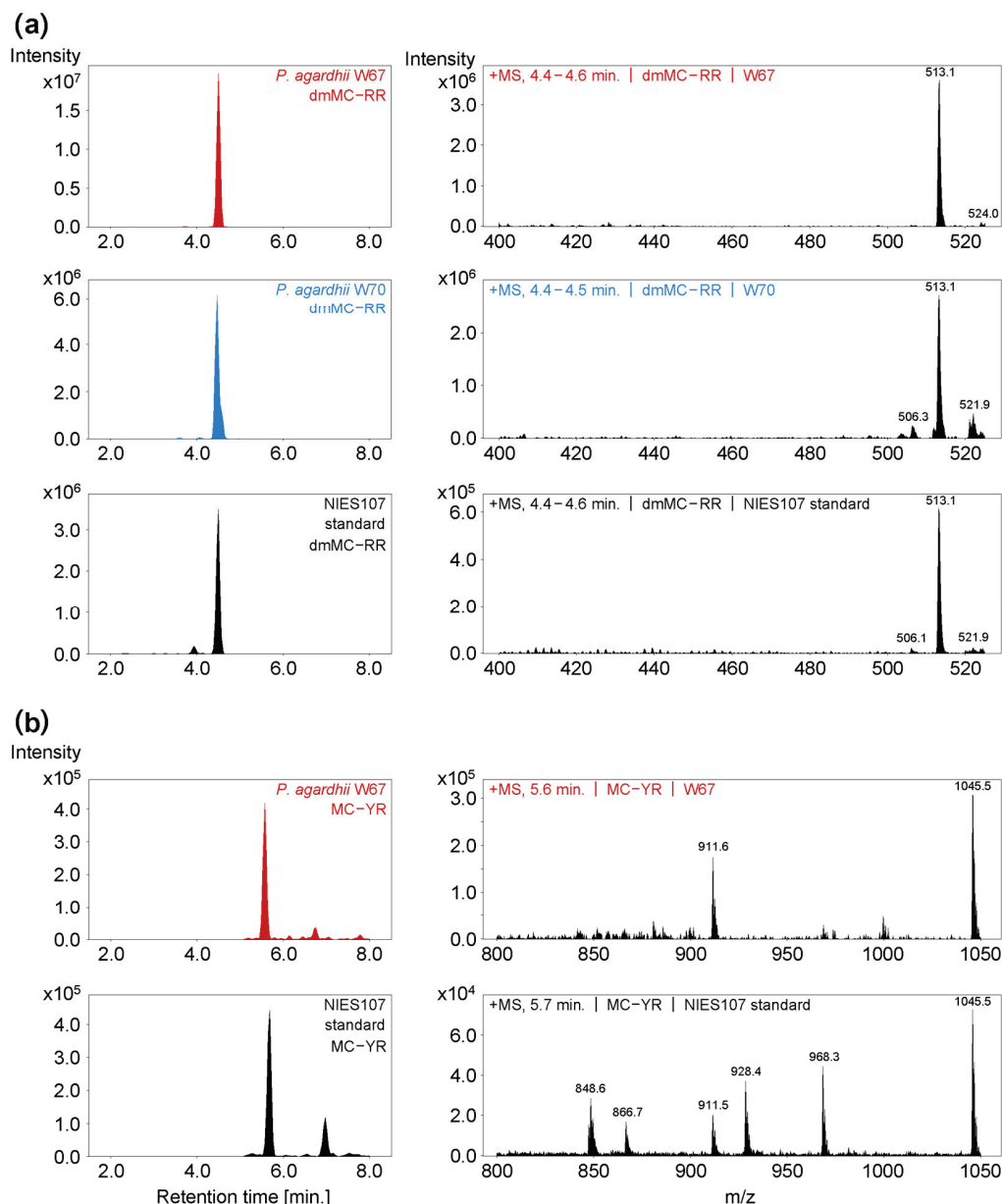


Figure 7. Visualized chromatograms and MS scan spectra of (a) dmMC-RR in the summer *P. agardhii* W67 (RT = 4.5 min), winter *P. agardhii* W70 (RT = 4.5 min), and NIES107 standard (RT = 4.5 min); and (b) MC-YR in *P. agardhii* W67 (RT = 5.6 min) and NIES107 standard (RT = 5.7 min). Summer and winter MCs-producing strains are marked, respectively, in red and blue.

3. Discussion

Cyanobacterial blooms in eutrophic north temperate lakes are a phenomenon that occurs frequently in summer and is becoming increasingly common in winter. Lubosińskie Lake has also experienced cyanobacterial blooms in recent decades due to its eutrophic nature [25,30,31,36]. High chlorophyll-*a* levels, sometimes exceeding 100 µg/L in both summer and winter [25], indicate the high productivity of this lake—conditions suitable for large cyanobacteria blooms [37]. In 2019/2020, Lubosińskie Lake continued to be “bloom friendly” for cyanobacteria, as these organisms were a dominant fraction of the phytoplankton in summer and winter. In previous studies (2006/2008), clear dominance of cyanobacteria (*Planktothrix agardhii*) was also observed in both summer and winter. The potential for the synthesis and actual concentration of microcystins was described, without distinguishing other metabolites of cyanobacteria [25,30].

The present study shows that cyanobacterial community in Lubosińskie Lake had a higher biomass and was richer in bloom-forming and potentially toxic taxa in summer than in winter. In both periods, the surface water was dominated by oscillatoriallean cyanobacteria: *Planktothrix* and *Limnothrix* in summer and *Limnothrix* in winter. Members of these genera, especially *P. agardhii* and *L. redekei*, are frequently dominant in eutrophic lakes of temperate zones and often compete with each other (e.g., see [22]). The winter population of cyanobacteria near the surface was clearly dominated by *Limnothrix redekei* and *Limnothrix planctonica* in February 2020, while the biomass of *P. agardhii* decreased significantly. The outcompeting of *P. agardhii* populations by *Limnothrix* sp. was previously reported in other shallow lakes and explained by the ability of *Limnothrix* to grow faster than *P. agardhii* at lower temperatures and limited light supply [22,38–40]. Interestingly, over a decade ago, previous studies reported that cyanobacterial blooms in Lubosińskie Lake were dominated by *P. agardhii* during all consecutive years of monitoring from 2006 to 2008 [30], while in contrast, *Limnothrix* spp. were reported to occur but not dominate the phytoplankton communities [25]. A shift in the cyanobacterial community with higher dominance of *Limnothrix* spp. over *P. agardhii* was described for the first time for winter in 2017 [31], dynamics that were also observed for the later winter in 2020 in the present study. More frequent monitoring of this particular lake in the near future, in terms of phytoplankton composition and physical and chemical changes in the water would help to explain the reasons for the observed shifts in species dominance between the seasons.

Summer conditions supported relatively high biomass of several nostocalean cyanobacteria, especially alien and invasive species such as *S. aphanizomenoides*, *R. raciborskii*, and *R. mediterranea*. These species have subcosmopolitan distribution and often constitute a substantial element of summer phytoplankton in eutrophic lakes [41–46]. However, the strains of these invasive species were not able to endure winter in Lubosińskie Lake, although it was mild, with a water temperature of approximately 4 °C. The same species were also absent in the lake in the winter of 2017, when ice cover was present [31]. These results indicate the rarity of ecotypes of the species that would be able to actively overwinter in the studied lake, while such ecotypes exist in other temperate lakes of Central Europe (e.g., [47]), and also in lakes on other continents (e.g., [48,49]), and can maintain relatively high biomass during mild winters. To our knowledge, there are no reports of active overwintering of *R. raciborskii* under ice or at temperatures near 0 °C, as some chroococcalean, nostocalean, and oscillatoriallean cyanobacteria are capable of that [50]. *R. raciborskii* is a common component of summer–autumn phytoplankton community in numerous temperate lakes (e.g., [43]). This raises the question of what the secrets of the success of *R. raciborskii* and other expansive species are in the already invaded waters of the temperate zone. The collapse of populations of this species in temperate lakes of Central Europe under harsh winter conditions and their subsequent renewal in summer suggest winter dormancy. Investing in the formation of akinetes [51,52] could be an effective strategy of this cyanobacterium in order to persist in the invaded waters. This could be an important reason for the stability of *R. raciborskii* populations in the temperate zone.

Both the summer and winter blooms consisted of potentially toxic cyanobacteria. Toxicological screening revealed the presence of hepatotoxins–microcystins (variants: MC-YR and demethylated forms of MC-RR and MC-LR) in lake water, but only during summer. Other studied cyanometabolites (anabaenopeptins, cylindrospermopsin, microcystins, and saxitoxins) were not detected in the lake during summer or winter. However, molecular analyses revealed the presence of *mcyE* gene, the marker for synthesis of microcystins, and the *cyrJ* gene, which is associated with CYN production, in both summer and winter samples. The presence of the *mcyE* and *cyrJ* genes suggests that potentially toxigenic MC- and CYN-producing cyanobacteria can survive in the winter community. In the case of MCs, this is also confirmed by the toxicological screening of cyanobacterial strains isolated from the lake, as dmMC-LR and dmMC-RR were detected in the winter isolate of *P. agardhii*. Despite the detection of MC-producing genotypes in winter, the microcystins themselves were undetected at that time in Lubosińskie Lake, which may suggest that MC-producing geno-

types accounted for a relatively low share in the cyanobacteria community. Considering that the biosynthesis of toxic cyanometabolites is regulated at the level of gene expression, which can vary throughout the season and decrease with decreasing temperatures (e.g., microcystins, [53]), the low efficiency of biosynthesis of a particular toxin could also explain its absence in the lake during winter. In the previous studies, microcystins were detected throughout the years 2006 to 2008 in Lubosińskie Lake [25,30]; however, their concentrations during winter seasons were lower than in the summer, e.g., on average, 17.7 $\mu\text{g/L}$ and 46.9 $\mu\text{g/L}$, respectively. Moreover, the potential toxigenicity of cyanobacterial blooms dictated by the gene *mcyE* was already observed to persist throughout the entire winter of 2007/2008 and summer of 2008 [30], similar to how it was observed for the present study. Overall, it is evident that the summer cyanobacterial bloom had a greater potential to contaminate lake water with toxic cyanometabolites, while the winter cyanobacterial bloom had a hidden potential for toxicity.

The first isolation of strains from the studied lake and their subsequent toxicological evaluation allowed us to gain deeper insight into the toxic potential of the summer and winter cyanobacterial communities in the studied lake. Thanks to this approach, we have also found genotypes that produce anabaenopeptins, which can exert inhibitory effects on various proteases, phosphatases, and carboxypeptidases [54–56] and may also play various biological roles for cyanobacteria [57–59]. Initial observations of strains and measurements in cultures during their maintenance under laboratory conditions indicate that strains from Lubosińskie Lake exhibit a wide variation in growth, and, at the same time, a high thermal tolerance of the winter-originated cyanobacteria isolates is noted. Originally collected from $\sim 4^\circ\text{C}$ lake water and subsequently routinely incubating under laboratory conditions at 20°C , the winter-originated strains are able to achieve similar or even much higher biomass than the summer-originated isolates within 40 days of cultivation (Figure 8). Interestingly, the highest biomass at 20°C was achieved by two winter isolates of *A. gracile* (W4, W58), although this species constituted only a small part of the winter community. Preliminary data from strain culturing indicate that the winter community may also contain cyanobacteria genotypes which are not psychrotolerant, perhaps having, in fact, summer or autumn origin. This observation raises the question of whether strains that achieve high biomass under summer conditions (“summer-adapted”) can also survive under winter conditions, and vice versa: whether “winter-adapted” strains can persist in the population during summer. It appears that they may, given that cyanobacteria exhibit wide thermal tolerance (e.g., winter strains from this study grew well, even at 20°C , under laboratory conditions) and the fact that high intra-/interspecific variability of strains may exist within a bloom [60–64].

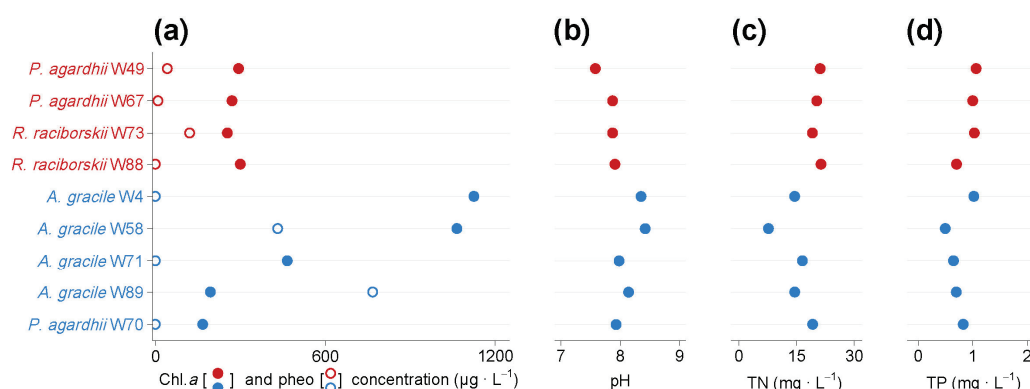


Figure 8. Preliminary data obtained from strain culturing about chlorophyll-*a* (filled circles) and pheophytin (empty circles) concentration (a), pH (b), total nitrogen concentration (c), and total phosphorus concentration (d) in 40-day-old cyanobacterial cultures. Red and blue colors indicate the origin of strains (summer and winter, respectively).

On the other hand, the lower growth rate of the summer-adapted strains in seemingly favorable conditions might come from their warm-optimized growth strategy aimed at improving the metabolism of reactive oxygen species. It has been demonstrated by Collins [65] and Lindberg and Collins [66] that temperature elevation can cause an increase in reactive oxygen species in phytoplankton organisms, and, hence, warm-adapted strains can reduce the growth rate at a higher temperature. Thus, the great diversity of strains in the population could facilitate the persistence of cyanobacteria as the main component of phytoplankton in eutrophic lakes, which does not decline even under limiting conditions, such as winter. Comparing thermal reaction norms for growth rate between the originated strains from summer and winter conditions is necessary to understand and explain more precisely the succession of cyanobacteria throughout the seasons in the studied lake.

4. Conclusions

This study demonstrates that biomass, community structure, and toxicity may differ greatly between summer and winter cyanobacterial blooms. Alien and invasive cyanobacteria, including *Sphaerospermopsis aphanizomenoides*, *Raphidiopsis raciborskii*, and *Raphidiopsis mediterranea*, were not able to actively overwinter in the studied lake even during mild winter. Investigation of strains from both sampling times revealed that toxigenic cyanobacteria appear in both summer and winter cyanobacterial communities. This study also is the second report in recent years demonstrating a dominance of *Limnothrix* over *Planktothrix* in the winter population of cyanobacteria in Lubosińskie Lake. More frequent eco-phyco-toxicological monitoring is required to understand the observed shifts in the cyanobacteria community structure and toxicity throughout the year in this lake and determine how far these changes are shaped by the incidences of mild winters.

5. Materials and Methods

5.1. Lake Location and Physicochemical Parameters

Lubosińskie Lake (Central–West Poland; 52°31′40″ N, 16°22′56″ E; mean depth = ~2.4 m, max depth = ~6.5 m) was sampled for physicochemical analyses, quantitative analyses of phytoplankton, and toxicological analyses on 2 September 2019 and 11 February 2020. Samples were collected from the lake surface using a bathometer (UWITEC, Mondsee, Austria). Additional plankton tows were collected for isolation of cyanobacteria using a plankton net with a mesh size of 40 µm. The concentrations of total nitrogen, total phosphorus, dissolved nitrogen forms, and total reactive phosphorus were determined spectrophotometrically, using a HACH DR/2010 spectrophotometer (Hach Company, Loveland, CO, USA). During sampling, water temperature, pH, and conductivity were determined in the field using a YSI multiparameter probe 556 MPS (YSI Inc., Yellow Springs, OH, USA), and Secchi depth was measured using a Secchi disc. Phytoplankton was identified and counted using a Fuchs-Rosenthal chamber. At least 400 cells or filaments were counted to reduce the error to less than 10% ($p = 0.05$). The biomass of specimens (fresh weight) was determined based on volumetric analyses of cells, using geometric approximation. Biomass computed in volume units was transposed to fresh biomass, assuming the density of phytoplankton to be 1 [67].

5.2. Biological Material

5.2.1. Isolation, Cultivation, and Identification of Cyanobacterial Strains and Sample Analyses

Isolation of cyanobacteria was performed at the Department of Hydrobiology at Adam Mickiewicz University within a few days after field sampling. The process involved picking cyanobacterial trichomes under the inverted-microscope Leica DM IL LED (Leica Microsystems, Wetzlar, Germany), using microcapillaries and their multiple transfers between drops of sterile WC medium [68] with imipenem antibiotic (100 µg/mL) until only a single trichome and no other phytoplankton organisms were visible in a drop of the medium. Subsequently, single trichomes were placed separately in vials filled with 2 mL of culture medium and incubated in a phytotron Conviron (Winnipeg, MB, Canada) at a

temperature of 20 ± 0.5 °C, light intensity of 50 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$, and photoperiod of 16 h:8 h (day/night). Successfully growing monoclonal strains were then identified based on their morphological features, following the determination keys [69,70].

To obtain information about the biomass yield of cyanobacterial strains in routine cultivation, strains were inoculated into the 175 cm² cell culture flasks (Nunc™ EasYFlask™, Thermo Scientific, San Jose, CA, USA) filled with 500 mL of WC medium. Cultures were incubated at 20 ± 0.5 °C, photoperiod of 16 h:8 h (light/dark), and light intensity of ~ 23 $\mu\text{mol photons m}^{-2}\text{s}^{-1}$. After 40 days of incubation, samples (100 mL) were collected and filtered (GF/C filters, Whatman, Maidstone, UK), and, subsequently, chlorophyll-*a* and phaeophytin concentrations were measured according to method ISO 10260:1992 [71]. The remaining filtrates were analyzed using a flow injection analyzer (FIA compact, MLE GmbH, Radebeul, Germany). Samples were filtered through membrane filters (0.45 μm , Sartorius), consequently determining the dissolved forms only. The total phosphorus was measured according to ISO 15681-1:2003 [72]. The total nitrogen method was analyzed according to ISO 29441:2010 [73].

5.2.2. Isolation of DNA and Phylogenetic Characterization of Cyanobacterial Strains

A culture volume of approximately 15 mL was used for DNA isolation. The procedure of DNA isolation was the same as in the case of lake-water samples. The gene *rpoB* (coding for the bacterial β subunit of RNA polymerase; [74]) was used for taxonomic characterization and phylogenetic analysis of the isolated cyanobacterial strains. This gene was selected over the typical gene, 16S rRNA, because it has shown variability in the size of amplicons and is recommended as marker for better differentiation of cyanobacterial taxa [75]. Two sets of primers amplifying the same region of the gene *rpoB* were selected (520–635 bp): (i) *rpoBF*/*rpoBR* (5'-ATGTGCCGCGAGGTGAAACCTAAT-3'/5'-RCMGCMGACGAAGAAGACG-3'), targeting cyanobacteria [74]; and (ii) *rpoBF1*/*rpoBF2* (5'-AGGAATTCACCACCACAACCT-3'/5'-ACCATCGGCTAATACCTG-3'), targeting cyanobacteria from the order Oscillatoriales [75]. Purification of PCR products was conducted using the QIAEX II Gel Extraction Kit (Qiagen, Hilden, Germany). Samples were sequenced by Genomed (Warsaw, Poland). The nucleotide BLAST tool was used to verify the identity of sequences with other published cyanobacterial strains. Sequences were aligned with the MUSCLE algorithm [76]. The final aligned dataset included 23 sequences, with 9 cyanobacterial strains isolated in the present study and the other 14 published strains obtained from NCBI databases for comparative purposes, with 460 aligned positions. Phylogenetic analyses were constructed with MEGA11 [77], using Neighbor Joining (NJ) and Maximum Likelihood (ML) methods for the *rpoB* aligned dataset. Bootstrap analyses with 1000 replicates (generated trees) were estimated to assess the robustness of the obtained topologies [78]. For ML analysis, the model that best fit the data was estimated with the tool "Find best DNA Models" implemented in MEGA11. The best-fitting model was K2+G+I, a Kimura 2-parameter model [79], and the following parameters were implemented: nucleotide frequencies as $f(A)$, $f(T)$, $f(C)$, and $f(G) = 0.250$; substitution rate matrix with $r(AT)$, $r(AC)$, $r(TA)$, $r(TG)$, $r(CA)$, $r(CG)$, $r(GT)$, and $r(GC) = 0.047$; and $r(AG)$, $r(TC)$, $r(CT)$, and $r(GA) = 0.156$. The proportions of sites were assumed to be invariable = 0, and gamma shape = 0.49. The final tree was constructed with coreDRAW X7 (Corel Corporation, Ottawa, ON, Canada), using the NJ topology and bootstrap support values for NJ and ML analyses obtained in MEGA11.

5.3. Toxicological Investigations

5.3.1. Enzyme-Linked Immunosorbent Assay (ELISA)

Lake-water samples (50 mL) were filtered through GF/C filters (Whatman, Maidstone, UK) and quantitatively analyzed for the presence of anabaenopeptins (APs) and saxitoxins (STXs), using Eurofins Abraxis ELISA kits (APs, Product no. 520070; STXs, 52255B; Warminster, PA, USA). Immunoassays were conducted on filtered water samples according to manufacturer protocols.

The ELISA was also used to analyze samples from 21-day-old strain cultures. The biomass of each strain, concentrated in glass vials, was homogenized by 15 min of ultrason-

ication in ultrasonic bath RK156 (Bandelin Sonorex, Berlin, Germany). Subsequently, ultrasonication (1 min using ultrasonic probe MS73 microtip and Bandelin Sonopuls HD2070 homogenizer, Bandelin electronic GmbH, Berlin, Germany) was applied. Extracted biomass was centrifuged (10 min at 15,000 rpm). Supernatants were then collected and filtered through a syringe filter with a pore diameter of 0.2 µm, with GHP membrane (PALL Corporation, New York, NY, USA), and stored at −20 °C before analysis. Immunoassays were performed on thawed filtrates using Eurofins Abraxis ELISA kits (Warminster, USA) for detection of the APs (Product No. 520070), ATX-a (520060), and STXs (52255B), according to manufacturer protocols.

5.3.2. Noncompetitive Time-Resolved Fluorescence Immunoassay (TRFIA)

The noncompetitive TRFIA was conducted following the assay concept described by Akter et al. [80], but with a slight modification. The assay detects both microcystin and nodularin, and this screening concerned the strains isolated from the lake. A culture volume of 100 mL of each strain was filtered through GF/C filters (Whatman, Maidstone, UK). The filters were then freeze-dried (Alpha 1-4 LSCplus freeze dryer, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany) and extracted in 75% methanol by ultrasonication, as in the case of samples for ELISA. The extracts were centrifuged (10 min at 15,000 rpm), and, subsequently, supernatants were collected and evaporated to dryness using a Reacti-Vap™ evaporator (ThermoFisher Scientific, Waltham, USA) with nitrogen gas (>99.99%) and a temperature of 50 °C. The reconstitution of dried extracts was performed with 150 µL of Milli-Q water. After reconstitution, the extracts were filtered through GHP Acrodisc 13 mm syringe filters (0.2 µm pore size). Prior to analyses, the prepared samples were stored at −20 °C. The 1x samples represent the extracted cyanotoxin (from original 100 mL culture) in final 150 µL of Milli-Q water. For TRFIA, each sample was diluted in Milli-Q as 1:10, and 25 µL of this diluted sample was used in each well. Briefly, on a 96-well streptavidin-coated plate, 25 µL of Assay Buffer (Kaivogen, Turku, Finland) was added to every well. Then, sample or standard (0.03–100 µg/L of microcystin-LR, prepared in Milli-Q) was added as 25 µL per well, in duplicates. For blank measurement (6 replicates), Milli-Q was added as 25 µL per well. After that, 25 µL of reagent mixture (containing the biotinylated capture antibody, immunocomplex antibody, and the Europium-labeled tracer antibody) was added to each well. The plate was incubated for 1 h, washed four times, and the time-resolved fluorescence signal of Europium was measured. Based on the blank + 3SD signal, the analytical detection limit was around 0.1 µg/L of microcystin-LR. Authentic standard NIES107 containing dmMC-RR, MC-RR, MC-YR, dmMC-LR, and MC-LR; and standard PCC8720 containing dmMC-LR, MC-LR, MC-LY, MC-LW, and MC-LF were used as positive controls in the immunoassay.

5.3.3. High-Performance Liquid Chromatography with Diode Array UV Detection (HPLC-DAD)

Lake-water samples were analyzed for the presence of microcystins (MCs) and cylindrospermopsin (CYN) using the HPLC-DAD system accessible at the UNESCO Chair on Ecohydrology and Applied Ecology at the University of Lodz (Poland). Biomass of cyanobacteria in a known volume was collected on Whatman GF/C filters. Further processing of the samples and chromatographic separation were conducted using the same instruments, equipment, and methodology as described previously [44]. Identification of cyanometabolites in the samples was based on the comparison of the retention times and UV spectra of the compounds to the standards (absorption maximum at 238 nm for MCs and at 262 nm for CYN). For calibration of HPLC analysis, MC-LR, MC-YR, MC-RR, and CYN standards provided by the TOXIC project (European Commission; contract number EVK1-CT-2002-00107) were used.

Cyanobacterial strains isolated from the lake were screened for the presence of ATX-a, CYN, MCs, and NOD using the Agilent 1100 series HPLC system (Agilent Technologies, Waldbronn, Germany) accessible at the Biochemistry and Cell Biology, Faculty of Science

and Engineering, Åbo Akademi University (Finland). Samples were processed using the same technique, instruments, and equipment as in the case of TRFIA immunoassay. The reconstitution of dried extracts was performed with either 150 µL of 75% methanol (MCs and NOD analyses) or 150 µL of Milli-Q water (CYN). After reconstitution, the extracts were filtered through GHP Acrodisc 13 mm syringe filters (0.2 µm pore size), and the prepared samples were stored at −20 °C until the analysis. Chromatographic separation of MCs and NOD was conducted on a Supelco Ascentis RP-Amide column (100 mm × 4 mm I.D., with 3 µm particles protected by a 4 × 2 mm C8 guard column), and ATX-a and CYN on a Merck Purospher Star RP-18e column (55 mm × 4 mm I.D., with 3 µm particles protected by a 4 × 4 mm C18 guard column). The mobile phase was the same as in the case of analyses of lake-water samples: solvent A was water, and solvent B was acetonitrile (both solvents with added 0.05% trifluoroacetic acid). The flow rate and linear gradient programs for particular cyanometabolite analyses were as follows: ATX-a and CYN = 0 min of 1% B, 20 min of 70% B, 22 min of 70% B, 22.1 min of 1% B, stop time of 30 min, and flow rate of 1 mL/min; and MCs and NOD = 0 min of 25% B, 7 min of 70% B, 10 min of 70% B, 10.1 min of 25% B, stop time of 15 min, and flow rate of 1 mL/min. Identification of cyanometabolites was conducted using chromatography data system ChemStation (Agilent Technologies, Waldbronn, Germany) and involved comparing the retention times and UV absorption spectra of the compounds to the standards. CYN and NOD were acquired from National Research Council (Halifax, N.S., Canada); ATX-a and MCs were present in-house standards prepared from cyanobacterial cultures ANA123 (ATX-a), NIES (MCs), and PCC7820 (MCs) originating from Finnish Environment Institute (Helsinki, Finland), National Institute for Environmental Studies (Tsukuba, Japan), and Pasteur Culture Collection of Cyanobacteria (Paris, France), respectively.

5.3.4. Liquid Chromatography–Mass Spectrometry (LC-MS)

Liquid chromatography coupled with mass spectrometry (LC-MS) was conducted on the samples from the strains using an Agilent 1200 Rapid Resolution LC coupled to a Bruker (Bruker Daltonik GmbH, Bremen, Germany) HCT ultra-ion-trap mass spectrometer with electrospray ion source. Separation of the cyanometabolites was conducted at 40 °C on a Supelco Ascentis C18 column (50 mm × 3 mm I.D. column, with 3 µm particles protected by a 4 × 2 mm C8 guard column). Extracts were injected in a volume of 5 µL, and the flow rate was 0.5 mL/min. The mobile phase was 99% water–1% acetonitrile–0.1% formic acid (solvent A) and 100% acetonitrile–0.1% formic acid (solvent B). The linear gradient solvent programs employed for each cyanotoxin were as follows: ATX-a = 0 min of 0% B, 5 min of 0% B, 7 min of 70% B, 9 min of 70% B, 9.1 min of 0% B, and stop time of 16 min; CYN, MCs, and NOD = 0 min of 0% B, 2 min of 0% B, 2.1 min of 25% B, 12 min of 70% B, 14 min of 70% B, 14.1 min of 0% B, and stop time of 21 min. The mass spectra were observed in the positive electrospray mode, with a dry temperature of 350 °C, dry gas flow of 10 L/min, nebulizer pressure of 40 psi, and capillary voltage of 4 kV. The MS scan range was m/z 160–190 (ATX-a), m/z 400–525 (CYN), and m/z 800–1050 (MCs and NOD). Identification of cyanotoxins was conducted using Compass 1.3 for HCT/esquire software. Retention times and the observed m/z values of the compounds were compared to the standards.

5.3.5. Isolation of DNA and Toxicity Assessments

DNA isolation was conducted on lake-water samples (100 mL) and on the samples from strain cultures (15 mL). Samples were filtered through nitrocellulose membrane filters (a pore diameter = 0.45 µm; Millipore, Burlington, MA, USA) and processed as described previously [81]. Isolated DNA was adopted as the template for qualitative (PCR, polymerase chain reaction) determination of the following genetic elements: *anaF* (467bp), *cyrJ* (578bp), and *mcyE* (405bp) (Table 2).

Table 2. Primers used in the present study.

Gene	Associated Metabolite	Primer Name [Primer Sequence (5'–3')]	Annealing Temperature	Reference
<i>anaF</i>	ATX-a	Atoaf [TCGGAAGCGCGATCGCAAAT] Atxar [GCTTCCTGAGAAGGTCCGCT]	57 °C	[82]
<i>cyrJ</i>	CYN	cynsulF [ACTTCTCTCCTTCCCTATC] cylnamR [GAGTGAAAATGCGTAGAACTTG]	57 °C	[83]
<i>mcyE</i>	MCs	mcyE-F2 [GAAATTTGTGTAGAAGGTGC] mcyE-R4 [AATTCTAAAGCCCAAAGACG]	56 °C	[84]

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/toxins16080357/s1>, Supplementary Information S1: Biomasses of phytoplankton taxa in Lubosińskie Lake during summer (2 September 2019) and winter (11 February 2020); Supplementary Information S2: Pairwise distance matrix between cyanobacterial strains isolated from summer and winter phytoplankton of Lubosińskie Lake and comparative sequences from GenBank, using the Kimura2-parameter model; Supplementary Information S3: The results of HPLC-DAD chromatography and ELISA immunoassay for lake-water samples; Supplementary Information S4: The results of ELISA immunoassay for extracts from cyanobacterial strains; Supplementary Information S5: The results of TRFIA immunoassay for extracts from cyanobacterial strains; Supplementary Information S6: The results of HPLC-DAD for extracts from cyanobacterial strains; Supplementary Information S7: The results of LC-MS for extracts from cyanobacterial strains; Supplementary Information S8: The results of toxigenicity assessment for extracts of summer and winter cyanobacterial strains from Lubosińskie Lake.

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