Does oligotrophication induce cyanotoxin production and accumulation in food webs of Arctic/alpine lakes?

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Abstract

Arctic/alpine lakes in Sweden are facing nutrient declines, i.e., oligotrophication, and intensified nitrogen (N)-limitation due to climate change and associated landscape alterations. These changes are expected to promote nitrogen-fixing cyanobacteria (N_2-fixers) in primary producer communities in benthic biofilms of the lakes. Many N_2-fixers are able to produce potent, harmful cyanotoxins. This study investigated whether increases in dominance of N_2-fixers affected the prevalence and accumulation of cyanotoxins – including microcystins and nodularins (as a group; MC), cylindrospermopsin, and anatoxin-a – in benthic biofilms and macroinvertebrates from nine Swedish Arctic/alpine lakes. Cyanotoxins were commonly detected in the samples. Cyanotoxin concentrations quantified by enzyme-linked immunosorbent assay (ELISA) and by liquid chromatography–mass spectrometry (LC-MS) were generally positively correlated. Data showed strong positive correlations of the relative biomass of N_2-fixers with the concentrations of cylindrospermopsin and anatoxin-a but not MC in biofilms. Most macroinvertebrate samples had higher cyanotoxin concentrations than the biofilms, indicating trophic transfer and bioaccumulation of cyanotoxins. Yet, cyanotoxin bioaccumulation did not differ among functional feeding groups of the benthic macroinvertebrates, possibly due to limited numbers of samples collected. Benthic macroinvertebrates are the key prey for fish including Arctic char and brown trout in Arctic/alpine lakes. Thus, with increases in dominance of N_2-fixers in benthic biofilms driven by lake oligotrophication and intensified N-limitation, fish likely will face increased exposure to cyanotoxins in diet or in water. This study highlights the importance of monitoring cyanotoxin risks in Arctic/alpine lakes, and the necessity of further investigating cyanotoxin bioaccumulation in the food web components especially fish.

Keywords : Oligotrophication, climate change, cyanobacteria, cyanotoxins, ELISA, LC-MS, bioaccumulation

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Abbreviations

Abbreviation	Description
ATX-a	Anatoxin-a
BAF	Bioaccumulation factor
CYN	Cylindrospermopsin
DIN	Dissolved inorganic nitrogen
dw	Dry weight
ELISA	Enzyme-linked immunosorbent assay
HDPE	High-density polyethylene
HRP	Horseradish peroxidase
LC-MS	Liquid chromatography–mass spectrometry
LD50	Lethal dose, 50%
MC	Microcystins and nodularins
N	Nitrogen
OD	Optical density
P	Phosphorus
PVDF	Polyvinylidene fluoride
SD	Standard deviation

1. Introduction

Global changes in climate and declines in atmospheric nitrogen deposition are altering the inputs of nutrients (i.e., nitrogen (N) and phosphorus (P)) from land to freshwater ecosystems in the northern high-latitude regions (Huser et al. 2018, Isles et al. 2018). Warming in the Arctic regions is particularly faster than the global average rate (IPCC 2021), accompanied by notable increases in terrestrial plant biomass (Elmendorf et al. 2012, Guay et al. 2014), i.e., a process named as greening of the Arctic. Warming and greening of the Arctic landscape enhances more efficient trapping of nutrients in terrestrial vegetation (Aerts et al. 2006). Concurrently, the Swedish Arctic/alpine region is facing declines in atmospheric N deposition linked to reductions in acid gas emissions (Isles et al. 2018, Ferm et al. 2019) attributed to successful global efforts in combating acid rains (Reis et al. 2012, Rafaj et al. 2014). Hence, the Arctic/alpine lakes in Sweden have been undergoing declines in N and P concentrations, i.e., oligotrophication, since the mid-1990s that these ecosystems are shifting toward ultra-oligotrophic and more N-limited conditions (i.e., with lower water N:P ratios) (Huser et al. 2018, Isles et al. 2018). Primary production in Swedish Arctic/alpine lakes has already been reported to be more limited by N than by P (Bergström et al. 2020). Ongoing oligotrophication and intensified N-limitation have a strong potential to further affect the development and the community composition of primary producers in the lakes.

Apart from having low nutrient levels, the Arctic/alpine lakes are also characterized by high water transparency so that photosynthesis can occur down to depths of >20 m (Vadeboncoeur et al. 2002, 2006). Benthic primary producers are therefore more significant contributors than phytoplankton to whole-lake primary production in these lakes (Vadeboncoeur & Steinman 2002, Ask et al. 2009). Benthic primary producers mainly consist of diatoms, green algae, and cyanobacteria in the Arctic/alpine lakes (Diehl et al. 2018), and they altogether can contribute up to 98% of total primary production in the lakes (Ask et al. 2009), constituting the major basal resource supporting the lake food webs (Vadeboncoeur et al. 2002, Karlsson et al. 2009). Littoral zones provide important habitats for primary producers and for both invertebrate and vertebrate consumers including aquatic insect larvae and fish (Olden et al. 2022). With ongoing oligotrophication and intensified N-limitation, the benthic primary producer assemblages, especially those in the littoral habitats, are expected to shift toward increased dominance by cyanobacteria. This is because certain cyanobacterial species are capable of fixing air nitrogen (N_2) (Paerl 2017), making them more competitive than other algae such as diatoms in nutrient- and N-limited conditions (Diehl et al.2018).

The massive growth (i.e., blooms) of cyanobacteria in pelagic habitats of aquatic ecosystems caused by nutrient pollution is a known environmental problem worldwide. However, the benthic cyanobacterial responses to climate change and oligotrophication, as well as their ecological impacts in the otherwise pristine Arctic/alpine lakes remain largely unknown. High, dense cyanobacterial mass can become significant problems for water quality and aquatic food webs, as many cyanobacterial species are capable of synthesizing various noxious compounds and/or potent toxins (Chorus 1999). Oligotrophication and intensified N-limitation (i.e., reduced lake water N:P ratios) potentially promote N_2-fixing cyanobacteria and their production of cyanotoxins as a survival or defense mechanism against competitors and/or grazing. Cyanotoxins released by cyanobacteria into the water column can be transferred to aquatic organisms including phytoplankton, zooplankton, plants, and animals. Cyanotoxins can also be taken up directly via ingestion by primary consumers, followed by bioaccumulation in subsequent trophic transfer in food webs, and consequently transferred to fish through predation (White et al. 2006, Ibelings & Havens 2008).

Cyanotoxins typically can be classified into four different categories based on the animal and human organs that they affect. These categories include hepatotoxins, neurotoxins, dermatotoxins, and cytotoxins (Corbel et al. 2014). Hepatotoxins such as microcystins mainly target the hepatocytes, which are the basic cell type in the liver. Microcystins can inhibit eukaryotic protein phosphatases and activate the enzyme phosphorylase b. This creates an excessive phosphorylation of cytoskeletal filaments, leading to death of hepatocyte and hepatic bleeding (Batista et al. 2003). Cylindrospermopsin is another hepatotoxin whose toxic effects include protein synthesis inhibition, membrane proliferation, fat droplet accumulation, and cell death (Terao et al. 1994). Neurotoxins directly affect the nervous system. For example, anatoxin-a is a neurotoxin that can attach to and overstimulate nicotinic acetylcholine receptors, forcing the sodium channels to stay open that allow excessive sodium to enter cells. Subsequent toxic effects for mammals include loss of coordination, muscle tremors, and respiratory failure (Huisman et al. 2018). Dermatotoxins such as lipopolysaccharides in cyanobacterial cell membrane can cause irritation to human skin (Huisman et al. 2018). Cytotoxins can attack cells in different organs, causing cell damages and death. The harmful effects of cyanotoxins on organisms can be acute (e.g., reduction in survivorship, feeding inhibition, paralysis) or chronic (e.g., reduction in growth and fecundity), and involving biochemical alterations (e.g., activity of phosphatases, proteases) and/or behavioural alterations (Ferrão-Filho & Kozlowsky-Suzuki 2011).

Cyanobacteria genera that produce hepatotoxins such as microcystins (MCs) and nodularins (NODs) include *Microcystis*, *Planktothrix*, and *Dolichospermum* (Wei et al. 2024). Instead, benthic mat-forming cyanobacteria genera such as

Nostoc may play a more significant role in local toxin dynamics (Jokela et al. 2017). The highest microcystin concentrations were found in invertebrates as maximum 9 μg g^{-1} dw in zooplankton and 10 μg g^{-1} dw in benthic invertebrates (Wei et al. 2024). In contrast, lower levels were recorded in the fish European smelt *Osmerus eperlanus*, with up to 2.7 μg g^{-1} dw in the liver and 0.10–0.18 μg g^{-1} dw in the muscle tissue. Cylindrospermopsin can be produced by the cyanobacteria *Cylindrospermopsis raciborskii* (Padisak 1997), *Nostocalean* spp., and *Oscillatoria* spp. (Seifert et al. 2007). Bioaccumulation of cylindrospermopsin has been recorded for snails, mussels, crayfish, fish, and tadpoles (Berry & Lind 2010). For example, the freshwater snail *Melanoides tuberculata* can accumulate cylindrospermopsin from *C. raciborskii* to reach a body cylindrospermopsin concentration up to 4.1 μg g^{-1} dry weight (White et al. 2006). Saker & Eaglesham (1999) reported that the Redclaw crayfish *Cherax quadricarinatus* harvested from a pond with *C. raciborskii* had accumulated cylindrospermopsin in hepatopancreas to a concentration of 4.3 μg g^{-1} dry weight.

Anatoxin-a (ATX-a) can be produced by widely distributed cyanobacterial genera such as *Anabaena*, *Dolichospermum*, *Aphanizomenon*, and *Cuspidothrix*, *Oscillatoria*, and *Planktothrix* (Edwards et al. 1992, Pawlik-Skowrońska et al. 2012, Plata-Calzado et al. 2024). Pawlik-Skowrońska et al. (2012) found that multiple lake fishes (i.e., roach *Rutilus rutilus*, Prussian carp *Carassius gibelio*, and European perch *Perca fluviatilis*) exposed to blooms of *Anabaena*, *Aphanizomenon*, and *Planktothrix* have accumulated ATX-a in muscles up to a concentration of 30 ng g^{-1} wet weight. All these findings imply possible bioaccumulation of cyanotoxins in lake food webs with increasing cyanobacterial dominance in primary producer communities.

Cyanotoxin concentrations in water and/or biota are commonly analysed using liquid chromatography–mass spectrometry (LC–MS) and enzyme-linked immunosorbent assay (ELISA) (Kulabhusan & Campbell 2021). Analysis by LC-MS can provide high sensitivity and accuracy, but it generally requires more time than ELISA. In contrast, ELISA is a ready-to-use test, which can be more time-efficient than chromatographic methods (Li et al. 2006). However, the direct use of ELISA kits for quantifying cyanotoxin concentrations in water samples without prior testing of the kits' performance may cause errors, because the concentrations of target analytes may exceed the detection limits and vary among samples (He et al. 2017). Nevertheless, previous research comparing ELISA and LC-MS have shown that the cyanotoxin results from these two methods generally match each other, with ELISA measurements were providing 26% closer to the true (theoretical) micocystin concentrations of fortified samples with known microcystin concentrations (Prescott et al. 2023).

1.1 Research aim

This study aims to investigate the effects of oligotrophication, characterized by nutrient declines and declining water N:P ratios (i.e., increasing N-limitation), on the prevalence and bioaccumulation of cyanotoxins in benthic habitats of nine Arctic/alpine lakes. Recent research has already shown that the relative biomass of N_2-fixing cyanobacteria in primary producer community of benthic biofilms is higher in lakes with lower water N:P ratios (i.e., stronger N-limitation) (Lau and Goedkoop 2025). In the current study, cyanotoxin concentrations in biofilms and macroinvertebrates collected from multiple benthic habitats in the lakes were analyzed using ELISA, and compared with cyanotoxin data that were previously obtained using LC-MS. I hypothesized that:

1. The cyanotoxin results from ELISA are positively correlated with those from LC-MS, although LC-MS may give higher concentrations than ELISA due to the higher sensitivity of LC-MS.

2. A higher dominance of N_2-fixing cyanobacteria is linked to higher cyanotoxin concentrations (measured by ELISA) in the biofilms.

3. A higher dominance of N_2-fixing cyanobacteria in benthic biofilms is positively correlated to the cyanotoxin accumulation in the benthic macroinvertebrates.

2. METHODOLOGY

2.1 Study lakes and sample collection

In total nine Arctic/alpine lakes in Sweden were sampled in August–September 2022, i.e., before this master thesis project was conducted (Appendix 1). Five lakes are in the Norrbotten county (Bananen, Diktar Erik, Valkeajärvi, Vassijaure, and Vuoskojaure) and four lakes in the Jämtland county (Dunnervattnet, Stor-Björsjön, Östra Helgtjärnen, Övre Fjätsjön). The lakes are situated between 62.2–68.4°N and at elevations of 315–744 m above sea level, with lake surface areas of 0.08–6.22 km². The lakes are generally oligotrophic but still cover gradients in surface water chemistry (0–4 m). Lake water concentrations of total P (TP) ranged 3–6 µg L^{-1}, total N (TN) 44–216 µg L^{-1}, dissolved inorganic N (DIN) 2–31 µg L^{-1}, dissolved organic carbon (DOC) 0.9–6.5 mg L^{-1} (Lau and Goedkoop 2025). The molar DIN:TP ratios, which indicate the degree of N-limitation (Bergström 2008), ranged between 1.2–13.2.

Epilithic biofilms (n = 2) were sampled from top surfaces of fist-sized stones randomly collected from shallow littoral habitats (≤0.4 m depth). For each sample, biofilm material from five stones was collected and combined using standardized brush methods (SS-EN 13946:2014 utg 2). The stones were photographed for estimating total top surface area and the density of primary producers. Epipelic biofilms were obtained from the top 0.5 cm of sediment cores (surface area = 33.18 cm^2) at three different habitats: 4 m, 6–9 m, and 12 m depths, each with two replicates. Lake Bananen has a maximum depth of 6 m, so epipelic biofilms were collected at 4 m and 6 m depths only. A subsample of each biofilm sample was immediately preserved with Lugol's solution for later analysis of taxonomic composition. The remaining biofilm materials were immediately kept at –20°C. The taxonomic composition of primary producers in biofilm samples was analyzed using microscopy in the lab, of which the methods are described in Lau and Goedkoop (2025).

Benthic macroinvertebrates were collected from the same habitats as for biofilm collection, i.e., littoral and at 4 m, 6–9 m, and 12 m depths. The invertebrates from littoral habitats were collected using kick-sampling (SS-EN-ISO 10870:2012), while those from deeper habitats were collected using Ekman grab samplers (SS 28190). Collected samples were immediately sorted on site to the lowest possible taxonomic level (i.e., species, genus, or family levels in general) and kept at –20°C. All biofilm and invertebrate samples were then freeze-dried and homogenized in the lab.

2.2 Cyanotoxin analysis using LC-MS

The LC-MS analysis of cyanotoxins in the biofilm and invertebrate samples was performed before this master thesis project. Detailed methods of the LC-MS analysis were reported in Lau and Goedkoop (2025). In brief, cyanotoxins from 2–3 mg dry weight of each sample was extracted using 0.5 mL 0.1% formic acid in nano-filtered water, followed by sonication and centrifugation. The extracted cyanotoxins were analyzed by LC-MS (6400 Series Triple Quadrupole LC/MS, Agilent Technologies) installed with an ACQUITY HSS T3 UPLC column. The limit of detection was 1 ng mL^{-1} in the sample solution. The analysed cyanotoxins include anatoxin-a, cylindrospermopsin, and different variants of microcystins (including microcystin-RR, microcystin-LR, microcystin-YR, microcystin-WR, microcystin-HilR, desmethyl-microcystin LR and desmethyl-microcystin RR). Cyanotoxin concentrations in the biofilm and invertebrate samples are reported as $\mu g\ g^{-1}$ dry weight (dw).

2.3 Cyanotoxin analysis using ELISA

2.3.1 Comparisons of extraction methods

Before running ELISA for toxin quantification in the samples, I compared four different methods for extracting individual cyanotoxins – i.e., microcystins (as a group with nodularins), cylindrospermopsin, and anatoxin-a – from the samples. These extraction methods included:

(i) Water + formic acid + sonication
(ii) Water + sonication
(iii) Water only
(iv) Water + heating

For anatoxin-a, five other extraction methods were compared (see below), as anatoxin-a and other neurotoxins are known to be sensitive to photodegradation and/or bacterial degradation (Osswald et al. 2007, Sundaravadivelu et al. 2022).

All these methods are commonly used for extracting cyanotoxins from solid biological samples based on the literature (e.g., Norris et al. 2001, Sundaravadivelu et al. 2022). For example, water (nano-filtered) is widely used in extraction solutions as it offers satisfactory extraction efficiency for hydrophilic cyanotoxins (Sundaravadivelu et al. 2022), while it is also environmental relevant (i.e., to reflect how easily the cyanotoxins can be released from the biological samples into natural water). Acidified methanol, acidified water is suggested to promote extraction of cyanotoxins from heterogeneous samples including water samples. Ultrasonication, centrifugation, and using heat treatments are commonly used during sample preparation to lyse cyanobacterial cells, promoting the release

of intracellular cyanotoxins into the extraction solution (Sundaravadivelu et al. 2022).

For the comparisons of extraction methods for individual cyanotoxins, I used two biofilm samples (each with two to three replicates) with contrasting cyanotoxin concentrations (high vs low; based on LC-MS data). One sample set included biofilm samples DP03, DP48, and DP43, which had high concentrations of microcystins and nodularins, cylindrospermopsin, and anatoxin-a. Another sample set included biofilm samples DP29, DP27, and DP48, characterized by low toxin concentrations. For each sample, 3.0±0.1 mg dw was weighed in Eppendorf tubes and the cyanotoxins were extracted using the different methods described below.

(i) Water + formic acid + sonication

1 mL of nano-filtered water with formic acid (0.1% final concentration) was added, followed by sonication in a water bath at maximum effect at room temperature for 15 min. The samples were then centrifuged for 30 min at 3000×g, filtered using PVDF syringe filters into small glass vials, and kept at -20°C until ELISA analysis.

(ii) Water + sonication

1 mL of nano-filtered water was added to the samples, followed by sonication, centrifugation, filtration, and storage as described in method (i).

(iii) Water only

1 mL of nano-filtered water was added, followed by vortexing for a few min and then standing for 15 min. Samples were then subjected to centrifugation, filtration, and storage as described in method (i).

(iv) Water + heating

1 mL of nano-filtered water was added, and the tubes were placed in a water bath at 80°C for 10 min before undergoing the same centrifugation, filtration, and storage steps as in method (i).

For anatoxin-a, a diluent solution was prepared by mixing 9 mL of nano-filtered water with 1 mL of 10X diluent. This diluent solution was used for preventing degradation of anatoxin-a in the samples and came with the kit. Similar extraction methods as (i) to (iv) were then used, except that the 1 mL of nano-filtered water was replaced by 100 µL diluent solution and 900 µL nano-filtered water with formic acid (0.1% final concentration) (for method (i)) or 100 µL diluent solution and 900 µL nano-filtered water (for methods (ii) to (iv)). An extra method, which used 1 mL of nano-filtered water without diluent solution,

i.e., identical to method (iii) described above, was also included for the comparisons.

After extraction, the samples were analyzed for cyanotoxin concentrations using ELISA within 24 hours. Data showed that the concentrations of cyanotoxins extracted by method (iii), which used water only, were higher than or did not differ from those extracted by the other methods (more detailed results of the method comparisons are presented in section 3). Thus, I proceeded to use method (iii) for extracting cyanotoxins from the biofilm and invertebrate samples for ELISA. Given the aquatic origin of cyanotoxins, extraction using MilliQ water only is considered as a more environmentally relevant method than the other extraction methods according to Selvaraj (2024). Cylindrospermopsin extraction was the most effective by using MilliQ water and alternative freezing/thawing (1.54 μg mg^{-1} dw). The highest anatoxin concentration (0.60 μg mg^{-1} dw) was recorded using MilliQ water and microwave treatment for 10–15 s (Selvaraj et al. 2024).

2.3.2 ELISA for microcystins and nodularins

Quantification of the total concentrations of microcystins and nodularins in the samples were conducted using the ABRAXIS® Microcystins-ADDA ELISA kit. This kit used an indirect competitive enzyme-linked immunosorbent technique. This kit quantified microcystins and nodularins by measuring light absorbance, which was inversely related to the cyanotoxin concentrations, using a standard curve. The kit included a microtiter plate (12 × 8 strips) coated with a microcystins analog-protein conjugate, six standards from Standard 0 to Standard 6 (0–5.0 ppb), a quality control standard (0.75 ± 0.185 ppb), antibody solution (6 mL), conjugate solution (12 mL), 5X wash buffer concentrate (100 mL), and stop solution (6 mL).

In the start, the extracted sample solutions were taken out from the freezer and kept at room temperature for 1 h until they returned to liquid form. Duplicates of 50 μL of the sample solutions, standard solutions, and control were added into individual wells of the microtiter plate. 50 μL of the antibody solution was then added into each well. The wells were covered with parafilm. The plate was then subjected to a rapid circular motion on the bench for 30 s to mix the content solutions, and incubated at room temperature for 90 min. Afterward, the parafilm was removed, and the solutions in the wells were discarded into a sink by shaking the plate vigorously. Wells were washed three times with 1X washing buffer solution (250 μL) and by shaking vigorously to remove the solution into a sink. Any remaining washing buffer in the wells was removed by patting the inverted plate on clean paper towels. 100 μL of the enzyme conjugate solution was then added to each well. The wells were again covered with parafilm. To ensure uniform mixing, the ELISA plate was gently agitated in a circular motion by

using hand on the bench and then incubated at room temperature for 30 minutes. Afterwards, the washing procedure was repeated three times as described above.

To start the colorimetric reaction, 100 µL of substrate/color solution was added to each well and the plate was incubated at room temperature for 25 min, protected from direct sunlight. 50 µL of stop solution was then added to each well to stop the reaction. The plate was kept in a calibrated microplate ELISA reader and the wavelength of 450 nm was used to measure the absorbance value of the wells. The relative absorbance value for each standard was then calculated:

%B/B0 = (Mean absorbance of Standard (B) / Mean absorbance of Standard 0 (B0)) X 100.

A standard curve was constructed by plotting the relative absorbance values (%B/B_0) of the standards against their corresponding cyanotoxin concentrations on a logarithmic scale. The concentrations of microcystins and nodularins in the samples were then determined by interpolating their OD values on the standard curve. All calculations were performed using the analysis software associated with the ELISA reader. To ensure data reliability, quality control samples and blank wells were included in the analysis. Samples with a concentration of microcystins and nodularins lower than standard 1 (0.15 ppb) were regarded inaccurate according to the kit's protocol, and they were excluded for subsequent data analysis. Samples showing a concentration higher than standard 5 (5.0 ppb) were diluted and tested again to obtain accurate results. The concentration of the positive control provided should be 0.75 ± 0.185 ppb (mean ± SD).

For microcystins and nodularins, in total 119 samples were analyzed by ELISA, with 84 biofilm samples and 35 macroinvertebrate samples. Results were obtained from 25 biofilm samples and 22 macroinvertebrates samples after excluding samples with invalid measurements (i.e., below the standard curve limits).

2.3.3 ELISA for cylindrospermopsin

The ABRAXIS® Cylindrospermopsin ELISA kit was used for quantifying cylindrospermopsin concentrations in the samples. The kit included a microtiter plate (12 × 8 strips) coated with a secondary antibody (goat anti-rabbit), seven standards from Standard 0 to Standard 6 (0–2.0 ppb), a quality control standard (0.75 ± 0.15 ppb), three glass vials of lyophilized cylindrospermopsin-HRP enzyme conjugate, conjugate diluent (12 mL), an empty amber HDPE bottle for preparing reconstitution enzyme conjugate, rabbit anti-cylindrospermopsin antibody solution (6 mL), 5X wash buffer concentrate (100 mL), and stop solution (12 mL).

Duplicates of 50 µL of the sample solutions, the standard solutions, and the quality control solution were added to the wells in the microtiter plate. The lyophilized enzyme conjugate was reconstituted by adding 3 mL of the conjugate diluent and vortexing well. The mixture was then let stand for at least 10 min, revortexed, and 50 µL of the reconstituted enzyme conjugate was added to each well, followed by adding 50 µL antibody solution. The wells were covered by parafilm, and the content solutions were mixed by rotating the plate. The plate was incubated at room temperature for 45 min. The parafilm was removed. Solutions in the wells were discarded into a sink, and the wells were washed four times with 250 µL washing buffer per well in the same way as in ELISA for microcystins and nodularins. Next, 100 µL substrate/color solution was added, and wells were covered, mixed, and incubated for 30–45 min in dark. After incubation, 100 µL stop solution was added, and the OD was measured at 450 nm using a calibrated ELISA reader. Determination of cylindrospermopsin concentrations was performed by calculating the relative absorbance value for each standard in the same way as for microcystins and nodularins. Samples with cylindrospermopsin concentrations below 0.05 ppb were regarded inaccurate according to the kit's protocol, and they were excluded for subsequent data analysis. Samples with concentrations exceeding 2.0 ppb were diluted and reanalyzed. Quality control samples, including the positive control (0.75 ± 0.15 ppb), were included in the analysis to ensure data reliability.

For cylindrospermopsin, in total 72 samples were analyzed, including 40 biofilm and 32 macroinvertebrate samples. Results were obtained from 34 biofilm samples and 28 macroinvertebrates samples after excluding samples with invalid measurements (i.e., below the standard curve limits).

2.3.4 ELISA for anatoxin-a

The ABRAXIS® Anatoxin ELISA Plate Kit was used for quantifying anatoxin-a concentrations in the samples. The kit was a direct competitive ELISA based on the recognition of anatoxin-a by a monoclonal antibody. Anatoxin-a degrades when exposed to natural or artificial light and under high pH conditions. The kit included a microtiter plate coated with a secondary antibody (anti-mouse) in a resealable pouch, three glass vials of lyophilized anatoxin-a-HRP enzyme conjugate, conjugate diluent (12 mL), three glass vials of lyophilized anti-anatoxin-a antibody, antibody diluent (12 mL), empty clear HDPE bottles and empty amber HDPE bottles for reconstitution, six standards from Standard 0 to Standard 5 (0–5.0 ppb), a control at 0.75 ± 0.185 ppb (1.5 mL), 5X wash buffer concentrate (100 mL), and stop solution (12 mL).

Duplicates of 50 µL of the sample solutions, standard solutions, and control, were added to the wells of the plate. Then, reconstituted enzyme conjugate and

reconstituted antibody solution were prepared following the same procedure as in ELISA for cylindrospermopsin. Afterward, 50 µL of reconstituted enzyme conjugate was added to the wells, followed by the addition of 50 µL of reconstituted antibody solution. The wells were covered by parafilm and incubated at room temperature for 60 min. After incubation, the same procedures for well washing using buffer solution, and the addition of color solution and stop solution as those for cylindrospermopsin were followed. The plate was incubated at room temperature for 20-30 min. After the absorbance was measured at 450 nm using a microplate ELISA reader, estimation of anatoxin-a concentrations was performed by calculating the relative absorbance value for each standard in the same way as for microcystins and nodularins. Samples with anatoxin-a concentrations lower than standard 1 (0.15 ppb) were regarded inaccurate according to the kit's protocol, and they were excluded for subsequent data analysis. Samples with concentrations higher than standard 5 (5.0 ppb) were reported as > 5.0 ppb or diluted and reanalyzed. The positive control concentration should be 0.75 ± 0.185 ppb.

For anatoxin-a, in total 83 samples were analyzed, with 51 biofilm and 32 macroinvertebrate samples. Results were obtained from 49 biofilm samples and 27 macroinvertebrates samples after excluding samples with invalid measurements (i.e., below the standard curve limits).

2.4 Data analyses

Cyanotoxin concentrations in the biofilm and invertebrate samples determined by ELISA are reported as µg g^{-1} dry weight. For each cyanotoxin (i.e., microcystins+nodularins, cylindrospermopsin, and anatoxin-a), the extraction methods were compared using mixed effect ANOVA. In the mixed effect ANOVA models, extraction method and sample identity were used as fixed factors, while sample replicate was used as a random factor nested under sample identity. The interaction between extraction method and sample identity was also included in the models. If the ANOVA detected significant effects of extraction method or sample identity, the post hoc Tukey's honest significance test was used for pairwise comparisons.

To address the first hypothesis, Pearson correlation was used to test for relationships between the ELISA and the LC-MS cyanotoxin data. Correlations were run for individual cyanotoxins (i.e., microcystins+nodularins, cylindrospermopsin, and anatoxin-a) and individual sample types (i.e., biofilms, macroinvertebrates, and biofilms and macroinvertebrates together). When data were not normally distributed, they were first $\log_{10}(x + 1)$ transformed. If the transformed data still did not follow a normal distribution, the non-parametric Spearman rank correlation was used instead. Paired t-test was also used to

compare between LC-MS and ELISA data and to quantify their differences for each cyanotoxin.

For the second hypothesis, Spearman rank correlation was used to test for relationships between the concentrations of individual cyanotoxins and the relative biomass of N_2-fixing cyanobacteria in the biofilms. Spearman correlation was used as the relative biomass data of N_2-fixing cyanobacteria did not follow a normal distribution.

To address the third hypothesis, the cyanotoxin bioaccumulation factor (BAF) was first calculated for the littoral macroinvertebrate samples based on the cyanotoxin concentrations of littoral biofilms in their corresponding lakes:

$$\mathrm{BAF} = X_{\mathrm{Invertebrate}} / X_{\mathrm{MeanBiofilms}}$$

Where $X_{\mathrm{Invertebrate}}$ is the concentration of a specific cyanotoxin in the littoral macroinvertebrate sample, and $X_{\mathrm{MeanBiofilms}}$ is the mean concentration of that cyanotoxin of the duplicate littoral biofilm samples from the same lake. BAF values greater than 1 indicate potential bioaccumulation, i.e., cyanotoxin concentrations are higher in the macroinvertebrates than in the biofilms. Spearman rank correlation was then used to assess whether the BAF was associated with the relative biomass of N_2-fixing cyanobacteria in the biofilms.

The littoral macroinvertebrates were further grouped based on their functional feeding: grazers, detritivores, generalists, and predators. One-way ANOVA (with post hoc Tukey's honest significance test whenever necessary) was then used to compare the BAF of individual cyanotoxins among the functional feeding groups across all lakes. This was to assess whether the cyanotoxin bioaccumulation increased in higher trophic levels. All statistical analyses were performed using Minitab® 19. Significance level was set to 0.05.

3. Results

3.1 Comparisons of extraction methods

3.1.1 Microcystins and nodularins (as a group)

There were significant effects of extraction methods on the concentrations of microcystins and nodularins (as a group) in the two selected biofilm samples (DP03 and DP29) (Table 1; Fig. 1). Cyanotoxin extraction using water + heating and water + sonication yielded higher concentrations than using water + formic acid + sonication (Table 2). Extraction using only water yielded concentrations of microcystins and nodularins that were not different from those obtained using other extraction methods (Table 2). Also, as expected, the two samples significantly differed in their concentrations of microcystins and nodularins, with higher concentrations detected in DP03 (overall mean = 0.62 µg g^{-1} dw) than in DP29 (0.25 µg g^{-1} dw) (Tables 1, 2; Fig. 1).

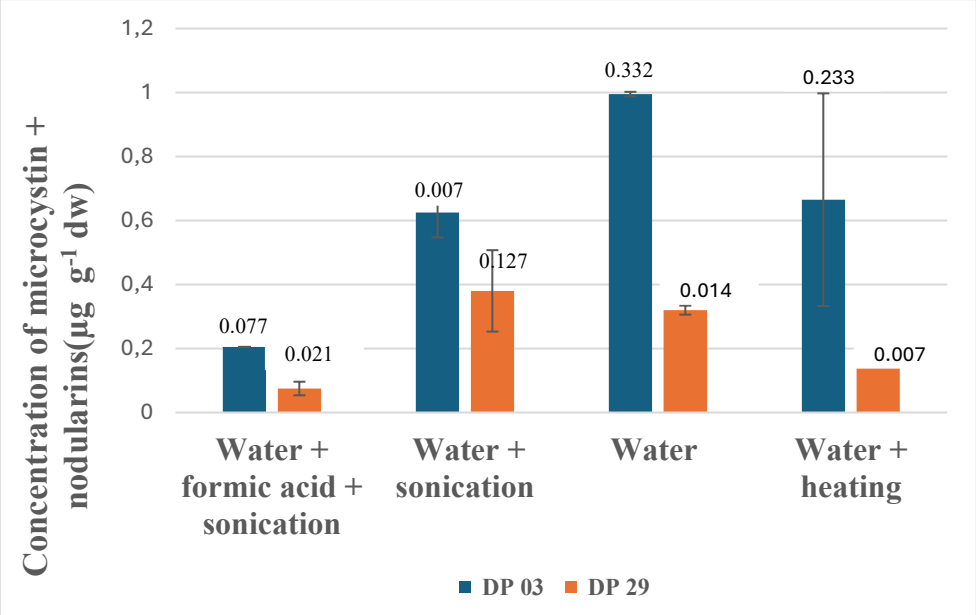

Figure 1. Concentrations (µg g^{-1} dw) of microcystins and nodularins measured by ELISA in two biofilm samples (DP03 and DP29) across the four extraction methods. DP03 and DP29 were selected for the method comparisons as they showed high and low concentrate respectively, based on LC-MS data. Bars show mean of two replicates ± SD (values).

Table 1. Mixed effect ANOVA results for the effects of extraction method, sample identity, and their interaction on the concentrations of microcystins and nodularins in two biofilm samples. DF Num = degree of freedom numerator; DF Den = degree of freedom denominator. P values smaller than 0.05 are boldfaced.

Factor	DF Num	DF Den	F value	P Value
Extraction Method	3.00	8.00	8.01	**0.009**
Sample identity	1.00	8.00	24.27	**0.001**
Extraction Method * Sample identity	3.00	8.00	2.47	0.136

Table 2. Tukey's Post Hoc Test for the effects of extraction method, on the concentrations of microcystins and nodularins in two biofilm samples. N=Number of samples.

Extraction Method	N	Mean	Grouping
Water	4	0.6575	A
Water + sonication	4	0.5025	A
Water + heating	4	0.4350	A B
Water + formic acid + sonication	4	0.1400	B

3.1.2 Cylindrospermopsin

No significant differences in cylindrospermopsin concentrations were found among the extraction methods (Table 3; Fig. 2). As expected, the two selected biofilm samples (DP27 and DP48) significantly differed in their cylindrospermopsin concentrations, with DP48 (overall mean = 0.14 µg g^{-1} dw) showing higher concentrations than DP27 (0.03 µg g^{-1} dw) (Tables 3,4; Fig. 2).

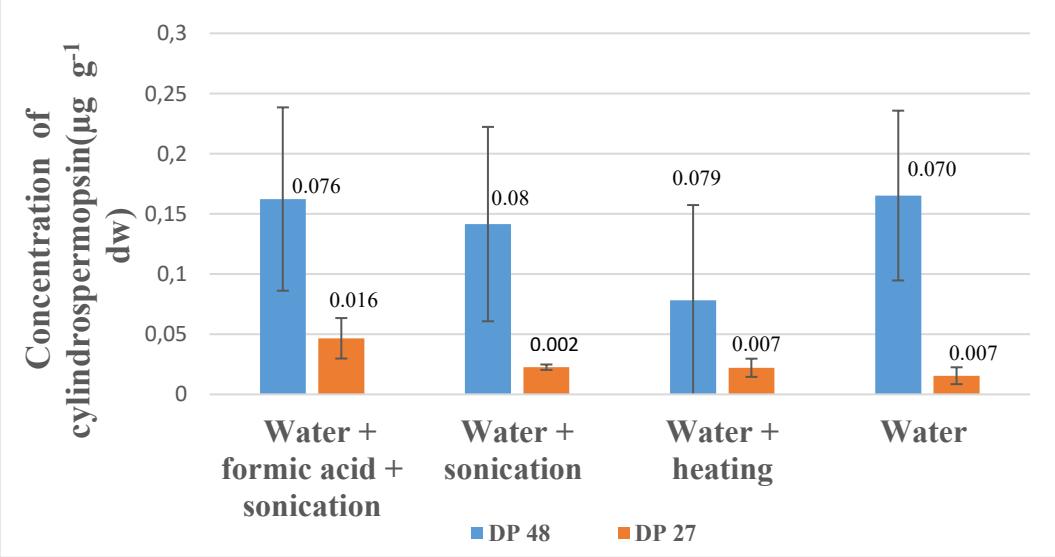

Figure 2.Cylindrospermopsin concentration (µg g^{-1} dw) measured by ELISA in two biofilm samples (DP27 and DP48) across the four extraction methods. DP27 and DP48 were selected for the method comparisons as they showed low and high cylindrospermopsin concentrations, respectively, based on LC-MS data. Bars show mean of two replicates ± SD (values).

Table 3. Mixed effect ANOVA results for the effects of extraction method, sample identity, and their interaction on the cylindrospermopsin concentrations in two biofilm samples. DF Num = degree of freedom numerator; DF Den = degree of freedom denominator. P values smaller than 0.05 are boldfaced.

Factor	DF Num	DF Den	F value	P Value
Extraction Method	3.00	8.00	0.71	0.575
Sample identity	1.00	8.00	16.20	**0.004**
Extraction Method * Sample identity	3.00	8.00	0.51	0.686

Table 4. Tukey's Post Hoc Test for the effects of extraction method, on the concentrations of Cylindrospermopsin in two biofilm samples. N=Number of samples.

Extraction Method	N	Mean	Grouping
Water + formic acid + sonication	4	0 .1045	A
Water only	4	0.0903	A
Water + sonication	4	0.0820	A
Water + heating	4	0.0501	A

3.1.3 Anatoxin-a

The extraction methods had significant effects on the measured anatoxin-a concentrations of the two selected biofilm samples (DP43 and DP48) (Table 5; Fig. 3). Concentrations of anatoxin-a extracted using water with diluent were not different from those extracted using water without diluent but were significantly higher than the concentrations obtained using the other methods (Table 6). Extraction using only water without diluent yielded similar concentrations as extraction using water (with diluent) + formic acid + sonication or using water (with diluent) + sonication (Table 6). Extraction using water (with diluent) + heating gave significantly lower concentrations than that using only water without diluent (Table 6). Again, as expected, the measured anatoxin-a concentrations

differed between the two samples, with higher concentrations detected in DP43 (overall mean = 4.30 µg g^{-1} dw) than in DP48 (0.32 µg g^{-1} dw) (Tables 5,6; Fig. 3).

The interaction between extraction method and sample identity was also significant (Table 5). Post hoc Tukey's comparisons showed that the differences in anatoxin-a concentration among the extraction methods were larger for the biofilm sample with a higher concentration (i.e., DP43) than that with a lower concentration (i.e., DP48) (Table 6).

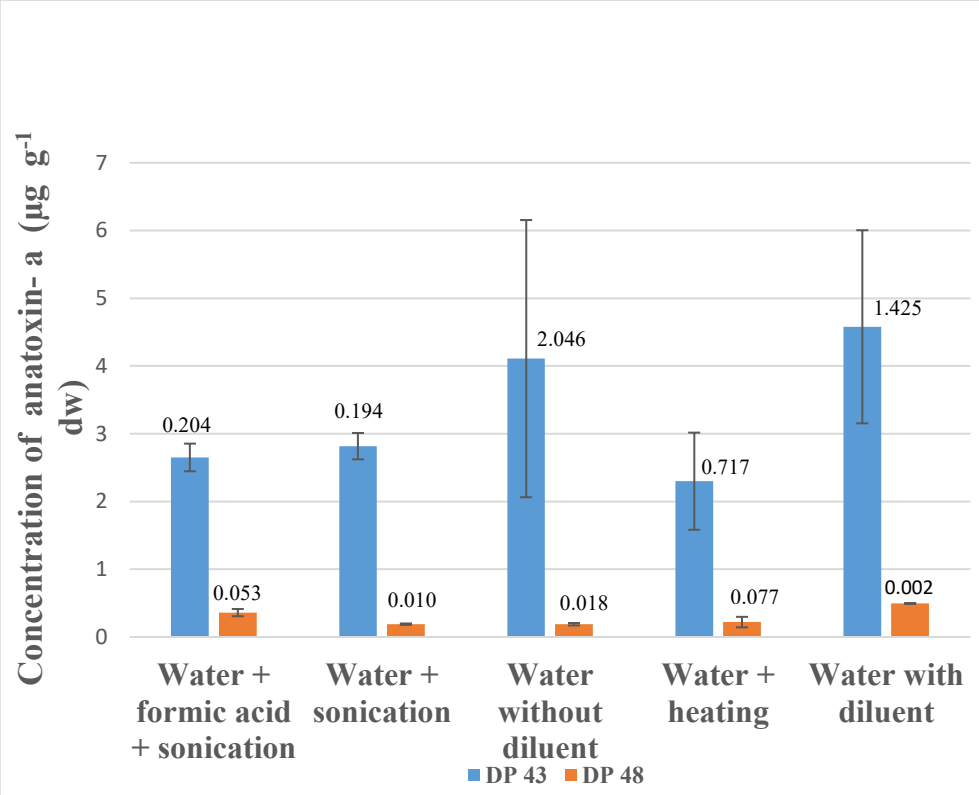

Figure 3. Anatoxin-a concentrations (µg g^{-1} dw) measured by ELISA in two biofilm samples (DP43 and DP48) across the five extraction methods. DP43 and DP48 were selected for the method comparisons as they showed high and low concentrations, respectively. based on LC-MS data. Note that these extraction methods were not the same as those used for the other cyanotoxins, see section 2.3.1 for details. Bars show mean of three replicates ± SD (values).

Table 5 . Mixed effect ANOVA results for the effects of extraction method, sample identity, and their interaction on the anatoxin-a concentrations in two biofilm samples. DF Num = degrees of freedom numerator; DF Den = degrees of freedom denominator. P values below 0.05 are in bold.

Term	DF Num	DF Den	F value	P Value
Extraction Method	4.00	18.00	11.45	**<0.001**
Sample 2	1.00	18.00	132.16	**<0.001**
Extraction Method * Sample 2	4.00	18.00	9.33	**<0.001**

Table 6. Tukey's Post Hoc Test for the effects of extraction method, on the concentrations of anatoxin-a in two biofilm samples. N=Number of samples.

Extraction Method	N	Mean	Grouping
Water with diluent	6	4.1430	A
Water without diluent	4	3.1603	A B
Water + formic acid + sonication	6	1.5051	B C
Water + sonication	6	1.4894	B C
Water + heating	6	1.2598	C

3.2 Comparisons between ELISA and LC-MS

3.2.1 Microcystins and nodularins

Concentrations of microcystins and nodularins measured by ELISA ranged 0.045-3.092 µg g^{-1} dw for biofilms and 0.012-6.730 µg g^{-1} dw for macroinvertebrates. Concentrations of microcystins measured by LC-MS were 0.000-9.909 µg g^{-1} dw for biofilms and 0.000-5.257 µg g^{-1} dw for macroinvertebrates. There was a significant positive correlation between ELISA and LC-MS measurements for biofilms (Table 7; Fig. 4). However, no correlations between ELISA and LC-MS measurements were found for macroinvertebrates or for the biofilm and macroinvertebrate samples together. Paired t-test showed no significant differences between ELISA and LC-MS measurements for the biofilm and macroinvertebrate samples together (Tables 8 and 9).

Table 7. Spearman rank (r) correlation results for measured cyanotoxin concentrations between ELISA and LCMS for the biofilms, benthic macroinvertebrates, and both biofilms and macroinvertebrates (all samples). P values smaller than 0.05 are boldfaced.

Sample type	Cyanotoxin	N	Spearman r	P Value
Biofilms	Microcystins and nodularins	25	0.453	**0.023**
Benthic macroinvertebrates	Microcystins and nodularins	22	0.072	0.750
All samples	Microcystins and nodularins	47	0.231	0.118
Biofilms	Cylindrospermopsin	34	0.566	**<0.001**
Benthic macroinvertebrates	Cylindrospermopsin	28	0.424	**0.025**
All samples	Cylindrospermopsin	62	0.636	**<0.001**
Biofilms	Anatoxin-a	49	0.356	**0.012**
Benthic macroinvertebrates	Anatoxin-a	27	0.543	**0.003**
All samples	Anatoxin-a	76	0.607	**<0.001**

Table 8. Concentrations (µg g^{-1} dry weight; mean ± SD) microcystins and nodularins, cylindrospermopsin, and anatoxin-a measured by ELISA and LC-MS for the biofilms and the macroinvertebrates.

Cyanotoxin	Number of samples	ELISA	LC-MS	Difference ELISA – LC-MS
Microcystins and nodularins	47	0.730 ± 1.209	0.510 ± 1.649	0.220 ± 1.659
Cylindrospermopsin	62	0.090 ±0.090	10.870 ± 19.290	-10.790 ± 19.230
Anatoxin-a	76	0.352 ± 0.756	1.330 ± 4.095	-0.978 ± 3.386

Table 9 . Results of paired t-test on cyanotoxin concentrations measured by ELISA and LC-MS. P values smaller than 0.05 are boldfaced.

Cyanotoxin	t Value	P value
Microcystins and nodularins	0.91	0.368
Cylindrospermopsin	-4.42	**0.000**
Anatoxin-a	-2.52	**0.014**

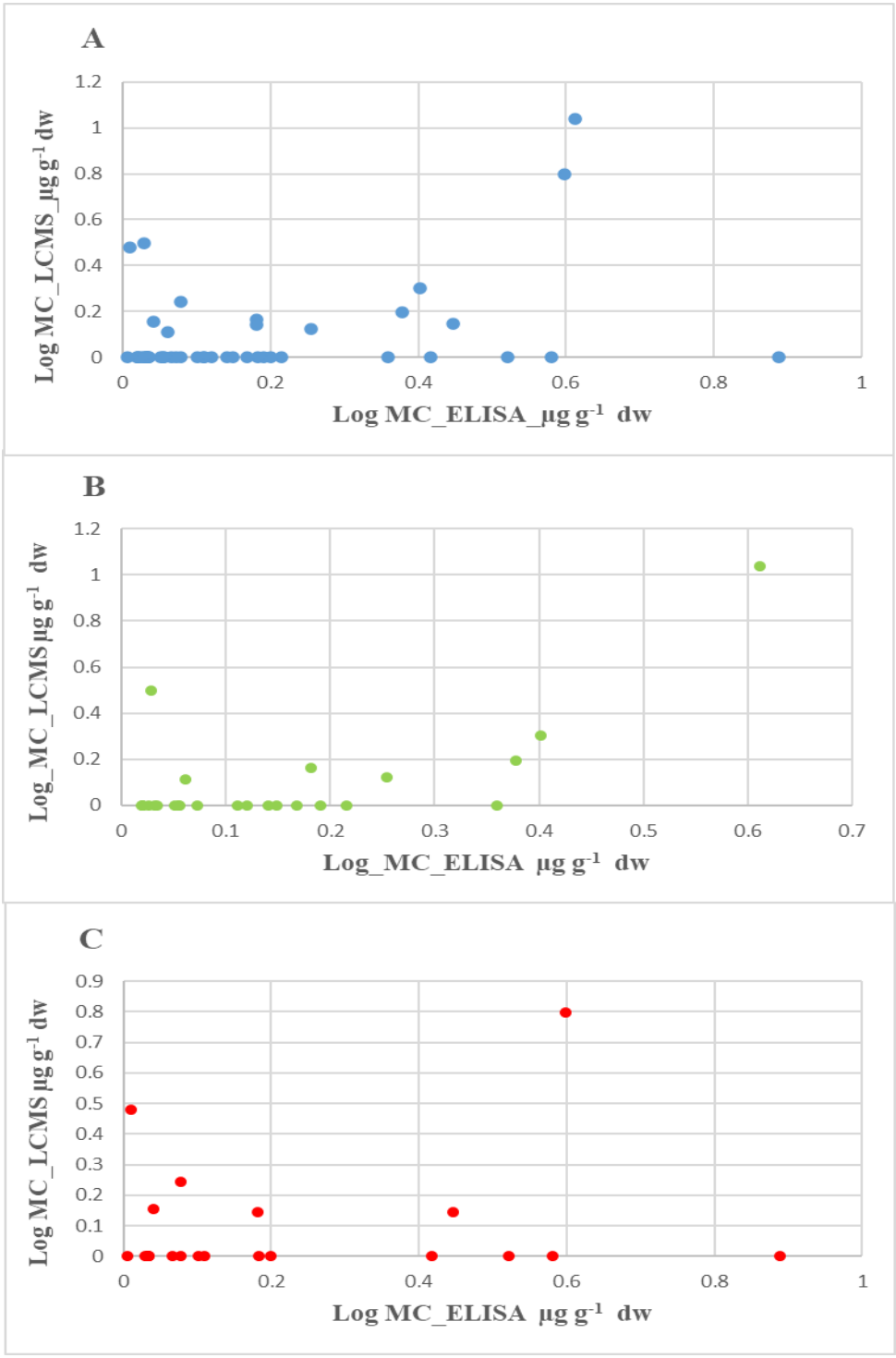

Figure 4. Concentrations of microcystins (MC; µg g^{-1} dry weight) measured by LC-MS against concentrations of microcystins and nodularins (µg g^{-1} dry weight) measured by ELISA for (A) all biofilm and macroinvertebrate samples, (B) only biofilms, and (C) only macroinvertebrates. Data were $log_{10}(x+1)$ transformed to enhance visualization.

3.2.2 Cylindrospermosin

Cylindrospermopsin concentrations measured by ELISA ranged 0.021-0.197 μg g^{-1} dw for biofilms and 0.023-0.388 μg g^{-1} dw for macroinvertebrates, while those measured by LC-MS were 0.000-8.257 μg g^{-1} dw for biofilms and 0.000-88.940 μg g^{-1} dw for macroinvertebrates. The ELISA measurements were positively correlated with the LC-MS measurements for biofilms and macroinvertebrates separately or altogether (Table 7; Fig. 5). Yet, there were significant differences between the ELISA and LC-MS measurements of cylindrospermopsin concentrations for all samples, with LC-MS yielding much higher concentrations than ELISA (Tables 8 and 9).

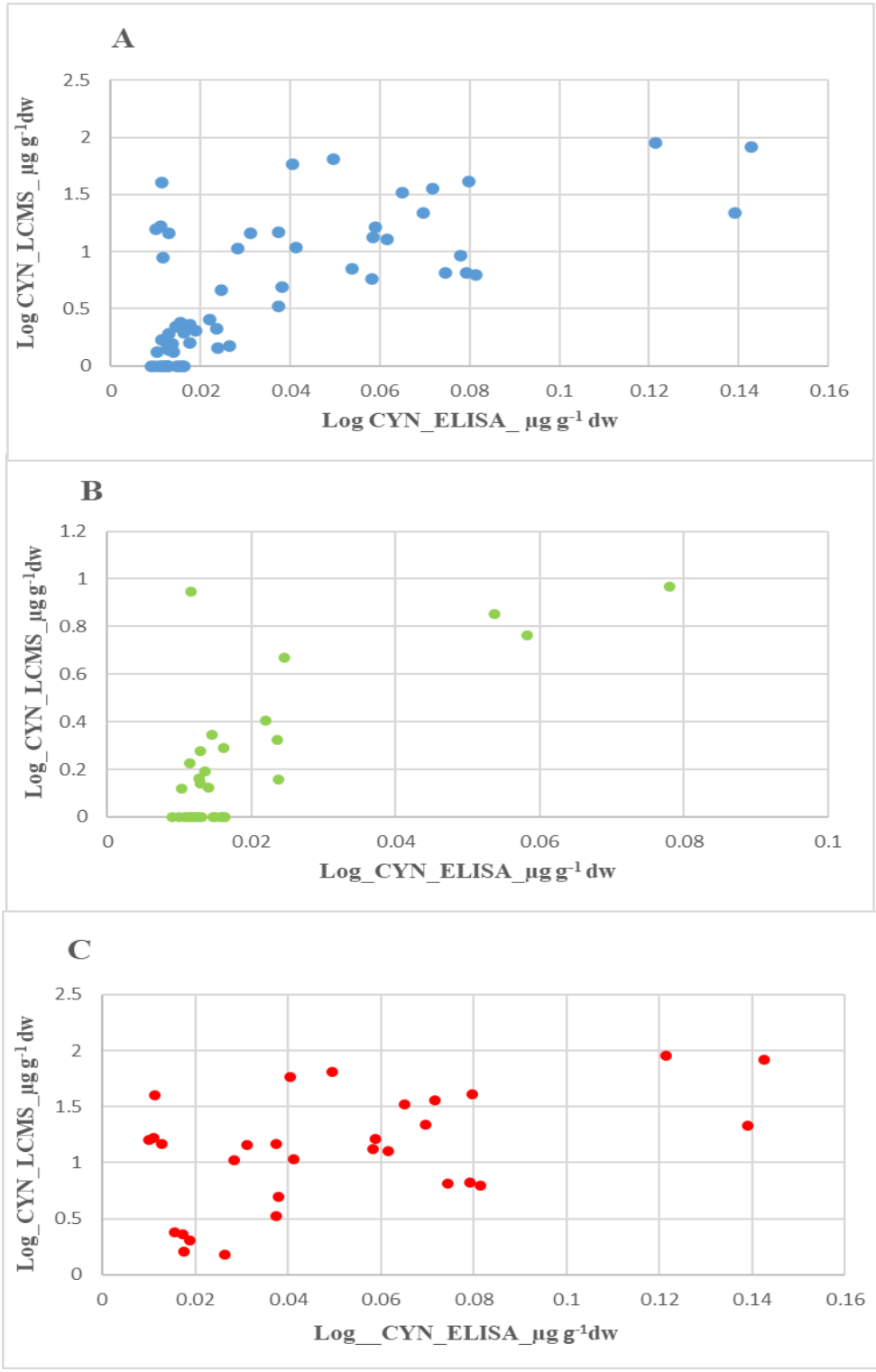

Figure 5. Concentrations of cylindrospermopsin (CYN; µg g^{-1} dry weight) measured by LC-MS against those by ELISA for (A) all biofilm and macroinvertebrate samples, (B) only biofilms, and (C) only macroinvertebrates. Data were log10(x+1) transformed to enhance visualization.

3.2.3 Anatoxin-a

Anatoxin-a concentrations measured by ELISA ranged 0.060-6.079 µg g^{-1} dw for biofilms and 0.128-0.928 µg g^{-1} dw for macroinvertebrates, while those measured by LC-MS were 0.000-28.036 µg g^{-1} dw for biofilms and 0.000-3.680 µg g^{-1} dw for macroinvertebrates. The ELISA measurements were positively correlated with the LC-MS measurements for biofilms and macroinvertebrates separately or altogether (Table 7; Fig. 6). However, there were significant differences between the ELISA and LC-MS measurements of anatoxin-a concentrations for all samples, with LC-MS yielding higher concentrations than ELISA (Tables 8 and 9).

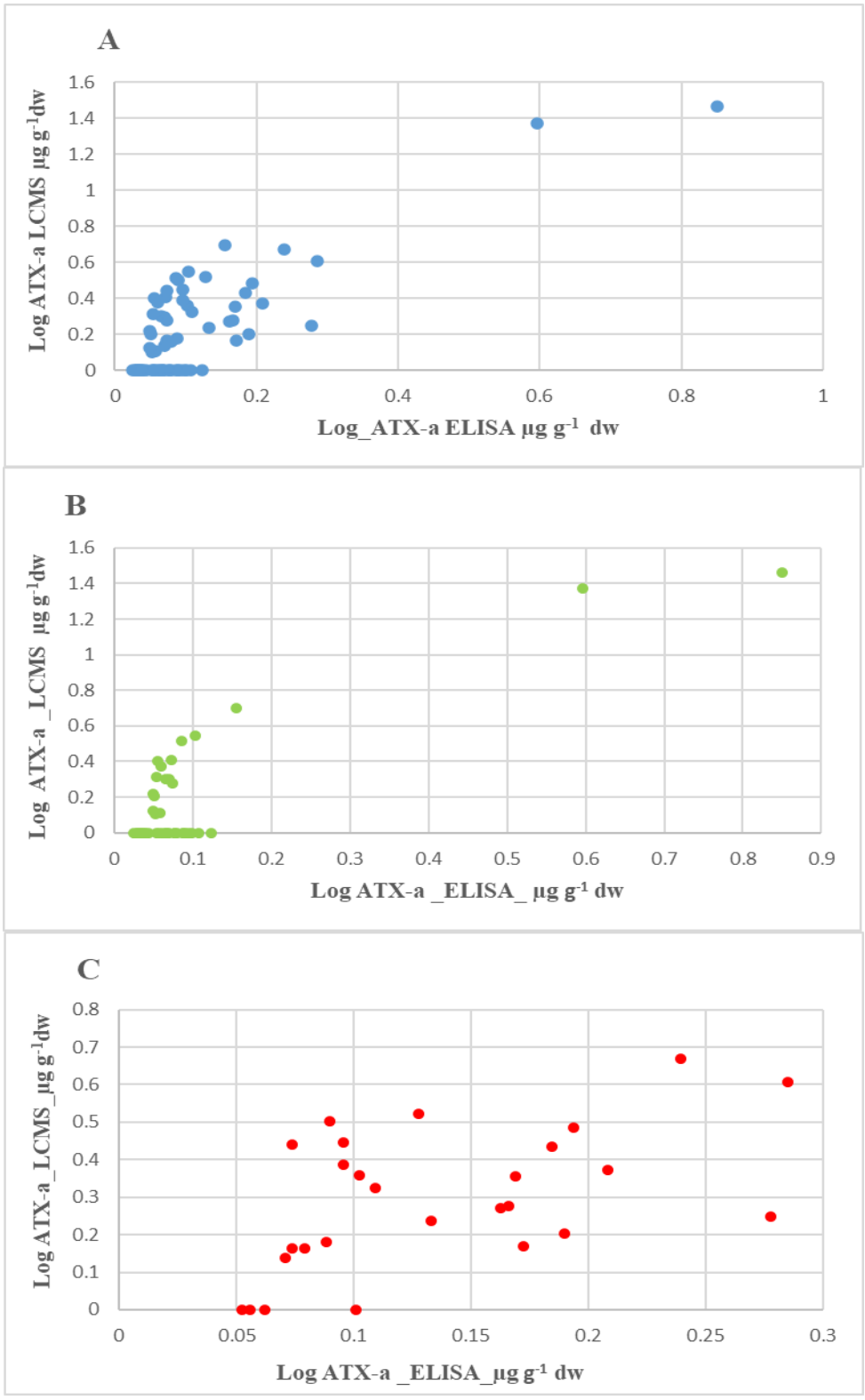

Figure 6. Concentrations of anatoxin-a (ATX; µg g^{-1} dry weight) measured by LC-MS against ELISA for (A) all biofilm and macroinvertebrate samples, (B) only biofilms, and (C) only macroinvertebrates. Data were log10(x+1) transformed to enhance visualization.

3.3 Links between N_2-fixing cyanobacteria prevalence and cyanotoxin concentrations

The relative biomass of N_2-fixing cyanobacteria in the primary producer community of benthic biofilms ranged from 0.0-78.2% in the littoral habitats, and 0.0-26.0% in the deeper habitats. The relative biomass of N_2-fixing cyanobacteria was positively correlated with the concentrations of cylindrospermopsin and anatoxin-a in the biofilms but was not correlated with the concentrations of microcystins and nodularins in the biofilms (Table 10; Fig. 7A, C, E). There were no significant correlations between the relative biomass of N_2-fixing cyanobacteria with the cyanotoxin concentrations in the benthic macroinvertebrates (Table 10; Fig. 7B, D, F).

All littoral benthic macroinvertebrates had a bioaccumulation factor (BAF) greater than 1 for all the cyanotoxins, i.e., cyanotoxin concentrations in the littoral benthic macroinvertebrates were higher than those in the littoral biofilms (Fig. 8). The BAF ranged 0.118-16.514 for microcystins and nodularins, 0.161-11.890 for cylindrospermopsin, and 0.079 – 5.917 for anatoxin-a. There were no significant correlations between the relative biomass of N_2-fixing cyanobacteria and the BAF for all three cyanotoxin groups (Table11; Fig. 8). The BAF of individual cyanotoxins also did not differ among functional feeding groups of the littoral benthic macroinvertebrates (Table 12).

Table 10. Results of Spearman rank correlation of the relative biomass (%) of N_2-fixing cyanobacteria in primary producer community of the biofilms with the cyanotoxin concentrations (µg g^{-1} dry weight) in the biofilms and macroinvertebrates.

Sample Type	Toxin Type	N	Correlation	P Value
Biofilms	Microcystins and nodularins	18	0.076	0.766
Benthic macroinvertebrates	Microcystins and nodularins	22	-0.406	0.061
Biofilms	Cylindrospermopsin	24	0.638	**0.001**
Benthic macroinvertebrates	Cylindrospermopsin	28	0.106	0.576
Biofilms	Anatoxin-a	45	0.389	**0.008**
Benthic macroinvertebrates	Anatoxin-a	27	-0.161	0.423

Table 11. Results of Spearman rank correlation of the relative biomass (%) of N_2-fixing cyanobacteria in primary producer community of the littoral biofilms with the bioaccumulation factors of individual cyanotoxins of the littoral benthic macroinvertebrates.

Toxin Type	N	Correlation	P Value
Microcystins and nodularins	21	-0.341	0.130
Cylindrospermopsin	28	0.04	0.840
Anatoxin-a	23	-0.230	0.290

Table 12. Results of ANOVA for comparing the bioaccumulation factors of individual cyanotoxins among functional feeding groups of the littoral benthic macroinvertebrates.

Cyanotoxin	DF	F value	P value
Microcystins and nodularins	3	1.31	0.305
Cylindrospermopsin	3	0.71	0.557
Anatoxin-a	3	2.39	0.101

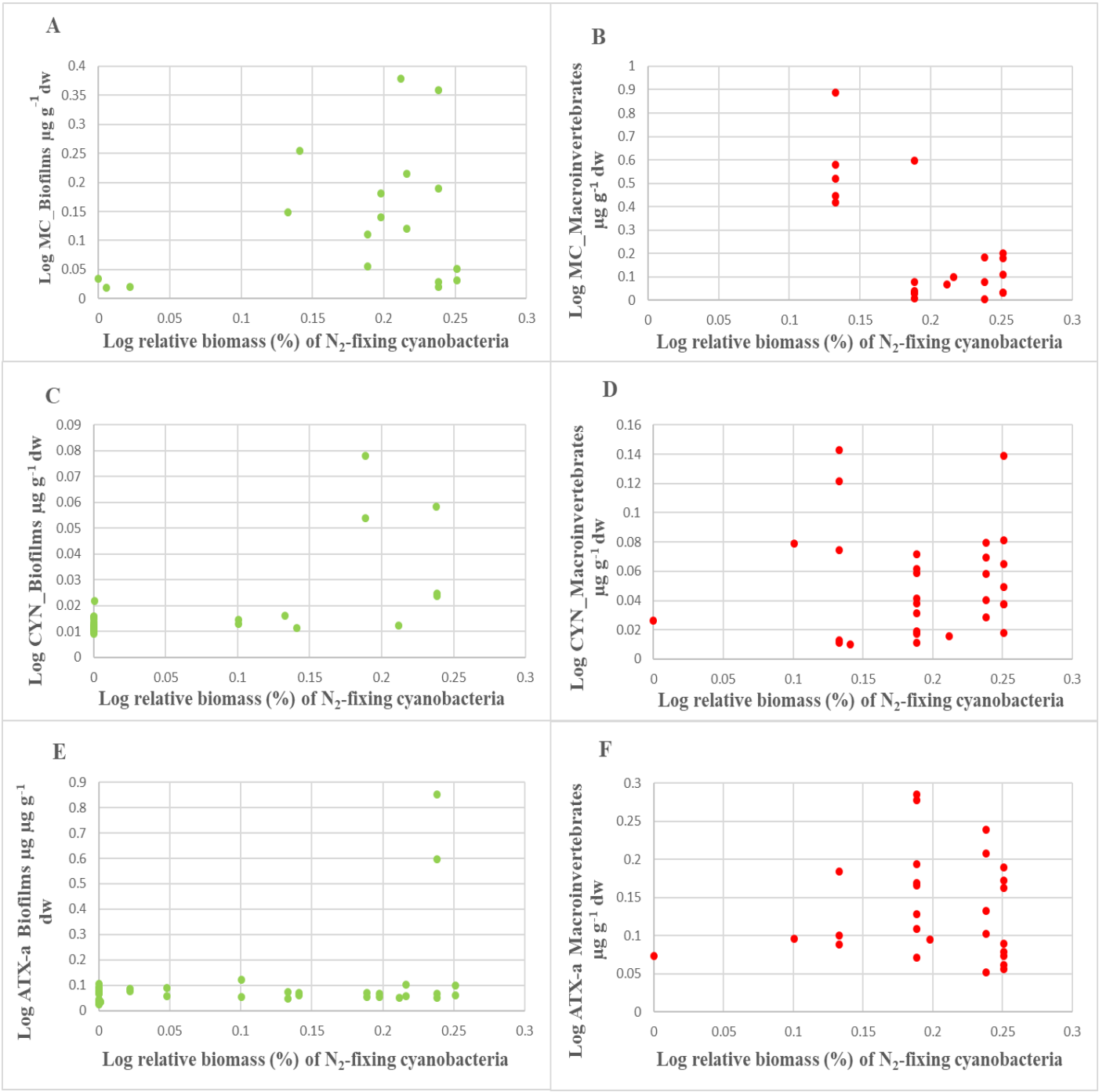

Figure 7. Cyanotoxin concentrations (µg g^{-1} dry weight) in benthic biofilms (A, C, E) and macroinvertebrates (B, D, F) measured by ELISA against the relative biomass (%) of N_2-fixing cyanobacteria in benthic biofilm primary producer community. (A, B) Microcystins and nodularins (MC), (C, D) cylindrospermopsin (CYN), (E, F) anatoxin-a (ATX). Data were $log_{10}(x+1)$ transformed to enhance visualization.

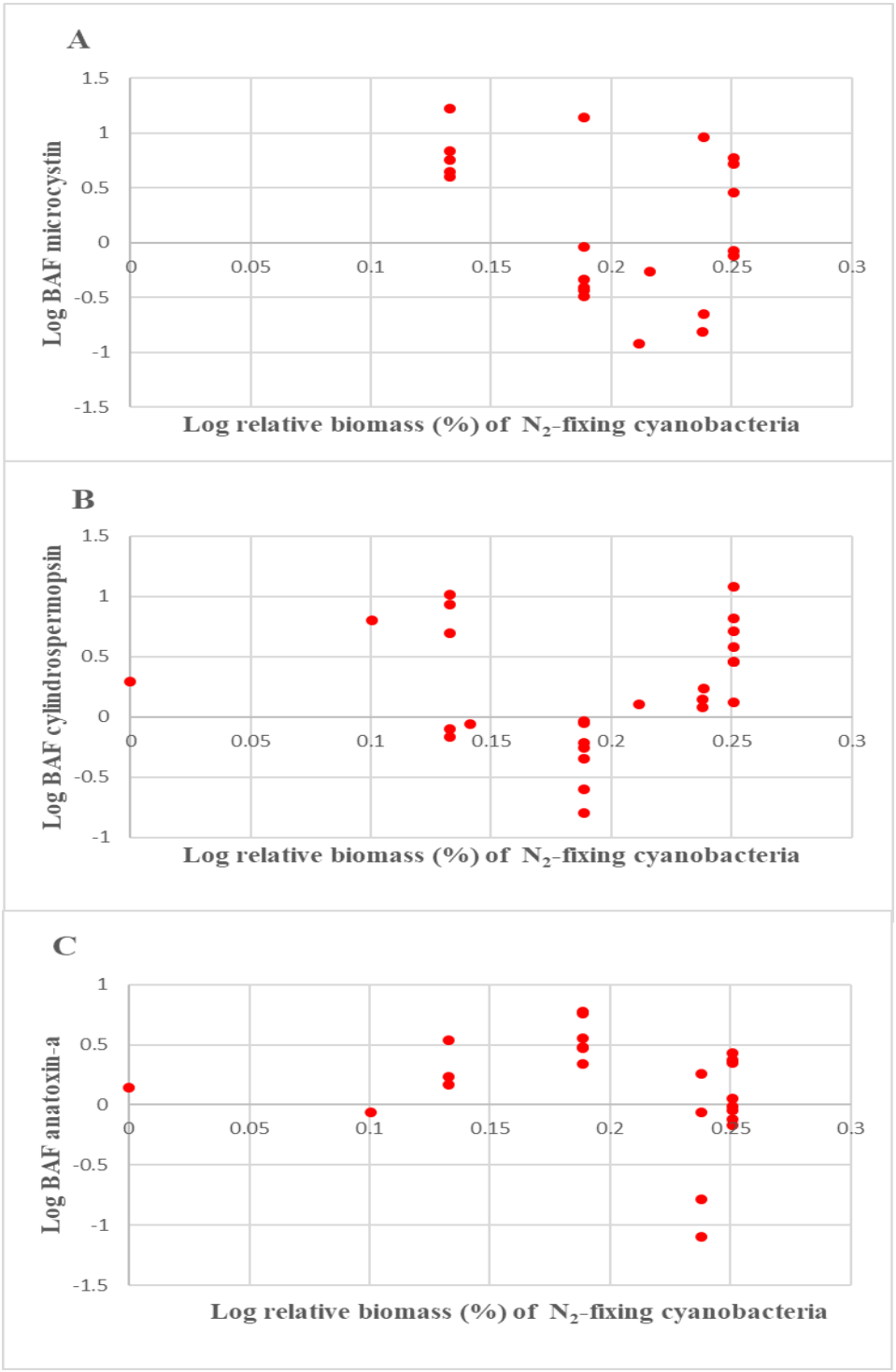

Figure 8. Bioaccumulation factors of individual cyanotoxins – (A) microcystins and nodularins, (B) cylindrospermopsin, (C) anatoxin-a – of littoral benthic macroinvertebrates against the relative biomass (%) of N_2-fixing cyanobacteria (in primary producer community of littoral biofilms. Bioaccumulation factors were $log_{10}(x)$-transformed and the relative biomass (%) of N_2-fixing cyanobacteria was $log_{10}(x+1)$-transformed to enhance visualization.

4. Discussion

This study investigated whether increases in nitrogen (N_2)-fixing cyanobacteria dominance in benthic biofilms, which is an expected consequence of lake oligotrophication and intensified N-limitation (Lau and Goedkoop 2025), were associated with increases in prevalence and bioaccumulation of cyanotoxins in benthic habitats of Arctic/alpine lakes. N_2-fixing cyanobacteria genera that were found in the biofilms included *Anabaena*, *Dichothrix*, *Nostoc*, *Scytonema*, *Stigonema*, and *Tolypothrix* (Lau and Goedkoop 2025). All these genera are able to produce cyanotoxins including anatoxin-a, cylindrospermopsin, and/or microcystins (Catherine et al. 2013, Luo et al. 2019, Wood et al. 2020, Nowruzi and Porzani 2021). The results showed that cyanotoxins were commonly detected in both biofilms and benthic macroinvertebrates. Cylindrospermopsin and anatoxin-a concentrations in biofilms were particularly positively associated with the relative biomass of N_2-fixing cyanobacteria in benthic biofilms. Cyanotoxin accumulation in benthic macroinvertebrates was evident, as 23 out of the 30 macroinvertebrate samples had a BAF greater than 1, although the BAF did not seem to differ among trophic levels (e.g., grazers and detritivores vs predators) possibly due to the limited number of macroinvertebrate samples. Since benthic macroinvertebrates are the main prey of fish in nutrient-poor northern lakes (Karlsson et al. 2009), these results suggest that oligotrophication and intensified N-limitation have a strong potential to increase the exposure of fish and their prey to cyanotoxins from benthic biofilms.

Cyanotoxin extraction from environmental and biological samples is a critical step for the detection, quantification, and assessment of cyanotoxin risks. This study first compared the efficiency of selected extraction methods for cyanotoxins including microcystins and nodularins, cylindrospermopsin, and anatoxin-a quantified by ELISA. Cyanotoxins are secondary metabolites that are enclosed by the cell membranes of cyanobacteria, such that extraction often aims to break the membranes to efficiently release the cyanotoxins (Haque et al. 2022). The method of using only water was selected for cyanotoxin extraction for the biofilm and macroinvertebrate samples in this study. Extraction using only water has advantages in terms of simplicity, cost-effectiveness (especially for hydrophilic cyanotoxins), and environmental relevance. Although sonication, heating and acidic conditions have been shown to increase cyanotoxin extraction efficiency for dried biological samples and water samples (Ranjan et al. 2010; Jiang-qi et al. 2013; Selvaraj et al. 2024), these methods did not improve cyanotoxin extraction as compared to using only water in this study. Also, the addition of diluent (for preventing anatoxin-a degradation) did not result in significantly higher concentrations of anatoxin-a than using only water without diluent. This result was likely attributed to that tested samples were freeze-dried, during which the

cell membranes could have already been broken (Sundaravadivelu et al. 2022). Thus, using only water could be an efficient cyanotoxin extraction method for freeze-dried samples.

Cyanotoxin concentrations measured by ELISA were generally positively correlated with those measured by LC-MS, except for the microcystins and nodularins in benthic macroinvertebrates. These results thus largely supported my first hypothesis. Yet, there were some differences between ELISA and LC-MS measurements for cylindrospermopsin and anatoxin-a. Compared to LC-MS, ELISA detected lower cylindrospermopsin and anatoxin-a concentrations. These differences could be due to the different extraction methods used for LC-MS and ELISA. For LC-MS, water with formic acid was used for the extraction, whereas only water was used in ELISA. LC-MS was regarded as having higher sensitivity than ELISA (Sundaravadivelu et al. 2022). Sensitivity and specificity of different analytical methods could also have contributed to the differences in cyanotoxin concentrations between ELISA and LC-MS (Cruz et al. 2012).

This study explored how dominance of N_2-fixing cyanobacteria in benthic biofilms influenced cyanotoxin production and accumulation in benthic food webs of Arctic/alpine lakes. It is relevant in the global context, as cyanobacterial blooms are increasing in frequency worldwide due to climate change (Huisman et al. 2018), and the increased risk of microcystins, cylindrospermopsin, and anatoxin-a is a growing concern for public health around the world (Farrer et al. 2015). In this study, no significant correlations between the relative biomass of N_2-fixing cyanobacteria in biofilms and the concentrations of microcystins and nodularins in either biofilms or benthic macroinvertebrates were found. The lack of correlation suggests that microcystins and nodularins were not the major cyanotoxins produced by the N_2-fixing cyanobacteria in the study lakes. In contrast, there were strong positive correlations of the relative biomass of N_2-fixing cyanobacteria with concentrations of cylindrospermopsin and anatoxin-a in the biofilms, suggesting that cylindrospermopsin and anatoxin-a were the major cyanotoxins produced by the N_2-fixing cyanobacteria found in the benthic biofilms, including *Anabaena*, *Nostoc*, *Scytonema* spp., *Tolypothrix* spp. (Lau and Goedkoop 2025). These results support my second hypothesis that increasing dominance of N_2-fixing cyanobacteria is likely to increase the cyanotoxin production in the biofilms.

Most of the benthic macroinvertebrate samples in this study had detectable levels of cyanotoxins. Importantly, a majority also had a bioaccumulation factor greater than 1, indicating increases in cyanotoxin concentrations from the biofilms to the benthic macroinvertebrates. Yet, no significant correlations were found between the cyanotoxin concentrations or BAF of benthic macroinvertebrates and the relative biomass of N_2-fixing cyanobacteria in biofilms, thus my third hypothesis was only partially supported. This result could be attributed by

multiple biotic and abiotic factors that influenced the cyanotoxin transfer from cyanobacteria to macroinvertebrates. As for biotic factors, primary consumers have the ability to cope with cyanotoxins via rapid excretion, metabolic detoxification (Pflugmacher et al., 1998), or biodilution, so the cyanotoxins do not necessarily bioconcentrate in food chains (Kozlowsky-Suzuki et al. 2012). Detoxification via metabolic transformation that reduces body cyanotoxin concentrations can occur in the benthic macroinvertebrates (Kozlowsky-Suzuki et al. 2012).

Cyanotoxins can cause different short- or long-term effects depending on how they enter the animal bodies. Animals can accumulate cyanotoxins through diet or exposure to dissolved cyanotoxins in the water. Toxic effects are considered more lethal or harmful when invertebrates are exposed to cell-free cyanotoxins rather than ingesting them via food. For example, significant mortality has been observed in the cladoceran zooplankton *Ceriodaphnia dubia* at mean lethal concentrations of 0.56-0.71 μg L^{-1} in water following exposure to anatoxin-a-producing *Phormidium* strains (Anderson et al. 2018). So, invertebrates could die before cyanotoxin accumulates in their bodies. As another example, Laurén-Määtä et al. (1995) found that larvae of the phantom midge *Chaoborus* had a higher mortality rate after ingesting the cladoceran zooplankton *Daphnia pulex* that had consumed microcystins produced by *Microcystin aeruginosa*. Although this result suggested cyanotoxin trophic transfer, no microcystins were detected in the *Chaoborus* larvae and the authors concluded that the ingested microcystins were mostly broken down or excreted. This indicates that exposure to cyanotoxins can have both lethal (for *Chaoborus*) and sub-lethal effects (for *Daphnia pulex*). Thus, exposure to increased cyanotoxin concentrations with increasing dominance of N_2-fixing cyanobacteria, as found in this study, may also have important negative impacts for the benthic macroinvertebrates in the Arctic/alpine lakes.

The bioaccumulation factors of cyanotoxins did not differ among the functional feeding groups of the benthic macroinvertebrates in the study lakes. This result could be due to the limited numbers of samples collected from different functional feeding groups in individual lakes (Appendix 2). Yet, the recorded cyanotoxin concentrations were high while comparing to literature findings. For example, cylindrospermopsin concentrations in the benthic macroinvertebrates measured by ELISA were 0.023-0.388 μg g^{-1} dw. The same concentration can be lethal in mammals, as a LD_{50} in mice has been reported as 2.1 μg g^{-1} at 24 hours and 0.2 μg g^{-1} dw at 5–6 days (Sivonen 2009). Also, in this study the cylindrospermopsin concentrations of grazers ranged 0.03–0.32 μg g^{-1} dw, much higher than the cylindrospermopsin concentration of 0.003 μg g^{-1} dw recorded for the apple snail *Pomacea patula catemacensis* from the more eutrophic Lake Catemaco (Mexico) (Berry & Lind 2010). Similarly, in this study the anatoxin-a concentrations in the benthic macroinvertebrates detected by

ELISA were in the range of 0.128-0.928 μg g^{-1} dw, which were also seemingly high, considering that the LD_{50} for mice was 0.20-0.25 μg g^{-1} dw (Sivonen 2009). Due to analytical limitations, matrix effects and cross reactivity with functionally similar compounds, ELISA might have overestimated the cyanotoxin concentrations in this study. Nevertheless, the cyanotoxin concentrations detected in the benthic macroinvertebrates in the Arctic/alpine lakes were non-trivial, potentially posing strong cyanotoxin risks for fish.

Although this study did not directly measure cyanotoxin concentrations in fish, previous studies have emphasized possible cyanotoxin bioaccumulation in fish through their macroinvertebrate prey. Also, cyanotoxin bioaccumulation can differ among species. For example, the microcystin concentrations in planktivorous European smelt *Osmerus eperlanus* and in the predatory fish European perch *Perca fluviatilis* were 2.86 and 0.11 times, respectively, of their diets in Lake IJsselmeer (Netherlands), indicating varying degrees of microcystin transfer to higher trophic levels (Ibelings et al. 2005). Fishes *Bramocharax caballeroi*, *Cichlasoma uropthalmus*, *Heterandria jonesii* had different cylindrospermopsin concentrations, i.e., 0.0008, 0.0003, and 0.0013 μg g^{-1} fresh weight, respectively, in Lake Catemaco (Berry et al. 2012). Anatoxin-a concentrations in muscles of the omnivorous roach *Rutilus rutilus* and Prussian carp *Carassius gibelio* were 0.03 μg g^{-1} fresh weight (Pawlik-Skowrońska et al. 2012). Thus, the capability of cyanotoxin bioaccumulation in fish coupled with the prevalence of cyanotoxins in their invertebrate prey may imply a strong potential of cyanotoxin transfer to higher trophic levels in the lake food webs.

5. Conclusions

Cyanotoxins were commonly detected in both benthic biofilms and macroinvertebrates in the Arctic/alpine lakes. Cylindrospermopsin and anatoxin-a concentrations quantified by ELISA were positively correlated with those quantified by LC-MS, although LC-MS generally detected higher concentrations than did ELISA. Concentrations of cylindrospermosin and anatoxin-a in biofilms increased with the relative biomass of N_2-fixing cyanobacteria. Most samples of benthic macroinvertebrates had higher cyanotoxin concentrations than the biofilms, indicating trophic transfer and bioaccumulation of cyanotoxins. Fish heavily rely on benthic prey in Arctic/alpine lakes. Therefore, increases in dominance of N_2-fixing cyanobacteria in benthic biofilms, as an expected result of lake oligotrophication and intensified N-limitation (Lau and Goedkoop 2025) and/or overall climate change (Huisman et al. 2018), will likely increase the exposure of fish to cyanotoxins through diet or dissolved cyanotoxins in water. Overall, this study highlights the importance of monitoring cyanotoxin risks in Arctic/alpine lakes, and the necessity of further investigating cyanotoxin bioaccumulation in the food web components especially fish.

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Popular science summary

Arctic/alpine lakes in Sweden, known for their clear, nutrient-poor waters, are becoming even more depleted in nutrients (oligotrophication) especially nitrogen due to climate warming. Intensified nitrogen-limited conditions may favor the dominance of nitrogen-fixing cyanobacteria (N_2-fixers) over other primary producers in biofilms growing on the lake bottoms (benthic habitats). This study investigated whether increases in dominance of N_2-fixers affected the prevalence and accumulation of cyanotoxins, including microcystins and nodularins, cylindrospermopsin, and anatoxin-a in benthic biofilms and macroinvertebrates from nine Swedish Arctic/alpine lakes. This study first compared the efficiency of selected extraction methods for the cyanotoxins quantified by ELISA. Water-based extraction showed high recovery of the cyanotoxins. Cyanotoxins in the biofilm and macroinvertebrate samples were then analyzed by ELISA, and these results were compared with the cyanotoxin data of the same samples previously analyzed by LC-MS. LC-MS gave higher measurements of cylindrospermopsin and anatoxin-a than did ELISA. These differences could be due to the different extraction methods and analytical sensitivities between LC-MS and ELISA. Cylindrospermopsin and anatoxin-a concentrations in the biofilms increased with increases in the relative biomass of N_2-fixers in biofilms. The benthic macroinvertebrates generally had higher cyanotoxin concentrations than the biofilms. The concentrations of cyanotoxins accumulated in the benthic macroinvertebrates did not change with the relative biomass of N_2-fixers in the biofilms, likely due to the limited number of samples and the inherent biological processes within invertebrates as a defense mechanism, including detoxification or selective feeding. The different feeding groups of macroinvertebrates, i.e., grazers, detritivores, generalists, and predators, were similar in their cyanotoxin accumulation. Benthic macroinvertebrates are the key prey for fish, including Arctic char and brown trout in the Arctic/alpine lakes. Thus, with increases in dominance of N_2-fixers in benthic biofilms driven by lake oligotrophication and intensified N-limitation, fish are likely to face increased exposure to cyanotoxins in diet or water. This study highlights the importance of monitoring cyanotoxin risks long-term in Arctic/alpine lakes and the necessity of further investigating cyanotoxin bioaccumulation in the food web components, especially fish.

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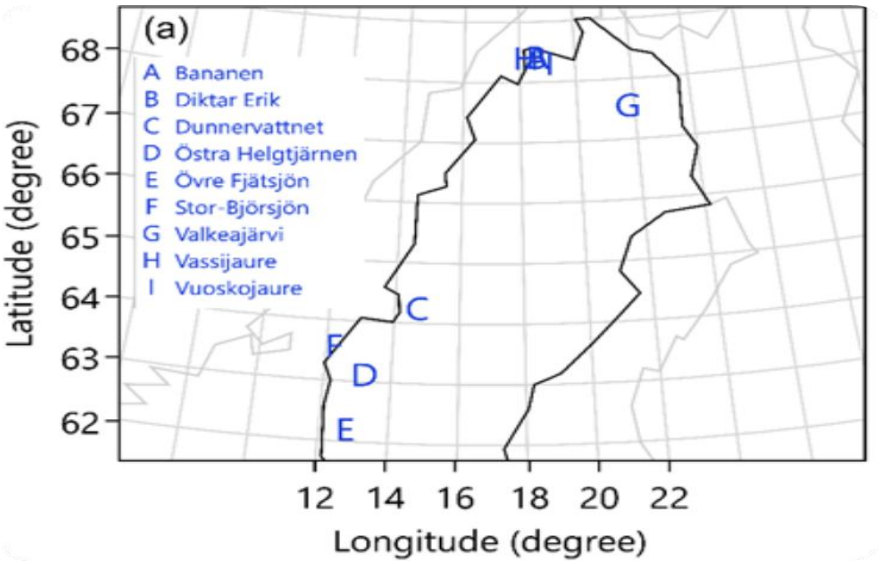
Appendix 1



Locations of the nine Swedish Arctic/alpine lakes that have been sampled for benthic algae and invertebrate grazers. Lakes Bananen, Diktar Erik, Vassijaure, and Vuoskojaure are located near Abisko.

Appendix 2

List of littoral macroinvertebrate taxon names collected from various lakes, their associated habitat type, and assigned functional feeding groups. N, number of samples.

Lake	Habitat	Taxon name	Functional feeding group	*N*
Bananen	Littoral	*Aeshna grandis*	Predator	1
Bananen	Littoral	Limnephilidae	Grazer	1
Bananen	Littoral	Phryganeidae	Detritivore	1
Diktar Erik	Littoral	Polycentropodidae	Predator	1
Valkeajärvi	Littoral	*Asellus aquaticus*	Generalist	3
Valkeajärvi	Littoral	Chironomidae	Generalist	1
Valkeajärvi	Littoral	Oligochaeta	Generalist	1
Valkeajärvi	Littoral	*Radix balthica*	Grazer	1
Vassijaure	Littoral	Ameletus	Grazer	2
Vassijaure	Littoral	Dytiscidae Adults	Predator	1
Vassijaure	Littoral	Dytiscidae Larvae	Predator	1
Vassijaure	Littoral	Oligochaeta	Generalist	2
Vassijaure	Littoral	Phryganeidae	Detritivore	2
Vassijaure	Littoral	Tipula	Detritivore	1
Dunnervattnet	Littoral	Oligochaeta	Generalist	1
Dunnervattnet	Littoral	Polycentropodidae	Predator	1
Stor-Björsjön	Littoral	Ameletus	Grazer	1
Stor-Björsjön	Littoral	Chironomidae	Generalist	1
Stor-Björsjön	Littoral	*Daphnia*	Grazer	1
Stor-Björsjön	Littoral	*Ephemera vulgata*	Grazer	1
Stor-Björsjön	Littoral	*Gammarus lacustris*	Generalist	2
Stor-Björsjön	Littoral	Oligochaeta	Generalist	1
Stor-Björsjön	Littoral	Polycentropodidae	Predator	1
Östra Helgtjärnen	Littoral	*Aeshna grandis*	Predator	2
Övre Fjätsjön	Littoral	Hirudinea/Glossiphoniidae	Predator	1
Övre Fjätsjön	Littoral	Polycentropodidae	Predator	1
Övre Fjätsjön	Littoral	*Radix*	Grazer	6

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