Eesti Maaülikool
Estonian University of Life Sciences

FROM MICROSCOPY TO GENES – TRACING TOXIC CYANOBACTERIA IN A SHALLOW EUTROPHIC LAKE

MIKROSKOOPIAST GEENIDENI – KUIDAS TUVASTADA TOKSILISI SINIVETIKAID MADALAS EUTROOFSES JÄRVES

KRISTEL PANKSEP

A Thesis
for applying for the degree of Doctor of Philosophy
in Applied Biology

Väitekiri
filosoofiadoktori kraadi taotlemiseks
rakendusbioloogia erialal

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"Don't limit yourself. Many people limit themselves to what they think they can do. You can go as far as your mind lets you. What you believe, remember, you can achieve."

Mary Kay Ash

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following papers, referred to in the text by the relevant Roman numerals. Papers are reproduced by kind permission from the publishers.

I. Laugaste, R., **Panksep, K.**, & Haldna, M. (2013). Dominant cyanobacterial genera in Lake Peipsi (Estonia/Russia): effect of weather and nutrients in summer months. Estonian Journal of Ecology, 62(4).

II. **Panksep, K.**, Tamm, M., Mantzouki, E., Rantala-Ylinen, A., Laugaste, R., Sivonen, K., Tammeorg, O., & Kisand, V. (2020). Using Microcystin Gene Copies to Determine Potentially-Toxic Blooms, Example from a Shallow Eutrophic Lake Peipsi. Toxins, 12(4), 211.

III. Agasild, H., **Panksep, K.**, Tõnno, I., Blank, K., Kõiv, T., Freiberg, R., Laugaste, R., Jones, R I., Nõges, P., & Nõges, T. (2019). Role of potentially toxic cyanobacteria in crustacean zooplankton diet in a eutrophic lake. Harmful algae, 89, 101688.

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ABBREVIATIONS

Chl *a*	Chlorophyll *a*
CCM	CO_2 concentrating mechanism
CDOM	Coloured dissolved organic matter
Ct	Threshold cycle in qPCR
FP	Phytoplankton
HAB	Harmful algal bloom
HPLC	High performance liquid chromatography
LC-MS	Liquid Chromatography-Mass spectrometry
LPS	Lipopolysaccharides
MC	Microcystin
*mcy*E	Gene encoding microcystin synthetase subunit
PCA	Principal component analysis
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
POM	Particulate Organic Matter
SIA	Stable Isotope Analysis
TN	Total nitrogen
TP	Total phosphorus
ZP	Zooplankton
WFD	European Union's Water Framework Directive
WHO	World Health Organisation

INTRODUCTION

Global warming, paired with eutrophication processes, is shifting phytoplankton communities towards the dominance of bloom-forming and potentially toxic cyanobacteria. Cyanobacterial blooms are considered an increasing threat in freshwater. Traditional monitoring predominantly relies on cyanobacterial biomass as an indicator of potential toxin presence, disregarding that toxin concentrations can rapidly increase even when cyanobacterial biomass is low. The concentration of toxins in the water is related to the abundance of toxin-producing species and the amount of toxin per cell (toxin quota). The research described here provides valuable information about cyanobacterial community composition, abundance of toxic genotypes, microcystin concentrations, microcystin quota, and environmental factors that promote toxic cyanobacterial blooms in large, shallow, freshwater Lake Peipsi. Moreover, this is the first study in Lake Peipsi to utilise molecular methods as a complement to routine monitoring to determine cyanobacterial toxicity potential. *In situ* studies on zooplankton taxon-specific ingestion of potentially toxic cyanobacteria are still limited. This study focused on the importance of cyanobacteria as a food source for dominant crustacean grazers. Using qPCR targeting cyanobacterial genus-specific *mcyE* synthetase genes in zooplankton gut contents, results show that potentially toxic strains of *Microcystis* can be ingested directly or indirectly by different zooplankton grazers. Results from this study expanded our knowledge on the ecology of toxic cyanobacteria, showed how molecular methods can improve traditional risk assessment concerning cyanobacteria abundance and their cyanotoxins, and broadened our knowledge of how targeted molecular tools can be further used in aquatic food-web studies.

1. REVIEW OF THE LITERATURE

1.1 Cyanobacteria

Cyanobacteria are a diverse group of photosynthetic prokaryotes that use CO_2, mineral nutrients, and light to produce organic compounds and oxygen (Sivonen and Jones, 1999; Humbert and Fastner, 2017). Cyanobacteria played a key role in the oxygenation of Earth's atmosphere 2.5 billion years ago (Schirrmeister et al., 2013). The evolution of oxygenic photosynthesis enabled multicellular life forms to appear (Sánchez-Baracaldo and Cardona, 2020). Today, cyanobacteria have a crucial role in the biological cycling of nutrients, carbon, and minerals (Codd et al., 2017). Cyanobacteria inhabit a wide variety of environments, prominently limnic and marine waters, but also soils and the surfaces of rocks and infertile substrates, such as desert sand and biocrust, volcanic ash, ice, and snow (Codd et al., 2017; Svirčev et al., 2019). Due to their long history on Earth, cyanobacteria are well adapted to diverse environmental conditions and extremes. They have a variety of cell types, specialised cell structures, and physiological strategies that make them ecologically successful (Marsac and Houmard, 1993; Vincent, 2009). Cyanobacterial community formation is determined by complex global, regional, local, and biological interactions. Functional traits, such as buoyancy control, atmospheric nitrogen fixation in some taxa, phosphorus storage, formation of resting cells (akinetes), and efficient use of low light give cyanobacteria a competitive advantage under some environmental changes or extreme conditions (Hyenstrand et al., 1998; Litchman et al., 2010; Paerl et al., 2011).

Several potentially toxic cyanobacteria (e.g., genera *Microcystis, Dolichospermum*, and *Aphanizomenon*) have gas vesicles that allow them to control vertical position in the water column and, therefore, gain an advantage in cases of thermal stratification or intensive solar radiation. Under a stable water column, these groups of cyanobacteria can place themselves at optimal depths for their growth. They can oppose sedimentation, float to the surface, induce a "shading effect" on other phytoplankton groups, or migrate to deeper layers to protect themselves from intense UV radiation (Rastogi et al., 2014; Humbert and Fastner, 2017; Duan et al., 2021). Some groups (exclusively Nostocales, Stigonematales) have heterocysts, which are specialized cells for the fixation of atmospheric nitrogen (N_2). These morphologically distinct cells develop under conditions of nitrogen

deprivation in the environment (Herrero et al., 2016). The ability to fix free nitrogen is an important advantage under N-limited conditions (Humbert and Fastner, 2017). Among diazotrophic cyanobacteria, species with heterocysts (e.g., *Aphanizomenon* spp*., Dolichospermum* spp*., Nodularia spumigena*) dominate in brackish and freshwater environments, while in tropical oceans, non-heterocystous, filamentous cyanobacteria (e.g., *Trichodesmium* spp.) are responsible for most of the N_2 fixation (Staal et al., 2003). Another beneficial trait for some genera from the order Nostocales is the ability to form dormant cells, also called akinetes. These thick-walled cells can survive adverse conditions and enable opportune proliferation (Kokociński et al., 2017). Also, colonies from the genus *Microcystis* can overwinter in sediments, re-emerge into the water column in spring or summer, and take advantage of ideal conditions for proliferation (Visser et al., 2005; Chen et al., 2013).

1.2 Cyanobacterial blooms

Anthropogenic nutrient loading causes eutrophication, which is a global environmental problem in aquatic systems (Jöhnk et al., 2008; Huber et al., 2012). Waterbodies provide a wide range of intrinsic ecosystem services (Schallenberg et al., 2013) valued by humans, such as biodiversity, complexity, beauty, spiritual significance, etc. (Sandler, 2012), or represent advantages that humans obtain from ecosystems, such as commercial and recreational fishing, recreation and tourism, hydroelectricity generation, drinking water supply, etc. (Mace et al., 2012). When waterbodies become enriched with nutrients, the phytoplankton community frequently shifts towards bloom-forming and potentially toxic cyanobacteria (Reynolds, 2006; Paerl and Paul, 2012; Paerl and Otten, 2013). Acute symptoms of eutrophication include cyanobacterial blooms causing discolouration, floating scums on the water surface (Figure 1), and foul smells (Ibelings and Chorus, 2007). Blooms can form at the surface or in the metalimnion of the waterbody.

In an environmental context, cyanobacterial blooms affect light distribution, thereby diminishing habitats for plants and animals, disrupting food web dynamics, creating hypoxic zones, and producing a wide variety of toxic secondary metabolites (Sivonen and Jones, 1999; Paerl et al., 2014). The oxygen demand of dense blooms is very high due to respiration activity at night and decomposition of high biomass. Anoxic conditions often cause cyanobacteria-related fish mortality (Havens, 2008). Unpleasant, irritating

odours from decayed biomass and secondary metabolites can also cause problems for beneficiaries of ecosystem services (Ibelings and Chorus, 2007). These changes can increase economic costs for fisheries and drinking water, as well as decreasing recreational, tourism, and property values (Steffensen, 2008). Despite nutrient load reductions in some systems and water pollution management efforts over 30 to 40 years, cyanobacterial blooms remain a principal concern in aquatic ecosystems (Mantzouki et al., 2018a; Fink et al., 2020).

Common bloom-forming cyanobacteria in surface waters are from the genera *Microcystis* and *Dolichospremum* in freshwater and *Aphanizomenon* and *Nodularia* in brackish water systems. Metalimnetic blooms are most commonly formed by species adapted to low light conditions (mainly *Planktothrix* spp.) but sometimes also species from genera *Aphanizomenon*, *Dolichospermum*, and *Raphidiopsis*. Several species from the above-mentioned genera can produce hepato- and neurotoxins (Oliver and Ganf, 2000). In favourable environmental conditions (e.g., warm temperatures, calm weather, and abundant light), the density of species that form surface scums can rapidly increase within hours (Chorus and Bartram, 1999; Ibelings et al., 2021). Surface scums can be concentrated along shorelines by wind activity, presenting health risks for people using the waterbody recreationally or poisoning wildlife, livestock, dogs, and birds (Ibelings et al., 2021).

Figure 1. Cyanobacterial bloom. Photos: R.Laugaste, K.Kangur, A. Rakko

1.3 Cyanobacterial toxins

Cyanotoxins are a diverse group of metabolites produced by cyanobacteria. These toxins are traditionally classified according to the effect of their toxicological target (i.e., hepatotoxins, neurotoxins, cytotoxins, dermatotoxins; Bláha et al., 2009) or according to their chemical structure (i.e., cyclic peptides, alkaloids, and lipopolysaccharides; Sivonen and Jones, 1999). According to their mode of action, microcystins and nodularin are classified as hepatotoxins; saxitoxins, anatoxin-a, homoanatoxin-a, and BMAA (β-methylamino-L-alanine) as neurotoxins; cylindrospermopsin as cytotoxin; and lyngbyatoxin and aplysiatoxin as dermatotoxins (Sivonen and Jones, 1999; Moreira et al., 2014). Cyanotoxins are not required for basic cell metabolism, so their primary function and role for cyanobacterial cells remain unclear (Vidal et al., 2021 and references therein). It has been assumed that they serve as chemical defence against grazing, provide a survival and competitive advantage over other microalgae and cyanobacteria under nutrient and light limitation (Paul, 2008; Chorus and Welker, 2021). Other possible roles of cyanotoxins that are hypothesized in the literature are attractants or repellents for heterotrophic microorganisms, nutrient uptake, protection against reactive oxygen species, carbon–nitrogen metabolism and overall maintenance of cell homeostasis (Beversdorf et al., 2015a; Dulic et al., 2022). In aquatic systems, cyanotoxins can be toxic to all trophic levels, including phytoplankton, zooplankton, fish, invertebrates, birds, and mammals (Christoffersen, 1996).

The most studied toxins produced by cyanobacteria are microcystins and nodularin. These cyclic peptides are commonly found in fresh and brackish waters worldwide (Chorus and Welker, 2021). The enzymes needed to synthesize these toxins are encoded by microcystin (*mcy*) and nodularin (*nda*) synthetase gene clusters (Moffitt and Neilan, 2004). The number and order of *mcy* genes can differ between microcystin-producing genera, but in general, they all have *mcyA-E* and *mcyG* genes (Rantala, 2007). All genes in the *mcy* gene cluster have their own function. In this study, the *mcyE* gene was chosen for detection and quantification of potential microcystin producers as this gene has a demonstrated role in microcystin production and is a reliable molecular marker for detection of microcystin producers (Rantala et al., 2006; Pacheco et al., 2016; Overlingė et al., 2021). Today, more than 250 structural variants of microcystins are fully characterised (Chorus and Welker, 2021). Most

commonly, microcystins are produced by freshwater cyanobacteria from the genera *Microcystis, Dolichospermum,* and *Planktothrix,* with less documented evidence from *Aphanizomenon, Limnothrix*, *Phormidium*, and *Nostoc*. Nodularin is produced by *Nodularia spumigena* (Catherine et al., 2017).

1.4 Environmental controls of cyanobacterial blooms and cyanobacterial toxicity

Cyanobacterial dominance is generally strongly related to the CO_2, temperature, light availability, and the accessibility of key nutrients, mainly phosphorus and nitrogen (Dignum et al., 2005; Merel et al., 2013; Ibelings et al., 2021).

Due to their long history on Earth, cyanobacteria have evolved to concentrate inorganic carbon for photosynthesis. This mechanism improves the carboxylation process and allows efficient operation of their otherwise inefficient Rubisco - CO_2-fixing enzyme (Badger and Price, 2003; Ibelings et al., 2021). Compared to other photosynthetic organisms, the CO_2 concentrating mechanism (CCM) in cyanobacteria is probably the most efficient, allowing an up to 1000-fold concentration of CO_2 (Badger and Price, 2003). CCM gives cyanobacteria an advantage in environments with limited CO_2 (Mangan et al., 2016), such as during bloom conditions with elevated pH.

Cyanobacterial prevalence, community composition, and bloom formation are affected by direct and indirect effects of temperature (Carey et al., 2012; Mantzouki et al., 2018a). Direct effects of water temperature affects mostly the timing and proportional dominance of cyanobacteria (Elliott, 2012). Compared to eukaryotic primary producers, the optimal growth rate of cyanobacteria is higher at higher temperatures giving them a considerable advantage in elevated temperatures (Paerl and Huisman, 2009). A survey where 143 lakes with varying trophic states were studied along a latitudinal gradient ranging from subarctic Europe to southern South America revealed much higher cyanobacterial biovolume in warmer climates, whereas total phytoplankton biomass remained unchanged (Kosten et al., 2012).

Some genera of cyanobacteria (e.g., *Aphanizomenon*, *Dolichospermum, Nodularia*) can fix atmospheric nitrogen and effectively, albeit only

temporarily, evade deficiencies of other nitrogen sources (Ibelings et al., 2021; Olofsson et al., 2021). Moreover, much of this newly fixed nitrogen can be remineralized as bioavailable nitrogen and therefore further stimulate primary production (Svedén et al., 2016; Olofsson et al., 2021). However, fixation of atmospheric nitrogen is energetically expensive and challenging; therefore, cyanobacteria usually do not fix N_2 in the presence of reactive nitrogen sources (Paerl, 2017; Olofsson et al., 2021). In contrast to nitrogen, phosphorus cannot be replenished biologically from the atmosphere, and it was long presumed that P controls the abundance of cyanobacteria in aquatic environments (Schindler, 1977; Dignum et al., 2005; Scott et al., 2019). High P concentration was thus considered the main risk factor for cyanobacterial blooms (Downing et al., 2001; Scott et al., 2019).

Whether solely phosphorus, or nitrogen and phosphorus both, are the drivers of eutrophication and cyanobacterial blooms has been debated for several decades (van Gerven et al., 2019). Several studies have shown that cyanobacterial growth is more often stimulated by the combination of P and N enrichment, and the dual control of nutrients is needed for water quality management (Paerl et al., 2011, 2016; Molot et al., 2014; Newell et al., 2019). In shallow lakes, P and N accumulated in sediments are resuspended to the water column due to wind and wave action and can repeatedly support phytoplankton growth during the vegetation period (P. Nõges et al., 2008; Bormans et al., 2016). Which nutrient limits cyanobacterial growth varies in different waterbodies temporally and geographically (Paerl et al., 2016). Several studies have demonstrated the significance of excessive anthropogenic N in promoting cyanobacterial blooms and proliferation of non-nitrogen-fixing species, such as *Microcystis* spp (Kosten et al., 2012; Paerl et al., 2016). The increasing dominance of non-nitrogen-fixing cyanobacteria in eutrophic systems is related to higher water temperatures, stable thermal stratification, and relatively high N:P ratios in external nutrient loads (Paerl et al., 2011, 2016).

The main factors associated with cyanobacterial dominance are widely understood. However, to what extent different environmental variables in natural systems contribute to toxin production is under debate (Beversdorf et al., 2015b). Experimental studies have demonstrated the various effects of nutrients, light, temperature, CO_2, UV, pH, and heavy metals in cyanotoxin production (Neilan et al., 2013; Beversdorf

et al., 2015b). However, there is still a lack of consistency, and in natural ecosystems, these effects are not straightforward (Mantzouki et al., 2018a). Field studies have shown the influence of nitrogen availability on microcystin production and congener composition (Chaffin et al., 2018). Also, the availability of nutrients, direct and indirect effects of temperature, and the stability of the water column are important factors impacting bloom toxicity (Chaffin et al., 2018; Mantzouki et al., 2018a).

1.5 Biological controls of cyanobacteria

Cyanobacterial population dynamics are affected by grazing, particularly by zooplankton (Ibelings et al., 2021), which can modulate bloom initiation, either via selective feeding (i.e., preferential grazing on eukaryotic phytoplankton competitors), or indirectly via trophic cascades (Rollwagen-Bollens et al., 2013; Urrutia-Cordero et al., 2015). Laboratory experiments have revealed substantial differences in grazing effects on cyanobacteria relative to zooplankton feeding modes. For example, among crustacean zooplankton, ambush or current feeding copepods (cyclopoids and calanoids, respectively), or filter-feeding cladocerans (such as *Daphnia*), vary in their abilities to discriminate and ingest different types of cyanobacteria (Ger et al., 2019; Leitão et al., 2021). Thus, the ingestion and control of cyanobacteria in natural environments depend largely on grazer community composition.

Top-down control of cyanobacterial growth has been a subject of interest, especially in terms of biomanipulation. Nevertheless, cyanobacterial blooms persist, even after biomanipulation, and large-bodied zooplankters fail to control cyanobacterial growth (Urrutia-Cordero et al., 2016). Cyanobacteria have evolved a variety of mechanisms to raise their resistance against grazing (Lürling, 2021). Filament and colony size larger than ingestion capacity, mucous colonies, toxin production, and viable gut passage are a few of the strategies to prevent grazing by zooplankton (Lewin et al., 2003; Ibelings et al., 2021; Lürling, 2021). Still, both proto- and metazooplankton can graze on both toxic and non-toxic strains of cyanobacteria (Davis and Gobler, 2016). Crustacean zooplankton (especially calanoid copepods) can cut filaments during ingestion to feed on shorter fragments (Kâ et al., 2012). *Daphnia*, a generalist feeding cladoceran, co-occurring with regular annual cyanobacterial blooms, can have an effective microcystin detoxification ability by using antioxidant systems that protect them

against cyanobacterial toxin accumulations (Wojtal-Frankiewicz et al., 2013). These traits of zooplankton facilitate their coexistence with cyanobacterial blooms. Still, examples of significant cyanobacterial bloom suppression by zooplankton grazing are rare in nature because grazer communities in eutrophic waters are often dominated by small-sized zooplankton. This trend is caused by intense fish predation in such lakes (Jeppesen et al., 2000).

Cyanobacterial blooms favour development of zooplankton communities with species capable of more selective feeding, such as cyclopoid copepods, small cladocerans, and rotifers (Kerfoot and Kirk, 1991; Kâ et al., 2012). Selective feeding facilitates grazer coexistence with toxic cyanobacteria by promoting grazing on less toxic algal species (DeMott and Moxter, 1991) or selecting for alternative, non-toxic prey (Ger et al., 2011). Some examples indicate that cyclopoid copepods and small cladocerans can suppress blooms of potentially toxic *Dolichospermum, Microcystis*, and *Planktothrix* as with a result of reduced fish predation in biomanipulation experiments in Lake Ringsjön (Sweden) (Urrutia-Cordero et al., 2015). However, feeding on toxic *Microcystis* can also lead to decreased grazer abundance due to the toxicity effect, as shown with the key zooplankton species *Pseudodiaptomus forbesi* in the San Francisco Estuary (Ger et al., 2018). Nevertheless, there is limited information on taxon-specific responses of grazers to potentially toxic cyanobacteria co-occurring in natural systems (Ger et al., 2018). Herbivorous zooplankton and their invertebrate predators also represent an important vector for cyanotoxin transfer to fish (Sotton et al., 2014). Therefore, understanding different capabilities of zooplankton to ingest potentially toxic cyanobacteria is important to help reveal trophic pathways of cyanotoxin accumulation in fish.

The role of bivalves in controlling cyanobacteria is not yet well understood: some studies have shown selective rejection of cyanobacterial colonies or viable gut passage through filter-feeding consumers, which could stimulate cyanobacterial growth (Vanderploeg et al., 2001, 2009), while other studies have demonstrated supression of cyanobacteria (reviewed in Ibelings et al., 2021).

Cyanobacterial population dynamics and abundance are also controlled by cyanophages, viruses that directly infect cyanobacteria (Mohiuddin and Schellhorn, 2020; Ibelings et al., 2021). These phages are broadly

abundant in aquatic environments (Gao et al., 2016) and act as vectors of gene transfer, contributing to new characteristics of cyanobacteria; therefore, the evolution and genetic diversity of cyanobacteria have been strongly affected by these viruses (Suttle, 2007). Fungal parasites (e.g., chytrids) can also infect cyanobacteria and help control cyanobacterial growth. Chytrid infection can be either lethal or promote grazing of colonial or filamentous cyanobacteria due to infection-induced fragmentation (Frenken et al., 2020).

1.6 Expansion of cyanobacteria blooms with eutrophication

Lake Peipsi *s.l.* (*sensu lato*), the largest transboundary lake in Europe, is a large, non-stratified, eutrophic lake on the border of Estonia (European Union) and the Russian Federation. It consists of three basins, which all differ in hydrology, morphology, and biota (Kangur et al., 2012; Tammeorg et al., 2014). Eutrophication processes have strongly influenced the ecosystem of Lake Peipsi (Kangur and Möls, 2008), and annual cyanobacterial blooms have occurred for several decades (Laugaste et al., 2007, 2013). A spatial north to south eutrophication gradient occurs across the lake basins (Kangur et al., 2013). Lake Peipsi *s.s.* (*sensu stricto*) is eutrophic, Lämmijärv is eutrophic/hypertrophic, and Pihkva is hypertrophic according to OECD 1982 classification, (Kangur et al., 2013). More than 80% of the nutrients are discharged into the lake by two main inflows – the rivers Velikaya and Emajõgi (Loigu et al., 2008; Nõges et al., 2010). The outflow of lake Peipsi – River Narva – is the drinking water source for Narva, the third-largest city in Estonia. River Narva drains into the eutrophic Gulf of Finland. The city of Tartu (approximately 93000 inhabitants) lies on the river Emajõgi has been the main source of pollution from the Estonian side. Wastewater from Tartu has been biologically and chemically treated since 2018. The efficiency of nitrogen and phosphorus removal has been over 50% and 85-90%, respectively (Blank et al., 2017). Wastewater from Pihkva (approximately 206000 inhabitants) is only biologically treated, and phosphorus is not separately extracted (Loigu et al., 2008).

A paleoecological survey indicated that Lake Peipsi *s.s.* was mesotrophic before the 1950s, and its productivity was relatively low (Heinsalu et al., 2007). The intense use of mineral fertilisers and industrial development during the Soviet time caused eutrophication of the lake, while changes in Lake Pihkva started in the 1930s (Heinsalu et al., 2007; Nõges et al.,

2007). Nutrient loadings decreased considerably in the late 1980s and early 1990s (Nõges et al., 2007, 2020). However, despite these substantial decreases in nutrient loads, Lake Peipsi is still eutrophic, conditions in the lake are deteriorating, and there is no clear evidence of improved water quality (Nõges et al., 2020), as evidenced by cyanobacterial blooms and fish kills during summer (Nõges, 2020).

The phytoplankton community of Lake Peipsi is dominated by diatoms in spring, and cyanobacteria become dominant in summer. The phytoplankton community in the lake has changed since 1954. Until 2000, the dominant diatom species in Peipsi *s.s.* was the large, filamentous *Aulacoseira islandica* (Piirsoo, 2020), which prefers oligo- and mesotrophic conditions. In the following years, *A. islandica* was replaced by *A. ambigua,* a diatom favoured by more eutrophic conditions (Laugaste et al., 2008). Since 2017, the diatom *Actinocyclus normanii f. subsalsus* has become dominant. In the cyanobacterial community, *Gloeotrichia echinulata*, which has been characteristic of Peipsi *s.s.*, is now also common in strongly eutrophic Lake Lämmijärv (Piirsoo, 2020). Cyanobacterial blooms have been common in the whole lake for six decades (Laugaste et al., 2013; Panksep et al., 2020). Principal genera comprising these blooms include *Gloeotrichia*, *Microcystis*, *Dolichospemum*, *Aphanizomenon*, and *Planktothrix*. *G. echinulata* is common in the north (Peipsi *s.s.*), while *Aphanizomenon flos-aquae* dominates in the south (Lämmijärv, Pihkva). In Peipsi *s.l.* (*sensu lato*), *Microcystis* and *Dolichospermum* blooms occur (Laugaste et al., 2013; Panksep et al., 2020). During the ice-free season, cyanobacteria are dominant in Peipsi *s.l.* Long-term (1992-2019) median cyanobacteria percentages of total phytoplankton biomass are 32% in Peipsi *s.s.*, 54% in Lake Lämmijärv, and 57% in Lake Pihkva (Nõges, 2020). Approximately 60% of cyanobacteria genera present in Lake Peipsi are bloom-forming, and there is a clear north to south distribution gradient (Nõges, 2020).

Lake Võrtsjärv (area 270 km^2, mean and maximum depths 2.8 m and 6 m, respectively) is located upstream from Lake Peipsi, in central Estonia. These two lakes are connected via the River Emajõgi, but phytoplankton community composition is essentially different (Nõges et al., 2020). During the ice-free period, Võrtsjärv is strongly influenced by winds and waves, and the phytoplankton community is affected by turbidity, a poor underwater light climate, and sediment nutrient releases from sediment resuspension (Janatian et al., 2021). Until the 1970s, the cyanobacterial community was dominated by *Planktolyngbya limnetica*.

Since then, the lake has been dominated by cold and shade tolerant, slow-growing *Limnothrix redekei* and *L. planktonica* (Nõges et al., 2020). Typical cyanobacterial species frequently blooming and dominating in downstream Lake Peipsi (*Gloeotrichia*, *Dolichospermum*, *Aphanizomenon*, and *Microcystis*) are not abundant in Lake Võrtsjärv (Laugaste et al., 2013; Nõges et al., 2020), comprising <10% of total cyanobacterial biomass (Nõges, 2020). Although *Limnothrix* spp. can account for up to 90% of total phytoplankton biomass in Lake Võrtsjärv, these species do not form surface cyanobacterial blooms (T. Nõges et al., 2008).

Increasing dominance of cyanobacteria in large, shallow lake systems has been observed worldwide (Paerl et al., 2014; Huisman et al., 2018; Qin et al., 2019, 2021). China's third-largest freshwater lake, Taihu (area 2,338 km^2, mean and maximum depths 1.9 and 2.6 m, respectively), is located in the Yangtze (Changjiang) River Delta (Qin et al., 2007). The catchment area of the lake is industrialised, agricultural, and heavily urbanised, with about 40 million inhabitants (Qin et al., 2021). Since the 1990s, *Microcystis* spp. has been dominant from spring through fall (average number of bloom days from 2007-2017 is 130), and almost half of the lake surface area is covered with intense toxic cyanobacterial blooms (Paerl et al., 2014; Qin et al., 2019). Moreover, these blooms may last also year-round in Lake Taihu (Ma et al., 2015).

In Lake Erie (area 25,667 km^2), the fourth-largest lake among the Laurentian Great Lakes (North America), cyanobacterial blooms formed by filamentous, heterocystous *Aphanizomenon* spp. and *Dolichospermum* spp., along with *Lyngbya* spp. and *Planktothrix* spp., were common from the late 1950s until the 1970s (Barnard et al., 2021). Since the mid-1990s, non-N_2 fixing *Microcystis* spp. blooms occur in the entire western basin, and *Planktothrix* spp. blooms in Sandusky Bay have been recurring annually (Steffen et al., 2014; Barnard et al., 2021).

1.7 Common methods to study cyanobacteria and cyanotoxins

1.7.1 Microscopy

Inverted light microscopy (Utermöhl, 1958) is the most common method employed to study cyanobacteria. If applied by highly trained personnel, this method provides detailed information about cyanobacterial biomass and community composition. Biomass data

can be used for rough estimates of maximum expected cyanotoxin concentrations (Ibelings et al., 2021) and, together with the taxonomic composition of cyanobacteria, provide a basis to predict potential bloom development and cyanotoxins. Cyanobacterial biomass should be used to support decisions for further direct toxin analyses (Ibelings et al., 2021). Microscopic quantification has several shortcomings: (1) it is time-consuming and highly skill-dependent; (2) results are more subjective and therefore less comparable between laboratories; and (3) as toxic and non-toxic cyanobacterial strains co-exist concurrently and are morphologically identical, they cannot be distinguished microscopically (Sivonen and Jones, 1999; Sanseverino et al., 2017).

1.7.2 Pigment-based methods

Cyanobacteria contain chlorophyll-a, phycocyanin, allophycocyanin, and phycoerythrin as photosynthesis pigments (Govindjee and Shavela, 2011). Chlorophyll-a (Chl *a*) is a general marker pigment for all phytoplankton groups and commonly used as a proxy for total phytoplankton biomass. Phycobilins are cyanobacterial auxiliary pigments and can be used for robust estimation of cyanobacterial biomass (Berman, 1972). For pigment analysis, routine and simple methods can be used in the field or lab using spectrofluorometric or spectrophotometric devices. Spectrophotometry can distinguish between different fat-soluble pigments: chlorophylls and carotenoids. Phycobilins are water-soluble, so they cannot be analysed along with fat-soluble pigments using spectrophotometry. Most often, spectrofluorometric devices are used for phycobilins, but they cannot be identified if the carotenoid signal is present (Zhao et al., 2011). If more detailed pigment analysis is needed, high-performance liquid chromatography (HPLC) is generally used (Salmaso et al., 2017). HPLC also provides an input for pigment-based chemotaxonomy (CHEMTAX), which is a beneficial, complementary tool for estimation of cyanobacterial biomass, since the cost of analysis is low, and the method is fast and sensitive (Tamm et al., 2019). Although pigment-based methods are efficient, robust, and can provide rapid results for cyanobacterial biomass, the potential toxicity of the samples remains unknown.

The spatial distribution and formation of cyanobacterial blooms over vast areas can be effectively tracked using airborne and satellite remote sensing (Hunter et al., 2017). Several models and algorithms,

mostly based on Chl *a* or phycocyanin, have been constructed to estimate cyanobacterial biomass using remote sensing data (Mishra et al., 2019). These techniques are particularly useful and straightforward in open ocean studies. Optical properties of shallow coastal areas and inland waters are more complex, often region-specific, and influenced by other parameters, such as coloured dissolved organic matter (CDOM), mineral particles, and non-algal particles; therefore, data interpretation and analysis are more challenging (Hunter et al., 2017; Tamm et al., 2019). There are also some limitations of using remote sensing observations: (1) for accurate and quantitative monitoring of cyanobacterial blooms, spatial and temporal *in situ* measurements are still needed; (2) cyanobacterial community composition and toxicity cannot be characterised; (3) even with a strong correlation between remotely estimated phycocyanin and *in situ* measured cyanotoxin, generalising bloom dynamics remains difficult in space and time; (4) distinguishing water colour changes caused by phytoplankton from changes caused by dissolved matter and sediments is difficult when all are present; and (5) cloud cover can shadow the surface of the waterbody and inhibit pigment estimation (Hunter et al., 2017; Mishra et al., 2019).

1.7.3 Molecular methods

Since the early 1990s, the use of DNA-based methods targeting specific genes involved in the biosynthesis of cyanotoxins has increased (Salmaso et al., 2017). The main advantages of molecular methods are cost-effectiveness, high sample throughput, high sensitivity, and specificity, making early detection of potentially toxic cyanobacteria possible before bloom events when toxin concentrations are too low for detection with physicochemical methods (Humbert, 2017). Specific PCR has been developed to detect genes encoding the synthesis of microcystin, nodularin, saxitoxin, anatoxin-a, and cylindrospermopsin (Moreira et al., 2014). Genus-specific PCR and qPCR allow identification and quantification of organisms producing toxins (Pacheco et al., 2016), and, therefore, the predictability of toxin concentrations increases (Padisák et al., 2021). The specificity, sensitivity, reliability, and speed of molecular tools are excellent for rapid assessments of the potential toxicity of blooms, thus representing a valuable complement for conventional methods (e.g., microscopy, HPLC, LC-MS) in routine monitoring and toxicity studies (Sanseverino et al., 2017).

There is a diverse range of methods available to detect and identify cyanotoxins in water. For rapid detection, commercially available Enzyme-Linked Immunosorbent Assay (ELISA) test kits are most commonly used. To confirm the presence or absence of cyanotoxins, semi-quantitative ELISA kits are useful, but quantitative ADDA-ELISA kits or other analytical methods should be used for more precise toxin analyses. The main advantages of using ELISA kits are their simple methodology and rapid results, but ELISA cannot differentiate between microcystin congeners and total microcystin concentrations. Also, results can be invalidated because antibodies detect congeners at different levels due to different cross-reactivities.

Phosphatase Inhibition Assay (PPIA) is a rapid, sensitive, commercially available method to detect hepatotoxins in water. The high sensitivity of this assay allows analysing of samples immediately without any pre-treatment, even at low MC-LR concentrations. This colourimetric assay is based on the phosphatase inhibition activity of microcystins and nodularin (Carmichael and An, 1999). If other compounds (i.e., okadaic acid - a natural marine toxin produced mainly by dinoflagellates; calyculin A - a cytotoxic compound from a marine sponge) that might cause phosphatase inhibition are present in the sample (Kaloudis et al., 2017; Metcalf et al., 2017), the selectivity of the assay might not be sufficient, resulting in toxin concentrations being over- or underestimated. Also, PPIA cannot be used to identify individual toxins or their variants. Therefore, PPIA should be considered as a "screening method", and further analysis with LC-MS/MS should be considered (Kaloudis et al., 2017).

1.7.4 Analytical methods

If the identification of toxin congeners is required, or the toxin concentrations are too low to measure with biochemical assays, further analysis with analytical methods is necessary. The most commonly used methods are HPLC and LC-MS. HPLC is an efficient analytical tool used for separation and determination of sample components (Hiskia et al., 2017; Lawton et al., 2021). Cyanotoxins with a UV chromophore (e.g., microcystins, nodularin, cylindrospermopsin, and anatoxin-a) can be detected with HPLC coupled with a Photodiode-Array (PDA) detector for Ultraviolet (UV) absorbance. This method is widely used due to its reasonable costs (lower compared to LC-MS), good sensitivity, and

automation capability (Hiskia et al., 2017). HPLC also has its limitations. HPLC cannot be used to measure toxicity, it is time-consuming, and, if other compounds with similar absorbance spectra are present, there is a risk of misidentification of analytes (Hiskia et al., 2017; Sanseverino et al., 2017). Furthermore, the method is dependent on the commercial availability of standard compounds for calibration (Hiskia et al., 2017). The standard method for detection, identification, and confirmation of cyanotoxins in environmental samples is LC-MS, which combines the separation capabilities of HPLC with the mass analysis capabilities of mass spectrometry (MS). Modification of LC-MS with an additional MS detector involves Liquid Chromatography-Triple Quadrupole Mass Spectrometry (LC-MS/MS) (Sanseverino et al., 2017). LC-MS/MS is an excellent tool for quantification of individual toxins and elucidation of the structure of unknown secondary metabolites in environmental samples (Sanseverino et al., 2017). One of the main advantages of LC-MS/MS is the simultaneous separation and identification of toxin variants without reference standards (Moreira et al., 2014). The main disadvantage of the method is the initial investment cost for the equipment and high annual maintenance costs (Lawton et al., 2021).

2. AIMS AND HYPOTHESIS OF THE STUDY

In the current thesis, I present a synthesis of spatial and temporal variability of potentially toxic cyanobacteria and the importance of cyanobacteria as a food source for crustacean zooplankton in a large, shallow lake. The thesis is based on three published papers, each dedicated to a different aspect of the whole (Figure 2).

In the present thesis, I have set the following aims:

1. To describe the spatial and temporal variability of potentially toxic cyanobacteria and microcystin concentrations and composition, and to determine the dominant microcystin producers in a large, shallow, eutrophic lake (**I; II**).

2. To elucidate the environmental variables related to the occurrence of potentially toxic cyanobacteria in an eutrophic freshwater environment (**I; II**).

3. To assess the importance of cyanobacteria as a food source for crustacean zooplankton, and to determine the ability of zooplankton to ingest toxic strains of cyanobacteria (**III**).

Hypotheses:

1. In large, shallow Lake Peipsi, temperature is a primary factor promoting the recruitment of different cyanobacterial genera (**I**);

2. The dominance of the genus *Microcystis* is mainly linked with total phosphorus content in the lake (**I**);

3. There is a significant relationship between the abundance of *mcyE* genes and microcystin concentrations (**II**), and the amount of *mcyE* genes could potentially be used as a predictor of microcystin concentrations (**II**);

4. Microcystin quota in Lake Peipsi is related to water temperature and total nitrogen and phosphorus concentrations (**II**);

5. In Lake Peipsi, cyanobacteria constitute a more significant diet source for filter-feeding cladocerans compared to calanoid copepods (**III**);

6. Cladoceran grazers are the major link transferring biomass of potentially toxic cyanobacteria through the food web in Lake Peipsi (**III**).

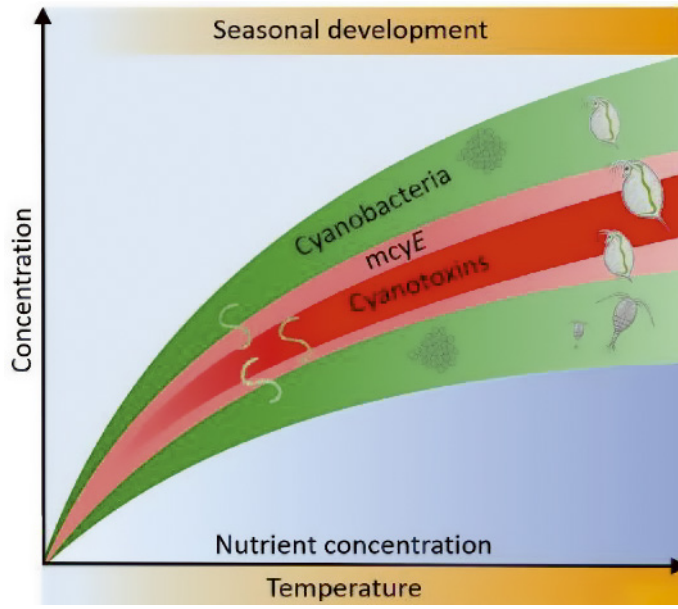

Figure 2. Graphical outline of the thesis. Schematic presentation of the relationships of concentrations of cyanobacteria, *mcyE* genes, and cyanotoxins with the main environmental factors and seasonality. Zooplankton, here cladocerans and copepods, are shown as the potential grazers on the cyanobacteria. Created in BioRender.com.

3. MATERIALS AND METHODS

A general list of the methods used in this study is in Table 1.

Table 1. Methods that were used in the study

Method	Paper
Microscopy	I, II, III
Zooplankton sorting	III
Cultivation of the strains used as external standards	II, III
DNA extraction from cyanobacterial strains, environmental samples, or zooplankton gut content	II, III
Planktothrix-specific primer and hydrolysis probe design	II
PCR detection of total cyanobacterial DNA and genus-specific mcyE PCR/qPCR	II, III
Toxin analyses by LC-MS/MS	II
Water chemistry analyses	I, II, III
Additional methods (pigment and stable isotope analysis)	III
Statistical analyses	I, II, III

3.1 Study sites and sampling

The surface area of Lake Peipsi *s.l.* is 3555 km^2, and the mean and maximum depths are 7.1 m and 15.3 m, respectively. The northern part (Peipsi *s.s.*) is eutrophic and the largest and deepest; the southern part is hypertrophic Pihkva, and these two basins are connected by Lämmijärv (Kapanen, 2018; Figure 3). Lake Peipsi is connected to the Gulf of Finland and the Baltic Sea via the Narva River. The catchment area of the lake is 47800 km^2 and is shared between Russia (58%), Estonia (34%), and Latvia (8%). In the lake drainage basin, semi-natural and forest areas mainly dominate, followed by agricultural areas (Jaani, 2001). Further information on the lake Peipsi ecosystem and biological and physicochemical properties can be found in papers (I; II; III).

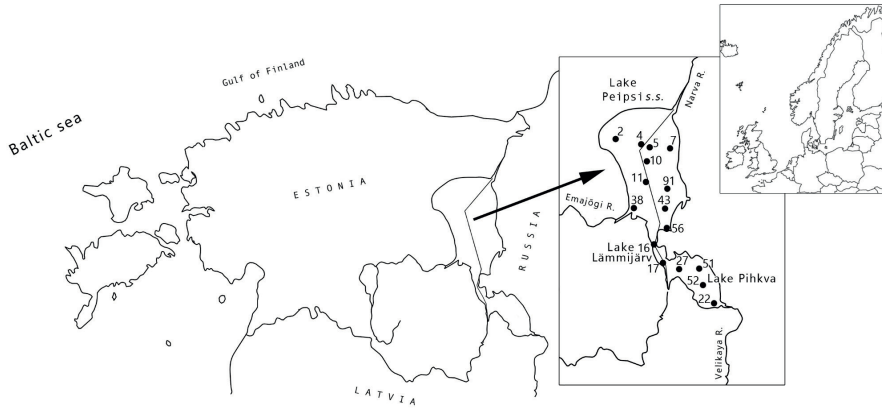

Figure 3. Location and sampling sites of Lake Peipsi. The figure is reprinted from paper II.

3.2 Microscopy

Phytoplankton samples were preserved with Lugol's (acidified iodine) solution and processed using the Utermöhl method (Utermöhl, 1958). Phytoplankton biomass was calculated from cell counts using a Nikon Eclipse Ti-S inverted microscope at 200× and 400× magnification. An aliquot of 3 mL was settled overnight. Species were identified to the lowest level possible using classifications described in the literature (Komarek and Anagnostidis, 1999, 2005; Komarek, 2013). Biovolume of algal cells, colonies, and/or filaments were calculated using assigned geometric shape dimensions and converted to biomass assuming specific density of 1 g/mL (Edler, 1979). Zooplankton samples were analysed in a Bogorov chamber using a Nikon SMZ1500 stereomicroscope at 120× magnification. Biomass was calculated as described in paper III.

3.3 Zooplankton sorting

Prior to molecular, pigment, and stable isotope analysis, frozen zooplankton samples were thawed and dominant cladoceran taxa (*Bosmina* spp., *Daphnia* spp., and *Bythotrephes longimanus*) and the copepod *Eudiaptomus gracilis* were sorted manually. For molecular analyses, 22 to 220 individuals of each species were separated and repeatedly rinsed with deionized water to minimise contamination from non-ingested algae. Samples were inspected visually under a microscope to verify that no external algal cells were stuck on animals and then collected into 1.5

mL microtubes in duplicate. The sorting technique is more precisely described in Tõnno et al. (2016) and in paper (III).

3.4 Cultivation of the Strains Used as External Standards

Cyanobacterial strains *Microcystis* sp. 205, *Dolichospermum* sp. 315, and *Planktothrix* sp. 49 used throughout this study (II, III) were grown and maintained in the HAMBI/UHCC Culture Collection during visits to the University of Helsinki. These strains were used as positive control material and for preparation of standard curves used in the qPCR analysis.

3.5 DNA extraction from cyanobacterial strains, environmental samples, and zooplankton gut content

DNA from environmental samples was extracted using the DNeasy PowerWater Kit (QiagenInc., Germantown, MD, USA) according to the manufacturer's instructions. Genomic DNA from ZP and cyanobacterial standard cultures (HAMBI/UHCC Culture Collection, University of Helsinki) were extracted using Dneasy® Blood and Tissue Kit (QiagenInc., Germantown, MD, USA) and E.Z.N.A.™ SP Plant DNA Kit (Omega Bio-Tek, Norcross, GA, USA), respectively. The quality and quantity of extracted DNA were controlled visually on gel and with a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). A more detailed description is provided in the original papers (II, III).

3.6 *Planktothrix*-specific primer and hydrolysis probe design

A new *Planktothrix*-specific primer pair and hydrolysis probe were designed to detect *Planktothrix*- specific *mcy*E genes in water samples (II). Specificity and sensitivity were optimised as described in Sipari et al. (2010).

3.7 PCR detection of total cyanobacterial DNA and genus-specific *mcy*E PCR/qPCR

Potential microcystin-producing genera in environmental samples and zooplankton gut contents were detected, identified, and quantified using

general and genus-specific PCR and qPCR methods. Amplifications were performed on an ESCO Swift™ Spectrum 96 Real-Time Thermal Cycler (ESCO, Singapore) and ABI 7500 Fast Real-Time PCR system (Thermo Fisher Scientific Inc, Waltham, MA, USA). Results were analysed using ABI 7500 Software. Detailed descriptions of the reaction conditions are presented in the original papers (II, III).

3.8 Toxin analysis by LC-MS

The identities and concentrations of microcystins in samples were determined by LC-MS according to their microcystin characteristic protonated molecular ions [M-H]−. Analyses were conducted using an Agilent 1100 LC-MS XCT+ Ion Trap System (Agilent Technologies, PaloAlto, CA, USA.). A more detailed description of the toxin analysis method is provided in the paper (II). Toxin analyses were performed during academic visits to the University of Helsinki.

3.9 Water chemistry analyses

Water chemistry analyses were performed by the state monitoring programme by the Estonian Environmental Research Centre following international and Estonian quality standards (ISO and EVS-EN ISO).

3.10 Additional methods

For phytoplankton marker pigment analysis, samples were filtered through Whatman GF/F filters and stored at -80 °C until further analysis. Pigments were extracted in 90% acetone, samples were sonicated in an ice bath, and extracted at -20 °C in the dark for 24 h. Extracts were filtered through 0.45 μm syringe filters and analysed with reversed-phase HPLC. Sorted algal and zooplankton samples for stable isotope analyses were dried in tin cups at 60 °C overnight, POM samples were sieved through a 100 μm net to remove larger ZP specimens, and filtered onto GF/F filters. SIA analyses were performed at the University of Jyväskylä (Finland). A more detailed description of the methods is presented in the original paper (III).

3.11 Statistical analysis

All statistical analyses were performed using R (R Core TEAM, 2020) packages and its extensions and STATISTICA (TIBCO Software Inc., PaloAlto, CA, USA). More detailed information about the packages and method used can be found in original papers (I, II, III).

4. RESULTS AND DISCUSSION

In this study, samples from Lake Peipsi and its basins were analysed for cyanobacterial community composition (I, II), presence and abundance of potentially toxic *Microcystis*, *Dolichospermum*, and *Planktothrix* (II), microcystin variants and their concentrations (II), and DNA-based composition of the crustacean zooplankton diet (III).

There are five potentially toxic cyanobacterial genera in Peipsi, with the most notable species: *Dolichospermum* spp. (*D. crassum*, *D. lemmermannii)*, *Aphanizomenon flos-aquae*, *Gloeotrichia echinulata*, *Microcystis* (*M. viridis*, *M. wesenbergii*), and *Planktothrix agardhii*. The biomass of the genus *Microcystis* exceeded other genera by multiple times and was dominant among potentially toxic algae (I; II).

This study showed a clear spatio-temporal distribution of cyanobacterial community composition across the three lake basins (I; II) (Figure 4). Hypertrophic lake basins, Lämmijärv and Pihkva, were comparable and varied considerably (permutation test for RDA, $n = 1000$, $p < 0.01$) from the eutrophic northern basin, L. Peipsi *s.s.* (Figure 5). Blooms in the northern part of Peipsi *s.s.* were mainly formed by *G. echinulata*, while, in the southern part of Peipsi *s.s.* and other basins, mixed blooms of *Microcystis* spp., *Aphanizomenon* spp., *Dolichospermum*. spp., and on a smaller scale, *Planktothrix* spp., were common. Temporal variations in *Gloeotrichia*, *Microcystis, Dolichospermum, Aphanizomenon*, and *Planktothrix* biomass were observed (I; II). *Gloeotrichia echinulata* attained its peak in July, *Dolichospermum* spp. had peaks at the beginning of July and in mid September, *Microcystis* spp. in August, *Aphanizomenon* spp. in September, and *Planktothrix* spp. in September and October (Figure 4). *Gloeotrichia echinulata* occured sporadically in Peipsi *s.s.* and sometimes in Lämmijärv, *Aphanizomenon* in all basins, but more numerous in Peipsi *s.s.*, and other genera were widespread with larger biomasses in southern lakes Lämmijärv and Pihkva.

Gloeotrichia echinulata inhabited moderately eutrophic Peipsi *s.s.*, and the presence of this species in the water column was related to water temperature, independent of nutrient concentrations (I). During spring, when water temperature was rising, germination of akinetes was triggered, and cells started assimilating and storing nutrients from

sediments (Karlsson-Elfgren et al., 2004). Due to low water transparency in Peipsi, germination probably occurred in shallow areas, and currents carried new colonies into open waters, where they inoculated visible *Gloeotrichia* blooms.

Major potential cyanotoxin producers in Lake Peipsi were species from the genus *Microcystis* (I; II; III). *Microcystis* spp. are the most pervasive cyanobacteria in freshwater ecosystems throughout the world (Harke et al., 2016; Cai et al., 2021), and their distribution range is continuously extending (Visser et al., 2016). In temperate climate zones, species from this genus overwinter mainly in sediments, and this "seed-bank" plays an important role in its ecological success (Cai et al., 2021). *Microcystis* spp. can survive under dark and cold conditions for a long period (Brunberg and Blomqvist, 2002). Before migration to the pelagic zone, *Microcystis* spp. assimilate P from sediments and use this internal storage to support growth (Šejnohová and Maršálek, 2012).

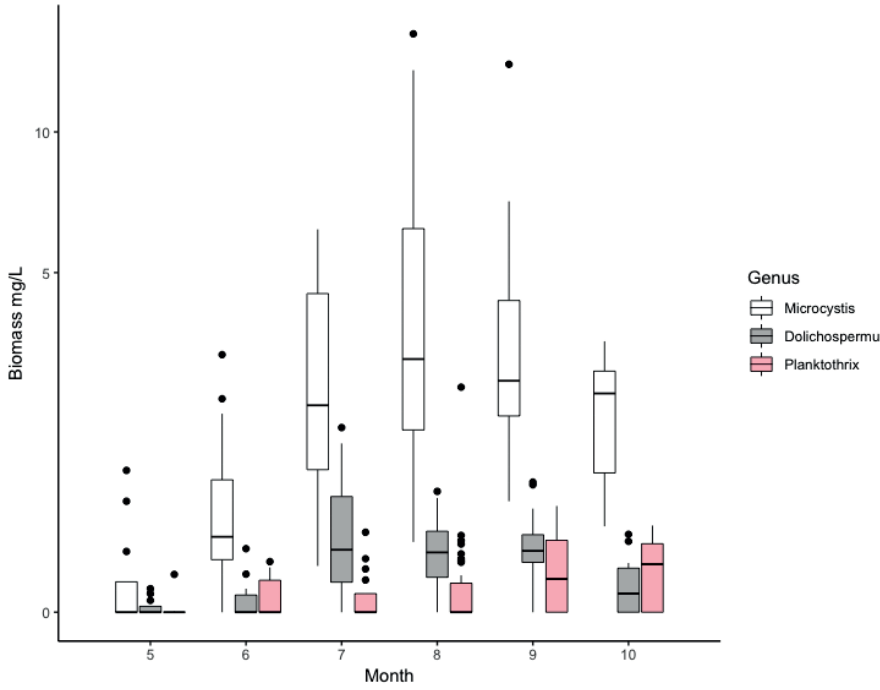

Figure 4. Temporal variation in *Microcystis*, *Dolichospermum*, and *Planktothrix* biomass (mgWW/L). Boxplots denote median biomass values across the basins of Lake Peipsi, and error bars represent spatial variation across sampling stations. The y-axis is plotted as a square root scale, and values of biomass remain unchanged. The figure is adapted from paper II.

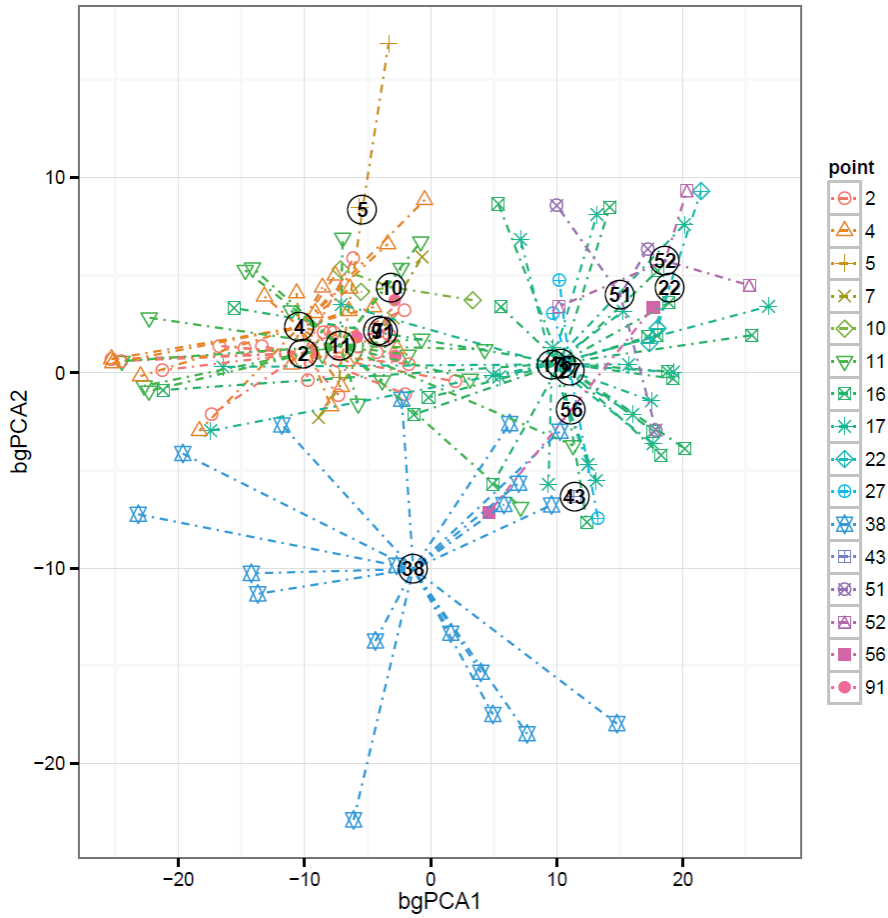

Figure 5. Cyanobacterial community composition in different basins (sampling stations) of Lake Peipsi based on 141 samples collected biweekly and monthly in 2011-2012, respectively. Sampling stations 2, 4, 5, 7, 10, 11, 38, 43, 56, and 91 are located in Peipsi *s.s.*; stations 16 and 17 in Lämmijärv, and 22, 27, 51, and 52 in Pihkva. The figure is adapted from paper II.

4.1 State the problem - microscopy cannot reveal if cells are toxic

Conventional monitoring programmes of phytoplankton and early warning systems for harmful cyanobacterial blooms are generally based on inverted microscopy and analysis of Chl *a*. The problem is that these methods are unsuitable for predictions of actual risks of cyanobacteria, and potential toxicity remains unknown, since toxic and non-toxic strains are morphologically indistinguishable and often co-exist (Sivonen and Jones, 1999; Merel et al., 2013; Pacheco et al., 2016). Analytical approaches for cyanotoxin analyses are highly evaluated, but still not

widely accessible in all countries, and therefore are limited for routine use. Molecular methods used in this study (II, III) enable early detection and monitoring of bloom development of toxic cyanobacteria. Another strength of the molecular approaches used (II, III) is the possibility of identifying and quantifying all of the principal microcystin producers and tracking their utilisation in the food web. Moreover, molecular assays used in this study provide high throughput (simultaneous analysis of up to 120 samples in a single qPCR run) and therefore reduce the amount of time for obtaining results.

4.2 Microcystins in Lake Peipsi (II)

In this study, we detected microcystins in all analysed samples (n = 69) collected monthly from Peipsi *s.l.* in 2012 (II). Compared to other large lakes (Võrtsjärv, Erie, Lake Michigan, Taihu, and Chaouhu), microcystin concentrations measured in integrated water samples were relatively low, generally less than 1 μg/L (author's data; Yu et al., 2014; Bartlett et al., 2018; Li et al., 2022). A total of 14 microcystin variants were found. The most abundant variants were MC-RR and MC-LR with structural congeners, found in 93% and 92% of samples, respectively. *P. agardhii* and *Planktothrix mcyE* genes were correlated with [D-Asp3]MC-RR (Figure 6) only in hypertrophic parts of the lake. In downwind, inshore areas, where cyanobacterial biomass can accumulate and where most human and animal activity occurs, microcystin concentrations were up to 2183 μg/L in a previous study (Tanner et al., 2005), even when microcystin concentrations in open water were relatively low, as in the present study. *mcyE* gene abundances in inshore areas were up to 57 million copies per mL, potentially representing a health risk for people using the lake recreationally. Even moderate or low concentrations of microcystins in open waters can pose a health risk for recreational users if, under favourable conditions, surface scums form and concentrate in shoreline areas (II).

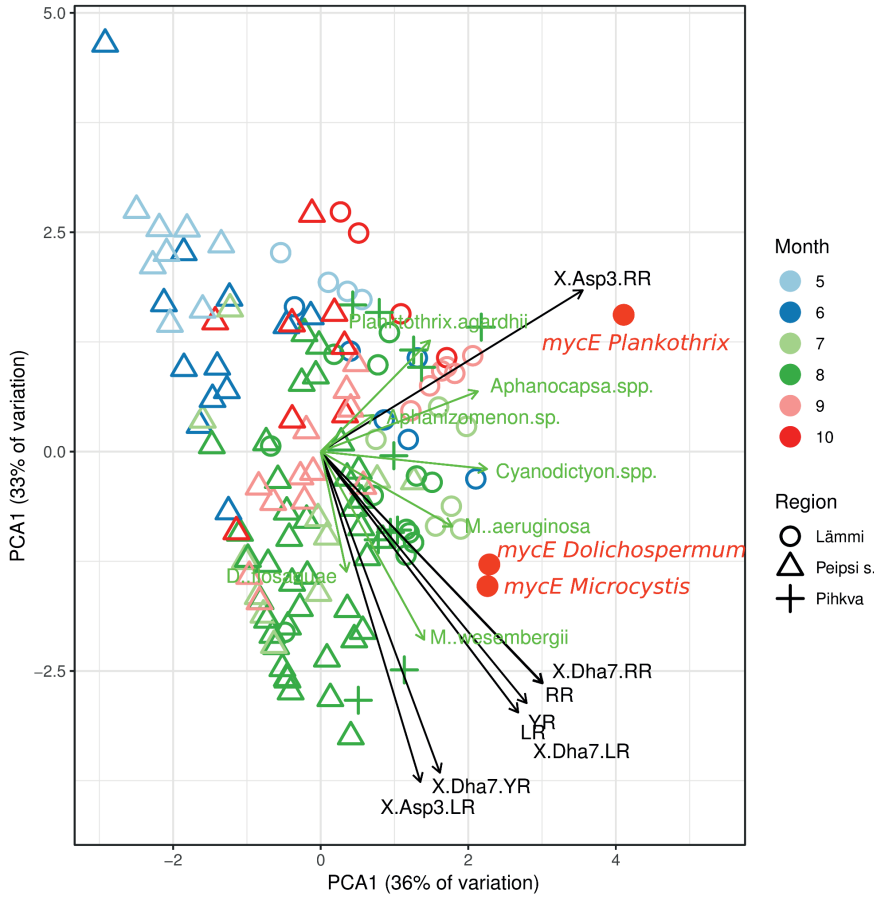

Figure 6. Multivariate comparisons of various *mcyE* gene abundances, a cyanobacterial community, and the presence/absence of MC variants. The significance ($p < 0.05$) of these linear fittings was obtained by a permutation test (1000 replicates). The length and direction of vectors indicate the strength and direction of the relationship. The figure is adapted from paper II.

4.3 Tracing toxic cyanobacteria by toxin concentration and gene copy number (II)

To implement modern molecular methods in parallel with existing environmental monitoring programmes, cell counts for the organism of interest are necessary. Whole-genome sequencing studies of cyanobacteria have shown that the *mcy* gene cluster appears only as a single copy per genome of cyanobacteria (Pacheco et al., 2016; Yuan and Yoon, 2021; Zupančič et al., 2021). Since this is a prerequisite to interference of cell numbers from *mcy* gene copies, the *mcyE* gene used

in our study can be used to evaluate the number of potentially toxic cells in water samples. In Lake Peipsi, we found microcystins and all of the main potential microcystin producers in all regions of the lake (II). In 80% of the analysed samples, all three genera appeared concurrently. *Microcystis* was the most frequently occurring genus, and the abundance of *Microcystis mcyE* genes was the highest throughout the study period (Wilcoxon pairwise test, $p < 0.01$), while *Planktothrix* and *Dolichospermum* contributed in small quantities (II). *Microcystis* biomass also exceeded other genera. Relationships between the sum of toxic *Microcystis, Dolichospermum*, and *Planktothrix* cells (assessed with qPCR) and total microcystin concentrations were strong ($r = 0.67$; $p < 0.01$; $n = 69$). Similar relationships have been shown by other investigators (Bukowska et al., 2017; Li et al., 2017; Lu et al., 2020; Yuan et al., 2020; Wood et al., 2021; Zupančič et al., 2021).

Within the European Multi Lake Survey (Mantzouki et al., 2018a, 2018b, 2018c), samples from 200 European lakes were analysed using genus-specific qPCR, and a positive relationship was observed between the abundance of *mcyE* genes and microcystin concentrations (author's unpublished data). Previous work showed a persistent, positive correlation between various *mcy* gene copies and microcystin concentrations in two-thirds of the studies ($n = 33$; years 2003–2015) reviewed (Pacheco et al., 2016). In studies where a positive relationship was not found, the *mcyE* gene was not used as the target in most cases (Pacheco et al., 2016). The present study showed that genus-specific qPCR of toxin genes is an effective and sensitive method to detect and quantify potential cyanotoxin producers. Detection of *mcyE* genes also has predictive value and can be used as an early warning tool to detect potential MC producers before bloom events, when toxin concentrations might be too low for detection with analytical methods. Even though toxin synthetase genes are obligatory for microcystin synthesis (Yuan and Yoon, 2021), and without those genes, toxin production is impossible, it should be noted that the presence of toxin synthetase genes unveil the potential to produce toxins, but do not prove that toxins are produced.

4.4 To what extent can we link the *mcy*E copy number to microcystin concentration and cellular quota?

Toxin concentrations in water are associated with intracellular microcystins per cell (toxin quota) and the abundance of toxin-producing species

(Horst et al., 2014; Wood et al., 2021). Traditionally, the toxin quota is calculated per unit of cyanobacterial biomass or Chl *a* (Horst et al., 2014; Mantzouki et al., 2018a). Here, the microcystin quota was calculated as the microcystin concentration per unit of cells with the *mycE* gene to elucidate the direct relationship between the abundance of toxin genes and MC concentrations. Examining the toxin quota provides additional information for toxicity risk assessments. Even though a positive relationship between MC concentration and *mcyE* gene abundances was observed in Lake Peipsi (II), the dynamics of toxin quota and toxin concentration in the water did not coincide. Contrary to expectations, a negative relationship between MC quota and the abundance of *mcyE* genes was observed. A lower microcystin quota occurred with higher MC concentrations and *vice versa*. When a lower amount of toxic cells can produce higher concentrations of microcystins, the public health concern is higher, as the toxicity potential is higher. Therefore, risk assessment based solely on *mcyE* gene abundance may under- or overestimate this threat. MC quota was negatively influenced by toxic cyanobacterial cell concentration (based on *mcyE* gene abundance). This outcome is contrary to that of others, who found a positive correlation between those parameters in Lake Rotorua Bay (Wood et al., 2021). However, taking into account samples from the whole lake, a much weaker relationship was observed. The possible explanation for this weaker connection was low microcystin quotas in stagnant microcystin scum samples. A positive relationship between *Microcystis* growth rates and microcystin quota was also described in Upper Klamath Lake (Eldridge and Wood, 2020).

Taken together, the hypothesis that *mcyE* gene copy numbers could be used as a direct predictor of MC concentrations was only partially supported and should be considered with caution due to variations in cellular quotas of microcystins. Toxin quota may be highly variable within the same species, as well as between different toxin-producing species (Sivonen and Jones, 1999). There is still no clear evidence of factors controlling toxin content of individual strains, but a genetic variation of different strains and synergetic effects of environmental variables could help explain the differences (Sivonen and Jones, 1999; Sukarji et al., 2022). Other investigators showed almost 20-fold changes in microcystin quota within five hours in the same bloom (Wood et al., 2011, 2012). In Lake Rotorua, a 148-fold variation of toxin quota was observed across the lake (Wood et al., 2021). Therefore, understanding

which factors explain high toxin quotas during low toxic cell abundances would be essential for lake managers for risk assessment.

4.5 Environmental variables that influence cyanobacterial community composition and the abundance of toxic genotypes

One of the aims of the thesis was to identify environmental variables related to potentially toxic cyanobacteria in a eutrophic freshwater environment. At the cyanobacterial community level, analyses revealed a weak relationship with environmental predictors (II). Nitrate and water temperature were the main variables associated with cyanobacterial abundance and community composition in the early growing season (II). From August to October, soluble reactive phosphorus (SRP) in Lake Peipsi *s.s.* and TN and TP in Lämmijärv were the most important environmental variables related to cyanobacteria (II). Other analysed environmental variables, like NH_4^+, NO_2^-, etc., were not correlated with the cyanobacterial community in general (II). This is not surprising, as these forms are much more reactive than NO_3^-, they are cycled rapidly and are immediately assimilated (Newell et al., 2019). However, at the genus level, the main bloom-forming cyanobacteria in Lake Peipsi were mostly affected by different environmental variables (I). *Gloeotrichia* sp. was related to water temperatures higher than 22°C and water level, *Microcystis* spp. with TP and Fe concentrations in the water, *Dolichospermum* spp. with water temperature and TN:TP ratio, and *Aphanizomenon* spp. with TN (I). Toxin quota per cell in Lake Peipsi was positively associated with water temperature (II). Water temperature as an important factor determining the toxin quota has been also shown by other authors (Wood et al., 2017; Mantzouki et al., 2018a), where no direct relationships were found with TN and TP.

In the second half of the summer, the dominance of cyanobacteria and the biomass of *Microcystis* spp., the main microcystin producer in Peipsi *s.s.*, was mostly controlled by P dynamics in the lake. Even though TP loading has declined since 1995, this decline has been hindered by internal loading from sediments, which currently exceeds the external P load (Nõges, 2020; Tammeorg et al., 2020). Internal loads of P from sediments contribute to the growth of cyanobacteria in summer, as it provides bioavailable P to the water column. Similar trends have been shown from other northern, temperate, shallow, eutrophic lakes

(Istvánovics et al., 2004; Steinman et al., 2009; Nürnberg and LaZerte, 2016; Tammeorg et al., 2016).

Nevertheless, the significance of N as a promotor of cyanobacterial blooms has been largely outshined by P (Newell et al., 2019). Nitrate at the beginning and TN, together with TP, in the late growing season, are the main predictors of cyanobacterial community composition, which indicates the necessity to consider nitrogen in lake water quality management plans as an important factor to reduce the proportion of cyanobacteria in phytoplankton biomass. The shift in the factors that regulate cyanobacteria in the early and late growing season is probably related to changes in the relative importance of the sources of nutrient supply. To conclude, due to climate change and ongoing anthropogenic eutrophication, both N and P loads should be reduced to improve water quality and achieve 'good' status, as required by the EU Water Framework Directive.

4.6 Tracing the cyanobacteria in the crustacean zooplankton diet in Lake Peipsi (III)

Crustacean zooplankton has a critical role in top-down control of phytoplankton and affect water quality and fish production; therefore, research on the role of zooplankton in suppressing algal biomass, including cyanobacterial blooms, has gained more interest (Ger et al., 2016). Laboratory studies show that species of *Daphnia* are tolerant of toxic cyanobacteria in their diet and can reduce cyanobacteria biomass and microcystins in the water (Sarnelle and Wilson, 2005; Wojtal-Frankiewicz et al., 2013). Still, natural grazer communities are composed of various species, leading to different zooplankton–cyanobacteria interactions. More knowledge from actual systems is needed to predict the ability of grazers to affect bloom dynamics in natural environments (Ger et al., 2014).

This study focused on dominant zooplankton taxa in Lake Peipsi that could affect food web dynamics the most. Dominant taxa were the herbivorous cladocerans *Daphnia* spp. and *Bosmina* spp. and the calanoid copepod *Eudiaptomus gracilis* (Blank et al., 2017). Additionally, a predatory cladoceran *Bythotrephes longimanus*, which is a favoured food object for juvenile and planktivorous fish in Peipsi (Ginter et al., 2018), was selected to investigate potential trophic links in transferring potentially

toxic cyanobacteria between primary grazers and higher organisms in the food web. To trace the role of cyanobacteria in the food web of Lake Peipsi, crustacean diet composition was analysed with HPLC, to determine algal marker carotenoid composition in general, and with qPCR, to determine the presence of potential microcystin-producing cyanobacteria in diet composition in more detail.

Grazing on potentially toxic cyanobacteria was evaluated by PCR analysis of the gut contents of sorted zooplankton. The presence of cyanobacterial 16S rDNA in zooplankton gut contents was revealed in all of the analysed taxa (*Bosmina* spp., *Daphnia* spp., *B. longimanus*, and *E. gracilis*) (III). Also, genus-specific qPCR affirmed the ingestion of potentially toxic, colonial *Microcystis* (III). Crustacean zooplankton ingest both filamentous and colonial cyanobacteria, with consumption of filamentous forms exceeding colonial cyanobacteria (Work and Havens, 2003). In the present study, qPCR analysis did not reveal the presence of potentially toxic filamentous cyanobacteria (*Dolichospermum* and *Planktothrix*) in gut contents of zooplankton. Water samples indicated potential microcystin production over the entire studied area (III). The principal potential microcystin-producing genus was *Microcystis*. *Planktothrix mcyE* genes were detected in water samples only in small amounts in August, and *Dolichospermum*-specific *mcyE* genes were not detected during the study period. The absence of cells with *mcyE* genes from these genera in zooplankton gut contents probably reflects almost nonexistent cells in the water column. Still, microscopic analysis of the cyanobacterial community recorded *Dolichospermum* spp. in water samples from July to September, probably indicating the presence of nontoxic genotypes. Crustacean zooplankton can fragment filamentous cyanobacteria to an ingestible size, thus facilitating grazing (Work and Havens, 2003; Kâ et al., 2012). Thus, it is presumed that grazing on non-toxic *Dolichospermum* occurred in Lake Peipsi.

4.7 The importance of cyanobacteria in the zooplankton diet. Interactions with potentially toxic cyanobacteria

According to pigment analysis with HPLC, the dominant crustaceans in Lake Peipsi differed in their feeding and selectivity for cyanobacteria (III). In filter-feeding cladocerans *Daphnia* spp. and *Bosmina* spp., approximately half of the ingested algae comprised cyanobacteria, which only comprised a small portion of ingested algae by the calanoid

copepod *E. gracilis*. For this copepod, cryptophytes were the preferred algae in the phytoplankton assemblage. In contrast, pigment-based gut content analyses of *Daphnia* spp. and *Bosmina* spp. were more closely representative of the phytoplankton community composition in Lake Peipsi, especially that of *Daphnia* spp., which exhibits lower feeding selectivity. Grazing selectivity of cladocerans is affected by the size of food objects (Feng et al., 2020), while herbivorous copepods (e.g., *E. gracilis*) seem capable of selecting food and sorting out the algae they prefer (Isari et al., 2013). Hence, these results confirm that *Eudiaptomus* and the cladocerans *Daphnia* spp. and *Bosmina* spp. have different feeding niches and energy transfer routes in Lake Peipsi.

Selectivity differences also affected ingestion of potentially toxic cyanobacterial cells by crustaceans in Peipsi. The highest number of potentially toxic *Microcystis* spp. cells was observed in the guts of *Daphnia* spp., which has little ability to avoid ingesting cyanobacteria (Lürling, 2003). In contrast, the gut pigment composition of *E. gracilis* in Peipsi supported the assumption that this copepod selectively avoids ingestion of cyanobacteria but cannot avoid all cyanobacterial cells, as shown previously in laboratory experiments (DeMott and Moxter, 1991; Ger et al., 2011). The $\delta^{15}N$ value of *E. gracilis* indicates one trophic level higher position in the food web compared to grazing cladocerans, so we may presume that some of the toxic *Micorcystis* cells could have originated from their prey organisms. *B. longimanus* is a raptorial, predatory cladoceran, and its gut pigment composition was mostly cyanobacteria and cryptophytes, reflecting feeding on cladocerans and copepod nauplii, as observed in other studies (Dumitru et al., 2001). *B. longimanus* was also able to transfer potentially toxic *Microcystis* in the food web, indicating that grazing cladocerans, including predatory cladocerans, could represent a plausible link to transfer cyanobacterial toxins through the food web of Lake Peipsi.

Active grazing of cyanobacteria by crustaceans raised the question regarding cyanobacterial support of zooplankton biomass. While molecular and pigment-based analysis of zooplankton gut contents provided an estimate of recently ingested food, long-term information about assimilated food sources can be provided by stable isotope composition analysis (Middelburg, 2014). In this study, qPCR revealed clear evidence of ingestion of *Microcystis* cells by crustacean zooplankton, especially by *Daphnia* spp. On the other hand, SIA analysis demonstrated

that ingested cyanobacteria (mostly *Microcystis* spp.) are not assimilated and do not contribute much to crustacean zooplankton biomass. Moreover, instead of top-down control, stimulation of cyanobacteria growth after gut passage through filter-feeding consumers, like cladocerans, mussels, and planktivorous fish, may occur (Lewin et al., 2003; Jančula et al., 2008; Vanderploeg et al., 2009; Zeng et al., 2014; Semenova et al., 2017). To obtain a more precise understanding of herbivorous zooplankton diets in Lake Peipsi, and to assess their assimilation of cyanobacteria, more source-specific biomarkers, such as fatty acid analyses, could be used (Taipale et al., 2011).

Compared to other phytoplankton groups, cyanobacteria are well equipped to increase their resistance to grazing (Ger et al., 2018; Ibelings et al., 2021). Mucilaginous sheaths or colonial/filamentous forms may diminish their nutritional quality and provide better defences against grazing by zooplankton (Ger et al., 2016, 2018). However, zooplankters also have adaptations against negative effects from cyanobacteria, such as selective feeding or stronger physical tolerance, especially in water bodies with a long history of cyanobacterial blooms (Ger et al., 2016). In Lake Peipsi, these adaptations appear to hold true. *Daphnia* spp. and other crustaceans studied have been exposed to annual cyanobacterial blooms over a long period and should be adapted to co-existence with toxic cyanobacteria, and *E. gracilis* seems to avoid ingestion of cyanobacteria, in favour of cryptophytes, via selective feeding.

CONCLUSIONS

In the present thesis, spatial and temporal variability of potentially toxic cyanobacteria was described, along with the occurrence of the cyanotoxin, microcystin, in a large, shallow, eutrophic lake. We elucidated the environmental variables related to potentially toxic cyanobacteria in a eutrophic freshwater environment and estimated the importance of cyanobacteria as a food source for crustacean zooplankton.

Based on the results of this thesis, the following conclusions can be drawn:

- There was a clear spatio-temporal distribution pattern of cyanobacterial species in the three lake basins. Hypertrophic lake basins, Lämmijärv and Pihkva were comparable and differed considerably from the eutrophic northern basin, L. Peipsi s.s.

- Major potential cyanotoxin producers in Lake Peipsi were from the genus *Microcystis* - the most pervasive cyanobacteria in freshwater ecosystems throughout the world. Microcystins and all main potential microcystin producers were widespread in all three parts of Lake Peipsi

- Genus-specific qPCR of toxin genes was an effective and sensitive method to detect and quantify potential cyanotoxin producers. Molecular detection of mcyE genes has predictive value and can be used as an early warning tool, to determine potential microcystin producers before bloom events, when toxin concentrations might be too low to measure with analytical methods. The application of this high throughput, highly specific and sensitive method is a valuable complementary tool for forecasting toxic blooms harmful to human and ecosystem health.

- Nitrate, together with water temperature, were the main predictors shaping cyanobacterial community composition at the beginning of the vegetation season, indicating the necessity to consider nitrogen in lake water quality management plans as an important factor to reduce the cyanobacteria and phytoplankton biomass. **This result supports the first hypothesis of the study "In large**

and shallow Lake Peipsi, the temperature is one of the main factors promoting the recruitment of different cyanobacterial genera (I)"

- The proportion of *Microcystis* spp. in total phytoplankton biomass is related to total phosphorus concentration. **The second hypothesis of the study "The dominance of the genus *Microcystis* is mainly linked with the total phosphorus content in the lake (I)" is supported. However, TP and biomass are expected to be related as they are not independent variables.**

The dynamics of toxin quota and toxin concentration might not coincide. When lower toxic cells in the lake produce higher concentrations of microcystins, the public health concern is higher, as the toxicity potential is higher. Therefore, risk assessment based solely on the abundance of *mcyE* genes may lead to under-or over-estimation of human health threats. **Taken together, our results suggest that our hypothesis, which states that *mcyE* copy number could be used as a direct predictor of MC concentration in the lake, is only partially supported and should be considered with caution due to the variation in the cellular quota of microcystins.**

Cyanobacteria, especially colonial forms, such as *Microcystis*, comprise a significant proportion of algae ingested by cladocerans. **These results indicate that grazing cladocerans, including predatory cladocerans, could represent a plausible link to transfer cyanobacterial toxins through the food web of Lake Peipsi and support the fifth and sixth hypotheses of the study.**

The copepod *E. gracilis* selectively avoided ingestion of cyanobacteria in favour of cryptophytes in its diet, thus exhibiting a different feeding niche and energy transfer routes compared to grazing cladocerans.

This thesis improves our knowledge of potentially toxic cyanobacteria and cyanotoxins in large, shallow, eutrophic lakes and also provides initial insights into *in-situ* consumption of toxic *Microcystis* by cladoceran and copepod grazers in Lake Peipsi. Knowledge gained from this study will guide further important questions that should be addressed in future research regarding food web function in Lake Peipsi. Phytoplankton community analysis using high throughput sequencing would allow

analysis of relationships between cyanobacterial community composition and concentrations and diversity of cyanotoxins. This approach would include picocyanobacteria which are largely excluded from most research. Moreover, to elucidate processes underlying cyanotoxin dynamics in more detail, further exploration focusing on expression (i.e., using RNA instead of DNA) of toxin genes, along with toxin concentrations, would be beneficial. Toxin gene expression would better indicate potential risks, especially in water bodies comprised of mixed assemblages of toxic and nontoxic cyanobacteria.

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SUMMARY IN ESTONIAN

„Morfoloogiast geenideni: kuidas otsida toksilisi sinivetikaid madalast eutroofsest järvest"

Sinivetikad ehk tsüanobakterid on üks edukaimaid elustikurühmi Maal ning ajalooliselt on nad täitnud ülitähtsat rolli rikastades Maa atmosfääri hapnikuga. Tsüanobakterite poolt põhjustatud toksilised veeõitsengud on aga kujunenud oluliseks keskkonnaprobleemiks kõikjal maailmas. Õitsengud mõjutavad veekogu ökosüsteemi ja selle pakutavaid ökosüsteemiteenuseid ning on ohuks inimeste ja loomade tervisele. Negatiivne mõju avaldub mitmetele majandussektoritele, sealhulgas põllumajandusele, kalandusele, vesiviljelusele ning puhke- ja veemajandusele. Kliima soojenedes ning veekogude eutrofeerumise jätkudes on oodata õitsengute intensiivistumist, mistõttu muutub järjest olulisemaks õitsengute varajane tuvastamine ja sellest tulenevate riskide hindamine. Traditsiooniliselt kasutatakse sinivetikate tuvastamiseks mikroskoopiat, aga morfoloogiliste tunnuste alusel ei ole võimalik toksilisi ja mittetoksilisi sinivetikaid teineteisest eristada. Seega on sellise seiremetoodikaga antud hinnangud õitsengute toksilisusele kaudsed, pole ennetavad ja ei täida oma eesmärki.

Toksiinide kontsentratsioon vees on otseselt seotud toksiini tootvate sinivetikate arvukusega ja toksiini kogusega raku kohta. Siiski pelgalt sinvetikate biomassile tuginedes võime toksiinidest tulenevat riski ebatäpselt hinnata. Oma doktoritöös uurisin tsüanobakterite koosluse koosseisu, toksiini tootvate sinivetikate arvukust ja toksiini kontsentratsiooni nii vees kui ka rakkudes ning hindasin toksiliste sinivetikate olulisust erinevate zooplankterite toiduobjektina Peipsi järves. Samuti andsin ülevaate keskkonnateguritest, mis mõjutavad toksiliste tsüanobakterite vohamist suures ja madalas järves. Oma uurimistöös kasutasin tsüanobakterite potentsiaalse toksilisuse hindamiseks molekulaarseid meetodeid – toksiiniproduktsiooniks vajalike geenide perekonnaspetsiifilist analüüsi ja analüütilisimeetodeid toksiinikontsentratsioonide mõõtmiseks. Kasutatud meetodid on väga tundlikud ja võimaldavad hinnata riski toksilise õitsengu tekkeks juba enne õitsengu algust.

Doktoritöö eesmärgid olid:

1. Hinnata mikrotsüstiini tootvate sinivetikate arvukuse ja mikrotsüstiini kontsentratsiooni ajalist ja ruumilist varieeruvust suures madalas eutroofses järves (**I; II**).
2. Selgitada välja peamised keskkonnategurid, mis on seotud potentsiaalselt toksiliste tsüanobakterite esinemisega madalas eutroofses järves (**I; II**).
3. Hinnata tsüanobakterite tähtsust zooplanktoni toiduallikana (**III**).

Töö tulemusena leiti järgmist:

Tsüanobakterite kooslus oli Peipsi järve osades erinev - hüpertroofse Lämmijärve ja Pihkva järve kooslus erines oluliselt Peipsi Suurjärvest. Lisaks eristus selgelt Võrtsjärve mõjutustega Emajõe sissevooluala (**I; II**). Vegetatsiooniperioodi alguses ennustab tsüanobakterite koosluse struktuuri kujunemist peamiselt nitraatide kättesaadavus ja vee temperatuur (**I; II**).

Tehti kindlaks, et Peipsi järves olid sinivetikate poolt toodetud maksamürgid - mikrotsüstiinid laialt levinud ja peamiselt tootsid neid mürke sinivetikad perekonnast *Microcystis*. Leiti, et enim mõjutab selle perekonna domineerimist fosfori kättesaadavus järves (**I; II**).

Doktoritöö tulemused näitasid, et koloonialised sinivetikad, peamiselt perekonnast *Microcystis*, moodustavad olulise osa Peipsi järves domineerivate filtreerivate vesikirbuliste toidust. Vesikirbulised, sealhulgas ka röövtoidulised vesikirbulised, võivad olla olulised sinivetikamürkide edasikandmisel toiduahela kõrgematele tasemetele (**III**). Seevastu aerjalgne *Eudiaptomus gracilis* väldib valikuliselt sinivetikaid ja eelistab toiduobjektidena neelvetikaid (**III**).

Uurimistöö tulemused annavad uudseid teadmisi potentsiaalselt toksiliste tsüanobakterite arvukuse ja tsüanotoksiinide kontsentratsiooni kohta suures ja madalas eutroofses järves. Töö on üks esimestest maailmas, mis annab ülevaate mikrotsüstiine tootvate sinivetikate tarbimisest veekogu toiduahelas ja nende olulisusest filtreerivate vesikirbuliste toiduobjektidena. Samuti annavad töö tulemused olulisi eelteadmisi

sinivetikamürkide võimaliku edasikandumise kohta mööda toiduahela lülisid kõrgematele troofilistele tasemetele.

Edasised fütoplanktoni koosluse uuringud, mis kasutavad kaasaegseid sekveneerimismeetodeid, võimaldavad analüüsida sinivetikate koosluse struktuuri veelgi detailsemalt. Analüüs võimaldab võtta arvesse ka väikesemõõdulised sinivetikad, mis traditsioonilisi seiremeetodeid kasutades hindamisest välja jäävad. Samuti võimaldavad kaasaegsed tehnoloogiad oluliselt suurendada analüüsitavate proovide hulka.

Käesolev uuring näitas, et tsüanotoksiinide tootmiseks vajalike geenide arvukuse hindamise meetod on oluliseks täienduseks praegu kasutatavatele seiremeetoditele andes ülevaate potentsiaalselt toksiliste sinivetikarakkude arvukuse kohta. Edasised toksiinigeenide ekspressiooniuuringud võimaldavad veelgi tõsta meetodi tundlikkust ja spetsiifilisust.

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I

ORIGINAL PUBLICATIONS

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Dominant cyanobacterial genera in Lake Peipsi (Estonia/Russia): effect of weather and nutrients in summer months

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Abstract. Hydrochemical and phytoplankton data from L. Peipsi (3555 km^2, mean depth 7.1 m) for July–September 1997–2011 (two lake basins) and for August 2003–2011 (three lake basins) were analysed. Our aim was to explain the impact of nutrient content and weather factors on the composition and species dominance of cyanobacteria. The share of cyanobacteria was on average 64% of the total biomass, maximum values amounted to 93% and 38 g m^{-3} in the areas of open water. Close to the lake shores these values reached 99% and 100 g m^{-3} in some cases. The most prevalent taxa affecting cyanobacterial biomass were *Gloeotrichia echinulata* in the littoral areas and *Microcystis* species in the open water. Principal component analysis placed all dominant genera (*Aphanizomenon*, *Anabaena*, *Gloeotrichia*, and *Microcystis*) separately from each other. Stepwise multiple analysis showed *G. echinulata* to be fairly independent of nutrients and related to the days with water temperature over 22 °C. The biomass of the genus *Microcystis* was found to have evidently the strongest positive connections with phosphorus and also with iron, as well as with all potentially toxic (vacuolated) forms of cyanobacteria. The other group of cyanobacteria, mainly small-celled colonial forms (*Aphanocapsa*, *Aphanothece*, *Cyanodictyon*, etc), constituted on average up to a fourth of the cyanobacterial biomass; its biomass showed a reasonably positive correlation with nitrogen and a negative correlation with water level. In summer, the succession of cyanobacterial genera in the lake started with *Anabaena*, then *Gloeotrichia* appeared (in the larger and deeper moderately eutrophic northern part of the lake), followed by *Microcystis* and *Aphanizomenon*.

Key words: cyanobacteria, succession, nutrients, water level, temperature.

INTRODUCTION

Considerable studies have been devoted to cyanobacterial blooms and to the mechanisms of their dominance in different bodies of water worldwide (Dokulil & Teubner, 2000; Salmaso, 2000; Oliver & Ganf, 2002, among others). Factors promoting blooms are well known and have been analysed thoroughly. However, according to Oliver & Ganf (2002), the distinct morphological, ecological, and physiological characteristics of individual species suggest that it is not possible to distinguish the role or principal factor promoting the dominance of particular

species. Besides the nutrient levels and characteristics commonly assessed, many other factors (e.g., mixing regime, CO_2 availability, differences in light requirements and growth rate) influence the emergence of individual species. Tan et al. (2008) connected the domination of cyanobacteria with their preferred germination conditions, and stressed that temperature, resuspension, and bioturbation are driving factors in their recruitment.

Although a trend of dominance of cyanobacteria in the case of a low N to P ratio is evident, it is not possible to establish strict threshold values for it. Havens et al. (2003) noted that the ratios of mineral forms of N to soluble reactive P (DIN : SRP) in Lake Okeechobee (USA) reveal stronger seasonal and year-to-year variability than the total N to total P (TN : TP) ratios. They found the critical ratio of TN to TP for proliferation of cyanobacteria to be 22 and suggested a DIN : SRP of 10 as favourable for the growth of N_2-fixing forms of cyanobacteria. Ni et al. (2012) hypothesized that a combination of high water temperature (>24 °C) and a high TP value (>0.06 mg L^{-1}) together with the regulation of TN : TP ratios (less than 40) enhance the growth of cyanobacteria, in particular *Microcystis*, in the eutrophic Lake Qingshan (southeastern China). According to Scheffer (1998), *Microcystis* blooms are not common in shallow well-mixed lakes (evidently, he considered very shallow *Limnothrix*-type lakes).

The majority of the lakes described in the literature are either very shallow (mean depth about 3 m), or relatively deep and stratified. According to Havens et al. (2003), cyanobacteria blooms in moderately deep, stratified eutrophic lakes are characterized by the dominating Nostocales and, in contrast, shallow lakes are dominated by the family Oscillatoriaceae (*Limnothrix*-type) owing to their ability to use low amounts of light in turbid water. These authors noted that high turbidity, high sharing stress, and low water column stability are co-occurring phenomena in shallow lakes. Our research site, Lake Peipsi, does not fit either group of lakes. Being not stratified but deeper (mean depth 8.3 m in its largest part), its bottom sediments are not so easily accessible to the action of the wind, and water turbidity is not high (mean Secchi value for the ice-free period being 1.8 m in its largest part). Thus, cyanobacteria dominance in L. Peipsi is somewhat different from that observed in deeper stratified as well as very shallow lakes.

The availability of long-term data on cyanobacterial genera/species dominance in L. Peipsi (Estonia/Russia) enabled us to study the conditions preferred by the different genera present. Formerly published data (e.g., Laugaste et al., 2001, 2008) show that the principal genera – *Gloeotrichia*, *Anabaena*, *Aphanizomenon*, and *Microcystis* – can co-exist in the lake. However, commonly one or two of these tend to dominate over the others. According to the data accumulated for the last five decades (Laugaste et al., 2001), a bloom of cyanobacteria occurred in the lake in all years studied, even in cool summers. The most conspicuous forms, visible with the naked eye, were *Gloeotrichia echinulata* (J. S. Smith) P. Richter in L. Peipsi *sensu stricto* (*s.s.*) and bundles of *Aphanizomenon flos-aquae* Ralfs in L. Pihkva (the southernmost part of L. Peipsi *sensu lato* (*s.l.*)). These two algal species dominated either alternately or in different lake parts. Patches of bloom,

consisting principally of *Anabaena* and *Microcystis* species, may occur even in the central part of L. Peipsi. Milky when decaying, these patches were particularly prominent in the southern part of L. Peipsi *s.s.*, in L. Lämmijärv, and L. Pihkva in August–September in the 1990s and 2000s. According to Tanner et al. (2005), in Lake Peipsi *s.s.*, the concentration of microcystins at a depth of 30–50 cm was 50 µg L^{-1} in the open area and up to 1074 µg L^{-1} in the nearshore area at the beginning of September 2002. Cyanobacterial blooms are more intensive in coastal areas. Thus, the biomass of *G. echinulata* on the surface can rise up to 800 g m^{-3}, as was the case in the Julys of 1965 and 1980 near the western shore and in 1997 in the northern, usually the least nutrient rich, part of the lake (Laugaste et al., 2001). *Aphanizomenon* has prevailed mainly in August–September; however, a maximum level of biomass was recorded in the late July of 1991: 250 g m^{-3} in the pelagial zone of the central part of L. Pihkva. A very intensive bloom was observed in the same location in 1972, when *Gloeotrichia* raised the values of biomass even over 100 g m^{-3} in July–August and *Aphanizomenon* did the same in September–October of that year (Laugaste et al., 2001).

In the present study we focused on the dominance of different cyanobacterial genera in summer, with special attention being paid to any possible connections with nutrients and weather conditions. We hypothesized that temperature would be one of the factors promoting recruitment of different genera; the prevalence of heterocystous forms might be linked with nitrogen limitation; domination of the genus *Microcystis* could be linked with the phosphorus content.

MATERIALS AND METHODS

Site description

Lake Peipsi (Peipus) proper or Peipsi *s.l.* is a large (area 3555 km^2) and shallow (mean depth 7.1 m), mainly unstratified lowland water body. Located on the Estonian–Russian border, L. Peipsi is the largest transboundary lake in Europe. Its volume of water is 25 km^3 at long-term mean water levels. The mean residence time of water is two years. Water level fluctuations in the lake are considerable with an average annual range of 1.15 m (Jaani et al., 2008). The catchment area of 47 800 km^2 is shared between Estonia (34%), Russia (58%), and Latvia (8%). Forest and semi-natural areas dominate in the lake drainage basin, agricultural areas cover around 14% of the basin (Jaani, 2001). The lake consists of three basins. The northern part, the largest and deepest, is L. Peipsi *s.s.*, the southernmost part is L. Pihkva (Pskov), which is connected with L. Peipsi *s.s.* by the narrow river-like L. Lämmijärv (Fig. 1). The largest inflows are the Velikaya River in Russia and the Emajõgi River in Estonia. The outflowing Narva River runs into the Gulf of Finland, part of the Baltic Sea. It should be also noted that the town of Narva draws drinking water from this river. The duration of ice cover is variable, from 2 weeks to 6 months. The water is the warmest (21–22 °C in open water on average) in July–August. The lake is well mixed by the wind and well aerated by

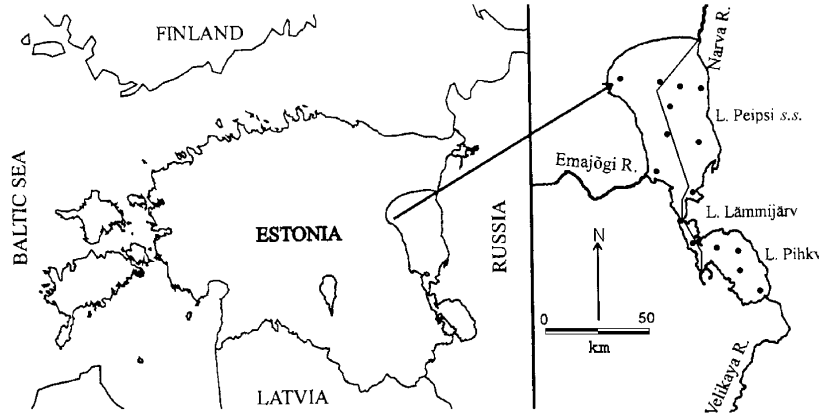

Fig. 1. Location of Lake Peipsi with sample sites. Peipsi *s.s.* – Peipsi *sensu stricto*.

waves and currents. There is no permanent stratification of temperature, oxygen content, or hydrochemical parameters in the ice-free period. Sometimes transient oxygen deficit occurs on the bottom of the lake. The water is alkaline, the mean pH in the ice-free period is 8.36 (Milius & Haldna, 2008). The main pollution source is the Velikaya River,which flows from the south into L. Pihkva (Haberman et al., 2010). The water volume of L. Pihkva is eight times less than that of L. Peipsi *s.s.* (Table 1). On the basis of the OECD (1982) classification, L. Peipsi *s.s.* is considered as an eutrophic water body, while L. Pihkva is hypertrophic at present. The increasing difference in total phosphorus (TP) concentrations between the northern and southern parts of the lake clearly shows that the input of P from the south is increasing (Kangur & Möls, 2008). A continuous and even accelerating deterioration of the quality of the lake water has occurred up to the present (Kangur & Möls, 2008).

Table 1. Some characteristics of different parts of L. Peipsi proper (Jaani, 2001). Asterisks (*) denote the geometrical mean values for August 2003–2011

Parameter	L. Peipsi *s.s.*	L. Lämmijärv	L. Pihkva
Area, km^2	2611	236	708
Mean depth, m	8.3	2.5	3.8
Maximum depth, m	12.9	15.3	5.3
Volume, km^3	21.79	0.60	2.68
Secchi depth, m*	1.6	0.8	0.6
TP, mg m^{-3}*	46	87	121
TN, mg m^{-3}*	657	1039	1136
Chl *a*, mg m^{-3}*	20.7	54.4	64.8

Sampling and processing

The material for 1997–2011 (June–September, once a month) was collected from six sampling sites only in L. Peipsi *s.s.* and L. Lämmijärv. The data for the whole lake (15 sampling sites) including L. Pihkva, located almost entirely in Russia, were available for August 2003–2011 (Fig. 1).

Well-mixed samples for phytoplankton analysis were obtained in parallel with hydrochemical data, by stirring the water in a large vessel. The water was collected at 1 m intervals through the entire water column using a two-litre Van Dorn sampler. Samples were preserved with Lugol's (acidified iodine) solution and processed applying the Utermöhl (1958) method. Hydrochemical samples (parameters in Table 2) were analysed in the Tartu Branch of the Estonian Environmental Research Centre. The data for water temperatures and water levels were obtained from the Institute of Meteorology and Hydrology at the Estonian Ministry of

Table 2. Analysed main parameters, data from July to September 1997–2011. Abbreviations: Phyto – phytoplankton; bm – biomass; Cy – cyanobacteria; Temp – temperature, everyday average for the warmest month, July, 1997–2011; COND – conductivity; DIN – sum of mineral forms of N (NO_2, NO_3, NH_4); Water level, everyday average for May–August, 1997–2011, 200 cm is equal to 30 m a.s.l.

Variable	Unit	*N*	Mean	SD	Minimum	Maximum
Phyto bm	G m^{-3}	280	12.23	8.68	1.42	47.69
Cy % in bm	%	280	60.84	18.39	5.25	97.27
Cy bm	g m^{-3}	280	7.9	7.16	0.56	38.13
Gloeotrichia bm	g m^{-3}	280	0.62	1.93	0	20.99
Anabaena bm	g m^{-3}	280	0.55	1.55	0	12.79
Aphanizomenon bm	g m^{-3}	280	0.86	1.76	0	14.12
Microcystis bm	g m^{-3}	280	2.85	5.17	0	35.47
Cy others bm	g m^{-3}	280	2.02	2.49	0	9.77
Temp	°C	434	20.4	2.1	16.3	25.9
pH		280	8.5	0.3	7.1	9.4
COND	µS cm^{-2}	280	269	28	104	394
HCO_3	meq L^{-1}	280	2.5	0.26	0.97	3.9
COD_{Cr}	mgO L^{1}	280	35.05	9.75	16	62
NH_4N	µg L^{-1}	280	34.27	31.25	5	350
DIN	µg L^{-1}	280	104	89	33	1203
PO_4P	µg L^{-1}	280	15.04	13.79	2	110
DIN : PO_4P		280	14.8	16.0	1.0	109.4
TN	µg L^{-1}	280	786	297	410	2100
TP	µg L^{-1}	280	65.8	42.2	15	360
TN : TP		280	14.45	7.07	1.94	58.67
SI	mg L^{-1}	247	1.32	1.05	0.1	6
FE	mg L^{-1}	251	0.24	0.26	0.02	2.3
CL	meq L^{-1}	280	0.18	0.03	0.04	0.28
SO_4	meq L^{-1}	280	0.26	0.06	0.06	0.37
Water level	cm	1722	209	37	108	304

Environment. The water levels for the months from May to August were analysed separately. The sum of the water temperatures taken daily for June and July, the sum of temperatures over 20 °C in these months, and the number of days with water temperature over 22 °C were also included. Besides the whole lake measurements, its individual basins were also analysed, particularly since *Gloeotrichia* and *Anabaena* preferred the northern eutrophic part, L. Peipsi *s.s.*, whereas *Aphanizomenon* and *Microcystis* favoured the southern hypertrophic part, L. Pihkva. It was found that as a result of this separation of data, the hydrochemical parameters became closer to normal distributions. The dominant four genera were analysed separately.

Log-transformed values for hydrochemical parameters and square root values for phytoplankton parameters were used for statistical analyses. To find out which chemical and weather factors had a statistically significant effect, the stepwise multiple regression model was used (significance level 0.001). For grouping cyanobacteria genera and the most effective environmental factors two first principal components of PCA were used. Spearman correlation analysis was applied to calculate the relationship between variables. Statistical analyses were run in R 2.14.0 (R Development Core Team, 2011).

RESULTS

As the results for the two southern lake basins, L. Lämmijärv and L. Pihkva, are comparable and markedly different from the northern basin, L. Peipsi *s.s.* (Table 1), hereafter they will be grouped together under the name L. Pihkva. In addition to the N_2-fixing heterocystous genera (*Gloeotrichia*, *Anabaena*, *Aphanizomenon*), a category consisting of potentially toxic cyanobacteria (all these three genera plus *Microcystis*) and another group of residual (others) small-celled and/or thin filamentous cyanobacteria (*Aphanocapsa*, *Aphanothece*, *Cyanodictyon*, *Radiocystis*, *Limnothrix*, etc.) were made. These three categories were analysed as individual groupings (Fig. 2). Some well-known potentially toxic cyanobacteria such as *Planktothrix agardhii* (Gomont) Anagnostidis & Komárek and *Woronichinia naegeliana* (Unger) Elenkin found intermittently in L. Peipsi (Laugaste et al., 1996) were not taken into account. It should be noted that the genus *Gloeotrichia* consisted of only one planktonic species in the lake (*G. echinulata*), while the genus *Aphanizomenon* was represented mainly by *A. flos-aquae* and small quantities *A. issatchenkoi* (Ussaczew) Proshk.-Lavr., *A. skujae* Komárk.-Legn. & Cronberg, and *A. gracile* Lemm. Two important species were present from the genus *Microcystis*: *M. viridis* (A. Braun) Lemm. and *M. wesenbergii* (Komárek) Komárek, while other *Microcystis* species (*M. botrys* Teiling, *M. aeruginosa* Kütz., *M. flos-aquae* (Wittr.) Kirchner etc.) occurred in smaller quantities. Approximately 10 *Anabaena* species were identified in the fresh material; however, establishing the dominant species when counting was difficult, as frequently only pieces of filaments without akinetes and heterocysts were found in the counting chamber. *Anabaena flos-aquae* G. S. West was the most common dominant in the lake

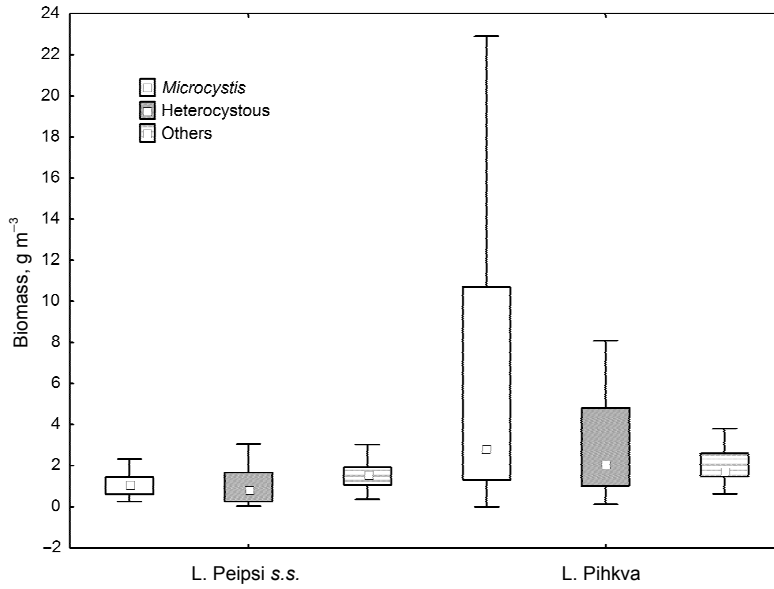

Fig. 2. Biomass of heterocystous forms, *Microcystis*, and the residual group (Others) of cyanobacteria (median, quartiles, and min–max range) in different parts of Lake Peipsi proper for August.

noted in earlier works (Laugaste et al., 2001), but in addition to this, species such as *A. circinalis* Rabenh., *A. perturbata* Hill, *A. curva* Hill, and *A. fusca* Hill have been identified more recently, and may even dominate. In some cases, *A. crassa* (Lemm.) Komark.-Legn. & Cronberg and *A. lemmermannii* P. Richter were also found to be among the dominants in the northern lake basin, L. Peipsi *s.s.*, while *A. compacta* (Nyg.) Hickel was frequently found (but was not dominant) in the southern parts. Patchiness of the distributions seems to be obvious, especially in the case of *Anabaena* and also *Gloeotrichia*, which occurred in samples only sporadically and was not found in open water at all in some years. At the same time, these cyanobacteria were abundant at some sites in the coastal region.

Principal component analysis placed all four genera (*Gloeotrichia*, *Aphanizomenon*, *Microcystis*, *Anabaena*) separately from each other (Fig. 3). Two main groupings of components explained 63% of the variance found. *Microcystis*, TP, and Fe formed a quite close group; *Aphanizomenon*, residual algae (others), and TN the other somewhat diffusive group, where *Anabaena* was linked with temperature and *Gloeotrichia* with water level and with temperatures over 22 °C. According to stepwise multiple analysis, the most distinguishable cyanobacterium in L. Peipsi, *Gloeotrichia echinulata*, is visible by the naked eye during two months each year, commonly from the end of June to mid-August, and it seems to be fairly independent of nutrients. The principal factor affecting all cyanobacterial genera taken together was temperature (33% of the variance), followed by nutrients (27%) and water

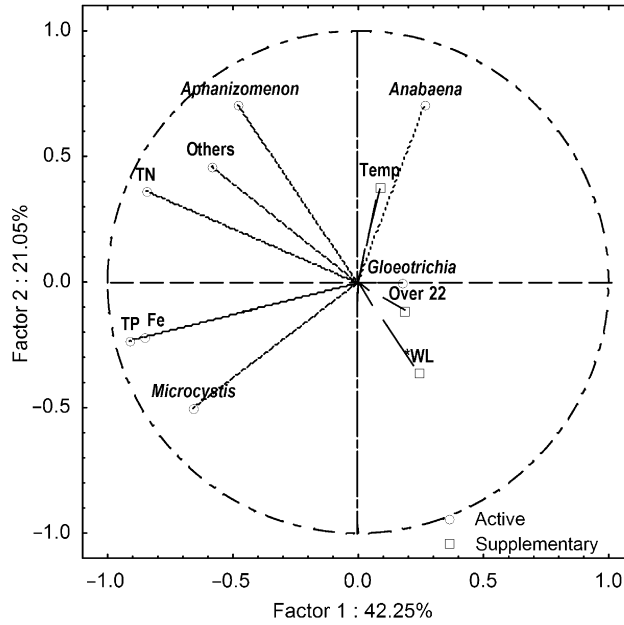

Fig. 3. Grouping of selected variables in PCA analysis. Abbreviations: TN – total nitrogen; TP – total phosphorus; WL – average water level for June, July, August; Temp – average of water temperatures in July–August; Over 22 – number of days over 22 °C.

level (15%). Factor 1 (temperature) was positively correlated with *Microcystis* ($p < 0.001$); factor 2 (nutrients) was positively correlated with *Microcystis*, *Aphanizomenon*, and the residual group ($p < 0.001$). *Anabaena* was negatively correlated with water level (factor 3, $p = 0.023$); *Aphanizomenon* was negatively correlated with temperature and water level ($p < 0.001$); *Microcystis* and the residual group were negatively correlated with water level ($p = 0.02$ and $p < 0.001$, respectively). Negative connections between *Aphanizomenon* and water temperature reflect the domination of *Aphanizomenon* at the end of summer and in autumn at lower temperatures (Fig. 4). Spearman correlation analysis displayed that most relationships concerning the heterocystous forms were influenced by *Aphanizomenon* ($r = 0.95$, $p < 0.001$), and those of all potentially toxic cyanobacteria by *Microcystis* ($r = 0.88$, $p < 0.001$). *Anabaena* had positive correlations with TN : TP ratios ($r = 0.39$, $p < 0.001$) and behaved in an opposite manner to the other bloom-forming genus *Microcystis*. At the same time, *Anabaena* and *Microcystis* were found to be present in the plankton mass together with all bloom-forming genera, and in some cases they were subdominants. Correlations between cyanobacterial genera and mineral forms of nutrients (DIN, PO_4, DIN : PO_4) were lacking, except for the correlation of *Aphanizomenon* and residual cyanobacteria with DIN. *Microcystis* and potentially toxic cyanobacteria were associated

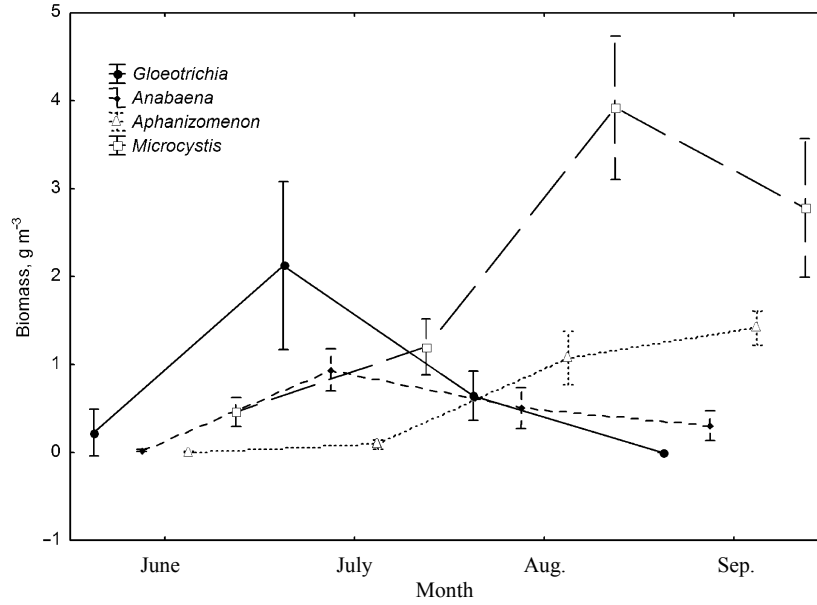

Fig. 4. Biomass of cyanobacterial genera (mean ± 0.95 conf. interval) in the whole lake in summer and early autumn.

with iron ($r = 0.67$ and 0.71, respectively, $p < 0.001$). The residual group showed a reasonably positive correlation with DIN and TN and with the ratio of mineral forms of N and P and a negative correlation with water level. This group links with other ions (Cl^-, SO_4^{2-}, K^+, Na^+, Si^{2-}; $r = -0.34$ to -0.42, $p < 0.001$). At the same time, quite remarkable negative correlations occurred in both lake parts between water level and the above-mentioned constituents ($r = -0.44$ to -0.58, $p < 0.001$), and also between the residual group and water levels ($r = -0.53$, $p < 0.001$). Evidently, any correlation of the residual cyanobacterial group with the measured constituents is mediated principally by water level. As to nutrients, TN and TP were weakly correlated with water level in all studied months ($r = -0.26$ to -0.45, p 0.02–0.006).

According to the data for 15 years, the main cyanobacterial genera appeared in the open water on the following days of the year: *Anabaena* 167th day (mid-June), *Microcystis* 176th day, *Aphanizomenon* 177th day, and *Gloeotrichia* 180th day (end of June). *Anabaena* and *Gloeotrichia* appeared earlier in the northern lake part, while *Microcystis* and *Aphanizomenon* were observed earlier in the southern part. However, *Anabaena*, *Microcystis*, and *Aphanizomenon* occurred among the dominants in both lake parts. The biomass of *Gloeotrichia* on the sampling day was positively affected most of all by water temperatures ten days prior to sampling, and the biomass of *Anabaena* and *Microcystis* was positively

affected by water temperatures 30 days prior to sampling ($p < 0.0001$). At the same time, the biomass of *Aphanizomenon* was negatively affected by water temperatures during the whole August ($p = 0.004–0.0009$) but not by those in earlier months.

DISCUSSION

According to the long-term data, the effects of water level, as well as the mechanical influence of wind and waves, are stronger in the shallower part, L. Pihkva, due to its 8-fold smaller volume than that of L. Peipsi *s.s.* (Milius et al., 2005). *Gloeotrichia echinulata* is common in well-mixed mesotrophic and eutrophic lakes found at temperate latitudes (Karlsson-Elfgren et al., 2003), and was recently found blooming in oligo- to mesotrophic lakes throughout the northeastern USA (Carey & Rengefors, 2010). It was found to inhabit mainly the moderately eutrophic northern basin of L. Peipsi, appearing into the littoral water along with *Anabaena*, however, later than other genera into the open water. This can be explained by the slower warming of the larger and deeper northern part of the lake, L. Peipsi *s.s.*, where *Gloeotrichia* dominates in midsummer. Among four studied genera it had also the strongest connections with temperature and was not related with nutrients. This may possibly be due to two reasons: firstly, the colonies were found only by chance in the open water due to their very irregular (patchy) distribution; secondly, its ability to assimilate large amounts of P from nutrient-rich sediments, above its immediate needs, and thus the colony can store P for subsequent growth. Thus, germinating colonies can sustain substantial growth even when nutrient supplies in water are low (Karlsson-Elfgren et al., 2004). Besides, the germination of this alga is light-dependent, and recruitment from shallow sediments forms the important seed for the pelagic population in Lake Erken (Karlsson-Elfgren et al., 2003). In L. Peipsi, where transparency is low (not more than 2–3 m in the spring clear-water period in the northern part), germination can take place in some shallow areas, and colonies would be carried into open water by currents. The structure of the currents is complicated, depending on the shape of the lake basin and the effects of prevalent winds (Jaani et al., 2008). Thus, the colonies can distribute in random and incalculable ways, being sometimes completely lacking in fixed monitoring sites. As a result, the observed relative importance of *G. echinulata* in the lake may be far less than the reality.

It is interesting that the genus *Anabaena*, as well as the N_2-fixing group, was found to have quite strong positive correlations with the TN : TP ratio and weak correlations with the nutrient forms. According to Reynolds (2006), for N_2 fixers nitrogen fixation response is a preferential reaction to the concentration of NH_4-N <0.5 μM N (<7 mg N m^{-3}). In August these values were on average 32 mg N m^{-3} in L. Pihkva and up to 49 mg N m^{-3} in L. Peipsi *s.s.* Reynolds stresses also that potential N_2 fixers can increase to significant levels without producing any heterocysts, which are produced abundantly by nitrogen limitation. Thus, the occurrence of significant numbers of *Anabaena* filaments without heterocysts in L. Peipsi may indicate that there was no nitrogen limitation in most cases. Nitrogen

fixation was measured in the lake in July to September 2005 and July to August 2006 (Laugaste et al., 2008). From 18 determinations, significant N_2 fixation was found in four sites in July 2005 (NH_4-N 22–87 mg m^{-3}, TN : TP 12–27; dominated by *G. echinulata*, *Anabaena lemmermannii*, and in southern parts, *Microcystis* spp.), and at one site in July 2006 (NH_4-N 33 mg m^{-3}, TN : TP 11; dominated by *Microcystis* spp. and in small quantities, *Aphanizomenon flos-aquae*). In August of 2003–2010, N_2 fixers achieved maximum biomass at a TN : TP mass ratio between 8 and 19, while *Microcystis* (not fixing N_2) occurred at a ratio below 10 in the lake. Ferber et al. (2004) stressed that *Anabaena* and *Aphanizomenon* can compensate for their N demand by vertical migrations into the hypolimnion or the sediments, thus avoiding the need to fix nitrogen (low DIN hypothesis). Thus, connections of heterocystous cyanobacteria with different forms of N in the water as well as with the TN : TP ratio are quite weak or lacking altogether. Ferber et al. (2004) suggested that the low DIN hypothesis is valid for both heterocystous and nonheterocystous vacuolated cyanobacteria. Consequently, we conclude that the domination of nitrogen-fixing cyanobacteria is far from being associated with real N fixing in the lake nor is it associated with the content of nitrogen in the water.

The response of N_2 fixers as well as *Microcystis* to the TN : TP ratio in L. Peipsi contradicted numerous data found in the literature: in our lake the biomass of heterocystous forms and their percentage in the cyanobacterial biomass increased with the TN : TP ratio, while the biomass of *Microcystis* fell (Fig. 5). Downing et al.

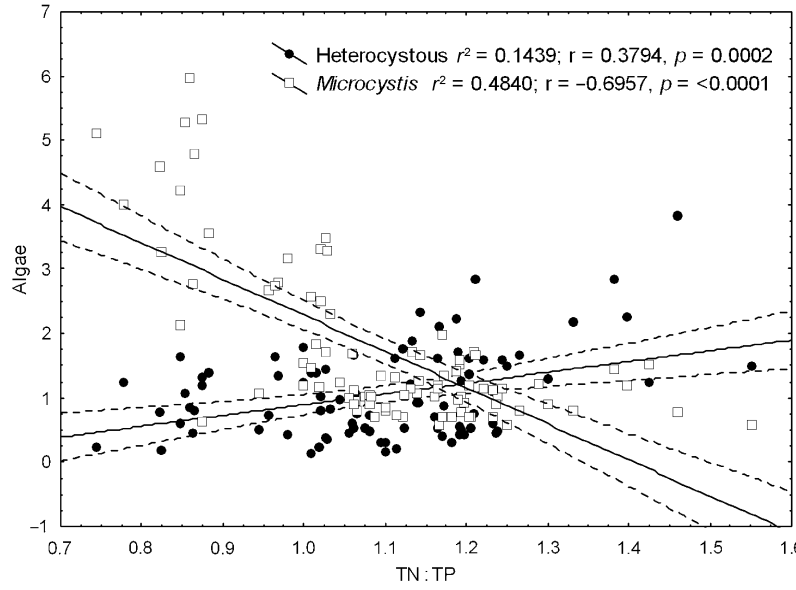

Fig. 5. Relationship between square root of heterocystous cyanobacteria and *Microcystis* biomass and the logarithmic TN : TP ratio in Lake Peipsi proper.

(2001) analysed 269 observations collected from 99 lakes around the world. These authors found the TN : TP ratio to be the poorest predictor of cyanobacterial dominance, while the probability for total P concentration to predict dominance is 30% higher. They noted that average summer P concentrations above 70 μg L^{-1} provide an 80% guarantee for cyanobacterial dominance. This threshold is appropriate for L. Pihkva but is too high for L. Peipsi *s.s.* Although such values are rare for the latter lake, cyanobacteria prevail in the summer months in all years. According to Nõges et al. (2008), the biomasses of all cyanobacteria and N_2-fixing species, as well as the proportion of cyanobacteria and N_2-fixing species found, achieved maximum values at a TN : TP mass ratio at or below 30 (for both total and mineral forms) in L. Peipsi. In our opinion, this threshold is not very strict, particularly for L. Peipsi *s.s.* The percentage of cyanophytes in the phytoplankton biomass had some correlations with nutrient concentrations in the northern part of the lake but not in the southern part L. Pihkva, evidently because that basin has permanent sufficiency of nutrients. Wang et al. (2008) could not identify a limiting nutrient with respect to TN : TP ratios in Chinese shallow lakes and fish ponds, and these authors suggested that total P is the primary factor regulating phytoplankton. Also some other authors have noted the importance of P and not the TN : TP ratio in cyanobacterial dominance (Trimbee & Prepas, 1987; Scheffer et al., 1997; Vrede et al., 2009; Arvola et al., 2011).

Reynolds (2006) pointed out that in experiments mineral N forms, particularly NH_4-N, are important for N_2 fixers and also for *Microcystis* growth. According to our data, *Anabaena*, *Microcystis*, and the residual group all had negative correlations with ammonium; N_2 fixers were positively correlated with TN and *Microcystis* with TP. Reynolds (2006) also noted that *Microcystis* is among the species that have a faster uptake capacity at a low P content, thus it has a greater affinity for P and a greater ability to compete for scarce resources. However, in L. Peipsi, long-term data indicate a phosphorus peak for August (Milius & Haldna, 2008) when *Microcystis* was seen to prevail. Marinho & Huszar (2002) noted a *M. aeruginosa* bloom in a tropical reservoir when DIN concentrations were <5 μM and TN : TP ratios below 10. In warm summers in Müggelsee in Germany, a bloom of *Aphanizomenon flos-aquae* is accompanied by the mass development of several *Microcystis* species (Teubner et al., 1999). Such a pattern of dominants is quite common also in L. Peipsi, particularly in warm weather.

The mineral iron showed moderate negative correlations with heterocystous forms and positive ones with *Microcystis* biomass in Lake Peipsi. Some authors (Ou et al., 2006; Li et al., 2009) have stressed the importance of iron for the growth of *M. aeruginosa*. Reynolds (2006) found the requirement of active N_2 fixers for iron to be relatively greater. Ou et al. (2006) found in laboratory conditions that the *Microcystis* growth kinetics with respect to changes in P and Fe reflect its competitive advantage in the natural environment. According to Tan et al. (2008), *Anabaena* and *Aphanizomenon* appear in plankton in late May–early June, *Gloeotrichia* from middle June to late July, *Microcystis* in late July, peaking in August in different lakes of middle latitudes. Unfortunately, it is

complicated to attempt to compare these data for different lakes with our study on one lake. In the shallow and medium-sized Shelburne Pond (USA), *Aphanizomenon flos-aquae* dominates in June–July, *Microcystis* species and *Planktothrix* dominate in July–August, and *Anabaena* occurs throughout the vegetation period with a maximum in autumn (Ferber et al., 2004). In Lake Balaton, *Aphanizomenon flos-aquae* appears and becomes dominant in the period characterized by low temperatures and high light intensities (late spring and early summer), and is followed by other heterocystous species such as *Anabaena* spp. (Kovács et al., 2012). In two Japanese lakes, the growth of *Aphanizomenon flos-aquae*, which usually blooms in summer but can also tolerate low temperatures in winter, is promoted at high temperatures (Yamamoto, 2009). The author also noted that nutrient concentrations and pH has no evident impact on *A. flos-aquae*, and further expansion of this cyanobacterium will be determined by the eutrophication process and by the absence of other cyanoprocaryotes. Such a pattern could be applied to L. Peipsi as well.

In summer months, the maximum biomass values of genera in L. Peipsi occurred in the following order: *Anabaena* + *Gloeotrichia*, *Microcystis*, *Aphanizomenon*. According to Laugaste et al. (2008), the dominance of the last genus continues up to November in some warm autumns. Evidently, unlike *Anabaena* filaments, the bundles of *Aphanizomenon* are rather resistant to waves. Jensen et al. (1994) found that in shallow lakes, *Anabaena* and *Aphanizomenon* prevail in early summer along with abundant N content, while *Microcystis* dominates at the end of summer. According to literature data, the period of the domination of *Aphanizomenon* appears to be extremely variable in different lakes.

It would be quite confusing to consider all these statements in the literature when analysing the succession of different algal genera in L. Peipsi. Jensen et al. (1994) stressed that in reality, the growth of organisms requires a complex of conditions, while we like simple explanations. As Oliver & Ganf (2002) noted, the occurrence and abundance of various types of gas-vacuolated cyanobacteria is not reliant on any particular environmental stimulus, but depends on a complex interplay of factors.

In conclusion, the succession of cyanobacterial genera in L. Peipsi started with *Anabaena*, then *Gloeotrichia* appeared (in the larger and deeper eutrophic northern lake part), followed by *Microcystis* and *Aphanizomenon*. The domination by *G. echinulata* was most affected by high water temperatures. Frequent occurrences of *Anabaena* species without heterocysts indicates the abundance of nitrogen in the lake. It seems also that *Anabaena* filaments are most susceptible to wave action. The connections of most heterocystous forms with nitrogen were weak. The biomass of the genus *Microcystis* was found to have evidently the strongest positive connections with phosphorus and also with iron, as well as with all potentially toxic (vacuolated) forms of cyanobacteria. *Aphanizomenon flos-aquae* was most frequent at the end of summer and in early autumn, while any connections between its presence and nutrients remained unclear. Further research is needed to establish whether altered P loading has brought about any change in the biomass or dominant species of cyanobacteria over a longer time period.

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toxins

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Article

Using Microcystin Gene Copies to Determine Potentially-Toxic Blooms, Example from a Shallow Eutrophic Lake Peipsi

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Abstract: Global warming, paired with eutrophication processes, is shifting phytoplankton communities towards the dominance of bloom-forming and potentially toxic cyanobacteria. The ecosystems of shallow lakes are especially vulnerable to these changes. Traditional monitoring via microscopy is not able to quantify the dynamics of toxin-producing cyanobacteria on a proper spatio-temporal scale. Molecular tools are highly sensitive and can be useful as an early warning tool for lake managers. We quantified the potential microcystin (MC) producers in Lake Peipsi using microscopy and quantitative polymerase chain reaction (qPCR) and analysed the relationship between the abundance of the *mcyE* genes, MC concentration, MC variants and toxin quota per *mcyE* gene. We also linked environmental factors to the cyanobacteria community composition. In Lake Peipsi, we found rather moderate MC concentrations, but microcystins and microcystin-producing cyanobacteria were widespread across the lake. Nitrate (NO_3^-) was a main driver behind the cyanobacterial community at the beginning of the growing season, while in late summer it was primarily associated with the soluble reactive phosphorus (SRP) concentration. A positive relationship was found between the MC quota per *mcyE* gene and water temperature. The most abundant variant—MC-RR—was associated with MC quota per *mcyE* gene, while other MC variants did not show any significant impact.

Keywords: cyanobacteria; qPCR; *mcyE*; microcystins; MC quota; Lake Peipsi

Key Contribution: This manuscript describes the relationship between cyanobacterial community composition, the abundance of the *mcyE* genes, MC concentrations, MC quota per *mcyE* gene and environmental variables in large and shallow north temperate lakes.

1. Introduction

Eutrophication of aquatic systems caused by anthropogenic nutrient enrichment is a critical environmental problem of the 21st century [1,2]. Extensive research has shown that increased nutrient-loading shifts phytoplankton communities towards the dominance of bloom-forming and potentially toxic cyanobacteria [3–5]. However, many symptoms of eutrophication are affected

and escalated by global warming, it was predicted that the extent and frequency of the harmful cyanobacterial blooms will increase in a warmer climate [6–10]. Anthropogenic pressures render shallow lake ecosystems especially vulnerable to environmental change and the subsequent boosting of cyanobacterial occurrence [11,12].

Cyanobacterial blooms pose a substantial health risk to humans and animal species due to the cyanotoxins they produce. These secondary metabolites are mainly stored inside the cyanobacterial cell and are released into the water during cell lysis, potentially leading to high toxin concentrations [13]. Aquatic life such as fish, crustaceans, mussels, and molluscs may also become toxic as cyanotoxins accumulate, moving up the food web [13–16]. Consequently, the dominance of cyanobacteria might cause considerable economic loss to fisheries and water supply companies, and decrease the recreational value of the water body and the market value of lakefront estates [10,17,18]. Rapidly changing environmental conditions may favor cyanobacterial growth over other species, with subsequent consequences such as habitat loss (e.g., hypoxic zones), disruption in energy flow along the food web, and decrease in biodiversity and ecosystem services [19–21].

Generally, cyanobacterial occurrence and dominance are significantly associated with three environmental variables and their interactions, nutrients, light and temperature, as reviewed in [22]. A high temperature stimulates cyanobacterial growth, with many species reaching their maximum growth rate above 25 °C [11,23]. As the nutrient dynamics in lakes is strongly affected by the external loading of phosphorus (P) and nitrogen (N) [24], the abundance and community composition of cyanobacteria are mainly linked to these two environmental factors [25]. Traditionally, high P concentration is considered as the main risk factor for cyanobacterial blooms [26]. In shallow lakes, the internal load is also considered as an important source of dissolved P that becomes directly available for phytoplankton growth [27,28]. Several studies have also demonstrated the significance of N on promoting cyanobacterial blooms. While there has been a debate about whether P or N control algal growth, the importance of the dual control of nutrients is widely recognized in water quality management [29–31]. Because the geographical distribution and both the magnitude and frequency of cyanobacterial blooms is increasing [32], it is urgent to accurately monitor potentially toxic cyanobacteria and improve our understanding of the environmental factors that promote bloom formation.

Conventional monitoring methods rely on inverted microscopy [33] and spectrophotometric measurements of chlorophyll-a (chl-a) to quantify phytoplankton, including cyanobacteria. Both of these methods are insufficient to detect toxin-producing cyanobacteria [34] because toxic and non-toxic strains within the same species are often morphologically identical and might co-exist within the same sample [13]. Traditionally, cyanobacterial biomass has been used as an indicator of potential toxin presence, disregarding the fact that toxin concentrations can rapidly increase even in a lower cyanobacterial biomass [34]. Measurements of chl-a and some other pigments provide a rapid estimation of total algal biomass (particularly, combined with remote sensing) [35], but, even if more group-specific photosynthetic pigments, such as zeaxanthin or phycocyanin, are used as a proxy for cyanobacterial biomass, the toxicity potential remains unknown.

To evaluate the risks to public health, data on potentially toxic cyanobacteria and cyanotoxins need to be provided as early as possible [11]. DNA based methods (e.g., polymerase chain reaction—PCR and quantitative PCR) comprise a valuable toolbox for both the detection and quantification of potentially toxic cyanobacteria [25,36–38]. However, the relationship between the cellular microcystin (MC) concentration and the copy number of *mcy* genes or MC producer's biomass is not straightforward. The amount of microcystin per unit of biomass (toxin quota per biomass) depends on various factors such as genotype, nutrient availability and temperature. The general understanding about the amount of toxin per cell under different environmental conditions is still poorly understood due to the availability of only a limited number of in situ studies. As the toxin quota per cell directly reflects the safety of the waterbody, it is important to clarify how the copy number of *mcy* genes, MC concentration and environmental factors are related to this specific parameter. Quantitative PCR (qPCR) is a time- and cost-effective tool to evaluate the proportion of potential toxin-producing genotypes over

the cyanobacterial population, as well as to investigate how environmental parameters determine toxicity potential at the spatio-temporal scale [25,34]. Consequently, qPCR can help us understand the mechanisms that trigger toxic blooms and can be used as an early warning tool for lake managers.

Lake Peipsi is the largest transboundary lake in Europe, located on the border of Estonia and Russia (Figure 1). This large (area 3555 km^2), shallow (mean depth 7.1 m) and unstratified lowland water body consists of three basins (Figure 1, Table A1). The northernmost Lake Peipsi *sensu stricto* (*s.s.*) with a very simple shoreline is the deepest part of the lake. The southernmost basin is Lake Pihkva and these two lakes are connected by riverlike Lake Lämmijärv [39]. These three basins are all different in trophic state, hydrology and morphometry [40]. The bottom topography of Lake Lämmijärv is remarkably different to the other basins and also the water temperature in spring and winter tends to be warmer than in Peipsi *s.s.* and Lake Pihkva [39]. According to the OECD (1982) classification, Lake Peipsi *s.s.* is considered as eutrophic and Lämmijärv and Pihkva basins are considered as hypertrophic parts of the lake (Table 1, Figure 1). Rivers Velikaya and Emajõgi, the main inflows, carry the majority (>80%) of nutrients into the lake [41,42]. The outflow from the lake, River Narva, discharges into the Gulf of Finland. Lake Peipsi has been strongly influenced by eutrophication and natural fluctuations in water level and temperature [40]. Due to these processes, massive cyanobacterial blooms have been common for several decades [43–45].

In this study, samples from Lake Peipsi and its basins (Figure 1; Table A1) were analysed for the presence and abundance of potentially toxic *Microcystis*, *Dolichospermum* and *Planktothrix* using qPCR. The concentration and variants of microcystins were analysed using liquid chromatography-mass spectrometry (LC-MS/MS). Microscopic analysis of the samples was conducted to analyse the cyanobacterial community composition. Although several studies on the occurrence of cyanobacteria in Lake Peipsi have been published [41,44,46–49], only one of these addresses the issue of toxicity and toxin concentrations [49]. In general, there are significantly less data and fewer studies on cyanotoxins from Eastern Europe, with Estonia being presented with only one publication [50]. The current study is the first to combine molecular tools with traditional methods to determine the potential for cyanobacterial toxicity in this large and shallow north temperate lake.

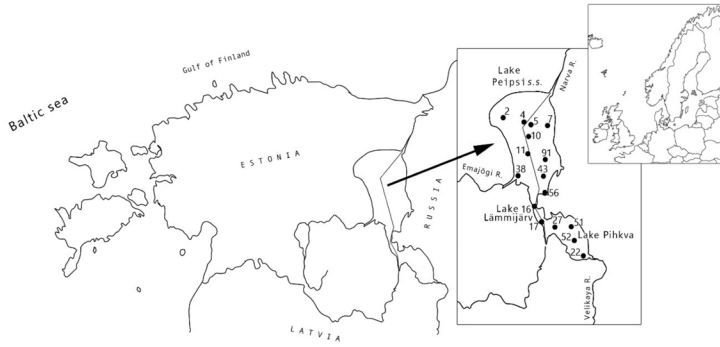

Figure 1. Location of Lake Peipsi (Estonia/Russia) and the sampling stations under study. Samples from Lake Pihkva (Russia) are collected in August only.

Here, we aim to (1) analyse cyanobacterial community composition in ecologically contrasting basins of Lake Peipsi and use molecular markers to identify and quantify the potential microcystin producers in the lake, (2) determine the relationship between toxin quota, the abundance of *mcyE* genes and MC concentrations, and (3) elucidate the environmental factors that promote toxic cyanobacterial blooms. Our first hypothesis is that the number of *mcyE* copies will follow an increase in toxin concentration. In our second hypothesis, we assume that in Lake Peipsi, *mcyE* gene copy number can

be potentially used as a predictor of MC concentration. Additionally, we hypothesize that specific toxin variants are directly related to certain cyanobacterial genera.

2. Results

2.1. Environmental Variables

During the study period, the basins of Lake Peipsi were characterized by different water quality parameters and general characteristics (Table 1). Across the lake basins, spatial gradients occurred in the trophic state. Total phosphorus (TP), soluble reactive phosphorus (SRP), total nitrogen (TN) and chl-a values increased from the northern basin towards the southern basins.

Table 1. Water quality characteristics for three basins of Lake Peipsi (Lake Peipsi *sensu stricto*, Lake Lämmijärv, Lake Pihkva.

Characteristic	Peipsi *s.s.* *		Lämmijärv *		Pihkva **	
	Mean	Range	Mean	Range	Mean	Range
Number of Samples	91		38		12	
Area, km^2	2611		236		708	
Mean depth, m	8.3		2.5		3.8	
Max depth, m	12.9		15.3		5.3	
Volume, km^3	21.79		0.6		2.68	
TP, mg/m^3	41	15–70	75	36–110	116	88–170
SRP, mg/m^3	12	2–49	13	3–25	28	13–79
TN, mg/m^3	701	460–1500	1001	410–1500	1147	950–1400
NO_3^-, mg/m^3	91	15–930	115	30–820	91	30–220
NO_2^-, mg/m^3	2	2–9	3	2–15	3	2–5
NH_4^+, mg/m^3	28	10–162	25	10–120	24	10–58
chl-a, mg/m^3	23.3	6.9–52.4	49.1	20.5–79	61.2	41.4–78.3
pH	8.5	8–8.9	8.6	8.3–9	8.9	8.4–9.2
Water temp, °C	18.2	5–23.9	17.9	10.3–24.7	22	19.7–25.6
Secchi depth, m	1.64	0.9–3.5	0.87	0.6–1.3	0.67	0.4–0.9
OECD classification	Eutrophic		Eutrophic/hypertrophic		Hypertrophic	

* mean values for growing season (2011–2012); ** mean values for August (2010–2012).

2.2. The Composition of the Cyanobacterial Community Based on Microscopy

During the growing season (May–October) across the basins of Lake Peipsi, cyanobacteria and diatoms prevailed in the phytoplankton biomass, while chlorophytes and cryptomonads prevailed in abundance. During the period of 2010–2012, cyanobacterial biomass varied from 0–16.03 mg wet weight/L (mgWW/L) and dominated in the summer months (July–August) or early autumn (September). The main potentially toxic cyanobacteria in the lake were from N_2-fixing heterocystous genera *Gloeotrichia*, *Dolichospermum*, *Aphanizomenon*, and non-heterocystous *Microcystis* and *Planktothrix*. The biomass of the genus *Microcystis* exceeded other genera manifolds (median 1.7 mgWW/L, maximum 14.5 mgWW/L in August), and was dominant among potentially toxic algae. *Planktothrix* attained a peak in September (maximum 2.2 mgWW/L) and *Dolichospermum* at the beginning of July, with maximum biomass of 2.4 mgWW/L (Figure 2). *Gloeotrichia* occurred sporadically in some regions of Peipsi *sensu stricto* (*s.s.*) and only a few times in Lämmijärv. *Aphanizomenon* was omnipresent in the lake, being more numerous in Peipsi *s.s.* Other potentially toxic genera appeared all over the lake with larger biomasses in southern basins, and lakes Lämmijärv and Pihkva [43]. Multivariate comparison between-groups principal component analysis (bgPCA) revealed a clear spatial distribution of cyanobacterial community composition in different basins of Lake Peipsi (permutation test, $p < 0.01$) (Figure 3). The cyanobacterial community composition in Peipsi *s.s.* varied considerably (permutation test, $p < 0.01$) from the communities in Lämmijärv and Pihkva. Additionally, the effect of the inflow from Emajõgi River was evident in station 38, which was significantly different (permutation test, $p < 0.01$) from the other areas (Figure 3).

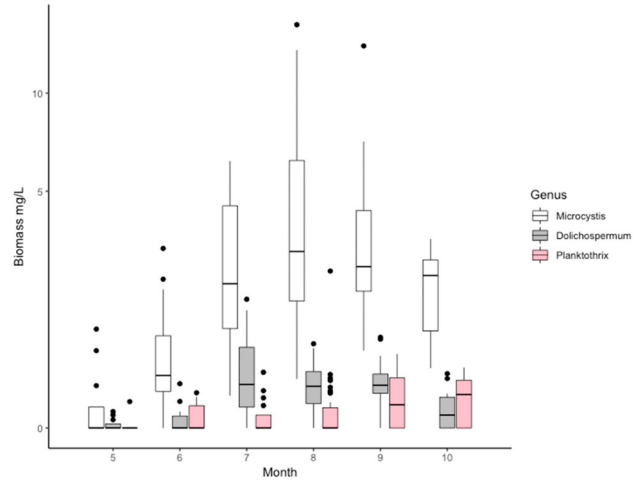

Figure 2. Temporal variation in *Microcystis*, *Dolichospermum* and *Planktothrix* biomass (mgWW/L). Boxplots denote median biomass values across the basins of Lake Peipsi and error bars represent spatial variation across the sampling stations. The y-axis is plotted as square root scale, values of biomass remain as original.

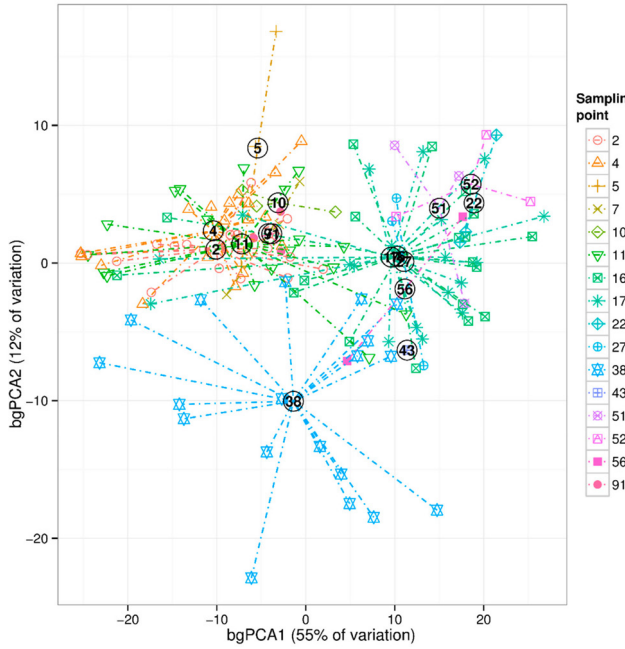

Figure 3. Cyanobacterial community composition in different basins (sampling stations) of Lake Peipsi. Sampling points 2, 4, 5, 7, 10, 11, 38, 43, 56 and 91 are located in Lake Peipsi *s.s.*; sampling points 16 and 17 in Lake Lämmijärv, and 22, 27, 51, 52 in Lake Pihkva.

2.3. Abundance of mcyE Genes

A simultaneous occurrence of the main MC producers was observed in all regions of the lake. In 80% of the samples, all three genera appeared concurrently. *Microcystis mcyE* genes were found in all of the samples ($N = 141$), *Dolichospermum mcyE* and *Planktothrix mcyE* were found in 95% and 83% of the samples, respectively. Compared to the other genera, *Microcystis mcyE* copy numbers were most abundant (Wilcoxon pairwise test, $p < 0.01$) over the entire growing season (median 1.89×10^5 gene copy/mL, minimum 0 and maximum 2.6×10^7 gene copy/mL, Figure 4). *Dolichospermum mcyE* and *Planktothrix mcyE* copy numbers were more comparable (median values 4.6×10^1 and 2.3×10^2 gene copy/mL; minimum 0 and 0, maximum values 4.05×10^4 and 7.46×10^4 gene copy/mL, respectively). Positive correlations were found between the biomass and the toxin-producing gene abundances for *Microcystis* ($r = 0.6$; $p < 0.01$), *Dolichospermum* ($r = 0.31$; $p < 0.01$) and *Planktothrix* ($r = 0.62$; $p < 0.01$).

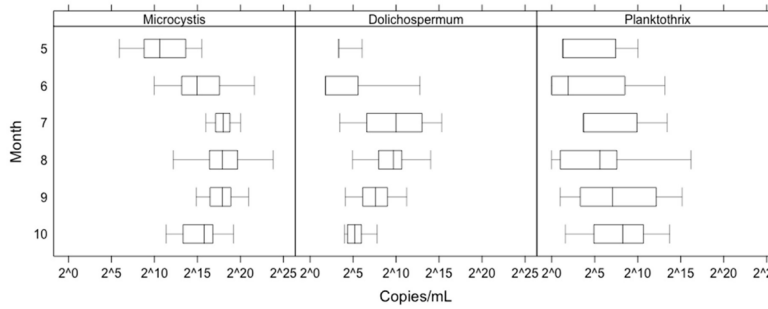

Figure 4. Temporal variation in *Microcystis*, *Dolichospermum* and *Planktothrix mcyE* gene copy number (*mcyE* gene/mL). Boxplots denote median *mcyE* copy numbers across the basins of Lake Peipsi. Error bars represent spatial variation across the sampling stations. On all three panels, the y-axis is plotted $\log_2$ scale and the values of *McyE* copies remain original.

The seasonal pattern of the values of toxic genotypes was consistent with the dynamics of the total MC concentrations (Figure 5a,b). The total microcystin concentration had a statistically significant positive correlation ($r_p = 0.67$; $p < 0.01$; $n = 69$) with the sum of *Microcystis*, *Dolichospermum* and *Planktothrix mcyE* gene copy numbers (Figure S1). The seasonal dynamics of the microcystin quota (particulate microcystin per unit of *mcyE* gene) was different from the seasonal dynamics of the MC concentration: a lower microcystin quota per *mcyE* gene occurred together with higher MC concentrations (Figure 5c) and vice versa. There was a strong negative correlation between MC quota per *mcyE* gene and *mcyE* gene copy number ($r = -0.75$; $p < 0.01$; $n = 69$). Logistic regression analysis demonstrated a correlation between MC-RR presence/absence and microcystin quota per *mcyE* gene ($z = 2.36$; $p < 0.05$); however, there was no statistically significant correlation between other MC variants and MC quota per *mcyE* gene. MC quota per *mcyE* gene was positively related to water temperature and pH ($r_s = 0.46$ and 0.31 respectively, $p < 0.05$; $n = 69$) negatively related to nitrate concentration ($r_s = -0.30$), yet no significant correlation was found between MC quota per *mcyE* gene and both TP and TN. The correlation with the toxin quota calculated per unit of chlorophyll-a resulted in similar relationships to these environmental parameters ($r_s = 0.47$ and 0.50, respectively, for temperature and pH, and $r_s = -0.38$ for nitrate; $p < 0.05$; $n = 69$).

According to the literature data [25,32], 36 species of cyanobacteria in Lake Peipsi are potentially toxic. A Mantel permutation test showed a significant relationship between the biomass of potentially toxic species and *mcyE* gene abundances ($r = 0.43$, $p < 0.01$, 999 permutations, $n = 141$). At the same time, there was no statistically significant correlation between the biomass of other non-toxin producing cyanobacteria and *mcyE* abundance ($r < 0.01$, $p = 0.53$, permutations $n = 999$, $n = 141$).

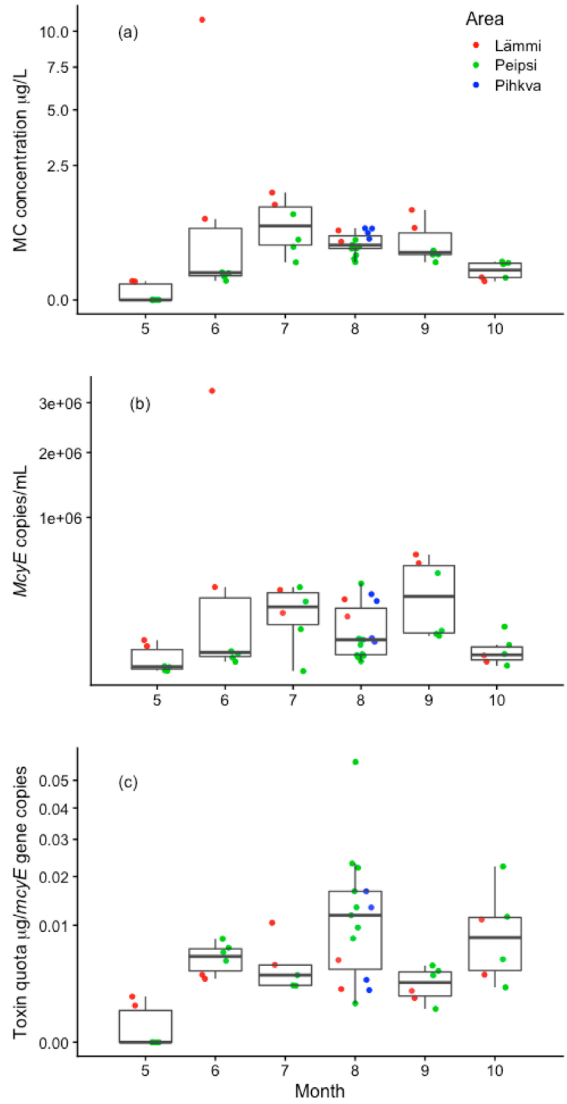

Figure 5. Temporal variation in total cell-bound microcystin (MC) concentration (**a**), the abundance of total *mcyE* genes (**b**) and toxin quota per *mcyE* gene—cell-bound MC per unit of *mcyE* gene (**c**) in the year 2012. Boxplots denote the median values of all basins and error bars represent spatial variation across all sampling stations. Points represent measurements in a specific lake basin. The y-axis is plotted as square root scale (MC concentration, *McyE* copies and Toxin quota per *mcyE* gene values remain original).

2.4. Microcystin- Concentrations and Variants

In this study, MCs were found in all samples analysed (n = 69). With a few exceptions, the microcystin concentration in the samples was relatively low, ranging from 0.001–10.9 µg/L, median 0.4 µg/L. The maximum concentration was measured in mid-July 2012 in Lämmijärv, when *Microcystis wesenbergii* (Komárek) Komárek ex Komárek dominated. Based on LC-MS/MS, a total of 14 MC variants were found and eight of them were identified (MC-RR; [D-Asp3]MC-RR; [Dha7]MC-RR; MC-LR; [D-Asp3]MC-LR; [Dha7]MC-LR; MC-YR; [Dha7]MC-YR). Microcystin-RR was the most abundant MC variant, found in 93% of samples, followed by MC-LR and its methylated variants in 92% of the samples. In 60% of the samples, all identified MCs co-existed. Both PCA analysis and linear fitting of environmental variables (Figure 6) demonstrate a clear pattern of the distribution of MC variants, gene copy numbers, and the biomass of potentially MC-producing species. During the entire growing season, all variants of MC were detected in samples from Lämmijärv, while all MC variants were detected in samples from Peipsi *s.s.* only during the late growing season. [D-Asp3]MC-RR was correlated with *Planktothrix agardhii* Gomont, Anagnostidis and Komarek in the southern basins (Lämmijärv and Pihkva) from July until the end of the sampling period. Other MC variants formed a close group with *M. wesenbergii*, *Microcystis aeruginosa* (Kützing) Kützing and *Dolichospermum flos-aquae* (Brébisson ex Bornet and Flahault) P. Wacklin et al. Concurrently, *Dolichospermum circinale* (Rabenh.) Wacklin *et al.*, and *M. wesenbergii* were also highly related to *mcyE* gene copy numbers.

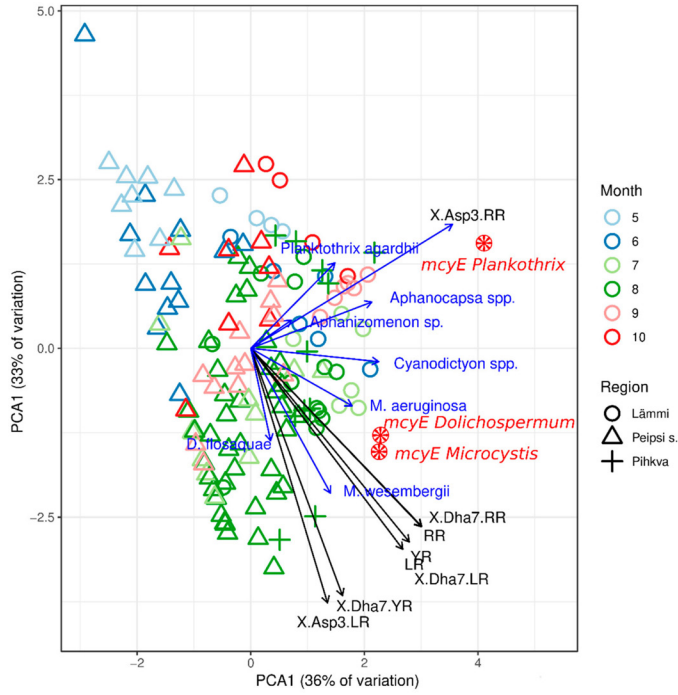

Figure 6. Multivariate comparisons of various *mcyE* gene abundances, cyanobacterial community and the presence/absence of MC variants. Significance ($p < 0.05$) of these linear fittings was obtained by a permutation test (1000 replicates). The length and direction of vectors indicate the strength and direction of the relationship.

2.5. Environmental Variables that Favour Potentially Toxic Cyanobacteria Genotypes

A principal component analysis was performed using the cyanobacterial community composition as variables and linearly fitting the environmental variables, such as basic nutrients (TP, SRP, TN, NO_3-, NO_2-, NH_4+) and temperature with a PCA ordination space. The analyses revealed a significant but mostly weak association with the distributions of environmental variables that favor cyanobacteria in Lämmijärv and Peipsi *s.s.* (Figure 7). In the early growing season, water temperature ($r^2 = 0.14$; $p < 0.05$) and nitrate ($r^2 = 0.19$; $p < 0.01$) were the main factors associated with cyanobacterial abundance in both lake basins. From August to October, in Peipsi *s.s.*, soluble reactive phosphorus (SRP) ($r^2 = 0.14$; $p < 0.05$), and in Lämmijärv total nitrogen ($r^2 = 0.28$; $p < 0.001$) and total phosphorus ($r^2 = 0.69$; $p < 0.01$) were the most important environmental descriptors related to the cyanobacterial community composition. Other environmental parameters (e.g., NH_4^+, NO_2^- etc.) were analysed as well, but no significant correlations were found with the cyanobacterial community.

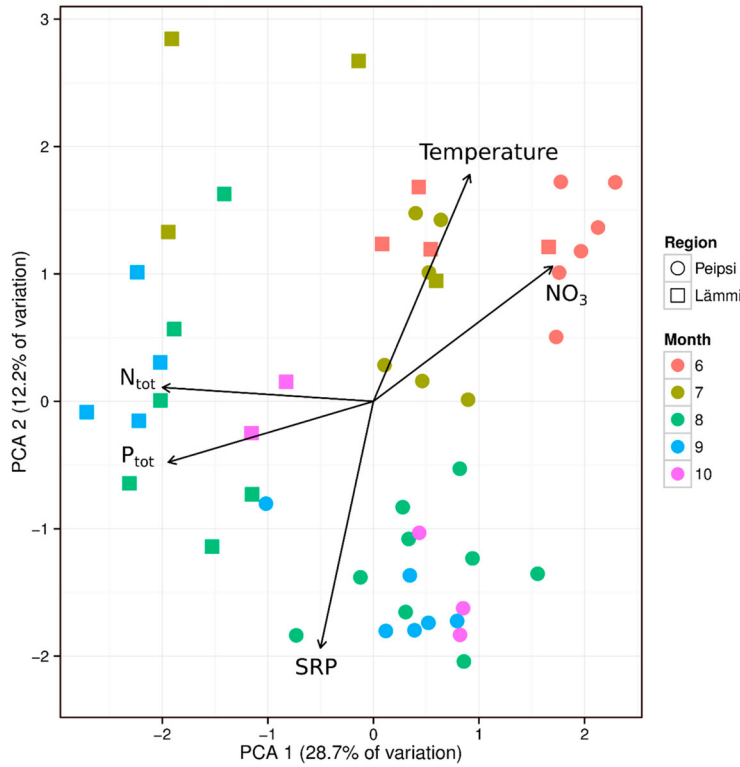

Figure 7. Multivariate comparisons of the abundance of cyanobacteria and environmental physico-chemical variables. Significance ($p < 0.05$) of these linear fittings was obtained by permutation test (1000 replicates). The length and direction of vectors indicate the strength and direction of the relationship.

3. Discussion

Cyanobacterial occurrence and dominance are mainly associated with eutrophication processes [4]. One of the initial aims of this study was to identify differences in the cyanobacterial community composition along a trophic gradient and to determine which cyanobacteria genus is the main potential

microcystin producer in the shallow eutrophic–hypertrophic Lake Peipsi. The current study revealed a clear spatial distribution of cyanobacterial community composition in ecologically contrasting basins of this large and shallow lake. The cyanobacterial community composition in the eutrophic part of the lake varied considerably from the communities in hypertrophic basins (Figure 3, Table 1, Table A1). This observation is in accordance with a previous study that demonstrated remarkable differences between Peipsi *s.s.*, Lämmijärv and Pihkva [44]. The most abundant species were *Microcystis viridis* (A. Braun) Lemm., *M. wesenbergii*, *Dolichospermum. circinale*, *D. crassum* (Lemm.) Wacklin *et al*., *D. lemmermannii* (Richter) Wacklin *et al*., *Planktothrix agardhii*, *Aphanizomenon flos-aquae* Ralfs and *Gloeotrichia echinulata* (J.S. Smith, P. Richter). A more detailed overview of species composition is provided in Table S1. Those findings are in agreement with data from systematic monitoring of Lake Peipsi, which has been carried out for six decades. According to the existing data [43], cyanobacterial blooms in the northern part of Peipsi *s.s.* are mainly attributed to *G. echinulata*. At the same time, several potential microcystin producers from genus *Microcystis*, *Dolichospermum* and *Planktothrix* occur in the southern part of Peipsi *s.s.* and Lämmijärv. *A. flos-aquae* has occurred in the lake in all years studied until the late autumn [43–46].

Another aim of this study was to use molecular markers to identify and quantify the potential microcystin producers in the basins of Lake Peipsi. For that purpose, genus-specific qPCR was used. Because microcystins are considered a major threat to human and animal health worldwide [51], we primarily focused on the genetic markers of the three most common microcystin-producing genera in Lake Peipsi (*Microcystis, Dolichospermum* and *Planktothrix*). Molecular analysis of potential MC producers revealed a simultaneous occurrence in all regions of the lake and 80% of the samples, with all three genera appearing concurrently (Figure 4). During the productive season (May to Oct), *Microcystis mcyE* copy numbers were the most abundant, while the abundance of *Dolichospermum mcyE* and *Planktothrix mcyE* copies were considerably lower. This result is not surprising because species from *Microcystis* are highly adaptive for various environments [52], and thus are the most commonly found microcystin producers in all eutrophic waters worldwide [13,51]. Although it is clear that the genus *Microcystis* was the dominant potential MC producer in Lake Peipsi, other genera also had their maximum peak periods (Figure 4). The ecological preferences of the different genera might provide an explanation. *Dolichospermum* had its peak in July, when the average water temperature was higher (mean 21.2 °C) and the water column more prone for stratification. Stratification periods give a competitive advantage for cyanobacterial species with gas-vacuoles (e.g., *Dolichospermum and Aphanizomenon*), as, due to their ability to regulate their position in the water column, they can optimise their use of nutrients and light [7]. However, it should be noted that with the given data it is difficult to assess the relative importance of direct temperature effects compared to the indirect effects or general climatic differences between seasons. In August, *Microcystis* dominated, and in September *Planktothrix* reached their peak. While water temperature in August was comparable with the temperature in July (mean 20.6 °C), in September the average temperature was only 14.4 °C. Compared to other genera, *Planktothrix* thrives in cooler water temperatures and is well adapted to lower temperatures [53]. This can explain its biomass peak in Lake Peipsi during the autumn months. Due to its low-light tolerance [54], *P. agardhii* can proliferate throughout a well-mixed shallow water column in the southern basins of the lake (Table A1), where the Secchi depth is significantly lower.

In late summer, the abundance of cyanobacteria was primarily associated with the concentration of SRP (Figure 7) in Lake Peipsi *s.s.*, suggesting that the dominance of cyanobacteria and biomass of the major microcystin producer is mainly controlled by P dynamics. Similarly, the recent studies in Lake Peipsi and other shallow eutrophic lakes of the north temperate region have shown that internal P loading provides considerable amounts of bioavailable P to the water column, which contributes to the growth of cyanobacteria in summer [48,55–59]. Under turbid conditions and warm water temperatures, cyanobacteria gain an advantage over eukaryotic phytoplankton groups, as they can control their buoyancy to maximise the light use, maintain growth rates in warmer temperatures, fix atmospheric nitrogen, and therefore take advantage of the use that is provided by internal loading during N-limited

periods [28,60]. Furthermore, several species of cyanobacteria are able to uptake and store bioavailable phosphorus, and thus the populations can sustain themselves on internal P storage [52]. Changes in the factors that regulate cyanobacteria abundance between early and late times of the growing season reported in the current study are most likely related to the changes in the relative importance of the sources of nutrient supply, as they are closely coupled to the seasonality of nutrient dynamics in Lake Peipsi [61,62]. Moreover, different cyanobacteria species may assimilate nutrients at different rates. This is supported by another of our findings: the clear spatial distribution of cyanobacterial community composition (Figure 3) in the basins of Lake Peipsi that we studied are characterized by the different trophy. The finding that NO_3^- (Figure 7) was the main driver shaping the cyanobacterial community composition at the beginning of the growing season in all basins of Lake Peipsi, and TN, together with TP, are influential factors during the late growing season in Lake Lämmijärv, may imply that N also has to be considered in lake water quality management aimed at reducing cyanobacteria.

3.1. MC Concentration Versus MC Variants

During the study period, MC concentrations measured from lake water samples were in a range (median 0.4 μg/L) comparable to other various large lakes such as Taihu [63,64], Chaohu [65], Green Bay of Lake Michigan [66] and Erie [67,68]. In a study where 143 lakes in New Zealand were investigated, the authors also reported rather low MC concentrations (<1 μg/L) in the majority of lakes [69]. Comparable MC concentrations were also found in another large and shallow Estonian lake, Võrtsjärv [70]. Our toxin concentrations, measured in Lake Peipsi, were comparable with MC concentrations from the year 2003, reported in a study by Tanner et al. [49]. Tanner and others [49] demonstrated that even when the MC concentration in the open water column is relatively low, the MC concentration was extremely high in the inshore areas where biomass may accumulate and most human and animal activity occurs (33 to 54 times higher compared to the open water). Inshore samples were not analysed during the current study because MC concentrations were not measured, however, *mcyE* copy numbers in inshore waters were extremely high, reaching 57 million gene copies per mL [70]. Species from *Microcystis* and *Dolichospermum* are able to form surface scums and, under favourable environmental conditions (e.g., abundant sunlight, warm temperature and still water-column), the density of potentially toxic cells can rapidly increase within a few hours [1]. If the wind sweeps these scums to the shore, it can present a very high risk for the people using the waterbody recreationally. In 2002, the MC concentration measured in the shoreline scum of Lake Peipsi was 2183 μg/L, even when the MC concentration in the open water was rather low [49]. Therefore, we can conclude that even moderate concentrations of microcystins in the open water area of the lake can pose a high risk for bathers if, under the right conditions, surface scums form and concentrate in shoreline areas.

In Lake Peipsi, a total of 14 MC variants were found and eight of them were identified. The most abundant MC variants were MC-RR, found in 93% of the samples, and MC-LR with its variants, found in 92% of the samples. MC-RR, together with MC-LR and MC-YR and their variants are the most commonly reported microcystins [51,71] and MC-LR is quite often mentioned as the most frequent MC.

More variants and a higher concentration of microcystins are often found in more eutrophic waters [66]. This is in accordance with our findings, showing that all analysed variants of MCs were detected in more eutrophic Lake Lämmijärv during the entire growing season, while in Lake Peipsi *s.s.* they were detected only during the late growing season. In the hypertrophic part of Lake Peipsi, *P. agardhii* was only significantly related with [D-Asp3]MC-RR (Figure 6) and the abundance of *Planktothrix mcyE* genes showed a significant correlation to this MC variant. This finding is in accordance with the study [72], where Sivonen and others found that *Planktothrix* isolates from Finnish lakes were able to produce only one of two types of microcystins ([D-Asp3]MC-RR or [Dha7]MC-RR), and not other MC variants. In Lake Peipsi, other MC variants formed a group with *M. wesenbergii*, *M. aeruginosa* in the southern parts of the lake and *D. flos-aquae* in Peipsi *s.s.* Thus, our third hypothesis that specific toxin variants are directly related to certain cyanobacterial genera was mainly supported. In Lake Peipsi, the presence of MC-RR was associated with MC quota per *mcyE* gene, while other

MC variants did not show any significant impact. One possible explanation for this might be that microcystin-RR was also the most abundant MC variant found in the samples.

The microcystin concentration displayed a statistically significant positive correlation with the sum of *Microcystis*, *Dolichospermum* and *Planktothrix mcyE* gene copy numbers (Figure S1). These results are in accordance with our first hypothesis. This demonstrates that *mcyE* gene abundance could be used to estimate toxin production. A review from Pacheco and others [34] regarding the use of qPCR to assess the toxicity of cyanobacterial blooms showed that 22 studies out of 33 (years 2003–2015) reported a persistent positive correlation between *mcy* gene copies and MC concentrations. In 80% of the studies that adopted *mcyE* gene detection, positive correlations were found [34]. Additionally, under the framework of the European Multi Lake Survey (EMLS) [73,74], where lakes across Europe were sampled once in a snapshot approach in 2015, a strong significant correlation between the abundance of *mcyE* gene and MC concentrations was found in 200 lakes [75]. In the current study, the advantage of *mcyE* gene abundance for the prediction of MC production was also confirmed by the analysis of the relationship between microscopy counts of cyanobacterial species that are known to produce toxins, MC concentrations and *mcyE* gene abundance. Still, it should be considered that, even though the correlation of the MC concentration and *mcyE* gene abundance is very strong, the number of gene copies merely reveals the potential to produce the toxin, and does not indicate if the genes of interest are actively expressed and toxins are produced [63,76]. Despite the positive detection of potentially toxic genotypes of cyanobacteria, mutations can inactivate the genes involved in the biosynthesis of toxins, and thus hinder toxin production [77,78]. Therefore, to confirm the presence and estimate the concentration of cyanotoxins in the water, chemical analytical methods, such as LC-MS/MS or HPLC, are still required [79]. In order to elucidate the processes underlying toxin dynamics in more detail in this freshwater system, further exploration focusing on measuring the expression of toxin genes along with toxin concentration and other lake parameters would be necessary.

3.2. Toxin Quota per McyE Gene

The concentration of toxins in the water is related to the abundance of toxin-producing species and the amount of toxin per cell. [80]. Generally, the toxin quota is described as the amount per unit of either biomass or chl-a [73,80]. In the current study, the microcystin quota was calculated as the microcystin concentration per unit of *mycE* gene, and we used this to elucidate the direct relationship between the abundance of toxin genes and MC concentration. Even though a significant positive connection between MC concentration and *mcyE* genes was found in Lake Peipsi, this study revealed that the dynamics of the MC quota per *mcyE* gene and MC concentration in the water was not concurrent (Figure 5). A lower microcystin quota per *mcyE* gene occurred together with higher MC concentrations and vice versa. A significant negative correlation was also found between MC quota per *mcyE* gene and the abundance of *mcyE* genes. Therefore, we found that our results only partly supported our second hypothesis, which states that *mcyE* copy number could be used as a direct predictor of MC concentration in the lake. Although there appears to be a correlation between *mcyE* gene numbers and microcystins, the variation in the cellular quota of microcystins may lead to under- or overestimation of the risk when merely based on *mcyE* gene numbers. This means that, in the situation where low numbers of *mcyE* genes correspond to a higher toxin quota per *mcyE* gene, the public health concern is higher, as it is subjected to a higher toxicity potential. If a low amount of toxic cells in the water can produce very high toxin concentrations in the water after cell lysis, then larger gene copies could rapidly reach extreme MC concentrations. This is in accordance with earlier observations where the MC quota is calculated with biomass or chl-a [73,80]. To estimate the risk for the water consumers, it is important to understand the reason behind the higher toxin quota when the biomass and *mcyE* gene copy numbers are rather low [80]. In response to environmental conditions, the toxin quota per cell in toxin-producing species can vary largely [13]. In our study, the MC quota per *mcyE* gene was related positively to water temperature and pH, and negatively to the concentration of nitrate, but no significant correlation was found with total nitrogen or total phosphorus. This finding broadly supports the work

of other studies in this area that link the dynamics of toxin quota per biomass with water temperature. A similar conclusion was also reported by Wood and others [81] in a shallow eutrophic lake in New Zealand, where MC quotas per biomass responded positively to surface water temperature. In another large-scale study, where 137 European lakes were analysed, the authors also report no direct impact of TP and TN on the toxin quota per chl-a, but found that water temperature is an important control factor [73]. Several other studies have shown the importance of water temperature as the regulatory factor of cyanobacterial biomass [23,31,82,83] even on the global scale [84]. While nutrients play an important role through supporting the cyanobacterial community and biomass, the temperature seems to be to discriminative when other conditions are rather equal. These results demonstrate that, as global temperatures are expected to increase [85], in addition to an increase in the distribution, intensity, and duration of cyanobacterial blooms [23], the toxin concentration per cell will get higher. However, further in situ research is required to refine our understanding of the complex interaction between toxins, toxin quota per toxin gene and nutrients, as the findings about the effect of NO_3^- on the toxin quota in general still seem contradictory. Our study suggests a negative correlation between nitrates and toxin quota per *mcyE* gene; one explanation for this inverse relationship can be the uptake of NO_3^- by toxin-producing cyanobacteria. The study by Horst and others [80] demonstrates a positive relationship between these variables and [81] did not detect any significant relationships between toxin quota and environmental parameters (including nitrates) other than the water temperature. However, as the role of nutrients is more complex, the absence of the significant relationship with nutrient-related parameters does not mean that the distribution and concentration of toxins are not influenced by nutrients. In addition, the mentioned studies used toxin quota calculated per biomass of cyanobacteria. We assume that, in general, the biomass of cyanobacteria or the concentration of chl-a are less reliable predictors of toxin concentration and the predictive power can be increased by measuring the absolute abundance of toxin production genes. Expanding this knowledge in further in situ studies would substantially contribute to appropriate lake management and risk assessment of the toxic blooms.

To conclude, we demonstrated that, even though the number of *mcyE* gene copies increased together with toxin concentration, the variation in the cellular quota of microcystins may lead to under- or overestimation of the risk when merely based on *mcyE* gene copy numbers, and therefore *mcyE* copy number should not be used as a single measure to predict MC concentration in Lake Peipsi. Additionally, we showed that specific toxin variants were directly related to certain cyanobacterial genera. *P. agardhii* was significantly related with only [D-Asp3]MC-RR and other MC variants formed a close group with *M. wesenbergii*, *M. aeruginosa* in *D. flos-aquae*. Further, nitrate was the only nutrient-related variable connected to MC quota per *mcyE* gene. A strong positive correlation between water temperature and MC quota per *mcyE* gene suggests that the warming trends might lead to more harmful cyanobacterial blooms in temperate shallow lakes.

4. Materials and Methods

4.1. Study Site and Field Surveys

During the growing season (May–Oct), water samples from the Estonian part of Lake Peipsi *s.s.* and Lämmijärv were collected biweekly in 2011 and monthly in 2012. In addition, samples from the whole lake were collected from 15 sampling stations in August during the Estonian–Russian joint sampling campaigns from the period 2010–2012. The coordinates of the studied sampling points are shown in Table S2. One hundred and forty-one depth-integrated water samples (depth range: surface to 0.5 m to the sediment) from 6–15 locations (Figure 1) were analysed.

Depth-integrated water was collected using a two-liter Van Dorn sampler at one-meter intervals and mixed in the collection bucket on the board of the research vessel. Integrated samples throughout the whole water column were collected due to the presence of buoyant cyanobacterial species. Subsamples for phytoplankton community composition analysis, molecular analyses of *mcyE* gene abundance, and toxin analysis were collected onboard. Subsamples were stored in an onboard refrigerator and

transported to the laboratory in the coolers for further processing. For DNA extraction, 100–2000 mL (depending on sampling point and time) of the depth-integrated water was filtered at a low vacuum (max. 0.2 bar) through 5 µm pore size Whatman Cyclopore Polycarbonate filters. For toxin analyses, 150–1200 mL of the water was filtered through Binder-Free Glass Microfiber Grade GF/C (pore size 1.2 µm, GE Healthcare, UK) filter. Until further analysis, filters were stored at −80 °C.

Water chemistry analyses were performed as a part of the state monitoring programme by Estonian Environmental Research Centre following international and Estonian quality standards (ISO and EVS-EN ISO).

4.2. Microscopic Analysis

Samples for phytoplankton community analyses (n = 141) were preserved with Lugol's (acidified iodine) solution and processed using the Utermöhl [33] method. Phytoplankton biomass was calculated from counts of cells using a Nikon Eclipse Ti-S inverted microscope at ×200 and ×400 magnification. Species were identified using classifications described in [86–88]. An aliquot of 3 mL was settled overnight. Biovolume of algal cells, colonies and/or filaments were calculated using assigned geometric shapes dimensions and converted to biomass assuming the specific density of 1 g/cm^3 in accordance with [89].

4.3. Detection of Microcystins (MCs)

MCs were identified from 69 environmental samples by LC-MS according to their microcystin characteristic protonated molecular ions $[M\text{-}H]^-$. The extracts were analysed (injection volume 5 µL) with an Agilent 1100 Series LC/MSD Trap System high-performance liquid chromatography (Agilent Technologies, PaloAlto, CA, USA.), which has an XCT Plus model ion trap as a mass detector. The ionization method used was electrospray ionization (ESI) in both positive and negative mode. The column used was Phenomenex Luna C8 (2) (150 by 2.0 mm, 5 µm) (Phenomenex, Torrance, CA, USA). Gradient was done with 0.1% formic acid in water (A) and 0.1% formic acid in 2-propanol (B) The gradient timetable was 20% B to 70% B over 37 min, after which the washing of the column was performed for in 100% B for 10 min and equilibrated in initial conditions for 12 min. The flow rate was 0.15 mL/min and the column temperature 40 °C. In ion source nebulizer gas (N2), pressure was 35 psi, desolvation gas flow rate 8 litres/min, and the desolvation temperature was 350 °C. The capillary voltage was set to 5000 V, the capillary exit offset was 300 V, the skimmer potential was 66 V, and the trap drive value was 73. Spectra were recorded 700–1500 m/z. The total microcystin concentration of the strain was approximated with a microcystin-LR standard (a gift from Z. Grzonka, Faculty of Chemistry, University of Gdansk, Poland) and microcystin-RR (Alexis, Farmingdale, NY, USA.) To avoid the underestimation of smaller concentration in environmental samples, standard curves were constructed with only the six most diluted standards. The identification of MCs was based on fragmentation patterns of the ions in MS2, ion masses and their retention times.

4.4. Cultivation of the Strains Used as External Standards

Microcystin-producing strains *Microcystis* sp. 205, *Dolichospermum* sp. 315 and *Planktothrix* sp. 49 were grown in HAMBI/UHCC Culture Collection, University of Helsinki in a Z8 medium under the continuous light at 20 ± 2. These strains were used in the preparation of standard curves for qPCR.

4.5. DNA Extraction

For DNA extraction, 40 mL of standard cultures were concentrated by centrifugation (5 min at 7000× g, at 4 °C) and DNA extracted immediately after that using E.Z.N.A.™ SP Plant DNA Kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's instructions. In addition to the protocol, the mechanical disruption of the cells with acid-washed glass beads (710–1180 µm; Sigma-Aldrich Co, St. Louis, MO, USA) and FastPrep®FP 120 bead-beater (MP Biomedicals, LLC, Irvine, CA, USA) was used. DNA from environmental samples was extracted using the DNeasy PowerWater Kit (Qiagen

Inc., Germantown, MD, USA) according to the manufacturer's instructions. The quality and quantity of extracted DNA were controlled with NanoDrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

4.6. Detection and Quantification of mcyE Genes in Environmental Samples

To exclude false-negative outcome caused by the possible presence of PCR inhibitors in environmental samples, cyanobacterium-specific 16S rRNA PCR [90] was performed. PCR conditions are shown in Table A2. In order to identify the dominant potential microcystin-producing genera in environmental samples, genus-specific qPCRs were carried out. In order to detect *Planktothrix mcyE* genes in water samples, a new *Planktothrix*-specific primer pair and hydrolysis probe was designed (Table S3). PCR conditions were optimized, and specificity (Table S4) and sensitivity (Figure S2) experiments were performed as described before in [76].

The external standards of MC-producing strains were used to quantify *mcyE* gene copy numbers. Standard dilutions of genomic DNA of the standards were prepared as described before [91]. To quantify *Microsystis mcyE* gene copies from lake samples, the standard dilution contained 10^6, 10^5, 10^4, 10^3, 10^2, 10^1 copies of *mcyE* genes. To determine *mcyE* gene copies from *Dolichospermum* and *Planktothrix*, standard dilutions contained 10^5, 2×10^4, 4×10^3, 8×10^2, 1.6×10^2, 3.2×10^1 and 10^1 copies of *mcyE* genes.

The reaction mixture in total volume of 20 µL included 5 µL of standard or environmental DNA, 1 × HOT FIREPol®Probe qPCR Mix Plus, with ROX (Solis BioDyne, Tartu, Estonia), 300 nM of both primers (Metabion international AG, Planegg, Germany) (Table A2) and 200 nM of TaqMan®probe (with an exception for *Planktothrix*, where 300 nM of TaqMan®probe was used). Environmental DNA samples were diluted 1:50 (to detect *Microcystis*) or 1:10 (to detect *Dolichospermum* and *Planktothrix*), MQ water was used as a negative control, and all the reactions were performed in three replicates. Amplifications were performed on an ABI 7500 Fast Real-Time PCR system (Thermo Fisher Scientific Inc, Waltham, MA, USA) using the following protocol: 95 °C for 12 min for initial denaturation, 40 cycles of 95 °C for 15 s and 62 °C (*Microcystis mcyE* and *Dolichospermum mcyE*) or 60 °C (*Planktothrix mcyE*) for 1 min. Results were analysed using 7500 Software version 2.0.5.

The microcystin toxin quota was calculated by dividing particulate microcystin concentration (µg/mL) by *mcyE* gene copies/mL.

4.7. Statistical Analyses

All statistical analyses were performed with the R package and its extensions [92] and STATISTICA 13 (TIBCO Software Inc., PaloAlto, CA, USA). To analyse cyanobacterial community composition and to compare the communities from different lake basins (sampling stations), we used a between-groups principal component analysis (bgPCA, R ade4) on the abundance data. The statistical significance ($p < 0.05$) of bgPCA was tested by a Monte Carlo permutation test (1000 replicates; [93].

Wilcoxon pairwise test was used to analyse the differences between gene abundance of *Microcystis mcyE*, *Dolichospermum mcyE* and *Planktothrix mcyE*. Pearson correlation (r_p) analysis on log-transformed data was used to test associations between *Microcystis*, *Dolichospermum* and *Planktothrix* specific *mcyE* gene abundance and total microcystin concentrations in the samples. In case the data lacked normal distribution even after logarithmic transformation, the Spearman rank-order correlation (r_s) coefficient was used.

Mantel test was performed to describe the relationship between matrices of microscopy counting data including cyanobacterial species potentially able to produce toxins and MC concentration. For this, the species list of cyanobacteria was compiled according to the historical records (provided by R. Laugaste) of phytoplankton species in Lake Peipsi. The potential ability to produce toxins was added from the list of toxin-producing cyanobacteria published by [1,25,32].

Multivariate comparisons of various *mcyE* gene abundances and presence/absence of MC variants were analysed using the subset of existing observations ($n = 69$) in PCA and linear fitting of independent

variables (envfit function in R vegan). A similar analysis was used to determine the relationship between the abundance of cyanobacteria and environmental physico-chemical variables. The significance ($p < 0.05$) of these linear fittings was obtained by permutation test (1000 replicates).

The microcystin quota was calculated by dividing MC concentration by *mycE* gene variants abundance (copy number/mL). Thereafter, the association between microcystin quota per *mcyE* gene and MC variant presence/absence was analysed by logistic linear modelling (glm function with binomial family in R base).

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6651/12/4/211/s1, Table S1: Species composition of cyanobacteria in Lake Peipsi, Table S2: Coordinates of the sampling points, Table S3: Sequences of the primers and hydrolysis probe designed to detect and quantify *Planktothrix mcyE* genes in environmental samples by qPCR assay, Table S4: Average threshold-cycle values (SD) of three replicate reactions obtained with cyanobacterial strains used to test the specificity of the genus-specific *Planktothrix mcyE* gene qPCR assay,. Figure S1: correlation between MC concentration and the sum of *Microcystis*, *Dolichospermum* and *Planktothrix mcyE* gene copy numbers. Figure S2: Average threshold cycles and standard deviations of triplicate *Planktothrix mcyE* targeted by qPCR assays. Assays were performed either with 10^4 or 10^2 copies of target *mcyE* gene and 0, 10^3, 10^4, 10^5, or 10^6 copies of competing *mcyE* genes of two other microcystin-producing strains. Strain abbreviations: P126/8 - *Planktothrix agardhii* 126/8; A90 - *Dolichospermum* sp. 90 and M7806 - *Microcystis aeruginosa*. PCC 7806.

Author Contributions: Conceptualization of this study was made by K.P., A.R.-Y. and K.S.; sampling design by K.P. and R.L.; data collection was performed by K.P. and O.T.; data analyses were performed by K.P.; M.T. and V.K.; K.P. prepared the original draft, supervised by V.K.; review and editing was performed by K.P., M.T., E.M., A.R.-Y., R.L., O.T., K.S. and V.K. All authors have read and agreed to the published version of the manuscript.

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

Appendix A

Table A1. Long term key characteristics of different basins of Lake Peipsi.

Characteristic	Peipsi *s.s.* *	Lämmijärv *	Pihkva **
Secchi depth, m	1.8 (1;2.8)	1 (0.6;1.8)	0.61 (0.4;0.8)
TP, mg/m^3	41 (21;80)	69 (40;130)	133 (57;201)
SRP, mg/m^3	8.6 (2;25)	11.8 (4;30)	25.6 (30;110)
TN, mg/m^3	672 (430;1500)	868 (600;1400)	1190 (948;1525)
NO_3-, mg/m^3	46 (10;240)	42 (10;300)	58 (30;110)
NO_2-, mg/m^3	1.7 (1;6)	1.8 (1;4)	2.4 (1.9;4.4)
NH_4+, mg/m^3	1.7 (1;6)	28 (10;99)	25 (15;33)
Chl a, mg/m^3	15.8 (5.6;40.5)	30 (9.6;70.5)	68 (33.7;123.5)
OECD classification	Eutrophic	Eutrophic/hypertrophic	Hypertrophic

* the geometrical mean values for growing season (1992–2012). ** the geometrical mean values for August (2003–2012) 95% quantiles in brackets.

Table A2. Primers and probes used in the study.

Target Gene	Primer	Primer Reference	PCR Program
Cyanobacterial 16S rRNA	CYA359F CYA781R	[90]	95 °C 5 min; 35 cycles: 95 °C 60s; 60 °C 60 s; 72 °C 60 s and 72 °C 10 min
Microcystis mcyE	127F 247R 186P	[76]	95 °C 15 min; 40 cycles: 95 °C 15 s; 62 °C 60 s;
Dolichospermum mcyE	611F 737R 672P	[76]	95 °C 15 min; 40 cycles: 95 °C 15 s; 62 °C 60 s;
Planktothrix mcyE	664F 744R 670P	Current study, Table S3	95 °C 15 min; 40 cycles: 95 °C 15 s; 60 °C 60 s;

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Role of potentially toxic cyanobacteria in crustacean zooplankton diet in a eutrophic lake

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ABSTRACT

The coexistence of potentially toxic bloom-forming cyanobacteria (CY) and generally smaller-sized grazer communities has raised the question of zooplankton (ZP) ability to control harmful cyanobacterial blooms and highlighted the need for species-specific research on ZP-CY trophic interactions in naturally occurring communities. A combination of HPLC, molecular and stable isotope analyses was used to assess *in situ* the importance of CY as a food source for dominant crustacean ZP species and to quantify the grazing on potentially toxic strains of *Microcystis* during bloom formation in large eutrophic Lake Peipsi (Estonia). *Aphanizomenon*, *Dolichospermum*, *Gloeotrichia* and *Microcystis* dominated bloom-forming CY, while *Microcystis* was the major genus producing cyanotoxins all over the lake. Grazing studies showed that CY, and especially colonial CY, formed a significant, and also preferred component of algae ingested by the cladocerans *Bosmina* spp. and *Daphnia* spp. while this was not the case for the more selective calanoid copepod *Eudiaptomus gracilis*. Molecular analyses confirmed the presence of CY, including *Microcystis*, in ZP guts. Further analyses using qPCR targeting cyanobacterial genus-specific *mcyE* synthase genes indicated that potentially toxic strains of *Microcystis* can be ingested directly or indirectly by all the dominant crustacean grazers. However, stable isotope analyses indicated that little, if any, assimilation from ingested bloom-forming CY occurred. The study suggests that CY, and particularly *Microcystis* with both potentially toxic and non-toxic strains, can be widely ingested by cladoceran grazers during a bloom event with implications for control of CY abundance and for transfer of CY toxins through the food web.

1. Introduction

Cyanobacteria (CY) blooms are a serious nuisance in marine and freshwater systems worldwide (Havens and Paerl, 2015) threatening aquatic ecosystems and challenging water resource management (Schindler, 2006). In freshwater systems *Microcystis*, *Dolichospermum*, *Planktothrix* and *Aphanizomenon* are the most common bloom-forming genera of CY which are also able to produce several cyanotoxins including hepatotoxic microcystin and its congeners (Sivonen and Jones, 1999; Vaitomaa et al., 2003). Zooplankton (ZP) is the primary link in top-town control of phytoplankton (PP) biomass in most freshwater food webs, and research into the role of ZP in grazing on CY and suppressing harmful algal blooms is gaining importance (Ger et al., 2016). Knowledge of trophic relationships between freshwater ZP and toxic CY is largely based on studies with *Daphnia* as a grazer model (e.g., Lampert, 1987; DeMott et al., 1991). Recent studies have shown that locally adapted clones of *Daphnia* can tolerate toxic CY in their diet (Sarnelle and Wilson, 2005; Wojtal-Frankiewicz et al., 2013) and are able to suppress CY biomass via grazing and thereby reduce the concentration of microcystins (Chislock et al., 2013). However, examples of efficient bloom control by *Daphnia* in nature are rare (Ger et al., 2014). In nature, multiple confounding factors influence the feeding interactions between ZP and bloom-forming CY, including variable morphological and biochemical properties of CY (e.g., DeMott and Moxter, 1991), co-occurring grazer composition and grazer behaviour (e.g., Urrutia-Cordero et al., 2015), ZP different capability for selectivity (e.g., Ger et al., 2011). Increasing problems with toxin-producing cyanobacterial blooms clearly highlight the importance of research in the field.

Analysis of a large data set has indicated that with increasing

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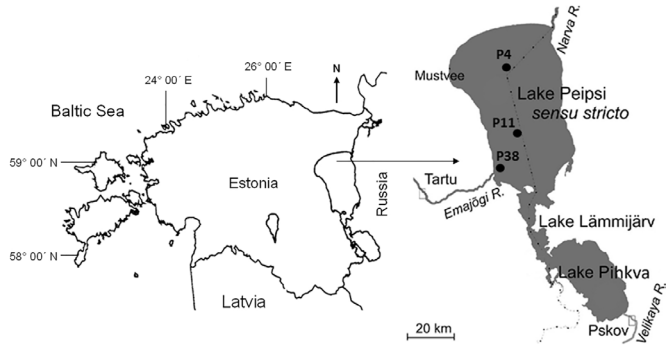

Fig. 1. Map of Lake Peipsi. Black dots mark the sampling stations (P4, P11, P38).

eutrophication of lakes, higher cyanobacterial biomass and level of fish predation, a shift toward smaller crustaceans occurs, especially among cladocerans (Zhang et al., 2013). Recent studies using natural ZP communities have shown that both micro- and mesozooplankton are capable of grazing with similar rates on toxic and non-toxic *Microcystis* strains (Davis and Gobler, 2011), and that a ZP community dominated by cyclopoid copepods and small cladocerans can suppress the blooms of potentially toxic *Dolichospermum, Microcystis* and *Planktothrix* species (Urrutia-Cordero et al., 2015). In contrast to the large generalist feeder *Daphnia* (Jürgens, 1994), smaller cladocerans and copepods are capable of more selective feeding based on size, type, taste and toxicity of food objects (Kerfoot and Kirk, 1991; Kâ et al., 2012). Smaller cladocerans, such as *Bosmina* can even develop a stronger tolerance for cyanobacterial toxins than that of *Daphnia* (Jiang et al., 2013a, b). For selective grazers like copepods, adaptive feeding strategies have been described allowing discrimination against microcystin producing strains of CY (Ger et al., 2011). Thus, the ingestion and control of toxic CY in natural environments can be significantly affected by the capacity of ZP taxa co-existing with a bloom for selective grazing or adaption to tolerate cyanotoxins (Ger et al., 2016). However, there is yet very limited information on the taxon-specific response of grazers to potentially toxic CY co-occurring in natural systems (but see Ger et al., 2018b).

During the past 15 years, several molecular methods have been developed to target genes that are involved in cyanotoxin synthesis (Humbert and Törökné, 2017). Advances in molecular techniques have facilitated the differentiation and quantification of potentially toxic strains of *Microcystis* by the presence or absence of the microcystin synthetase genes (Hisbergues et al., 2003; Rantala et al., 2006; Baxa et al., 2010; Humbert et al., 2010; Salmaso et al., 2017). Microcystin synthetase genes can also be detected from the guts of potential consumers (Davis and Gobler, 2011) thereby enabling direct estimates of the grazing rates and tracing the transfer of toxic CY in the food web. However, to the authors knowledge, only a few studies have used molecular quantification for assessing ZP grazing on CY and there is limited information about potentially toxic CY in ZP diet in natural systems (Motwani and Gorokhova, 2013; Ger et al., 2018b).

Crustacean ZP feeding and selection for PP taxa was studied in Lake Peipsi, Estonia, during the period of cyanobacterial bloom formation, focusing on crustacean consumption of potentially toxic *Microcystis*. The feeding was measured for the dominant taxa of Lake Peipsi – the cladocerans *Daphnia* spp. and *Bosmina* spp. and the calanoid copepod *Eudiaptomus gracilis* (Blank et al., 2017). These taxa could have a major effect on the grazing food chain in Peipsi. Additionally, the large predatory cladoceran *Bythotrephes longimanus*, a preferred food for fish in Lake Peipsi (Ginter et al., 2018), was assessed as a potential trophic link for transferring CY toxins from prey organisms to higher trophic levels.

It was hypothesised that CY, both non-toxic and potentially toxic, constitute a diet source for the dominant cladoceran grazers, such as *Daphnia* spp. and *Bosmina* spp., while this resource is less utilized by the more selectively feeding calanoid copepod *E. gracilis*. To assess the algal diet composition of crustaceans, high-performance liquid chromatography (HPLC) was used to determine algal marker carotenoid composition in the grazers' guts. A shortcoming of the HPLC method is its low taxonomic resolution, providing information only at class level (Roy et al., 2011), so DNA-based diet analysis was used to determine the dietary composition of CY more precisely. Highly sensitive quantitative polymerase chain reaction (qPCR) enabled identification and quantification of the food source of interest, including potentially toxic strains of CY, in ZP gut content. Stable isotope analyses of dominant ZP and PP taxa were performed to explore the trophic structure and relationships between the crustacean ZP and CY in the food web of Lake Peipsi.

2. Material and methods

2.1. Study site

Lake Peipsi *s.l.* (*sensu lato*) (57°51′-59°01′N, 26°57′-28°10′E, 30 m a.s.l.), consisting of three basins from north to south (Peipsi, Lämmijärv and Pihkva), is a large non-stratified eutrophic lake between Estonia and Russia with a surface area of 3555 km^2 and with a mean and maximum depth of 7.1 m and 15.3 m, respectively. The lake is typically ice-covered from December to April and its water level is not regulated (Kangur and Möls, 2008). The present study is based on data collected from the largest basin, Peipsi proper (Fig. 1), hereafter referred to as Peipsi. While the lake as a whole is eutrophic, its major basin is considered moderately eutrophic with total N and P concentrations of 694 mg N m^{-3} and 46 mg P m^{-3}, respectively. The mean chlorophyll *a* content in Peipsi is about 24.0 mg m^{-3}, Secchi depth is 1.5 m and mean pH is 8.4 (Blank et al., 2017). The lake is characterized by annually recurring cyanobacterial blooms, dominated by potentially toxic genera of *Microcystis*, *Dolichospermum* (formerly *Anabaena*), *Aphanizomenon* and *Gloeotrichia*. Species of *Microcystis* are the major potential toxin-producing CY in Lake Peipsi (Laugaste et al., 2013; K. Panksep., unpublished) causing microcystin concentrations up to 50 µg L^{-1} in the open water area and more than 2000 µg L^{-1} in inshore scum

accumulation areas during late summer (Tanner et al., 2005).

2.2. Sampling

Samples were collected monthly from May to September 2014 from the routine monitoring stations (P4, P11, P38) in Peipsi (Fig. 1). Additional samples for stable isotope analyses were collected from June to September in 2015 at the same stations. P4 and P11 represent the pelagic area of Peipsi while P38 is influenced by the inflowing River Emajõgi (Fig. 1). To obtain depth-integrated samples from sampling points, water was collected at 1-m intervals with a Van Dorn sampler from the entire water column and mixed in a tank. Subsamples were taken from this depth-integrated water to analyse PP composition and biomass and to detect and quantify potentially toxic CY with molecular methods. For molecular analyses, 200 to 1000 mL of the depth-integrated water was filtered at low vacuum through Whatman Cyclopore Polycarbonate filters (pore size 1 µm) which were stored at −80 °C until further analysis. To identify ZP composition and biomass, 20 L of the depth-integrated water was filtered through a 48-µm mesh plankton net and concentrated into a 200 ml sample jar. PP and ZP samples were fixed with acidified Lugol's solution at a final concentration of 1% and kept in the dark until further analysis. For analysing phytoplankton pigments (PPig) and potentially toxic CY in ZP gut, depth-integrated samples were collected with vertical tows of a plankton net (300 µm mesh) until sufficient material was obtained. The collected ZP was instantly rinsed with filtered lake water to clean the ZP sample from PP as much as possible. The cleaned ZP was anesthetized in carbonated water and concentrated in a small volume of filtered lake water. Thereafter the samples were stored frozen (-20 °C) in darkness. Samples for stable isotope analyses were collected with a plankton net of 48 µm mesh until sufficient ZP and PP material was obtained. Particulate organic matter (POM) samples were taken from the same depth-integrated lake water used for PP samples. The collected samples were kept cool until further analyses in the laboratory.

2.3. Phyto- and zooplankton biomass and zooplankton sorting for analyses

PP cells were enumerated with an inverted microscope (Nikon Eclipse Ti-S) at x400 magnification using Utermöhl's technique (Utermöhl, 1958). PP taxa were identified to the lowest practicable level. Each counted taxon was converted to biovolume by measuring cell/ trichome/ colony dimensions and then approximating each taxon with a simple geometric shape. Biomass of PP was expressed as mg WW L^{-1} (milligram of wet weight in one litre of lake water).

ZP biomass and community composition were analysed under a stereomicroscope (Nikon SMZ1500, up to x120 magnification) in a Bogorov chamber. Crustacean length was converted to wet weight as described by Studenikina and Cherepakhina (1969), and Balushkina and Winberg (1979). The individual wet weights of rotifers were estimated from average lengths, according to Ruttner-Kolisko (1977). ZP taxa accounting for 20% or more of the number or biomass were considered, respectively, as abundance and biomass dominants (Haberman, 1977).

Prior to PPig and molecular analyses, frozen ZP samples were thawed to separate the most abundant ZP species. Three dominant cladoceran taxa – *Bosmina* spp., *Daphnia* spp. and *B. longimanus* – and the copepod *E. gracilis* were separated. The technique for separation of ZP individuals is more precisely described in Tõnno et al. (2016). From each subsample 6 to 220 individuals of each species were separated and rinsed to minimize contamination from non-ingested algae. For pigment analysis, zooplankters were collected on Whatman GF/F glass microfiber filters (pore size 0.7 µm). Whenever possible, replicate samples were analysed. For DNA-based gut content analysis individuals from dominant ZP taxa were rinsed repeatedly with deionized water, inspected visually to verify that no external algal cells were stuck on animals and then collected into 1.5 mL microtubes in two parallel groups for immediate DNA extraction. For *Daphnia* spp. and *E. gracilis*, each group consisted of 50 individuals, and for *Bosmina* spp. and *B. longimanus* 35 to 50 and 19 to 22 individuals, respectively. For ZP feeding, samples from May, June, July and August were analysed separately.

2.4. DNA extraction and molecular analyses

Genomic DNA from ZP and cyanobacterial standard strains were extracted using DNeasy® Blood and Tissue extraction kit (Qiagen Inc.), and from integrated water samples using DNeasy® PowerWater Kit (Qiagen Inc.) according to the manufacturer's instructions. Quality and quantity of extracted DNA were controlled visually on a gel and with NanoDrop 2000 UV–vis spectrophotometer (Thermo Fisher Scientific Inc.). DNA was stored at –80 °C until further analysis. As very intensive sorting and purification effort is needed for DNA sample materials, DNA analyses from ZP were performed only from sampling site P11. Located in the centre of Lake Peipsi, P11 represents the large open water area of this lake.

For preliminary detection of total cyanobacterial DNA and the toxic and non-toxic *Microcystis* spp. genotypes (water and ZP guts), conventional PCR reactions with cyanobacterium-specific primers targeting 16S rRNA genes and phycocyanin operon of *Microcystis* (Table 1) were performed (Nübel et al., 1997; Kurmayer and Kutzenberger, 2003). The reaction mixture (total volume 20 µL) included 1x FIREPol® Master Mix Ready to Load with 7.5 mM $MgCl_2$ (Solis BioDyne, Estonia), 0.3 µM or 0.5 µM of primers (PC-IGS and 16S rRNA, respectively) and 2 µL of template DNA. PCR conditions are shown in Table 1. In order to quantify potential microcystin producers among genera *Microcystis*, *Planktothrix* and *Dolichospermum* in the samples, genus-specific qPCR was performed using an absolute quantification method with an internal standard curve, constructed as described before by Vaitomaa et al. (2003) and Koskenniemi et al. (2007). For the standard curve construction, information about the approximate genome size of *Microcystis*, *Dolichospermum* (Castenholz, 2001) and *Planktothrix* (Herdman et al., 1979) strains was used. The qPCR amplifications of the *mcyE* synthase genes (primers are shown in Table 1) were performed with ESCO Swift™ Spectrum 96 Real Time Thermal Cycler (ESCO, Singapore) in 3 replicates. Ten-fold dilutions of environmental DNA were used as a template and MQ water was used as a negative control. The reaction mixture (total volume 20 µL) included 1x HOT FIREPol®

Table 1
Primers used to detect and quantify potentially toxic cyanobacteria in the water and ingested by the zooplankters in Lake Peipsi from sampling station 11 (May 2014- September 2014).

Cyanobacterial 16S rRNA	Primer	Primer reference	PCR program
Cyanobacterial 16S rRNA	CYA359F CYA781R	Nübel et al. (1997)	95 °C 5 min; 35 cycles: 95 °C 60 s; 60 °C 60 s; 72 °C 60 s and 72 °C 10 min
Phycocyanin operon (PC)	188F 254R	Kurmayer and Kutzenberger (2003)	95 °C 5 min; 45 cycles: 95 °C 20 s; 58 °C 30 s; 72 °C 20 s and 72 °C 10 min
Microcystis mcyE	127F 247R 186P	Sipari et al. (2010)	95 °C 15 min; 40 cycles: 95 °C 15 s; 62 °C 60 s;
Dolichospermum mcyE	611F 737R 672P	Sipari et al. (2010)	95 °C 15 min; 40 cycles: 95 °C 15 s; 62 °C 60 s;
Planktothrix mcyE	664F 744R 670P	Rantala-Ylinen et al., unpublished	95 °C 15 min; 40 cycles: 95 °C 15 s; 60 °C 60 s;

Probe® qPCR mix plus (no ROX) (Solis BioDyne, Estonia), 0.3 µM of *mcyE* primers and TaqMan probes and 5 µL of diluted template DNA. DNA based methods were not used in order to detect and quantify *Aphanizomenon* spp. and *Gloeotrichia* in water and ZP gut samples.

2.5. Pigment extraction and HPLC analyses

In this study, CY were represented by the marker carotenoid echinenone (Echin; Leavitt and Hodgson, 2002) while the carotenoids zeaxanthin and canthaxanthin (Zea & Cantha, respectively) were mainly from colonial CY (Bianchi et al., 2002). The carotenoids fucoxanthin (Fuco), diadinoxanthin (Diadino) and diatoxanthin (Diato) represent diatoms (Roy et al., 2011). Two of these diatom marker pigments were presented together (Diadino + Diato), as in their xanthophyll cycle Diadino can be transformed to Diato at high light (Roy et al., 2011). Lutein (Lut) and chlorophyll *b* (Chl *b*) were analysed as proxies of chlorophytes, while alloxanthin (Allo) and peridinin (Peri) represented cryptophytes and dinophytes, respectively (Roy et al., 2011; Waters et al., 2013). Chlorophyll *a* (Chl *a*) was selected as a proxy of total PP biomass (Waters et al., 2013).

PPig were analysed following the slightly modified recommendations of Leavitt and Hodgson (2002) and Lie and Wong (2010). Briefly, depth integrated water samples (100–250 mL, depending on water turbidity) and rinsed ZP suspensions were filtered through Whatman GF/F 0.7 µm pore size filters and stored at -80 °C in the darkness until PPig analysis. To extract PPig, 90% acetone (by volume) with the internal standard (trans-*β*-apo- 8′-carotenal (Sigma cat. # 10810; Reuss and Conley, 2005) was added to frozen GF/F filters, after which samples were sonicated (Branson 1210) for approximately 10 min in an ice-bath under dim light and extracted at -20 °C in the dark for 24 h. Before chromatographic analysis, extracts were clarified by filtration through a 0.45 µm filter (Millex LCR, Millipore).

Reversed–phase HPLC was applied, using a Shimadzu Prominence (Japan) series binary gradient system with a photodiode–array (PDA) and fluorescence detector to separate PPig. A fluorescence detector with excitation wavelength set at 440 nm and emission at 660 nm was used to confirm correct identification and low concentrations of Chl a (Airs et al., 2001). Ammonium acetate (0.5 M) as an ion-pairing reagent was added in a volume ratio of 2:3 to all samples before the injection. To avoid chemical decomposition of PPig, the autosampler was cooled down to +5 °C (Reuss and Conley, 2005). Separations were performed in a reversed-phase mode by using two Waters Spherisorb ODS2 3 µm columns (150 mm × 4.6 mm I.D.) in-line with a precolumn (10 mm × 5 mm I.D.) containing the same phase. For more details see Tamm et al. (2015). For peak identification and quantification, commercially available external standards from DHI (Denmark) were used.

Published pigment degradation rates in ZP guts are somewhat controversial: some studies have shown that pigments fully preserve in the guts of copepods, cladocerans and other small zooplankton (Quiblier-Lloberas et al., 1996; Poister et al., 1999), while others have revealed a remarkable degradation of carotenoids (Head and Harris, 1992, 1994). Like in our earlier study (Tõnno et al., 2016), we assumed that the Chesson's selectivity index (Chesson, 1983) can be considered a snapshot of PPig transferred through ZP, and that this transfer is presumably much faster than digestive changes in the composition of carotenoid pigments. Therefore, the pigment degradation information could be considered non-crucial (Pandolfini et al., 2000; Thys et al., 2003).

2.6. Stable isotope analyses

In the laboratory the collected bulk plankton samples were first left to settle until the large filamentous and colonial forms of CY (*Aphanizomenon* spp., *Gloeotrichia echinulata*, *Microcystis* spp.) were floating on the surface. The floating CY were then collected with a micropipette and sorted manually at genus level under a dissecting microscope. Sorted algal subsamples were rinsed gently with deionised water and checked for purity under the dissecting microscope. The algal materials were then transferred to foil cups as a thin layer and dried at 60 °C overnight (16 h). The remaining bulk sample was filtered through a 300 µm mesh plankton net to retain crustaceans. The dominant copepod and cladoceran taxa were separated manually under the dissecting microscope, rinsed with deionised water, transferred to pre-weighed tin cups and dried at 60 °C overnight. POM samples were obtained by filtering the integrated water sample onto precombusted (550 °C, 3 h) GF/F filters (Whatman Inc.) after sieving through a 100 µm net to remove larger ZP. Due to high inorganic carbonate concentration in Peipsi, prior to $\delta^{13}C$ analysis the dried material was held for 24 h in concentrated HCl fumes to remove inorganic carbon from samples and then re-dried for 1 h at 60 °C. Untreated material was used for parallel $\delta^{15}N$ analyses. Stable isotope analyses were performed at the University of Jyväskylä (Finland) using a Flash EA 1112 elemental analyser connected to a DELTAplus Advantage IRMS (Thermo Finnigan). Stable isotope ratios are expressed as delta values ($\delta^{15}N$, $\delta^{13}C$) in parts per thousand (‰). The reference material used (International Atomic Energy Agency standard, NBS-22) was a secondary standard of known relation to the international standard (Pee Dee belemnite). The analyses were run using dried and homogenized birch leaves for PP and white muscle tissue of pike for ZP material as internal laboratory working standards. Due to seasonally varying lipid concentration in animal tissues, the $\delta^{13}C$ values of ZP were lipid-normalised using mass balance correction models according to Smyntek et al. (2007).

2.7. Statistical analyses and Chesson selectivity index

To test, how the studied pigments in water column corresponded to the phytoplankton taxonomic group's biomasses by microscopy, we used Spearman rank correlation analyses (Table 2). Differences between measured variables were tested for significance using a one-way ANOVA or Kruskal–Wallis one-way analysis of variance on ranks if the data did not meet the requirement of normality or equality of variance. Crustacean zooplankton diet differences according to gut pigment composition between sampling sites were analysed by principal components analysis (PCA). All variables (gut pigment compositions of *Daphnia* spp., *Bosmina* spp. and *E. gracilis*) were analysed after centering and standardization. PCA was performed with the function "prcomp". To examine differences between PPig proportions in PP and ZP, an independent sample *t*-test was performed. If $t < 0$ then PPig proportion is lower in ZP than in PP (not preferred food) and if $t > 0$, then pigment proportion is higher in ZP than in PP (preferred food). All statistical analyses were performed using R (R Development Core Team, 2018).

The alpha selectivity index of Chesson was used to assess feeding selectivity of ZP (Chesson, 1983). The formula used for calculations is:

Table 2
Spearman rank order test between water column pigments and dominant phytoplankton groups ($p < 0.05$) in Lake Peipsi (May 2014- September 2014). The relationship between Allo & Cryptophytes was not significant. Chl *a* – Chlorophyll *a*; Echin–echinenone; Zea–zeaxanthin; Cantha–canthaxantin; Fuco–fucoxanthin; D + D – diadinoxanthin + diatoxanthin; Lut–lutein; Chl *b* – Chlorophyll *b*; Allo–alloxanthin.

Pair of characteristics	r
Echin & Total Cyanobacteria	0.91
Zea & Colonial Cyanobacteria	0.89
Cantha & Colonial Cyanobacteria	0.89
Fuco & Diatoms	0.78
D + D & Diatoms	0.80
Lut & Chlorophytes	0.82
Chl *b* & Chlorophytes	0.79
Allo & Cryptophytes	0.45
Chl *a* & Total phytoplankton biomass	0.61

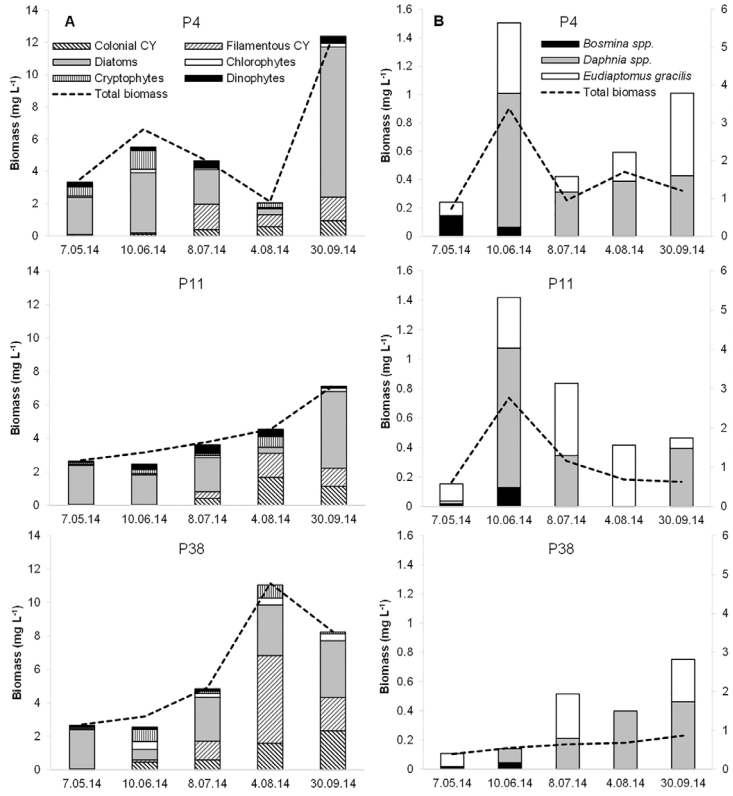

Fig. 2. Dynamics of different groups of phytoplankton (A) and zooplankton (B) at three sampling stations (P4, P11, P38) of Lake Peipsi from May to September 2014. Colononial CY – colonial cyanobacteria; Filamentous CY – filamentous cyanobacteria; Total biomass – total phytoplankton or zooplankton biomass.

$$\alpha_i = \frac{r_i/p_i}{\sum_{i=1}^{n} r_i/p_i};\ i = 1...n$$

where r_i is the percentage of i-th PPig in ZP guts, p_i the percentage of the same PPig in the lake water and n the total number of pigments analysed. Chesson's selectivity index was calculated for nine PPig (except Chl *a*). When $a = 1/n$ (in present study $1/n = 0.111$), ZP feeding is non-selective. Values of $a_i > 0.111$ or $a_i < 0.111$ represent selection and avoidance of pigments and respective PP groups by ZP, respectively.

3. Results

3.1. Plankton seasonal dynamics

At all investigated sampling stations total PP biomass started to increase in June and peaked in August or September (Fig. 2A). PP was dominated by diatoms (*Stephanodiscus* sp., *Cyclostephanos* sp., *Asterionella* sp.) from May to July. Diatoms (*Aulacoseira* sp., *Stephanodiscus* sp.) were also the dominant PP group in September in the northern and central area of Peipsi (P4 and P11, respectively; Fig. 2A). CY started to increase from July forming the highest biomasses in August (P11 and P38) or September (P4), while bloom conditions (CY > 50% of total PP biomass) occurred over the lake in August. CY dominated by filamentous *Dolichospermum* spp., *Gloeotrichia* sp. and colonial *Microcystis* spp. (Figs. 2A, 3). *Microcystis* spp. dominated in August and September forming up to 44% of the cyanobacterial biomass (Fig. 3). Among minor algal groups, dinophytes had generally highest biomasses at P11 and chlorophytes at P38, while cryptophytes indicated increased biomass at P4 in May and June and at P11 and P38 in August (Fig. 2A).

PPig had the highest concentration in lake water at all sampling stations in September. The marker pigments of diatoms (Fuco, Diadino + Diato) and CY (Echin, Zea, Cantha) dominated (Fig. 4) and were strongly correlated with biomass (Table 2) while the concentrations and correlations of other investigated carotenoids remained

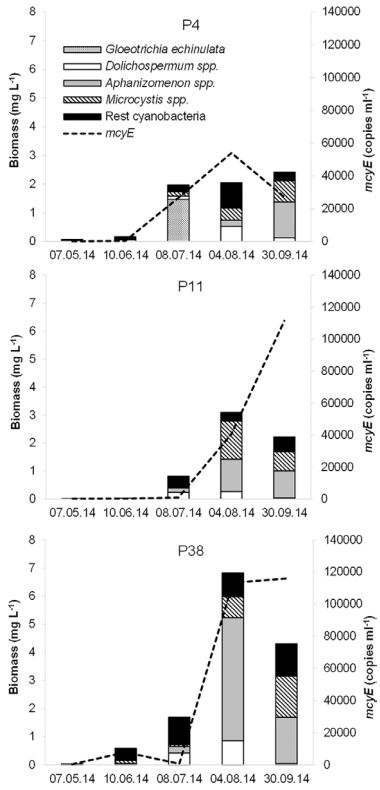

Fig. 3. Dynamics of potentially toxic cyanobacteria and *mcyE* synthase genes at three sampling stations (P4, P11, P38) of Lake Peipsi from May to September 2014. Rest cyanobacteria – ohter non-toxic cyanobacteria.

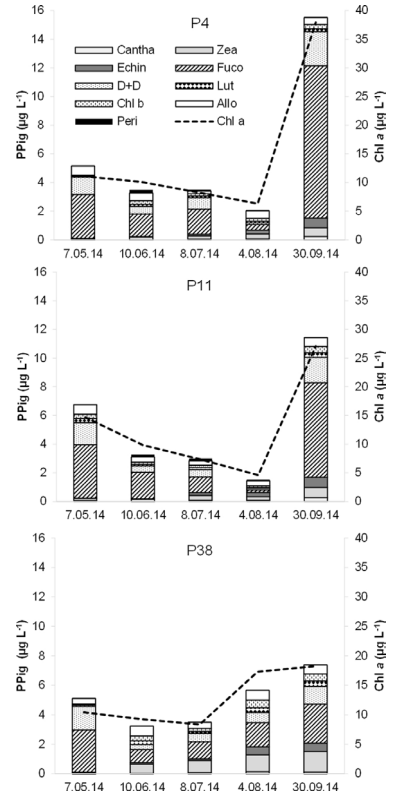

Fig. 4. Dynamics of phytoplankton pigments in depth-integrated water samples from three sampling stations (P4, P11, P38) of Lake Peipsi from May to September 2014. Chl *a* – chlorophyll *a*; Cantha – canthaxantin; Zea – zeaxanthin; Echin – echinenone; Fuco – fucoxanthin; D + D – diadinoxanthin + diatoxanthin; Lut – lutein; Chl *b* – chlorophyll *b*; Allo – alloxanthin; Peri – peridinin.

modest generally following the PP community structure in Peipsi. However, at P11 the Chl *a* and PP biomass trends were not coherent from May until August as Chl *a* was decreasing and biomass was increasing during this period. Similar temporal discrepancies have been observed also in Lake Võrtsjärv where these have been explained by changing underwater light conditions influencing the cellular content of light-harvesting pigments (Nõges et al., 2011).

During the study period, ZP biomass was dominated by crustaceans. Among those, *E. gracilis* and *Daphnia* spp. were the major taxa, forming approximately half or more of the total ZP biomass except in May when the biomass was dominated by cylopoid copepodites and rotifers (Fig. 2B). ZP biomass maxima were detected in June (P4 and P11) and September (P38; Fig. 2B). In May and June the population of *Daphnia* was dominated by *D. cucullata* while *D. galeata* prevailed from July to September. The genus *Bosmina* was represented by the species *B. berolinensis*, *B. gibbera* and *B. longirostris*. Other cladoceran species such as *Bythorephes longimanus*, *Chydorus* spp., and *Diaphanosoma brachyurum* occurred occasionally.

3.2. Feeding selectivity of zooplankton

The PCA of the sampling sites according to gut pigment composition described 60.8% of the variance in the data in first two PCA axes (Fig. 5) showing algal diet differences between the crustaceans. As the ordination of the PCA revealed no spatial differences in gut pigment composition in studied crustacean taxa, the ZP results from different sampling sites were pooled. The PPig concentrations measured from lake water were generally strongly correlated with the measured PP biomass suggesting that PPig provides a valid reflection of PP community structure in the lake and can be used to explore feeding by ZP. Throughout the study period, the guts of the calanoid copepod *E.*

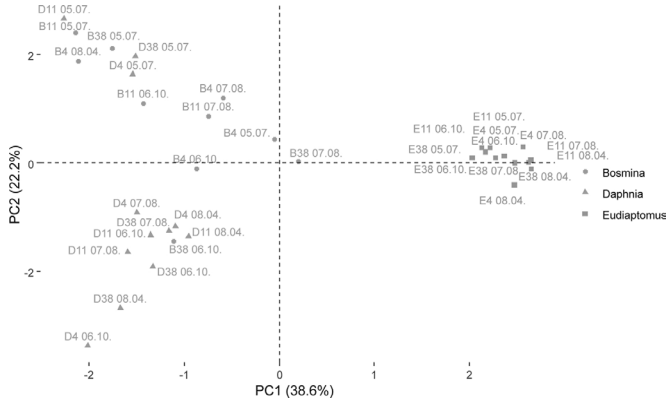

Fig. 5. PCA ordinaton of sampling sites according to gut pigment compostion. Sample names are abbreviated accordingly: genus name (B, D, E refers to the *Bosmina*, *Daphnia* and *Eudiaptomus*, respectively), sampling site 4, 11 or 38 and sampling date.

gracilis contained predominantly the cryptophyte marker pigment Allo (Fig. 6A). Chesson's selectivity index (hereafter selectivity index) and statistical analyses also demonstrated a clear preference of *E. gracilis* for cryptophytes (Tables 3 and 4). The cladoceran *Daphnia* spp. gut had a variable pigment content dominated by marker pigments of diatoms (Fuco, Diadino + Diato) and CY (Zea, Cantha, Echin). In their diet, *Daphnia* spp. preferred colonial CY (Zea, Cantha), chlorophytes (Lute) and diatoms (Diadino + Diato; Fig. 6B; Tables 3 and 4). *Bosmina* spp. guts contained mainly Zea and Echin, and less Fuco and Allo. Based on the selectivity index, *Bosmina* spp. demonstrated a clear preference for CY but also cryptophytes and dinophytes (Peri) in their diet (Fig. 6C; Tables 3 and 4). The gut pigment composition of the predatory cladoceran *B. longimanus* was assessed in July and August, and showed major contributions from cryptophytes and colonial CY (Zea, Fig. 6D; Table 3).

3.3. Presence of potential microcystin-producing cyanobacteria in water samples and zooplankton guts

The presence of *mcyE* genes in all studied water samples indicated potential microcystin production over the study area. According to the qPCR, the major potential producers of microcystins were *Microcystis* spp. (N = 15, mean 3.3×10^5 copies mL^{-1}; max 1.16×10^5 copies mL^{-1}) while *Planktothrix* contributed only a small portion of microcystins (approximately 10%) in August in the southernmost sampling area., *Dolichospermum* spp. with *mcyE* genes were not detected during the study period. Both the biomass of potentially toxic CY and the abundance of *mcyE* genes in lake water peaked in August-September and were highest in the southernmost sampling area in the Peipsi proper (Fig. 3).

Molecular analyses revealed the presence of cyanobacterial 16S rDNA in gut contents of all sorted ZP taxa (*Bosmina* spp., *Daphnia* spp., *B. longimanus* and *E. gracilis*) in Peipsi. *Microcystis* DNA was detected in most of the analysed ZP guts whereas the abundance of *Microcystis mcyE* gene copies varied from 0 to 414 per specimen (Table 5). The maximum number of *Microcystis* cells with *mcyE* genes was found in *Daphnia* spp. gut content in August. *MycE* genes from the genera *Dolichospermum* and *Planktothrix* were not detected in qPCR analysis of ZP gut contents. In brief, the genus-specific qPCR analyses confirmed the consumption of both, non-toxic and potentially toxic cells of colonial *Microcystis* by all studied ZP taxa while no ingestion of potentially toxic filamentous *Dolichospermum* and *Planktothrix* was confirmed.

3.4. Trophic positions of dominant zooplankton and phytoplankton taxa

As the $\delta^{15}N$ and $\delta^{13}C$ signatures of the most abundant sample types (*E. gracilis*, *Daphnia* spp. and POM) varied only little between the lake areas and sample dates, the obtained ZP and PP results were integrated as a single planktonic food web structure of Peipsi (Fig. 7). A substantial difference was observed in the isotopic compositions of the large colonial *G. echinulata* and *Aphanizomenon* spp. compared to those of *Microcystis* spp., POM and the crustacean ZP compartment. The cladoceran grazers (*Daphnia* spp., *B. berolinensis* and *B. gibbera*) had only small isotopic differences indicating similar diet composition. The predatory cladoceran *B. longimanus* and the copepod *E. gracilis* had clearly higher $\delta^{15}N$ values than those of grazing cladocerans and apparently occupied approximately one level higher trophic position in the food web.

4. Discussion

4.1. Algal diet and feeding selection of crustacean zooplankton

In this study, the questions of CY as a possible food source for the dominant crustaceans and the transfer of potentially toxic CY in the food web of a eutrophic lake were addressed. The results provided evidence that CY, and especially colonial CY, constitutes a significant part of the algal food ingested by the dominant cladocerans *Daphnia* spp. and *Bosmina* spp. in Peipsi during the period of cyanobacterial biomass increase. Both potentially toxic and non-toxic strains of colonial CY *Microcystis* spp., the major microcystin producing algae in Peipsi, were ingested directly or indirectly by the studied crustaceans (*Daphnia* spp., *Bosmina* spp., *E. gracilis* and *B. longimanus*).

However, the study revealed a considerable difference in ingested algal diet composition and selection between the calanoid copepod *E. gracilis* and grazing cladocerans (Fig. 6). During the bloom formation, CY comprised only a small portion of the algae ingested by *E. gracilis* while cryptophytes, a group of small flagellates, provided the major algal content. Several studies have shown *E. gracilis* preference for cryptophytes (Knisely and Geller, 1986; Ger et al., 2018a). The higher $\delta^{15}N$ value of this species and inferred trophic position in the food web

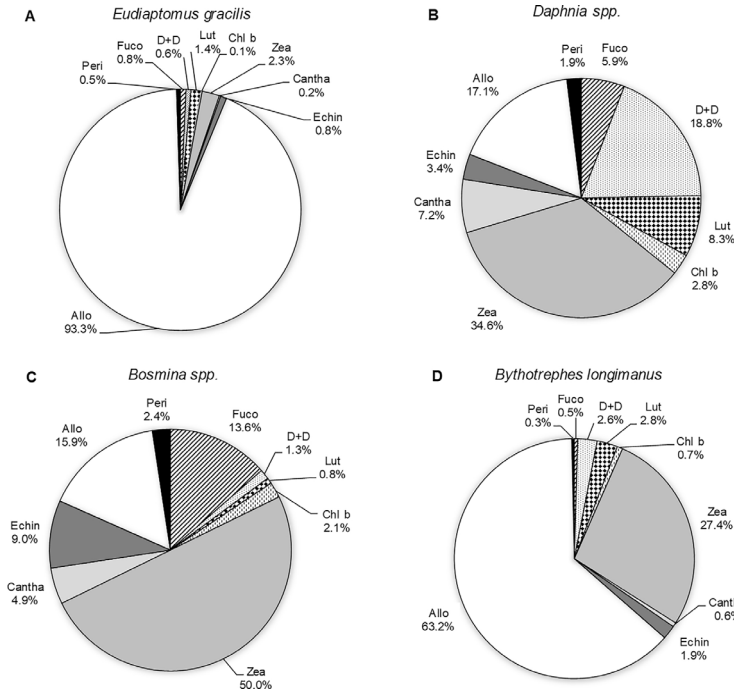

Fig. 6. Percentage contribution of phytoplankton pigments in calanoid copepod and cladocerans guts. A) *Eudiaptomus gracilis*; B) *Daphnia* spp.; C) *Bosmina* spp.; D) *Bythotrephes longimanus*. Abbreviations of phytoplankton pigments as in Fig. 4.

(Fig. 7), actually suggest considerable reliance on other zooplankters in Peipsi. Therefore, *E. gracilis* seem to have omnivorous feeding with strong reliance on two sources, cryptophytes and smaller zooplankters. This raises the possibility that part of the PP pigments detected in their guts may also originate indirectly from prey organisms such as protozoa and rotifers (Agasild et al., 2012; Šorf and Brandl, 2012) that have fed on cryptophytes. Hence *Eudiaptomus* in Peipsi seem to have different feeding niche and energy transfer routes compared to the grazing cladocerans.

For the two filtering cladocerans *Bosmina* spp. and *Daphnia* spp., a clear preference for CY was observed and approximately half of their ingested algae comprised different forms of CY. This selection for CY was clearly stronger for *Bosmina* spp. than for *Daphnia* spp. Generally more than half of *Bosmina* spp. diet comprised CY, mostly colonial forms; and this share increased towards August forming up to 70% of the gut pigment composition. Compared to *Daphnia, Bosmina* has a more selective grazing with a dual-feeding mode using filtering for small particles and grasping for large particles (Bleiwas and Stokes, 1985; Kerfoot and Kirk, 1991). This enables the bosminids to exploit widely varying sizes of food particles in the environment. *Daphnia* spp. had a more variable diet composition and exhibited generally lower selectivity than other crustacean species studied (Table 3). However, the ingested food of *Daphnia* spp. reflected more closely the ambient PP composition than that of other crustaceans making *Daphnia* the genus most influenced by PP seasonal fluctuations in Peipsi. Similar findings were reported for *Daphnia* from gut pigment studies by Tõnno et al. (2016) from shallow eutrophic Võrtsjärv, and gut micropscopic evaluation showed that approximately 70% of PP consumed by *Daphnia* spp. from eutrophic Lake Okeechobee (USA) belonged to CY (Work and Havens, 2003).

Bythotrephes longimanus is most abundant in Peipsi in July-August, typically co-occurring with increasing CY and decreasing number of grazing cladocerans (Haberman et al., 2008; Laugaste et al., 2008). *B. longimanus* is a raptorial predatory cladoceran and so its gut PPig composition presumably reflects the food consumed by its prey organisms. From its trophic position (Fig. 7) and given that the guts of *B. longimanus* contained mostly pigments belonging to CY and cryptophytes (Fig. 6D), the dominant cladocerans *Bosmina* and *Daphnia* may be its major prey in Peipsi while the cryptophyta pigment may originate from the consumption of copepod nauplii (Hansen and Santer, 1995; Dumitru et al., 2001). The presence of PP pigments in *B. longimanus* as a second order predator indicates a tight temporal coupling of feeding and energy flow between CY, grazing cladocerans and predatory ZP in Peipsi. *Bythotrephes* can exert several times higher predation impact on ZP than that of fish and its food consumption can exceed the prey production during its population peak in summer (Dumitru et al., 2001; Bunnell et al., 2011; Hoffman et al., 2011). Thus, *Bythotrephes* can be responsible for an important part of secondary PP transfer, including

Table 3
Chesson's selectivity index of zooplankton taxa for phytoplankton pigments in Lake Peipsi (May 2014-August 2014). Values indicating positive selection ($a_i > 0.111$) are marked in bold. 0 – phytoplankton pigment was not detected in ZP guts. Fuco – fucoxanthin; D + D – diadinoxanthin + diatoxanthin; Zea–zeaxanthin; Cantha–canthaxantin; Echin–echinenone; Lut–lutein; Chl *b* – Chlorophyll *b*; Allo–alloxanthin; Peri–peridenin.

	Site	Date	Fuco	D + D	Zea	Cantha	Echin	Lute	Chl *b*	Allo	Peri
Bosmina spp.	P4	7.05.2014	0.007	0	**0.838**	0	0.083	0	0	0.034	0.039
		10.06.2014	0.030	0	**0.500**	0	**0.264**	0	0.029	0.096	0.081
		8.07.2014	0.008	0	**0.427**	**0.181**	**0.149**	0	0	**0.220**	0.140
		4.08.2014	0.026	0	**0.270**	**0.392**	**0.122**	0	0	0.058	**0.132**
	P11	7.05.2014	0.007	0.004	**0.173**	**0.305**	**0.472**	0	0	0.014	0.024
		10.06.2014	0.014	0.005	**0.270**	**0.377**	**0.280**	0	0.008	0.026	0.020
		8.07.2014	0.002	0.005	**0.536**	**0.192**	**0.118**	0	0	**0.132**	0.016
	P38	7.05.2014	0.005	0.002	**0.174**	**0.267**	**0.536**	0	0	0.009	0.008
		10.06.2014	0.039	0	**0.211**	**0.158**	**0.183**	0.052	0.08	0.066	**0.212**
		8.07.2014	0.000	0	**0.407**	0	**0.113**	0	0	**0.263**	**0.217**
Daphnia spp.	P4	7.05.2014	0.006	0.009	**0.162**	**0.223**	**0.584**	0	0	0.011	0.005
		10.06.2014	0.025	0.028	**0.302**	**0.270**	0.086	**0.154**	0.080	0.020	0.034
		8.07.2014	0.010	0.104	**0.268**	**0.287**	0.075	0.089	0.007	**0.126**	0.035
		4.08.2014	0.018	**0.112**	**0.213**	**0.213**	0.026	**0.112**	0.033	0.088	0.074
	P11	7.05.2014	0.013	0.006	0.078	**0.383**	**0.486**	0	0	0.020	0.014
		10.06.2014	0.008	0.037	**0.342**	**0.365**	0.107	0.10	0.018	0.018	0.010
		8.07.2014	0.009	**0.190**	**0.220**	**0.247**	0.036	**0.16**	0.016	0.088	0.034
		4.08.2014	0.015	**0.178**	**0.234**	**0.151**	0.022	**0.15**	0.054	**0.132**	0.067
	P38	7.05.2014	0.003	0.003	**0.146**	**0.207**	**0.619**	0	0	0.016	0.007
		10.06.2014	0.028	0.099	**0.115**	**0.457**	0.061	**0.116**	0.025	0.056	0.043
		8.07.2014	0.009	**0.113**	**0.224**	**0.298**	0.080	**0.204**	0.003	0.060	0.009
		4.08.2014	0.020	0.102	**0.173**	**0.330**	0.000	**0.261**	0.033	0.076	0.006
B. longimanus	P4	8.07.2014	0	0.014	**0.151**	0	0.043	0.045	0	**0.741**	0.005
		4.08.2014	0.019	0.058	**0.217**	0	0.046	0.060	0	**0.561**	0.039
	P11	8.07.2014	0	0.024	**0.111**	0	0.058	0.064	0	**0.739**	0.004
		4.08.2014	0	0.046	**0.341**	0.039	0.000	0.087	0.029	**0.447**	0.011
	P38	8.07.2014	0	0.025	**0.155**	0	**0.182**	0.059	0	**0.542**	0.037
		4.08.2014	0.008	0.035	**0.196**	0	0.053	**0.124**	0.012	**0.544**	0.029
E. gracilis	P4	7.05.2014	0.002	0.003	**0.349**	0.066	0.069	0	0	**0.454**	0.058
		10.06.2014	0.006	0.004	0.073	0.082	0.102	0	0.006	**0.689**	0.037
		8.07.2014	0.001	0.000	0.026	0	0.030	0.002	0	**0.938**	0.004
		4.08.2014	0.003	0.021	0.012	0.010	0.002	**0.158**	0	**0.761**	0.035
	P11	7.05.2014	0.003	0.003	0.106	**0.165**	**0.171**	0.007	0	**0.535**	0.011
		10.06.2014	0.002	0.003	0.073	0	**0.278**	0	0.001	**0.602**	0.041
		8.07.2014	0.000	0.005	0.014	0.002	0.009	0.020	0	**0.946**	0.005
		4.08.2014	0.003	0.015	0.009	0.006	0.001	0.069	0.002	**0.883**	0.012
	P38	7.05.2014	0.001	0.001	**0.135**	**0.127**	**0.262**	0	0.01	**0.436**	0.025
		10.06.2014	0.018	0.008	0.036	0	**0.134**	0	0.02	**0.550**	**0.230**
		8.07.2014	0.001	0.005	0.011	0	0.063	0.051	0.00	**0.865**	0.004
		4.08.2014	0.005	0.005	0.008	0.009	0.000	0.009	0.00	**0.930**	0.029

Table 4
Results of *t*-tests ($p < 0.05$ are marked in bold) between proportions of phytoplankton (PP) pigments in phytoplankton and cladocerans (*Daphnia* spp., *Bosmina* spp.) and the copepod *E. gracilis* in Lake Peipsi (May 2014- August 2014). $t < 0$ – pigment propotion is lower in zooplankton (ZP) than in PP; $t > 0$ – pigment proportion is higher in ZP than in PP. Abbreviations of phytoplankton pigments as in Table 3.

		Fuco	D + D	Zea	Cantha	Echin	Lut	Chl *b*	Allo	Peri
Daphnia spp.	*t*	**−4.41**	0.24	**5.45**	**5.76**	0.24	1.79	**−2.69**	−0.27	−1.72
	p	< 0.05	NS	< 0.05	< 0.05	NS	NS	0.0133	NS	NS
Bosmina spp.	*t*	**−4.00**	**−6.01**	**8.05**	**2.25**	1.49	**−5.28**	**−3.53**	1	−0.31
	p	0.0007	< 0.05	< 0.05	0.036	NS	< 0.05	0.002	NS	NS
E. gracilis	*t*	**−8.11**	**−7.27**	**−3.02**	**−3.01**	**−2.14**	**−5.45**	**−6.52**	**36.44**	**−2.97**
	p	< 0.05	< 0.05	0.0062	0.0064	0.044	< 0.05	< 0.05	< 0.05	0.0071

*NS–not significant.

toxic CY, up the food web in Peipsi.

4.2. Grazing on potentially toxic cyanobacteria

Molecular analysis of the ZP guts confirmed the results from the pigment study proving the ingestion of CY by crustaceans throughout the investigation period. However, more specific analyses using genus-specific qPCR confirmed no ingestion of the potentially toxic filamentous CY *Dolichospermum* and *Planktothrix* by any of the analysed ZP taxa. Although, *Planktothrix* spp. were not detected in the PP community with traditional microscopy, a low number of *Planktothrix* spp. cells with *mcyE* genes were detected with genus-specific qPCR. This discrepancy could simply reflect the sample volumes used for analysis. In our study, 3.2 mL of the sample volume was used for microscopy, whereas 200–1000 mL was filtered for molecular analyses increasing the chance of including less abundant species of PP (Rodríguez-Ramos et al., 2013). In the present study, therefore, the lack of *Planktothrix* spp. cells with *mcyE* genes in ZP guts most probably reflects a low

Table 5
Occurrence of cyanobacterial DNA in zooplankton specimen gut contents collected from Lake Peipsi sampling station 11 (May 2014- August 2014). CY 16S – PCR based detection of cyanobacteria (16S rDNA gene); MIC PC – total population of *Microcystis* (phycocyanin operon); MIC *mcyE* synthase gene–qPCR based detection and quantification of potentially toxic *Microcystis* spp.; '+' means detected; '–' not detected.

Date	*Bosmina* spp.			*Daphnia* spp.			*B. longimanus*			*E. gracilis*		
	CY 16S	MIC PC	MIC *mcyE*	CY 16S	MIC PC	MIC *mcyE*	CY 16S	MIC PC	MIC *mcyE*	CY 16S	MIC PC	MIC *mcyE*
7.05.2014	+	–	–	NA	NA	NA	NA	NA	NA	+	–	–
10.06.2014	+	+	34	+	–	–	NA	NA	NA	+	+	–
8.07.2014	+	+	DET	+	–	–	+	+	15	+	+	124
4.08.2014	NA	NA	NA	+	+	414	+	+	DET	+	+	40

*DET–below to the detection limit set (less than 10 *mcyE* copies).
**NA–not analysed.

encounter rate due to the small number of cells in the water column.

Dolichospermum spp. were present in the cyanobacterial community of Peipsi from July to September, but no cells with the *mcyE* gene were detected with molecular methods indicating the existence of only nontoxic genotypes in the *Dolichospermum* population in the lake during the study period. This explains why *Dolichospermum* spp. cells with the *mcyE* gene were not detected in ZP guts, although grazing on nontoxic *Dolichospermum* could still occur in Peipsi. Previous field and laboratory studies (Work and Havens, 2003; Kâ et al., 2012) have shown that *Dolichospermum* filaments can be fragmented to an edible size and ingested by crustacean ZP.

Even though several bloom-forming cyanobacterial genera occur in Peipsi (Laugaste et al., 2013), *Microcystis* species are the major microcystin producers all over the lake (Panksep et al., unpublished). Present study revealed that the potentially toxic *Microcystis* was ingested by both the cladocerans and the calanoid copepod. Ingestion of the potentially toxic *Microcystis* cells by crustaceans was first observed (via *mcyE* gene detection) in June and higher ingestions was recorded during July and August along with increasing *Microcystis* biomass and number of toxic cells in the lake water (Fig. 3). Recently a study in the upper San Franscisco Estuary by Ger et al. (2018b), the first to use the *mcyE* gene for evaluating *in situ* ingestion of toxic *Microcystis* strains, reported that the ingestion by *Pseudodiaptomus forbesi* increased together with enhancing bloom intensity. A similar trend of increasing consumption of toxic *Microcystis* during cyanobacterial blooms could probably occur also in Peipsi with maxima expected in August or September depending on location within the lake (Fig. 3). Although CY contributed most to the algae ingested by *Bosmina* spp., the highest number of potentially toxic cells of *Microcystis* spp. was observed in the guts of *Daphnia* spp. which according to its generalist feeding has little ability to avoid ingesting CY (Lürling, 2003; Ghadouani et al., 2004).

Quantifying ZP grazing via qPCR amplification of prey DNA from the consumers' guts is a promising method to improve the understanding of taxon level interactions of ZP with bloom-forming and potentially toxic CY. As of yet there is only limited research applying this methodology (e.g. Nejstgaard et al., 2008; Engström-Öst et al., 2011; Durbin et al., 2012) and only very few studies have involved field research assessing ZP ingestion on CY (Motwani and Gorokhova, 2013; Ger et al., 2018b). In present study, these first *in situ* ingestion measurements via qPCR targeting *Microcystis* specific *mcyE* gene in cladocerans *Daphnia* spp., *Bosmina* spp., *B. longimanus* and the calanoid copepod *E. gracilis,* although limited in numbers, give some idea of the possible ingestion of potentially toxic *Microcystis* in the natural environment during blooms. Moreover, the evidence of consumption of toxic *Microcystis* by dominant grazers raises further important questions regarding the functioning of the Peipsi food web: firstly, what would be the consequences of ingestion of toxic *Microcystis* cells to the crustaceans; and secondly, how would the ZP grazing affect *Microcystis* during the bloom.

Since 2001, a significant decline in the biomass of all ZP groups (rotifers, cladocerans, copepods and *Dreissena polymorpha* veligers) and in the mean body weight of cladocerans has been observed in Peipsi in parallel with increasing cyanobacterial biomass caused mainly by *Microcystis* and *Aphanizomenon* (Haberman et al., 2010). One possible

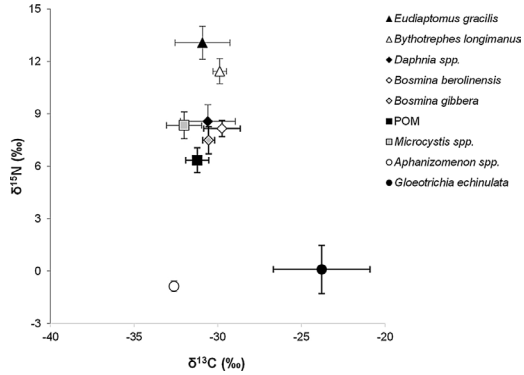

Fig. 7. Stable isotopic composition of cyanobacteria, particulate organic matter (POM) and crustacean zooplankton in Lake Peipsi. The values are expressed as means (± SD) of all sample dates and sampling sites 4, 11 and 38.

cause for this long-term decline is a negative effect of cyanotoxins on ZP, as proposed by Haberman et al. (2010). An *in situ* study by Ger et al. (2018b) found an inverse relationship between the abundance of the copepod *P. forbesi* and its gut *Microcystis* content, indicating toxic cyanobacterial ingestion as a potential factor influencing ZP abundance in nature. Indeed, given the range of ingested toxic *Microcystis* cells in crustacean gut content in the present study (15–414 cells ind^{-1}) and assuming an approximate gut filling time of 20–30 minutes (Chesney et al., 2019), the ingestion rate for toxic *Microcystis* could yield rather high numbers, exceeding 1000 cells $ind^{-1}\,h^{-1}$ in the case of *Daphnia* spp. Microcystin is known to inhibit ZP growth and reproduction and can even cause mortality (DeMott et al., 1991). The present study cannot prove the ingestion of toxic algae as the major cause for the observed ZP decrease in Peipsi, but does confirm the contribution of potentially toxic *Microcystis* cells to the diets of dominant crustaceans. The long-term decline of ZP biomass since 2001in Peipsi (Haberman et al., 2010) might also be due to simultaneously increased feeding pressure by juvenile fish as suggested by Ginter et al. (2018). Most probably, these two factors are co-acting to shape ZP dynamics in the lake.

Recent laboratory experiments using local ZP populations in grazing studies have provided evidence of a physiological detoxification of cyanotoxins which is more efficient in generalist feeders like *Daphnia* (Ger et al., 2016; Macke et al., 2017). For copepods, prey selectivity is the major adaptation for tolerating cyanobacterial blooms (Ger et al., 2016). It has been shown that *E. gracilis* is capable of selecting between potentially toxic and non-toxic *Microcystis* strains, preferring the latter (Ger et al., 2011). Therefore, the *Daphnia* spp. and *E. gracilis* genotypes (and also those of the other crustacean species studied) living in Peipsi and exposed to annual cyanobacterial blooms are presumably adapted to tolerate the presence of toxic CY in their diet (e.g. Hairston et al., 2001; Sarnelle and Wilson, 2005) or to avoid the ingestion of toxic strains via selective feeding.

The gut pigment composition of *E. gracilis* in Peipsi supported the assumption that this copepod selectively avoids ingestion of CY. Still, the DNA analyses revealed the presence of *Microcystis*, including toxic cells, in the guts of *E. gracilis*. It is very likely that despite its high selectivity, *Eudiaptomus* cannot totally avoid all cyanobacterial cells, as shown in the laboratory experiments by DeMott and Moxter (1991) and Ger et al. (2011). Ger et al. (2011) proposed that cellular microcystin causes the cue-based selective avoidance of toxic CY in *E. gracilis* and such behaviour could explain the limited ingestion of CY by *E. gracilis* in Peipsi. Nevertheless, it cannot be excluded that some CY (and toxic *Microcystis* cells) found in *E. gracilis* originate from their prey organisms. The same applies for cladocerans such as *Daphnia* and *Bosmina* that are known to actively consume ciliates in Peipsi (Agasild et al., 2012). Although, the feeding of microzooplankton (ciliates, rotifers) was not assessed in this study, still, their role of transferring CY in food web can presumably be significant as ciliates and protozoa-dominated microzooplankton have been reported as important grazers on CY (Davis and Gobler, 2011).

The qPCR method also demonstrated indirect transfer of potentially toxic *Microcystis* by the large predatory cladoceran *Bythotrephes*. This implies transfer of toxic CY and possibly also microcystins through multiple trophic levels in the Peipsi food web. Similar transfer of cyanobacterial *Planktothrix rubescens* DNA and microcystins to zooplanktivorous *Chaoborus* larvae was reported for Lake Hallwil (Switzerland) by Sotton et al. (2014). Overall, the DNA analyses indicated that among crustacean ZP in Peipsi, cladocerans may have a greater potential for toxic *Microcystis* consumption and possible transfer in the food web.

4.3. Potential impact of crustacean grazing on toxic cyanobacteria and cyanotoxin transfer to fish

Contrary to the snapshot estimations of the ZP diet based on gut content analyses for PPig and DNA, the stable isotope composition provides information on food sources assimilated into consumer biomass over a longer period (Middelburg, 2014). Moreover, in the present study the stable isotope analyses enabled separation and analysis of potentially toxic and bloom forming *Aphanozomenon* and *Gloeotrichia* for which grazing quantification via qPCR is currently constrained due to methodological limitations. Based on differences in $\delta^{15}N$ (equivalent to approximately two trophic levels), the results suggested that *Aphanizomenon* spp. and the large colonial *G. echinulata* were neither ingested nor assimilated by the dominant crustacean grazers in Peipsi (Fig. 7). Correspondingly low $\delta^{15}N$ signatures were measured for these nitrogen-fixing cyanobacterial taxa from meso- and eutrophic lakes from Finland (Vuorio et al., 2006). According to the stable isotope compositions of POM, composed mainly of algae smaller than 100 µm, this mixed algal pool is clearly the main carbon source for grazing ZP in Peipsi. This fraction also contains cells and small colonies of *Microcystis*. The $\delta^{13}C$ of pure *Microcystis* spp. is rather similar to that of POM. However, the $\delta^{15}N$ signature of the non-nitrogen-fixing *Microcystis* spp. is much higher than that of the nitrogen-fixing CY and is actually similar to that of grazing cladocerans and hence is not consistent with substantial assimilation of this specific CY by grazing cladocerans (Post, 2002). Despite the clear evidence from qPCR of ingestion of *Microcystis* cells by the crustaceans, and especially considering the relatively high number of cells in the gut of *Daphnia* spp., it appears that *Microcystis*, although readily ingested, does not contribute much to the crustacean biomass. This could reflect poor digestibility of *Microcystis* because of the mucilaginous membranes protecting the cells (Lewin et al., 2003).

Numerous studies have reported gut passage of undigested *Microcystis* species followed by stimulated photosynthetic activity due to nutrient uptake in the guts of many consumers, such as zebra mussel (Vanderploeg et al., 2009), silver and bighead carp (Jancula et al., 2008; Zeng et al., 2014), roach (Lewin et al., 2003) and filter-feeding cladocerans (Semenova et al., 2017). *Microcystis* colonies are often depleted of inorganic phosphorus during their bloom formation in water bodies (Harke et al., 2012), so nutrient uptake during viable passage through a nutrient-rich consumer digestive tract can increase their growth on excretion back into water (Kolmakov, 2014). In Peipsi in July 2014, the colony sizes of *Microcystis* spp. ranged from 48 to 96 µm in diameter with overwhelming domination of smaller colonies; by September the share of larger colonies had increased (Kersti Kangro, Estonian University of Life Sciences, unpublished data). Hence in July and August apparently a large portion of *Microsystis* spp. colonies were of ingestible size for both *Daphnia* spp. and *Bosmina* spp. (Jarvis et al., 1987). Moreover, for these cladocerans, the gut pigment composition suggested a considerable ingestion of colonial CY. Thus, it is likely that *Microcystis* is not directly contributing to ZP secondary production but instead its growth is promoted if grazed by cladocerans (Kolmakov, 2014). If this is the case, the ingestion and subsequent excretion by crustaceans, and especially by *Daphnia*, could help to maintain and support the development of the potentially toxic *Microcystis* strain in Peipsi in the second half of summer. Grazer selectivity can facilitate the dominance of toxic PP as was recently shown in laboratory experiments with selectively feeding copepod *E. gracilis* (Ger et al., 2018a). Though cladoceran preference for colonial CY was demonstrated in present study further research is needed to clear the effect of generally less selectively feeding cladoceran grazing on toxic cyanobacterial dominance in nature.

Herbivorous zooplankton and their invertebrate predators represent an important vector for microcystin transfer to fish (Sotton et al., 2014). Release of intracellular microcystins may also occur during the digestion by fish of zooplankton prey which have ingested toxic CY (Kozlowsky-Suzuki et al., 2012). Based on the results, the predation on cladocerans, and particularly on *Daphnia*, may represent a significant route for transfer of microcystins to young-of-the-year and planktivorous fish in Peipsi. *D. galeata*, larger *Bosmina* species (*B. c. coregoni*, *B. gibbera*) and *B. longimanus* form essential food items for new

recruitment of commercial fishes in Peipsi, such as perch (*Perca fluviatilis*), bream (*Abramis brama*), and also for planktivorous smelt (*Osmerus eperlanus eperlanus* m. *spirinchus*) and vendace (*Coregonus albula*) comprising more than half of their diet (Ginter et al., 2018). Higher ingestion of ZP (relative to fish body mass) occurs during August-October (Ginter et al., 2018) matching the months of potentially toxic blooms. Although massive summer fish kills have been documented in Peipsi co-occurring with cyanobacterial blooms (Kangur et al., 2005, 2013), the connection with transfer of cyanotoxins to fish (Ibelings et al., 2005; Sotton et al., 2014; Pham and Utsumi, 2018) have not been studied. The combination of multiple stress factors, such as increased water temperature accompanied with decreased oxygen level and increased concentrations of ammonium has so far been proposed as the main reason for fish kills in Peipsi (Kangur et al., 2005).

In conclusion, this study demonstrates that CY, especially colonial forms like *Microcystis*, form a significant part of the algae ingested by cladocerans and that grazing cladocerans could represent the major link transferring cyanobacterial toxins through the food web in Peipsi. Based on qPCR analyses, the results indicated that during cyanobacterial bloom formation and bloom event in Peipsi toxic *Microcystis* was ingested by all dominant crustacean zooplankters. Moreover, quantifying *mcyE* genes in ZP species belonging to different trophic levels provided unique results on taxon-specific ingestion on toxic CY. Still, stable isotope analyses suggested that little, if any, assimilation occurred from ingested bloom-forming CY by grazing cladocerans. Similar relationships are likely to occur in other eutrophic lakes dominated by CY. The findings of this study have important implications for understanding of the food web functioning in eutrophic lakes during cyanobacterial blooms but also emphasize the need for further research on toxic *Microcystis* and ZP interactions to clarify the potential stimulating effect on growth of toxic CY and the transfer of cyanotoxins to fish.

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CURRICULUM VITAE

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2010-2022 PhD in Environmental Sciences and Applied Biology Estonian University of Life Sciences, (Academic leave since 2014)
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2003-2007 BSc in Biology, University of Tartu
2000-2003 Hugo Treffner Gymnasium, Department of Life Sciences

Employment:

2020-… University of Tartu, Institute of Technology, Laboratory Specialist in Molecular Biology
2009- … Estonian University of Life Sciences, Chair of Hydrobiology and Fishery, Specialist, Senior Specialist
2019-2021 Estonian University of Life Sciences, Chair of Aquaculture, Junior Research Fellow
2017-2018 University of Tartu, Institute of Ecology and Earth Sciences, Junior Research Fellow
2010-2014 Solis BioDyne Ltd, Laboratory Assistant
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Research interests:

Cyanobacteria, Cyanotoxins, Molecular detection methods, eDNA, eDNA biomonitoring, global and continental studies of cyanobacteria and aquatic fungi, early warning surveillance of SARS-CoV-2 based on wastewater analysis

Professional short-term training:

2019 "COST Leadership Workshop", COST Association AISBL, Brussels, Belgium

2018 "Validation & reporting of single-species eDNA analyses", University of Innsbruck, Austria

2018 "Estonia-Taiwan Workshop on Remote Sensing for Environmental Monitoring and Geodetic Applications, Estonian University of Life Sciences, Tartu, Estonia

2018 "Applied biostatistics in biological sciences using R", Estonian University of Life Sciences, Tartu

2018 "Next generation monitoring of phytoplankton diversity, with special focus on toxic cyanobacteria", University of Geneva, Switzerland

2017 "Hands-on qPCR", TATAA Biocenter, Prague, Czech Republic

2017 "Sample preparation and quality control of nucleic acids", TATAA Biocenter, Prague, Czech Republic

2017 "Experimental design and statistical data analysis for qPCR", TATAA Biocenter, Prague, Czech Republic

2017 "Practical aspects of laboratory work", University of Tartu, Estonia

2015 "From A to Z: Methods for a successful Multi-lake survey on cyanobacterial blooms", CyanoCOST-NETLAKE training school, Evian, France

2013 NOVA PhD course "Molecular typing and next generation sequencing of food- and waterborne pathogens", University of Helsinki, Finland

2012-2013 "Molecular detection and quantification of toxic cyanobacteria", Dora PhD student mobility scholarship, University of Helsinki

2011 "Applying molecular Biology to Microalgal Identification", University of Las Palmas de Cran Canaria, Spain

2010 "Winter Ecology", University of Jyväskylä, Finland

2010 "Introductory Course of Freshwater Algal Identification", University of Durham, United Kingdom

Supervised dissertations:

Anna Tkadlecova, MSc, 2020, (sup) Pavel Beran, Kristel Panksep, "Detection of toxic cyanobacteria in Estonian large lakes and coastal waters", University of South Bohemia in České Budějovice, Czech Republic

Carmen Kivistik, MSc, 2018, (sup) Kristel Panksep "Toxic cyanobacteria in Estonian large lakes", Estonian University of Life Sciences.

R&D related managerial and administrative work:

2022 –… Board member of Mycology and Microbiology Center (MMC)

2021 –… COST Action CA20125: Applications for zoosporic parasites in aquatic systems. Grant Awarding Coordinator, Core Group Member, Member of Management Committee

2021 –… Scientific Organizing Committee member of "12th International Conference on Toxic Cyanobacteria" Toledo, USA

2020 – … Organisation of "Symposium of Aquatic Microbial Ecology - SAME17", Tartu, Estonia.

2019 – … COST Action CA18225: Taste and Odor in early diagnosis of source and drinking Water Problems. Member of Core Group, Management committee and STSM coordination committee

2019 – … PI and coordinator of FunAqua - global DNA-based inventory of aquatic fungi for documenting global fungal biodiversity in water and sediments.

2018 – … Member of Global Lake Ecological Observatory Network (GLEON)

2018 – … Member of Global Microcystin Aggregation (GMA) project

2016 – 2021 COST Action CA15219: Developing new genetic tools for bioassessment of aquatic ecosystems in Europe. Member of Management committee

2015- ... Estonian ambassador in CyanoCOST/NETLAKE joint project European Multi-lake Survey

2012 – 2016 COST Action ES1105: Cyanobacteria blooms and toxins in water resources: Occurrence, impacts and management (CYANOCOST). Member of Management committee

List of publications:

Scholarly articles indexed by Web of Science Science Citation Index Expanded:

Pawlowski, J.; Bruce, K.; **Panksep, K.**; Aguirre, F.I.; Amafitano, S.; Apothéloz-Perret-Gentil, L.; Baussant, T.; Bouchez, A.; Carugati, L.; Cermakova, K.; Cordier, T.; Corinaldesi, C.; Costa, F.O.; Danovaro, R.; Dell'Anno, A.; Duarte, S.; Eisendle, U.; Ferrari, B.J.D.; Frontalini, F.; Frühe, L. ... Fazi, S. (2022). Environmental DNA metabarcoding for benthic monitoring: A review of sediment sampling and DNA extraction methods. The Science of The Total Environment, 151783. DOI: 10.1016/j.scitotenv.2021.151783.

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Tamm, M., Ligi, M., **Panksep, K.**, Teeveer, K., Freiberg, R., Laas, P., ... & Nõges, T. (2019). Boosting the monitoring of phytoplankton in optically complex coastal waters by combining pigment-based chemotaxonomy and in situ radiometry. Ecological Indicators, 97, 329-340.

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1.2 Peer-reviewed articles in other international research journals with an ISSN code and international editorial board:

Leese, F., Altermatt, F., Bouchez, A., Ekrem, T., Hering, D., Meissner, K., ..., **Panksep, K**., … & Steinke, D. (2016). DNAqua-Net: Developing new genetic tools for bioassessment and monitoring of aquatic ecosystems in Europe. Research Ideas and Outcomes, 2, e11321.

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2.1 Scholarly monographs:

Bruce, K., Blackman, R., Bourlat, S. J., Hellstrom, A. M., Bakker, J., Bista, I., ..., **Panksep, K**., ... & Deiner, K. (2021). A practical guide to DNA-based methods for biodiversity assessment. Pensoft Advanced Books, 1. DOI: 10.3897/ab.e68634.

2.4 University textbooks:

Ott, Ingmar; Timm, Henn; Järvalt, Ain; Järvekülg, Rein; Kisand, Anu; Mäemets, Helle; **Panksep, Kristel**; Pedusaar, Tiia; Tammert, Helen; Tuvikene, Arvo; Tuvikene, Lea; Tõnno, Ilmar; Vilbaste, Sirje (2020). Siseveekogud. Eesti Loodusfoto.

5.2 Conference abstracts

Panksep K., Agasild H.,Tõnno I., Blank K., Freiberg R., Laugaste R., Nõges P., Nõges T (2019). Detection of cyanobacteria in the diet composition of crustacean zooplankton: a multiproxy study. Abstract book for ICTC11: 11th International Conference on Toxic Cyanobacteria, Krakow, Poland, 5–10 May, 2019

Panksep, Kristel; Mantzouki, Evanthia; Lurling, Miguel; Fastner, Jutta; Visser, Petra; Tammert, Helen; Ibelings, Bastiaan (2018). A continental scale multi-lake survey of cyanobacteria, toxin synthetase genes and toxins during a heatwave summer. Abstract book for ICHA 2018: 18th International Conference on Harmful Algae, Nantes, France, 21-26 Oct. 2018. Nantes, France: ICHA2018, 176.

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Panksep, Kristel; Ylinen, Anne; Laugaste, Reet; Buhvetsova, Olga; Kahre, Olev; Sivonen, Kaarina (2012). Detection of hepatotoxin-producing cyanobacteria in Lake Peipsi (Estonia/Russia). 3rd European Large Lakes Symposium, October 8-12, 2012, Konstanz, Germany, 22.

Panksep, Kristel; Laugaste, Reet (2011). The dominance of potentially toxic cyanobacteria in large shallow polymictic Lake Peipsi (Estonia/Russia). The 7th International Shallow Lake Conference, April 24-28, 2011; Wuxi, China. , 130−131.

ELULOOKIRJELDUS

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2010-2022 Eesti Maaülikool, Keskkonnateadus ja rakendusbioloogia, (PhD)
2007-2009 Eesti Maaülikool, Rakendushüdrobioloogia, Diplom cum laude (MSc)
2003-2007 Tartu Ülikool, Bioloogia, (BSc)
2000-2003 Hugo Treffneri Gümnaasium, Loodusteaduste suund

Töökohad ja ametid:

2021-… Tartu Ülikool, Loodus- ja täppisteaduste valdkond, tehnoloogiainstituut, molekulaarse diagnostika spetsialist
2009- … Eesti Maaülikool, Põllumajandus- ja keskkonnainstituut, hüdrobioloogia ja kalanduse õppetool, spetsialist, peaspetsialist
2019-2021 Eesti Maaülikool, Veterinaarmeditsiini ja loomakasvatuse instituut, vesiviljeluse õppetool, nooremteadur
2017-2018 Tartu Ülikool, Loodus- ja täppisteaduste valdkond, ökoloogia ja maateaduste instituut, veeseente bioloogia nooremteadur
2010-2014 Solis BioDyne OÜ, laborispetsialist
2007-2009 Eesti keskkonnauuringute Keskus, laborispetsialist

Huvid:

Sinivetikad, sinivetikate toksiinid, molekulaarsed meetodid keskkonateadustes, keskkonnaDNA (eDNA), eDNA põhinev elurikkuse seire, globaalsed ja kontinentaalsed uuringud sinivetikate ja veeseente elurikkuse kirjeldamiseks

Koolitused:

2019	"COST Leadership Workshop", COST Association AISBL, Brüssel, Belgia
2018	"Validation & reporting of single-species eDNA analyses", University of Innsbruck, Austria
2018	"Estonia-Taiwan Workshop on Remote Sensing for Environmental Monitoring and Geodetic Applications, Eesti Maaülikool, Tartu, Eesti
2018	"Applied biostatistics in biological sciences using R", Eesti Maaülikool, Tartu, Eesti
2018	"Next generation monitoring of phytoplankton diversity, with special focus on toxic cyanobacteria", Genfi Ülikool, Šveits
2017	"Hands-on qPCR", TATAA Biocenter, Praha, Tšehhi
2017	"Sample preparation and quality control of nucleic acids", TATAA Biocenter, Praha, Tšehhi
2017	"Experimental design and statistical data analysis for qPCR", TATAA Biocenter, Praha, Tšehhi
2017	"Practical aspects of laboratory work", Tartu Ülikool, Eesti
2015	"From A to Z: Methods for a successful Multi-lake survey on cyanobacterial blooms", CyanoCOST-NETLAKE programmide ühiskoolitus, Evian, Pransusmaa
2013	NOVA doktorikursus "Molecular typing and next generation sequencing of food- and waterborne pathogens", Helsingi Ülikool, Soome
2012-2013	"Molecular detection and quantification of toxic cyanobacteria", DoRa doktorantide semester välismaal, Helsingi Ülikool

2011 "Applying molecular Biology to Microalgal Identification", Las Palmase Ülikool, Hispaania

2010 "Winter Ecology", Yväskylä Ülikool,Soome

2010 "Introductory Course of Freshwater Algal Identification", Durhami Ülikool, Inglismaa

Juhendatud väitekirjad:

Carmen Kivistik, magistrikraad, 2018, (juh) Kristel Panksep, Toksilised sinivetikad Eesti suurjärvedes, Eesti Maaülikool.

Anna Tkadlecova, master degree, 2020, (juh) Pavel Beran, Kristel Panksep, "Detection of toxic cyanobacteria in Estonian large lakes and coastal waters" Lõuna-Boheemia Ülikool, Ceske Budejovitse, Tšehhi

Teadusorganisatsiooniline ja -administratiivne tegevus

2022−... Mükoloogia ja Mikrobioloogia keskuse (MMC) juhatuse liige

2021 −... COST Action CA20125: Applications for zoosporic parasites in aquatic systems. Eesti esindaja korralduskomitees, tuumikgrupi liige, COST grandisüsteemi koordinaator, hindamiskomisjoni liige

2021 −... Teaduskonverentsi "12th International Conference on Toxic Cyanobacteria" Toledo, USA, korraldusmeeskonna liige

2020 −... Teaduskonverentsi "Symposium of Aquatic Microbial Ecology - SAME17", Tartu, Estonia, korraldaja

2019 −... COST Action CA18225 - Taste and Odor in early diagnosis of source and drinking Water Problems. Eesti esindaja, lühiajaliste välisvisiitide hindamiskomisjoni liige

2019 −... Projekti FunAqua "global DNA-based inventory of aquatic fungi for documenting global fungal biodiversity in water and sediments" - juht ja koordinaator

2018 −... Global Lake Ecological Observatory Network (GLEON) liige

2018 −... Global Microcystin Aggregation (GMA) projekti liiige

2015 −... Eesti esindaja CyanoCOST/ NETLAKE ühisprojektis European Multi-lake Survey (EMLS)

2016 − 2021 COST Action CA 15219: Developing new genetic tools for bioassessment of aquatic ecosystems in Europe. Eesti esindaja korralduskomitee liikmena

2016 – 2021 COST Action ES1105: Sinivetikate õitsengud ja toksiinid veekogudes. CYANOCOST. Eesti esindaja korralduskomitee liikmena

Peamised publikatsioonid:

Teadusartiklid, mis on kajastatud Web of Science andmebaasides:

Pawlowski, J.; Bruce, K.; **Panksep, K.**; Aguirre, F.I.; Amafitano, S.; Apothéloz-Perret-Gentil, L.; Baussant, T.; Bouchez, A.; Carugati, L.; Cermakova, K.; Cordier, T.; Corinaldesi, C.; Costa, F.O.; Danovaro, R.; Dell'Anno, A.; Duarte, S.; Eisendle, U.; Ferrari, B.J.D.; Frontalini, F.; Frühe, L. ... Fazi, S. (2022). Environmental DNA metabarcoding for benthic monitoring: A review of sediment sampling and DNA extraction methods. The Science of The Total Environment, 151783. DOI: 10.1016/j.scitotenv.2021.151783.

Sagova-Mareckova, M., Boenigk, J., Bouchez, A., Cermakova, K., Chonova, T., Cordier, T., ..., **Panksep, K**., ... & Stoeck, T. (2021). Expanding ecological assessment by integrating microorganisms into routine freshwater biomonitoring. Water Research, 191, 116767. DOI: 10.1016/j.watres.2020.116767.

Donis, D; Mantzouki, E; McGinnis, D F.; Vachon, D; Gallego, I; Grossart, H-P; Senerpont Domis, L; …; **Panksep, K.,** ... Ibelings, B

W. (2021). Stratification strength and light climate explain variation in chlorophyll a at the continental scale in a European multilake survey in a heatwave summer. Limnology and Oceanography, 1−20. DOI: 10.1002/lno.11963.

Tedersoo, L., Mikryukov, V., Anslan, S., Bahram, M., Khalid, A. N., Corrales, A., ..., **Panksep, K.**, ..., & Abarenkov, K. (2021). The Global Soil Mycobiome consortium dataset for boosting fungal diversity research. Fungal Diversity, 1-16. DOI: 10.1007/s13225-021-00493-7.

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Tamm, M., Ligi, M., **Panksep, K.**, Teeveer, K., Freiberg, R., Laas, P., ... & Nõges, T. (2019). Boosting the monitoring of phytoplankton in optically complex coastal waters by combining pigment-based chemotaxonomy and in situ radiometry. Ecological Indicators, 97, 329-340.

Mantzouki, E., Campbell, J., Van Loon, E., Visser, P., Konstantinou, I., Antoniou, M., ..., **Panksep, K.**, … & Stević, F. (2018). A European Multi Lake Survey dataset of environmental variables, phytoplankton pigments and cyanotoxins. Scientific data, 5(1), 1-13.

Mantzouki, E., Lürling, M., Fastner, J., de Senerpont Domis, L., Wilk-Woźniak, E., Koreivienė, J., ..., **Panksep, K.**, … & Walusiak, E. (2018). Temperature effects explain continental scale distribution of cyanobacterial toxins. Toxins, 10(4), 156.

Tammeorg, O., Niemistö, J., Möls, T., Laugaste, R., **Panksep, K.**, & Kangur, K. (2013). Wind-induced sediment resuspension as a potential factor sustaining eutrophication in large and shallow Lake Peipsi. Aquatic sciences, 75(4), 559-570.

1.2 Teadusartiklid teistes rahvusvahelistes teadusajakirjades, millel on registreeritud kood, rahvusvaheline toimetus, rahvusvahelise eelretsenseerimine

Leese, F., Altermatt, F., Bouchez, A., Ekrem, T., Hering, D., Meissner, K., ..., **Panksep, K**., … & Steinke, D. (2016). DNAqua-Net: Developing new genetic tools for bioassessment and monitoring of aquatic ecosystems in Europe. Research Ideas and Outcomes, 2, e11321.

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Bruce, K., Blackman, R., Bourlat, S. J., Hellstrom, A. M., Bakker, J., Bista, I., ..., **Panksep, K**., ... & Deiner, K. (2021). A practical guide to DNA-based methods for biodiversity assessment. Pensoft Advanced Books, 1. DOI: 10.3897/ab.e68634.

2.4 Kõrgkooliõpikud:

Ott, Ingmar; Timm, Henn; Järvalt, Ain; Järvekülg, Rein; Kisand, Anu; Mäemets, Helle; **Panksep, Kristel**; Pedusaar, Tiia; Tammert, Helen; Tuvikene, Arvo; Tuvikene, Lea; Tõnno, Ilmar; Vilbaste, Sirje (2020). Siseveekogud. Eesti Loodusfoto.

VIIS VIIMAST KAITSMIST

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PISTESÄÄSKLASTE (DIPTERA: CULICIDAE) MITMEKESISUS EESTI LINNAKESKKONNAS JA LOODUSES
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