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Detection of toxic cyanobacteria in Estonian large lakes and coastal waters

Master thesis

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Abstract

This master thesis deals with the detection of toxic cyanobacteria in Estonian lakes (Peipsi, Lämmijärv and Võrtsjärv) and coastal waters. Using the molecular qPCR method, the *mcyE* gene encoding the microcystin toxin (MC) was detected in 3 potential producers – *Microcystis, Dolichospermum* and *Planktothrix.* Toxin analysis was performed by LC-MS/MS, the results were compared with results of qPCR and statistically evaluated. A positive correlation was found between the concentration of the *mcyE* gene and the detected MCs. The highest measured values of the occurrence of the *mcyE* gene were detected in August, with slight deviations depending on the detected organism and the environmental conditions of the lake. The highest occurrence of MCs in the studied waters was detected in September. A positive correlations were also found between the concentration of the *mcyE* gene, eventual MCs and the amount of cyanobacterial biomass or chlorophyll-a content in water. The results of this work provide an overview of the development of cyanobacteria and their toxins in the Estonian large lakes and in the Baltic Sea during year 2018.

Key words: Microcystin, *mcyE,* cyanobacteria, qPCR, Estonia, lake

Abstrakt

Tato diplomová práce se zabývá detekcí toxických cyanobakterií v estonských jezerech (Peipsi, Lämmijärv a Võrtsjärv) a v pobřežních vodách. Pomocí molekulární metody qPCR byl detekován gen *mcyE* kódující toxin microcystin u 3 potenciálních producentů – *Microcystis, Dolichospermum* a *Planktothrix*. Chemickou metodou LC-MS/MS byla provedena analýza toxinů, jejíž výsledky byly porovnány s výsledky qPCR a statisticky vyhodnoceny. Mezi koncentrací výskytu genu *mcyE* a detekovanými microcystiny byla nalezena pozitivní korelace. Nejvyšší naměřené hodnoty výskytu genu *mcyE* byly detekovány v srpnu, s lehkými odchylkami v závislosti na detekovaném organismu a environmentálních podmínkách jezera. Nejvyšší výskyt microcystinů ve studovaných vodách byl detekován v září. Pozitivní korelace byla rovněž nalezena mezi koncentrací *mcyE* genu, případně microcystinů a množstvím cyanobakteriální biomasy či obsahem chlorofylu-a ve vodě. Výsledky této práce poskytují přehled o vývoji studovaných cyanobacterií a jejich toxinech v estonskách jezerech a v Baltském moři v průběhu roku 2018.

Klíčová slova: microcystin, *mcyE,* sinice, qPCR, Estonsko, jezero

List of abbreviations

Ach – acetylcholine

Adda – (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid

b.w. – body weight

C_q – quantification cycle

CNS – central nervous system

C_t – treshold cycle

CYN – cylindrospermopsin

DNA – deoxyribonucleic acid

dNTP – deoxyribonucleotide

e.g. – for example

ELISA – enzyme-linked immunosorbent assay

esc. – et cetera

et al. – et alii

FISH – fluorescent in situ hybridization

HAB – harmful algal bloom

HILIC – hydrophilic interaction liquid chromatography

HPLC – high-performance liquid chromatography

i.p. – intraperitoneal

LC-MS – liquid chromatography – mass spectrometry

MC – microcystin

Mdha – N-methyldehydroalanine

Mdhb – 2-(methylamino)-2-dehydrobutic acid

MeAsp – D-erythro-β-methyl-aspartic acid

MS – mass spectrometric

OATPs – organic anion transporting polypeptides

PCR – polymerase chain reaction

PDA – photodiode array

PP – protein phosphatase

PPIA – protein phosphatase inhibition assay

PSP – paralytic shellfish poisoning

PST – paralytic shellfish toxin

qPCR – quantitative polymerace chain reaction

RNA – ribonucleic acid

RP-HPLC – reersed-phase high-performance liquid chromatography

rRNA – ribosomal ribonucleic acid

RT-PCR – real time polymerase chain reaction

STX – saxitoxin

Taq – *Thermus aquaticus*

TN – total nitrogen

TP – total phosphorus

UV – ultraviolet

VFDF – very fast death factor

WHO – world health organisation

Table of content

1. Introduction

Water is one of the essential parts of Earth. It covers more than 71% of the Earth's surface and is home to countless organisms. Almost 3% of the total water is freshwater. One of the oldest organisms that inhabited the aquatic environment are cyanobacteria and algae, collectively referred to as phytoplankton. Phytoplankton is one of the most important factors affecting the climate and life on Earth. Its essential role is photosynthesis, in which the captured carbon dioxide from the atmosphere converts into oxygen, an essential element for life. Phytoplankton also plays an important role in food chain as a base of food for other organisms, from microscopic zooplankton to human.

There are many changes due to global warming on planet Earth. The warming of the oceans and the overall pollution and eutrophication of water bodies have proved to be a major problem. With the increasingly unpretentious development of industry and agriculture, a significant phenomenon, the so-called algal bloom, has been developing in recent decades. The algal bloom consists of overgrown algae, but mainly cyanobacteria. Ideal conditions for the overgrowth of these organisms are due to the abundance of various forms of nitrogen and phosphorus in the waters. These elements are disproportionately discharged into the water due to increasing industrialization, improper use of mineral fertilizers and high consumption of phosphate detergents or dishwashing detergents.

The cyanobacterial blooms in freshwater reservoirs, rivers and coastal waters pose many problems. They arise during May and remain present until the end of October, depending on various factors. Cyanobacteria produce several secondary metabolites. One of them is cyanotoxins, dangerous toxins threatening the health and lives of people and animals. Due to their stability and resilience, they are particularly dangerous in drinking water tanks and therefore their early detection is important.

Cyanobacteria and their toxins can be detected by various methods, but each has its advantages and disadvantages. Molecular biological methods offer, in comparison with other methods (microscopy, chemical methods, biochemical methods) accurate, fast and especially early detection of potentially harmful cyanobacteria and thus significantly help the monitoring of water bodies. Reliable

detection is one of the main ways to prevent threats to people, animals and economic interests, so it is important to address and further develop this area.

The main aim of my study is detect and quantify potential toxin producers with molecular methods, especially qPCR. *McyE* gene was chosen for this study. Comparing the results of molecular analysis with results of chemical method LC-MS/MS is another step to last but not less important aim, which is finding relation between toxin concentration and toxin synthetase genes.

2. Cyanobacteria and their toxins

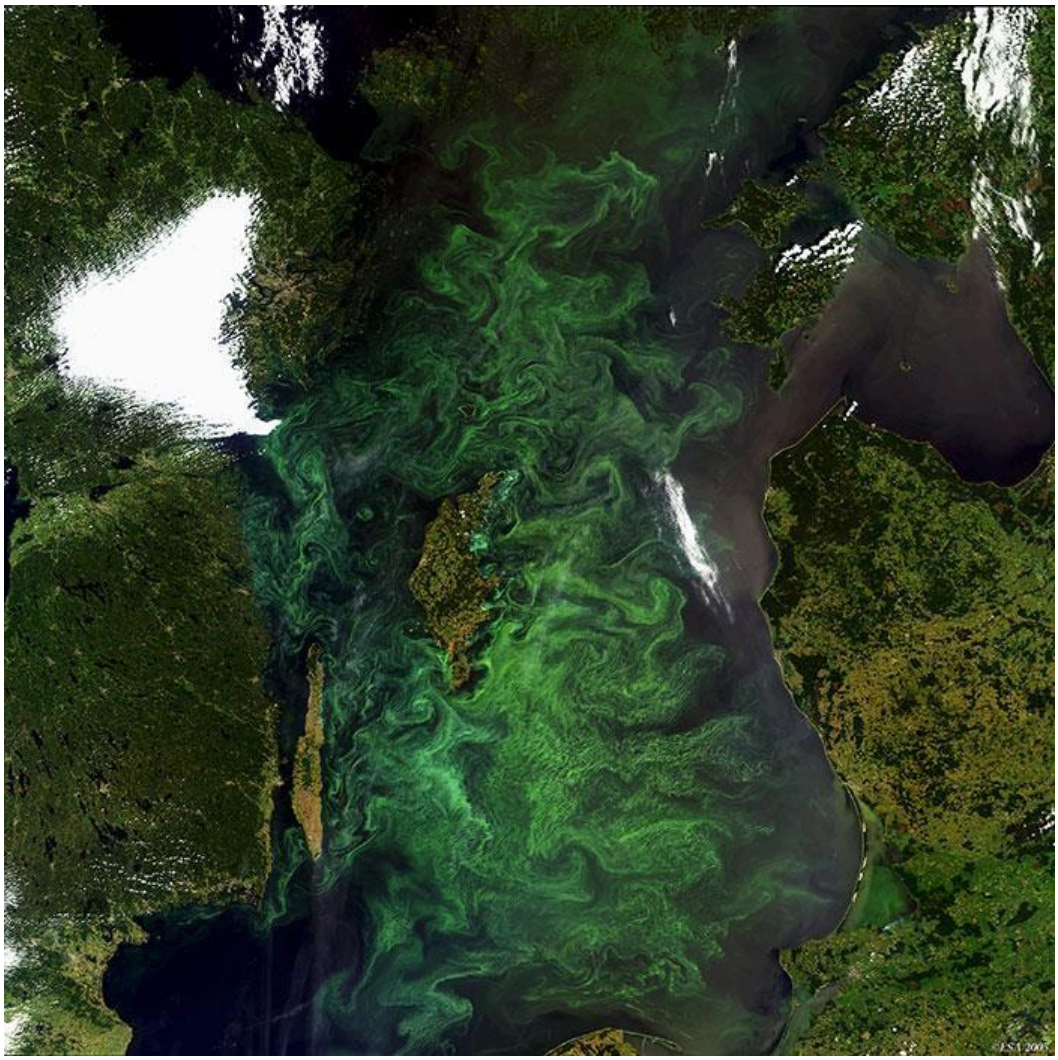
Cyanobacteria, originally called to as the ‚blue-green algae', are Gram-negative photosynthesizing prokaryotic microorganisms very similar to bacteria. The similarity with bacteria is primarily due to a cellular structure, the absence of a nuclear membrane or membrane-bound organelles present (Percival *et al*., 2014). More than 3500 million years ago, they have been found in a variety of environments, most often in fresh waters, brackish and marine waters, also on illuminated surfaces of rocks and soils (Meriluoto *et al*., 2017). Their ability to survive has been proven in extreme environments including dessert sand or volcanic ash (Chorus and Bartram, 1999).

Cyanobacteria are a photoautotrophic organism. They are able to perform photosynthesize through the green pigment, chlorophyll-a. Several species can also grow heterotrophically. These species are using organic compounds (especially phosphorus and nitrogen) as a source of carbon (Halm *et al*., 2011). In addition to chlorophyll, they also often produce blue pigments (phycobilins or phycocyanins) and red to brown pigments (phycoerythrins) (Gupta, 2015).

Cyanobacteria are morphologically diverse. Five cyanobacterial morphotypes were described – unicellular colonies with binary fission, unicellular colonies with multiple fission, multicellular colonies, multicellular colonies with differentiated cells, and branched multicellular colonies with differentiated cells (Schirrmeister *et al*., 2011). Cells of cyanobacteria are larger than most other bacteria (Singh and Montgomery, 2011). Colonies are often clearly visible by the naked eye and can form formation referred to as algal (cyanobacterial) blooms or, if they cause harmful effects, harmful algal blooms (HABs) (Figure 1) (Gupta, 2015).

The main reason for the occurrence of algal blooms is anthropogenic eutrophication (increasing the concentration of phosphorus and nitrogen nutrients) but also other environmental factors, including stagnant water conditions, higher pH values, and higher temperature. The presence of the algal blooms may indicate water quality problem, especially as many species of cyanobacteria are able to synthesize a wide range of substances, including potent toxins (Bláha *et al*., 2009). It has been found that about 75 % of cyanobacterial blooms are toxic (Chorus *et al*., 2000). The production of toxins is significantly higher during warm weather. Toxins production during HABs can be extremely high (Gupta, 2015).

Figure 1 – Satellite picture of Baltic sea, Harmful algal bloom (July 2005).



Cyanobacteria are producing several groups of toxins with different effects – hepatotoxins, neurotoxins, cytotoxins, dermato toxins and irritant toxins (Wiegand and Pflugmacher, 2005). Some toxins may exhibit more toxic effects on different structures (Svrcek and Smith, 2004).

2.1. Hepatotoxins

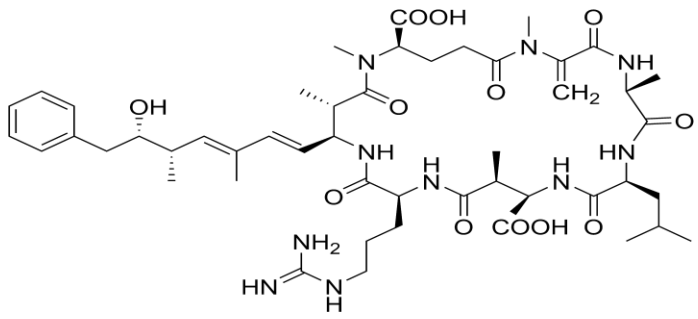
2.1.1. Microcystins

Microcystins (MCs) are the most frequently reported cyanotoxins produced by several freshwater genera of cyanobacteria – *Microcystis, Dolichospermum (*formerly reffered as *Anabaena), Planktothrix, Nostoc, Oscillatoria* and *Hapalosiphon* (Gupta, 2015; Dittman *et al*., 2013). Microcystins belong to the group cyclic heptapeptides. The general structure is cyclo – (D-Ala-L-X-D-MeAsp-L-Y-Adda-D-Glu-Mdha), in which X and Y represent different L amino acids. D-MeAsp is D-erythro-β-methyl-

aspartic-acid, Adda is (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid and Mdha is N-methyldehydroalanine (Figure 2) (Bláha *et al*., 2004). More than 80 variants have been described, mostly differing in amino acid positions X and Y, also in positions 2 and 4 or demethylation amino acids in positions 3 and 7 (Bláha *et al*., 2004; Chorus and Bartram, 1999). One of the most common variants is microcystin-LR which contain leucine and arginine (Gupta, 2015). Microcystins are extremely stable, hydrophile, water soluble and non-volatile (Bláha *et al*., 2004).

Suominen *et al*. (2016) found out that closely related genotypes have distinct nutrient physiologies. Phosphorus is more important for toxic genotype, non-toxic genotype prefer growing under phosphorus limitation. The dominance of toxic genotype increased after pulses of nitrogen and phosphorus. This competition between toxic and non-toxic genotypes of microcystins can be an important factor in determining cyanobacterial bloom toxicity. The ability of microcystins to adapt hight light conditions and protect themselves from oxidative stress might be their primary role.

Figure 2 – Microcystin chemical structure



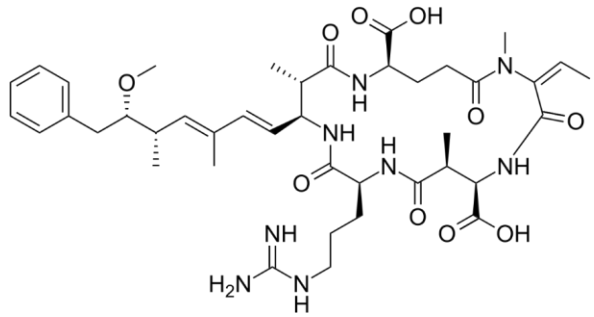
Microcystins have been shown very toxic (acutely and also chronically) to animals and humans. The value LD_{50} in mice (i.p.) is for most of the structural variants between 50–300 $\mu g.kg^{-1}$ b.w. (Chorus and Bartram, 1999). The mechanism of toxicity microcystins is based on irreversibly inhibit serine/threonine protein phosphatases (PP) 1 and 2A (Yoshizawa *et al*., 1990). Microcystins have primarily hepatotoxic effects. The acuteness of microcystin poisoning depends on the route of exposure, the level of exposure and also the mixture of components involved in the exposure. Low exposure levels to microcystins lead to skin irritation, respiratory and gastrointestinal

problems. Higher exposure levels (especially oral exposures) causes liver damage, which can lead to liver failure (Briand *et al*., 2003; Gupta, 2015). The first signs of liver damage which are possible to detect are increasing concentrations of liver enzymes in the blood and liver swelling. The symptoms of intoxication appear within hours of exposure and may include inappetence, depression, vomiting and diarrhea (Gupta, 2015). On a base several animal experiments were microcystins considered as a potential tumour promotion factor (Bláha *et al*., 2009).

2.1.2. Nodularins

Nodularins are toxins produced by filamentous bloom-forming cyanobacteria *Nodularia spumigena* (Moffitt and Neilan, 2004). *Nodularia* is found in brackish water and freshwater, worldwide. The blooms contain this cyanobacteria are common in late summer in Baltic sea and can cover very large areas, around 60 000 km^2 (Laamanen *et al*., 2001). Nodularins belong to the group cyclic pentapeptides. The general structure is cyclo – (D-MeAsp-L-Arg-Adda-D-Glu-Mdhb), in which Mdhb represent 2-(methylamino)-2-dehydrobutyric acid (Figure 3) (Rinehart *et al*., 1988). About 10 variants of nodularins have been discovered. The most abundant form of these group of toxins is Nodularin-R (Chen *et al*., 2013).

Figure 3 – Nodularin chemical structure



Nodularin has a potent hepatotoxic activity. The value LD_{50} in mice (i.p.) is between 30-50 $\mu g.kg^{-1}$ b.w. (Dittman *et al.*, 2013). Nodularin may enter the food-chain. Intoxication by nodularin may occur after ingestion contaminated water, fish or seafood, especially mussels and prawns. The hepatotoxin enters cells by active transport via transmembrane multispecific organic anion transporting polypeptides (OATPs) (Van Apeldoorn *et al*., 2007). The mechanism of toxicity is quite similar to that of microcystins but in a contrast to microcystin-LR, nodularin does not bind

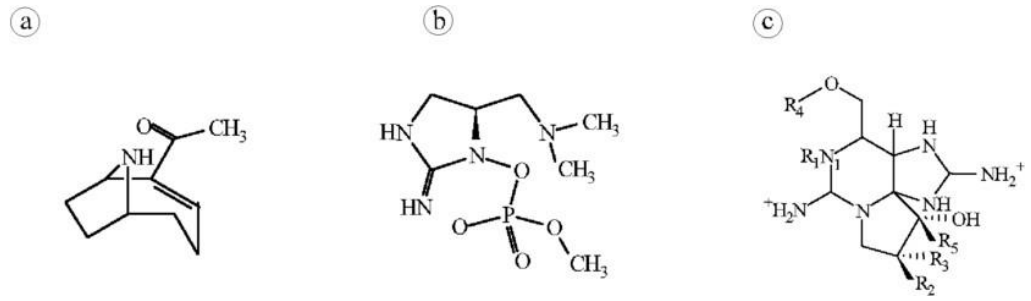
covalently to PP1 or PP2A (Lindwall *et al*., 1997). Nodularin is expressed in liver, kidneys, gastrointestinal tract and brain (Van Apeldoorn *et al.*, 2007). Besides to liver toxicity, nodularin also has initiating and tumor promoting activity. Nodularin has been identified as a liver carcinogen and is suspected to induce hepatocellular carcinoma (Ohta *et al.*, 1994, Meili *et al.*, 2016).

2.2. Neurotoxins

2.2.1. Anatoxin-a

Anatoxin-a is one of the more common, potent, fast-acting neurotoxin produced by several genera of cyanobacteria, specifically *Dolichospermum, Aphanizomenon, Microcystis, Planktothrix, Raphidiopsis, Arthrospira, Cylindrospermum, Phormidium, Nostoc* and *Oscillatoria.* It has been detected worldwide in freshwaters. (Osswald *et al*., 2007). The chemical structure of anatoxin-a is a different than a structure of hepapatoxins. Anatoxin-a is a bicyclic amino alkaloid, 2-acetyl-9-azabicyclo[4.2.1]non-2-ene (Figure 4a) (Duy *et al*., 2000). In a contrast with microcystins, phosphorus levels have no effect for production of anatoxin-a (Chorus and Bartram, 1999). Anatoxin-a is susceptible in the presence of sunlight and to microbial degradation (Rapala *et al*., 1994, Van Apeldoorn *et al*., 2007).

Figure 4 – Chemical structure of (a) Anatoxin-a, (b) Anatoxin-a(s) and (c) Saxitoxin



Anatoxin-a is classified as Very Fast Death Factor (VFDF) due to its lethality. The value LD_{50} in mice (i.p.) is 375µg/kg b.w. Death can occur within minutes to a few hours depending on the species, the amount of toxin ingested, and the amount of food in stomach (Carmichael, 2001, Chorus and Bartram, 1999, Spoerke and Rumack, 1985). This potent neurotoxin is a stereospecific postsynaptic cholinergic agonist that binds to nicotinic ACh (acetylcholine) receptors at neuromuscular junction at the same

position as ACh. It cause persistent stimulation and results in uncoordinated muscle contraction, paralysis of respiratory muscles and leading to death by asphyxiation (Gupta, 2015, Van Apeldoorn et al., 2017). Anatoxin-a do not cause characteristic blood chemistry changes or histological lesions which would be associated with poisoning (Rogers et al., 2005).

Anatoxin-a is a potentially biological weapon, which can be very dangerous because of its high potency, fast action and occasional availability of highly lethal concentrations of toxin during HABs. However, this toxin is very susceptible to rapid bacterial breakdown under non sterile storage conditions and thermolabile. No cases of use have been reported to date (Gupta, 2015, Zhang, 2014).

2.2.1.1. Homoanatoxin-a

A structural analogue of anatoxin-a is called homoanatoxin-a. It has a propionyl group at C-2 instead of the acetyl group in anatoxin-a (Van Apeldoorn *et al*., 2007). The producers are *Planktothrix formosa* and *Phormidium formosum* (Chorus and Bartram, 1999). The LD_{50} in mice (i.p.) is 250µg/kg bw. Symptoms of intoxication are similar to anatoxin-a including body paralysis leading to death within 7-12 minutes (Chorus and Bartram, 1999). Homoanatoxin-a is a relatively rare neurotoxin, but its lethal effects have been seen, for example, in Ireland (Furey et al., 2003) or New Zealand (Wood et al., 2007).

2.2.2. Anatoxin-a(S)

Anatoxin-a(S) is a potent neurotoxin produced by *Dolichospermum (Anabaena)* species – *A. flosaquae* and *A. lemmermannii* (Chorus and Bartram, 1999). It has been detected especially in North America (Mahmood *et al*., 1988), Denmark (Henriksen *et al*., 1997) and Scotland (Chorus and Bartram, 1999). Anatoxin-a(s) is a cyclic guanidine methyl phosphate ester which is pharmacologically and chemically distinct from anatoxin-a (Figure 4b) (Hyde and Carmichael, 1991). Structural variants of anatoxin-a(S) have not been described. The toxin is relatively stable under neutral or acidic conditions, but susceptible to rapid degradation under alkaline conditions (Chorus and Bartram, 1999).

The LD_{50} value in mice (i.p.) for anatoxin-a(S) is 40 – 60µg/kg b.w. and death can occur within minutes after exposure (Mahmood and Carmichael, 1986). Anatoxin-a(S) is a noncompetitive inhibitor of acetylcholinesterase with similar mechanism of

action to the organophosphorus insecticides. It is activated via oxidative metabolism (Gupta, 2015). Clinical signs of poisoning are indiscernible from organophosphate poisoning, including hypersalivation, urinary incontinence, defecation, muscle tremors, fasciculations, convulsions and respiratory failure (Mahmood and Carmichael, 1986). Although the anatoxin-a(S) is very similar to anatoxin-a and can be potential threat, its use requires sophisticated procedures and equipment to implement (Gupta, 2015).

2.2.3. Saxitoxins

Saxitoxins (STXs) are a group extremely potent neurotoxins collectively referred to as paralytic shellfish toxins (PSTs) (Cusick and Sayler, 2013). These toxins are produced in freshwater and marine environments Several genera of cyanobacteria, especially *Dolichospermum, Aphanizomenon, Planktothrix, Cylindrospermopsis, Lyngbya* and *Scytonema*, are responsible for a production in freshwater systems. In marine waters, the producers are eukaryotic dinoflagellates, belonging to the genera *Alexandrium, Gymnodinium* and *Pyrodinium* (Cusick and Sayler, 2013; Smith *et al*., 2012; Wiese *et al*., 2010). Saxitoxins are nonvolatile, highly polar, tricyclic perhydropurine alkaloids derived from imidazoline guanidinium (Figure 4c) (Schantz *et al*., 1975). At least 57 analogues of saxitoxin has been described (Wiese *et al*., 2010). Saxitoxins are resistant to heat and highly water-soluble, tasteless and odorless. They are not destroyed by normal food preparation methods (Trevino, 1998).

Saxitoxin is the most toxic toxin from STXs group and one of the most potent natural neurotoxins known (Wiese *et al*., 2010). The value LD_{50} in mice (i.p.) is 10µg/kg b.w., 263µg/kg oral and 3,4µg/kg intravenous (Wilberg and Stephenson, 1960). The mechanism of action is based on blocking of sodium channels. Saxitoxins cross the blood-brain barrier and sodium channel blockade in the CNS causes paralysis (Borison and McCarthy, 1977; Gupta, 2015). The symptoms of paralytic shellfish poisoning (PSP) are highly dependent on the amount of toxin ingested. Low level of intoxication causes moderate paresthesia, often described as a tingling sensation around the mouth and extremities. High levels of exposure are generally associated to ingestion of toxin-accumulating shellfish or fish and may cause muscle paralysis and respiratory failure leading to death (Garsia *et al.*, 2004; Gupta, 2015).

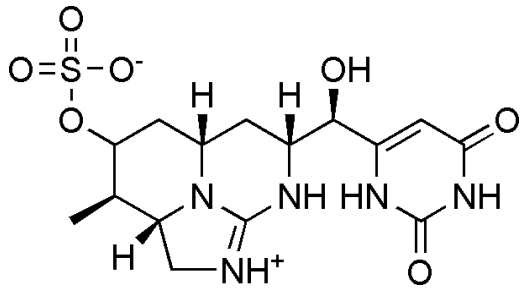
Saxitoxin is the only one of the cyanotoxins listed in the Chemical Weapons Convention Schedule 1 due to its danger, stability, and capability to be produced synthetically (Gupta, 2015).

2.3. Cytotoxins

2.3.1. Cylindrospermopsin

Cylindrospermopsin (CYN) is a cyanotoxin with cytotoxic, hepatotoxic and neurotoxic effects produced by several freshwater cyanobacteria species, especially by *Cylindrospermopsis raciborskii,* but also *Umezakia natans, Aphanizomenon sp., Raphidiopsis curvata, Lyngbya wollei* and *Anabaena bergii* (Pearson *et al*., 2010). CYN is a cyclic sulfated guanidine alkaloid (Figure 5). The molecule of this toxin is zwitterionic and highly water-soluble (Ohtani *et al.*, 1992). Two structural variants have been described – deoxycylindrospermopsin, which is less toxic, and more toxic 7-epicylindrospermopsin. CYN is resistant to high temperatures, sunlight and pH extremes (Chiswell *et al*., 1999).

Figure 5 – Chemical structure of cylindrospermopsin



The LD value in mice (i.p.) for cylindrospermopsin is 2100 µg/kg b.w. The mechanism of action is based on inhibition of glutathionine, protein synthesis and cytochrome P450, but the exact mechanism of action has not been described (Bazin *et al*., 2010; Pearson *et al*., 2010). Symptoms of cylindrospermopsin intoxication were first described after mass poisoning of humans in Palm Island (Australia) in 1979. Symptoms included hemorrhagic diarrhea, fever, vomiting, dehydration, hepatomegaly, electrolyte imbalances, acidosis and hypovolemic shock (Hawkins *et al*., 1985). Intoxication by cylindrospermopsin can lead to liver, kidney, thymus and heart damage (Terao *et al.*, 1994).

3. Commonly used detection methods of cyanobacteria

3.1. Light microscopy

Light microscopy is a basic and traditional method for the identification of cyanobacteria. This inexpensive method is very useful for getting a lot of information about morphology and cellular differentiation of the morphotypes. It is also very suitable for counting a cyanobacteria present in a sample (Kurmayer *et al*., 2017). The identification and enumeration of cyanobacteria is usually carried out using the Utermöhl method (Utermöhl, 1931). The principle of this method is based on a sedimentation of measured aliquots of the sample (which was preserve by formaldehyde or Lugol's solution) into counting chambers with a determined volume. After sedimentation, eukaryotic phytoplankton and cyanobacteria are identified and counted with an inverted microscope (Kurmayer *et al*., 2017).

Light microscopy is an important step to obtain a general overview of the sample under study. However, this method is time consuming and requires a high level of operator knowledge. Moreover, this method cannot be used to determine the toxin produced, to distinguish between species that are morphologically similar or toxic from non-toxic microalgal populations (Eckford-Soper and Daugbjerg, 2014; Kurmayer *et al*., 2017).

3.2. Molecular methods

Since the early 1990s, molecular methods have been used in the detection of cyanobacteria. These methods are based on the detection of genes present in cyanobacteria and genes associated with cyanotoxin synthesis. Compared to classical methods (e.g. light microscopy), immunological or chemical methods, they are characterized by higher specificity, reliability and speed. These techniques allow early detection of toxic cyanobacteria and thus represent a very useful method for monitoring. Most of the molecular techniques are based on PCR technique (conventional PCR, multiplex PCR, qPCR and others), but non-PCR-based techniques (FISH, DNA microarray) are also used (Moreira *et al*., 2014; Sanseverino *et al.*, 2017).

3.2.1. PCR

Polymerase Chain Reaction (PCR) is the most frequent, widespread molecular method, which was developed by Karry Mullis in 1983 (Shafique, 2012). This

technique allows the creation of multiple copies of specific gene fragments. The PCR method is used to identify targeted small DNA fragments that serve as a template for the design of primers. Primers are specific regions of 18-25 bp DNA that complementarily bind to template single-stranded DNA. Subsequently, new copies of the defined DNA region are formed between the bound primers by the enzyme thermostable polymerase. The most common thermostable polymerase is *Taq* polymerase isolated from thermophilic bacterium *Thermus aquaticus*. Other indispensable components of the PCR reaction are deoxynucleotide triphosphates (dNTPs) and a suitable buffer to ensure an optimal reaction environment (Kurmayer *et al*., 2017; Šmarda *et al*., 2005).

The principle of the PCR method is based on alternating temperatures that are repeated in multiple cycles. The first step is denaturation, which separates double stranded DNA to single strands. This is followed by the annealing step in which the primers are bound to DNA strands. During the final elongation step, dNTPs are attached by polymerase and a new strand is synthetized. The amplification results in millions of copies of the targeted DNA sequences. Visualization of the obtained products is performed by gel electrophoresis (Šmarda *et al*. 2005).

The PCR is commonly used method for the detection of cyanobacteria. It is used to generally detect the presence of cyanobacteria in water samples by amplifying genes for 16S ribosomal RNA (rRNA), a gene generally used for the identification of prokaryotes (Sanseverino *et al*., 2017). Similarly, this method can also detect toxins-producing cyanobacterial genera and their genes for toxins such as microcystin, nodularin, saxitoxin, anatoxin-a or cylindrospermopsin. Specific primers have been developed for detection of genes present in the gene clusters encoding these biosynthetic enzymes (Moreira *et al*., 2014). In addition to conventional PCR, when only one gene can be amplified per reaction, multiplex PCR is being developed. Thus, it is possible to identify multiple genes simultaneously in one reaction, e.g. to identify different genes associated with cylindrospermopsin production or to distinguish microcystin producing from non-producing *Microcystis* strains (Fergusson and Saint, 2003; Ouahid *et al*., 2005; Sanseverino *et al*., 2017). The disadvantage of the PCR method is often insufficient sensitivity and inability to quantify the toxin in the sample (Moreira *et al*., 2014).

3.2.2. qPCR

Quantitative PCR (qPCR) or also Real Time PCR (RT-PCR) is an advancement of conventional PCR, although the basic principle of the methods is the same. Compared to traditional PCR, where the PCR products (amplicons) are analyzed after the final PCR cycle, the amount of target sequence of DNA is measured after each cycle during qPCR. Fluorescent markers incorporated to amplicons emit a fluorescent signal that is captured in the real time. Two most frequently fluorescent reporter systems are used – intercalating dyes (e.g. SYBR Green) and probe based system (TaqMan hybridization probe system) (Martins and Vasconcelos, 2011). The principle of qPCR is based on increasing amount of targeted sequence in each cycle. Basically, the more target sequence, the earlier the amplification exceeds a set a threshold. The cycle in which is threshold exceeded is called threshold cycle (C_T) or quantification cycle (C_q-value) (Kurmayer *et al*., 2017).

The qPCR method is widely used molecular method in microbial ecology (Martins and Vasconcelos, 2011). It can be used to measure gene copy numbers, studying sample series (e.g. seasonal dynamics of toxigenic cyanobacteria), study of influence of variable environment on gene transcript levels, etc. (Kurmayer *et al*., 2017). A several qPCR assays have been developed for quantification of toxin genes. Most of these assays have been developed for quantification of different microcystin synthetase genes (e.g. *mcyA, mcyB, mcyD and mcyE*) (Pacheco *et al.,* 2016). The quantification of other toxin genes are not so widespread as in the case of microcystins. A few assays have been designed for quantification of cylindrospermopsin biosynthetic genes (*cyrA,cyrC, cyrJ, rpoC1*) (Pacheco *et al.*, 2016), saxitoxins biosynthetic genes (*stxA, stxG* and *stxB*) (Savela *et al*., 2015) and anatoxin biosynthetic genes (*anaC* and *anaF*) (Rantala-Ylinen *et al*., 2011).

During the cyanobacterial bloom, several species of cyanobacteria may occur at the same time and produce different classes of cyanotoxins. Multiplex qPCR is an effective solution how to quantificate more different toxins in one reaction, save time, samples and costs. This method was first described by Al-Tebrineh *et al*. (2012). Four different toxin biosynthesis gene clusters were targeted (microcystin, nodularin, cylindrospermopsin and saxitoxins) and 16S rRNA gene was used as an internal control. The selected biosynthetic genes were *mcyE, ndaF, cyrA* and *stxA*. The assay for detection of different target genes includes TaqMan probes with different

fluorophores with different fluorescence spectra at 5'end and a quench group at the 3'end. This method has many advantages – high sensitivity, fast results, cost-effectiveness and high throughput capacity. The disadvantage of multiplex qPCR is complicated design of assay (compatible primers, probes and reaction conditions) (Al-Tebrinah *et al*., 2012; Pacheco *et al.*, 2016).

The qPCR is a widely applicable method. Due to its sensitivity, specificity and ability to quantify the targeted gene, it is an indispensable tool for monitoring HABs. Another advantages are costs and time saving. Disadvantages are the possibility of inhibiting the reaction by the presence of inhibitors in the sample and thus showing false negative results and the inability of the method to distinguish between toxic and non-toxic variants of the gene. The results of this method are recommended to be verified by other methods, e.g. chemical or biochemical (Pacheco *et al.*, 2016; Sanseverino *et al*., 2017).

3.3. Chemical methods

Chemical methods are an essential tool for detection and quantification of a toxin in environmental samples. Most of the chemical methods used for analysis of cyanobacterial toxins are liquid-based separations. These methods, included HPLC (High-Performance Liquid Chromatography) and LC-MS (Liquid Chromatography Mass Spectrometry), are the most common (Moreira *et al.*, 2014).

3.3.1. HPLC

High-Performance Liquid Chromatography (HPLC) is a method used to separate sample components and determine their presence and concentration in the sample. The sample represent a mixture of analytes, which are passing through a column filled with adsorbent (stationary phase) and transport of analytes is facilitated by a flow of liquid (mobile phase). This method uses polarity and charge, rarely also size differences between the analytes (Hiskia *et al.*, 2017). Important part of the HPLC is a detector. Detectors provide detection of analytes. HPLC with photodiode array (PDA) or ultraviolet (UV) absorbance are useful tools for detection of cyanotoxins that have a UV chromophore, (e.g. microcystins, nodularins, cylindrospermopsin and anatoxin-a) (Sanseverino *et al*., 2017). Mass spectrometric (MS) detection is useful for almost all cyanotoxins (Hiskia *et al.*, 2017).

Currently, the most often applied HPLC method for detection of cyanotoxins is reversed-phase HPLC (RP-HPLC). In this modification, the stationary phase has a nonpolar character and the mobile phase has a polar character. The stationary phase used in RP-HPLC is based on modified silica gel to which hydrophobic alkyl chains (mainly C18) are attached (Sanseverino *et al*., 2017). RP-HPLC often involves the use of gradient mobile phases consisting of acetonitrile in water (Meriluoto, 1997). This modification of HPLC can be used for detection of microcystins, nodularins, cylindrospermopsin and derivatized anatoxin-a. Saxitoxins can be separated by this technique if ion-pairing reagents are used to increased their retention. Another variant of HPLC, Hydrophilic Interaction Liquid Chromatography (HILIC) can be used for very hydrophilic cyanotoxins (e.g. underivatized anatoxin-a, cylindrospermopsin, saxitoxins) (Hiskia *et al*., 2017).

HPLC is a standard, commonly used method, although it has its disadvantages and limitations. The technique is time consuming, does not measure toxicity, does not provide definite identification and the results may be distorted by the presence of other compounds with similar absorbance spectra. In addition, the poor commercial availability of compounds required for standardization and calibration and lack of standards makes using this method more difficult. Despite all the disadvantages, this method is still the most widely used due to its sensitivity (0,02μg/l), efficiency and automation capability (Hiskia *et al*., 2017; Kaushik and Balasubramanian, 2013; Mathys and Surholt, 2004).

3.3.2. LC-MS

Liquid Chromatography –Mass Spectrometry (LC-MS) is basically HPLC unit with MS detector attached to it. Modification of this method, Liquid Chromatography-Triple Quadrupole Mass Spectrometry (LC-MS/MS), which has an additional MS detector, is due to its excellent sensitivity and selectivity a perfect tool for unambiguous identification and quantification of different and unknown toxins in environmental samples (Sanseverino *et al*., 2017). A standard for the precise quantification of toxins is not necessary, which is a great advantage over other methods (Moreira *et al*. 2014). This method is relatively expensive for routine screening, and therefore initial analysis of the samples is performed by different method (ELISA) (Van Apeldoorn *et al*., 2007).

3.4. Biochemical methods

Biochemical methods are useful for easy, cost-effective and rapid monitoring of cyanotoxins concentration. Among those method, the most commonly used method is ELISA (Enzyme-Linked Immunosorbent Assay). This technique may serve as complementary to molecular methods to confirm the presence of toxin in water (Sanseverino *et al*., 2017).

3.4.1. ELISA

The Enzyme-Linked Immunosorbent Assay (ELISA) is a method used to determine the occurrence of toxin and its quantification in the sample. This technique is an antibody based assay, which can use monoclonal or polyclonal antibodies. ELISA method is useful as primary quantification screening tool with detection limit 0,1 µg/L (Sanseverino *et al*., 2017).

Antibodies have been developed against the cyanotoxins such as microcystins, nodularins, saxitoxins or cylindrospermopsin. These assays can be used for detection of toxins not just in the water samples, but also in the samples such as wastewater, cyanobacteria biomass and blooms or plant and animals contaminated with toxins (Moreira *et al*., 2014).

ELISA is quite reliable method. False negatives results are not frequent, but the occurrence of false positive is possible (Rivasseau *et al*., 1999). The main advantages of this method are high sensitivity, low detection limits, rapid detection of the toxin and minimal capital set-up cost. The disadvantage is the inability to identify the toxin producer, but only the toxin already present in the sample (Elliot *et al*., 2013; Gaget *et al*., 2017).

3.4.2. PPIA

Protein Phosphatase Inhibition Assay (PPIA) is a rapid, sensitive, simple and reproducible method. This colorimetric assay using protein phosphatase 2A and substrates such as p-nitrophenyl phosphate for determining hepatotoxins (microcystin and nodularin) in water. Due to high sensitivity, the samples of water do not need any treatment and the assay can be used immediately. This assay is able to determine MC at concentrations below the provisional WHO (World Health Organisation) guideline (1µg/L of microcystin-LR). Unclear results may be obtained if too many unknown

substances are present in the sample, which may lead to an overestimation or underestimation of the toxin concentration. The assay is unable to distinguish between microcystin and nodularin, nor the individual forms of these toxins. If the test result is positive, further analyzes should be considered. PPIA test is commercially available (Heresztyn and Nicholson, 2001; Sanseverino *et al*., 2017).

4. Risk assessment and case studies

Scientific studies have already clarified much about cyanobacteria, but they are still unable to safely predict and assess the risk of human exposure to them. Risk assessment is based on identification of cyanobacteria, the assessment of possible harmful effects on individuals or populations, the assessment of all possible routes of exposure and the effects of the toxin dose. Human exposure to cyanobacteria and their toxins may occur in several ways, most commonly oral (ingestion of contaminated water) and skin exposure to toxins during recreational activities, or inhalations. Cases of intoxication during hemodialysis treatment have been also reported (Churro *et al.*, 2012).

Due to changes occurring on the planet and leading to water eutrophication, HABs develop and cyanotoxin poisonings are more common. The first mention about cyanotoxin poisoning dates back to 1878 from South Australia when cattle died after the ingestion of contaminated water from Lake Alexandrina. Since then, more than 80 cases of animal deaths have been reported, most commonly cattle, wild animals, sheep, pigs, birds and fish and less often horses and dogs (Codd and Poon, 1988; Duy *et al.*, 2000).

Human poisoning by cyanotoxins has been reported on all continents except Antarctica. The most serious cases leading to damage to human health or death were reported after ingestion of contaminated water. The first reported case of cyanotoxin-related human intoxication occurred in the United States in 1931, when 5000 people got sick by gastroenteritis. The reason was the massive cyanobacterial bloom *Microcystis* found in the Ohio River, which supplied many cities with water (Tisdale, 1931). In Australia, a large invasion of *Cylindrospermopsis raciborskii* was observed in the Palm Island Dam in 1979. After algicidal intervention, the toxins were massively released into the water and as a result more than 140 people, mostly children, became ill (Byth, 1980). In Brazil in 1988, more than 2000 cases of gastroenteritis were reported during 42 days after filling the Itaparica dam, which was a source of drinking water. Eighty-eight people died. Subsequent analyzes of water detected high concentrations of toxic cyanobacteria, especially *Anabaena* and *Microcystis* (Teixera *et al*., 1993).

Intravenous intoxication occurred in 1996 in Caruaru (Brazil), when 130 patients of the hemodialysis center were intoxicated. 56 of them died as a result of poisoning. The symptoms of all patients were very similar – visual disturbances, nausea, muscle weakness and painful hepatomegaly, which in some cases led to acute liver failure. Microcystins were subsequently detected in the blood serum of exposed patients and the liver tissue of dead patients. The poisoning was caused by the presence of cyanobacteria of the genera *Aphanizomenon* and *Oscillatoria* in the reservoir serving as a source of water for the dialysis center (Jochimsen *et al.*, 1988).

Poisoning associated with recreational exposure (swimming and water sports) has also been reported. These intoxications did not have as serious consequences as intoxication due to ingestion of contaminated water. The most common symptoms were local allergic manifestations, headaches, nausea, diarrhea, eye and ear irritation (Chorus and Bartram, 1999).

5. Aims

Here, in my master thesis, I aim to (1) detect and quantify potential toxin producers with molecular method qPCR, (2) compare the results with traditional methods, which is in the case of this study chemical method LC-MS/MS and (3) find the relation between toxin concentration and toxin synthetase genes.

6. Materials and methods

6.1. Site description

Samples for analysis were collected from lakes in Estonia. More than 1400 lakes are present in Estonia. Lake Peipsi is the largest (surface area 3555 km^2) lake in Estonia and the fourth largest lake in Europe. It is classified as an eutrophic lake (Nõges *et al.*, 2007). With a mean depth of 7,1 m and maximum depth of 15,3 m, it is one of the shallow lakes. The water level in the lake fluctuates in an average annual range of 1,15 m. The water volume is 25 km^3. The lake is located on the Estonian-Russian border and is the largest transboundary lake in Europe (Jaani, 2001).

The catchment area consists of three basins with a total area of 47 800 km^2 and is shared between Estonia (34%), Russia (58%) and Latvia (8%). The largest and deepest part of the area is the Lake Peipsi on the north. The southern part of the area, Lake Pihkva, is connected to Peipsi by a narrow river-like lake Lämmijärv. The largest inflows are Emajõgi River in Estonia and Velkaya River in Russia. The outflow from the lake is Narva River, running into the Gulf of Finland, part of the Baltic sea. The Narva River is a source of drinking water for the city of the same name (Jaani, 2001; Kangur and Möls, 2008).

Lake Võrtsjärv is the second largest lake in Estonia and the largest lake belonging entirely to Estonia. Surface area of the lake is 270 km^2 and mean depth 2,8 m. The watershed of Lake Võrtsjärv is 3 104 m^2 and lies within the Lake Peipsi catchment. Lake Võrtsjärv is, like Peipsi, characterized as eutrophic. The largest inflow of Võrtsjärv is Väike Emajõgi, and outflow is River Emajõgi runs to the Lake Peipsi (Nõges et al., 2010).

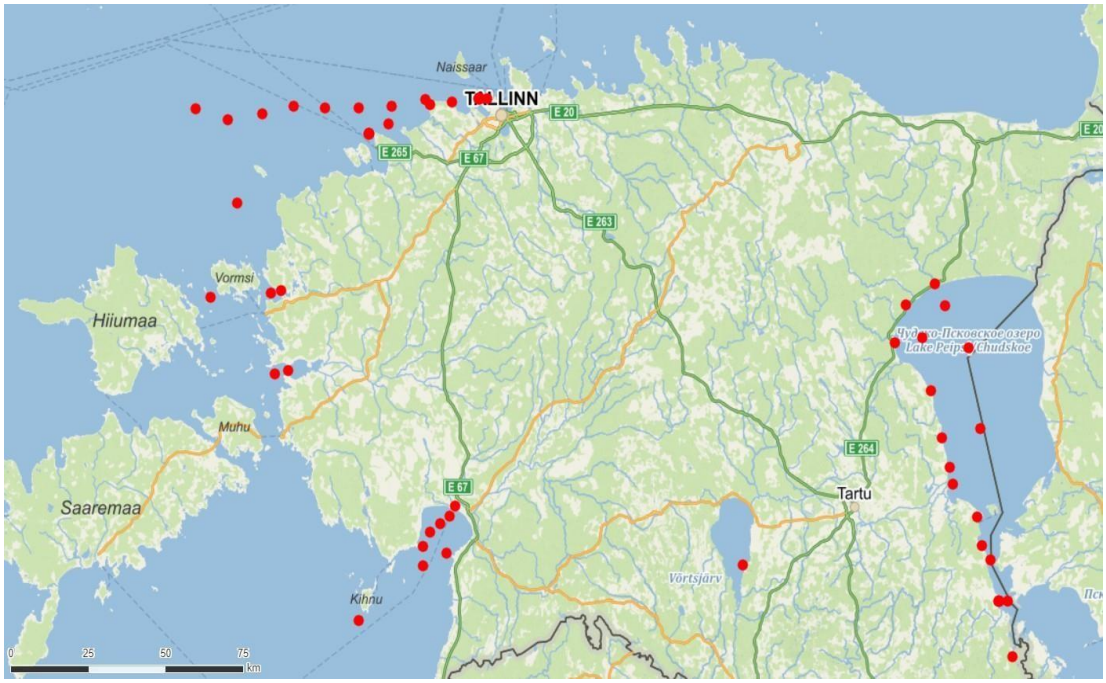
The Baltic Sea is mediterranean sea of the Atlantic ocean with catchment area 1 641 650 km^2. The sea is located between Denmark, Sweden, Estonia, Finland, Latvia, Lithuania, Germany, Poland and Russia. Surface area of the sea is 377 000 km^2 and average depth 55 m (maximum depth 459 m). The Baltic Sea is the world's largest inland brackish sea with low salinity (average 3,5%) (Cohran *et al.*, 2019).

6.2. Sampling

Sampling took place between 2018 and 2019 at several sites (Figure 6). Samples from Lake Peipsi were collected during May to October 2018 at 7 sampling

sites (5 sampling sites Peipsi and 2 Lämmijärv), samples from the littoral parts of the lake were collected in August 2018 at 10 sampling sites. Samples from Lake Võrtsjärv were collected between February 2018 and January 2019 at one site. In August 2018, samples of 7 sites in Pärnu were collected. Samples from the Baltic Sea were collected at 17 different sites in April, May and July 2018. The coordinates of the sampling points are in Appendix 1.

Figure 6 – Map of Estonia with sampling points



Samples were collected from integrated water and stored in the refrigerator on board of a boat. During the transportation to the lab the samples were stored in cool box. The samples were filtered through Whatman® mixed cellulose ester membrane filters, pore size 0,45µm (Sigma Aldrich, St. Louis, Missouri, USA) and stored in 96% molecular grade ethanol solution in –80°C until DNA extraction.

6.3. DNA extraction

DNA extraction was performed using the DNeasy PowerWater Kit (Qiagen, Hilden, Germany) according to the manufacturer‘s instructions. Each DNA extraction was performed in triplicate. DNA quality and quantity was measured using NanoDrop 2000 UV-Vis spectrophotometer (Thermofisher Scientific, Waltham, Massachusetts, USA).

6.4. qPCR

Identification and quantification of potential toxin producing genera of cyanobacteria in environmental samples was performed by genus-specific qPCR. External standards *Microcystis sp*. 205, *Dolichospermum sp*. 315 and *Planktothrix sp*. 49 from HAMBI Culture Collection (University of Helsinki) were used to quantify *mcyE* gene copy numbers.

The standard dilutions containing 10^6, 10^5, 10^4, 10^3, 10^2 and 10^1 copies of the mcyE gene were prepared with genomic DNA. In order to calculate and prepare the standard dilutions was necessary to know the size (bp) and mass (g) of the genome as well as the DNA concentrations of the strain used. The genome size of the particular strains used to prepare the standards is not known, therefore, estimated values or determined for other strains of each genus, were used instead. The mass of the genomes of *Dolichospermum*, *Microcystis* and *Planktothrix* (Table 1) were calculated according to the following equation. The calculation assumes that the molecular weight of 1 bp is 660 g.mol^{-1}. The Avogadro's constant (6,02214086 x 10^{23} mol^{-1}) is used in the equation.

$$\frac{Genome\ size\ (bp)\ x\ 660\ g/mol}{6{,}02214086\ x\ 10^{23}\ mol^{-1}} = Genome\ size\ (bp)\ x\ 1{,}096\ x\ 10^{-21}\ g/bp$$

Table 1: Data for calculation of the standard curves used in the qPCR

Strain	Size of genome (Mb)	DNA concentration (ng/µl)
Dolichospermum sp. 315	5,17 (Teikari *et al.*, 2019)	104
Microcystis sp. 205	5,84 (Kaneko *et al.*, 2007)	37,1
Planktothrix sp. 49	5,05 (Christiansen *et al.*, 2003)	149

The qPCR was performed in a 384 well-plate with 2,5µl environmental DNA and 7,5µl of mastermix. The mastermix for *Microcystis* and *Dolichospermum mcyE* gene was prepared with HOT FIREPol®Probe qPCR Mix Plus (ROX) (Solis Biodyne,

Tartu, Estonia), 300nM of both primers (Metabion International AG, Planegg, Germany) and 200nM of probe. The concentration of primers for *Planktothrix mcyE* gene was 900nM and probe 100nM. The sequences of used primers and probes are shown in Table 2.

Table 2: Primers and probes used in the qPCR reactions

Gene	Primer	Sequence	Reference
Microcystis-mcyE	127F	5'-AAGCAAACTGCTCCCGGTATC-3'	(Sipari *et al*., 2010)
	247R	5'-CAATGGGAGCATAACGAGTCAA-3'	
	186P	5'-GCAATGGTTATGGAATTGACCCCGGAGAAAT-3'	
Dolichospermum-mcyE	611F	5'-CTAGAGTAGTCACTCACGTC-3'	(Sipari *et al*., 2010)
	737R	5'-GGTTCTTGATAGTTAGATTGAGC-3'	
	672P	5'-CAAGTTCCCACAATTCTTGGATTAGCAGC-3'	
Planktothrix-mcyE	664F	5'- CATTGCCGGATTAGGCGT-3'	(Panksep *et al*., 2020)
	744R	5'- AGGATTTTTGCAGAGGAATTGTG-3'	
	670P	5'-TAAAAGTGAATATGATTATCAAGAACCCATCCCC-3'	

Samples of environmental DNAs were diluted 1:10. All the reactions were performed in triplicates. MQ water was used as a negative control.

Amplification was performed in a QuantStudio™ 5 System (Thermofisher Scientific, Waltham, Massachusetts, USA) following the protocol: an initial preheating step of 15 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C

and 62°C (*Microcystis-mcyE* and *Dolichospermum-mcyE*) or 60°C (*Planktothrix-mcyE*) for 1 minute.

The amplification results were analyzed using the Standard Curve and Relative Quantification application on ThermoFisher Connect™ web interface (Applied Biosystems).

6.5. LC-MS/MS

The concentration and variants of microcystins were analysed using liquid chromatography mass spectrometry (LC-MS/MS). The analysis was provided by Demokritos (Greece) according to the standard methods.

7. Results

Chapter “7. Results” is not available in the online version of the thesis.

8. Discussion

Chapter “8. Discussion” is not available in the online version of the thesis.

9. Conclusion

Global warming, together with the increasing negative impact of industry and agriculture on water bodies, presents us with a new phenomenon, harmful algal blooms, formed by a cyanobacteria producing dangerous toxins. The literature part of this diploma thesis dealt with the toxins that cyanobacteria produce, methods of their detection and risk assessment and case studies. Cyanotoxins are divided into several categories according to their effect on hepatotoxins, which include, for example, microcystins, the detection of which I dealt with in this work, as well as neurotoxins and cytotoxins. Metods for detecting cyanobacteria and their toxins have been described. Molecular methods and their use in the detection of cyanobacteria were given the most space, especially qPCR, which was used in this study. Risk assessment and case studies were mentioned at the end of the literature part.

The main aims of the thesis were to detect and quantify potential producers of cyanotoxins using the molecular method qPCR and then compare the results with the results of chemical analysis performed by LC-MS/MS. The samples used for this work were collected during year 2018 in the Estonian lakes Peipsi, Lämmijärv, Vortsjärv, and also in the Baltic Sea. DNA was subsequently extracted from these samples and qPCR analysis was performed to amplify the *mcyE* gene from three potential producers - *Microcystis*, *Dolichospermum* and *Planktothrix*. Toxin analysis was performed by LC-MS/MS. The results of both analyzes were statistically evaluated.

Using the qPCR method, the *mcyE* gene was detected in most of the analyzed samples, except of the samples from the Baltic Sea, where the presence of this gene was not confirmed. The analysis showed the highest concentration of the target gene during the summer months, when cyanobacteria are most active. The highest values were measured in Lake Lämmijärv, which is classified as highly eutrophic. The most represented producer of microcystins was *Microcystis*, the remaining studied producers were represented in several times smaller quantities.

Toxin analysis showed the presence of 5 variants of microcystins, the total amount of which was positively correlated with the detected *mcyE* gene copy numbers. The most common variant of microcystins was the MC-RR variant. The highest incidence of toxins was also found in Lake Lämmijärv. No microcystins were detected

in the Baltic Sea. This fact is confirmed by the results of the analysis of samples from the Baltic Sea using qPCR.

Qualitative parameters of water from the studied areas were also included in the analysis, the results of which were also compared. A positive correlation was statistically significant between the concentration of the *mcyE* gene copy numbers and amount of cyanobacterial biomass or chlorophyll-a. Subsequently, a positive correlation was also statistically significant between the concentration of MC and cyanobacterial biomass, as well as the amount of chlorophyll-a. The effect of water temperature or the correct ratio of nitrogen and phosphorus on the occurrence of individual species of cyanobacteria in the studied areas was also observed.

The results of this work provide an overview of the development of cyanobacteria and their toxins in the Estonian large lakes and in the Baltic Sea during year 2018 and confirm and meet the set aims.

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Figures

11. Appendices

Chapter “11. Appendices” is not available in the online version of the thesis.