Master Thesis

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Water Quality, Cyanobacteria and Cyanotoxins in Two Freshwater Reservoirs on the Coast of Georgia

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Abstract

Cyanobacteria constitute a diverse group of photoautotrophic bacteria that inhabit a wide range of aquatic and terrestrial environments. Several aquatic bloom-forming species of cyanobacteria produce toxic secondary metabolites that are hazardous to humans and animals. This thesis presents the study of cyanobacterial communities and cyanotoxins in relation to water quality parameters in two coastal lagoons - Lake Paliastomi and Lake Nurigeli situated in Georgia. Microscopic, immunological (ELISA) and molecular techniques (PCR) combined with physical-chemical parameters were applied to evaluate ecological conditions of Lake Paliastomi and Lake Nurigeli with the focus on cyanobacteria and cyanotoxins. Cyanobacteria species identified by microscopy in these lakes belong to genera *Anabaena*, *Anabaenopsis* and *Microcystis*. Microscopic analyses were in accordance with PCR amplification, which was positive for oxyphotobacteria specific primers. Negative results on PCR when amplifying *mcy* gene regions could also be linked to low concentrations of microcystin. Toxin analyses demonstrated low concentrations of microcystin and saxitoxin and high concentrations of anatoxin-a in two lakes. On the bases of total phosphorus concentrations (0,1-0,2 mg/L), temperature (25,2 - 29,2°C) and pH (7,4 - 9,2) Lake Paliastomi and Lake Nurigeli provide good environments for cyanobacteria.

Keywords: Cyanobacteria, cyanotoxins, water quality, Lake Paliastomi, Lake Nurigeli, Georgia

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Acknowledgments

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1. Introduction

Cyanobacteria are morphologically diverse group of gram negative bacteria that inhabit the earth ecosystem from ancient time. They are oxygenic photoautotrophic organisms and play major role in nutrient cycle. However, more often cyanobacteria are infamous for their ability to produce toxic secondary metabolites. They are common in aquatic environments and become dominant during high nutrient availability and temperature, resulting in cyanobacterial blooms. These blooms are in most cases toxic and are considered as a global environmental problem (Bitton 2002).

The consequences drawn by cyanobacterial blooms are significant to humans and animals worldwide. Apart from the odor and taste cyanobacteria give to the water, such water could be poisonous while consumed by livestock. The health risk effects vary according to the concentration and type of cyanotoxins from skin irritation to severe damage of intestines, liver, or even death (Nybom 2013).

Cyanobacterial blooms and toxin production are influenced by various environmental factors. Temperature, pH, light intensity, nutrients and environmental pollutants in general may have impact on metabolism and development of cyanobacteria (Boopathi and Ki 2014). Therefore, monitoring the condition of aquatic environment in line with occurrence of cyanobacteria and their toxins is essential.

Two freshwater reservoirs Lake Paliastomi and Lake Nurigeli on the coastal zone of the Black Sea in Georgia were studied for water quality, cyanobacteria species composition and cyanotoxins for this thesis. The present lakes are both Black Sea lagoons, situated in the centers of two different cities L. Nurigeli in Batumi and L. Paliastomi in Poti.

Coastal lagoons are interesting from ecological point of view for their sensitive ecosystems. They are often vulnerable to pollution and anthropogenic influence (Dassenakis et al 2006). The above mentioned lakes are not exceptions for such influence. Like many waterways, these lakes and especially L. Paliastomi underwent changes due to anthropogenic influence. This includes discharge of pollutants, overfishing and direct connection to the Black Sea. The latter factor altered water composition of L. Paliastomi, turning it into brackish water (Komakhidze 2006). These lakes have been previously studied for limnological parameters as well as for phytoplankton composition. Cyanobacterial blooms have been reported in

L.Paliastomi by Zivert in 1931. In his work he reported the bloom caused by *Nodularia spumigena*. Blooms caused by cyanobacteria in L. Paliastomi were also reported in 1964-66, this time the dominant species were *Nodularia spumigena* and *Anabaenopsis elenkini* (Chkhaidze 1969). In overall, registered species of cyanobacteria in L. Paliastomi are *Anabaena contorta* (Bachman), *Anabaena spiroides* (Klebahn), *Anabaenopsis elenkini*, *Aphanizomenon flos-aquae*, *Aphanizomenon elenkinii* (Kisselev), *Microcystis aeruginosa*, *Microcystis pulwerea* and *Oscillatoria planctonica* (Chkhaidze 1969; Ministry of Environment and Natural Resources of Georgia 1998).

According to the studies reported by Kalandadze and Geradze (2009) cyanobacteria have been present in L. Nurigeli as well, however the study for composition of cyanobacteria species have not been reported.

Since the ecosystems tend to change through natural and anthropogenic influence, observation of microbial community response to these changes is essential in order to understand the dynamics of certain ecosystem. For identification of cyanobacterial cells in water samples, morphological characterization based on microscopic study is generally used. On the other hand, for the estimation of cyanobacterial toxins several diagnostic methods are used such as mass spectrophotometry, enzyme linked immunosorbent assay, etc. However, molecular techniques, especially those based on polymerase chain reaction (PCR) increase in popularity in this field of study. In addition, for their high sensitivity, these methods make it possible to distinguish toxic and nontoxic strains among the same species (Ouellette et al 2006).

Ecological condition and microbial contamination of fresh surface waters are poorly investigated in Georgia. Cyanotoxins are insufficiently studied as well. Previous studies of L. Paliastomi and L. Nurigeli have presented morphological characterization of cyanobacterial cells. However it does not contribute sufficient picture of cyanotoxin occurrence in these fresh surface waters. This is the first study of cyanobacteria and cyanotoxins in these lakes based on techniques such as enzyme-linked immunosorbent assay and PCR.

The main aim of this study is to provide data on ecological status of L.Paliastomi and L. Nurigeli with the focus on cyanobacteria and cyanotoxins.

2. Background

2.1 Cyanobacteria and cyanobacterial blooms

Cyanobacteria constitute large heterogeneous group of prokaryotic organisms, which dominated on earth over 1,5 billion years ago (Bitton 2002). They are common in aquatic environments and play major role in oxygen, nitrogen and carbon dynamics. Cyanobacteria are evolutionarily the oldest group of oxygenic photosynthetic organisms and have wide tolerance to climatic and environmental conditions. The major habitat for them are fresh and marine environments, however they can be found in hot or cold springs, volcanic ashes, rocks and others (Chorus and Bartram 1999).

Morphologically they occur as unicellular, colonial or multicellular filamentous forms. The type of reproduction of cyanobacteria is asexual, binary fission. The cells of cyanobacteria range in diameter from 0,5-1 µm to 40 µm. They lack nuclei and other organelles as all prokaryotes. Cyanobacteria have two types of DNA- chromosomal and extra chromosomal DNA. The genome size varies among the unicellular and filamentous forms (Dworkin et al 2006). Like all gram-negative bacteria, the cell wall of cyanobacterial cell is composed of peptidoglycan (Madigan et al 2012).

Adaptation factors such as nitrogen fixation, photosynthesis, buoyancy and differentiated cells for reproduction and rest give cyanobacteria advantage over other organisms (Kaebernick and Neilan 2001). Photosynthesis is a major source of energy for these organisms. As photoautotrophs, cyanobacteria require only water, carbon dioxide, light and inorganic substances for existence (Bellinger and Sigee 2010).

Despite the benefits cyanobacteria have on the ecosystem, the metabolic products they produce cause problems to the environment. They often form massive blooms in water bodies affected by eutrophication (Kaebernick and Neilan 2001). In most cases cyanobacterial blooms are promoted by elevated temperature, weak water mixing and richness of nutrients such as nitrogen and phosphorus (Reichwalt and Ghadouani 2012). However some species can survive in nutrient limited conditions as well. They can tolerate different environmental stresses and often respond to such conditions by producing more toxins (Boopathi and Ki 2014).

In fact, cyanobacterial blooms are common in eutrophic water reservoirs worldwide. Such blooms are mainly produced by the organisms which belong to genera *Anabaena, Aphanizomenon, Cylindrospermopsis, Microcystis, Nodularia* and *Planktothrix (*Chorus et al 2000). In temperate zones they occur in late summer and early autumn, while in trophic zones all year round (Chorus and Bartram 1999). Cyanobacterial blooms cause chemical and biological changes in water bodies. In addition such water could have specific odor and taste. Toxins produced by cyanobacterial blooms could be poisonous to organisms that inhabit or consume such water.

2.2 Cyanobacterial bloom occurrence in Georgia

Despite the richness of freshwater resources, the quality of fresh surface waters in Georgia does not often correspond to European water quality standards. Fresh surface water is not used for drinking water in Georgia. In fact, 90% of the population in Georgia is supplied with drinking water from groundwater, however, 43 freshwater reservoirs are directly used for irrigation and hydropower (United Nations 2003).

The main causes of pollution are untreated municipal wastewater discharge and agricultural runoffs. These nutrients cause intensive growth of microorganisms and algae diminishing freshwater quality directly or indirectly. The Georgian National Environmental Agency monitors surface freshwater quality of rivers and lakes. According to these monitors, some freshwaters in Georgia are suffering from high ammonia concentrations and others that are used for bathing are of poor quality. In addition, the monitoring program covers just a small part of the fresh surface waters and overall situation is difficult to overview (Ministry of Environmental Protection and Natural Resources of Georgia 2010).

High levels of nutrients in lakes develop high microbial and cyanobacterial activities. Among studied lakes in Georgia, Lake Kumisi suffered from massive cyanobacterial blooms during the period 2006-2008. Registered species included *Anabaena flos-aqua, Anabaenopsis elenkini, Microcystis aeruginosa, M. pulverea, Oscillatoria planctonica, O. limnetica, O. brevis, and Spirulina minima*. Massive fish mortality in Lake Kumisi was probably caused by toxic cyanobacteria species (Jaiani et al 2013).

Similarly, cyanobacterial bloom occurred in Lake Paravani which was mainly caused by *Anabaena flos-aquae* and *Aphanizomennon flos-aquae* in late 1979-1980. Several fish death and livestock poisons have been reported due to this event (IOC-UNEP-WHO-FAO 1995). More recent studies concerning occurrence of cyanobacteria in Lake Paravani are not available.

Cyanobacterial blooms in Georgian surface freshwaters have been associated with unpleasant smell, livestock poisons and fish mortality. Human health impairments related to cyanotoxins have not been recorded in the country (IOC-UNEP-WHO-FAO 1995).

2.3 Cyanotoxins

Cyanobacterial secondary metabolites are called cyanotoxins and they are toxic in most cases. These are a diverse group of compounds regarding chemical and toxicological properties. Majority of them are stable chemical compounds and resistant to boiling. Cyanotoxins are responsible for acute and chronic toxicity among wild and domestic animals as well as in humans (Boopathi and Ki 2014). Occurrence of cyanotoxins in drinking and recreational waters is a public health concern. Therefore control of fresh and marine environments as well as removal of cyanobacteria and their toxins is essential.

The first record of livestock death by cyanotoxins was registered in Australia in 1878. Since then these toxic compounds are reported to be responsible for death of several animals like fish, birds, dogs, cattle, etc., around the world (Apeldoorn et al 2007; Manganelli et al 2012).

Humans are also exposed to cyanotoxins. The direct poisoning in humans is associated with drinking or recreational waters. Humans may also be exposed to cyanotoxins indirectly by consumption of food from contaminated water (Manganelli et al 2012). Several authors suggest that microcystins might be one of the factors originating primary liver cancer in humans (Drobac et al 2013; Svirčev et al 2013).

The most acute incident related to cyanotoxins which caused human fatalities occurred in Brazil in 1996, when hemodialysis patients were treated with water contaminated by microcystins. Most of the patients, 100 out of 126 developed acute liver failure and over 60 patients died (Pouria et al 1998; Carmichael et al 2001).

According to their toxicity, cyanotoxins are divided into the following groups: neurotoxins, hepatotoxins and dermatotoxins (WHO 2003; Boopathi and Ki 2014). Several types of toxins may be produced by one species. Furthermore, there are genotypes within species which might not produce toxins (Funari and Testai 2008).

Hepatotoxins damage the liver tissue in the organisms exposed. Lethal dose of hepatotoxins depends on the route of exposure, type of hepatotoxins and organism exposed. This group of natural toxins includes microcystins and nodularins. Hepatotoxins and especially microcystins appear to be the most frequent among cyanobacterial toxins. First isolated from *Microcystis aeruginosa*, microcystins are water soluble, cyclic heptapeptides. They cannot directly penetrate lipid membranes, instead they are transported by bile acid type transporters (Nybom 2013).Microcystins are produced by the species of genera *Anabaena, Anabaenopsis, Microcystis, Nostoc,* and *Plantothrix*. There are over 85 variants of microcystins identified so far (Boopathi and Ki 2014). The most widespread type of this toxin is Microcystin-LR which is the most studied compound as well (Figure 1a). Microcystin-LR is included in the guideline list for drinking water by World Health Organization with threshold level of 1µg/L (WHO 2011).

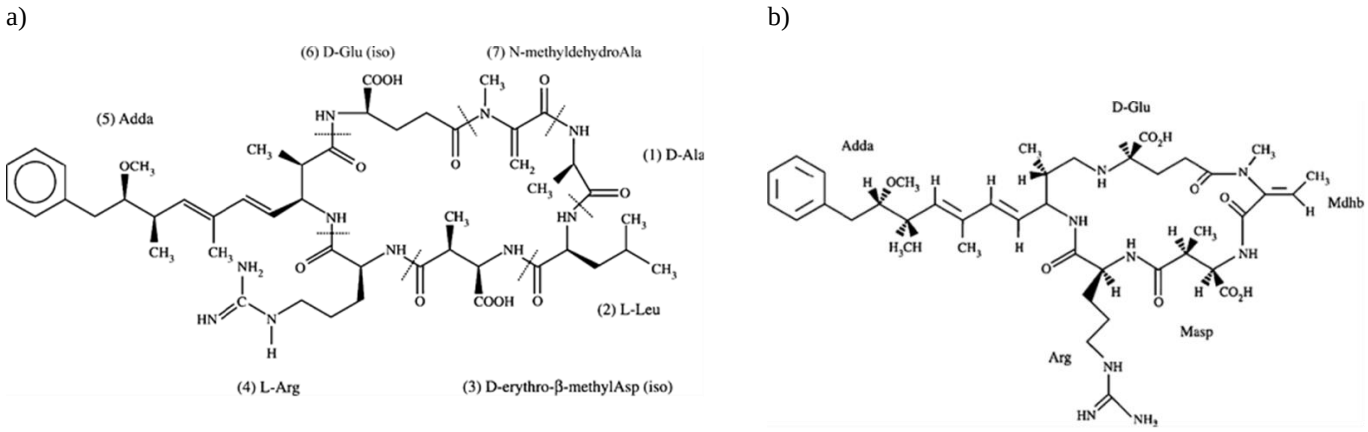

Figure 1. Chemical structures of (a) microcystin-LR and (b) nodularin (Funari and Testai 2008)

Nodularins (Figure 1b) are found to be similar to microcystins regarding chemical structure and toxicity. Both microcystins and nodularins possess special ADDA amino acid, which plays major role in their toxicity (Huisman et al 2005). The toxins are synthesized by non-ribosomal peptide synthetase (NRPS) and polyketide synthase (PKS) enzymes which are

encoded in *mcy* (in *Microcystis*) and *nda* (in *Nodularins*) gene clusters (Pearson and Neilan 2008). Microcystin producing gene *mcy* varies among species.

According to the United States Environmental Protection Agency (US EPA 2014) several U.S. states have maintained guidelines for cyanobacterial toxins in drinking and recreational waters, as well as tolerable daily intake values (TDI). Among them, Oregon Health Authorities (OHA) proposed TDI of 0,05 µg/kg-day and guideline value for recreational water of 10,0 µg/l for microcystins (US EPA 2014; Farrer et al 2015).

Another major group of cyanotoxins is neurotoxins. Neurotoxins are alkaloid toxins and include anatoxin-a and homoanatoxin-a, anatoxin-a (S) and saxitoxin.

Anatoxin-a (Figure 2a) has been produced by the species of genera *Anabaena*, *Aphanizomenon* and *Plantotrix*. This toxin has a low molecular size and causes depolarization of neuronal cells and overstimulation of muscles. Homoanatoxin-a is a structural analog of anatoxin-a, toxicological properties are also similar. These chemical compounds are highly toxic. They cause muscular paralysis and respiratory failure within short period of time (US EPA 2006, Funari and Testai 2008). Lethal dose of anatoxin-a according to WHO (2003) is 250 µg/kg of pure toxin in mice. Based on the OHA guideline values TDI of anatoxin-a for humans is 0,1 µg/kg-day and for recreational waters 20,0 µg/L (US EPA 2014; Farrer et al 2015).

a) b)

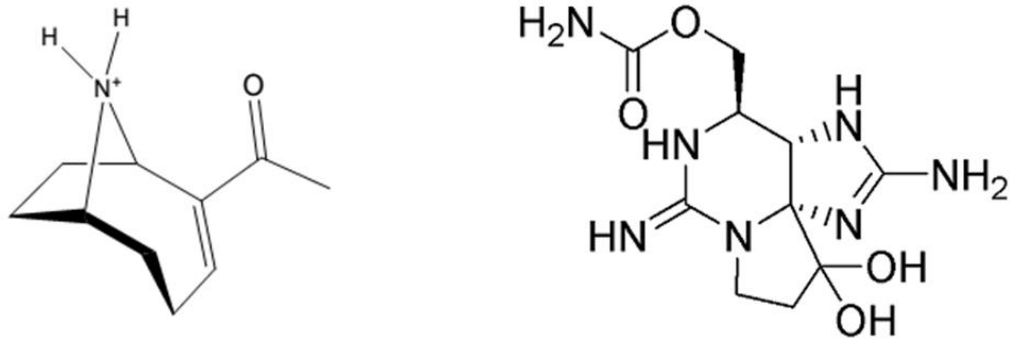

Figure 2. Chemical structures of neurotoxins: (a) anatoxin-a (US EPA 2006) and (b) saxitoxin (Faber 2012)

Saxitoxins (Figure 2 b) are also known as paralytic shellfish poisoning (PSP). PSP includes up to 20 several congeners, which differ from each other by their toxicity. Among them, saxitoxin is the most toxic compound (Funari and Testai 2008). Saxitoxins cause paralyses

and respiratory failure by blocking neuronal transmission. They are produced by the species which belong to genera *Anabaena, Aphanizomenon, Cylindrospermopsis* and *Lyngbya.* Saxitoxins are heat stable, water soluble molecules (Faber et al 2012). Guideline values for this toxin are relatively limited. TDI of saxitoxin is 0,05 µg/kg-day in humans, while threshold level for recreational water is 10,0 µg/L according to OHA (US EPA 2014; Farrer et al 2015).

Cytotoxin or cylindrospermopsin (Figure 3) has been found in *Cylindrospermopsis raciborskii,* in some species of *Anabaena, Aphanizomenon* and *Lyngbya* (Pearson et al 2010). This toxin hinders protein synthesis, causing failure of liver, kidney, lungs, intestine and adrenals (WHO 2003). It has small molecular size and is highly hydrophilic. Therefore absorption of cylindrospermopsin is achieved by active bile acid transport systems (Funari and Testai 2008). Various animal and human poisonings have been associated with this toxin. However, the most notable is Palm Island mystery disease, which occurred in Australia in 1979. 139 children and 10 adults were intoxicated after consuming the drinking water treated by algicidal copper sulfate compound (Griffiths and Saker 2002; Boopathi and Ki 2014). Copper sulfate was used to control cyanobacterial bloom in Salomon Dam water supply system, which caused the lyses of the cells of *Cylindrospermopsis raciborskii.* As reported later, this water contained toxic *Cylindrospermopsis raciborskii* and nontoxic *Anabaena circinalis* (Griffiths and Saker 2002).

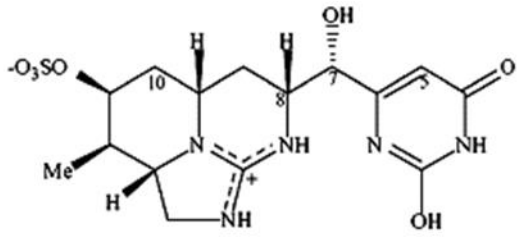

Figure 3. Chemical structure of cylindrospermopsin (Funari and Testai 2008)

According to OHA, TDI of cylindrospermopsin for humans is 0,03 µg/kg-day and guideline value for recreational water is 6,0 µg/L (US EPA 2014; Farrer et al 2015).

Dermatotoxins are skin irritating compounds and are produced by fresh and marine species of cyanobacteria which belong to the genus *Lyngbya.* High level of toxins produced by these filamentous cyanobacteria cause skin itching, redness, burning and swelling (Rzymski and Poniedzialek 2012).

Cyanotoxins are resistant to environmental changes. Their persistence depends on type of toxin and location. In general, hepatotoxins are more stable compared to neurotoxins and cylindrospermopsins. In darkness, these compounds may remain toxic for several months or years.

Cyanotoxins are a serious concern regarding drinking and recreational waters. They have ability to bioaccumulate in the food web, thus quick methods to determine the presence of cyanotoxins are essential. Among several methods, ozonation is effective, however biodegradation might be the best treatment for removal of these toxins (Nybom 2013).

Several methods have been developed in order to determine the contamination of cyanotoxins in the environment. Identification and quantification of cyanobacterial toxins may be achieved by several chemical methods such as high-liquid pressure chromatography –HPLC and enzyme-linked immunosorbent assay- ELISA. Identification of species of cyanobacteria and toxin producing species is achieved by molecular methods such as polymerase chain reaction (PCR) and DNA sequencing (Moreira et al 2014).

2.4 Interaction between cyanobacteria and heterotrophic bacteria

Cyanobacteria and heterotrophic bacteria are important parts of aquatic environments and play important roles in nutrient cycling. The abundance of specific populations of organisms is determined by chemical, biological and physiological factors including temperature, nutrients, pH and salinity. Mass cyanobacterial blooms and their metabolites can alter pH and oxygen composition of aquatic ecosystem, changing concentrations of organic compounds and nutrients in surrounding environment. Such alterations could be harmful for certain organisms, however, organisms such as heterotrophic bacteria often benefit from these alterations. Moreover, these organisms interact with each other, compiling symbiotic relationship. During such symbiosis both cyanobacteria and heterotrophic bacteria utilize the products produced by one another (Cai et al 2013).

Apart from the oxygen, nitrogen and carbon heterotrophic microorganisms get from cyanobacteria, the latter also provides good surface for attachment and protection. Nutrient rich polysaccharide sheaths produced by cyanobacteria is good surface for attachment for

bacteria. Colonial or filamentous cyanobacteria often have bacterial cells attached to them, more often to their heterocysts (Salomon et al 2003).

Several studies (Eiler et al 2007; Rigosi et al 2014) reveal that heterotrophic bacterial cell densities are higher in the presence of cyanobacterial blooms rather than in their absence. The species of the genus *Anabaena* compose specialized environment for development of certain bacteria. Heterocysts of *Anabaena* sp. was found to be good surface for attachment of gram negative rod shaped bacteria *Rhizobium* sp. in the study conducted by Stevenson and Waterbury (2006). Furthermore, several pathogenic microbes have been reported to be associated with cyanobacterial blooms in freshwaters such as *Vibrio* strains (Eiler et al 2007) and *Legionella* sp. (Taylor et al 2009; Cai et al 2013).

On the other hand, microbial interaction often limit growth of cyanobacterial cells by mean of producing algicidal compounds (Manage et al 2001; Salomon et al 2003). Several groups of bacteria such as *Cytophaga* sp. have been reported to produce different types of enzymes which cause the lysis of cyanobacterial cells in nutrient rich aquatic environments (Rashidan and Bird 2001).

2.5 Description of study areas

Georgia is a small country in South Caucasus. The territory of the country is 70 000 km^2 and population about 4 million people. Georgia is situated on the eastern coast of the Black Sea and shares border with 4 countries: Armenia, Azerbaijan, Russia and Turkey (Figure 4).

Georgia is mountainous country with diverse landscapes, such as sub-alpine grasslands of Greater and Lesser Caucasus, mountain forests, humid lowland forests in Western part and arid steppe and semi-deserts in Eastern part of the country. The rugged mountains of Caucasus constitute 85% of total area of Georgia with highest peaks -Mount Skhara (5069 m) and Mount Kazbegi (5037 m). On the western part of the country, along the coastal zone of the Black Sea there are lowlands with protected areas, generally below 100 m above sea level (United Nations 2003).

Because of mountainous nature and high level of precipitation (1338mm annually) Georgia is rich in freshwater resources. Among post Soviet Union countries Georgia takes the first

place for water resources, while among European countries it is only behind Norway, Switzerland and Austria.

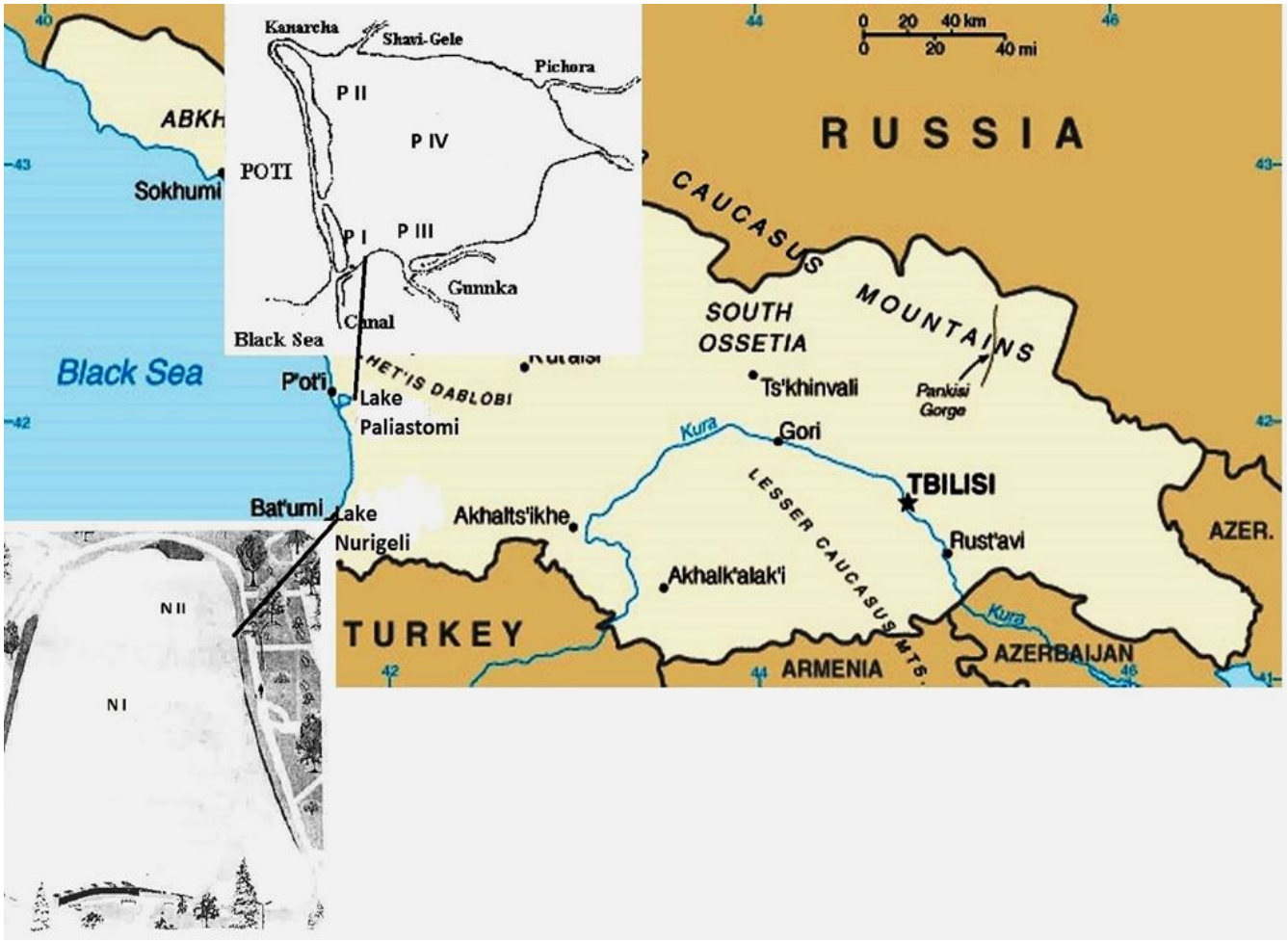

Figure 4. Georgia with study areas: Lake Paliastomi and Lake Nurigeli. The lakes are displayed with sampling stations: P1 to P IV –Lake Paliastomi and NI and N II –Lake Nurigeli (adopted from Dassenakis et al 2006 and Kalandadze and Geradze 2009)

There are about 25 000 rivers and 860 lakes in Georgia. Most of the rivers (76%) belong to the Black Sea basin and the rest of them (24 %) to the Caspian Sea basin. The two largest rivers- Mtkvari (or Kura) with length of 384 km and Rioni with 327 km of length flow in opposite directions. Total surface area of lakes is 175 km^2 and total volume 400 million m^3. The biggest lake in Georgia is Lake Paravani with surface area of 37,5 km^2 while Lake Tabatskuri has the largest volume of 221 million m^3 (Ministry of Environmental Protection and Natural Resources of Georgia 2010).

2.5.1. Lake Paliastomi

Lake Paliastomi (Figure 4) is situated at the south-eastern part of the city of Poti (population 50 000 people), on the left bank of the river Rioni and close to the Black Sea, 0.5m above sea level. It is an old lagoon of the Black Sea with average depth of 2.2 m and maximum depth of 4 m. Water volume of the lake is about 40 million m^3 (Table 1). Because of small depth and big surface area even slight winds cause waves on the lake. The northern and south-eastern parts of L. Paliastomi are surrounded by marshes. The marshes are cross-sectioned by the rivers Shavtskala, Pichora, Gurinka, Maltakva and small Paliastomi. These rivers flow into L. Paliastomi. The major supply for the lake is river Pichora (Barach 1964).

Table 1. Information about the morphometry and catchment area of Lake Paliastomi and Lake Nurigeli (Komakhidze 2006; Kalandadze and Geradze 2009)

	Lake Paliastomi	**Lake Nurigeli**
Surface area	18 km^2	0.07 km^2
Water volume	40 mln m^3	0,24 mln m^3
Average depth	2.2 m	5 m
Maximum depth	4 m	7-8 m
Catchment area	547km^2	

L. Paliastomi used to be connected to the Black Sea through the river Kapartcha, which went from the north-western part of the lake reservoir into the sea with the length of 9 km. Because of floods of L. Paliastomi, an artificial channel was made in 1924. The channel connected the lake to the sea directly. Due to increased salinity the ecosystem of the lake has changed as a result. Plankton biomass and benthic populations have decreased dramatically (Komakhidze 2006).

L. Paliastomi is an important ecosystem of the coastal zone of the Black Sea. It is a part of the ecologically important Kolkheti lowland (Dassenakis et al 2006). For its unique flora and fauna, Kolkheti lowland is in the list of Ramsar Convention since 1997. It is an

important refugium for local and migratory waterfowls, fish, vertebrates and others (Komakhidze 2006).

L. Paliastomi has been one of the biggest natural reservoirs for fish resources in Georgia. It has also been characterized as a highly productive water basin regarding natural food resources (Mikashavidze et al 2006). But environmental factors altered its ecosystem turning it into brackish water which affected species composition of the lake. In the 1940s community of fish accounted 40 species in the lake (Puzanov 1940). However, the number of fish species has been reduced to 24 since the change of hydrological regime. This is mainly due to elevated salinity (Komakhidze 2006).

Occurrence of cyanobacterial blooms in L. Paliastomi was first reported by Zivert in 1931, in his work he reported a bloom caused by *Nodularia spumigena*. Blooms caused by cyanobacteria in this lake was also reported in the period of 1964 to 1966, this time the dominant species were *Anabaenopsis elenkini* and *Nodularia spumigena* (Chkhaidze 1969). Fish mortality in the lake was reported to be due to harmful cyanobacterial blooms. Observed cyanobacteria species in L. Paliastomi are *Anabaena contorta, Anabaena spiroides, Anabaenopsis elenkini, Aphanizomenon flos-aquae, Microcystis aeruginosa, Microcystis pulvera, Oscillatoria planctonica* and *Worochinia naegliana* (Chkhaidze et al 1976; Ministry of Environment and Natural Resources of Georgia 1998). However, dynamics in the phytoplankton community of the L. Paliastomi varies from season to season, demonstrating shift from freshwater to marine phytoplankton species. In the studies by Mikashavidze et al (2006) the dominance of marine phytoplankton was obvious in the lake.

2.5.2. Lake Nurigeli

Lake Nurigeli is situated in the center of the city of Batumi (population 190 000 people), on the shoreline of the Black Sea (Figure 4). It is a small lake of lagoon origin and is nourished by groundwater. The shape of the lake is elongated oval, with the circumference of 1030 m and length of 105m. The average depth of the lake is 5 m, maximum depth 7-8 m, surface area of 0.07 km^2 and water volume 0,24 million m^3 (Table 1).

Scientific data about this lake are relatively limited. The first hydrobiological study of this lake was conducted in 1924. By that time the lake was a eutrophic freshwater reservoir with much organic matter in the sediments. Because of richness in aquatic plants it was impossible to get through the lake even by boat. Since 1970-1980 the lake has been restored by introducing phytophagous fishes *Ctenopharyngodon idella* and *Hypophthalmichthys nobilis* (Barach 1964; Kalandadze and Geradze 2009).

According to the studies conducted in 2009 the phytoplankton community of L. Nurigeli was dominated by species of Bacillariophyta (35%) and Xantophyta (25%) while cyanophyta compiled just 5% of the total phytoplankton biomass (Kalandadze and Geradze 2009). Occurrence of cyanotoxins in this lake has not been investigated. L. Nurigeli is now a closed freshwater body, because the only channel which earlier connected it to the Black Sea is closed. Thus the lake is susceptible to gradual nutrient-enrichment and water degradation.

Situated in the center of the city, L. Nurigeli is popular place among locals for pleasure and recreation. Moreover, fish in the lake is a food source for some citizens of Batumi and it is often visited by local fishermen.

3. Materials and Methods

3.1 Sample collection

Samples for this study were collected during several field trips at L. Paliastomi and L. Nurigeli in summer 2014. From L. Paliastomi samples were collected at four sampling stations (PI, P II, P III and P IV) in June, July and August and from L. Nurigeli at two sampling stations (N I and N II) in July.

Two liters of surface water was collected in plastic bottles. All samples were collected between 12:00 AM-14:00 PM. Water samples were immediately taken into the laboratories for filtration and chemical analyses.

Standard limnological analyzes were undertaken at the following water laboratories: “Black Sea Monitoring Institute, the National Agency for Environment of Georgia”, “Black Sea

Flora and Fauna Scientific Research Centre" both situated in Batumi and Scientific research Firm " Gamma" situated in Capital city Tbilisi.

3.2 Chemical Analysis of Water

Water was filtered with glass microfiber filters (GF-5 Grade) with pore size of 0,7 µm and analyzed within 2-3 hours in the two laboratories in Batumi for the following physical-chemical parameters with common methodologies: Temperature and pH (EW-35634-30 pH Tester), salinity (Titration by the method of Mohr), total phosphorus (Ascorbic Acid Method 10209-10210) and ammonia, nitrite and nitrate (Continuous flow nutrient Analyser). For analysis of TOC (ISO 6060), conductivity (ISO 7888), color (ISO 7887) and iron (EPA3005A) water samples were placed on ice and immediately sent to the Scientific Research Firm "Gamma" in Tbilisi. For iron analyses 200-300 ml of water was treated with 2,5 ml of concentrated nitric acid (HNO_3).

3.3 Isolation of Cyanobacteria

150-200 ml of water was filtered through the glass-fiber filters of 1.2 µm pore size (Whatman GF/C) by use of vacuum pump. Filters were dried at room temperature in plastic petri dishes. They were kept in darkness at room temperature.

3.4 Microscopy and species/genus identification

Samples for species/genus characterization were collected by planktonic net (25 µm pore size). The samples were preserved with Lugol`s solution and stored in darkness at room temperature.

Samples were investigated by using OLYMPUS GX22LED microscope at magnification - 100 and 400X. Phytoplankton species and genera were identified by use of the following taxonomic literature: Tikkanen and Willén (1992) and Komárek and Zapomèlová (2007).

3.5 Detection of Toxins

Subsamples for cyanotoxins were collected in glass tubes and frozen down after collection. Samples were frozen and thawed two times before analysis for microcystins, anatoxin-a and saxitoxin using the enzyme-linked immunosorbent assay (ELISA) technique and ABRAXIS- KITs according to the method description for different KITs.

3.6 DNA Isolation

DNA was extracted from frozen filters by using the xanthogenate method (Jungblut and Neilan 2006). 1/8 of the filter was cut out using sterile scissors and pincers. Filters were placed in eppendorf tubes and 50 µl TER and 750µl of XS buffer (1/PEX 1g; TrisHCl 10 ml; 20mM EDTA 0,74448g; 1% SDS 1g; 800mM AA 6,166g) were added to each tube. The samples were incubated at 70ºC for 2 hours, followed by vortexing for 10 seconds. The samples were placed on ice for 30 minutes afterwards and centrifuged at 14000 rpm for 10 minutes. Supernatant was placed in new tubes and 750 µl of isopropanol was added. Samples were incubated at room temperature for 10 minutes and centrifuged at 12000 rpm for 10 minutes. Supernatant was removed and samples were washed with 70% ethanol twice. Samples were left overnight to let ethanol evaporate thoroughly. 50 µl of TE buffer was added to each sample afterwards.

3.7 Determination of Purity and Concentration of DNA

Samples with DNA (25µl) were checked for purity and concentration by use of PicoDrop spectrophotometer at wavelength 260 and 280 nm.

3.8 PCR

A PCR reaction was performed using universal primer pair CC-CG for oxyphotobacteria. For this purpose 16S rRNA regions V6 to V8 (606 bp) were amplified according to Rudi et al (1997).

The gene was amplified in 25 µl reaction containing 1 µl DNA + 24 µl PCR mix (dH_2O 15,8µl; 10x PCR Buffer 2,5µl; 25 mM MgCl 2µl; 2 mM dNTP 2,5µl; 10mM Primer F (5'TGTAAAACGACGGCCAGTCCAGACTCCTACGGGAGGCAGC3') 0,5µl; 10mM Primer R (3' GGCTCGGCACGGCATCGATTGCGC 5') 0,5µl; 5U/µl Taq 0,2µl).

Amplification of DNA was performed with 31 PCR cycles. Thermocycling conditions included an initial denaturation step at 94°C for 4 minutes and extension step at 72°C for 7 minutes. Cycling parameters were 96°C for 15 seconds, 70°C for 30 seconds and 72°C for 1 minute for final polymerization.

The PCR products were electrophoresed in 6% Polyacrylamide gel (TEMED 10 µl and APS 80µl) at 150V for 45 minutes using 5µl PCR product + 3µl loading buffer and 1µl Ladder +1µl loading buffer.

Two PCR reactions were run with genus specific primer pairs also. For amplification of *mcyB* region of microcystin synthetizing gene in the genus *Microcystis* Tox4 F and Tox4 R primers were used (Kurmayer et al 2002).

The 1312 bp region of the gene was amplified in 25 µl reaction containing 1 µl DNA + 24 µl PCR mix (dH2O 15,8µl; 10 x PCR Buffer 2,5µl; 25 mM MgCl 2µl; 2 mM dNTP 2,5µl; 10mM Primer F (5'GGATATCCTCTCAGATTCGG3') 0,5µl; 10mM Primer R (5'CACTAACCCCTATTTTGGATACC3') 0,5µl; 5U/µl Taq 0,2µl).

Amplification of DNA was performed with 35 PCR cycles. Thermocycling conditions included an initial denaturation step at 94°C for 10 minutes, followed by denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds and polymerization at 72°C for 1,5 minute.

For amplification of *mcyE* region of microcystin producing gene in the genus *Anabaena* primer pair mcyE-F2 and AnamcyE-12R were used (Vaitomaa et al 2003).

370bp region of the gene was amplified in 25 µl reaction containing 1 µl DNA + 24 µl PCR mix (dH2O 15,8µl; 10x PCR Buffer 2,5µl; 25 mM MgCl 2µl; 2 mM dNTP 2,5µl; 10mM Primer F (5'GAAATTTGTGTAGAAGGTGC3') 0,5µl; 10mM Primer R (5'CAATCTCGGTATAGCCGC3') 0,5µl; 5U/µl Taq 0,2µl).

Amplification was performed with 30 PCR cycles. Thermocycling conditions included denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds and polymerization at 72°C for 1 minute. Final extension step was for 10 minutes at 72°C.

The PCR products were electrophoresed by use of 6% Polyacrylamide gel (TEMED 10 µl and APS 80µl) at 150V for 40 minutes using 5µl PCR product + 3µl loading buffer and 1µl Ladder +1µl loading buffer.

4. Results/Discussion

4.1 Physical-Chemical parameters

Inland freshwater basins are fragile ecosystems and they are susceptible to biological and artificial interactions, responding to them by changing their ecological status. Hence, study of limnological and biological parameters give good indications about the ecological conditions of certain freshwater bodies. Nutrient concentrations are good indicators of changes in the freshwater bodies. Phosphorus and nitrogen are important compounds essential for growth and biomass of algae in lakes. However, enrichment of natural waters with these inorganic nutrients leads to eutrophication process (Sigee 2005). The nutrients enter the freshwaters from natural sources- by atmospheric deposition and microbial activities, or artificially- with agricultural runoffs, sewage effluents and municipal discharges. In line with nutrients, climate change and extensive environmental disturbance further affect the ecological status of freshwater basins. In addition, good indicators of changes are organisms living in certain aquatic environment. Thus biological monitoring provides a good picture of the lake condition as well (Lyche-Solheim et al 2013).

Proper care and investigation of freshwater resources is a big challenge for Georgia. Despite the richness of water resources, many lakes in Georgia are strongly affected by environmental and anthropogenic factors. Quality standards for freshwater ecosystems are set based on EU norms in the country. According to the data provided by National Environment Agency, concentrations of ammonia in Georgian fresh surface waters exceed guideline values for protection of human health. Furthermore, nitrite concentrations are higher than acceptable standards for aquatic life (Ministry of Environmental Protection and Natural Resources of Georgia 2010). Poor condition of freshwater basins is the result of inadequate industrial and municipal wastewater treatment, as well as agricultural runoffs (United Nations 2003).

The results of the physical and chemical analyses from L. Nurigeli and L. Paliastomi are summarized in Table (2). The temperature in L. Paliastomi varied from 25,6 C° in June to 29,2 C° in August. The mean temperature value in L. Nurigeli during sampling was 25,8C° in July. Water temperature is one of the most important abiotic factors in aquatic environment. It controls different parameters including microbial growth and activity. With elevated temperatures like as in L. Paliastomi and L. Nurigeli cyanobacteria outcompete other primary producers and dominate over them (Paerl and Huisman 2009). Elevated temperature increases stability in water bodies, resulting in inhibition of proper mixing. Such conditions are favorable for buoyant cyanobacteria such as *Microcystis* sp. (Visser et al 2005). Production of toxins by cyanobacteria also increases between temperatures 20-25°C (Kaebernick and Neilan 2001).

According to the pH values, which varied from 7, 4 to 8,5 water mass in L. Paliastomi is neutral to light alkaline. These values might be linked with high photosynthetic activity of

Table 2. Physical-chemical parameters of Lake Paliastomi (P) and Lake Nurigeli (N) sampling stations in the period from June to August 2014

Sampling Station	**T°C** min-max average	**pH** min-max average	**Salinity ‰** min-max average	**Total Posphorus mgC/L** min-max average	**NO_2^- mgN/L** min-max average	**NO_3^- mgN/L** min-max average	**NH_4^+ mgN/L** min-max average	**Fe^+ mg/L** min-max average	**TOC mg/L** min-max average	**EC µS/cm** min-max average	**Color mg Pt/L** min-max average
P I	25,6 ±28,2	8,4 ±8,5	5,1 ±9,8	0,1 ±0,2	0,001 ±0,01	0,000 ±0,2	0,01±0,03	0,2 ±0,4	9,6 ±9,8	800 ±1700	10 ±20
	27,37	8,4	7,4	0,1	0,01	0,1	0,02	0,3	14,5	1200	16,7
P II	25,7 ±28,8	7,4 ±8,3	2,9 ±5,3	0,1±0,2	0,001 ±0,3	0,000 ±0,2	0,004 ±0,05	0,1 ±0,2	5,7 ±12,6	400 ±700	10 ±40
	27,37	7,7	4,0	0,2	0,09	0,1	0,03	0,2	8	600	26,7
P III	25,7 ±29,2	7,9 ±8,3	5,3 ±6,9	0,1 ±0,2	0,002 ±0,1	0,000 ±0,1	0,01 ±0,03	0,2 ±0,3	5,7 ±13,4	800 ±1100	15 ±20
	27,67	8,1	6,1	0,2	0,04	0,1	0,02	0,2	8,3	1000	18,3
P IV	25,7 ±29,2	8,3 ±8,4	4,1 ±7,1	0,1 ±0,2	0,002 ±0,1	0,000 ±0,13	0,01 ±,03	0,1 ±0,3	9,2 ±13,4	700 ±1100	10 ±30
	27,47	8,4	5,8	0,1	0,02	0,1	0,02	0,2	8,2	900	20
N I	26,4	9,2	4,9	0,2	0,2	0,2	0,04	-	-	-	-
N II	25,2	9,2	4,2	0,2	0,2	0,04	0,1	-	-	-	-

phytoplankton and salinity in the lake. The highest value (pH 8,5) was recorded in sampling station PI which corresponds to raised salinity in this sampling station as well. It is noteworthy that pH drops to 6,7-6,9 (Table 3) in winter and spring due to increased freshwater flow into the lake (Dassenakis et al 2006). High pH values (9,2) recorded in both sampling stations in L. Nurigeli indicated high photosynthetic activity in water column. However, according to the studies by Kalandadze and Geradze (2009) pH values were relatively lower (8,6 and 8,8) during the same season in 2009.

In L. Nurigeli salinity values were 4,9 and 4,2 ‰ in sampling stations N I and NII respectively. In L. Paliastomi salinity varied between 2,9‰-9,8‰. Brackish waters are characterized to have higher salinity than fresh waters and lower than marine waters, in fact, salinity in brackish waters range from 3 to 10‰ (El-Dessouky and Ettouney 2002). The highest value of salinity in L. Paliastomi (9,8‰) was in sampling station PI. This sampling station is at the estuary to the Black Sea and it was characterized with high salinity compared to the other sampling stations. The center of the lake (sample station P IV) had relatively high level of salinity too (7 ‰). This coincides with the fact that water flows from the sea estuary towards the center. High salinity in this lake might be influenced by seawater flow, which mainly occurs in summer. In autumn and spring salinity reduces (Table 3) because of more freshwater inflow, especially from river Pitchora (Dassenakis et al 2006).

Conductivity in L. Paliastomi varied from 400 to 1200 µS/cm, which might point out possible contamination of lake by inorganic compounds. It may also be attributed to relatively high salinity and water temperature.

Increased salination of fresh surface waters changes hydrological regime of water. Moreover, it affects aquatic organisms by inhibiting their normal physiology. Salinity is thought to be important factor in controlling the growth of nitrogen fixing organisms (Moisander et al 2002). Nitrogenous compounds increase salt tolerance in cyanobacteria (Rai and Tiwari 1999). Many species of cyanobacteria from genera *Anabaena, Microcystis* and *Nodularia* are tolerant to high salinity (Paerl and Huisman 2009). In addition, high salinity and conductivity was reported to increase saxitoxin production in *Cylindrospermopsis raciborskii* (Pomati et al 2004).

Values of water color ranged from 10 mg pt/L to 40 mg Pt/L and TOC concentration varied from 5,7 to 13,4 mg/L in L. Paliastomi. The highest values of TOC (13,4 mgC/L) were recorded in sampling stations PIII and PIV respectively. Color of water is derived from dissolved organic matter. Similarly, TOC is a measure of the content of organic matter, which consists of dissolved and particulate matter including phytoplankton (Wetzel 1983). According to the Norwegian classification guidelines (Iversen and Sandøy 2013) water with TOC between 5-15 mgC/L and water color between 30-90 mg Pt/L corresponds to humic water.

Table 3. Physical-chemical parameters from previous studies in Lake Paliastomi and Lake Nurigeli (Ministry of Fish Industry USSR 1990; Dassenakis et al 2006; Kalandadze and Geradze 2009)

Lakes, stations And sampling period	**T°C**	**pH**	**Salinity‰**	**NO_3^- mg/L**	**NO_2^- mg/L**	**NH_4^+ mg/L**	**PO_4^{3-} mg/L**
Lake Paliastomi July 2000							
PI	29,8	8,2	11,5	0,01	0,001	0,13	0,003
PIV	29,4	8,5	10	0,001	0,0003	0,2	0,001
Lake Paliastomi November 2000							
P I	10,5	6,9	9,5	0,1	0,003	0,13	0,0003
P IV	12,5	6,9	5,1	0,1	0,004	0,1	0,002
Lake Paliastomi July 1990							
P I	26,0	7,6	10,4	0,04	0,004	-	0,01
P IV	27,0	7,6	8,6	0,02	0,001	-	0,01
Lake Paliastomi May 1990							
P I	16,6	7,6	7,2	0,01	0,000	-	0,001
P IV	16,5	7,6	8,7	0,02	0,000	-	0,003
Lake Nurigeli July 2009							
N I	17,3	8,6		0,02	0,01	0,1	0,4
N II	17,4	8,8		0,13	0,003	0,1	0,3

Iron concentration varied from 0,1 to 0,4 mg/L in L. Paliastomi, being highest (0,4 mg/L) in sampling station P I. Iron is important compound for physiological processes in cyanobacteria. It is essential for respiration, photosynthesis, chlorophyll-a synthesis and nitrogen fixation (Holland and Kinnear 2013). It also limits the rate of cell division in cyanobacteria. Iron availability in water is determined by pH, humic content and redox potential (Wetzel 1983). Thus, neutral to slightly alkaline pH in L. Paliastomi could limit iron uptake by cyanobacteria. It has been reported that, microcystin producing cyanobacteria show more effectiveness in iron uptake than cyanobacteria, not producing this toxin (Utkilen and Gjølme 1995; Sevilla et al 2008).

Based on nutrient data total phosphorus concentration varied between 0,1 to 0,2 mg/L in L. Paliastomi, being relatively similar in all sampling stations. In L. Nurigeli total phosphorus was 0,2 mg/L. The amount of total phosphorus in L. Paliastomi and L. Nurigeli corresponds to the values typical for eutrophic lakes (Sigee 2005).

Nitrate level in L. Paliastomi was 0,1-0,2 mg/L and was absent in samples from June in all 4 sampling stations. This might be caused by active consumption of nitrates by cyanobacteria or other phytoplankton species during vegetation period (Komakhidze 2006). Data from July and August showed relatively similar concentrations of nitrates in all sampling stations. Ammonium concentration in L. Paliastomi ranged from 0,004 to 0,05 mg/L, being highest in sampling station P II (0,05 mg/L). According to the previous studies nitrate concentration in L. Paliastomi was highest (0,1 mg/L) in November 2000 (Table 3). Similarly, ammonium concentration in L. Paliastomi was highest in July and November 2000 (Table 3, Dassenakis et al 2006).

Nitrate concentration in L. Nurigeli was higher in the center of the lake (0,2 mg/L) than in the sampling station N II (0,04 mg/L). Ammonium concentrations were 0,04 and 0,1mg/L in sampling stations N I and N II correspondingly. In comparison with previous studies, phosphate concentration was high (0,4 mg/L and 0,3 mg/L) in the lake during the same summer period in 2009. On the other hand concentrations of nitrogen compounds showed more or less similar values to our results with nitrate ranging from 0,02 to 0,13 mg/L and ammonium 0,1 mg/L in 2009 (Table 3, Kalandadze and Geradze 2009).

It should be noted that cyanobacteria are good competitors for nitrogen which could be limiting factor in cyanotoxin production. However, exact role of nitrogen and nitrate in the

process of cyanotoxin production is quite contradictory. Several authors suggest that non-nitrogen-fixing cyanobacteria produce highest levels of cyanotoxins in nitrogen rich environments (Kaebernick and Neilan 2001; Boopathi and Ki 2014). Nitrogen-fixing cyanobacteria can also produce cyanotoxins in the absence of nitrogen (Kaebernick and Neilan 2001). However, relationship between nitrogen fixing and non-nitrogen fixing species of cyanobacteria is more complex. Nitrogen fixing cyanobacteria will switch to nitrogen fixation only in the absence of nitrogen, otherwise they will consume ammonium and nitrate from surrounding environment. Moreover, nitrogen fixing species may promote growth of non-nitrogen fixers by means of releasing fixed nitrogen in the water column (Agawin et al 2007).

High temperature, pH and total phosphorus values signify poor condition of waters in L. Paliastomi and L. Nurigeli. Elevated concentrations of total phosphorus in L. Paliastomi might be linked to the river freshwater discharges. The four rivers flowing into the lake contribute to both natural and anthropogenic nutrient loadings. Samples from L. Nurigeli demonstrated high concentration of total phosphorus and even higher pH values compared to L. Paliastomi. The lack of well mixing of water is disturbing factor for L. Nurigeli. As reported by Kalandadze and Geradze (2009) the lake is resided by macrophytes, which might further complicate the state of this freshwater basin.

4.2 Phytoplankton-Cyanobacteria community

Qualitative net samples revealed the dominance of mainly two genera of cyanobacteria (*Microcystis* and *Anabaena*) in L. Paliastomi and two genera *(Anabaena* and *Anabaenopsis*) in L. Nurigeli (Table 4).

Several species of *Anabaena* such as *Anabaena circinalis, A. compacta, A. flos-aqua* and *A. spiroides* were found in sample from June in L. Paliastomi. Anabaena caspica and Anabaenopsis sp. where present in sample from L. Nurigeli. Species of genus *Anabaena* represent a heterogenous group of planktonic cyanobacteria. They are common in eutrophic waters and occur in different regions of the world. The best season for growth of most *Anabaena* species has been reported to be early summer, however, they can be present in water in winter as well. Notably, the cyanobacterium *Anabaena caspica* which was found

in L. Nurigeli, is known to be halophilic and common only in Caspian Sea (Komárek and Zapomèlová 2007; Guiry and Guiry 2015). The occurrence of this cyanobacterium in freshwater basin is questionable and might be linked to the uncertainty in determination.

Table 4. Cyanobacteria species occurrence in Lake Paliastomi and Lake Nurigeli from June to August 2014. (?) mark indicates an uncertain identification of species

Cyanobacteria species	**Paliastomi** 27/06/2014	**Paliastomi** 23/07/2014	**Paliastomi** 07/08/2014	**Nurigeli** 10/07/2014
Anabaena caspica				+ (?)
Anabaena circinalis	+	+	+	
Anabaena compacta	+			
Anabaena flos-aqua	+	+	+	
Anabaena spiroides	+			
Anabaena vigueri			+	
Anabaenopsis sp.				+
Microcystis aeruginosa		+	+	
Microcystis reinboldii			+	
Microcystis viridis			+	
Snowella atomus		+		

Another cyanobacteria found in L. Nurigeli was from genus *Anabaenopsis*. This solitary filamentous free-floating cyanobacterium is distributed in temperate, tropical and subtropical regions. However, the main environment for it is alkaline saline or fresh water basins (Ballot et al 2008). Alkaline nature of L. Nurigeli might be corresponding habitat for *Anabaenopsis* sp. This was obvious from the sample which was rich in *Anabaenopsis* sp. under microscopic identification.

Species from genera *Microcystis (Microcystis aeruginosa, M. circinalis, M. reinboldii* and *M. viridis)* along with *Anabaena* (*Anabaena circinalis, A. compacta, A. flos-aqua* and *A. spiroides)* were abundant in samples from July and August in L. Paliastomi. *Microcystis* is

widespread in aquatic environments and is known to form blooms in variety of regions. These organisms are dominant in many nutrient rich lakes because they can grow at high pH and can readily use inorganic carbon. The ability of buoyancy further makes them superior among other phytoplankton species (Visser et al 2005). The fact that *Microcystis* species in samples from L. Paliastomi occurred only in July and August might be linked to the increased water temperature. As reported by Visser et al (2005) the growth rate of *Microcystis* species is 9 fold higher during high temperature conditions compared to the rate of other genera such as *Aphanizomenon* and *Planktothrix*, with just 3-4 fold enhanced growth rate.

The presence of cyanobacteria in L. Paliastomi and L. Nurigeli can be explained by high temperatures and nutrient loadings in the water columns of the lakes. However, composition of species might vary according to environmental changes. Since different species of cyanobacteria produce different types and amount of cyanotoxins, such alterations may lead to changes of cyanotoxin concentrations as well. The changes of species composition in L. Paliastomi have been noticed earlier due to alteration of hydrological regime. This lake has been characterized by cyanobacterial blooms since the 1930s and the composition of phytoplankton varied from year to year according to the hydrological regime. In studies by Devidze (2000) conducted in 1998-1999 freshwater phytoplankton species dominated over brackish or marine cyanobacteria species in L. Paliastomi. The cyanobacteria species *Anabaenopsis arnoldi*, *Anabaenopsis elenkinii* and *Nodularia spumigena f.litorea*, were registered by that time. On the other hand, according to the studies by Mikashavidze et al (2006) dominance of marine phytoplankton in all sampling stations was obvious in 2006.

4.3 Cyanotoxin analysis

Results for cyanotoxins in L. Paliastomi and L. Nurigeli are presented in Table 5. Microcystin concentration in all samples and in both lakes were 0,2 µg/l.

Saxitoxin concentrations in 2 samples from June and July in L. Paliastomi were close to the detection limit 0,02 µg/l and sample from August was positive with 0,03 µg/l respectively. Sample from L. Nurigeli was close to the detection limit 0.02 µg/l as well.

Table 5. The amount of microcystin, saxitoxin and anatoxin –a in samples from June to August 2014 in Lake Paliastomi and Lake Nurigeli

	Paliastomi 27/06/2014	**Paliastomi** 23/07/2014	**Paliastomi** 07/08/2014	**Nurigeli** 10/07/2014	Negative
Microcystin µg/l	0.2	0.2	0.2	0.2	< 0.10 µg/l
Saxitoxin µg/l	0.02	0.02	0.03	0.02	< 0.02 µg/l
Anatoxin -a µg/l	52,5	10,3	24,6	29,8	< 10 µg/l

Results for anatoxin–a showed higher concentrations compared to microcystins and saxitoxins in samples. Sample from June in L. Paliastomi showed an average result of 52,5 µg/l and the sample from August 24,6 µg/l, while average value of the sample taken in July revealed 10,3 µg/l of anatoxin-a. This toxin was present in the sample from L. Nurigeli as well with an average concentration of 29,8 µg/l.

Determination of cyanotoxins with ELISA kits is widely used for toxicity evaluation by several laboratories. It is rapid and relatively low-priced method to determine cyanotoxins from environmental samples, as well as from organisms and tissues. However, this method only evaluates total value of toxin and it is impossible to determine different congeners of the toxin. Moreover, false positive results have also been detected due to high cross-reactivity. Therefore, cross-checking of results with other methods are recommended (Koreivienè and Belous 2012; Moreira et al 2014). Notably, analyses of saxitoxin and anatoxin-a with ELISA kits are relatively new, thus limited studies are available. Additionally, analysis of saxitoxin using ELISA kit has been developed to improve the

detection methods of this toxin. Mouse bioassay (MBA) has been used as the reference method in EU for determination of Paralytic shellfish poisonings i.e. saxitoxins (European Commission 2005). According to the studies by Garet et al (2010) ELISA could be used as an alternative method in detection of saxitoxin because of it has more precise detection limit compared to the other methods.

The risk of cyanotoxin exposure to humans and animals is a concern for public health. Concentration of microcystin and saxitoxin in L. Paliastomi and L. Nurigeli are low, however, concentrations of anatoxin-a in these lakes are quite high. They exceed the guideline value of 20,0 µg/L for recreational waters (Farrer et al 2015). High anatoxin-a concentration in recreational waters are hazardous for wild and domestic animals. Several dogs have died immediately after consuming the river water contaminated with anatoxin-a in USA (Farrer et al 2015). The situation is a problem for L. Paliastomi as well. The water of this lake is consumed by livestock from surrounding catchment area, several animals could be noticed consuming the water during the field trips in summer 2014 as well.

Despite the fact that these lakes are not used by humans for drinking supply, aquatic life is exposed to cyanotoxins. From this point of view, fish are more vulnerable to cyanobacterial toxins, as being on top of the aquatic food chain (Drobac et al 2013). Fish get affected by these toxins by abnormal embryonic development and retardation in growth rate. Thus productivity of the fish in lakes might be reduced. The composition of the fish community in L. Paliastomi has already been reduced by alteration of hydrological regime. The factor of cyanotoxins can further diminish the fish productivity of the lake. The fish from these lakes and especially from L. Paliastomi are food source for locals. Since microcystin in fish mainly accumulates in liver, digestive tract, gonads, gills, kidneys, consumption of meat from such fish might not be hazardous. However accumulation of microcystin in muscles with small amounts has also been described (Drobac et al 2013). Not only humans but all organisms consuming contaminated fish might be exposed to cyanotoxin poisonings. Kolkheti lowland and L. Paliastomi are refugium for local and migratory fish, waterbirds, mammals and other organisms. Therefore the chance of bioaccumulation of cyanotoxins is high.

Air transmission of microcystin and cyanotoxins in general might be problematic in lakes during recreation activities (Backer et al 2009). This is especially true for L Paliastomi,

which is characterized with wavy nature. Thus, even small amount of toxin in water could cause human exposure.

The fate of cyanotoxins in aquatic environments is of a big interest. Among several degradation mechanisms, biodegradation of these undesirable compounds appears to be the most effective. Heterotrophic bacteria present in natural aquatic ecosystems use cyanotoxins, and especially microcystins as a carbon source (De La Cruz 2011; Nybom 2013). This natural process is reliable and does not involve use of harmful chemicals to remove cyanotoxins from waters.

Biodegradation of cyanotoxins and especially hepatotoxins have been reported by several authors. Strains of *Sphingomonas* sp. were reported to degrade different types of microcystin during 4 to 6 days (Harada et al 2004; De La Cruz et al 2011). Similarly, several authors (Manage et al 2001; Rapala et al 2005; Hu et al 2009; De La Cruz 2011) have reported that different groups of heterotrophic bacteria, such as *Arthrobacter, Brevibacterium, Rhodococcus* sp., and *Methylobacillus* sp. could effectively degrade hepatotoxins.

4.4 Molecular analysis

Molecular methods and especially PCR based methods are popular and useful techniques in the study of cyanotoxins. These techniques aim to identify cyanobacteria and their potential to produce toxins. The amplification of gene regions using taxon-specific primers identifies cyanobacteria species. Moreover, the detection of gene clusters responsible for synthesis of certain cyanobacterial toxins gives clear toxicological profile (Moreira et al 2014).

Table 6. Concentration and Purity of extracted DNAs from Lake Paliastomi (P) and Lake Nurigeli (N) from June to August 2014. Extractions performed 11/11/2014 and 26/02/2015 and Positive samples for oxyphotobacteria specific primers (CC-CG)

Sampling Station		**Extracted 11/11/2014**		Positive samples for CC-CG primers	**Extracted 26/02/2015**		Positive samples for CC-CG primers
		Concentration ng/µl	Purity A260/A280		Concentration ng/µl	Purity A260/A280	
P1	27/06/14	16,2	1,8		19,8	1,9	+
	23/07/14	19.1	2,3	+	33,4	2,2	+
	07/08/14	7,9	1,4	+	40,2	1,9	
P II	27/06/14	23,7	1,9		26	1,7	+
	23/07/14	12,6	3,5		58,7	1,8	
	07/08/14	4,7	-2,3	+	22,8	2	+
P III	27/06/14	8,4	3		15,4	2,7	+
	23/07/14	5,2	-3,1		37,2	2	+
	07/08/14	19,5	1,8	+	56,5	2,1	
P IV	27/06/14	17,9	1,9	+	70,1	1,8	+
	23/07/14	32,6	2	+	45,5	2	+
	07/08/14	16,9	1,8	+	73,1	2	
N I	10/07/14	14,3	1,8	+	39,4	2	+
N II	10/07/14	10,0	1,8	+	17	2,6	+

Spectrophotometric results of extracted DNA samples from L. Paliastomi and L. Nurigeli are presented in Table 6. The extraction was performed twice with xanthogenate method. Most of the DNA samples extracted 11/11/14 showed low concentrations and purity. Only some samples PIV-23/07/14 and PII-27/06/14 had relatively high concentrations - 32,6 ng/µl and 23,7 ng/µl respectively. On the other hand, DNA samples extracted 26/02/15 showed high quantity of DNAs. The purity of these samples also revealed better results.

The CC-CG PCR successfully amplified the 606 bp fragments of cyanobacterial DNA. Samples extracted 11/11/14 showed positive amplification in 9 samples out of 14 (Figure 5, Table 6) and with samples extracted 26/02/15 ten samples revealed positive amplification out of 14 samples (Figure 6, Table 6). Positive control (*Plantotrix* sp.) showed good amplification during all runs as well.

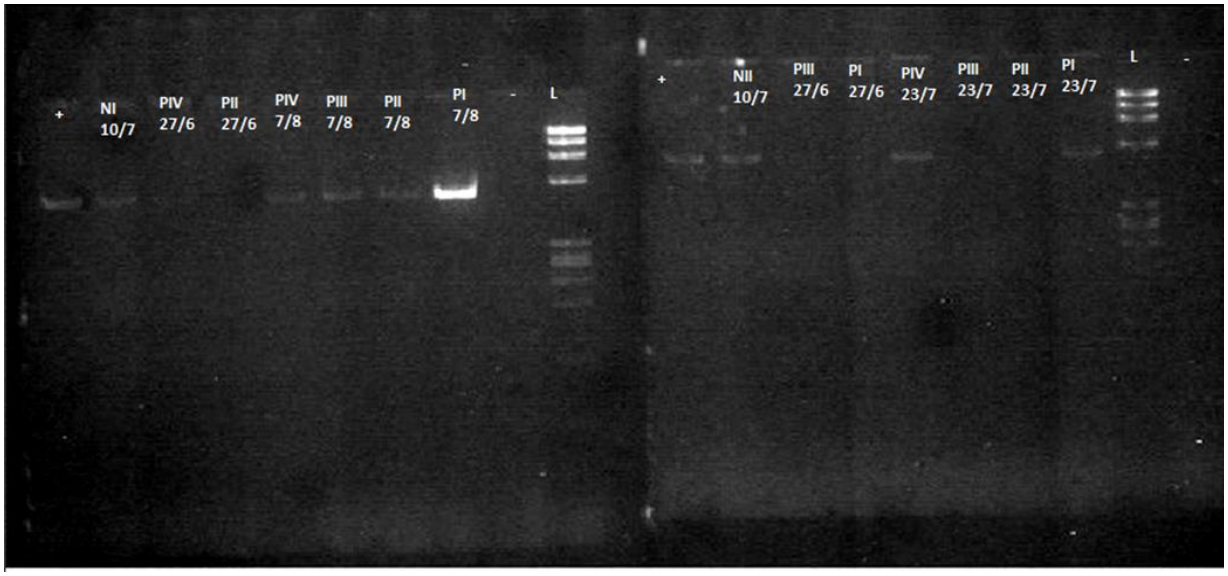

Figure 5. PCR products with oxyphotobacteria specific primers, DNA extracted on 11/11/14. (+) positive control, (-) negative control, (L) Ladder.

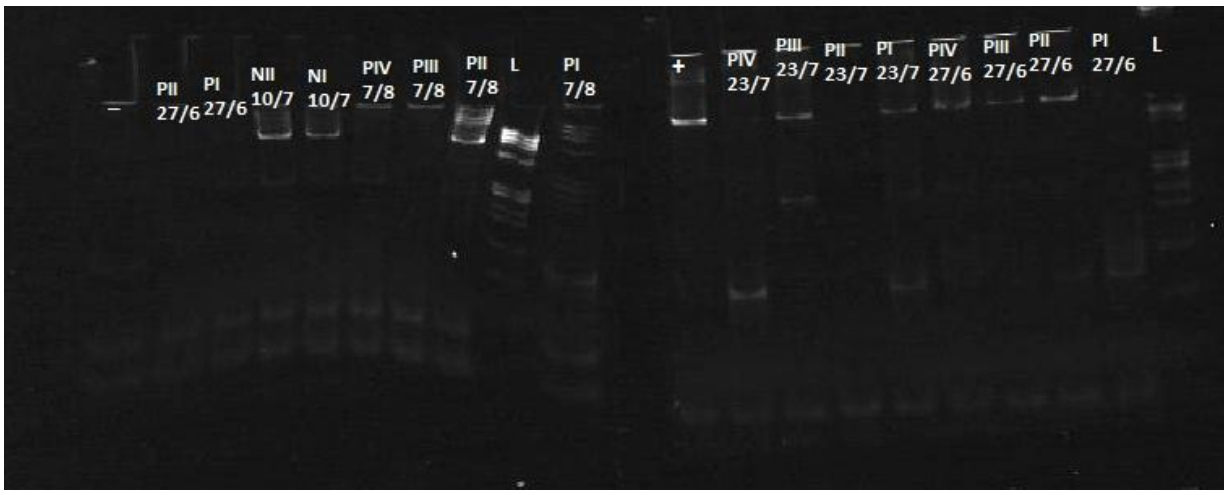

Figure 6. PCR products with oxyphotobacteria specific primers, DNA extracted 26/02/2015. (+) positive control, (-) negative control, (L) Ladder.

In overall the sampling station PI which is at the estuary of the Black Sea, with two runs of PCR using universal primers for oxyphotobacteria showed positive amplifications in June, July and August. This sampling station is characterized with highest salinity during the whole period. Sample PII 07/08/14 was positive after PCR runs for both extractions. The same sampling station showed positive band in sample taken in June, but no amplification

was seen in July, in neither extractions. P II sampling site is at the estuary of river Shav-Ghele and this part of the lake is surrounded by marshes. The sampling station PIII which is in the estuary of another river Gurinka, revealed the presence of cyanobacterial DNA in June, July and August from both extractions. The sampling station PIV demonstrated positive amplification of cyanobacterial DNA in all samples as well. This sampling station, which is in the center of the L. Paliastomi had the highest concentrations of DNA during second extraction (Table 6).

Samples from L. Nurigeli, were also positive for cyanobacteria by using oxyphotobacteria specific primers. The samples from both extractions had different concentrations and purity, being better in second extraction. Both sampling stations NI and NII showed positive results in PCR runs using universal primers (Figures 5 and 6).

Positive amplifications of oxyphotobacteria gene regions confirm presence of cyanobacteria. Therefore, microscopic/morphologically based description of abundance of cyanobacterial cells in L. Paliastomi and L. Nurigeli are in line with PCR results.

PCR reactions using genus specific primer pairs to amplify the regions of gene responsible for microcystin synthesis (*mcyB* in *Microcystis* sp. and *mcyE* in *Anabaena* sp.) were performed several times. Nested PCR reaction with 1µl of PCR product was also performed. However no amplifications in neither runs could be detected. *Microcystis* sp. (Figure 7) and *Anabaena* sp. (Figure 8) used as positive controls showed good amplification during all runs signifying that PCR reaction and cycle conditions were well formulated. The fact that genus specific primers failed to amplify any bands in PCR, was thought to be caused by low quantity and quality of DNAs extracted 11/11/14. However, even high concentration of DNA in samples did not change the results.

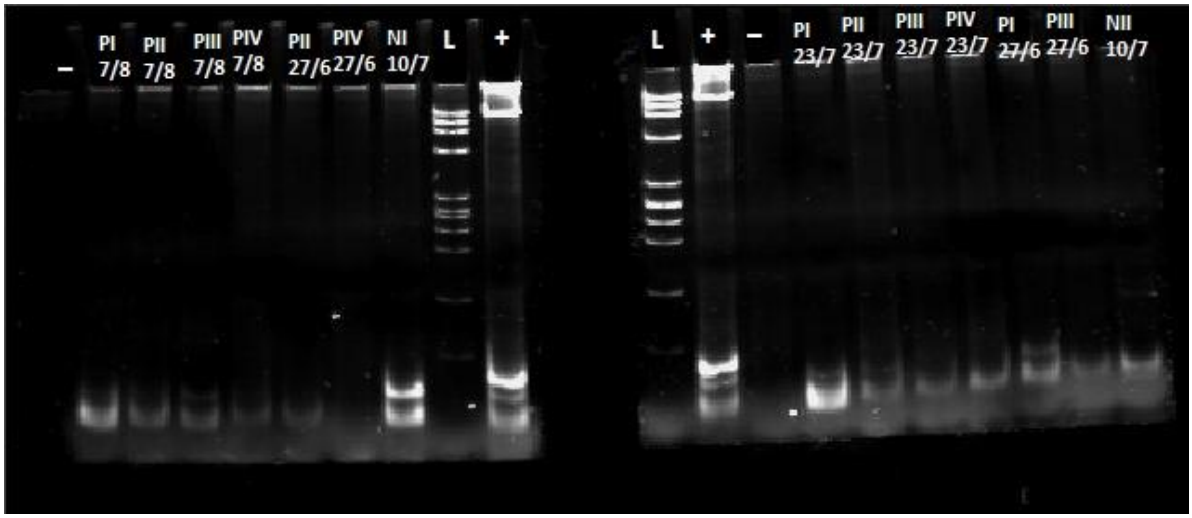

Figure 7. PCR results for detection of toxic *Microcystis* in L. Paliastomi and L. Nurigeli using *Microcystis* sp. *mcyB* genus-specific primers. (+) positive control, (-) negative control, (L) Ladder.

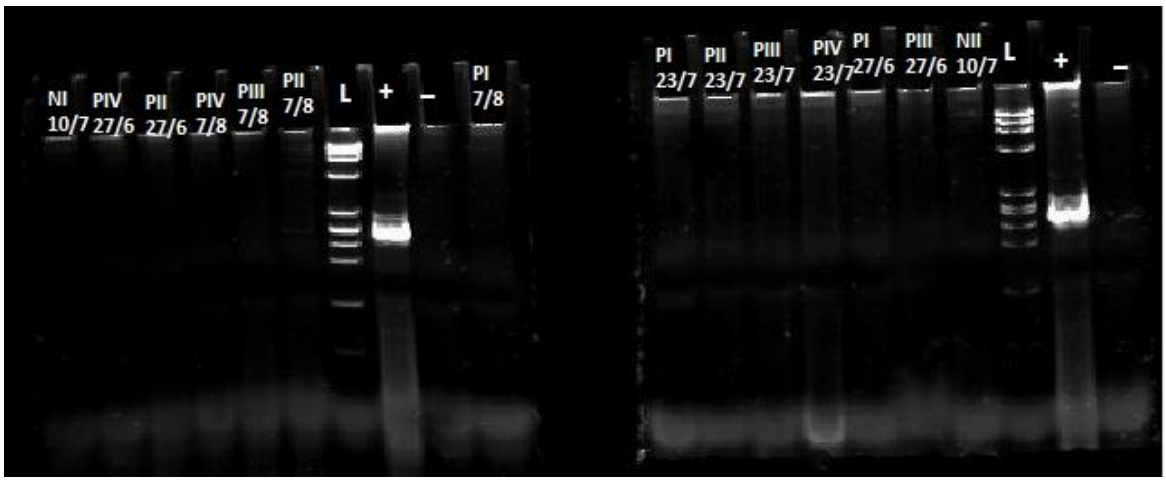

Figure 8. PCR result for detection of toxic *Anabaena* in Lake Paliastomi and Lake Nurigeli using Anabaena sp. *mcyE* genus-specific primers. (+) positive control, (-) negative control, (L) Ladder.

Negative results in PCR reaction using genus specific primers could be in agreement with low microcystin concentrations in L. Paliastomi and L. Nurigeli measured by ELISA. Thus cyanobacterial cells identified during microscopic analyses and CC-CG PCR might be non microcystin producers.

Another possible explanation of these results could be the fact that, blooms in the same water column can show variation in toxicity from year to year (Kaebernick and Neilan 2001) which might be the case in these lakes as well. Furthermore, amplification of only one part of *mcy* gene might not be enough to determine the presence of microcystin producing genes. Several authors used different PCR primer pairs to amplify various *mcy* gene regions to check the possibility of false positive results from ELISA (Valério et al 2009).

More research could be performed in future to elucidate cyanobacterial profiles in L. Paliastomi and L. Nurigeli. PCR optimization could also be effective. Remarkably, extraction of DNA of high quality from cyanobacterial cells have been reported to be complicated (Fiore et al 2000; Tillet and Neilan 2000; Singh et al 2011). The multilayered cell wall of cyanobacteria and mucilaginous sheath around cells impede efficient DNA extractions (Singh et al 2011). Moreover, environmental samples are often associated with humic acids, sands, etc., which accompany DNA after extraction and inhibit PCR reactions (Yilmaz et al 2009). During this study, spectrophotometric results revealed protein contamination of several samples (PI 23/07/14; PII 07/08/14; PIII 27/06/14; PIII 23/07/14; PIV 23/07/14; NII 10/07/14) with purity of 2 A260/A280 or over. Therefore even high concentration of DNA might not give good results on PCR because of inhibition. Dilution of samples which is thought to decrease PCR inhibitors in samples (Ouellette et al 2006) was not successful in our case. Maybe, in future improved sampling and laboratory work could better explain present results.

Furthermore, long term analyses might explain the nature of cyanobacteria community in these lakes. As reported by Vèzie et al (2002) low nutrient concentrations, especially nitrogen promote growth of nontoxic *Microcystis* cells compared to toxic ones. Similarly, low concentrations of nitrogen compounds in L. Paliastomi and L. Nurigeli are in agreement with cyanobacteria and cyanotoxins characterization in these lakes.

5. Conclusion

Based on physical and chemical parameters L. Paliastomi and L. Nurigeli belong to eutrophic water basins. The microscopic identification, as well as PCR amplification revealed the existence of cyanobacterial cells. The absence of PCR bands corresponding *mcy* gene regions might indicate that the cells of cyanobacteria in L. Paliastomi and L. Nurigeli are not producing microcystins. However, the present state of lakes along with possible future climate warming may further promote growth of toxic rather than non-toxic cyanobacteria in these lakes (Davis et al 2009). Toxin analyses demonstrated low concentrations of microcystin and saxitoxin and high concentration of anatoxin-a in the lakes. Although these lakes are not used as drinking source, recreational activities and consumption of fish from these waters have to be emphasized by the locals and authorities in the regions around L. Paliastomi and L. Nurigeli.

Current condition of the lakes is the result of environmental and artificial interference. Furthermore, the lack of proper management of water resources in the regions impede restoration processes in these lakes. In case of L. Paliastomi, the most disturbing factors are the change of its hydrological regime and discharge of municipal and agricultural runoffs from catchment area. In L. Nurigeli eutrophication processes might be further promoted by the absence of proper water mixing. Closure of the channel connecting the lake to the sea can lead to the enrichment of nutrients in the freshwater body.

Continued monitoring of the physical-chemical parameters together with cyanobacteria abundance and cyanotoxin concentrations in L. Paliastomi and L. Nurigeli could aid in the improvement of current conditions of these lakes. Moreover, since shift of toxic and nontoxic cyanobacteria is frequent in aquatic environments, restoration strategies for these lakes should be implemented to prevent development of toxic cyanobacterial species in future.

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