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CYANOTOXINS AND THEIR ACCUMULATION IN THE CURONIAN LAGOON

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INTRODUCTION

Scope of the study

Mass occurrences of cyanobacteria have been reported to increase in frequency, as well as in intensity, due to eutrophication (Finni et al., 2001; Poutanen and Nikkilä, 2001; Stewart et al., 2006). Certain cyanobacteria form nuisance blooms, due to its dense occurrence in open water or in coastal areas close to beaches. In addition, some of the bloom-forming species produce toxins, called cyanotoxins, which are products of their secondary metabolism. Most of the cyanotoxins that have been identified to date appear to be more hazardous to terrestrial mammals than to aquatic biota (Stewart et al., 2006). Researchers have primarily focused on compounds that impact upon humans and livestock, either as toxins or as pharmaceutically useful substances (Sivonen and Börner, 2008).

The first reports on algal blooms in the basin of the Baltic Sea have been known since nineteen century. Generally, they are caused by two species of filamentous nitrogen-fixing cyanobacteria, *Nodularia spumigena* and *Aphanizomenon flos-aquae*. Since the end of twentieth-century, cyabobacteria blooms in the Baltic Sea have been reported regularly (Balode and Purina, 1996; Lehtimäki, 1997; Moffitt and Neilan, 2001). They are dominated mostly by toxic *Nodularia spumigena*, *Aphanizomenon flos-aquae* and some species of genus *Dolichospermum* (Karlsson et al., 2005; Halinen et al., 2007).

The Curonian lagoon, the largest coastal lagoon in Europe, is mostly freshwater water body where autotrophic plankton communities are mainly dominated by cyanobacteria, plagues by such eutrophication relataed issues as heavy planktonic blooms and hypoxia (Žilius et al., 2012). Species of the genera *Aphanizomenon*, *Planktothrix* and *Microcystis* are the main contributors and initiators of the algal blooms in the Curonian lagoon (Olenina, 1998). They lead to the production of bioactive secondary metabolites, degradation of the hydrochemical properties of the water along with increased fish mortality, disease incidences among the main target species of the local fisheries (Belykh et al., 2013). Detailed studies of the Curonian Lagoon ecosystems date back to the beginning of the 20th century.

According to Olenina (1998), Pilkaitytė (2003) and Dmitrieva (2007), the seasonal dynamics of phytoplankton communities in Curonian Lagoon is typical for eutrophic freshwaters of the temperate zone, but not much is known on the toxicity of the cyanobacteria from Curonian Lagoon. In the Russian part of the Curonian lagoon two toxins of unknown structure were detected (Carmichael et al., 1993). Their toxicity was identified using biotests on mice and HPLC. Recently, because of the global climate change, the impact of cyanobacteria and their secondary metabolites become more important for all ecosystem function (Razinkovas et al., 2008; Paerl and Huisman, 2008).

The aim of this work was to contribute to the present knowledge by studying the toxins produced by cyanobacteria and their impact on the Curonian lagoon food web including mussels and fish.

The goal of the study

The study aims to assess the hepatotoxins produced by cyanobacteria and their accumulation in mussels, fish and bottom sediments in the Curonian lagoon.

The main objectives:

1. To identify toxic cyanobacteria species and to document the occurrence of cyanotoxins in the Curonian lagoon;
2. To evaluate the importance of temperature and salinity to the cyanotoxin production *in situ*;
3. To estimate the level of microcystin bioaccumulation in different size groups of mussels (*Dreissena polymorpha*) and fish (*Rutilus rutilus*);
4. To assess the microcystin bioaccumulation and biodegradation in sediments and related processes *in situ*.

The uniqueness of the study

For the first time cyanobacterial secondary metabolites in the Curonian lagoon were detected. The concentrations of hepatoxins determined in seston exeeded the World Health Organization's guideline value of 1 $\mu g\ l^{-1}$ for drinking. MC-LR is the most abundant toxin, which concentrations excceded normatives for recreational

waters (20 μg l^{-1}). MC-LY was detected, one of the rare but highly toxic variant, which could make harm to hydrobionts and humans. The presence of nodularin was recorded in Curonian lagoon for the first time. This is the first study reporting the hepatotoxins accumulation in mussels and fish. Futuremore, the study results points towards the cyanotoxins resuspension as a possible mechanism of secondary contamination in the lagoon.

Scientific and practical significance of the results

This study provides information on the secondary metabolites produced by cyanobacteria in the Curonian lagoon. This knowledge significantly contributes to the general understanding of massive fish kills during the midsummer algal "bloom" period and might be useful in effective management of the ecosystem. Findings suggest that cyanotoxin accumulation in mussels could be a good indicator of their production and presence in the lagoon. The results of this study indicate that bottom sediments have potential to remove, retain or release toxins and their derivatives from/to the water column, and consequently to contribute to secondary contamination of ecosystem.

Defensive statements

1. Toxic cyanobacteria species *Nodularia spumigena* from the Baltic Sea and local *Microcystis aeruginosa* are important part of autotrophic plankton and may affect the water quality in the Curonian lagoon.

2. Toxic cyanobacterial bloom and microcystins presence in midsummer are mainly related to the temperature, while salinity is the main factor determining the presense of nodularin.

3. Cyanotoxin's accumulation in zebra mussels could be considered as an indicator of hepatotoxin presence; their bioaccumulation capacity depends on individual size. Accumulation and elimination capacity of microcystins in roach (*Rutilus rutilus*) strongly depends on fish size.

4. Microcystins bioaccumulation and biodegradation in sediments is related to sediment type. Secondary contamination is possible via resuspension effects on previously buried microcystins.

Scientific approval

The results of this study were presented at nine conferences:

1. International conference "World and nature", Šiauliai, Lithuania April 7-8, 2006;

2. International simpozium "Research and management of eutrophication in coastal ecosystems", Nyborg, Denmark June 20-23, 2006;

3. The second regional student conference on "Biodiversity and functioning of aquatic ecosystems in the Baltic Sea region", Klaipėda, Lithuania October 7-8, 2006;

4. X- Lithuanian hidroecology conference „Water ecosystems function and fluctuation", Molėtai, Lithuania September 29-30, 2007;

5. The second Lithuania scientific and practical conference on „Masrine and Coastal Researh", Palanga, Lithuania April 9-11, 2008;

6. The third regional student conference on "Biodiversity and functioning of aquatic ecosystems in the Baltic Sea region", Juodkrantė, Lithuania, October 9-13, 2008;

7. Estuarine Coastal Schelf Association 50th International Symposium, Klaipėda, Lithuania, September 23-27, 2012;

8. The seventh Lithuanian scientific and practical conference on „Marine and Coastal Research", Palanga, Lithuania April 3-5, 2013;

9. Baltic Sea Science Congress, Klaipėda, Lithuania August 26-30, 2013.

Publications on the dissertation topic:

1. Paldavičienė, A., Mazur-Marzec, H., and Razinkovas A. (2009). Toxic cyanobacteria bloom in the Lithuanian part of the Curonian Lagoon. Oceanologia, 51(2), pp.203-216.

2. Ianora, A., Bentley, M., Caldwell, G., Casotti, R., Cembella, A., Engström-Öst, J., Halsband, C., Sonnenschein, E., Legrand, C., Llewellyn, C., Paldavičienė, A., Pilkaitytė, R., Pohnert, G., Razinkovas, A., Romano, G., Tillmann, U. and Vaičiūtė, D. (2011). The Relevance of Marine Chemical Ecology to Plankton and Ecosystem Function: An Emerging Field, Marine Drugs, 9, pp. 1625-1648.

3. Paldavičienė A., Zaiko A., Mazur-Marzec H., Razinokvas-Baziukas A. (2015). Bioaccumulation of microcystins in invasive bivalves: A case study from the boreal lagoon ecosystem. Oceanologia 57, pp.93-101.

Volume and structure of the thesis

The dissertation is presented in the following chapters: Introduction, Literature Review, Study Area, Material and Methods, Results, Discussion, Conclusions, References and Appendix. The dissertation volume is 156 pages; it contains 21 figures and 16 tables. It is written in English with a Lithuania summary.

Abbreviations used in the study

B.W. - body weight;
BMAA - ß- N-methylamino-L-alanine;
Chla *a* - Chlorophyll *a*;
D.W. - dray weight;
ELISA- enzime-linked immunosorbent assay;
Hepatotoxins - liver toxins;
HPLC- high-performance liquid chromatography;
HUFA - highly usaturate fatty acids;
i.p. - intraperitoneal;
IARC - The International Agency for Research on Cancer;
LC-MS - liquid chromatography-mass spectrometry;
LD_{50} - lethal dose;
MC - microcystin;
MW - molecular weight;
N – Nitrogen;
Nod - nodularin;
NPLD - net pen liver disease;
P – Phosphor;
PAR - photosynthetically active radiation;
PCR - polymerase chain reaction;
PPIA - protein phosphatase inhibition assay;
PUFA - polyunsaturate fatty acids;
RDA - redundancy analysis;
ROS - reactive oxygen species;
SAFA - saturate fatty acids;
sGST - soluble glutathione S-transferase;
SXT – Saxitoxin;
TDI - tolerable daily intake;
UV - ultraviolet;
UVR - ultraviolet radiation;
W.W. - Wet weight;
WHO - World Health Organization;

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1. LITERATURE OVERVIEW

1.1. Ecology of cyanobacteria

Cyanobacteria (blue-green algae) are prokaryotes capable of carrying out a plant-like oxygenic photosynthesis; they represent one of the oldest known bacterial lineages, with fossil evidence suggesting an appearance around 3–3.5 billion years ago (Dietrich and Hoeger, 2005). Some Cyanobacteria combine the fixation of CO_2 and the fixation of N_2 which are the two most important biogeochemical processes on Earth. They are globally important primary producers and contribute greatly to the global nitrogen budget (Karl et al., 2002). Cyanobacteria are essential players in the Earth's present and past ecosystems.

Cyanobacteria can be found in all ecosystem habitats on Earth; from the freshwater lakes and rivers, through the sea to the oceans, but also include extreme habitats, such as alkaline hot springs (Miller et al., 1998), arctic freshwaters (Quesada et al., 2006), marine and hypersaline environments like the intertidal zone or evaporates (sediments left after evaporation of sea water) (Sørensen et al., 2005), rice fields (Adhikary and Sahu, 2000), hot arid areas (Scherer et al., 1984) and terrestrial rock surfaces (Pattanaik and Adhikary, 2002). At all these places, the cyanobacteria have to adapt to great changes and frequent fluctuations of extreme environmental conditions.

Many planktonic filamentous cyanobacteria organize themselves into colonies or aggregates. This is the case for oceanic N_2-fixing cyanobacterium *Trichodesmium* that is found as "puffs" (round aggregates) or "tufts" (elongated aggregates) (Davis and McGillicuddy, 2006), and similar aggregates are produced by the brackish heterocystous cyanobacterium *Nodularia* and the freshwater *Aphanizomenon* (Walsby et al., 1997). Generally, large filamentous cyanobacteria colonize first, most probably because of their possession of thick extracellular sheaths or mucus layers, which improve the water retention properties. Since mat systems are

considered as joint ventures of different taxa, these organisms are beneficial to each other. Large colonies or filaments of cyanobacteria may also be difficult to handle for zooplankton, because their size and shape interfere with the filtering system of zooplankton (Gliwicz and Lampert, 1990). In marine habitats, such as the hypersaline Laguna Guerrero Negro, Mexico, the extensive cyanobacterial mats are dominated by the filamentous *Lyngbya aestuarii* and *Calothrix* sp. (Javor and Castenholz, 1984).

The presence of gas vacuoles allows cyanobacterial cells to adjust their vertical position in the water column, either by floating toward light sources or sinking to deeper waters to access higher nutrient concentrations or avoid photooxidative damage (Walsby and Booker, 1980; Klemer et al., 1996). Buoyancy of the two most common Baltic cyanobacteria, *Nodularia spumigena* (Mertens in Jürgens, 1822) and *Aphanizomenon flos-aquae* (Linnaeus) Ralfs ex Bornet and Flahault, 1888), is regulated by gas vesicles and changes in cellular concentrations of carbohydrates. The gas vesicles of these species survive mixing down to 60 m depth (Walsby et al., 1997). *N. spumigena* seems to be more strongly buoyant than *A. flos-aquae* (Niemistö et al., 1989). Buoyancy regulation allows the cyanobacteria to position themselves favorably within gradients of physical and chemical factors (Mur et al., 1999; Mitrovic et al., 2001) and can be an important advantage in competition with other phytoplankton organisms, especially during non-turbulent conditions (Mur et al., 1999).

These planktonic cyanobacteria sinking and floating regulated by gas vacuoles also constitutes a protective strategies against UVR which is a primary cue for the vertical movement (Reynolds et al., 1987). To escape from high solar radiation, motile cyanobacteria in mats often migrate up- and down-wards depending on the spectral waveband (Bebout and Garcia-Pichekl, 1995). Ramsing and Prufert-Bebout (1994) reported a downward movement of motile *Oscillatoriales* from microbial mat surfaces into the mat matrix or into soft sediments during periods of high insolation. Some species can migrate several meters per hour, and therefore, blooms may be formed in a short time (Walsby et al., 1992**).** The speed of floatation

is a function of the cell (colony) size. It would take the small single cells of *Microcystis* much longer to migrate a certain distance in the water column than the colonies that contain several l0s to 100s of cells (Seckbach, 2007).

1.1.1. Ecosystem effects

The presence of Cyanobacteria (e.g., *Dolichospermum circinalis, Aphanizomenon flos–aquae, Cylindrospermopsis raciborskii, Microcystis aeruginosa*) may lead to a wide array of biological impacts, including allelopathic effects on other phytoplankton (Suikkanen et al., 2004); suppression of zooplankton grazing, leading to reduced growth and reproduction rates and to changes in phytoplankton structure (Ferrao–Filho et al., 2000, Ghadouani et al., 2003).The accumulation of cyanotoxins in tissues of invertebrates (Liras et al., 1998, Lehtiniemi et al., 2002) and vertebrates (Prepas et al., 1997; Matsunaga et al., 1999; Magalhćes, 2001) as well as hepatotoxic effects on fish can also occur (Andersen et al., 1993);

Cyanobacteria blooms may affect grazing process in zooplankton by mechanical interference with the filtration apparatus. It has been suggested that the high C:P ratios that occur during blooms may lead to growth limitation of zooplankton taxa like *Daphnia*, which have a high P requirement (Hessen, 2005). It is of interest that large *Daphnia*, which are generally considered the most effective grazers of algae and the taxa responsible for such things as the spring clear water phases in eutrophic water bodies, are most sensitive to chemical stressors, including cyanobacterial toxins and most sensitive to mechanical interference. Given these biological changes, it is important to consider what effects, if any, frequent or persistent cyanobacterial blooms have on biomass and taxonomic structure of fish, in particular the commercially and recreationally important species. The answer may depend on Latitude. In temperate and boreal regions, where piscivores (salmonids) require a cold water refuge during summer, eutrophication may eliminate those fish if the hypolimnion becomes anoxic (Colby et al., 1972).

Physical, chemical and biological processes occurring in the water column of lakes, rivers and estuaries have large influences on the sediments and their associated biota and nutrient cycles (Palmer et al., 2000). Systems with frequent and/or prolonged blooms of cyanobacteria display benthic responses. Because the particle size, nutrient stoichiometry and other properties (e.g., presence of toxins) of settling seston is altered when cyanobacteria become dominant, compared with diatoms or other algae.This affects the benthic chemistry and the biota that use settled organic material as substratum or food. The enhanced organic export to sediments that occurs in eutrophic lakes leads to sediment anoxia, and this alters the taxonomic structure of benthic invertebrates and reduces the extent to which Fe binds to PO_4 at the sediment water interface. This may lead to increased diffusive internal P loading. Cyanobacterial blooms themselves may directly enhance internal P loading to surface waters if vertically migrating algae pick up P near the sediment surface and then move up into the epilimnion. Estimates of P loading in lake Mendota (Wisconsin, USA) by this process range from 2.0 to 3.6 mg P m^{-2} d^{-1} (Head et al., 1999). Where such loading occurs at a high rate, there may be long–term consequences for lake rehabilitation. Head et al. (1999) noted that "following reduction of external P loading, utilization of such internal P sources may delay expected reductions of bloom–forming cyanobacterial communities, and consequently delay improvements in other aspects of water quality.

Several of the hypothesized mechanisms probably contribute to the success of cyanobacteria simultaneously. According to Havens (2008), simple conceptual model summarizes ecological effects of cyanobacteria blooms. Their potential adverse impacts may partly explain why single relationships have failed or dominated in various aquatic systems (Hyenstrand et al., 1998).

1.2. Chemical structure of cyanotoxins

Cyanobacteria are well known for their ability to produce a large number of diverse secondary metabolites, compounds that are probably not essential for primary cell metabolism (Vining, 1992). Secondary metabolites are not used in the primary metabolism of an organism and include compounds that can operate as allelochemicals, antibiotics, hormones or toxins (Carmichael, 1992; Welker and Von Döhren, 2006).

Cyanotoxins are a diverse group of natural toxins, both from the chemical and the toxicological points of view. Cyanobacteria produce a variety of unusual metabolites, the natural function of which is unclear upon other biota. A lot of research has primarily focused on compounds that have impact upon human and livestock, either as toxins or as pharmaceutically useful substances (Ruhoy and Daughton, 2008). Further ranges of non-toxic products are also being found in cyanobacteria and the biochemical and pharmacological properties of these are totally unknown (WHO, 1999; WHO, 2011).

Mechanisms of cyanobacterial toxicity currently described and understood are very diverse and range from hepatotoxic, neurotoxic and dermatotoxic effects to general inhibition of protein synthesis (Funari and Testai, 2008; WHO, 2011). For the assessment of specific hazards related to cyanobacterial toxins it is necessary to understand their chemical and physical properties, to recognize their occurrence in waters used by live organisms, to know the factors regulating their production and transportation through the food chains in water ecosystem (WHO, 1999; WHO, 2011).

Cyanotoxins are classified into three broad groups of chemical structure: cyclic peptides, alkaloids and lipopolysaccharides (LPS), that reflect their strongest biological effects on the systems and organs (Codd et al., 2005 a, b) (Table 1.1). Each cyanotoxin can be produced by more than one cyanobacteria species; likewise, the same species is able to produce more than one toxin (Table 1.2). Moreover, within a single species, different genotypes occur, some of which possess the gene for a given toxin and others that do not.

This was first demonstrated for microcystins (MCs) (Kurmayer et al., 2002). In 50-75% of cyanobacterial blooms, the toxicity is associated with a simultaneous production of diverse cyanotoxins (Carmichael, 1994), whose relative importance and spatial distribution are subjected to a wide variability. The toxicity of a given bloom is determined by its strain composition, i.e., the relative share of toxic versus nontoxic genotypes (Kurmayer et al., 2011).

Globally, the cyclic peptide toxins of the microcystin and nodularin family are the most frequently found cyanobacterial toxins in blooms from fresh and brackish waters. They pose a major challenge for the production of safe drinking water from surface water bodies containing cyanobacteria with these toxins. In mouse bioassays, which traditionally have been used to screen toxicity of field and laboratory samples, cyanobacterial hepatotoxins (liver toxins) cause death by liver hemorrhage (Williamson, 2013). Microcystins have been characterized from planktonic species from *Dolichospermum, Microcystis, Oscillatoria (Planktothrix), Nostoc,* and *Anabaenopsis* genera, and from terrestrial *Hapalosiphon* genera genus (Williamson, 2013). Nodularin, with one exception, has been characterized only from *Nodularia spumigena* (Gehringer et al., 2012).

The cyclic peptides are comparatively large natural products, with molecular weight (MW) ≈ 800-1,100, although small compared with many other cell oligopeptides and polypeptides (proteins) (MW > 10,000). The cyanobacterial hepatotoxins contain either five (nodularins) or seven (microcystins) amino acids, with the two terminal amino acids of the linear peptide being condensed (joined) to form a cyclic compound. They are water soluble and, except perhaps for a few somewhat more hydrophobic microcystins, are unable to penetrate directly the lipid membranes of animal, plant and bacterial cells. Therefore, to elicit their toxic effect, uptake into cells occurs through membrane transporters which otherwise carry essential biochemicals or nutrients. In aquatic environments, according to WHO (1999; 2011), these toxins usually remain within the cyanobacterial cells and are only released in substantial amounts on cell lysis. Their high chemical stability and their water solubility

have important implications for their environmental persistence and exposure to other live organisms in water bodies.

The first chemical structures of cyanobacterial cyclic peptide toxins were identified in the early 1980s and the number of fully characterized toxin variants has greatly increased during the 1990s. The first such compounds found in freshwater cyanobacteria were cyclic heptapeptides (with seven peptide-linked amino acids) with the general structure of: cyclo-(D-alanine1-X^2-D-MeAsp^3-Z^4-Adda^5-D-glutamate^6-Mdha^7) in which X and Z are variable L amino acids, D-MeAsp is *D-erythro*-β-methylaspartic acid, and Mdha is *N*-methyldehydroalanine. The amino acid Adda, (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10 phenyldeca-4,6-dienoic acid, is the most unusual structure in this group of cyanobacterial cyclic peptide toxins. These compounds were first isolated from the cyanobacterium *Microcystis aeruginosa* and therefore the toxins were named microcystins (Carmichael, 1992). Structural variations have been reported in all seven amino acids, but most frequently with substitution of L-amino acids at positions 2 and 4, and demethylation of amino acids at positions 3 and/or 7. About 80 structural variants of microcystins have been characterised so far from bloom samples and isolated strains of cyanobacteria (Table 1.2).

Table 1.1. Toxigenic cyanobacteria from marine, brackish and freshwaters (Codd et al., 2005 a,b).

Toxin group[1]	**Primary target organ in mammals**	**i.p. LD_{50}**[4] **(µg/kg b.w.)**	**Main producing cyanobacteria**[2]
Cyclic peptides			
Microcystins	Liver	50-1200	*Microcystis* spp., *Planktothrix* spp., *Dolichospermum, Nostoc, Synechocystis, Cyanobium bacillare, Arthrospira fusiformis, Limnothrix redekei, Phormidium formosum, Hapalosiphon hibernicus, Anabaenopsis*
Nodularin	Liver	50	*Nodularia spumigena*
Alkaloids			
Anatoxin-a	Nerve synapse	375	*Anabaena* spp., *Microcystis* spp., *Planktothrix* spp., *Aphanizomenon flos-aquae, Aphanizomenon issatschenkoi, Cylindrospermum, Raphidiopsis mediterranea*
Anatoxin-a(S)	Nerve synapse	20-40	*Anabaena flos-aquae, Anabaena lemmermannii*
Aplysiatoxins	Skin		*Lyngbya majuscula* (*marine waters*), *Schizothrix, Oscillatoria nigro-viridis*
Cylindrospermopsins (CYN)	Liver[3]	2100 (24 h) 200 (6 days)	*Cylindrospermopsis raciborskii, Umezakia natans, Aphanizomenon ovalisporum, Aphanizomenon flos-aquae, Rhaphidiopsis curvata, Anabaena lapponica, Anabaena bergii*
Lyngbyatoxin-a	Skin, gastro-intestinal tract	330	*Lyngbya*
Saxitoxins (PSP)	Nerve axons	10-20	*Anabaena, Aphanizomenon, Lyngbya, Cylindrospermopsis* spp.
Lipopolysaccharides (LPS) (endotoxins)	Potential irritant; affects any exposed tissue	40-190 mg/kg bw	All cyanobacteria

[1] Many structural variants may be known for each toxin group;
[2] Not produced by all species of the particular genus

[3] Whole cells of toxic species elicit widespread tissue damage, including damage to kidney and lymphoid tissue
[4] i.p. (intraperitoneal) LD_{50} **(**lethal dose**);**

In one species of brackish water cyanobacterium, an identically acting and structurally very similar, cyclic pentapeptide occurs. It has been named as nodularin after its producer, *Nodularia spumigena*. The chemical structure of nodularin is cyclo-(D-MeAsp[1]-L-arginine[2]-Adda[3]-D-glutamate[4]-Mdhb[5]), in which Mdhb is 2-(methylamino)-2- dehydrobutyric acid. A few naturally occurring variations of nodularins have been found: two demethylated variants, one with D-Asp instead of D-MeAsp, the other with DMAdda instead of Adda; and the non-toxic nodularin which has the 6Zstereoisomer of Adda (Namikoshi et al., 1994). The equivalent 6Z-Adda stereoisomer of microcystins is also non-toxic. In the marine sponge, *Theonella swinhoei*, a nodularin analogue called motuporin has been found. It differs from nodularin only by one amino acid, having hydrophobic L-Val in place of the polar L-Arg in nodularin (De Silva et al., 1992). The toxin might be cyanobacterial in origin because the sponge is known to harbour cyanobacterial symbionts (WHO, 1999).

The toxicity of microcystins and nodularins to mammals is mediated through their strong binding to key cellular enzymes called protein phosphatases. In solution, microcystins and nodularins adopt a chemical "shape" that is similar, especially in the Adda-glutamate part of the cyanotoxin molecule (Rudolph-Böhner et al., 1994; Annila et al., 1996). Studies showed that this region is crucial for interaction with the protein phosphatase molecule, and hence it is crucial for the toxicity of these cyanotoxins (Barford and Keller, 1994). Microcystins show an additional characteristic of forming a covalent bond between the Mdha residue and the protein phosphatase molecule (Harada et al., 1990 a, b; Rinehart et al., 1994).

Most of the structural variants of microcystin and nodularin are highly toxic in mouse bioassay within a comparatively narrow range (intra-peritoneal (i.p.) 50-300 μg kg^{-1} body weight (bw)). Only a few non-toxic variants have been identified. In general, any structural modifications to the Adda-glutamate region of the toxin molecule,

such as a change in isomerisation of the Adda diene (6(*E*) to 6(*Z*)) or acylation of the glutamate, renders microcystins and nodularins non-toxic. Microcystins and nodularin have been characterised from axenic cyanobacterial strains and genes encoding the biosynthesis of theses peptides were identified in cyanobacteria, thus the origin of these compounds is clear (Harada et al., 1990 a, b; Rinehart et al., 1994).

Table 1.2. The microcystins (MCYST) reported in WHO (2011).

Microcystin	**Molecular weight**	**Toxicity LD_{50}[2]**	**Organism[3]**	**Reference(s)**
MC-LA	909	50	*M. aeruginosa*[s], *M. viridis*[s]	Botes et. al., 1984; Kaya and Watanable, 1990
MC-LAba	923	NR	*M. aeruginosa*[s]	Gathercole and Thiel, 1987
MC-LL	951	+	*M. aeruginosa*[b]	Craig et al., 1993
MC-AR	952	250	*Microcystis spp.*[b]	Namikoshi et al., 1992a
MC-YA	959	NR	*M. aeruginosa*[s]	Botes et al., 1985
[D-Asp[3], Dha[7]]MC-LR	966	+	*M. aeruginosa*[s], *Anabaena sp.*[s]	Harada et al., 1991b Sivonen et al., 1992a
[D-Asp[3], Dha[7]]MC-EE(OMe)	969	+	*Anabaena sp.*[s]	Namikoshi et al., 1998
MC-VF	971	NR	*M. aeruginosa*[s],	Bateman et al., 1995
[D-Asp[3]]MC-LR	980	160-300	*A.flos-aquae*[s], *M. aeruginosa*[s], *M. viridis*[b], *O. agardhii*[s]	Krishnamyrthy et al., 1989; Cremer and Henning, 1991; Harada et al., 1990b; 1991a; Luukkainen et al., 1993
[Dha[7]]MC-LR	980	250	*M. aeruginosa*[s], *Anabaena sp.*[s], *O. agardhii*[s]	Harada et al., 1991b Sivonen et al., 1992a Luukkainen et al., 1993
[DMAdda[5]]MC-LR	980	90-100	*Microcystis spp.*[b], *Nostoc sp.*[s]	Namikoshi et al., 1992a Sivonen et al., 1992b
[Dha[7]]MC-EE(OMe)	983	+	*Anabaena sp.*[s]	Namikoshi et al., 1998
[D-Asp[3], Dha[7]]MC-E(OMe)E(OMe)	983	+	*Anabaena sp.*[s]	Namikoshi et al., 1998
MC-LF	985	+	*M. aeruginosa*[s]	Azavedo et al., 1994
MC-LR	994	50	*M. aeruginosa*[s], *A. flos-aquae*[s], *M. viridis*[s]	Botes et al., 1985; Krishnamyrthy et al., 1989;

Microcystin	Molecular weight	Toxicity LD_{50}[2]	Organism[3]	Reference(s)
[D-Asp[3], D-Glu(OCH_3)[6]]MC-LR	994	NR	*A. flos-aquae*[s]	Sivonen et al., 1992b
[(6Z)-Adda[5]]MC-LR	994	>1.200	*M. viridis*[b]	Harada et al., 1990a, b
[Dha[7]]MC-E(OMe)E(OMe)	997	+	*Anabaena sp.*[s]	Namikoshi et al., 1998
[L-Ser[7]]MC-LR	998	+	*Anabaena sp.*[s]	Namikoshi et al., 1992c
MC-LY	1.001	90	*M. aeruginosa*[s]	Stoner et al., 1989
[L-Ser[7]]MC-EE(OMe)	1.001	+	*Anabaena sp.*[s]	Namikoshi et al., 1998
[D-Asp[3], Ser[7]]MC-E (OMe)E(OMe)	1.001	+	*Anabaena sp.*[s]	Namikoshi et al., 1998
MC-HilR	1.008	100	*Microcystis spp.*[b]	Namikoshi et al., 1995
[D-Asp[3],ADMAdda[5]] MC-LR	1.008	160	*Nostoc sp.*[s]	Sivonen et al., 1990a; Namikoshi et al., 1990
[D-Glu(OCH_3)[6]] MC-LR	1.008	>1,000	*A. flos-aquae*[s], *Microcystis sp.*[s]	Sivonen et al., 1992d; Bateman et al., 1995; Rinehart et al., 1994;
[D-Asp[3], Dha[7]]MC-RR	1.009	+	*O. agardhii*[b], *Anabaena sp.*[s], *M. aeruginosa*[s]	Krishnamyrthy et al., 1989; Sivonen et al., 1992a;
[D-Asp[3],ADMAdda[5], Dhb[7]] MC-LR	1.009	+	*Nostoc sp.*[s]	Beattie et al., 1998
[L-MeSer[7]]MC-LR	1.012	150	*Microcystis spp.*[b]	Namikoshi et al., 1992a; 1995
[Dha[7]]MC-FR	1.014	NR	*Microcystis sp.*[b]	Luukkainen et al., 1994
[L-Ser[7]]MC-E(OMe)E(OMe)	1.015	+	*Anabaena sp.*[s]	Namikoshi et al., 1998
[ADMAdda[5]] MC-LR	1.022	60	*Nostoc sp.*[s]	Sivonen et al., 1990a;Namikoshi et al., 1990
[D-Asp[3],ADMAdda[5]] MC-LHar	1.022	+	*Nostoc sp.*[s]	Sivonen et al., 1992b
[D-Asp[3]]MC-RR	1.023	250	*O. agardhii*[s], *Anabaena sp.*[s], *M. aeruginosa*[s]	Meriluoto et al., 1989; Sivonen et al., 1992a; Luukkainen et al., 1994
[Dha[7]]MC-RR	1.023	180	*M. aeruginosa*[s], *Anabaena sp.*[s], *O. agardhii*[s]	Kiviranta et al., 1992; Sivonen et al., 1992a; Luukkainen et al., 1993
MC-LW	1.024	NR	*M. aeruginosa*[s]	Bateman et al., 1995
MC-FR	1.028	250	*Microcystis spp.*	Namikoshi et al., 1992a

Microcystin	Molecular weight	Toxicity LD_{50}[2]	Organism[3]	Reference(s)
			[b]	
MC-M(O)R	1.028	700-800	*Microcystis spp.*	Namikoshi et al., 1992a
[Dha[7]]MC-HphR	1.028	+	*Anabaena sp.*[s]	Namikoshi et al., 1992b
[D-Asp[3], Dha[7]]MC-HtyR	1.030	+	*Anabaena sp.*[s]	Namikoshi et al., 1992b
[Dha[7]]MC-YR	1.030	+	*M. aeruginosa*[s]	Sivonen et al., 1992c
[D-Asp[3]]MC-YR	1.030	+	*Microcystis spp.* [b]	Namikoshi et al., 1992b
MC-YM(O)	1.035	56	*M. aeruginosa* [b]	Botes et al., 1985; Elleman et al., 1978
[ADMAdda[5]] MC-LHar	1.036	60	*Nostoc sp.* [s]	Sivonen et al., 1990a; Namikoshi et al., 1990
MC-RR	1.037	600	*M. aeruginosa*[s], *M. viridis*[s], *Anabaena sp.*[s]	Kusami et al., 1987; Painuly et al., 1988; Watanabe et al., 1988; Sivonen et al., 1992a
[(6Z)-Adda[5]]MC-RR	1.037	>1,200	*M. viridis*[b]	Harada et al., 1990a, b
[D-Ser[1], ADMAdda[5]]MC-LR	1.038	+	*Nostoc sp.* [s]	Sivonen et al., 1992b
[ADMAdda[5], MeSer[7]]MC-LR	1.040	+	*Nostoc sp.* [s]	Sivonen et al., 1992b
[L-Ser[7]]MC-RR	1.041	+	*Anabaena sp.*[s], *M. aeruginosa*[s/b]	Namikoshi et al., 1992c; Luukkainen et al., 1994
[D-Asp[3], MeSer[7]]MC-RR	1.041	+	*O. agardhii*[s]	Luukkainen et al., 1993
MC-YR	1.044	70	*M. aeruginosa*[s], *M. viridis*[s]	Botes et al., 1985; Watanabe et al., 1988
[D-Asp[3]]MC-HtyR	1.044	160-300	*A.flos-aquae*[s]	Harada et al., 1991a
[Dha[7]]MC-HtyR	1.044	+	*Anabaena sp.*[s]	Namikoshi et al., 1992b
MC-(H_4)YR	1.048	NR	*Microcystis spp.* [b]	Namikoshi et al., 1995
[D-Glu-$OC_2H_3(CH_3)OH$[6]] MCYST-LR	1.052	> 1.000	*Microcystis spp.* [b]	Namikoshi et al., 1992a
[D-Asp[3],ADMAdda[5], Dhb[7]] MC-RR	1.052	+	*Nostoc sp.* [s]	Beattie et al., 1998
MC-HtyR	1.058	80-100	*A.flos-aquae*[s]	Harada et al., 1991a
[L-Ser[7]]MC-HtyR	1.062	+	*Anabaena sp.*[s]	Namikoshi et al., 1992b
MC-WR	1.067	150-200	*Microcystis spp.* [b]	Namikoshi et al., 1992a
[D-Asp[3],ADMAdda[5],	1.073	+	*Nostoc sp.* [s]	Beattie et al., 1998

Microcystin	Molecular weight	Toxicity LD_{50}[2]	Organism[3]	Reference(s)
Dhb[7]] MC-HtyR				
[L-MeLan[7]] MC-LR	1.115	1.000	*Microcystis spp.* [b]	Namikoshi et al., 1995

Aba Aminoisobutyric acid
ADMAdda *O*-Acetyl-*O*-demethylAdda
Dha Dehydroalanine
Dhb Dehydrobutyrine
DMAdda *O*-DemethylAdda
E(OMe) Glutamic acid methyl ester
(H4)Y 1,2,3,4,-tetrahydrotyrosine
Har Homoarginine
Hil Homoisoleucine
Hph Homophenylalanine
Hty Homotyrosine
MeLan *N*-Methyllanthionine
M(O) Methionine-*S*-oxide
MeSer *N*-Methylserine
'NR' denotes 'Not reported'
(6Z)-Adda Stereoisomer of Adda at the double bond
[2] Toxicity determined i.p. mouse (μg kg^{-1}); the LD_{50} value is the dose of toxin that kills 50% of exposed animals; a '+' denotes a toxic result in a non-quantitative mouse bioassay or inhibition of protein phosphatase
[3] An's' denotes toxins isolated from culture samples and a *'b'* denotes toxins isolated from bloom samples

The degree of severity of MC-induced toxicity depends on the levels and duration of exposure, determined by the balance between MC absorption, detoxification, and excretion. Acute toxicity is highly variable among MC variants: some of them, such as MC-LA, MC-YR and MC-YM, show intraperitoneal (i.p.) LD_{50} similar to MC-LR.; For the other congeners, LD_{50} are spread in a wide range of values (from 50 up to 1200 μg kg^{-1}) (Table 1.2), due to the presence of different substituents (Funari and Testai, 2008).

The International Agency for Research on Cancer (IARC) recently reviewed available data on MC-LR; however, on the basis of data on tumor promoting mechanisms, IARC has classified MC-LR as possibly carcinogenic to humans (IARC, 2006).

The maximum allowable concentration for microcystin in drinking water is 1 μg l^{-1} (Falconer, 1994). According to World Health Organization (WHO) for recreational water bodies the allowable microcystin concentrations are < 20 μg l^{-1}. However, there

are even more strict requirenments in some countries (e.g. Washington State department of Health has set provisional recreational guidance levels of 6 μg l^{-1} (Hardy, 2008)). Based on this limit, World Health Organization (WHO) established 0.04 μg kg^{-1} of body weight per day as a tolerable daily intake (TDI) of microcystins (Chorus and Bartram, 1999). Magalhães et al. (2001) data confirmed the accumulation and the persistence of MC in *Tilapia rendalli* muscles tissue and demonstrated a risk from the consumption of this fish, since 71.7 % of the muscle samples were above the recommended TDI. According to the published data, hepatoxins concentration in the fish tissues can sometimes reach or even exceed the TDI level recommended by WHO.

1.3. Toxins production

Not all species of cyanobacteria produce toxins, yet some species produce several types of toxin. Even within a single species, some strains are toxic while others are not. This makes the prediction of toxicity an arduous task and certainly more difficult than simply predicting bloom occurrence. In most cases, however, toxic and non-toxic strains of a species occur simultaneously. The concentration of toxin(s) is dependent on the density of toxin-producing species in water body. Intensity and species composition of blooms varies both over time and with location in water body, as a result the toxicity will vary as well. Lakes, rivers, water reservoirs that have never had a problem can suddenly become toxic and, conversely water bodies that have shown toxicity in the past may not experience toxic blooms for several years. Some water bodies could become toxic while others could remain safe. The reasons for cyanotoxin production are not clear. Several hypotheses have been put forward to explain the intensity of algal toxins production: biosynthesis (Gallon et al., 1990, 1994; Turner and Tester, 1997), genetic regulation of cyanotoxin production (Bittencourt-Oliveira, 2003; Ross et al., 2006;), abundance and colony size (Scheffer et al., 1997; Chorus and Bartram, 1999; Kurmayer et al., 2003); grazer repellence (DeMott, 1999; Lampert, 1987; DeMott and Moxter, 1991; Kirk and Gilbert, 1992; Carlsson et al., 1995),

regulation by chemical and physical factors (Orr and Jones, 1998; Sivonen and Jones, 1999; Oh et al., 2000).

1.3.1. Genetic regulation of cyanotoxin production

In some bloom-specific cases, one species can be morphologically identical to the next yet may vary in toxicogenicity (Baker et al., 2001). In other cases, some species are known to upregulate or downregulate their toxicity under varying laboratory conditions (Kaebernick and Neilan, 2001). It is not known why such natural variations in toxicity exist. For toxins such as MC, NOD and CYN, the molecular machinery involved in their production is already elucidated and the presence of toxin-coding genes has been considered an important tool for the study of the potential toxicity of cyanobacteria (Pearson and Huisman, 2008). All these toxins are produced by a wide range of cyanobacteria genera that have been involved in severe poisoning episodes all over the world (Codd et al., 2005 a, b). The use of molecular probes that target toxin-associated genes, in conjunction with immunoassays, have led to advancements in the identification of toxic strains (Bittencourt-Oliveira, 2003). Several genes responsible for cyanotoxin production have been identified in *M. aeruginosa* (Kaebernick and Neilan, 2001). The first molecular biological studies on toxic cyanobacteria investigated the possible involvement of plasmids in toxin production. According to Schwabe et al. (1988), four toxic strains of *Microcystis aeruginosa* contained plasmids, and no plasmid could be shown in one toxic and in several nontoxic strains. More recently, a similar study in Australia found no evidence for plasmid involvement in microcystin synthesis (Bolch et al., 1996). Gallon et al. (1994) studied an anatoxin-a producing *Anabaena* strain NCR 44-1, which spontaneously became non-toxic. They found that the size of a plasmid was reduced in that non-toxic clone, but this work has not been repeated or confirmed.

Several cyclic and linear peptides, often with D-amino acids, are known to be produced nonribosomally by multi-domain peptide synthetases via the so-called thiotemplate mechanism in bacteria and

lower eukaryotes. The best characterised are the synthesis of gramicidin S and tyrocidin by *Bacillus.* Peptide synthesis genes have been detected and sequenced (partly) in *Microcystis aeruginosa* (Meissner et al., 1996) and in *Anabaena* (Rouhiainen et al., 2004). Analogous polymerase chain reaction (PCR) products to the peptide synthesis genes have been shown by using DNA from *Microcystis* (Jacobs et al., 1995; Arment and Carmichael, 1996) and *Nodularia* as a template. Dittman et al. (2000) showed, in knockout experiments, that peptide synthesis genes are responsible for microcystin production. Ross et al. (2006) data confirms that the presence of the *mcy*B gene, responsible for the production of microcystins, was present in the St. Lucie River *M. aeruginosa* assemblage. At least some strains which produce hepatotoxins also produce other small cyclicpeptides (Namikoshi and Rinehart, 1996; Weckesser et al., 1996; Welker and von Döhren, 2006) which are likely to be produced by nonribosomal peptide synthesis.

Rantala *et al.* (2004) suggested an ancient origin of MC and reported the loss of *mcy* genes and, as a consequence, a loss in the ability of some strains to produce toxin. It is known that the transfer of fragments of DNA is common among prokaryotes (Tsuge et al., 2001) and, in the particular case of cyanobacteria, genes responsible for toxin production might be subjected to horizontal gene transfer, gene losses (Mikalsen et al., 2003), transposition, mutagenesis, deletion and recombination (Moffitt and Neilan, 2001). As described by Kaebernick *et al.* (2001), mutations within the mcy gene cluster might occur during cultivation leading to a decrease in toxicity. Without involving molecular analysis, it was already reported a decrease in the toxicity of strains when maintained in culture. Cyanobacteria seem to be more toxic in the natural environment than under laboratory conditions (Prati et al., 2002).

Among the mcy genes, a region of the mcyE has been used as a reliable molecular marker for the detection of MC producers (Rantala et al., 2006). This gene is related to enzymes that are involved in the synthesis of Adda and addition of D-glutamate, which are both essential amino acids for the toxicity of MC. In phylogenetic studies it was shown that mcyE sequences from

different producer genera form their own clusters and remain excluded from horizontal gene transfer (Rantala et al., 2006). This fact may explain the prevalence of this gene in the strains and the loss of others.

1.3.2 Abundance and colony size

Cyanobacterium frequently forms mass developments and surface scums in eutrophic water reservoirs. The majority of these formations contain toxins. Variation in cyanotoxins concentration is related to the contribution of toxin-producing genotype to cyanobacterial community, cyanobacterial abundance, biomass, and season. In eutrophic lakes, cyanobacterial biomass starts to rise from late spring or early summer till it reached the maximum peak mostly in the end of summer – autumn (Jungmann et al., 1996). At the same time, toxins concentration is increasing when the biomass grow. Scheffer et al. (1997) detected strong positive relation between microcystins concentration and toxic cyanobacterial biomass. According to Mazur-Marzec (personal opinion) not all the time the toxins production could be related to the occurrence of high biomass.

The morphological characteristics like cell size, colony size are very important and related with toxin concentration. Kurmayer et al. (2002), Kurmayer and Kutzenberg (2003) and Via-Ordorika et al. (2004) detected positive correlation between the concentration of cyanotoxins and morphological feature of toxin-producing cyanobacteria. The percentage of the mcy gene and microcystin concentration was significantly higher in cyanobacteria forming bigger colonies.

In addition, toxin production rate was positively related to the growth rate of the larger colony size classes forms (>100 μm). According to Kurmayer et al. (2002), 42 to 73% of cyanobacteria with the large colony size (>500 μm) belonged to the microcystin-producing genotype, compared to only 10 to 15% of the small colonies (< 500 μm). Other works also indicate that toxins are mainly produced by larger colonies. These findings have important implications for interactions between cyanobacteria and grazers.

1.3.3. Induction by grazers

Increased toxin production can also be used as a defence against device zooplankton grazing by inhibiting feeding rate (DeMott, 1999). Grazer repellence is probably the most studied hypothesis (Lampert, 1987; DeMott and Moxter, 1991; Kirk and Gilbert, 1992; Carlsson et al., 1995). According to Pajdak-Stós et al. (2001), cyanobacteria are highly resistant to grazing by protozoans, copepods and cladocerans, due to millions of years of co-evolution with these herbivores. It has, however, been pointed out that the occurrence of co-evolution is hard to assess in the marine environment (Hay, 1991). There are only suggestions from studies of copepods (Turner and Tester, 1997) and bivalve molluscs (Shumway and Cucci, 1987) that coevolutionary experience or periodic exposure to toxic phytoplankton blooms may have conferred some ability to consume toxic phytoplankton with no ill effects. Much of the disparity of effects is due to the variety of phytoplankton toxins. The sensitivity of grazers to cyanobacteria toxins are species-specific or even strain-specific. Different grazers also exhibit different responses to different toxic phytoplankton species or to different clones or blooms of the same species (Turner and Tester, 1997). Notably, very few grazers are specialized on cyanobacteria, with the exception of several ciliate strains, e.g. *Nassula* sp. and *Pseudomicrothorax* sp. (Canter and Gregory, 1994; Fialkowska and Pajdak-Stós, 1997), due to the grazing-decreasing characteristics of cyanobacteria, other than toxins (Canter and Gregory, 1994). Furthermore, according Engström-Öst (2002), there is not known when toxin production of cyanobacteria evolved. Variations in phytoplankton toxicity result in complex and inconsistent interactions between toxic phytoplankters and their grazers. There is also variation in physiological responses of organisms to algal toxins in terms of binding or recognition the initial event in the onset of toxicity (Baden and Trainer, 1993). An overall synthesis of interactions between toxic phytoplankton and their grazers is elusive because blooms and grazer interactions are situation-specific. Many contradictions are due to a variety of toxins and other cyanobacterial metabolites which may have different physiological effects on

consumers and to differences in toxin potency or intracellular concentrations due to genetic variability, uncontrolled culture conditions, or environmental variations that are not accounted for in natural blooms. Engström-Öst (2002) argues that the fossil record does not support the hypothesis that herbivory would generate strong selective pressure on the algal species, although grazing is usually intense and universal in the marine environment.

According to Mazur-Marzec et al. (2013) microcystins, nodularins and other non-ribosomal peptides produced by cyanobacteria were suggested to play some role in competition with eukaryotic algae (Suikkanen et al., 2004), or as defense agents against grazers (Rohrlack et al., 2004; Czarnecki et al., 2006). The potential role of the compounds in cyanobacteria bloom termination through viral lysis (Sedmak et al., 2008) or in the interaction with chytrid fungi infecting cyanobacteria (Sønstebø et al., 2011) was also considered. To sum up, the ecological purpose of the secondary metabolites produced by planktic organisms, e.g. phytoplankton is not much known (Carmichael, 1992; Verity and Smetacek, 1996). Although a certain compound may affect copepod grazing adversely, it does not necessarily imply that it has evolved as a feeding deterrent (Hay, 1996). Indeed, certain zooplanktons ingest various toxic phytoplankters with impunity, whereas for others, deleterious effects may occur. Putative explanations for toxin production other than grazing deterrence include precursors for subcellular organelles, cell-wall degradation products, nucleic acid synthesis, nitrogen storage, or inhibition of competing, co-occurring phytoplankton species (Engström-Öst, 2002).

1.3.4. Chemical and physical factors

Laboratory studies with pure strains of cyanobacteria have found that environmental factors can induce changes in toxicity or toxin concentration (on a per unit biomass basis), but usually by a factor of no more than three or four. On a per cell basis, the changes in toxin content are probably even smaller. These environmentally-induced changes are far less than the range of more than three orders of

magnitude in toxin content measured between individual strains grown in culture under identical conditions. This gives support to the assumption that much, if not most, of the variation in toxicity of "monospecific" natural blooms is the waxing and waning of strains of the same species, but with varying toxin quotas. The factors that control the growth and toxin content of individual strains are as yet unknown.

Some cyanobacteria are known to exhibit high or low toxicity under different laboratory conditions (Kaebernick and Neilan, 2001). The production of toxin by a single cyanobacterial strain seems to be consider as the spontaneous and permanent loss of toxin production has been seldom reported. The effects of several environmental factors on growth and toxin production by cyanobacteria have been studied in batch and continuous culture experiments. Culture age in batch cultures, and temperature, are the parameters most frequently examined, followed by light, pH, nutrients, salinity, and micronutrient concentrations. Studies have been done on hepatotoxic *Microcystis, Oscillatoria (Planktothrix), Dolichospermum* and *Nodularia;* anatoxin-a producing *Anabaena, Aphanizomenon* and *Planktothrix;* and saxitoxin producing *Aphanizomenon* and *Anabaena circinalis.* Microcystins and anatoxin-a are largely retained within cells when the conditions for the growth of the organism are favorable. The amount of microcystin in a culture increases during the logarithmic growth phase, being highest in the late logarithmic phase (Watanable, 1996). Light and temperature influence both growth rates and toxin production in many species of cyanobacteria (Sivonen, 1990; Rapala et al., 1997). Several studies have shown that toxin production in *Microcystis aeruginosa* increases with irradiance under light-limited conditions (Utkilen and Gjolme, 1992, Weidner et al., 2003). Microcystin composition of *Planktothrix agardhii* changed toward a higher proportion of a more toxic variant with increased light intensity (Tonk et al., 2005).

Temperature is also an important factor affecting both growth and synthesis of secondary metabolite by cyanobacteria (Sivonen, 1990; Lehtimaki et al., 1997; Rapala et al., 1997). Strains and species differ slightly in their optimum growth temperatures. The toxin content in

most studies was highest at temperatures between 18 °C and 25 °C, whereas at low (10 °C) or very high temperatures (30 °C) toxin content decreased. Temperature gradients caused two- to three-fold difference in toxin content. The temperature optimum reported for *Aphanizomenon* varies within 15-28ºC, and the minimum temperature at which growth ceases lies below 10ºC. The optimum growth temperature of *N. spumigena* strains isolated from the Baltic Sea was 20-25ºC; no growth was observed at 10ºC (Lehtimaki et al., 1997; Rapala et al., 1997). Temperature affects the life cycle of *N. spumigena* as well, since the akinetes germinate at temperatures above 16ºC (Huber, 1984). Pliński and Jóźwiak (1999) observations confirmed the fact that *N. spumigena* prefers a temperature higher than the optimum for *A. flos-aquae* growth. Westhuizen and Eloff (1985) showed that cells of slower-growing cultures are more toxic except for the very slow-growing culture at 16ºC.

Variations in phytoplankton toxicity are also related to physiological responses of organisms (Baden and Trainer, 1993). Cyanobacteria can persist under the harmful solar radiation, while cells undergo stress and (can) start to release toxins. Ross et al. (2006) results indicated that when cultures of *M. aeruginosa* were irradiated by full sunlight, the UV absorbing material UF 96UV prevented any elevation in hydrogen peroxide (H_2O_2) release. *M. aeruginosa* placed under UV transmitting material significantly increased production of H_2O_2. However, the biological production of H_2O_2 may reflect an imbalanced state of redox within the chloroplast and thus serves as a proxy for cellular stress (Twiner and Trick, 2000; Choo et al., 2004). This observation supports the hypothesis that ultraviolet radiation triggers the formation of reactive oxygen species (ROS) in *M. aeruginosa*. More importantly, elevated levels of H_2O_2 were accompanied with a 40% increase in toxin release into the surrounding media, whereas *M. aeruginosa* protected by UV-blocking material showed no sign of H_2O_2 increase or toxin elevation. According to Ross et al. (2006), toxin release could be associated with oxidative stress and subsequent programmed cell death (PCD). Upon exposure to selected stressors, cell bound toxins were capable of being released into the immediate vicinity at levels

over 90% above what was normally secreted by dense assemblages of *M. aeruginosa* (Ross et al., 2006). Physical stressors induce oxidative stress, which results in PCD and a concomitant release of toxin into the surrounding media (Ross et al., 2006).

Other very important factor for cyanotoxins production is pH. In the study where mouse bioassay was used to detect effects of pH on toxin production, cells were found to be more toxic when grown at high and low pH. At high pH, cyanobacteria, especially *Spirulina*, occur massively in alkaline lakes. Some of the organisms are obligate alkaliphiles while in acidic environments cyanobacteria are seldom (Seckbach, 2007).

Phosphorus limitation and high oxygen saturation in the medium can cause increased toxicity (Igarashi et al., 1995). In high concentrations of phosphorus, hepatotoxic strains produced more hepatoxins, but for anatoxin-production phosphorus had no effect. The differences in microcystin production induced by low and high phosphorus concentrations vary between two- and fourfold. Similarly, in field studies, a positive correlation of total phosphorus with microcystin-LR concentration in cells of *Microcystis aeruginosa* or in bloom material of *Microcystis* spp. has been found by Khattar, et al. (2009). Non-nitrogen fixing species, like *Microcystis* and *Oscillatoria*, produce more toxins under nitrogen-rich conditions. Nitrogen fixing species are not dependent on the nitrogen in the media for their toxin production (Lehtimäki et al., 1997; Rapala et al., 1997). On the other hand, in continuous cultures when the toxins were expressed in relation to cell protein rather than to dry weight, Utkilen and Gjølme (1992) found that nitrogen and phosphorus limited conditions had no effect on the toxin content of *Microcystis aeruginosa.*

Indications regarding the role of iron are contradictory (Utkilen and Gjølme, 1992; Lyck et al., 1996). According to Paerl and Zehr (2000), iron (Fe) and trace metal micronutrients are essential for cyanobacterial growth. Iron is a cofactor of enzymes involved in photosynthesis, electron transport, energy transfer, N (NO_3^- and NO_2^-) assimilation and N_2 fixation. Iron limitation of cyanobacterial growth has been demonstrated in freshwater and marine ecosystems

(Paerl and Zehr, 2000). In oceanic and coastal regions without terrigenous or atmospheric Fe inputs, diazotrophic growth may be mediated by Fe availability. Iron impact on cyanotoxins production is not known and needs future researches. While studying the effect of trace metals on growth and on toxin content of *Microcystis aeruginosa,* Lukac and Aegerter (1993) found that in batch cultures only zinc was required for both optimal growth and toxin production.

Salinity, specifically ionic composition and strength, can inhibit growth and toxin production among freshwater cyanobacteria (e.g., *Microcystis, Cylindrospermopsis*). However, salinity not necesary can be a limiting factor and across-the-board barrier to cyanobacterial growth and proliferation, as witnessed by the presence of diverse epiphytic and epibenthic N_2-fixing (e.g., *Calothrix*, *Lyngbya*, *Nostoc*, *Scytonema*) and non-N_2-fixing genera (e.g., *Oscillatoria*) in estuarine and coastal ecosystems (Potts, 1994). Species of some benthic cyanobacteria genera, including *Lyngbya* and *Oscillatoria* are well adapted to freshwater or saline (including hypersaline) conditions. N_2- and CO_2-fixing activities in some freshwater cyanobacteria may be inhibited at increasing salt concentrations (Moisander et al., 2002). Cyanobacteria are abundantly found at high salt concentrations, but seldom develop massively at salt concentrations above 250 gl^{-1} (Seckbach, 2007). Salinity may be one of the stressors which stimulate cyanotoxins production. According to Ross et al. (2006), toxin levels were relatively low in dense natural assemblages (3.5 $\mu g\ l^{-1}$) of cyanobacteria. The toxin levels increased by 90% when *M. aeruginosa* was stressed by an increase in salinity. In an attempt to use salinity alterations as a bloom elimination strategy, specimens of *M. aeruginosa* were found to be viable at salt concentrations up to 9.8 psu (Atkins et al., 2000). Salinity values surpassing this critical mark were accompanied with a reduction in total viable cell concentration and increase in cell lysis (Atkins et al., 2000; Orr et al., 2003). Ross et al. (2006) showed nearly 80 % increase in toxin release when cells were transferred to water with a salinity of 32 ‰. This increase was concomitant with an increase in secreted H_2O_2, showing that oxidative stress correlated with toxin release (Ross et al., 2006). Culture experiments on two toxic Nodularia strains isolated from the Baltic Sea

(BY1 from the Arkona Sea and HEM from the Gulf of Finland; Lehtimäki et al., 2002) indicated that the effect of salinity (test range 3-11 psu) on toxin production and growth of strain BY1 was significant. Further studies by Lehtimäki et al. (1997) showed that growth of strain BY1 was optimal at 10 psu, while intracellular nodularin content was highest at 15 psu. Contradictory results were obtained *by* Moisander et al. (2002) who found that the growth rate of Nodularia strain FL2f from the Baltic Sea did not change over the test range of 0-30 psu NaCl. Blackburn et al. (1996) investigating the effect of salinity (test range 0-35 psu NaCl) on growth and toxin production of six Australian strains of *Nodularia* isolated from three water bodies of different salinity found that the growth rate of all strains at 0 psu NaCl was significantly lower than at the higher salinities. The optimal growth rates were recorded at 12 and 24 psu NaCl. The acclimation Nodularia NSPI-05 to a wide range of salinities (0-35 psu) was also confirmed in the current experiments. These results showed a clear difference in the response of the Baltic and Australian strains. According to Mazur-Marzec et al. (2005) culture experiments on the toxic *Nodularia spumigena* strain NSGG-1 isolated from the Gulf of Gdańsk showed a significant effect of salinity on growth and nodularin production. Growth of the NSGG-1 strain was optimal between 7 and 18 psu, lower at 3 and 24 psu and was significantly inhibited at the extreme salinities of 0 and 35 psu. Nodularin content of *N. spumigena*, estimated by the NOD/Chla ratios, correlated positively with salinity and increased from 0 to 35 psu. The NOD/Chla ratio on day 10 of growth was high, and, reached the maximum at day 30. A sudden increase in salinity from 7 to 18 and 35 psu resulted in plasmolysis of Nodularia cells. In general, the results into the effect of salinity on toxin production by cyanobacteria are controversial and require future research.

1.4. Cyanotoxins bioaccumulation in aquatic organisms

Direct poisoning of animals by cyanobacteria can occur by two routes: through consumption of cyanobacterial cells from water, or indirectly through consumption of other animals that have themselves fed on cyanobacteria and accumulated cyanotoxins.

Cyanotoxins are known to bioaccumulate in common aquatic invertebrates and vertebrates, including zooplankton mussels and fish. Consequently, there is considerable potential for toxic effects to be transferred in aquatic food chains. Such toxin transfer is well known for anthropogenic pollutants such as heavy metals and pesticides. There is no reason to suspect that the situation would be any different with natural cyanotoxins. It is difficult to ascribe the deaths of natural populations of aquatic animals, especially fish, unequivocally to cyanotoxin poisoning. The collapse of a large cyanobacterial bloom can lead to very low concentrations of oxygen in the water column as a consequence of bacterial metabolism. As a result, fish deaths due to the anoxia may occur. The best evidence for the potential of cyanobacteria to cause toxic effects on aquatic organisms comes from controlled laboratory experiments. In these experiments, animals are exposed to toxic cyanobacteria, cell-free extracts or solutions of cyanotoxins (Ferrão-Filho and Kozlowsky-Suzuki, 2011). According to published data, microcystins bioaccumulate in common aquatic organisms, including zooplankton (Watanabe et al., 1992), mussels (Ericson et al., 1989; Falconer et al., 1992; Prepas et al., 1997; Watanabe et al., 1997) fish (Carbis et al., 1997; Beattie et al., 1998) and birds (Sipia et al., 2006; 2008).

1.4.1. Bacteria and aquatic plants

The influence of cyanobacterial toxins on other bacteria is not fully understood and the scientific literature gives a number of contradictory statements. According to some authors neither an extract of *Microcystis aeruginosa* nor pure MC-LR has a biological effect on *Bacillus subtilis, Staphylococcus aureus, Escherichia coli* or *Pseudomonas hydrophila* (Foxall and Sasner, 1988). However, these limited tests should not be seen as general indicators of the potential impacts of cyanotoxins on aquatic bacteria. The majority of aquatic bacteria are yet to be cultured, and studies with common mammalian pathogens or "laboratory" bacteria should not be taken as all encompassing. It is quite possible that cyanotoxins impact on some species of aquatic bacteria. Certainly, microcystins are not

toxic to all bacteria because several species are known to degrade quite high concentrations of these toxins (Bourne et al., 1996; Bourne et al., 2006; Maruyama et al. 2006, Amé et al. 2006, Tsuji et al. 2006).

It is even possible that the slow release of cyanotoxins from the cell surface or from senescent cells may stimulate associations of particular bacterial types which may even act as symbionts. Attempts have been made to use bacterial toxicity tests (based on inhibition of bacterial phosphorescence) to screen for the presence of cyanotoxins, especially microcystins. However it appears that the inhibition of bacterial phosphorescence is not related to the commonly known cyanotoxins. It has been suggested that the negative effect may be related to the presence of unidentified LPS endotoxins in the cell wall of the cyanobacterial cells (WHO, 1999).

Additionally, hepatotoxins can have indirect negative effects on extensive aquaculture productivity, where the growth of cultured species depends largely upon thriving populations of lower trophic levels (i.e., aquatic plants, macroalgae, microbes, zooplankton, microcrustaceans, and more edible or nutritious phytoplankton species). Cyanotoxins impact macrofytes growth and occurrences. Pflugmacher (2002) exposed macroalgae *Cladophora sp.* to ecologically relevant concentrations of MC-LR (0.5 μg L^{-1} and 1.0 μg L^{-1}). As a result, significantly reduced photosynthesis and growth rates were observed. Another macroalgae, *Fucus vesiculosus*, underwent oxidative stress after exposure to nodularin-containing extract (Pflugmacher et al., 2007). The microbial food web may also suffer in the presence of a hepatotoxic cyanobacterial bloom, as exposure decreased the survival of the protozoan, *Tetrahymena pyriformis* and reduced the growth rate of nanoflagellates (Christoffersen, 1996).

1.4.2. Zooplankton

In the presence of hepatotoxin-producing cyanobacteria, the zooplankton community in extensive aquaculture ponds can shift to less desirable species e.g., species that are more evasive or are

smaller than the preferred food size. Theses changes can be induced by the reduced fecundity, decreased feeding rates, and increased mortality of large-bodied species (Engström et al., 2000; Liu et al., 2006). Hepatotoxic cyanobacteria also have proven harmful to crustaceans, they caused decreasing grazing rates in the amphipod, *Gammarus zaddachi*, and mysid shrimp, *Mysis mixta* (Engström et al., 2001; Korpinen et al., 2006). In fairy shrimp, *Thamnocephalus platyurus*, (Keil et al., 2002), brine shrimp, *Artemia salina* (Metcalfe et al., 2002), and the microcrustacean, *Kalliapseudes schubartii* (Montagnolli et al., 2004) an increased mortality was observed.

Evidence of the potential effects of cyanotoxins on zooplankton, which mainly comes from laboratory studies, is complex and inconsistent. Overall, it appears that cyanobacteria may exhibit a deleterious effect on zooplankton, but the effect is highly variable between genera and species, and even between clones of individual zooplankton species (Ferrão-Filho and Kozlowsky-Suzuki, 2011; Agrawal and Agrawal, 2011). One of the main questions yet to be resolved is whether the observed inhibitory effects are due to the putative poor nutritional value of cyanobacteria, to the known cyanotoxins, or to other unidentified compounds. There is evidence in the literature to support all three effects as being significant, at least with particular species under experimental growth conditions. A major difference in study design is whether organisms are exposed to cyanotoxins dissolved in water, or fed with toxic cyanobacteria. The latter is likely to lead to a substantially higher dose. Furthermore, Jungmann and Benndorf (1994) reported that exposure of *Daphnia* to dissolved microcystins showed effects only at concentrations several orders of magnitude above those found in field samples. They did, however, observe toxicity to *Daphnia* by unidentified metabolites other than microcystins from *Microcystis.* There is dramatic variation among zooplankton species in their response to toxic (and even non-toxic) cyanobacteria. For example, DeMott et al. (1991) showed that the of four species zooplankton (copepod and three of *Daphnia*) differed in their sensitivity to hepatotoxins by almost two orders of magnitude, but toxic effects were observed only at very high concentrations that are scarcely

encountered in natural water bodies (48 h LD_{50} of microcystin ranging from 450 to 21,400 $\mu g l^{-1}$). Snell (1980) found that there was a genotype-dependent response of the rotifer *Asplanchna girodi* to toxic *Anabaena flos-aquae* and *Lyngbya* sp. Hietala et al. (1997) reported a variation in susceptibility of more than three orders of magnitude in the acute toxicity of *Microcystis aeruginosa* to 10 clones of *Daphnia pulex*. Both DeMott et al. (1991) and Laurén-Määttä et al. (1997) suggested that clone and species differences between zooplankton susceptibilities to toxic cyanobacteria may lead to selection pressures in favour of resistant strains or species in water bodies where toxic cyanobacteria occur frequently. A lack of evident effects on zooplankton, feeding on toxic algae, may be more general than potential adverse effects (Turner et al. 1998). Zooplankton can avoid harmful algae by selective feeding (Turriff et al., 1995, Turner et al., 1998) and vertical migration (Forsyth et al., 1990). Laboratory trials show that copepods may benefit from cyanobacteria when provided with appropriate mixtures of cyanobacteria and good quality food (Schmidt and Jónasdóttir, 1997). Furthermore, the quality of cyanobacteria as food for zooplankton may change or even improve when the bloom is starting to age and decay. This may take place because the toxin concentration of blooms decreases during the senescent phase (Kankaanpää et al., 2001), and because a decaying *Nodularia spumigena* bloom is known to attract a diverse community of bacteria, flagellates, microzooplankton and crustaceans. Finally, Repka et al. (1998) showed that detritus derived from cyanobacteria is good quality food for aquatic herbivorus organisms.

Large zooplankton species seems to be negatively affected by microcystin while smaller zooplankton was less affected. In a laboratory experiment Gustafsson (2007) found that the ability of a large zooplankter, *Daphnia*, to feed on and tolerate toxic algae is improved if the animal has been previously exposed to cyanotoxins. Pre-exposed individuals show higher survival rates and higher reproductive output compared to individuals that have not encountered toxic cyanobacteria before. Further studies revealed that this tolerance is an induced defence developed during an individual's lifetime. Moreover, this trait could be transferred from mother to

offspring, a phenomenon called “maternal effects”. Daughters from mothers that had experienced toxic cyanobacteria had higher fitness compared to daughters of non-exposed mothers. Tolerance to toxic cyanobacteria differs among populations from different lakes, among clones inhabiting the same lake, and also among individuals within a clone. Difference in tolerance among populations and clones probably reflect the animals, historical experience of toxic cyanobacterial blooms. The difference in tolerance among individuals, however, is more difficult to explain and needs to be studied further (Gustafsson, 2007). Benndorf and Henning (1989) found that the toxicity of a field population of *Microcystis* was increased by the feeding activity of *Daphnia galeata* over a period of a few months. A possible explanation for this phenomenon is offered by DeMott et al. (1991) who demonstrated that a number of zooplankton species will avoid grazing on toxic cyanobacteria, but continue to graze on non-toxic species. Similar results have also been shown for grazing by the phytoplanktivorous fish tilapia and silver carp. Thus, grazing pressure from zooplankton and some fish may lead to the selective enrichment of toxic cyanobacterial strains over time. It is likely that under natural conditions in water bodies, certain species and strains of zooplankton may be affected by cyanotoxins, whereas others will be unaffected. As such, cyanotoxins and other cyanobacterial metabolites may influence the zooplankton community structure, especially during times when cyanobacteria are dominant within the phytoplankton (Rohrlack et al., 2004; Czarnecki et al., 2006; Von Elert et al., 2012).

According to published data (Porter and Orcutt, 1980; Lamper, 1987), cyanobacteria have commonly been considered as low quality food for zooplankton, due to their morphology, low nutritional value and toxin content. Many cyanobacteria have a filamentous or colonial morphology, and form aggregates, which could reduce feeding rates, or clog the feeding appendages of suspension feeding zooplankton. Taste of a food species, i.e. bad taste or bad odour is also considered to be a characteristic of poor food quality. The low nutritional content of cyanobacteria, demonstrated as a low reproductive response, may be due to assimilation inhibited by some

classes of cyanopeptides (reviewed by Lampert ,1987; Rohrlack et al., 2004; Czarnecki et al., 2006; Von Elert et al., 2012) or lack of essential compounds (Müller-Navarra et al., 2000; Brett et al., 2006), such as highly unsaturated fatty acids (HUFA). Saturated fatty acids (SAFA) are important due to their high calorific content and are mainly utilized for energy, whereas polyunsaturated fatty acids (PUFA) affect the production of eicosanoids, which are crucial for numerous physiological functions related to reproduction in invertebrates, e.g. egg production and egg hatching (Brett et al., 2006). Previously, the egg production and egg hatching success of copepods, provided with both toxic and non-toxic cyanobacterial monocultures, have been shown to be low. Deformed egg sacs have also been reported (Koski et al., 1999). Finally, zooplankton feeding may be inhibited, or zooplankton mortality increased, due to algal toxins or other secondary metabolites (DeMott et al., 1991).

Despite the above stated negative effects, evidence has started to accumulate that cyanobacteria may also have neutral or even positive effects on grazers. Engström-Öst (2002) studied grazing on cyanobacteria in the Baltic Sea and showed that copepods fed more actively on cyanobacteria when the blooms were dense and also during later growth stages. Rolff (2000) recognized strong signals of cyanobacteria in samples of stable isotopes dominated by rotifers and cladocerans in the Baltic proper. O'Neil and Roman (1994) suggested that pelagic harpacticoid copepods may be able to affect the distribution and species composition of *Trichodesmium* sp. by grazing.

1.4.3. Mussels

As primary consumers, molluscs constitute an important link between primary producers (potentially toxic cyanobacteria) and higher consumers (crayfish, fish, waterfowl (Dilllon, 2000)). They could be very good water quality biomarkers. Due to their generally wide distribution and abundance, close association with benthic sediments, and relatively sedentary nature, physiological ability, molluscs are potentially ideal indicator species of water contamination (Lefcort et al., 2002).

In the context of increasing freshwater pollution a lot of researchers studied the impact of cyanobacterial toxin on different age life-traits (survival, growth and fecundity) and locomotion of mussels. According to literature published data (Gerard and Poullain, 2005; Lance, 2007; Lance et al., 2009; Gèrard et al., 2008; Ferrão-Filho and Kozlowsky-Suzuki, 2011), cyanotoxins induced a decrease in survival, growth and fecundity but had no effect on locomotion of *Gastropods* representing different life-stages. The estimated survival rate was reduced when exposed snails were young. Moreover, the toxin exposure significantly reduced the number of snail embryos in the oviduct pouch. The age of exposed snails (juveniles, subadults, adults), microcystin-LR induced a decrease in survival, growth and fecundity but had no effect on locomotion. In the cases of *Potamopyrgus antipodarum* toxicity of MC-LR was most significant for juveniles than for other life stages (subadults, adults). Opposite results were obtained by Gèrard et al. (2008) who demonstrated that MC accumulation in *situ* was greater in adults (40±13 ng g^{-1} fluctuation of body weight (FW)) versus juveniles (12±6 ng g^{-1} FW). When juveniles and adults of *Lymnaea stagnalis* consumed the same density of toxic cyanobacteria in experimental conditions, juveniles accumulate 66% of ingested MCs versus 47% MC for adults (Lance et al., 2006). The higher MC accumulation in juveniles was probably due to their less developed and competent immune system (Dikkeboom et al., 1985), and thus, less efficient detoxification. Here, juveniles born mostly during spring did not experience cyanobacteria exposure before bloom. Consequently, in the field, one can assume that the higher MC value in adults (versus juveniles) were due to their age, long exposure - probability during their whole life - to both high densities of toxic cyanobacteria (summer proliferations) and dissolved MC concentrations (autumn collapse).

At different stages of a toxic bloom, exposure may result in toxic stress to specific organs in the mussel. In mussels, the highest microcystin concentrations are found in the hepatopancreas, and in vertebrates they are found in the liver. Davies et al. (2005) provided evidence that during the marine bloom of *N. spumigena* different organs of the green lipped mussel (*Perna viridis*) may come under

toxic stress. During the growth phase of the bloom, and when toxin levels are low in the water, intoxication may come mainly from consumption of *N. spumigena* cells leading to a toxic stress in the hepatopancreas before redistribution to other organs. During post-bloom conditions, toxicity to mussels may be initiated from organs in close contact with high levels of *N. spumigena* lysis in the water. Finally, antioxidative and detoxification enzymes responses were often opposite, depending on whether the treatment was a lysis of *N. spumigena* or bloom densities of *N. spumigena* cells. These opposing responses may be due to prevalent toxins acting on the mussel. They can represent an intoxication route to predators in food web. Intoxication of molluscs may occur via feeding on toxic cyanobacteria and absorption of cyanotoxins dissolved in water or adsorbed to particles and food. Consumption of toxic cyanobacteria and absorption of dissolved MC have been experimentally demonstrated in gastropods, resulting in MC accumulation and detrimental effects on life trails (Gérard and Poullain, 2005; Gérard et al., 2005; Lance et al., 2006). Both intoxication routes have been suggested for gastropods in the fields (Zurawell et al., 1999; Zhang et al., 2007; 2008). Moreover, recurrent proliferations of toxic cyanobacteria have been shown to coincide with decline in gastropods demonstrating that toxic cyanobacteria could induce changes in gastropod community structure (Gérard et al., 2009).

In addition, large bivalves (*Unio, Anodonta, Dreissena)* may contain a great quantity of cyanotoxins in the field, but seem insensitive to the compounds (Watanable et al., 1997; Ibeling et al., 2005). Long-life molluscs are more negatively affected by toxic cyanobacteria than short-life ones. For bivalves, field studies have demonstrated that large long-life (10-15 years) species (*D. polymorpha, Anodonta woodiana, Unio douglasiae*) exposed to severe recurrent blooms accumulate MCs up to 30 μg/g dry weight (DW), thus present contamination danger to food webs (Ibelings et al., 2005). Instead, immersion studies have shown that freshwater and marine bivalves survived days of exposure to high concentrations of toxic cyanobacteria (108 cells L^{-1} or 100 mg DW L^{-1}) containing 2.5–300 μg L^{-1} of hepatotoxin, suggesting that these

species are tolerant to ecologically relevant concentrations of the compound, for at least short periods of time. In the Baltic Sea, the concentration of nodularin in soft tissues of *Mytilus edulis* correlated with the *N. spumigena* biomass and during cyanobacteria bloom it reached the highest average values of 210.1 ng g^{-1} DW. Mussels with the smaller shell (<2 cm) tended to accumulate lower amounts of nodularin (NOD) during *N. spumigena* bloom (Duinker et al., 2006; Mazur-Marzec et al., 2013). Watanabe et al. (1997) showed that some freshwater mollusks such as *Anodonta wodiana*, *Unio douglasia* and *Cristaria plicata* accumulated microcystin in a range of 1-5 μg/mussel during a heavy microcystis bloom. Amorim and Vasconcelos (1999), showed that under laboratory conditions *Mytilus galloprovincialis* can accumulate up to 140 μg g^{-1} DW of microcystin.

When high density of cyanobacterial cells were provided to the mussels, a decrease in the filtration activity resulting in lower amounts of toxins ingested should be expected (Vasconcelos, 1995). As bivalves filter particulate organic matter from water column using mucus-lined gills and sort appropriate food according to size. Although suspension-feeding bivalves are usually assumed to be herbivores with phytoplankton being the major nutrition source, there is also evidence that zooplankton and occasionally animals 3-6 mm in size can be consumed (Wong and Levinton, 2005). Consequently, most bivalves cannot totally avoid consuming toxic cyanobacteria or cyanotoxin intoxicated herbivorous zooplankton (Bontes et al., 2007). Selective filter feeding can occur in large species such as *Dreissena polymorpha* that expel large amounts of cyanobacteria in pseudofaeces (Naddafi et al., 2007). Hepatoxins may inhibit ion transport in gill leading to loss of homeostasis or even death (Malbrouck and Kestemont, 2006). *In vivo* saltwater mussels, *Mitylus edulis*, were fed with cyanobacteria, *Microcystis aeruginosa*, that contained a high concentration of microcystins. The mussels were killed on a periodic basis over the course of 2 months (Williams et al., 2005). Direct evidence was provided for the existence of covalently bound microcystins when transferred to untreated seawater. Mussel mortality was low during the uptake

experiment, although it increased slightly during the depuration period. Falconer et al. (1992) did not report data on the mortality of *M. edulis* during their study on the occurrence of toxic blooms of *Nodularia*. On the other hand, Eriksson et al. (1989) stateed that *Anodonta* intoxicated with *Oscillatoria* did not seem to be affected by the toxins. Similar experiments done with bivalves molluscs and toxic dinoflagellates showed that mortality of the organisms is dependent upon time following exposure (Lesser and Shumway, 1993), age, as well as the prior exposure history.

Variation in MC accumulation has been reported according to species, mainly due to uptake routes and detoxification abilities (Zurawell et al., 2005). Part of cyanotoxins taken up by aquatic organisms may be rejected as faeces while another portion may be irreversibly bound to protein phosphatases or they can be metabolized (Vasconcelos, 1995). MC accumulation will be also different among molluscs according to feeding habits (grazing, filtering), respiration mode (aerial, aquatic), ecology and physiology and life history strategy (Dillon, 2000; Gérard et al., 2009). According to Vasconcelos (1995), the presence of cyanobacterial blooms in estuaries where bivalves are growing naturally can constitute a health hazard in that these organisms can retain microcystins and transfer them through the food chain.

Intoxication may occur in benthic sediment feeding or detritofagus molluscs due to ingestion of toxic cyanobacteria and cyanotoxins adsorbed to sediment and detritus. Thus, it can explain cyanotoxins occurrences in molluscs from waters where cyanobacteria have not been recorded or were present at very low concentration. Gèrard et al. (2009) presented several hypothese explaining the occurrences of MC in water were cyanobacteria were not detected. First, toxic cyanobacteria were present but not detected due to low density, benthic habitat and/or great spatiotemporal variability. Through winter in temperate lakes, low numbers of cyanobacteria vegetative filaments are observed in plankton (e.g., with potentially toxic *Planktothrix agardhii* and *Aphahanizomenon flos aquae* as dominant species). Filaments and or akinets constitute small benthic overwintering populations which represent the primary

source of inoculate for large summer planktonic populations. Such overwintering cyanobacteria may be responsible for MC intoxication of molluscs before bloom. Second, MC-intoxicated molluscs came from neighboring waters inhabited by toxic cyanobacteria. Indeed, migration has been demonstrated via passive aerial dispersal, mostly by waterfowl, and very efficient for small molluscs (<3 mm) (Streit et al., 1997). All detoxification processes, specific or not, are more or less efficient depending on molluscan taxon, age and environmental conditions (Yokoyama and Park, 2003), resulting in different MC accumulation.

1.4.4. Fish

In aquatic systems, fish stand at the top of the aquatic food chain, and are possibly affected by exposure to toxic cyanobacteria. Trophic transfer also has been demonstrated under laboratory conditions in which hepatotoxins were transferred from zooplankton to fish (Engström-Öst et al., 2002; Karjalainen et al., 2007; Smith and Haney, 2006). Magalhães et al. (2001) results showed a rapid transference of MC from seston to fish.

Studies dealing with fish–cyanobacteria interactions have mainly been concerned with fish development (Oberemm et al., 1999), excretion (Sahin et al., 1996), foraging behavior (Keshavanath et al., 1994) and growth (Bury et al., 1995). Few papers have focused on the detection of toxins in fish tissue.

Different toxins caused histopathological damage depending on fish species, their habitats, and behaviors, e.g. salmonids are much more sensitive than channel catfish (Tencalla et al., 1994; Bury et al., 1995; Snyder et al., 2002), and goldfish were found to be nearly 30 times less susceptible to i.p. microcystin than mice (Sugaya et al., 1990). Lower concentrations of hepatotoxins have been measured in field-collected salmon and herring (0.7-6.5 ng g^{-1} DW) and in experimentally spiked individuals (6–48 ng g^{-1} DW) (Engström-Öst et al., 2002; Sipiä et al., 2001: Kankaanpää et al., 2002), while the highest concentrations of hepatotoxin have been detected in cod and flounder (25–400 ng g^{-1} DW) (Sipiä et al., 2001).

Usually the investigations of lethal effects in fish during cyanobacterial blooms indicated that the cause of death was mostly due to damage of the gills, digestive tract, liver (Rodger et al., 1994). The gill damage is probably caused by the high pH induced by cyanobacterial photosynthesis activity prior to the bloom collapse, together with the higher level of ammonia arising from the decomposition of the cyanobacteria. However, gill damage may have enhanced microcystin uptake and thus led to liver necrosis. Damage to gills by dissolved MC-LR has been shown experimentally in tilapia and trout (Gaete et al., 1994; Bury et al., 1996). According to Rodger et al. (1994) the cyanobacterial extract may affect gill permeability. Epithelial lifting has been detected in dying brown trout retrieved after a toxic cyanobacterial bloom. This would seriously disturb osmoregulation. Unidentified microcystin-producing organisms produce a progressive degeneration of the liver in salmon smolts placed into open-water net pens (Anderson et al., 1993). The disease, referred to as Net Pen Liver Disease (NPLD), has resulted in significant economic losses for the mariculture industry.

Other pathological symptoms ascribed to toxic cyanobacterial blooms include damage to the blood, heart, kidney, skin and spleen (Råbergh et al., 1991). The latter study highlighted degenerative changes in kidney tubules and glomeruli. The effect of microcystins on fish under natural field conditions has been described by Carbis et al. (1997) as atrophy of hepatocytes, gills with pinpoint necrosis, epithelial ballooning, folded lamellar tips, exfoliation of the lamellar epithelium, elevated asparate aminotransferase activity and serum bilirubin concentrations. Physiologically, the fact that in silver carp MC-LR was not detectable in the blood suggests that little MC-LR could be transported across the intestines. Exposure of fish to lysed *M. aeruginosa* cells (24-42 μg l^{-1}MC-LR; 288 μg chlorophyll a L^{-1}) caused an increase in plasma cortisol levels. That fish responded physiologically to the presence of lysed *M. aeruginosa* cells and exhibited a classic stress response (Bury et al., 1996).

Rapid toxin uptake by fish during the cyanobacterial bloom could couse serve tissues damage or even sometimes fish death. In laboratory experiments with rainbow trout, Tencala and Deitrich

(1997) found that MC-LR was quickly taken up into the liver 1 h after start of the exposure. In field studies, Wiegand et al. (1999) observed a rapid uptake of MCs by fish, since the toxin was detected in viscera, liver and muscles shortly after the beginning of the cyanobacterial bloom. Tencala et al. (1994) exposed yearling rainbow trout to an oral dose of 6600 ng g^{-1} and found that 100% of fish died between 72 and 96 h. A rapid senescence of cyanobacterial (blue-green algal) bloom in the field (at the Thedy Waser Commercial Fish Hatchery in Andelfingen, Switzerland) occurred (within about 24 h) and about 1000 dead and dying brown trout, *Salmo trutta* L., were found during the next 2 days.

However, toxins can limit fish feeding on cyanobacteria. Beveridge et al. (1993) showed suppression in filtration rate and growth of two tilapia species, *Hipophthalmichthys molitrix* and *Oreochromis niloticus*, in presence of toxic *Microcystis aeruginosa.* Potentially toxic *Microcystis* respond to a chemical signal or feeding related activity of fish by microcystin production, even though both fish species appeared not to feed vigorously on toxic *Microcystis*. It is postulated that, in the context of toxic cyanobacterial ecology, *M. aeruginosa* has an inducible defence by increasing toxin production in response to the presence of fish (Jang et al., 2004).

The fish may learn to avoid harmful metabolites, though hunger limits this avoidance (Thacker et al., 1997, Nagle and Paul, 1998). Grazing studies with herbivorous fish have shown that feeding decreases when the percentage of toxic cyanobacteria cells increases (Keshavanath et al., 1994). Hansson (1997) suggested that herring larvae tend to avoid water containing dense *Nodularia spumigena* blooms in the Baltic Sea. Therefore the larvae are forced to stay in colder and deeper water, possibly reducing their rate of growth and increasing mortality. Some phytoplanktivorous fish species tended to avoid consuming toxic *Microcystis* cells (Magalhães et al., 2001). Tilapia and carp can reduce their grazing activity when the percentage of toxic cyanobacteria increases (Beveridge et al., 1993; Keshavanath et al., 1994).

Another important defense mechanism in fish is the hormonal spew during the critic phase of toxic blooms. Plasma catecholamine

and corticosteroid levels have been widely used as indicators of stress in fish (Barton and Iwama, 1991) and these hormones control energy mobilization and water and ion balance reestablishing ion homeostasis, which may be perturbed by pollutants. Inability to adapt to the stress may lead to the compensatory responses becoming maladaptive, resulting in a reduction in growth, reproductive capacity, and immunosuppression (Barton and Iwama, 1991). The present findings of a stronger cortisol response in fish exposed to toxic cyanobacteria, show that the former elitic the greater stress response. The low cortisol levels thereafter may indicate physiological adaptation, although changes in secretion and clearance rates may also have occurred (Redding et al., 1984). Hyperglycemia occurs in response to increased energetic demands imposed on fish at times of stress (Wedemeyer et al., 1990).

Both, toxic and non-toxic cyanobacterial cells reduced fish growth, although the toxic cells suppressed fish growth much more than non-toxic. This reduction of growth rate might be attributed to an enhanced energy demand related to the induction of detoxication enzymes (Wiegand et al., 1999), increased plasma levels of cortisol and glucose (Bury et al., 1995), inactivated ion regulation (Bury et al., 1995; Carbis et al., 1997) and increasing swimming activity (Bagnaz et al., 1998). Best et al. (2002) data shows that most fish fed with toxic *Microcystis* lost weight and growth rate was also negative a greater extent than non-toxic cells. Experiments by Kamjunke et al. (2002) also showed that there were no differences in motility of omnivorous fish (e.g. roach) among *Microcystis, Daphnia* and *Aphanizomenon* diets.

Laboratory studies indicate that cyanotoxins (dissolved microcystins) may affect fish egg (Mazur-Marzec et al., 2007); development of embryos, larvae (Oberemm et al., 1999) and behavior of fish (Baganz et al., 1998). According to Mazur-Marzec et al. (2007) extracts of the *N. spumigena* bloom sample had a significant effect on *Danio rerio* egg development. The mortality of eggs exposed to the *Nodularia* extract was observed only in the first 24 hours of incubation, and mainly in the solutions with the highest concentration of NOD. Fish eggs and offspring are even more threatened by toxic *Nodularia*, as these organisms cannot escape from the area of the boom. The influence of cyanobacterial toxins

on egg hatching time was recorded by Oberemm et al. (1999) in experiments with rainbow trout. The eggs of the fish hatched earlier when treated with microcystin. On the other hand, the hatching time was delayed when the effect of neurotoxic saxitoxin was investigated. Apart from egg mortality and changes in hatching time, the *Nodularia* extract caused some malformations in *D. rerio* larvae. Bury et al. (1995) revealed that cyanobacterial secondary metabolites may deter juvenile fish from feeding on them. Catfish, for example, ingest surface scum while feeding on floating food pellets, thereby increasing their potential exposure to cyanobacterial metabolites (Zimba et al., 2001).

The phytoplanktivorous fish are especially important because of their role in aquatic ecosystems as direct consumers of phytoplankton primary production, and their potential role in biological management of algal blooms (Opuszynski and Shireman, 1995; Xie and Liu, 2001). Hepatotoxins were transferred to Atlantic salmon through the consumption of toxin-containing biofouling organisms and crab larvae, resulting in net pen liver disease (Andersen et al., 1993; Kent et al., 1996).

The vulnerability of fish to toxins, from an evolutionary point of view, is probably influenced by the natures of habitat and feeding mode. Species native to oligotrophic habitats are more vulnerable than species native to eutrophic habitats where microcystins exposure is more frequent (Snyder et al., 2002). Phytoplanktivorous fish are probably more resistant to microcystins exposure than other fishes.

Fish exposed to microcystin by various routes including dermal rout, inhalation, oral and intravenous, may initiate microcystin detoxification mechanisms which may help fish to survive the stress induced by toxic cyanobacteria. Pflugmacher et al. (1998) showed the enzymatic conjugation of microcystin-LR to glutathione catalysed by the soluble glutathione *S*-transferase (sGST) in fish. This conjugate seems to be the first step in microcystin detoxication pathway, as well as it can contribute to in vivo metabolism and detoxification of numerous other compounds (Best et al., 2002). Sahin et al. (1996) have shown that cyanobacterial toxins are excreted into the bile of the fish, rather than accumulating in liver. Detoxication mechanism which is active in most fish species

collapses, when the concentration of toxins, and possibly other active compounds present in the extracts, is very high. Many authors presume that at the beginning of exposure to very high concentration of toxins, the activity of enzymes taking part in detoxication of xenobiotics is just increasing and the liver is not ready to metabolize the whole pool of toxins. As in muscles the activity of enzyme is generally low (if any), toxins transported from liver to muscle may temporarily accumulate there to high concentration. Further studies are required to confirm this hypothesis.

Oberemm et al. (1999) showed that crude extract of cyanobacteria cells had much more pronounced effects on fish development than pure toxin alone. They also hypothesized that other substances produced by these microorganisms might be responsible for the adverse effects on fish. Mazur-Marzec et al. (2007) laboratory experiments with *Danio rerio* exposed to toxic *N. spumigena* supported the hypothesis that it is not only nodularin, but probably also other cell components of the cyanobacterium that is responsible for the observed changes in development of the fish.

Summarizing, the effects of toxins on the embryonic, juvenile, and adult life stages of fish include histopathological damage in the liver, kidneys, gills, intestines, heart or spleen, disrupted osmoregulation, altered serum biochemistry, malformation of embryonic-larval alimentary system, reduced growth rate, stress response, and modified swimming behavior (Malbrouck and Kestemont, 2006). The exact mechanism of cyanotoxins action on different stages of fish development has not been fully recognized yet.

Thus, hepatotoxins can limit aquaculture yield indirectly by reducing the productivity of prey (food supply) or altering the community structure towards less nutritious prey species. Hepatotoxins accumulate in pelagic and benthic animals in both natural and aquaculture systems throughout the world (Smith et al., 2008).

Common advice given by water authorities is that the viscera of the fish, which contain cyanobacteria and/or their toxins, should not be eaten.Special caution should be taken in all cases where major toxic blooms occur.

2. THE STUDY AREA

2.1. Geographical location and geology of the Curonian lagoon

Curonian Lagoon is situated along the Baltic Sea coast of Lithuania and the Kaliningrad region of Russia. The total area of the lagoon is approximately 1600 km^2. Total volume of water of the lagoon is approximately 6.2 km^3, and the average depth is about 3.8 m (Pustelnikovas, 1998), with the maximum (5.8 m) in the southern part of the waterbody (up to 14 m in artificially deepened Klaipėda port area) (Gasiūnaitė, 2000). Curonian Lagoon is a complex ecosystem with many interacting processes. It is semi-enclosed system, influenced by the fresh waters of Nemunas and other smaller rivers and saline waters from the Baltic Sea. The lagoon has only one narrow outlet (width ~ 400 m) - Klaipėda Strait with the depth of 8–14 m. It is separated from the Baltic by a narrow (~ 1-3 km) sandy spit - the Curonian Spit (Fig. 2.1). The largest city of the region, the industrial town of Klaipėda (in 2013 population 158.541) is situated in the Northern part of the lagoon along the strait connecting the lagoon to the Baltic Sea. The area of land draining into the Curonian Lagoon covers 100,458 km^2, of which 48% lies in Byelorussia, 46 % in Lithuania, and 6 % in the Kaliningrad, with a total population of about 5 million inhabitants (Ferrarin et al., 2008).

Grain size and organic matter content composition are major criteria characterizing the Curonian lagoon sediments (Pustelnikovas and Gulbinskas, 2002). Distribution of fine grain size fractions in the sediment is determined by hydrodynamic factors and accumulation of thin dispersed autochthonous and allochthonous material from water column (Pustelnikovas, 1998). Sediments include a broad spectrum of sands, silts and clay. Fine sand is the most widespread and dominant fraction in the northern and central parts of the Curonian lagoon (Fig. 2.1). In the western part, fine sand is separated from the coast by belt of medium sand or silty sediments (Trimonis et al., 2003). Distribution of fine silty mud is restricted to the main sedimentation zones, situated in the frontal area of the Nemunas

River delta, relatively deep sheltered bays along the Curonian spit (e.g. Nagliu Bay) and open southern part. Bottom areas in the central part of the lagoon are mainly predominantly covered by shell deposits (more than 50 % sediment volume) and predominated by *D. polymorpha* (Trimonis et al., 2003; Zaiko, 2009).

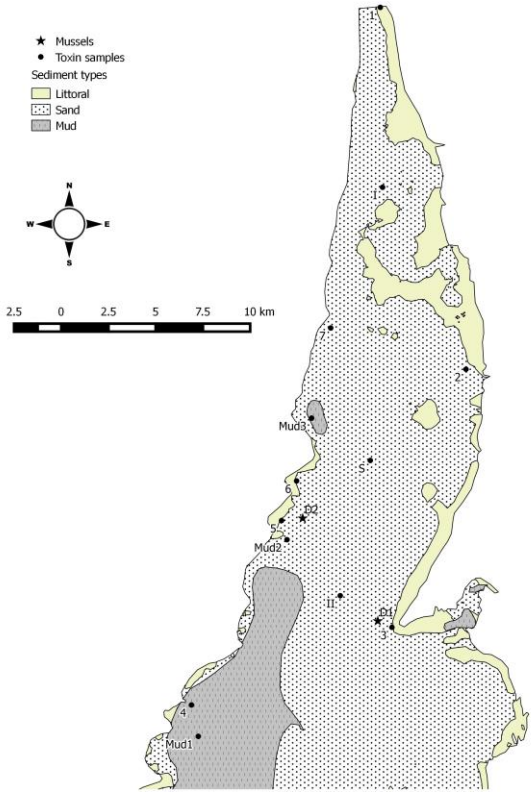

Figure 2.1. Curonian Lagoon study stations (1-7 phytoplankton samples; D1, D2 - mussels samples; I, II - fish samples; Mud1, Mud2, Mud3, S - sediment samples).

2.2. Climatic and hydrological characteristics

Curonian Lagoon is a temperate estuarine lagoon. Water temperature varies between 0.1–29.0°C. The lowest water temperatures (0.0–0.1° C) occur during January to February, when the lagoon could be covered with ice. The highest temperature 25–29° C is observed mostly in July – August (Žaromskis, 1996). Thermal stratification of the water column is weak and unstable. The temperature of the near-bottom water layer is lower than that in the upper layer by 1–2°C (Pustelnikovas, 1998). The Klaipėda strait during the winter time is always ice free, while the rest of the lagoon is ice covered for 110 days, on average (min 12; max 169) (Žaromskis, 1996). Western and southern winds predominate in the lagoon area. Usually winds are not strong, just 1–5 m s^{-1} (55%). The winds stronger than 15 m s^{-1} are more frequent during autumn – winter period. The wind induced waves, due to the small depth, are short and the fetch is not so important. The wave height is mostly determined by the wind speed. Mathematical model simulation results show that the brackish Baltic Sea water (average salinity around 7 psu) is pushed into the lagoon in case of strong north or north-west winds (Ferrarin et al., 2008).

The Curonian Lagoon, from hydrological point of view, could be divided into three principal areas (Ferrarin et al., 2008): 1. *Transitory northern part* with active sea-lagoon water exchange and river runoff. From the Baltic Sea water inflows to the Curonian lagoon contained 5.1 km^3 $years^{-1}$ or 4.4% of the average annual runoff (Gailiušis et al., 2005). Typically, seawater inflows take place on about 29% days per year in the northern part of the lagoon. 2. *Stagnant or limnic part* characterized by poor water renewal and intensive sedimentation which result in extensive fine sediments bottoms; and 3. *Intermediate and complex central part*, directly exposed to the freshwater runoff. Fresh water runoff to the Curonian Lagoon is dominated by the Nemunas River, averaging 22.1 km^3 $year^{-1}$, or 96% of the average annual runoff (Dailidienė and Davulienė, 2008). Precipitation on average 740 mm $year^{-1}$ (Bukantis, 1994) has considerably lower inputs to the lagoon and constituts 18.7% of the average annual runoff (Gailiušis et al., 2005).

2.3. Water chemistry and hydrochemical characteristics

Curonian lagoon is a shallow eutrophic water body. Shallow depths, wind and waves influence sediment resuspension and water transparency. Very low water transparency and narrow fotic zone limit the distribution and abundance of macrophytes (Razinkovas and Pilkaitytė, 2002). Seasonal changes in pH and water transparency fluctate conversely in the lagoon: the highest value of pH (9.1) is observed during summer, when the transparency is the lowest (0.3 m). Contrary in autumn-spring when the Secchi depth is 2.2 m, the decrease in pH to 8.1 is observed (Razinkovas and Pilkaitytė, 2002; Pilkaitytė, 2003).

Dissolved oxygen saturation of the water column fluctuates spatially and temporally (both diurnally and seasonally) and generally varies between 20 % and 115 % (Ferrarin et al., 2008). Oxygen concentrations decrease during the ice cover period as well as during calm weather days in summer, when elevated water temperatures and intensive microalgae vegetation facilitate the transient establishment of hypoxia or anoxia, particularly during night hours (Jurevičius, 1959; MRC, 2006; Žilius et al., 2012).

The annual cycle of the biogenic compounds follows the pattern typical for temperate and boreal transitional waters with strong riverine inputs. The highest concentrations of nutrients are observed in winter (Total N 210.6 μmol l^{-1}, Total P 4.1 μmol l^{-1}) and early spring (Ferrarin et al., 2008). The concentration of phosphate decreases rapidly in April and starts to increase in early summer due to decomposition of organic material. The nitrogen concentration can decrease to analytical zero in May; nitrate concentration tends to increase from midsummer, whereas ammonium concentrations have no pronounced seasonal pattern (Razinkovas and Pilkaitytė, 2002). In summer, during vegetation period, N_2–fixing cyanobacteria can contribute up to 43% of nitrogen brought by river runoff (Langas et al., 2010). The silica concentration is lowest during spring after the diatom bloom. It remains low throughout the summer and starts to increase again in early autumn (Razinkovas and Pilkaitytė, 2002). Seasonal nutrient variation in the lagoon is mainly associated with

the river runoff, microalgae vegetation periods, nutrients remineralization and sediment resuspension (Razinkovas and Pilkaitytė, 2002; Žilius et al., 2012).

Seasonal variations in salinity depend on regional climate, affecting river discharge and wind driven brackish water inflows from the Baltic Sea (Daunys, 2001; Dailidienė and Davulienė, 2007, 2008). Due to restricted water exchange with the Baltic Sea and the dominance of the river runoff, salinity fluctuations range from freshwater up to 8 psu, and are limited to the northern part of the lagoon (Dailidienė and Davulienė, 2007). Seawater intrusions can rarely reach the central part of the lagoon (Dailidienė and Davulienė, 2007; Jurevičius, 1956). Therefore, the average annual salinity varies from 2.6 psu at the entrance to the sea down to 1.2 psu in the northern part, and to less than 0.1 psu in the central part (Dailidienė and Davulienė, 2007; Jurgelėnaitė and Šarauskienė, 2007).

2.4. Phytoplankton

Salinity and turbulence are the main factors considered to be critical for the development of algae blooms (Pilkaitytė and Razinkovas, 2006) as well as for the structure and function of the whole plankton community (Gasiūnaitė, 2000; Gasiūnaitė and Razinkovas, 2004). Representatives of marine, brackish and fresh-water species could be found in the lagoon. In total, 438 phytoplankton species are observed in the Curonian lagoon (Olenina, 1997; Gasiūnaitė et al., 2005). The lagoon is a eutrophic system, with repeated diatoms and cyanobacteria blooms. Diatoms dominate during the spring. When the temperature rises up to 15-16ºC, the cyanobacteria appear and after some period become dominant. In total, 62 cyanobacterial species were recorded in Curonian lagoon. *Aphanizomenon flos-aquae, Anabaena spiroides, Chroococcus limneticus, Coelosphaerium kuetzingianum, Cyanodiction sp., Gomphosphaeria pusilla, Merismopedia punctata, M. warmingiana, Microcystis aeruginosa, M. incerta, M. wesenbergii, M. viridis, Nodularia spumigena, Limnothrix planktonica, Snowella lacustris, Woronichinia compacta* are cyanobacterial species recorded in

summer (Olenina, 1997). The main bloom forming species is *Microcystis aeruginosa, Aphanizomenon flos-aquae* (Pilkaitytė, 2003). During the summer, the maximal chlorophyll *a* concentrations range from 117 μg l^{-1} in the northern part (Pilkaitytė and Razinkovas, 2007) to 219 μg l^{-1} in the southern stagnant part (Semenova and Aleksandrov, 2009). When strong west wind blows from the sea, the cyanobacterial community is dominated by *Limnothrix redekei, Planktothrix agardhii, Anabaena sp.* instead of *Aph. flos-aquae.* Generally, cyanobacteria can be abundant till the end of September, and sometimes even till October (Olenina, 1997; Olenina, 1998; Pilkaitytė and Razinkovas, 2007). In autumn, *Aph. flos-aquae, Chroococcus limneticus, C. turgidus, Microcystis aeruginosa, M. grevillei, M. incerta, Snowella lacustris, Phormidium sp., Woronichinia compacta* are the main cyanobacterial species (Olenina, 1997). Diatoms dominate in the Nemunas river phytoplankton community from April to July; green algae are most abundant during May-September, while dominance of cyanobacteria is characteristic only of late summer. The variation of the cyanobacteria/diatoms ratio throughout the seasons is markedly lower in the river than in the lagoon. No important changes in phytoplankton abundance were found in the medium-scale river lagoon gradient; however, a quantitative gradient could be observed on a larger spatial scale, especially during cyanobacterial blooms. Structural changes along the gradient are well expressed during the cyanobacteria-dominated period in the lagoon (June–October): a high cyanobacteria/diatoms ratio was typical for the central part of the lagoon, whereas a low ratio is characteristic of Nemunas river distributaries and lagoon stations situated closer to the river mouths (Gasiūnaitė et al., 2012). Large scale distribution of phytoplankton in surface waters is mainly determined by the lagoon circulation system and the highest concentrations of phytoplankton are typically found in active sedimentation zones along the western-south sector (Giardino et al., 2010).

In the upper sediment layer (0–5 cm), 120 diatom taxa were recorded. The largest amounts of planktonic species *Actinocyclus normanii, Aulacoseira islandica and Stephanodiscus rotula* (60–80

%) accumulated in the sediments in the western part of the lagoon (Kasperovičienė and Vaikutienė, 2007). In spring *Skeletonema marinoi, Thalassiosira sp.* and *Fragilaria sp*. dominated in central part of the lagoon while in summer epipsammic (*Fragilaria heidenii, Martyana martyi*) and epipelic (*Navicula scutelloides, Amphora pediculus*) benthic diatoms dominated. However, in benthos, the colony-forming cyanobacterium, *Microcystis sp.,* dominated (Žilius et al., 2012).

2.5. Fish

Of the 57 fish species recorded in the Curonian Lagoon, 46 are of freshwater origin. The most common are roach (*Rutilus rutilus*), perch (*Perca fluviatilis*), redeye (*Scardinius erythrophalmus*), white bream (*Blicca bjoerkna*) and common bream (*Abramis brama*) (Repečka et al., 1996). The main commercial fishes are roach, perch and bream; their commercial catches are between 700 and 1.680 t a^{-1} (Virbickas *et al.,* 1996).The juvenile fish assemblage is dominated by smelt (*Osmerus eperlanus*) and pikeperch (*Sander lucioperca*) in the lagoon pelagic habitats, while roach, perch, three-spined stickleback (*Gasterosteus aculeatus*) and gudgeon (*Gobio gobio*) prevail in the littoral (Žiliukienė, 1998; Žiliukas, 2003). Fish fry dynamics has a pronounced seasonal pattern: three-spined stickleback dominate in the littoral in spring, roach and gudgeon in summer and perch in autumn (Žiliukas, 2003).

2.6. Benthic macrofauna

The litorale zone of the Curonian Lagoon is inhabited by approximately 280 benthic macrofauna species (Zettler and Daunys, 2007). In the west part of the lagoon, benthic communities are dominated by *Neries diversicolor, Marenzelleria neglecta,* oligochaets and chironomids, *Balanus improvisus, Cordylophora caspia, Mya arenaria, Macoma baltica and Mitilus edulis* (Gasiūnaitė et al., 2008). The eastern part is dominated by large native unionids (*Unio tumidus*), alien invasive species *Dreissena*

*polymorpha (*Daunys et al., 2006), *Marenzelleria neglecta* and Ponto-Caspian and North American aphipods (mainly *Obessogammarus crassus*, but also *Pontogammarus robustoides* and *Gammarus tigrinus*). A large part of the muddy bottom is covered by shell deposits formed mainly by *Valvata* species with an admixture of *Bithynia spp*., *Radix spp*., *D. polymorpha* and *Potamopyrgus antipodarum*. In areas less exposed to saline water inflows, clumps of living zebra mussels may also be found (Gasiūnaitė et al., 2008).

The bivalve *D. polymorpha* dominates the benthic communities in the central and southern freshwater parts of the lagoon (Olenin and Leppäkoski, 1999). Due to the habitat engineering activity of *D. polymorpha*, the community of co-occurring species has the highest biodiversity (up to 29 species per sample, and about 50 species in total). The total biomass (up to 11 kg m^{-2}) and abundance (up to 10^5 ind. m^{-2}) are the highest for the entire Curonian Lagoon (Olenin and Daunys, 2004).

3. MATERIALS AND METHODS

3.1. Sample collection

Samples of cyanobacteria were collected at seven different stations (Fig. 2.1) in the Curonian lagoon from July to October (2006 – 2007), from May to October (2008) with bucket from the littoral zone. Water samples (300 ml for cyanobacteria taxonomic identification and 100 ml x 3 for toxins analysis) were taken every two weeks and were taken every week during the summer "bloom" period. Water temperature and salinity were measured with MultiLineF/Set- (WTW, USA) at the same time when phytoplankton samples were taken.

Samples were preserved with acid Lugol's solution according to the Utermöhl method (Edler, 1979). Taxonomic cyanobacteria species composition was identified and abundance was counted using inverted microscope (MEIJI; 200x, 400x) according to identification keys (Tikkanen and Willen, 1992; Hindák, 2002; Komárek and Anagnostidis, 2005). Cyanobacteria counts (units: filaments of 100 μm length, colonies of 100 cells) and biomass (using stereometrical formulae) were calculated according to HELCOM (2001).

For intracellular cyanotoxins analyses water samples (100 ml x 3) were filtered through Whatman GF/C filters, which were kept in freezer at -20˚C until toxin analysis.

Accumulation of toxins were analyzed in mussels (*Dreissena polymorpha*), fish (*Rutilus rutilus*), and upper layer (~ 5 cm) of bottom sediments. Mussels were collected during three years (2006–2008) in summer and autumn at two locations (Fig. 2.1). Mussles were separated into size groups (< 10 mm; 10–30 mm; > 30 mm); three replicates of 1g DW tissues were taken from each group and kept in freezer at -20˚C until toxin analysis. Fish (*Rutilus rutilus*) were collected (by Ventės and Juodkrantės fishermen with gill nets) for two years (2007–2008), in summer and autumn, from two locations (Fig. 2.1). Fish were divided into age-groups according to their weight (I (Q=8–20.3 g); II-III (Q=20.3–33.85 g); IV-VI (Q=59.76–134.08 g), following the practice for the fishery stock

analysis in the Curonian lagoon; three replicates of 1 g DW fish muscle were taken from each group and kept in frozen at -20˚C until toxins were analyzed. Sediment samples were (collected by CORPI scientific workers: Mindaugas Žilius and Tomas Ruginis) taken in triplicate from upper sediment layer (0–5 cm), from four different locations (Fig. 2.1). A sample (5 cm^3) of the upper 0–5 cm section of each core was collected and kept at -20˚C until toxins were analyzed.

Analyses of chlorophyl *a* concentration in sediments were determined at department of Marine Biology and Ecology, Oceanography Institute, University of Gdansk. Chlorophyll *a* was extracted by adding 90% ethanol to sediment samples (Jespersen and Christoffersen, 1987). Then, the samples were centrifuged and the obtained supernatants were analyzed spectrophotometrically (UV-1202, Shimadzu) according to Lorenzen (1967).

Table 3.1. Field samples collected during investigation period 2006-2008.

Investigate	Year	Month	Station	Sample number	Material for toxin analyses
Cyanotoxins	2006 2007 2008	July-October July-October May-October	1-7	112 109 85	Each sample (100 cm^3) was filtered through Whatman GF/C filters
Mussels	2006-2008	July/October	D1, D2	108	Tissue 1g DW
Fish	2007-2008	July/Octber	I and II	36	Muscle 1g DW
Sediment	2008	July/October	Mud1, Mud2, Mud3, S	24	Sediment 1 g DW from each station

Mussel, fish and sediment extracts were lyophillised (TEGA, Germany). Then, 30 ml of methanol (analytical grade) was applied for 1 g DW of mussel, fish and sediment sample. The material was disrupted by sonication (1 min; HD 2070 Sonopuls ultrasonic disrupter equipped with a MS 72 probe; Bandelin, Berlin, Germany; 20 kHz, 25% duty cycle) and centrifuged for 15 min at 10,000 × *g* at 20ºC. The solvent was removed by rotary evaporation and the residue was re-dissolved in 1 ml of MiliQ water. After that, samples

were vortexed for 1 min and then centrifuged for 15 min at 12,000 × *g* and 20ºC. The obtained extracts were subjected to solid phase extraction on Sep-Pak Vac C18 cartridges (200 mg, Waters, Massachusetts, USA).

3.2. Analysis of toxins: HPLC, ELISA, PPIA

Liquid chromatographic method (HPLC)

The filters with cyanobacterial material were suspended in 90% methanol (1 ml) and sonicated for 5 min. Then, another portion of 90% methanol (1 ml) was added. The samples were treated with bath sonication for 10 min (Sonorex, Bandelin, Berlin, Germany), vortexed for 1 min and left for 2 hours at room temperature. After centrifugation at 12,000 *g* for 15 min the supernatant was separated and analyzed with Waters HPLC system (Milford, MA, USA). The system consisted of a model 626 pump with model 600S controller, model 917plus auto-sampler, model 996 photodiodarray detector (PDA). Isocratic elution was performed on a Waters Symetry RP-18 column (5 μm; 150 mm × 3.9 mm I.D.) using a mobile phase of acetonitril : water (32 : 68), both containing 0.05% TFA, and at flow rate of 1 ml min^{-1}. The auto-injection volume was 20 μl. Microcystins (RR, LR, LA, LF, and LW) and nodularin standards were purchased from Alexis Biochemicals (San Diego, CA, USA). Concentrated solutions of toxins (10 μg ml^{-1}) were prepared in methanol. All reagents used for the analyses were of the HPLC grade. Methanol and acetonitrile were purchased from J.T. Baker (Deventer, The Netherlands). Deionized-distilled water was obtained using the Mili-Q ultra pure water system (Millipore, Bedford, MA, USA). The cyanotoxins were identified by their retention time and characteristic absorption spectrum with maximum at 238 nm (Fig. 3.1). All HPLC data was collected and processed using the Waters Millennium 32 Software. The quantitative analysis of hepatotoxins was carried out using a calibration curve based on peak area measurements for standard solutions.

Immunological assay (ELISA)

In the study were used 3 different sets of ELISA tests to detect MC/Nod (Abraxis, United States), SXT (Abraxis LLC, United States) and BMAA toxins (Abraxis, United States). The tests were preferred according Meriluoto ans Spoof (2008). As far are described methodology hepatotoxins which were found during investigations. Cyanobacterial, mussels, fish and sediment extracts were diluted in MilliQ water (10–5.000 times) and analyzed by enzyme-linked immunosorbent assay (ELISA). The ELISA assay was carried out on a 96-well microplate. Three strips were be used to run the negative control and six calibrators. Add 100 µL of Negative Control (NC) and each calibrator (0.1 ppb; 0.2 ppb; 0.4 ppb; 0.56 ppb; 0.8 ppb; 1.6 ppb MC-LR calibrator) and 100 µl of each samples. The wells were covered with tape or parafilm to prevent evaporation and incubate at ambient temperature for 30 minutes. After incubation, the covering was carefully removed and 100 µl of microcystins-enzyme conjugate was added to each well. The contents of the wells were throughly mixed by moving the strip holder in a rapid circular motion on the benchtop. The wells were covered again with a new tape or parafilm to prevent evaporation and incubated at ambient temperature for 30 minutes. After incubation, the covering was carefully removed, and the contents of the wells were discard into a sink. After vigorously washing the wells with distilled water, 100 µl of substrate was added to each well beginning with the Negative Control (NC) and calibrators (C1 to C6), then the samples. The microplate was incubated at ambient temperature for 30 minutes, and after addition of Stop Solution (100 µl; 1.0 N HCl) to each well the results were recorded with a microtiter plate reader at 450 nm (and 650 nm as reference.

Protein phosphatase inhibition assay (PPIA)

The cyanobacterial, mussels, fish and sediment extracts prepared for ELISA were also analysed by colorimetric protein phosphatase 1 inhibition assay (PPIA). The PPIA assay was carried out on a 96-well microplate according to the method described by Rapala et al. (2002). Bovine serum albumin (BSA) was obtained from Sigma-

Aldrich (St. Louis, MO, USA). Dithiothreitol (DTT), $MgCl_2 \times 6H_2O$, $MnCl_2 \times 4\ H_2O$, Na_2SO_4, *p*-nitrophenyl phosphate (*p*-NNP), tris-(hydroxymethyl)-aminomethane (Tris) were of analytical grade. The substrate and enzyme buffers were prepared immediately before the test. Catalytic subunits (2.5) of commercially available enzyme (PP1; New England Biolabs, USA) were diluted in 1.5 of the enzyme buffer. Subsequently 10 µl of standard solutions or sample were added to the well and mixed with 10 µl of PP1 in buffer. After 5 min incubation, 200 µl of *p*-NPP in buffer solution was added to each well. The content of the wells was mixed by swirling the plate sideways. After 2-hour incubation at 37°C the absorbance of the solutions was measured. The plates with ELISA and PPIA tests were read on VERSA max microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 450 nm (ELISA) or 405 nm (PPIA), respectively.

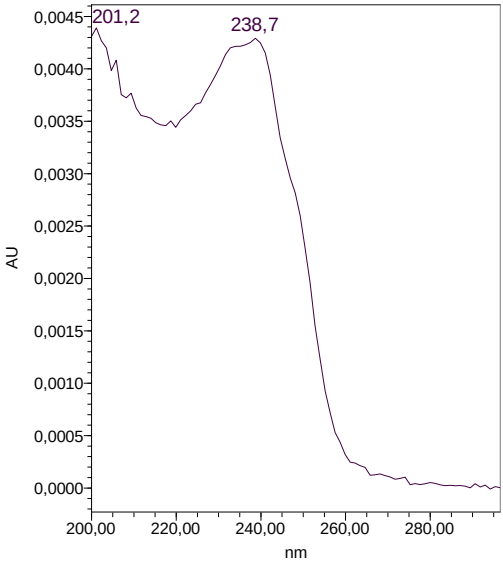

Figure 3.1. Absorption spectrum of MC-LR obtained by dioda array detector for sample from Curonian Lagoon.

3.3. Experimental study of MC-LR bioaccumulation in mussels

Zebra mussels were collected in June 2013, using a trawl, from the Curonian lagoon. Water and mussel samples were taken for toxins control. Mussels were divided into three size classes <10 mm, 10-30 mm, and >30 mm length. For the experiment, 200 mussels from each size group were placed in 2 l container with lagoon water filtered through 0.2 μm mesh size. Before being transferred to the containers, the mussels were gently cleaned with a brush under running de-ionized water and pre-incubated for 2 weeks. Grazing and assimilation experiments were performed with different amount of toxic cyanobacterium *Microcystis aeruginosa* (CCNP1101, medium Z8) (containers A and B Fig. 3.2). The food concentration in "A" series approximately was similar to one during the algal bloom in the Curonian Lagoon, while in "B" series it was over two times higher and "C" was left as a control with no feeding (Fig. 3.2). Cells density was calculated every day using inverted microscope by R. Pilkaitytė. The water was renewed everyday and the grazing/assimilation experiment were run for 2 weeks. The mussels were fed everyday with toxic culture for 14 days. Every five days 40 mussels (from each size group) were randomly chosen from each container for cyanotoxins analyses. After 2 weeks, mussels from each treatment were left on a diet without food for the depuration of MC-LR from the tissue (Fig. 3.2). The temperature (22±1°C), day/night (12 : 12) regime was constant through all experiment.

This depuration process was run for the next 2 weeks and every week 40 mussels from each size group were taken for toxin analyses. The concentration of cyanobacteria chlorophyll *a* was measured every day before and after feeding. For this purpose a fluorometer (FluoroProbe II, bbe-Moldaenke, Germany) was used. For the determination of MC-LR concentration, the pooled mussels from each size group (n= 40) were homogenized in 1 cm^3 75% MeOH in an ultrasonic bath (30 min), followed by 1 min probe sonification (Branson Sonifier II W-250 ultrasonic disruptor equipped with a micro tip, 30% duty cycle, output setting 2). Hereafter, 300 μl of the

samples was centrifuged at 10,000 × g for 10 min; 200 µl was then transferred to vials for liquid chromatography-mass spectrometry (LC-MS) analyses.

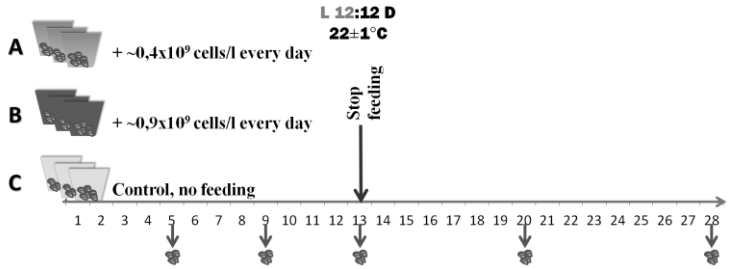

Figure 3.2. Exposure of mussels to *Microcystis aeruginosa* – experimental set-up (Picture by R. Pilkaitytė)

The analyses of cyanotoxins in animal tissues were performed with Agilent 1200 (Agilent Technologies, Waldboronn, Germany) coupled online to a hybrid triple quadrupole/linear ion trap mass spectrometer (QTRAP5500, Applied Biosystems, Sciex; Concorde, ON, Canada). As a mobile phase a mixture of A (5% acetonitrile in water containing 0.1% formic acid) and B (100% acetonitrile containing 0.1% formic acid) was used. Separation was performed on a Zorbax Eclipse XDB-C18 column (4.6×150 mm; 5 µm) (Agilent Technologies, Santa Clara, California, USA). Phase B was linearly increased from 15% to 75% in 5 min and then to 90% in the next 5 min. This composition of the mobile phase was held for 5 min and brought back to 15% B in 1 min. The column oven temperature was 35°C, the flow rate was 0.6 ml min^{-1} and the injection volume was 5 µl.

The structures of cyanobacterial toxins were characterized using the QTRAP LC-MS/MS system equipped with a turbo ion source (550°C; 5.5 kV). The experiments were run in a positive mode using the information dependent acquisition method (IDA). In addition, the enhanced ion product spectra (EIP) were acquired from 50 to

1200 Da with a collision energy (CE) of 60 V and collision energy spread (CES) of 20 V. Declustering potential (DP) was set at 80. Data acquisition and processing were accomplished using Analyst QS® 1.5.1 software. For the quantitative analysis of microcystins and nodularin, the multiple reaction monitoring mode was used. The total concentration of microcystins in the analysed samples was calculated as MC-LR equivalents using a calibration curve prepared with a standard of MC-LR (Alexis Biochemicals, Lausen, Switzerland).

3.4. Statistical data analysis

The data were analyzed using non-parametric KrusKal-Wallis, Mann-Witney-Wilkokson tests, factorial analysis of variance (ANOVA) and other standart statistics methods.

The concentration of cyanobacterial toxins were related to the environmental variables using redundancy analysis (RDA). "Redundancy" analysis shows how much of the variance in one set of variables can be explained by the other. In this case, RDA was considered to be more appropriate than CCA (canonical correspondence analysis) since there were many zero values and in general the relationship between different toxins and explanatory variables was quite linear. The implementation of RDA in the Brodgar software (Highland Statistics Ltd.) statistical package was used for the analysis. Confidence level of 0.05 was accepted for statistical tests to differentiate between statistically significant and insignificant relationships.

An exploratory data analysis showed that the values of microcystin concentrations in *D. polymorpha* tissues (including zero values) had considerably right-skewed distributions with several very apparent outliers. Therefore it was decided to apply log-transformation for the data, in order to minimize the effect of outliers. Since general assumptions of the parametric analysis methods (Shapiro-Wilk normality test, $p<0.014$; Fligner-Killeen test of homogeneity of variances, $p<0.05$) were not met neither before

nor after transformation applied, the further data analysis was performed using non-parametric methods.

The multivariate effects of the studied factors - time of sampling (combining year and month), sampling site and mussel size - on the concentration of microcystins in the mussel tissues were analyzed by statistical program PRIMER 6 & PERMANOVA (Anderson 2001, 2005). The test-statistic is a multivariate analogue to Fisher's F-ratio and is calculated directly from any symmetric distance or dissimilarity matrix. P-values are then obtained using permutations. In current study the Euclidean distance similarity measure was used to construct the similarity matrices. The statistical differences between the factor levels were assessed by four-way PERMANOVA with “time of sampling” (6 levels), “size” (3 levels) and “location” (2 levels) as factors. The permutation of raw data was used as this method is recommended in the case of relatively small sample size (Anderson and Robinson, 2001). When a factor and/or interaction was identified as significant ($P < 0.05$), post-hoc PERMANOVA pair-wise tests were conducted to detect which levels were responsible for significant differences. Multiple regression was applied to the log-transformed microcystin concentrations data from the zebra mussel tissues with mussels size and sampling time as explanatory variables.

4. RESULTS

4.1. Seasonal and spatial distribution of cyanobacteria

Cyanobacteria composition, abundance and biomass were analysed at seven different stations in the Curonian Lagoon during the study period (2006–2008). In total 29 cyanobacteria species were detected, 12 of them are potentially toxic and can produce toxins (Table 4.1).

In the first year (2006) of investigation species from *Microcystis, Merismopedia, Woronichinia, Chroococcus, Planktothrix* and *Nodularia* genera were detected. Out of the 18 found cyanobacterial species, 9 belong to the group of potential toxin producing species. *M. aeruginosa* was the dominant species which formed blooms during summer-autumn period (VII-X) (Table 4.2). The density of the population ranged from $0.96\pm0.83 \times 10^6$ unit l^{-1} (VII, 1 station) to $11.62\pm1.20 \times 10^6$ unit l^{-1}(VIII, 4 station) and the biomass from 8281±6439 μg l^{-1} (VII, 1 station) to 93273±7445 μg l^{-1} (VIII, 4 station). Brackish water cyanobacterial species, *N. spumigena*, was observed mostly in the northern part of the Curonian lagoon (stations 1, 7). The highest *N. spumigena* density (1.437×10^6 unit l^{-1}) and biomass (7220 μg l^{-1}) were recorded in the middle of August (VIII 17, 1 station). Also this species was detected in the south-western part (station 4) of the study area. The density of *N. spumigena* population ranged there from $0.04\times0.09 \times 10^6$ unit l^{-1} (IX) to $0.14\pm0.37\times 10^6$ unit l^{-1} (VIII) and biomass ranged from 207±507 μg/l (VIII) to 213±427 μg/l (IX).

During the second year of investigation (2007) cyanobacteria from the genera *Planktothrix, Chroococcus, Aphanizomenon, Microcystis, Woronichinia* were detected, but they did not form bloom in the Curonian lagoon. In 2007, 28 cyanobacterial species were detected, 11 of them belong to potentially toxic. The filamentous *Planktothrix agardhii* was the most abundant species. The highest density ($2.38\pm0.67 \times 10^6$ unit l^{-1}; 73% on average of the total) and biomass of the cyanobacterium (2923±890 μg/l; 62% on average of the total) were detected in September. Cyanobacteria from

Chroococcales order, *Aphanocapsa holsatica, Aphanocapsa incerta, Microcystis aeruginosa,* were the most abundant species in the north-western part of the study area (stations 1, 5, 6, 7). *Aph. flos-aquae, Chroococcus limneticus, Microcystis wesenbergii* were mostly detected in the south-eastern part (station 4, 5) of the Curonian lagoon (Table 4.2). The brackish water species *N. spumigena* was mainly observed in August in station 1.

During the last year (2008) of the study 19 cyanobacteria species were detected, 7 of them were potentially toxic. The most abundant genera were: *Anabaena* (*Dolichospermum), Aphanizomenon, Chroococcus, Woronichinia* (Table 4.2). *Aph. flos-aquae* was the main bloom forming cyanobacteria species in the Curonian lagoon. Its density ranged from 0.27×10^6 unit l^{-1} (11.85% on average of the total) recorded in May at station 4 to $28.11\pm5.81 \times 10^6$ unit l^{-1} (87.30 % on average of the total) in July at station 1. The biomass of *Aph. flos-aquae* varied from 751 µg/l (14.10% on average from the total) in May at station 4 to 61542±13140 (95.48% on average from the total) in July at station 1. The estuarine toxic species *N. spumigena* was detected in a few samples collected in the northern part of the Curonian lagoon. At station 6, *N. spumigena* was detected only in one sample collected at the end of August. The density of *N. spumigena* population was 0.124×10^6 unit l^{-1} (0.55% on average of the total in station 7) and the biomass reached 623 µg/l (0.53% on average of the total in 7 station). The highest density (0.689×10^6 unit l^{-1}; 1.71% on average of the total) and biomass (3455 µg/l; 3.34% on average of the total) of the toxic species was recorded in samples collected at station 1.

Table 4.1. List of cyanobacteria found during the investigation period (2006-2008).

Cyanobacterial species	Ecological characteristics of the species				Year		
	S	**T**	**N**	**X**	**2006**	**2007**	**2008**
Anabaena spp. (*Dolichospermum spp.*)	N/L	W	P	T		+	+
Aphanizomenon flos-aquae Ralfas ex Bornet & Flahault 1886	N	W	P	T	+	+	+
Aphanocapsa delicatissima W. West & G.S. West, 1912	L	W	P			+	+
Aphanocapsa holsatica (Lemmermann) G. Cronberg & J. Komárek, 1994	M					+	+
*Aphanocapsa incerta (*Lemmermann) Cronberg & Komárek, 1994						+	+
Aphanothece spp.						+	
Aphanothece minutissima (W. West) Komárková-Legnerová & Cronberg, 1994			P	+		+	
Chroococcus dispersus (Keissler) Lemmermann 1904	L		P		+	+	+
Chroococcus minutus (Kützing) Nägeli, 1849	L	W	P				+
Chroococcus turgidus (Kützing) Nägeli, 1849	K		E		+	+	+
Chroococcus spp.					+	+	
Cyanodictyon reticulatum (Lemmermann) Geitler, 1925	L		P		+	+	+
Dolichospermum spiroides (Kleb.) Wacklin, L.Hoffm. & Komárek 2009	N/L	W	P	T		+	
Gomphosphaeria aponina Kützing, 1836	N		E			+	
Limnococcus limneticus (Lemmermann) Komárková, Jezberová, Komárek & Zapomělová, 2010	N	W	P		+	+	+
Limnothrix planctonica (Wołoszyńska) Meffert, 1988	M		P		+	+	
Limnothrix redekei (van Goor) M.E.Meffert 1988	L		P	E			+
Lyngbya C.Agardh ex Gomont, 1892	M			T		+	
Merismopedia punctata Meyen, 1839	N		P		+	+	+
Merismopedia warmingiana Lagerheim, 1883	N		E		+	+	+
Merismopedia spp.	N		E		+	+	
Microcystis aeruginosa (Kützing) Kützing, 1846	N	W	E	T	+	+	+
Microcystis flos-aquae (Wittrock in Wittrock & Nordstedt) Kirchner, 1898	N		E	T	+	+	

Cyanobacterial species	Ecological characteristics of the species				Year		
	S	T	N	X	2006	2007	2008
Microcystis viridis (A.Braun) Lemmermann, 1903	N		P	T	+	+	
Microcystis wesenbergii (Komárek.) Komárek, 1968	N		P	T	+	+	+
Microcystis spp.	N	W	P	T	+	+	
Nodularia spumigena Mertens ex Bornet & Flahault, 1886	R	W	E	T	+	+	+
Nostoc Vaucher ex Bornet & Flahault, 1886				T	+	+	
Oscillatoria spp.				T	+	+	
Planktolyngbya limnetica (Lemmermann) J.Komárková-Legnerová & G.Cronberg 1992	N		E				
Planktothrix agardhii (Gomont) Anagnostidis & Komárek 1988	N/L	W	E	T	+	+	+
Planktothrix spp.				T	+	+	
Pseudanabaena limnetica (Lemmermann) Komárek,1974	N	W	P			+	
Snowella lacustris (Chodat) Komárek & Hindák, 1988	N	W	P	T	+	+	+
Woronichinia compacta (Lemmermann) Komárek & Hindák, 1988	N		E		+	+	+
Woronichinia naegeliana (Unger) Elenkin, 1933	N		P	T		+	+

S – species adaptation to salt water: marine (M); marine-brackish (R); brackish (B); limnic-brackish (N); limnic (L); marine-brackish-limnic (cosmopolitic) (K)) according to Algaebase and WoRMS (World Register of Marine Species);
T – Adaptation to different temperature regime: warm water species >10° C (W);
N – Environmental niche: planktic(P); planktic-benthic (E) ;
X- Potentially toxic or toxic species (T);
"+" – detected species during investigation;

Table 4.2. The seasonal occurrence of dominant cyanobacteria species in the Curonian lagoon

Month	Year		
	2006	2007	2008
V	-	-	***Anabaena spp.*** (4, 6) (*Dolichospermum*) ***Woronichinia naegelina*** (5, 6, 7) ***Aph. flos-aquae*** (4,5) *Chroococcus limneticus* (5)
VI	-	-	*Chroococcus limneticus* (A) ***Aph. flos-aquae***(A) ***Woronichinia naegelina*** (A) ***Anabaena spp***. (2,4) (*Dolichospermum*)
VII	***M. aeruginosa*** (A) ***Microcystis sp.*** (1, 2, 5, 6, 7) ***Nodularia spumigena*** (1)	***Planktothrix agardhii*** (A) ***Aph. flos-aquae*** (2,3) *Chroococcus limneticus* (2,3,4) ***M. aeruginosa*** (1, 2, 4, 5, 6 ,7) ***M. wesenbergii*** (1,4,5)	***Aph. flos-aquae*** (A) *Chroococcus limneticus* (4)
VIII	***M. aeruginosa*** (A) ***Microcystis sp.*** (1, 2, 5, 6, 7) ***M. wesenbergii*** (1, 3) ***Nodularia spumigena*** (1, 7)	***Planktothrix agardhii*** (A) *Chroococcus limneticus* (2, 3, 4) ***Aph. flos-aquae*** (3) ***Microcystis aeruginosa*** (A) ***M. wesenbergii*** (4)	***Aph. flos-aquae*** (A) *Chroococcus limneticus (*4, 6*)*
IX	***M. aeruginosa*** (A) ***Microcystis sp.*** (1, 2, 6) ***M. wesenbergii*** (1, 3)	***Planktothrix agardhii*** (A) ***Aph. flos-aquae*** (3, 4, 5) *Chroococcus limneticus* (2, 3) ***M. aeruginosa*** (2, 4, 5, 7) ***M. wesenbergii*** (6)	***Aph. flos-aquae*** (A) *Chroococcus limneticus* (6)
X	***M. aeruginosa*** (A) ***Microcystis sp.*** (1, 2, 5, 6) ***M. wesenbergii*** (2, 3)	-	***Aph. flos-aquae*** (A) ***Woronichinia naegelina*** (7) ***Planktothrix agardhii*** (6,7)

"-"no data ;() -number in bracket means station where the species was observed, A – in all stations; bold words indicate that the species can potentially produce toxins;

4.2. Secondary metabolites produced by cyanobacteria

During the study period (2006–2008), neurotoxins, cytotoxins and dermatotoxins were not detected. In the analyzed samples, four different microcystin analogues – MC-LR, MC-RR, MC-LY, MC-YR and one not identified MC analogous - were detected in year 2006. Cyanotoxins were detected in 91 (80%) out of 112 samples collected at all sampling stations in 2006. During the study, MC-LR was the most abundant cyanotoxin. Its concentration varied from below 0.1 μg l^{-1} to 134.25 μg l^{-1}. MC-LR was found in samples from all investigated stations (Table 4.3). In August, MC-LR concentrations in all stations exceeded 1 μg l^{-1} (Table 4.3). The highest MC-LR concentrations were observed in the western part of the Curonian lagoon (at stations 4, 5, 6, 7) (Table 4.3). At these stations, from July till the end of August the MC-LR concentration increased from 3 to 14 times. The maximum concentration of this toxin (134.25 μg l^{-1}) was detected in phytoplankton samples collected at station 4 in mid-August (VIII 11) (Table 4.3). The lowest concentration of MC-LR were detected in eastern part of the lagoon at station 1 (an average 0.24±0.11 μg l^{-1}), station 2 (0.30±0.18 μg l^{-1}) and station 3 (0.24±0.19 μg l^{-1}).

MC-RR and MC-YR were detected mostly in the western part of the lagoon (stations 4, 5, 6, 7). The concentration of these toxins varied from below 0.1 μg l^{-1} to 30.7 μg l^{-1} in the case of MC-RR (highest concentration measured at 30 of July, station 4) and from below 0.1 to 20.67 μg l^{-1} in the cases of MC-YR (station 4 in 18 of August) (Table 4.3).

A very low concentration of MC-LY (0.61 μg l^{-1}) was detected in the only sample collected from station 5 in August (VIII 11) (Table 4.3). In samples from all stations there was one microcystin analogue that could not be identified (MC-?) on the basis of HPLC (Table 4.3). This microcystin analog was found at all stations and in all seasonal samples (Table 4.3). MC-? was detected in all samples from stations 2 and 3 in August (Table 4.3), but the highest concentration of the unidentified MC (17.35 μg l^{-1}) was determined in sample collected at station 4 on 11 August in 2006. In general, the highest microcystin concentrations were detected in southern part of the lagoon at station

4 during July-August. In October no microcystin was observed in the collected phytoplankton samples (Table 4.3).

In 2006, the brackish cyanobacterium *N. spumigena* was detected in the northern part of the Curonian Lagoon (stations 1, 6, 7). Nodularin was detected in 24% of all samples collected that year. The concentration of the toxin ranged from below 0.1 μg l^{-1} to 0.97 μg l^{-1} at station 6 and from below 0.1 μg l^{-1} to 5.82 μg l^{-1} at station 7. The highest concentration was determined in the middle of August (5.82 μg l^{-1} at station 7; 5.56 μg l^{-1} at station 1). In the southern part of the Curonian Lagoon nodularin was detected only in samples collected on 6 September from stations 4 and 5; the concentration of the toxin was 0.85 μg l^{-1}(station 4) and 1.14 μg l^{-1}(station 5) (Table 4.3).

In 2007, nodularin was detected only in 4.6% of samples (in 5 out of 108 samples) they were collected at the end of August-September. In samples from station 1 the concentrations of the toxin ranged from 0.1 to 284.6 μg l^{-1} and in one sample from station 7 it ranged from below 0.1 to 7.89 μg l^{-1}. That year, dmMC-RR (7.5 μg l^{-1}) was also detected in one sample collected at station 6 on 14 August (Table 4.3).

During the last year of the study (2008), *Aph. flos-aquae* was the dominant species. In neither of the investigated planktonic samples (85) cyanotoxins were detected.

The application of HPLC equipped with a diode array detector, ELISA and PPIA revealed the presence of microcystins in 80% of bloom samples collected in 2006. To compare the concentrations of microcystins determined by HPLC, ELISA and PPIA in environmental samples of cyanobacteria we applied nonparametric Kruskal-Wallis test. In addition, to observed any relation between the methods the multiple regression was used. The results indicated no significant differences (KW-H=3.49; p=0.18) between microcystins (MCs) concentrations detected by HPLC, ELISA and PPIA methods.

Strong positive correlation between the microcystins concentration determined by HPLC and ELISA ($y=0.0081+ 1.2483*x$; $R =0.92$; $p < 0.005$) and PPIA ($y=1.8007+0.4218*x$; $R=0.43$; $P=0.123$) were observed.

Table 4.3. Hepatotoxins concentrations in water samples from all investigated stations in Curonian Lagoon in 2006-2007.

Toxins Concentration ($\mu g\ l^{-1}$)	Stations																				
	1			2			3			4			5			6			7		
	I_{min}-I_{max}	$\overline{X}$	S^2	I_{min}-I_{max}	$\overline{X}$	S^2	I_{min}-I_{max}	$\overline{X}$	S^2	I_{min}-I_{max}	$\overline{X}$	S^2	I_{min}-I_{max}	$\overline{X}$	S^2	I_{min}-I_{max}	$\overline{X}$	S^2	I_{min}-I_{max}	$\overline{X}$	S^2
2006																					
MC-?	n.d.-5.49	2.09	1.71	n.d.-4.16	1.76	1.54	n.d.-4.62	1.55	1.57	n.d.-17.35	2.41	4.06	n.d.-3.44	0.80	1.23	n.d.-2.12	0.59	0.77	n.d.-1.37	0.23	0.36
MC-LR	n.d.-1.19	0.24	0.42	n.d.-1.96	0.30	0.69	n.d.-2.59	0.24	0.70	n.d.-134.25	15.16	32.47	n.d.-21.22	2.66	5.72	n.d.-13.54	4.24	4.89	n.d.-20.96	3.26	6.32
MC-YR	n.d.-1.99	0.14	0.53	-	-	-		-	-	n.d.-20.27	1.19	4.79	n.d.-1.35	0.21	0.42	n.d.-0.85	0.05	0.20	n.d.-1.91	0.11	0.45
MC-RR	n.d.-19.32	1.38	5.16	-	-			-	-	n.d.-30.71	8.91	11.75	n.d.-18.91	3.11	5.59	n.d.-9.18	1.28	3.02	n.d.-3.69	0.39	1.12
MC-LY	-	-	-	-	-	-		-	-	-	-	-	n.d.-0.61	0.03	0.14	-	-	-	-	-	-
NOD	n.d.-5.57	2.03	2.26	-	-	-	-	-	-	n.d.-0.85	0.05	0.20	n.d.-1.14	0.06	0.27	n.d.-0.97	0.12	0.29	n.d.-5.82	0.99	1.67
E (%)	92			78.57			57			66.67			66.67			83.33			88.89		
2007																					
dmMC-RR	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	n.d.-7.5	0.75	2.31	-	-	-
NOD	n.d.-284.6	30.63	82.06	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	n.d.-7.89	0.42	1.81
E (%)	17			-			-			-			-			8.33			5.26		

n.d. – not detected; I_{min}-I_{max} - minimum and maximum toxins concentration in station, S^2 - dispersion; $\overline{X}$ - abundance index, toxins average in all investigated samples; E (%) – number of samples where toxins were detected;

4.3. Impact of environmental conditions (salinity, temperature) on growth and production of toxins by cyanobacteria

Data on physical parameters measured during cyanobacterial bloom (July–September in 2006–2007; from May–October during 2008), at 7 different stations of the Curonian Lagoon are presented in Table 4.4. In 2006, temperature ranged from 17.4˚C to 28.7˚C (an average 22.08±2.33˚C), in 2007, the temperature varied from 12.0˚C to 26.4˚C (an average 18.98±3.15˚C), while in 2008, it was from 10.1–24˚C (an average 19.49±3.34). The highest temperature (28.7˚C) was recorded in the middle of August 2006 at station 5, the lowest temperature (10.1˚C) was measured in October 2008 at station 1 (Table 4.4). On the other hand, among the sampling stations no significant (KW-H=3.93; p=0.69) differences in water temperature were observed in 2006–2008 (Table 4.4). The largest water temperature variations were observed in the northern part of the Curonian Lagoon (stations 1, 2, 6, 7) (Table 4.4).

Statistically significant differences in PSU among the investigated years (2006–2008) were observed (KW-H=108.60; p=0.00). In 2006, salinity ranged from 0.00 to 6.00 PSU, in 2007, the salinity varied from 0 to 4.4 PSU, while in 2008, it was from 0–7.2 PSU. In 2006–2008, the highest salinity values were recorded in the end of August and they reached 6.0 PSU (station 7), 4.4 PSU (station 1) and 7.2 PSU (station 1), respectively. Statistically significant differences in salinity were also recorded among the sampling stations (KW-H=48.22; p=0.00) Higher salinity was mostly observed in the northern part of the Curonian Lagoon (stations 1, 7, 6) (Fig. 4.1) while the traces of brackish water could be found as far as the central part of the lagoon (station 4). During all investigation period most of the increased salinity values were observed in the end of August–September.

Table 4.4. Physical characteristics of Curonian Lagoon (2006–2008).

	Stations																				
	1			2			3			4			5			6			7		
	2006																				
	n	min-max	Me	n	min-max	Me	n	min-max	Me	n	min-max	Me	n	min-max	Me	n	min-max	Me	n	min-max	Me
T °C	14	16.8-27.0	23	14	16.0-24.0	22	14	18.0-26.3	23.75	18	16.0-25.0	22.4	16	16.0-26.8	22.25	18	16.2-26.2	22.1	18	14.8-24.4	22
Salinity PSU	14	0.0-6.4	0.40	14	0.0-0.4	0	14	0.0	0.0	17	0.00-0.04	0.0	18	0.0-0.08	0.01	15	0.0-0.6	0.01	18	0.0-6.0	0.4
	2007																				
T °C	12	16.0-25.4	19	12	15.0-24.2	22	12	16.0-25.5	20	10	19.0-26.4	21	10	15.0-24.6	21	10	16.0-25.1	20	10	14.0-24.6	19.6
Salinity PSU	12	0.00-4.4	0.8	12	0.00-1.2	0.2	12	0.00	0.0	10	0.00	0.00	10	0.0-0.7	0.0	10	0.0-1.4	0.2	10	0.0-4.4	0.4
	2008																				
T °C	14	10.1-21	19.53	12	11.2-22.1	19.1	12	12-24	19.5	12	12-23.3	20.8	12	11.8-23.8	20.5	12	10.58-23.8	19	12	16.7-20.8	18
Salinity PSU	14	1.35-7.2	1.35	12	0.0-2.4	0.55	12	0.0-0.7	0.35	12	0.0-0.3	0.10	12	0.1-0.4	0.3	12	0.1-2.6	0.3	12	0.2-5.3	0.6

T °C – water temperature; Me- median; n- number of investigated samples;

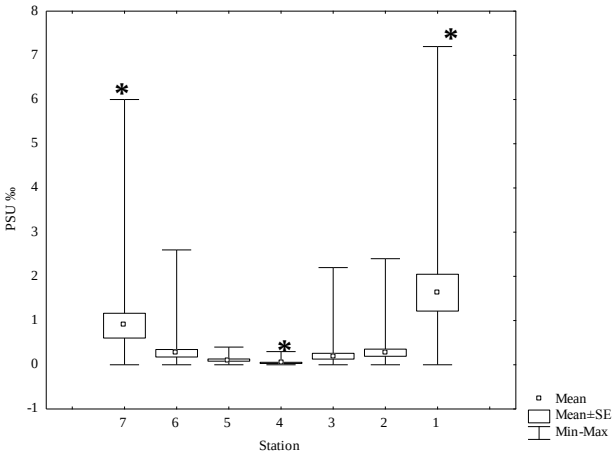

Figure 4.1. Salinity of water at different stations of the Curonian Lagoon during 2006-2008.

Most of the samples which contained cyanotoxins were collected in 2006, so the redundancy analysis (RDA) was used to evaluate the relationship between temperature, salinity, main dominant cyanobacteria genera and cyanotoxins abundance only for data collected in this year. RDA analysis showed that cyanotoxins (MC-LR, MC-RR, MC-YR, MC-LY and the unknown microcystin) concentrations were positively related to the water temperature, biomass of *M. aeruginosa*, *M. wesenbergii*, *Microcystis sp.*, while nodularin concentrations was positively related to the salinity (PSU) and biomass of *N. spumigena*. Nine explanatory variables (salinity, water temperature, the biomass of the dominant cyanobacteria genera: *Chroococcus, Merismopedia, Planktothrix, Microcystis* and species *M. aeruginosa, M. wesenbergii*) explained 35 % of the variation in the concentrations of six different toxins, as could be seen from the 2-dimentional plot (23% on axis 1 and 12 % on axis 2) (Fig. 4.2). The results of a forward selection and permutation tests, presented in Table 4.5, indicated that cyanotoxins (both microcystins and nodularin) were well associated with water temperature (p=0.005), salinity (p=0.005) and with biomass of *M. aeruginosa* (p=0.005) and *N. spumigena* (p=0.005).

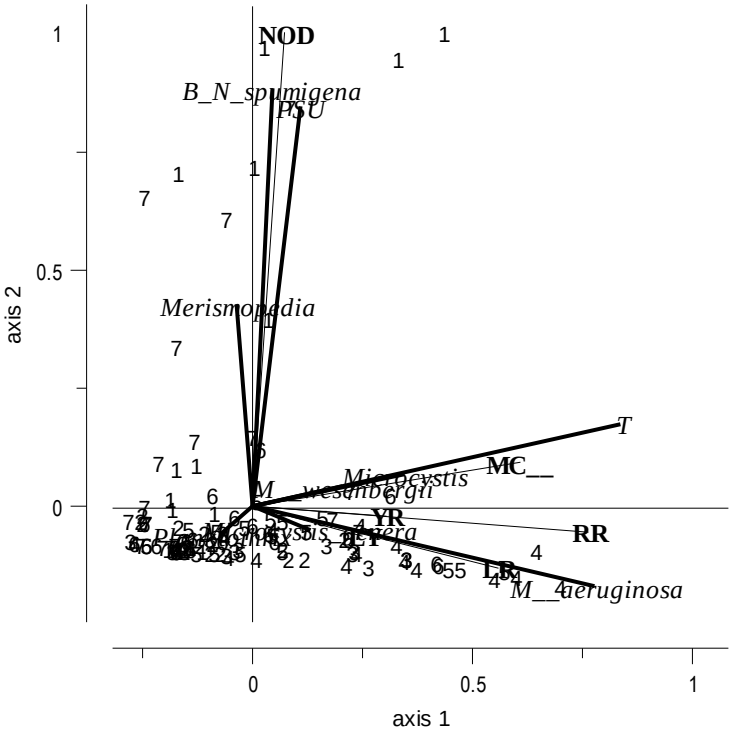

Figure 4.2. Biplot obtained by Redundancy Analysis. Light lines represent the response of variables: MC-LR, MC-RR, MC_?, MC-YR, MC-LR, MC-LY, nodularin; italic line is for explanatory variables: salinity (PSU), temperature (T), the biomass of the dominant cyanobacteria genera: *Chroococcus*, *Merismopedia*, *Planktothrix*, *Microcystis*, species *M. aeruginosa*, *M. wesenbergii* and number of investigated stations.

Table 4.5. Conditional effects for the different cyanotoxins (microcystins and nodularin) concentration data. The total sum of all eigenvalues is 0.35 and the total inertia is 1. The second column shows the increase in explained variation due to adding an extra explanatory variable. The third column shows an eigenvalue as % using only explanatory variable

Variable	Cond. effects	Eigenvalue as %	F-statistic	p-value
Salinity (PSU)	0.03	25.68	7.644	0.005
Temperature (T)	0.15	43.17	20.774	0.005
Chroococcus	0.01	2.33	1.531	0.180
Merismopedia	0.01	6.53	1.154	0.390
Planktothrix	0.001	0.59	1.202	0.320
Microcystis	0.01	7.51	1.624	0.052
M. wesenbergii	0.01	3.64	1.256	0.653
M. aeruginosa	0.03	37.03	8.963	0.005
N. spumigena	0.10	27.43	15.090	0.005

4.4. Bioaccumulation of microcystins in mussels (environmental studies)

Cyanotoxins concentrations in mussel tissues were investigated in samples collected at two locations (station D1 and station D2) (Fig. 2.1) in the beginning of summer (June) and in autumn (September) during three subsequent years (2006–2008). The concentration of the toxin measured in mussel tissues using ELISA varied from values below the detection limit to 139 ng g^{-1} DW (September 2006; station II; size group > 30 mm). When PPIA was used, the concentrations ranged from below the detection limit to 284 ng g^{-1} DW (September 2006, station I; size group > 30 mm). The highest microcystin concentrations were detected in samples collected in 2006, compared with other investigated years (*Man Whitney U Test* U=867; Z=6.97; p=0.00) (Fig. 4.3). In 2007 and 2008, the concentration of toxin varied from below the detection limit to 1.17 ng g^{-1} DW and to 0.90 ng g^{-1} DW, respectively. The

results indicated differences in the concentration of cyanotoxins between mussels collected in June and September (*Man Whitney U Test* U=118; Z=5.97; p=0.00) (Fig. 4.3). In September 2006, mussels contained higher amounts of the toxin (an average 49.07±10.30 ng g^{-1} DW) than in June 2006 (0.21±0.06 ng g^{-1} DW) (*Kruskal-Wallis test* H=35.63; p=0.00) (Fig. 4.6). Similar situation was observed in 2007; the average concentration of the toxins in mussels collected in September was 0.22±0.06 ng g^{-1} DW and in June 0.02±0.01 ng g^{-1} DW (*Kruskal-Wallis test* H=13.69; p=0.00). The opposite situation was observed in 2008. The detected concentrations of cyanobacterial toxins were significantly higher in June (an average 0.10±0.04 ng g^{-1} DW) as compared to September (an average 0.002±0.001 ng g^{-1} DW) (*Kruskal-Wallis test* H=6.94; p=0.01).

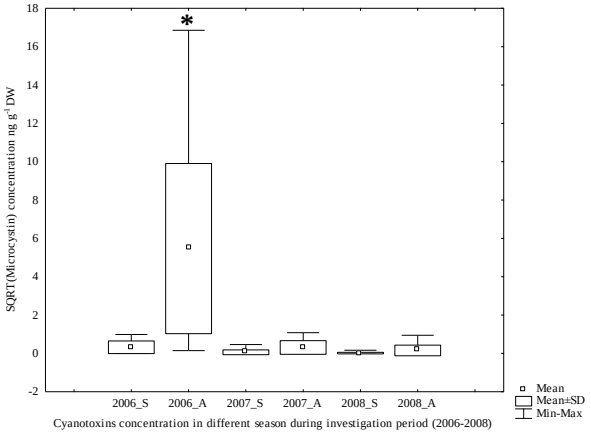

Figure 4.3. Mean concentration of microcystins in mussels tissues determined in different season (S – summer (June); A – autumn (September)) during investigation period (2006-2008).

Although the pair-wise comparison of the two applied methods of cyanotoxin analysis, ELISA and PPIA, has shown no significant differences in the obtained results (W=1.13, p=0.26), the average

values of microcystins concentration determined by PPIA tended to be approximately 10 ng g^{-1} DW higher than by ELISA (Fig. 4.4).

In order not to lose any data and minimize the undesirable bias, the results of both tests were considered in the multivariate analysis as response variables. The location of sampling station was the only environmental factor recorded along with the sampling date and the size of the analyzed mussels. In 2006, the highest toxin concentration of 284.06 ng g^{-1} DW (an average 31.72±10.43 ng g^{-1} DW) was determined in mussels from station D1. Mussels from station D2 accumulated up 32.13 ng g^{-1} DW of cyanotoxins (an average 17.561±5.354 ng g^{-1} DW), but the differences between the station were not statistically significant (*Kruskal-Wallis test* H=0.03; p=0.87). In 2007 and 2008, higher concentrations of cyanotoxins were recorded in mussels from station D2 (an average 0.20±0.06 ng g^{-1} DW (2007) and 0.10±0.04 ng g^{-1} DW (2008)) comparing with station D1 (0.03±0.01 ng g^{-1} DW (2007) and 0.003±0.001 ng g^{-1} DW (2008)), but the differences were insignificant (2007: *Kruskal-Wallis test* H=2.01; p=0.16; 2008: *Kruskal-Wallis test* H=2.10; p=0.15).

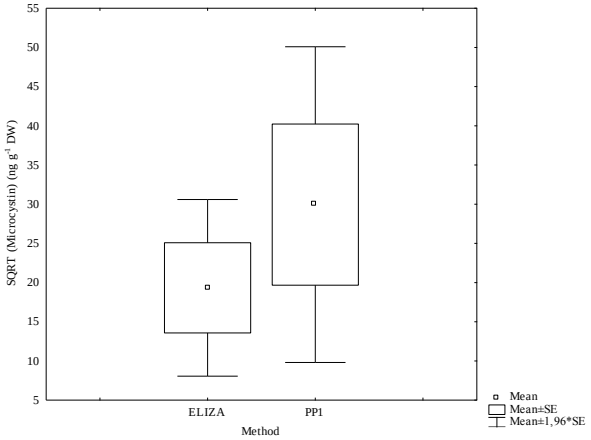

Figure 4.4. Mean concentration of microcystins in mussels determined by ELISA test and PPIA

The microcystins concentrations were analyzed in different size groups of mussels: < 10 mm, 10–30 mm, > 30 mm. Significant differences in the concentration of the toxins between different size groups of mussels were recorded (*Kruskal-Wallis test* H=11.776; p=0.003) (Fig. 4.5). The higest concentration of microcystins (in 2006: varied 0.03 to 284.06 ng g^{-1} DW, station D1; in 2007: n.d.–1.17 ng g^{-1} DW, station D2; in 2008: n.d.–0.89 ng g^{-1} DW, station D2) were detected in mussels bigger than 30 mm during all investigated years 2006-2008 (Tab. 4.6). The lowest concentration (microcystin concentration varied in mussles < 10 mm 2006: n.d.–0.67, station D1; 2007: n.d.–0.22 ng g^{-1} DW, station D2) were recorded in mussels smaller then 10 mm in 2006–2007. In 2008, cyanotoxins were not detected in any mussels sample < 10 mm (Tab. 4.6).

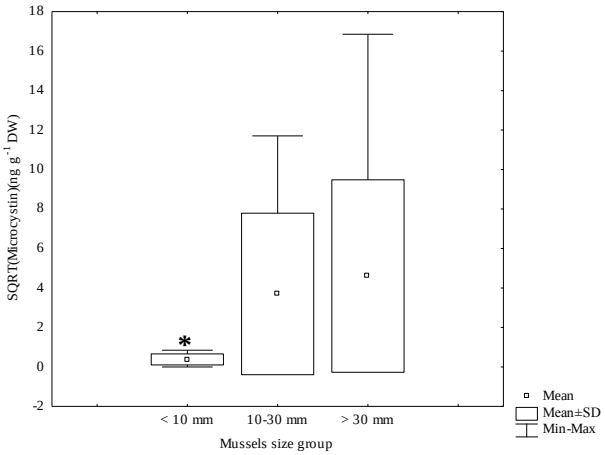

Figure 4.5. Mean concentrations of microcystins determined in different size group of mussels during investigation period 2006-2008.

Among different size groups of mussels collected in June and in September no significant differences in MCs concentrations were recorded (In mussels: < 10 mm: KW-H=1.57; p=0.21; 10–30 mm KW-H=12.36; p=0.42; > 30 mm KW-H=1.71; p=0.19) (Tab. 4.6). Also

between mussels collected at stations D1 and D2, in a given month, there were no statistically significant differences in microcystins concentrations (In mussels: < 10 mm: KW-H=1.47; p=0.23; 10–30 mm KW-H=0.32; p=0.57; > 30 mm KW-H=0.87; p=0.35) (Tab. 4.6).

The post-hoc PERMANOVA pair-wise test indicated significant differentiation in the data collected in 2006 ($p<0.05$), as well as in the data got from the smallest (<10 mm) mussel size group ($p<0.05$) (Fig. 4.6). The statistical significance of the factors and their interactions obtained with PERMANOVA analysis are presented in Table 4.7.

Table 4.6. Mean values of microcystin concentrations determined in different size groups of mussels (± standard error of mean, n = 3) in 2006-2008.

Year	Station D1 [microcystin concentration ng g^{-1}]					
	June			September		
	< 10 mm	10-30 mm	> 30 mm	< 10 mm	10-30 mm	> 30 mm
2006	0.031± 0.015	0.029± 0.012	0.333± 0.179	0.386± 0.103	90.639± 10.479	98.897± 45.421
2007	0.02± 0.017	n. d.	0.027± 0.023	0.023± 0.017	0.024± 0.021	0.112± 0.051
2008	n. d.	n. d.	0.009± 0.05	n.d.	0.004± 0.03	0.007± 0.004
Year	Station D2 [microcystin concentration ng g^{-1}]					
	June			September		
	< 10 mm	10-30 mm	> 30 mm	< 10 mm	10-30 mm	> 30 mm
2006	0.183± 0.113	0.007± 0.003	0.676± 0.133	0.276± 0.086	28.159± 6.251	76.065± 14.803
2007	0.067± 0.034	n. d.	n. d.	0.194± 0.099	0.368±0. 227	0.590± 0.142
2008	n. d.	0.256± 0.157	0.326± 0.138	n. d.	n. d.	0.003± 0.001

The highest concentrations of microcystins were determined in mussels longer than 30 mm collected in 2006 (Table 4.7). Then, in the following years, a consistent reduction in the MC concentration was noticed (Fig. 4.7). In 2008, the toxins concentrations were

detected only in two size groups: 10-30 mm (0.07±0.04 ng g^{-1}) and larger than 30 mm (0.15±0.07 ng g^{-1}). The concentrations were very low, close to the detection limit.

Table 4.7. PERMANOVA based on Euclidean distances of the measured microcystins concentrations in zebra mussels in response to the time of sampling, location, size group and their interactions.

Source	***df***	**MS**	**Pseudo-*F***	***P*(perm)**
Time	5	43.26	393.01	0.001
Location	1	0.26	2.19	0.132
Size	2	9.98	83.74	0.001
Time × Location	5	0.58	4.89	0.003
Time × Size	10	9.38	78.69	0.001
Location × Size	2	0.44	3.72	0.03
Time × Location ×Size	10	0.52	4.41	0.001
Residual	72	0.12		
Total	107			

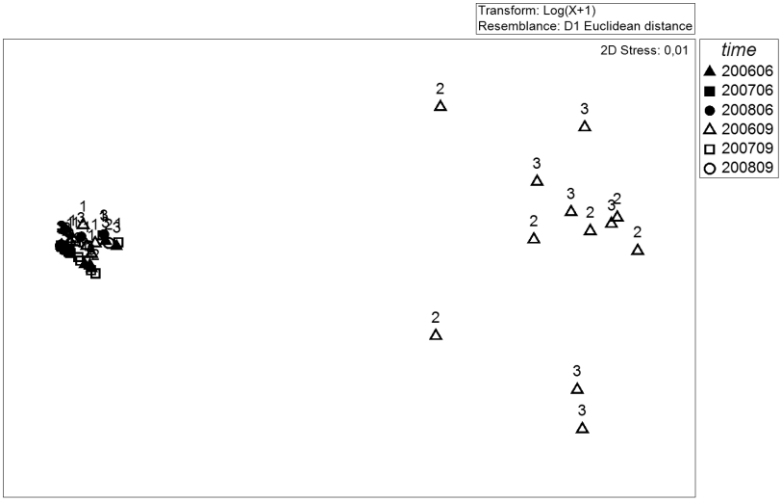

Figure 4.6. A non-metric MDS plot visualizing the multivariate distance among microcystin concentrations in mussels of different sizes (1 - <10 mm length; 2 – 10-30 mm; >30 mm), sampled during 3 sampling sessions (June 2006; September 2006; June 2007; September 2007; June 2008; September 2008).

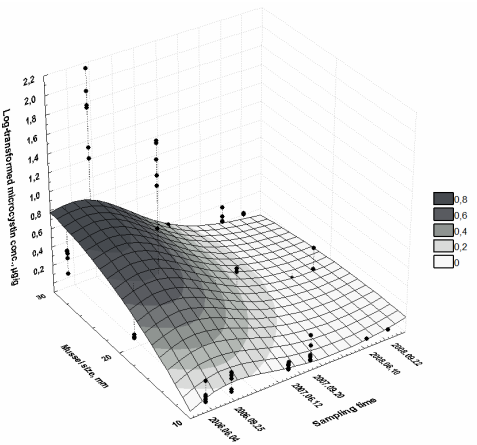

Figure 4.7. Log-transformed microcystin concentrations obtained from zebra mussel tissues as a function of mussel size and sampling time. Circle spots indicate actual measurements and the surface represents the regression model, color scale indicates the respective microcystin concentration levels.

4.4.1. Bioaccumulation and biodegradation of MC-LR in different size groups of mussels (laboratory experiments)

The grazing on cyanobacteria and assimilation of microcystin by mussels were studied in laboratory experiments with different amounts of toxic *Microcystis aeruginosa* (CCNP1101, medium Z8). The *Microcystis aeruginosa* culture contained 3.1 µg MC-LR l^{-1} as measured by HPLC. Cyanotoxins were not recorded in water and mussels before the experiment. The chlorophyll *a* concentrations in container A (were *Dreissena polymorpha* fed ~0.4×10^9 cells/l) and B (fed ~0.9×10^9 cells/l) was measured every day before and after feeding. The measurements showed a decrease in Chl *a* concentration, which indicated that zebra mussels consumed the toxic *Microcystis aeruginosa* cells. In containers A *Microcystis* was consumed more or less evenly; every day, Chl *a* concentration was reduced by 81.8% of the total, on average. The exception was on day 4th when only 42.9% was consumed. However, in containers B, the consumption rate varied in much higher range (25.1–94.0% of total biomass per day) (Fig. 4.8).

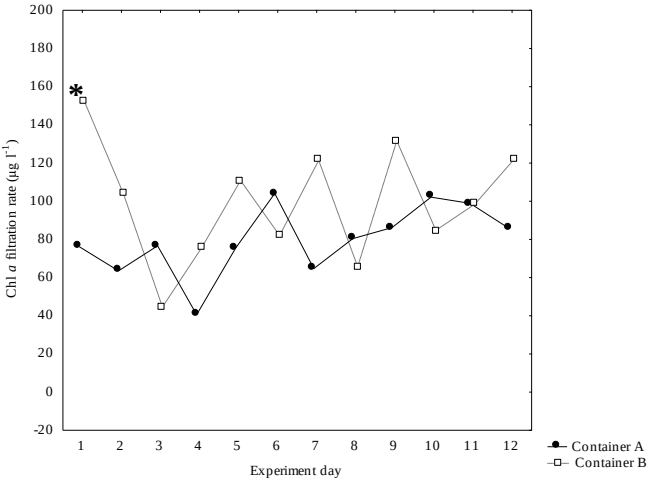

Figure 4.8. Changes in Chl *a* concentration in the containers A and B with *Dreissena polymorpha* exposed to *Microcystis aeruginosa*.

Consumption of cyanobacteria, expressed as Chl *a* changes per day, was significantly higher in mussels from container B compared with container A (Fig. 4.8). Data analysis revealed stistically significant differences in filtration rate of Chl *a* among mussels from container A and B in all experiment days (Fig. 4.8) (Table 4.8).

Table 4.8. Two-way ANOVA results . Df – degrees of freedom, SS – sum of squares, MS – mean square; F – F value, p – significance level, where $p < 0.05$.

Factor	Df	SS	MS	F	*P*
Container	1	6765.8	6765.8	12.720	0.0008
Experiment day	11	20787.9	1889.8	3.553	0.001
Container x	11	20695.1	1881.4	3.537	0.001
Experimental day	48	25531.9	531.9		
Residual	71	73780.7			
Total					

The results of LC-MS analysis showed the accumulation of cyanotoxin MC-LR in different size groups of *Dreissena polymorpha* mussels. In the experiment, the concentration of MC-LR in the smallest mussels (<10 mm) was 2.91±0.72 ng/g, and was higher than in the mussels belonging to the 10–30 mm and > 30 mm size groups where it amounted to 1.30±0.38 ng/g and 0.73±0.24 ng/g (*T-test*, p = 0.01), respectively (Fig. 4.9).

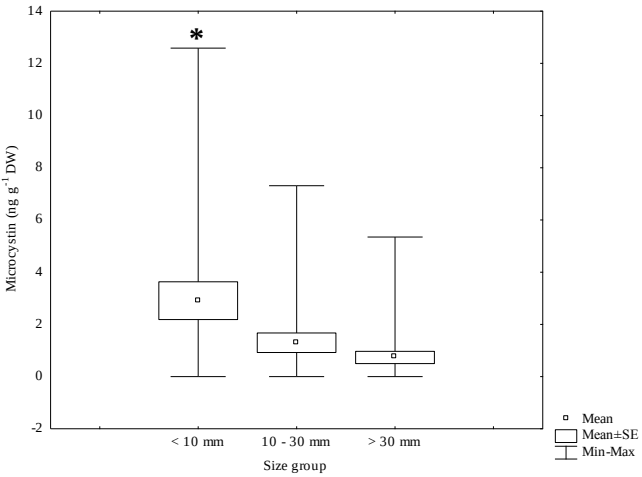

Figure 4.9. Mean concentration of microcystins in different size groups of *Dreissena polymorpha* exposed to the toxic *Microcystis aeruginosa*.

In the experiment mussels were incubated in the culture of *M. aeruginosa* characterized by different cell density (containers A and B). However, the concentrations of MCs in mussels' tissue did not differ significantly between the two experimental variants: A and B (*T-test*, p=0.53).The highest toxin concentrations were detected on days 9 and 13 of the experiment in all size groups of mussels (Fig. 4.10). At the end of the 28-day experiment, very small amounts of microcystin were detected only in mussels belonging to 10–30 mm and bigger than 30 mm size group and exposed to the higher concentrations of *M. aeruginosa* cells.

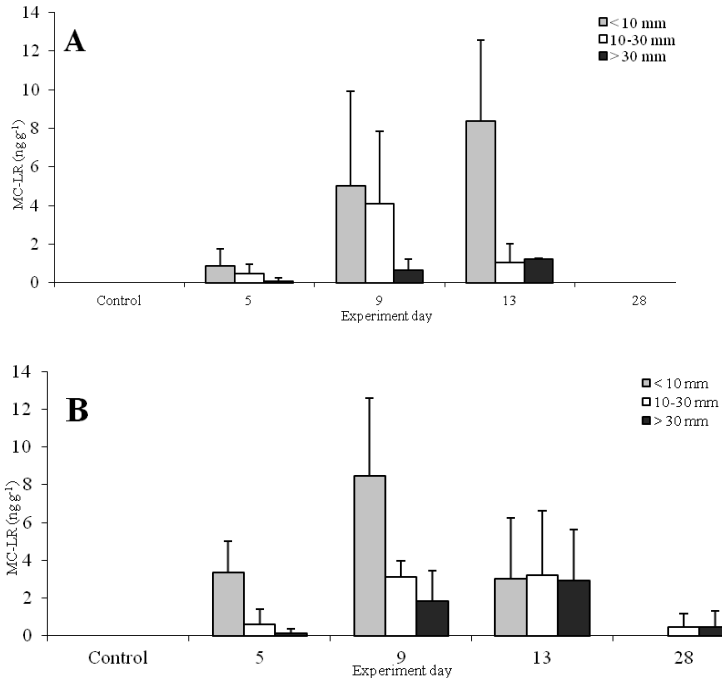

Figure 4.10. MC-LR accumulation in different size groups of mussels fed by the culture of toxic *Microcystis aeruginosa* characterized by different cell density (A (0.4 x 10^9 cell/l) and B (0.9 x 10^9 cell/l)).

Significant differences in microcystin accumulation were detected between the different size groups of mussels on different days of the experiment (Time) (Tab. 4.9), but cell density of the toxic culture did not have any significant influence (Fig. 4.11, Table 4.9).

Table 4.9. Differences in MC-LR accumulation between different size groups of mussels („Time“ and different containers (A and B) are independent variable.

Mussels size group	Kruskal-Wallis test			
	Microcystin accumulation in the course of the experiment Time“		Microcystin accumulation in mussels from different containers (A and B)	
	H	p	H	p
< 10 mm	18.306	0.001	0.594	0.817
10–30 mm	14.515	0.006	0.586	0.444
> 30 mm	13.062	0.011	0.371	0.542

The results of the regression analysis show significantly week relation between M. aeruginosa consumption (measured as the decrease in Chla concentration) by *D. polymorpha* and microcystin accumulation in the mussel tissues (R^2=0.0595; r=0.244; p=0.002; y= 76.7014+4.335+X) (Fig. 4.12).

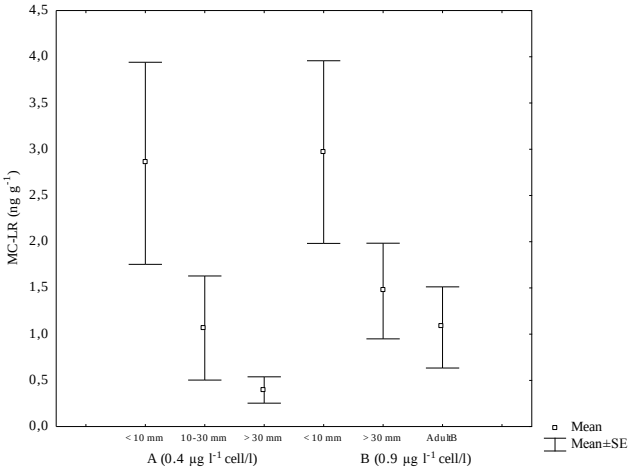

Figure 4.11. Microcystin accumulation in different size groups of *Dreissena polymorpha* exposed to different amount of toxic *M. aeruginosa* cells.

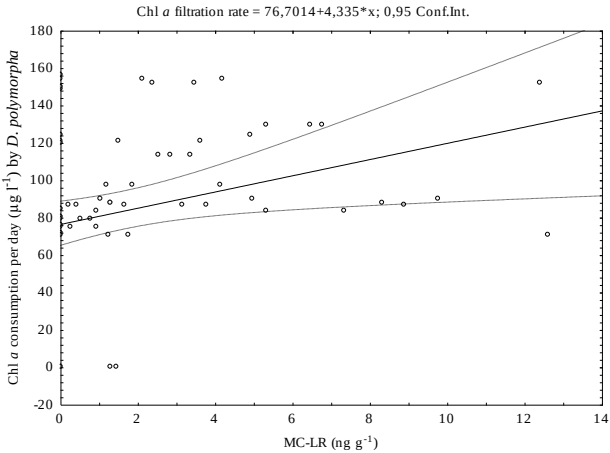

Figure 4.12. Microcystin accumulations in mussels' tissues depend on the Chl *a* consumption per day.

4.5. Bioaccumulation of microcystins in fish

Cyanotoxin concentrations in the muscle of *Rutilus rutilus* were analyzed in samples collected at two locations (station 1 and station 2) in the beginning of summer (June) and in autumn (in the end of September) for two subsequent years (2007–2008). In fish muscle, microcystin was detected. The concentration of the toxin varied from values below the detection limit to 196.4 ng g^{-1} DW, when measured with ELISA test (June 2007; station I; age group IV–VI), and from values below the detection limit to 176.4 ng g^{-1}DW, when measured with PPIA (June 2007; station I; age group IV–VI). The pair-wise comparison of the two applied methods of sample analysis, ELISA and PPIA, has shown no significant differences in the obtained results (W=1.168, p=0.69), the average values of microcystin concentration determined by ELISA tended to be approximately 20 ng/g DW higher than by PPIA. In order not to lose any data and minimize the undesirable bias, the results of both the tests were summed in the analysis as response variables.

The cyanotoxins concentrations were analyzed in different age groups of fishes' I (an average weight Q = 8–20.3 g), II-III (an average weight Q = 20.3–33.85 g), IV-VI (an average weight Q = 59.76–134.08 g). Significant differences in the concentration of microcystin between different age groups of fishes were recorded (*Kruskal-Wallis test* H=75.22; p=0.00) (Fig. 4.13). Cyanotoxins were not detected in size group I during 2007–2008. In II-III size group microcystin concentration an average 13.33±23.96 ng g^{-1} were observed only 2007. The highest microcystin concentration 70.8 ng g^{-1} was detected in II-III size fish which were collected in June at station II. In September that year no cyanotoxin was detected in II-III size fish. Microcystin concentration in this size group did not differ between investigated stations (*Kruskal-Wallis test,* H=1.23; p=0.27). The microcystin concentrations varied from 52.5 to 70.8 ng g^{-1} in samples from station II (an average 62.36±6.80), at the same time in samples from station I the concentrations were 32.42–52.47 ng g^{-1} (an average 43.38±7.73) (Tab. 4.10).

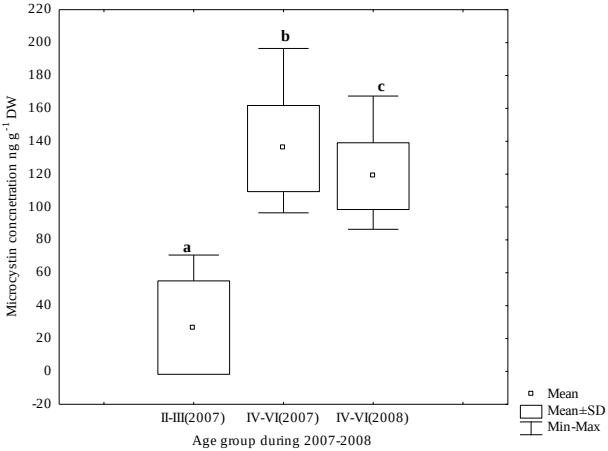

Figure 4.13. Microcystin concentration in muscle from different age groups of fish exposed to *Microcystis aeruginosa during* 2007-2008.
a, b, c – statistical significant different;

During the investigation period, the highest microcystin concentrations 127.17±24.68 ng g^{-1} were detected in IV-VI age group of fish. In this size group of fish significant differences (Tab. 4.11) in the concentration of microcystin between years of investigation - were observed. In 2007, microcystin concentrations varied between 96.46 – 196.44 ng g^{-1} and from 86.48–167.46 ng g^{-1} in 2008. The highest microcystin concentrations 196.44 ng g^{-1} DW were detected in IV-VI age group of *Rutilus rutilus* in 2007 June.

In this age group of fish, cyanotoxins concentration (Tab. 4.10) measured in samples collected in June did not differ statistically from toxins concentration detected in September during investigation years (Table 4.11). The research indicated different seasonal trends in the mean toxins concentrations in fish between the samples collected at two stations.

Table 4.10. An average values of MC-LR concentrations [ng g^{-1} dw] determined in different age groups of fish (*Rutilus rutilus*) (± standard deviation of mean, n=6) in years 2007-2008.

Year	MC-LR concentration at I station [ng/g dw]					
	June			September		
	I	II - III	IV - VI	I	II - III	IV - VI
2007	n. d.	44.28± 7.26	149.03 ± 13.33	n. d.	n. d.	105.84± 2.76
2008	n. d.	n. d.	102.94 ± 4.88	n. d.	n. d.	104.10± 4.25
Year	MC-LR concentration at II station [ng/g dw]					
	June			September		
	I	II - III	IV - VI	I	II - III	IV - VI
2007	n. d.	62.36± 6.80	138.87 ± 16.44	n.d.	n. d.	148.43± 9.43
2008	n. d.	n. d.	131.48 ± 4.60	n.d.	n. d.	136.62 ±8.02

The toxin concentrations in fish from the IV-VI age group were significantly higher in samples from station II (in 2007: 148.87±9.43 ng g^{-1} DW; 2008: 136.62±8.02 ng g^{-1} DW) collected in September compared with mussels from station I (in 2007:

105.84±2.76 ng g^{-1} DW; 2008: 104.10±4.25 ng g^{-1} DW) (Fig. 4.14). Interestingly, the toxins were recorded only in IV-VI age group of *Rutilus rutilus* in 2008 (Tab. 4.10). Almost all factors except season influence toxins accumulation in IV-VI age fish muscle (Tab.4.11).

Table 4.11. Factorial ANOVA results. Df – degrees of freedom, SS – sum of squares, MS – mean square; F – F value; p – significance level, where $p < 0.05$.

Factor	Df	SS	MS	F	*p*
IV - VI					
Season	1	564,5	564,5	1,863	0,180
Station	1	6571,0	6571,0	21,686	<0,001
Year	1	3380,6	3380,6	11,157	0,002
Season x Station	1	2404,9	2404,9	7,937	0,008
Season x Year	1	1202,8	1202,8	3,970	0,053
Station x Year	1	610,2	610,2	2,014	0,164
Season x Station x Year	1	1775,8	1775,8	5,861	0,020
Residual	40	12120,1	303,0		
Total	47	28630,0			

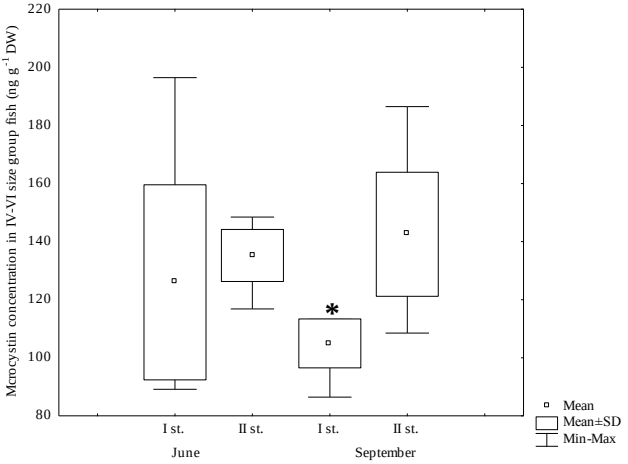

Figure 4.14. Microcystin concentration in IV-VI year fish collected in the Curonian Lagoon from stations I and II in June and September (2007-2008).

4.6. Microcystin bioaccumulation in sediments

The presence of microcystin was detected in sediments from all four investigated stations (Mad1, Mad2, Mad3, and Sand) (Table 4.12). Toxins concentrations were measured by ELISA and PPIA (Table 4.12).

Table 4.12. The average values of microcystin (MC) and chlorophyll a concentrations in the sediments collected from the Curonian Lagoon (2008). Toxin concentration [ng g^{-1} DW] was measured by ELISA and PPIA.

Season/Station		Mud1	Mud2	Mud3	Sand
Summer					
Chl *a* (μg/m^2 sed)		3028±860	3218±102	3995±828	2211±771
MC concentration (ng g^{-1})	ELISA	17.83±2.10	18.09±2.19	25.68±3.07	2.01±0.82
	PPIA	10.50±2.27	5.14±6.39	25.41±19.64	2.68±2.33
Autumn					
Chl a (μg/m^2 sed)		6586 ± 993	6363 ± 792	7780 ± 1869	2615 ± 819
MC concentration (ng g^{-1})	ELISA	2.22 ± 0.31	3.14 ± 1.07	6.02 ± 1.77	0.93 ± 0.18
	PPIA	0.56 ± 0.99	1.25 ± 1.17	3.02 ± 2.72	0.17 ± 0.17

Microcystin concentrations measured in sediments in 2008 varied between 1.17 and 28.15 ng g^{-1} DW – when measured by ELISA, and between 0.16 and 48.06 ng g^{-1} DW – when measured by PPIA (Fig. 4.15).

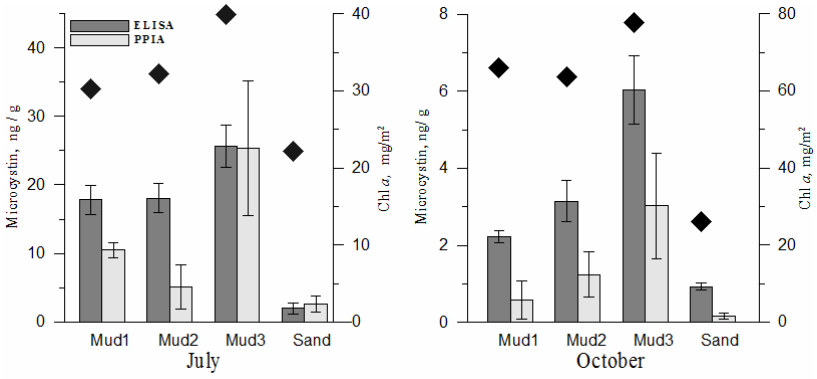

Figure 4.15. Microcystin and Chl *a* (♦) concentration in the upper sediment layer from the Curonian Lagoon in 2008.

Yet, the pair wise comparison of the results obtained by the two applied methods of cyanotoxin measurement, has not shown any significant difference (*T test*, t=1.26; p=0.22; F=1.25; p=0.53). The results of the two methods were multiplied for later analyses.

Chlorophyll *a* concentration at the surface sediment layer varied between 22.11 mg/m^3 (in sandy sediment, bottom) and 39.94 mg/m^3 (in muddy sediment, bottom) in July 2008, and between 26.19–77.91 mg/m^3 in October 2008 (in sandy and muddy bottom respectively). Microcystin concentration was insignificantly lower if compared with chlorophyll *a* concentration (r^2=0.049; r=-0.22; p=0.13). Significantly higher microcystin concentrations were observed in the beginning of summer compared with the autumn *T-test*, t=4.83; p=0.00) (Fig. 4.16).

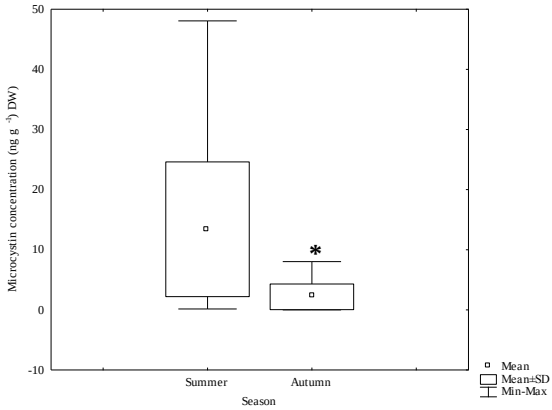

Figure 4.16. Microcystin concentration in sediments from the Curonian Lagoon in different seasons.

In samples collected at individual stations, the concentrations of cyanotoxins were different. Significantly higher concentrations were observed in muddy bottom habitats, compared to the sandy ones, (KW-H=13.29; p=0.004) (Fig. 4.17).

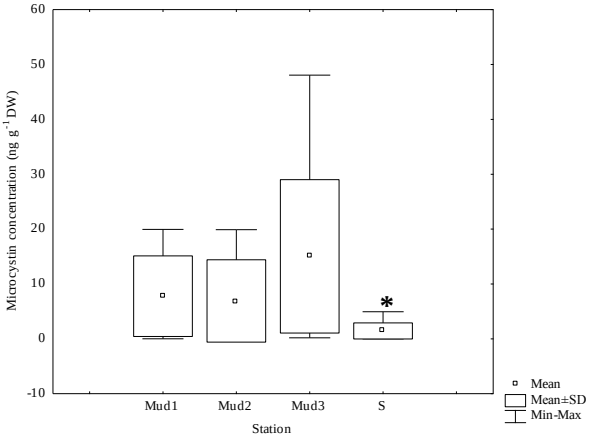

Figure 4.17. Microcystin concentration in sediments from different stations of the Curonian Lagoon. * Statistical significant differences;

Moreover, the results demonstrated that in summer cyanotoxins contents in sediments from individual stations were significantly different, in autumn they were placed within a narrow range (Table 4.13).

This finding indicates higher rate of cyanotoxins biodegradation than their supply to the sediments in autumn. During summer and autumn the highest microcystin concentrations were detected in sediments from station Mud3 (in summer: 48.07 ng g^{-1} DW; in autumn 8.03 ng g^{-1} DW), while in the sandy sediments cyanotoxins concentration was lowest both in summer (0.31 ng g^{-1} DW) and in autumn (0.02 ng g^{-1} DW).

Table 4.13. Two-way ANOVA results. Df – degrees of freedom, SS – sum of squares, MS - mean square; F – F value, p – significance level, where $p < 0.05$

Factor	**Df**	**SS**	**MS**	**F**	***p***
Station	3	1122.159	374.053	8.60282	<0.001
Season	1	1518.557	1518.557	34.92519	0.000
Station×Season	1	2914.208	67.024	67.024	0.000
Residual	43	1869.651	43.480		
Total	47	4510.364			

5. DISSCUSION

5.1. Toxic cyanobacteria blooms in Curonian lagoon, Lithuanian part

Cyanobacteria blooms in the Baltic Sea basin have been known since XIX century. Generally, they are caused by two species of filamentous nitrogen fixing cyanobacteria, *N. spumigena* and *Aph. flos-aquae* (Jonasson et al., 2008). In the recent years, the coastal waters of the Baltic Sea suffered the world's largest algal blooms spanning over 100000 km^2 and caused by *N. spumigena*, *Aph. flos-aquae*, and species of genus *Dolichospermum* (Halinen et al., 2007; Karlson et al., 2005). In the recent decades, abundance of toxic cyanobacteria was found in Vistula (Mazur-Marzec et al., 2010) and Curonian lagoons (Paldavičienė et al., 2009). In the Curonian lagoon the most frequent bloom-forming cyanobacteria belong to *Microcystis, Planktothrix, Dolichospermum, Aphanizomenon and Woronichinia* genera (Pilkaitytė, 2003; Olenina, 1997). In 2006, the assemblages of cyanobacteria were dominated by different *Microcystis* species, including *M. aeruginosa* which was most abundant and formed high toxic biomass. Huge bloom of *Aph. flos-aquae* was recorded in 2008, however, no cyanotoxins were detected. In 2007, no cyanobacteria bloom occurred, presumably due to the unfavorable weather conditions. Only in several samples, *N. spumigena* filaments were present. So, Lithuania didn't escape being added to the list of countries experiencing toxic cyanobacteria blooms (Chorus and Bartram, 1999).

During the studies, the extracellular toxins were not analized. This is because according to literature (Sivonen and Jones, 1999; Meriluoto and Codd, 2005) cyanotoxins investigation in field studies and laboratory experiments with toxic cultures of cyanobacteria have shown that in healthy growing cells more than 80-90 % of microcystins or nodularins are intracellular. The release of the toxins occurs during senescence of the cultures or disruption of cell caused e.g. by grazers. According to the published data (Sivonen and Jones, 1999; Meriluoto and Codd, 2005), the measurements of the

extracellular toxins were done less frequently, as they require more sensitive detection methods. The concentrations of the extracellular toxins depend on multiple factors, including the rate of their release, dilution, photolysis, microbial degradation or sorption to particles and sediments (Meriluoto and Codd, 2005).

Different methods of toxin analysis are characterized by different sensitivity and selectivity. Some of them (e.g. PPIA) provide the information on toxicity of bloom; some are important for quantitative analysis of individual toxins. Other methods are important for analysis of MC and Nod when their concentrations are below detection limit of HPLC. Using HPLC, PPIA and ELISA methods in the analyses of samples from Curonian lagoon we detected five microcystin variants (MC-LR, MC-LY, MC-RR, MC-YR, dmMC-RR) and nodularin. Microcystins were detected in 75% of the analyzed bloom samples collected in 2006. This percentage is within the range of results from other European surveys, e.g. 66% in Denmark (Henriksen, 1996), 66% in Germany (Fastner et al., 2001), 60% in Portugal (Vasconcelos, 2001), 53% in Belgium and Luxembourg (Willame et al., 2005). In some regions higher ratio may occur, e.g. 90% in Czech Republic (Maršalek et al., 2001). Of all samples analyzed in the current study and (co-)dominated by *Microcystis spp.*, 72% were found to contain at least one microcystin variant, while for the other co-dominating genera (*Planktothrix, Dolichospermum and Woronichinia)* it ranged from 7 to 12%. As documented by Sivonen and Jones (1999) microcystin-LR was the most frequently encountered MC variant in many countries. Also in phytoplankton samples from the Curonian lagoon MC-LR was the most abundant MC variant. Other microcystins, MC-LY, one of the rare but highly toxic variant, which could make harm to hydrobionts and humans (Sivonen and Jones, 1999), was detected only in one sample (station 5, 2006). In 2007, we detected only one microcystin variant (dmMC-RR) in 1 of all the investigated samples.

As *N. spumigena* is a estuarine species it is not surprise that the nodularin concentration tended to follow a north-south pattern along the salinity gradient, where the highest concentration was observed

in northern part, and the lowest concentration was detected in southern-central part (station 4 in 2006).

According to published data, nutrients, physical and hydrological factors could influence abundance and biomass of different cyanobacteria species (Chorus and Bartram, 1999; Yunes et al., 1994, 1998; Lehtimaki et al. 1994; Pliński and Dziopa, 2001; Pliński and Jóźwiak, 1999; Pilkaityte, 2003; Pilkaityte and Razinkovas, 2007; Rapala and Sivonen, 1998; Romo, 1994; Sivonen et al., 1989; Shapiro J., 1990; Stal et al., 2003; Song et al., 1998; Tonk et al., 2005; Olenina I., 1997; Van der Westhuizen and Eloff, 1985). However, only several works (Salomon et al., 2003; Tonk et al., 2005; Rapala and Sivonen, 1998) have described how the environmental factors can affect cyanobacteria growth and toxin production. According to Pilkaitytė and Razinkovas (2007), summer phytoplankton community in the Curonian lagoon is characterized by the nitrogen limitation, while phosphorus occurs as the secondary limiting factor. In general, inorganic nutrient supply to the hypereutrophic Curonian lagoon are too high to limit total plankton biomass, which is controlled mostly by the ambient physical factors.

Cyanobacteria have generally higher temperature optima for growth than other phytoplankton organisms and the temperature has been considered as the most important factor contributing to cyanobacterial dominance (Van der Westhuizen and Eloff, 1985; Rapala, 1998). This can explain why in temperate and boreal water bodies most of cyanobacteria bloom in summer (Pliński and Dziopa, 2001). Our study also pointed to the temperature as a key factor related to the abundance of cyanobacteria and may be connected to the presence of toxic cyanobacteria in the Curonian lagoon. According to Rapala and Sivonen (1998) temperature affected the production of MC-RR. In vitro experiments (Rapala et al., 1993) showed the highest microcystin production by *Anabaena* spp., *Aphanizomenon* sp., and *M. aeruginosa* at 20°C. In the Baltic Sea, microcystins were analytically detected by various authors, e.g., in the coastal waters of Poland (Mazur-Marzec and Pliński, 2003) and the Gulf of Finland (Karlsson et al., 2005). In the Lithuanian part of the Curonian Lagoon during the *M. aeruginosa* bloom of 2006, all

five microcystin variants detected in our samples strongly correlated with temperature, while nodularin correlation with temperature was comparably low.

Salinity is another important factor controlling the growth of cyanobacteria (Stal et al., 2003). Studies in the Patos lagoon showed that *M. aeruginosa* blooms are formed in freshwater and under discharge conditions they reach the estuarine area with a salinity gradient from the Atlantic Ocean. The intracellular MC concentrations were lower as salinity increased (Yunes et al., 1994, 1998). In the Curonian lagoon the correlation between microcystins concentration and water salinity was relatively low. To explain the fact two things should be considered: salinity could regulate the structure of cyanobacteria community in the lagoon; e.g. the increase in salinity could reduce the population of microcystin producing species. The other option is that salinity could have influenced toxin production of certain cyanobacteria species. As for *N. spumigena*, the toxic brackish water species, salinity is the main factor determining its distribution, growth, biomass and toxins production (Lehtimaki et al., 1994; Mazur-Marzec et al., 2005). Despite the fact that strong blooms of cyanobacteria are a frequent phenomenon in the Baltic Sea, they do not occur in neighboring waters with salinity outside the range regarded as optimal for their growth i. e. from 5 to 13 psu (Sivonen et al., 1989; Lehtimaki et al., 1994). This could be one of the reason why we detected *N. spumigena* mainly in the northern part of the Curonian lagoon (stations 1, 7) were the salinity occasionally reaches even 7 psu. Very low concentrations of nodularin were detected in southern-western part of the Curonian lagoon (station 4, 5, 6) which might be due to the wind induced north-south hydraulic transport. The study results showed strong positive significant relationship between salinity and nodularin concentration. Belykh et al. (2013) found that the population of *Microcystis* spp. in the Curonian Lagoon (Russian part) is genetically heterogeneous with six different *mcy*E genotypes of capable of microcystin synthesis. *Microcystis* cells with *mcy* genes capable of microcystin synthesis inhabit 70% of water bodies. The species of the genera *Planktothrix* and *Dolichospermum* are less common, they are found in 63 and

37% of water bodies, respectively (Rantala et al., 2006). The species of the genus *Microcystis* are the most common source of microcystins and often cause hepatotoxic blooms in highly productive waters. Toxigenic cyanobacteria of the genus *Nodularia* was not detected in the Russian part of the Curonian lagoon (Belykh et al., 2013). In the Lithuanian part of the lagoon, however, both *N. spumigena* trichomes and its toxic secondary metabolite nodularin were found (Paldavičienė et al., 2009).

The preliminary study concerning the toxin concentration and toxicity of cyanobacterial blooms in the Curonian lagoon was carried out in 2006–2007. Mass toxic blooms of *M. aeruginosa* were recorded. Quite high microcystin and nodularin concentrations found in the Curonian lagoon, which is widely used for the recreation and fishing activities, could cause a threat to human health. So far, the knowledge about cyanobacteria producing toxins in the Curonian lagoon is very poor. An inclusion of cyanotoxins measurements in the monitoring program would be beneficial both for the water management and ecological studies in the water body.

5.2. Bioaccumulation and effects of cyanotoxins on food web

5.2.1. Accumulation and elimination of microcystins in mussels

The abilities to accumulate cyanotoxins might differ among mollusks, due to their feeding habits (grazing, filtering), respiration mode (aerial, aquatic), specific ecological, physiological traits and life history strategy (Dillon 2000; Gérard et al. 2008). The higher risk of contamination with cyanotoxins is related to the direct exposure of mollusks to the heavy cyanobacteria blooms (Amorim and Vasconcelos, 1999).

In the current study, the highest concentrations of microcystin were detected in large mussels (>30 mm length) collected in 2006. It probably related with huge toxins concentration detected in phytoplankton samples that year. These findings are consistent with the results of toxicological plankton study conducted in 2006-2008

(Paldavičienė et al., 2009). In 2006, in the Curonian Lagoon heavy cyanobacteria bloom, mostly formed by *M. aeruginosa*, was reported. That year, intracellular microcystin (predominantly microcyctin-LR) was detected in 75% of the samples collected during the bloom, with concentrations ranging from <0.1 to 134.2 $\mu g l^{-1}$. In 2007, cyanobacteria from the genera *Planktothrix, Limnothrix, Woronichinia* were present, but they did not form a bloom in the Curonian Lagoon. That year, cyanotoxins were detected only in 4% of all investigated samples. In the next year (2008), *Aph. flos-aquae* dominated the cyanobacteria community. However, no cyanotoxins hepatotoxins were reported in the samples. Therefore, the results showed that MC content in mussels coincided well with the production of toxins by cyanobacteria, and was reduced gradually due to depuration and natural shift of mussels in the population (Fig. 5.1). Quite different results were obtained in laboratory experiment where the highest microcystin concentration was detected in small size mussels (< 10 mm). According to Daunys et al. (2006), *Dreissena polymorpha* juveniles have intensive filtration rate and they do not have settled immunity response (Hudnell, 2008). Because of this, small size mussels can accumulate faster higher amount of contaminants and experience higher death-rate.

At the end of the laboratory experiment conducted within this work (on day 28), very small amounts of MC-LR were detected only in mussels belonging to the size group 10-30 mm and bigger than 30 mm and in those exposed to the higher concentrations of *M. aeruginosa* cells. These results confirmed the suggestions of Amorim and Vasconcelos (1999) that the size of the organisms may also play an important role in bioaccumulation process as it is related to the filtration and depuration rates. In addition, at different stages of development, the activity of enzymes involved in the toxin degradation process is also different. Generally, the organisms at early stages of development are characterized by lower activity of the enzymes involved in biotransformation of toxins. Thus, there could be at least several explanations of the *in situ* results showing higher microcystin concentrations in larger mussels comparing to the small ones. Adult zebra mussels can exploit cyanobacteria as a food in the

water column, irrespective of the size, shape, and the toxicity of these phytoplankton species. It is also known that large mussels even seem to prefer cyanobacteria over other phytoplankton groups and detritus (Woller-Skar, 2009; Fahnenstiel et al., 1995; Vanderploeg et al., 2002). Mussel larvae, on the contrary, can effectively filter and utilize small-sized cyanobacteria only if the latter do not contain microcystin (Naddafi, 2007). The larvae show higher mortality, decrease in growth and fecundity rates when they were fed upon MC containing strains of cyanobacteria (Gérard and Poullain, 2005; Lance et al., 2007; 2008; Gèrard et al., 2009).

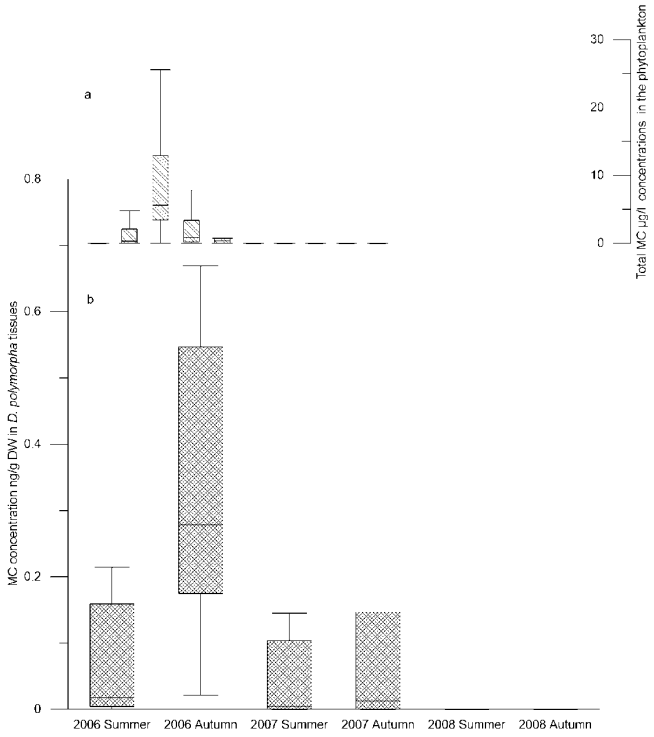

Figure 5.1. Microcystin concentration in water ("a" upper figure) of the Curonian Lagoon and in *Dreissena polymorpha* during the in situ ("b") investitation in 2006-2008 (Paldavičienė et al., 2015).

In contrast, the adult mussels easily survive on a diet of toxic cyanobacteria (Dionisio Pires et al., 2004). Probably, due to better developed detoxication system (Dionisio Pires et al., 2004). The toxic bloom in 2006 was reported in mid-August (Paldavičienė et al., 2009), after the first settlement peak of zebra mussels spat in June, and well before the late settlement (in August–September). It means that in September (when the highest microcystin concentrations were detected in zebra mussel tissues) there was a higher probability to find among newly settled mussels (< 10 mm length) those that have not been (or have been marginally) exposed to the toxic bloom during their larval and post-veliger stages.

Large bivalves (like *Unio, Anodonta* and *Dreissena*) may accumulate a notable quantity of cyanotoxins in the field, but seem to be rather insensitive to them (Watanable et al., 1996; Ibelings et al., 2005; Bij de Vaate et al., 2010). The larger and older mussels could have experienced contact with toxic cyanobacterial blooms more than once or for a longer period during their life. Thus, there is a probability to find some residual concentrations of cyanotoxins in mollusk tissues a certain time after exposure due to incomplete depuration (Mazur-Marzec et al., 2006). Our results, as well as results of other studies (Amorim and Vasconcelos, 1999; Yokoyama and Park, 2003), confirm the long-lasting persistence of microcystin in environment and filter-feeding organisms. Even devoid of cyanotoxins in water, a certain amount of toxins have been detected both in sediment samples and zebra mussel tissues two years after exposure to the toxic bloom. The permanent contamination of mussels with cyanotoxins might be linked to their presence in sediments due to slower biodegradation at low water temperatures or/and due to their binding to sediment particles (Mazur-Marzec et al., 2006). As we did find considerable concentrations of microcystins in the bottom sediments at all sites sampled in 2008 (Fig. 4.17), when no toxic bloom was detected, it can be hypothesized that microcystins absorbed to the sediment particles could have persisted from previous years. That is consistent with a number of studies (Lahti et al., 1997; Chen et al., 2006; Zakaria et al., 2007; Latour et al., 2007) stating that microcystins and their

degradation products could persist in bottom sediments for more than a decade (Pawlik-Skowrońska et al., 2010). Therefore, considering the resuspension as one of the most common phenomena in a shallow Curonian lagoon (Pilkaityte and Razinkovas, 2006), residuals of toxic compounds could be uptaken by mussels with resuspended sediment particles not only in 2006 but also in 2007 and 2008 when no toxic blooms were detected. Resuspension also could explain the presence of comparatively high microcystin concentrations in mollusks well after the toxic blooms the same year (Fig. 5.1) as zebra mussels is known for quite high depuration rates (Dionisio Pires et al., 2004). Also, it is known that in temperate waters, vegetative filaments or akinets of potentially toxic cyanobacteria may form benthic overwintering populations (Gèrard et al., 2009).

Due to their feeding behavior, generally wide distribution and abundance, close association with benthic sediments and relatively sedentary nature, zebra mussels are considered as a proper indicator of water contamination (Salanki, 2000; Lefcort et al., 2002). Based on the current results and taking into account that mussels accumulate microcystins both by grazing upon toxic phytoplankton and uptake of the dissolved (extracellural) toxins (Prepas et al., 1997), we suggest zebra mussels as a good biomarker of cyanotoxins in the ecosystem. Toxic compounds bind in mussel tissues may have important implications for the good environmental status of ecosystem, socio-economic aspects and even human health.

Another implication is related to the potential use of zebra mussels in water quality remediation and subsequent utilization of the cultured biomass. Our data suggest that utilization of *D. polymorpha* cultured under toxic bloom conditions may pose some risk for husbandry or add to intoxication of economically important aquatic species. Due to higher bioaccumulation capacity and incomplete depuration long time after exposure, larger mussels are of a higher concern comparing to the young ones. Therefore for remediation of coastal lagoons, the seasonal (May to October) zebra mussel cultivation approach should be considered. This would ensure sufficiently effective extraction of nutrients by newly settled

mussels, avoiding the risk of sever contamination with cyanotoxins. Anyway, proper monitoring of cyanotoxin concentration in the water during the cultivation season should be undertaken.

5.2.2. Accumulation and effects of microcystin on fish

The studies on the accumulation of cyanotoxins and effects of cyanobacteria on vertebrates have focused mostly on fish, the main secondary consumers in aquatic food webs (Ferrão-Filho and Kozlowsky-Suzuki, 2011). In fish cyanotoxins may be accumulated via different ways: throug direct feeding on phytoplankton (phytoplanktivorous species), uptake of dissolved microcystin via epithelium (gills, skin) or exposure via the food web. Generally it is believed that the oral route is the most important (Ernst et al., 2001).

The highest concentrations of cyanotoxins were found in planktivorous fish that feeds on phytoplankton cells. They also have a great potential for MCs accumulation. Huge toxin concentrations were detected in: liver of smelt *Osmerus eperlanus* (MCs 874 $\mu g\ g^{-1}$ DW, Ibelings et al., 2005) and intestines of silver carp (*Hypophthalmichthys molitrix*) (MC 137 $\mu g\ g^{-1}$). The maximum concentration of MC in a carnivorous fish was 51 $\mu g\ g^{-1}$ DW MC in the liver of perch (*Perca fluviatilis*) (Ibelings et al., 2005). Carnivorous fish, as top predator, had lower average MC contents than other organisms from aquatic trophic system, which suggests no tendency of biomagnifications along the food chain. According to a large data set on aquatic consumers of different trophic levels (Ibelings et al., 2005; Kozlowsky-Suzuki et al., 2006), the biodilution, not biomagnifications of MCs is the predominant process in aquatic food webs. However, the zooplantivorous fish could possibly accumulate high amounts of MCs (Semyalo, 2009). According to feeding spectrum variability, among the omnivores there is the highest number of cyanotoxin-accumulating fish species.

Roach (*Rutilus rutilus*) is one of the most popular, abundant and widely distributed fish species. It is an ecologically tolerant species, well adapted even in eutrophicated and polluted waters (Leonardos et al., 2005). The ability of roach to use cyanobacteria as food item is

generally believed to be one of the reasons of the dominance of roach over perch in eutrophic European lakes (Horppila et al., 2000). In the Curonian lagoon, roach is one of the most popular and abundant fish species (Ložys, 2013). From the Curonian Lagoon it is known that zebra mussels are consumed mostly by vimba (*Vimba vimba*), white bream (*Blicca bjorkna*), roach (*Rutilus rutilus*) (Kublickas, 1959), so this species of fish is very important for trophic food web. According to Papadimitriou et al. (2012), fish species differ in their sensitivity to MC. Cyprinids have been shown to be up to 50-times more sensitive than trout (Fisher and Dietrich, 2000). Due to the wide distribution and feeding habits and sensitivity to microcystin, roach (Ferrão-Filho and Kozlowsky-Suzuki, 2011) was chosen for the experiments with toxin-producing *Microcystis aeruginosa*. Bioaccumulation of MC in the fish is a dynamic process depending on uptake and metabolism/elimination (Malbrouck and Kestemont, 2006). Those processes are strongly related and changeable with the size of the organism. According to the Papadimitriou et al. (2012), MC accumulation in *Rutilus panosi* is size depended. The authors revealed that fish with lower total length, accumulated higher amounts of MC, in comparison with fish of higher total length. A possible explanation could be that different total length classes of *R. panosi* have different dietary components. Species of the genus *Rutilus* are omnivorous cyprinid species feed on zooplankton, zoobenthos, detritus, epiphytes, phytoplankton, macrophytes, and mussels (Brabrand, 1985; Rask, 1989; Horppila and Peltonen, 1994). According to Papadimitriou et al. (2012), the higher accumulation of MC in smaller sized (instead of large sized) *R. panosi* may be also due to more efficient detoxification system in older organisms. The results obtained in this work were quit opposite: we detected 53.32±11.58 ng g DW MC in second (an average weight Q = 20.3–33.85 g) and 127.18±24.68 ng g DW MC in the third class (an average weight Q=59.76–134.08 g) of *Rutilus rutilus*. The results obtained in this work did not show any MC accumulation in the first age group of the roach (an average weight Q =8–20.3 g). Due to the fact that toxic blooms in the lagoon were present in 2006, the older fish had a greater chance to be in contact with the cyanotoxins. In

2007–2008, no cyanotoxins were detected in phytoplankton samples. Detection of MC in fish tissues in 2007–2008 collected in June, before cyanobacteria massive development indicates its long-lasting persistence in the environment and showed that depuration from animal tissue is not complete. Cyanotoxins accumulated in animal tissues can be metabolized into less harmful compounds after conjugation with such endogenous compouns as glutathion, resulting in the excretion or physiological degradation. The variability in glutathione transferase activity (the activity of the enzyme that katalyses detoxication reations via conjugation) in fish species is predominantly related with the fish size (Egaas et al., 1999).

A further complicating factor is the time dependent nature of toxin accumulation vs. depuration in fish (and other biota). The concentrations of cyanotoxins found in fish are very much dependent on the short to medium or long term history of exposure to cyanobacteria toxins (e.g. (Kankaanpää et al., 2002), and hence are rather unpredictable. In studies on *Tilapia rendalli* it was shown that accumulation of MC produced by *Microcystis* cells was dependent on general feeding conditions for the fish (Soares et al., 2004). Interestingly, the presence of MC in fish mussels was found during the depuration period, when fish were no longer exposed to toxic cyanobacteria. The results indicated that fish may contain toxins even when it was caught in the absence of toxic blooms. Unfortunately, the comparison of data from different studies (Kankaanpää et al., 2002; Soares et al., 2004; Xie et al., 2005) is not possible, because the toxin concentrations, exposure routes and a range of other biotic and abiotic factors differs between sites and studies. The results from the Curonian lagoon showed that MC accumulation in roach of different size is significantly related with factors like: season (June/September), sampling station and years (2007–2008).

Due to the high human consumption of fish, it is very important to analyze cyanotoxins transport and accumulation through the aquatic food web. According to WHO recommendations (WHO, 1998), the daily total ingestion (TDI) of food containing MC is 0.04 μg/kg per body weight. In roach muscle samples analyzed in this

work, MC concentrations varied between 32.42–196.44 ng/g. Considering the results, the average of daily fish consumption (for example for 60 kg person) not more than 120 g per day is safe and not exceed the TDI, usually it is one portion of fish. Assuming a dry weight to wet weight ratio of 0.1, MCs in the stomach content (Chen et al., 2009).

According to Lopes and Vasconcelos (2011), the available data on MC accumulation in sea food from brackish-water environments are very limited. According to Papadimitriou et al. (2012), the seasonal accumulation of MC in *Rutilus panosi* tissues is size depended. MC concentrations detected in the larger sized *R. panosi* were close to the TDI recommended by WHO.

Some fish species cannot avoid MC-producing cyanobacteria but they can reduce grazing, suggesting that this may be used as a defense mechanism against the transfer of MC *via* seston. Zhang et al. (2008) showed that high MC concentrations retarded egg development (2–10 h delays) and larval growth, reduced hatching rate (up to 45%) and caused high malformation rate (up to 15 %) and hepatocytes damage.

5.2.3. Microcystin accumulation and biodegradation in sediments and its effect on benthic-pelagic systems

Some aquatic animals, including different invertebrates and fish, live in or feed on sediment materials and become in contact with sedimented cyanotoxins and cyanobacterial cells. Microcystins were found to interact with humic and fulvic substances, with suspended particle matter and sediments (Meriluoto and Spoof, 2008). Sediments might affect the fate of microcystins by their sorption from water column in dissolved form. Sediments can also accumulate toxins either from benthic cyanobacterial mats or from settling biomass of planktonic cyanobacteria. Moreover, biodegradation of microcystins can take place in sediments.

Bottom sediments including contaminated resuspended sediments in water column are known to be filtered by bivalve suspension feeders (Bruner et al., 1994; Gossiaux et al., 1998).

According to multiple studies (Kankaanpää et al., 2001; Mazur-Marzec et al., 2007; Meriluoto and Codd, 2005; Toruńska et al., 2008) about 10 % of cyanobacteria cells are transported to the bottom, while the majority of the biomass decomposed in the upper layers of the water column. Information on microcystin concentrations in natural sediments and their spatial or temporal variability remains limited. However, secondary metabolites of cyanobacteria, namely microcystins are known to persist in bottom sediments and were routinely found in many freshwater (Tsuji et al., 2001; Babica, 2006; Chen et al., 2006) and even marine systems (Kankaanpää et al., 2001; 2009). Microcystins were detected in several samples of sediments from Japanese (0.08–2.33 μg g^{-1} DW) (Tsuji et al., 2001), Chinese (0.12–0.99 μg g^{-1} DW) (Chen et al., 2006), Czech Republic (0.003–0.380 μg g^{-1} DW) (Babica, 2006) and German (several μg l^{-1} up to about 300 μg l^{-1}) (Ihle et al., 2005) water bodies or in sediments from the Baltic Sea (nodularin, 90 μg/L; MC-LR 0.56 μg $m^{-2}d^{-1}$) (Kankaanpää et al., 2001; 2009). Lower concentrations of microcystins and/or nodularins were detected in sediments of prawn farm in Australia (0.0005–0.00471 μg g^{-1} DW) (Kankaanpää et al., 2005). But the most important and useful work regarded seasonal changes of microcystin levels in sediments was published by Ihle et al. (2005). Investigation of microcystin distribution and dynamic in sediments is important for understanding their environmental fate and could contribute to elucidation of their ecophysiological function. Microcystin function in overwintering cyanobacteria and spring reinvasion has been proposed.

We have measured cyanotoxins concentrations in sediments of the Curonian lagoon (in Lithuanian part) and particular attention was paid to their spatial and seasonal changes. The higher concentrations of microcystins were detected in the beginning of June, before cyanobacterial bloom and further declined during the season. This trend might correspond to the end of *Microcystis* overwintering phase and to the beginning of reinvasion, which is usually accompanied with decrease in microcystin concentrations (Ihle et al., 2005). Because of tight adsorption of microcystins to sediment

particles, the declining trend in toxin concentration during the spring could be rather attributed to their biodegradation than to their release from sediments. However, also microcystin removal by sediment resuspension as well as by spring reinvasion of cyanobacterial cells into water column should be taken into account. According to the Misson and Latour (2012), sediment mixing was very important factor, indicating that passive resuspension plays a much more important role in the recruitment of *Microcystis* than light and even temperature. According to Ihle et al. (2005), the concentrations of microcystins in sediments were shown to increase again in the autumn, when the overwintering of *Microcystis* starts and huge amount of cyanobacterial bloom settles down to the bottom of reservoir. In our results, the difference in microcystin concentrations between the investigated stations was more pronounced in the beginning of summer (in June before the *Aphanizomenon flos-aquae* bloom) and it could be the result of better conditions for overwintering of cyanobacterial cells and slower decay of cyanobacteria as well as microcystins. According to Mazur-Marzec et al. (2007), in the surface layers of the sediments nodularin (2.3 ng g^{-1} DW) was also detected in March when small part of *N. spumigena* akinets are present in sediments. According to Brunberg and Blomqvist (2002), during the ice-covered winter period, the cell numbers of *Microcystis* were reduced by 30% and 24 % in the shallow and deep areas, respectively (calculated from samples collected on 3 December and 16 March) or by 48% and 40% (calculated for samples collected between 1 December and 1 April). This indicates high persistence of cyanotoxins in sediments. It has also been shown that benthic *Microcystis* can survive for several years in the sediment, located a few centimeters beneath the sediment surface, without an annual return to the planktonic phase, but nevertheless preserving all they need to return to growth subsequently (Latour et al., 2007). According to Misson and Latour (2012), benthic *Microcystis* colonies buried in sediments for almost 3 years and are still able to contribute in the recruitment process. Although the recruitment rate of this oldest population was low, it does confirm the long-term resistance of these organisms confronted

by unfavorable conditions (darkness, low temperature and anoxic conditions), as previously suggested by the work of Latour et al. (2007). According to the Mazur-Marzec et al. (2007), the increased stability of nodularin might result from slower biodegradation at low temperature or/and from binding of the toxin to sediment particles.

Microcystins were present in the surface layers of sediments in significant concentrations, which could result from their strong adsorption on sediment matter. Hepatotoxins sorption to sediments could occur through complex mechanisms including electrostatic interactions, hydrogen bonding and non-specific Van der Waals forces (Toruńska et al., 2008). Only minor adsorption of microcystins on to suspended particulate matter and sand sediments has been reported (Rivasseau et al., 1998; Hyenstrand et al., 2003). Nevertheless, natural clay particles adsorbed effectively (about 80%) microcystins from aqueous phase (Morris et al., 2000). Many papers revealed importance of microcystin sorption onto sediments (Tsuji et al., 2001; Chen et al., 2006); adsorption of microcystins onto soils was demonstrated as well (Miller et al., 2001). It has been suggested that the clay particles are the most active binding components in soils and sediments. According to Toruńska et al. (2008), sorption of Nod on sediment particles strongly depended on the size of sediment particles. In the laboratory experiment, the sediment samples which contained 56% small particles (< 0.063 mm) were characterized by a higher sorption capacity. According to Mazur-Marzec et al. (2007), the higher toxins concentration were detected in muddy sediments of Gulf of Finland comparing to the coastal part of the Gulf of Gdańsk, where mostly sandy sediments occur. Also our results showed higher microcystins concentrations in fine silty mud sediments comparing with sandy sediments (Sand station) (Fig. 4.17). The decline in microcystin concentration observed in September indicated possible biodegradation in natural sediments.

Resuspension, especially wind driven is well known to be important feature in many shallow systems. Suspension filtering bivalves are tended to ingest large amounts of resuspended material which could include both overwintering cyanobacterial cells and sediment particles contaminated with cyanotoxins. One year after the

large *Microcystis* bloom in the Curonian lagoon there were found elevated concentrations of microcystin in the tissues of *Dreissena polymorpha* (Paldavičienė et al., 2015), while no toxic cells were found in the water column. It could be suggested that the resuspension as a driving mechanism of secondary contamination of food web even in absence of toxic meroplanktonic cyanobacteria bloom in water column. That phenomena is expected to be important in many shallow systems featuring both heavy cyanobacteria blooms and resuspension.

CONCLUSIONS

1. During this study for the first time cyanotoxins were found in the Curonian lagoon. Five microcystin variants (MC-LR, MC-RR, MC-LY, MC-?, MC-YR and dmMC-RR (in 2007)) and nodularin were detected. In 2006, MC-LR was detected in 75% of the samples collected during the "bloom" period, in concentrations from < 0.1 to 134.2 μg l^{-1}. MC-RR and MC-YR were mostly detected in the western part of the lagoon, in concentrations from <0.1 to 30.71 μg l^{-1} and <0.1 to 20.27 μg l^{-1} accordingly. Rare but highly toxic to hydrobionts and humans MC-LY was detected in the only sample with concentration of 0.61 μg l^{-1}. Unidentified MC-? was found at all stations in concentrations range from <0.1 to 17.35 μg l^{-1}. It was the first record of nodularin presence in the Curonian lagoon (concentrations from <0.1 to 284.6 μg l^{-1}). DmMC-RR was detected only in one sample (7.5 μg l^{-1}). The concentrations of hepatoxins determined in seston exeeded the WHO guideline value of for drinking water (1 μg l^{-1}) and recreational needs (20 μg l^{-1}), while no neurotoxins, cytotoxins and dermatotoxins were found during the study period.

2. Microcystin-producing cyanobacteria were found when temperature is an average 22.34°C (mostly in August) while salinity is the indicator of the presence of toxic cyanobacterium *Nodularia spumigena* in the Curonian lagoon during the summer-autumn period.

3. The large zebra mussels (> 30 mm) and larger roach (IV-VI) specimens from the Curonian Lagoon have higher bioaccumulation capacity and slower depuration rates after long time exposure to toxic cyanobacteria, as compared to the smaller individuals (In large mussels: up to 284.06 ng g^{-1} DW (2006); up to 1.17 ng g^{-1} DW (2007); up to 0.89 ng g^{-1} DW (2008); in large roach: 96.46–196.44 ng g^{-1} (2007); 86.48–167.46 ng g^{-1}(2008); in mussels < 10 mm: up to 0.67 (2006); up to 0.22 ng g^{-1} DW (2007); not detected (2008); No cyanotoxins were found in roach juveniles during 2007–2008).

4. Bottom sediments were found to be the secondary repository of cyanotoxins in the Curonian lagoon, while the concentrations in

muddy sediments (ELISA: 12.16±9.23 ng g^{-1}; PPIA: 7.65±11.40 ng g^{-1}) were higher than in sandy ones (ELISA: 2.01±0.82 ng g^{-1}; PPIA: 2.68±2.33 ng g^{-1}). Even after the decline in sediment concentrations in the autumn, cyanotoxins were found next year present in mussel tissues in absence of this contaminant in the pelagic phase. This phenomenon points towards the high probability of secondary contamination via resuspension process of cyanotoxins stored in the bottom sedimentary system.

RECOMMENDATIONS

1. Zebra mussel would be used for the removal of nutrient, we suggest considering seasonal (May to October) zebra mussel cultivation approach. This would ensure sufficiently effective extraction of nutrients by newly settled mussels, minimizing the risk of severe cyanotoxins accumulation.

2. We suggest the inclusion of cyanotoxins into the water quality monitoring practices in the Curoninan lagoon to supplement the tradional microscopic examinations with molecular genetic tests and analytical methods for assessing their impact on the whole ecosystem food chain.

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