QUANTIFICATION OF TOXIN BIOSYNTHESIS GENES IN CYANOBACTERIA AND DINOFLAGELLATES –

Genetic Factors as Predictors of Toxin Production in the Environment

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“The true delight is in the finding out rather than in the knowing.”

― Isaac Asimov

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, referred to in the text by their Roman numerals (I-IV):

I Henna Hautala, Urpo Lamminmäki, Lisa Spoof, Sonja Nybom, Jussi Meriluoto, Markus Vehniäinen (2013) Quantitative PCR detection and improved sample preparation of microcystin-producing *Anabaena*, *Microcystis* and *Planktothrix*. *Ecotoxicol. Environ. Saf.* **87**:49–56.

II Henna Savela, Markus Vehniäinen, Lisa Spoof, Sonja Nybom, Jussi Meriluoto, Urpo Lamminmäki (2014) Rapid quantification of *mcyB* copy numbers on dry chemistry PCR chips and predictability of microcystin concentrations in freshwater environments. *Harmful Algae*. **39**:280–286.

III Henna Savela, Lisa Spoof, Niina Perälä, Mikko Preede, Urpo Lamminmäki, Sonja Nybom, Kerstin Häggqvist, Jussi Meriluoto, Markus Vehniäinen (2015) Detection of cyanobacterial *sxt* genes and paralytic shellfish toxins in freshwater lakes and brackish waters on Åland Islands, Finland. *Harmful Algae*. **46**:1–10.

IV Henna Savela, Kirsi Harju, Lisa Spoof, Elin Lindehoff, Jussi Meriluoto, Markus Vehniäinen, Anke Kremp. Quantity of the dinoflagellate *sxtA4* gene and cell density correlates with paralytic shellfish toxin production in *Alexandrium ostenfeldii* blooms. Accepted for publication in *Harmful Algae*.

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ABBREVIATIONS

A	adenine
ACN	acetonitrile
ACP	acyl carrier protein
Adda	(2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid
bp	base pair
BSA	bovine serum albumin
C	cytosine
cDNA	complementary DNA
Cq	quantification cycle
CYN	cylindrospermopsin
D-Asp	D-aspartic acid
DAD	diode-array UV detection, in HPLC-DAD
dcGTX	decarbamoyl gonyautoxin
dcSTX	decarbamoyl saxitoxin
ddPCR	droplet digital PCR
Dha	dehydroalanine
Dhb	dehydrobutyrine
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
D-MeAsp	D-*erythro*-β-methyl-aspartic acid
dNTP	deoxyribonucleotide
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ESI	electrospray ionization
EST	expressed sequence tag
FISH	fluorescence *in situ* hybridization
FLD	fluorescence detection, in HPLC-FLD
G	guanine
Gb	gigabase, 1×10^6 DNA bases
gDNA	genomic DNA
GTX	gonyautoxin
HAB	harmful algal bloom
HGT	horizontal gene transfer
HILIC	hydrophilic interaction chromatography
HPLC	high-performance liquid chromatography
IAC	internal amplification control
LAMP	loop-mediated isothermal amplification
LC-MS	liquid chromatography–mass spectrometry
LC-MS/MS	liquid chromatography–tandem mass spectrometry
LD_{50}	median lethal dose
LNA	locked nucleic acid

LSU	ribosomal RNA coding gene, large subunit
LWT	*Lyngbya wollei* toxin
MATE	multidrug and toxic compound extrusion protein
MC, MCYST	microcystin
Mdha	N-methyldehydroalanine
MT-PCR	multiplex parallel polymerase chain reaction
NeoSTX	neosaxitoxin
NMT	N-methyl transferase
NOD	nodularin
NRPS	non-ribosomal peptide synthetase
OATP	organic anion-transporting polypeptide (human)
ORF	open reading frame
PAPS	3'-phosphoadenosine-5'-phosphosulfate
PC-IGS	phycocyanin intergenic spacer
PCR	polymerase chain reaction
PKS	polyketide synthase
PP	protein phosphatase
PST	paralytic shellfish toxin
PTFE	polytetrafluoroethylene
qPCR	quantitative polymerase chain reaction
qRT-PCR	quantitative reverse transcription polymerase chain reaction
rDNA	ribosomal RNA-coding sequence
RNA	ribonucleic acid
rRNA	ribosomal RNA
RT-PCR	reverse transcription polymerase chain reaction
sd	standard deviation
SHA	sandwich hybridization assay
STX	saxitoxin
T	thymine
THF	tetrahydrofuran

ABSTRACT

Harmful algal blooms (HABs) are events caused by the massive proliferation of microscopic, often photosynthetic organisms that inhabit both fresh and marine waters. Although HABs are essentially a natural phenomenon, they now cause worldwide concern. Recent anthropogenic effects, such as climate change and eutrophication via nutrient runoff, can be seen in their increased prevalence and severity. Cyanobacteria and dinoflagellates are often the causative organisms of HABs. In addition to adverse effects caused by the sheer biomass, certain species produce highly potent toxic compounds: hepatotoxic microcystins are produced exclusively by cyanobacteria and neurotoxic saxitoxins, also known as paralytic shellfish toxins (PSTs), by both cyanobacteria and dinoflagellates. Specific biosynthetic genes in the cyanobacterial genomes direct the production of microcystin and paralytic shellfish toxins. Recently also the first paralytic shellfish toxin gene sequences from dinoflagellate genomes have been elucidated.

The public health risks presented by HABs are evident, but the monitoring and prediction of toxic events is challenging. Characterization of the genetic background of toxin biosynthesis, including that of microcystins and paralytic shellfish toxins, has made it possible to develop highly sensitive molecular tools which have shown promise in the monitoring and study of potentially toxic microalgae. In this doctoral work, toxin-specific genes were targeted in the developed PCR and qPCR assays for the detection and quantification of potentially toxic cyanobacteria and dinoflagellates in the environment. The correlation between the copy numbers of the toxin biosynthesis genes and toxin production were investigated to assess whether the developed methods could be used to predict toxin concentrations.

The nature of the correlation between gene copy numbers and amount of toxin produced varied depending on the targeted gene and the producing organism. The combined *mcyB* copy numbers of three potentially microcystin-producing cyanobacterial genera showed significant positive correlation to the observed total toxin production. However, the presence of PST-specific *sxtA*, *sxtG*, and *sxtB* genes of cyanobacterial origin was found to be a poor predictor of toxin production in the studied area. Conversely, the dinoflagellate *sxtA4* was a good qualitative indicator of a neurotoxic bloom both in the laboratory and in the field, and population densities reflected well the observed toxin concentrations. In conclusion, although the specificity of each potential targeted toxin biosynthesis gene must be assessed individually during method development, the results obtained in this doctoral study support the use of quantitative PCR -based approaches in the monitoring of toxic cyanobacteria and dinoflagellates.

TIIVISTELMÄ

Mikroskooppiset organismit muodostavat säännöllisesti haitallisia, yleisemmin leväkukintoina tunnettuja massaesiintymiä niin merissä kuin makeissakin vesissä. Perimmiltään ilmiö on luonnollinen, mutta aiheuttaa huolta maailmanlaajuisesti. Viimeaikaiset ihmistoiminnan vaikutukset, kuten ilmastonmuutos sekä vesistöjen rehevöityminen ravinnevalumien seurauksena ovat tehneet massaesiintymistä yleisempiä ja haitoiltaan merkittävämpiä. Sinilevät, eli syanobakteerit, sekä panssarisiimalevät, eli dinoflagellaatit, ovat tavallisia haitallisten massaesiintymien aiheuttajia. Sen lisäksi, että näiden organismien lisääntyessään tuottaman biomassan määrä voi aiheuttaa monenlaista haittaa, osa syanobakteereista ja dinoflagellaateista voi tuottaa voimakkaita myrkkyjä. Mikrokystiinit ovat maksaa vahingoittavia myrkkyjä, joita tuottavat tietyt syanobakteerit. Saksitoksiinit, joita kutsutaan myös halvaannuttaviksi simpukkatoksiineiksi, ovat hermomyrkkyjä, joita voivat tuottaa sekä syanobakteerit että dinoflagellaatit. Tietyt biosynteettiset geenit syanobakteerien genomeissa ohjaavat sekä mikrokystiinien että saksitoksiinien tuotantoa. Ensimmäiset saksitoksiinigeenisekvenssit on viime vuosina selvitetty myös dinoflagellaateista.

Myrkyllisten syanobakteerien ja dinoflagellaattien muodostamat massaesiintymät ovat kiistaton uhka ihmisten ja eläinten terveydelle, mutta esiintymien ennustaminen ja seuranta on hankalaa. Myrkkyjen, kuten mikrokystiinien ja saksitoksiinien, biosynteesin geneettisen taustan selvittyä on tullut mahdolliseksi kehittää herkkiä molekulaarisia menetelmiä myrkkyjä tuottavien organismien havainnointia ja seurantaa varten. Tässä väitöskirjatyössä geneettistä tietoa hyödynnettiin kehittämällä PCR-menetelmiä haitallisten syanobakteerien ja dinoflagellaattien havaitsemiseen. Menetelmien avulla luonnonvesistä kerätyistä näytteistä määritettyjä biosynteesigeenin kopiolukumääriä vertailtiin mitattuihin myrkkymääriin, ja tutkittiin, oliko näiden tekijöiden välillä merkittävää yhteyttä. Tulosten perusteella tehtiin päätelmiä menetelmien hyödyllisyydestä leväesiintymien myrkyllisyyden arvioinnissa.

Tutkimuksessa havaittiin, että positiivinen yhteys tai sen puute oli riippuvainen kustakin kohdegeenistä ja tuottajaorganismista tapauskohtaisesti. Vesinäytteistä tutkittiin mikrokystiinigeeni *mcyB*:n kopiolukuja kolmessa kohteena olleessa syanobakteerisuvussa. Havaittiin, että yhteenlaskettu kopioluku korreloi merkitsevästi tuotetun myrkkymäärän kanssa. Syanobakteerien tuottamien saksitoksiinien, ja niiden tuottoon tarvittavien geenien (*sxtA*, *sxtG* ja *sxtB*) välillä samanlaista yhteyttä ei kyetty osoittamaan. Toisaalta vastaavan dinoflagellaattigeenin, *sxtA4*:n, läsnäolo ennusti poikkeuksetta hermomyrkkyjen tuottoa sekä laboratorio-olosuhteissa että luonnossa, ja populaatiotiheys heijasti hyvin myrkkyjen määrää. Vaikka jokaisen mahdollisen kohdegeenin soveltuvuus myrkyllisyyden ennustamiseen on tutkittava erikseen, väitöskirjatyössä saadut tulokset tukevat olettamusta, jonka mukaan myrkyllisten syanobakteeri- ja dinoflagellaattiesiintymien valvonta voisi hyötyä geneettisestä testauksesta.

1 INTRODUCTION

Harmful algal blooms (HABs) are causing worldwide concern in the scientific community, government authorities, and the general public. The adverse effects of HABs can be caused by the large biomass produced by the rapid proliferation of bloom-forming microalgae, which can severely damage the entire ecosystem by depleting oxygen and nutrient reserves in the water (Hallegraeff 2014). The other major cause for concern is the production of various toxic metabolites. Toxins can contaminate drinking water sources, and accumulate in the food chain, resulting in sometimes fatal poisonings of humans, animals, and aquatic organisms (Sivonen and Jones 1999, Kuiper-Goodman *et al*., 1999, Anderson *et al*., 2002). Significant public health costs, as well as economic losses to fish and shellfish farming, and the recreational industry are caused by HABs (Hoagland and Scatasta, 2006). Although bloom events are a natural phenomenon, and have occurred throughout known history, the anthropogenic impact on water ecosystems during the last few decades, and the warming global climate have contributed to the observed increase in their frequency and severity worldwide (Hallegraeff 2003, Anderson *et al*., 2002).

Cyanobacteria, also called blue-green algae, are photosynthetic prokaryotes and some of the most important primary producers in the world's oceans and freshwater environments in which their mass occurrences, or blooms, are very common (Dittman and Wiegand, 2006). The harmful nature of cyanobacterial blooms has been known for a long time (Bartram *et al*., 1999). The earliest scientific report on a toxic cyanobacterial mass occurrence, and the deaths of domestic animals that followed from the consumption of the contaminated water, dates to 19th century Australia (Francis, 1878). Since then, toxins of cyanobacterial origin have been implicated in numerous poisoning events involving wild and domestic animals, as well as humans (Kuiper-Goodman *et al*., 1999; Stewart *et al*., 2008). Severe human poisoning incidents have occurred: for example, in Brazil, water contaminated with cyanobacterial toxins killed 60 patients admitted to a dialysis clinic (Pouria *et al*., 1998).

Dinoflagellates are a group of unicellular, mostly marine eukaryotes. Some species depend on photosynthesis for survival, and some are exclusively heterotrophic, but mixotrophy is also common, also in HAB species (Anderson *et. al*., 2002). Dinoflagellates, like cyanobacteria, are an important food source for marine life, but are also the causative organisms behind the HAB events colloquially known as "red tides" (Hallegraeff, 2003). A description of a red tide is thought to have been recorded for the first time in the Bible, in which the waters of the River Nile are described to have turned red as blood, and poisonous to fish and humans (Hallegraeff, 2003). Fewer than a hundred dinoflagellate species are known to produce an array of varied and often structurally complex potent toxins (Hallegraeff, 1993). Dinoflagellate toxins, especially neurotoxins such as paralytic shellfish toxins, are responsible for thousands of food poisoning cases annually (Hallegraeff, 2003). Seafood toxin content is continuously

monitored in many countries, and fishing and shellfish harvesting closures are enforced to minimize the risks of contaminated food ending up on the market (Hungerford, 2014).

Due to the risks posed by toxic cyanobacteria and dinoflagellates, monitoring demands are increasing. The possibility to use nucleic acid amplification and hybridization in the detection and quantification of harmful microalgae, such as cyanobacteria and dinoflagellates, was recognized already in the early 1990s (Anderson, 1995). The polymerase chain reaction, PCR, is the most commonly used method, and its inherent specificity and sensitivity make it well-suited for the analysis of complex environmental samples. Other potential benefits include the possibility of designing systems that incorporate a simple sample preparation and reaction setup in the assay, of adapting the methods to both low- and high-throughput settings, and of developing automated applications. Species-specific genetic markers, such as the ribosomal RNA encoding genes, were the first to be used as target sequences in the development of nucleic acid-based methods for the detection of HAB-causing microorganisms, the earlier examples including assays for cyanobacteria by Neilan *et al*. (1995; 1997). However, the specificity in terms of the co-occurrence of the chosen genetic marker and toxin production was not always optimal; it is common to encounter both toxic and non-toxic strains of the same species in one location (Dittmann, 2013; Anderson *et al*., 2012). Therefore, the subsequent discovery of toxin biosynthetic genes, beginning with the cyanobacterial toxin microcystin (Tillett *et al*., 2000; Christiansen *et al*., 2003; Rouhiainen *et al*., 2004), opened up improved possibilities for method development. Indeed, the new genetic information has been efficiently utilized in the development of both qualitative and quantitative PCR assays, as well as hybridization methods.

2 LITERATURE REVIEW

2.1 Harmful algal blooms

The term harmful algal bloom does not have a strict scientific definition, but is generally used to include any microalgal bloom that is detrimental to human health or socioeconomic interests (Hallegraeff, 2014). In the recent decades, the worldwide prevalence and intensity of harmful algal blooms have undergone an apparent rise (Anderson *et al.*, 2002). The increased awareness of toxic microalgae may be a contributing factor; the reporting and monitoring of HAB events is more common, and the issue easily receives media coverage.

Coastal waters are more and more utilized for commercial fish and shellfish culture operations, and the industry is very susceptible to the adverse effects of harmful microalgal blooms, particularly those of dinoflagellates (Hallegraeff, 2014). The discharge of untreated domestic, agricultural, and industrial wastes rich in nutrients has been linked to the increased incidence of HABs (Hallegraeff, 2003), although the relationship between nutrient availability and HAB species composition can be complex (Glibert and Burkholder, 2006). Changes in nutrient ratios, such as the availability of nitrogen in relation to phosphorus, and the effects of land usage also cause concern, as they have been implicated in the changes in phytoplankton populations, which in turn have been observed to favor nuisance species (Anderson *et al.*, 2002, Heisler *et al.*, 2008). The extremely varied survival strategies of different species of harmful microalgae make almost any environment susceptible to HABs (Hallegraeff, 2014). Human activities can result in the unintended introduction of harmful species to new habitats around the world, *e.g.*, via ship ballast water (Scholin *et al.*, 1995, McMinn *et al.*, 1997, Doblin *et al.*, 2007), or transport of shellfish stocks (Schwinghamer *et al.*, 1994). Climate change is expected to further complicate the issues related to HAB events. Studies on fossil dinoflagellate cyst deposits have indicated that some potentially harmful or related species have been more widespread during warmer climate conditions than they are today (McMinn, 1989, Fjellså and Nordberg, 1997), and it is known that cyanobacteria can benefit from warmer growth conditions, increased atmospheric CO_2 levels, and weather extremes (Paerl and Paul, 2012).

Harmful algal blooms can detrimentally affect the environment in several ways. Some HABs are composed of essentially non-toxic species which are capable of sudden significant increases in biomass, *e.g.*, the dinoflagellate *Noctiluca scintillans* (Hallegraeff, 2003). These very dense blooms can lead to nutrient and oxygen depletion, killing fish and invertebrates, as well as potentially preventing light from reaching but the very surface of the water, thus harming photosynthetic organisms (Paerl and Paul, 2012). Some HAB species cause damage mainly to farmed fish by producing substances that clog fish gills, or compounds that are exclusively toxic to fish and invertebrates (Hallegraeff, 2003). These types of harmful algal blooms have notable economic impacts (Hoagland and Scatasta, 2006). Water discoloration and formation of foul-

tasting and malodorous substances hamper both drinking water production as well as the recreational use of water (Ho *et al*., 2009). Possibly the greatest issue, however, are the many toxins that HAB-forming species can produce: these pose significant health risks and can harm and kill not only humans, but fish, birds, and other aquatic and terrestrial animals (Hallegraeff, 2014).

2.2 Cyanobacterial and dinoflagellate toxins

Both cyanobacteria and dinoflagellates are prolific producers of varied and structurally complex biologically active secondary metabolites, many of which are toxic, but some may also have potential beneficial uses, *e.g*., as therapeutic agents (Burja *et al*., 2001, Kita *et al*., 2010). The exposure to toxic compounds produced by cyanobacteria and dinoflagellates can occur via many routes. Cyanobacterial toxins are major drinking water contaminants, and numerous occurrences of human and animal poisonings have been caused by toxin-containing water (Kuiper-Goodman *et al.*, 1999; Stewart *et al.*, 2008). In Brazil, approximately 2000 cases of gastroenteritis, including 88 fatalities, were caused by cyanobacteria-contaminated drinking water (Teixeira *et al.*, 1993). In Australia, 150 poisoning cases requiring hospitalization were attributed to the copper sulfate-induced termination of a cyanobacterial bloom in a drinking water source (Bourke *et al.*, 1983). The recreational use of waters with cyanobacterial mass occurrences has also been reported to have led to various incidences of human poisoning (Dillenberg and Dehnel, 1960; Turner *et al.*, 1990; Pilotto *et al.*, 1997). Fish and seafood can accumulate a number of cyanobacterial toxins, introducing them to the food chain, possibly even up to the level of human consumption (reviewed by van Apeldoorn *et al.*, 2007). Agricultural irrigation may present a less-acknowledged exposure route, as the toxins may accumulate onto and even in the tissues of edible plants, if there are toxic cyanobacteria present in the irrigation water (Codd *et al.*, 1999; Hereman and Bittencourt-Oliveira, 2012).

Toxins produced by marine dinoflagellates are notorious for their ability to cause severe seafood-related illnesses, including paralytic, diarrhetic, neurotoxic, and amnesic shellfish poisoning, among others (Hallegraeff, 2014). The annually reported poisonings number in the thousands, the paralytic shellfish poisoning syndrome alone causing approximately 2000 cases, with an estimated mortality rate of 15% (Hallegraeff, 2003). The traditional vectors of the various poisoning syndromes are bivalves, such as clams and mussels (Shumway, 1990). However, several other−although rarer−vector species, such as gastropods, crustaceans, and fish have been reported (Deeds *et al.*, 2008). Marine dinoflagellate toxins have not traditionally been an issue in terms of drinking water but may yet become one, if desalination is to be taken into wider use in drinking water production (Caron *et al.*, 2010).

Cyanobacterial toxins can be structurally classified into peptides and alkaloids, the hepatotoxins microcystins and nodularin belonging to the peptide group (van Apeldoorn *et al.*, 2007). Alkaloid toxins can be further divided into neurotoxins, epithelial irritants, and cytotoxins. The first group includes saxitoxins (or paralytic shellfish toxins, PSTs),

anatoxin-a, and anatoxin-a(S), the second includes aplysiatoxin, debromoaplysiatoxin, and lyngbyatoxin-a, and the final group consists of cylindrospermopsins (van Apeldoorn *et al.*, 2007). Non-ribosomal synthesis of a multitude of metabolites is common in prokaryotes, and a survey of 126 cyanobacterial genomes revealed that 70% contained non-ribosomal peptide synthesis or polyketide synthesis gene clusters (Shih *et al.*, 2013). These numbers include the hepatotoxic microcystins, which are produced via a hybrid non-ribosomal-polyketide synthesis route by cyanobacteria encountered worldwide in mainly freshwater habitats (Tillett *et al.*, 2000; Christiansen *et al.*, 2003; Rouhiainen *et al.*, 2004; Fewer *et al.*, 2013; Shih *et al.*, 2013). Microcystin biosynthesis has been characterized both in free-living planktonic and benthic, as well as symbiotic cyanobacterial genera: *Anabaena*, *Microcystis*, *Planktothrix*, *Nostoc* (Sivonen *et al.*, 1990a; Luukkainen *et al.*, 1993; Oksanen *et al.*, 2004; Kaasalainen *et al.*, 2009), *Phormidium* (Izaguirre *et al.*, 2007), *Hapalosiphon* (Prinsep *et al.*, 1992), and *Fischerella* (Fiore *et al.*, 2009). In most cases, the planktonic, bloom-forming genera cause the most severe hepatotoxic occurrences, due to their ability to achieve high local cell densities (Dittmann and Wiegand, 2006).

Dinoflagellate toxins are the cause of nearly all classical seafood poisoning syndromes, and exhibit remarkable structural complexity and diversity (Kellmann *et al.*, 2010). Polyether toxins come in linear, macrocyclic, and ladder-frame forms and they include the diarrhetic shellfish poisoning toxins okadaic acid and dinophysistoxins, which are potent phosphatase inhibitors (Bialojan and Takai, 1988), as well as compounds called azaspiracids, which cause similar symptoms, (Satake *et al.*, 1998). The polyether ladder-type brevetoxins, ciguatoxins, maitotoxin, and yessotoxin are extremely potent neurotoxins (Kalaitzis *et al.*, 2010). Dinoflagellate neurotoxins also include the macrocyclic imines spirolides (Cembella *et al.*, 2000) and gymnodimines (Seki *et al.*, 1995), as well as the alkaloid saxitoxin (Llewellyn, 2006) and its various structural analogues. The overall majority of dinoflagellate toxins are of polyketide origin (Rein and Snyder, 2006).

Saxitoxin and other paralytic shellfish toxins are produced by both dinoflagellates and cyanobacteria, in marine and freshwater environments, respectively. PST production has been confirmed in species and strains of cyanobacteria belonging to the genera *Anabaena* (Llewellyn *et al.*, 2001), *Aphanizomenon* (Ballot *et al.*, 2010; Ledreux *et al.*, 2010), *Cylindrospermopsis* (Lagos *et al.*, 1999), *Planktothrix* (Pomati *et al.*, 2000), *Lyngbya* (Carmichael *et al.*, 1997; Lajeunesse *et al.*, 2012), *Raphidiopsis* (Yunes *et al.*, 2009), *Microcystis* (Sant'Anna *et al.*, 2010), and *Scytonema* (Smith *et al.*, 2011). The dinoflagellate genus *Alexandrium* contains most of the marine PST producers identified to date (Anderson *et al.*, 2012). Additionally, two other dinoflagellate species, *Gymnodinium catenatum* (Oshima *et al.*, 1993), and *Pyrodinium bahamense* (Usup *et al.*, 1994), are known to synthesize these toxins.

2.2.1 Toxic cyanobacteria and dinoflagellates in Finnish lakes and the Baltic Sea

Toxic cyanobacterial blooms are common in Finnish lakes; in a survey conducted in the 1980s, 44% of samples collected elicited either hepato- or neurotoxicity in mice (Sivonen *et al.*, 1990b). Later, genetic analysis of cyanobacterial populations has indicated that the prevalence of potentially microcystin-producing cyanobacteria in freshwater lakes would be as high as 70–90% (Rantala *et al.*, 2006). Other cyanobacterial toxins detected include PSTs, saxitoxin in particular, which have been observed to co-occur with *Anabaena* spp. (Rapala *et al.*, 2005), and anatoxin-a and its variants, which have been detected not only in freshwater lakes (Sivonen *et al.*, 1989a), but recently Baltic Sea coastal waters as well (Rantala-Ylinen *et al.*, 2011). Thus, not only are toxic cyanobacteria widespread, they produce several types of potent toxins in Finnish lakes.

Cyanobacterial blooms of *Nodularia spumigena* and *Aphanizomenon* sp. have been observed in the coastal areas and even in the open Baltic Sea already in the 19th century (Finni *et al.*, 2001), and the investigation of the sediment fossil record has revealed that cyanobacteria have formed mass occurrences in the Baltic Sea for over 7000 years (Bianchi *et al.*, 2000). The observations of open sea blooms have increased since the Second World War, and the prevalence of cyanobacterial carotenoid pigments in sediments has indicated an increased incidence of blooms since the 1960s (Finni *et al.*, 2001; Poutanen and Nikkilä, 2001). In the Baltic Sea, *N. spumigena* dominates the cyanobacterial blooms, which are extensive enough to be easily visible from space (Kahru *et al.*, 2007). The production of the hepatotoxin nodularin by Baltic Sea *N. spumigena* was confirmed in 1989 by Sivonen *et al.* (1989b). More recently, also the widespread occurrence of microcystin-producing *Anabaena* in the Baltic Sea has been reported (Karlsson *et al.*, 2005; Halinen *et al.*, 2007; Fewer *et al.*, 2009).

Dinoflagellates form a major part of the Baltic Sea phytoplankton (Klais *et al.*, 2011). Toxic dinoflagellates from the Baltic Sea include the PST-producing *Alexandrium ostenfeldii* (Kremp *et al.*, 2009) and *Dinophysis* spp., which produces the diarrhetic shellfish poisoning toxins dinophysistoxins and pectenotoxins (Kuuppo *et al.*, 2006). Although there are earlier records of *A. ostenfeldii* in the Baltic Sea dating back to the beginning of the 20th century (Ostenfeld, 1913), an increase in coastal blooms and cell densities has been reported since the late 1990s, and the production of PSTs in strains of *A. ostenfeldii* isolated from the Baltic Sea was confirmed in 2009 (Kremp *et al.*, 2009).

2.2.2 Microcystins

Microcystins are cyclic peptides that consist of seven amino acid residues, produced by cyanobacteria belonging to both free-living and symbiotic genera (see section 2.2). The general structure contains several non-proteinogenic amino acids and is cyclo(D-Ala-X-D-MeAsp-Z-Adda-D-Glu-Mdha), where D-MeAsp is D-*erythro*-β-methyl-aspartic

acid, Mdha is N-methyldehydroalanine and Adda is (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(E),6(E)-dienoic acid (Botes *et al.*, 1984; Rinehart *et al.*, 1988). The letters X and Z represent variable L-amino acids which are indicated with their respective single letter codes at the end of the microcystin variant's name: for example, microcystin-LR (MC-LR, Figure 1) contains L-leucine and L-arginine residues (Carmichael *et al.*, 1988). To date, over a 100 microcystin variants have been characterized in free-living and symbiotic cyanobacteria (Sivonen, 2009; Kaasalainen *et al.*, 2012). The structural differences mainly stem from the variable L-amino acids and the presence or absence of methyl groups on the D-MeAsp and Mdha residues (Namikoshi *et al.*, 1998). Side-chain substitutions can, however, occur in all of the amino acid residues of the microcystin molecule (Sivonen and Jones, 1999).

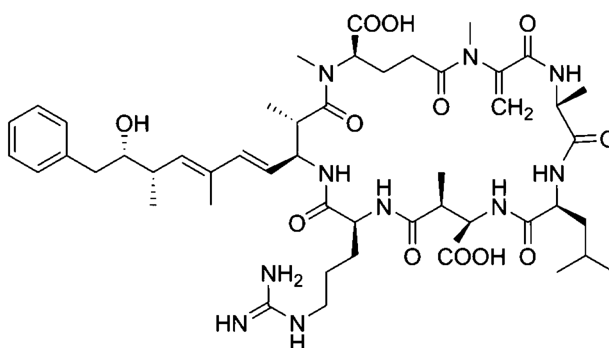

Figure 1. Microcystin-LR. Microcystins are cyclic peptides, the general structure of which is cyclo(D-Ala-X-D-MeAsp-Z-Adda-D-Glu-Mdha), where D-MeAsp denotes D-erythro-β-methyl-aspartic acid, Mdha N-methyldehydroalanine, and Adda is (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(E),6(E)-dienoic acid (Botes *et al.*, 1984; Rinehart *et al.*, 1988). The letters X and Z represent variable L-amino which here are L-leucine and L-arginine residues, hence the name microcystin-LR (Carmichael *et al.*, 1988).

Microcystins are hepatotoxins, *i.e.*, they cause damage to liver tissue in mammals, including humans. A LD_{50} value of 50–60 μg kg^{-1} (measured in mice using intraperitoneal administration) is generally accepted, and has been determined using microcystin-LR (Kuiper-Goodman *et al.*, 1999). The toxicity of microcystin variants is dependent on the structure, the variation sometimes being over 10-fold (Sivonen and Jones, 1999). Although microcystins can damage multiple organs, including kidneys, lungs, and intestines, the toxic effects are concentrated in the liver (Kuiper-Goodman *et al.*, 1999). Hepatocytes express multispecific organic anion-transporting polypeptides (OATP) on the cell surface, which are normally involved in the transport of bile salts and steroids, among others (Hagenbuch and Meier, 2003). Liver cells have been shown to exhibit active uptake of microcystin via these polypeptides (Runnegar *et al.*, 1991; Fischer *et al.*, 2005). Microcystins do not pass through the cellular membrane without active transport, demonstrated by prevention of MC-LR toxicity by introduction of known OATP substrates (Fischer *et al.*, 2005). Interestingly, microcystin is also taken up by a type of OATP expressed by the endothelial cells of the blood-brain barrier

(Fischer *et al.*, 2005), and a few recent studies have provided preliminary indications of possible neurotoxic and neurodegenerative effects induced by microcystin exposure (Myhre *et al.*, 2013).

In the cell, microcystins act by selectively inhibiting protein phosphatases (PP) 1 and 2A (MacKintosh *et al.*, 1990). Several of the amino acids making up microcystins, including Adda, D-Glu and Mdha (the last of which forms an irreversible covalent bond with a cysteine residue in the PP catalytic domain), have been shown to participate in the binding to the active site of the protein phosphatases (MacKintosh *et al.*, 1995; Runnegar *et al.*, 1995; Gulledge *et al.*, 2002). Due to their structural differences nodularin, the microcystin-related cyclic pentapeptide toxin, doesn't form a covalent bond with the PPs like microcystins do, but despite this nodularin is as toxic as MC-LR, due to the high affinity of Adda to the PP active site, demonstrating the importance of Adda to microcystin toxicity (Honkanen *et al.*, 1991; Lanaras *et al.*, 1991; Craig *et al.*, 1996; Bagu *et al.*, 1997). Certain modifications to Adda and D-Glu, such as the isomerization of the Adda-diene (Harada *et al.*, 1990a; Harada *et al.*, 1990b), or acylation of the glutamate (Rinehart *et al.*, 1994), result in practically non-toxic microcystin variants, providing further evidence for the importance of this structural region for the toxicity of microcystins. The cyclic structure also plays a role: the linearization of the molecule significantly decreases the toxicity (Choi *et al.*, 1993; Bourne *et al.*, 1996).

The inhibition of PP1 and PP2A in the affected hepatocytes leads to the excessive phosphorylation of the cytoskeletal proteins, which in turn causes the characteristic cell deformation, triggering apoptosis and cell death (Eriksson *et al.*, 1990; Bøe *et al.*, 1991; Falconer and Yeung, 1992). Cell disruption is followed by the loss of liver sinusoidal structure; the resulting tissue damage leading to potential internal hemorrhaging in the organ and, in extreme cases, to hemodynamic shock and death (Kuiper-Goodman *et al.*, 1999). There is some evidence that in addition to their immediate toxicity, microcystins may have harmful properties also at subacute concentrations, especially if the exposure is continuous, *e.g.*, through drinking water; epidemiological evidence from China and Serbia has indicated an increased incidence of liver cancer in areas in which the drinking water is commonly contaminated with the toxins (Yu, 1995; Svirčev *et al.*, 2009). Sub-lethal exposure results in increased liver enzyme activities in serum, indicating damage to the organ (Falconer *et al.*, 1983). Animal studies have shown that microcystins can act as tumor promoters (Nishiwaki-Matsushima *et al.*, 1992), and although final conclusive evidence remains to be presented, microcystin-LR has been classified as a potential human carcinogen on the basis of the current knowledge (IARC, 2010).

2.2.3 The *mcy* gene cluster and microcystin biosynthesis

Microcystins are produced through a non-ribosomal, sequentially proceeding synthesis route, and the structure of the toxin is determined by the suite of biosynthetic enzymes encoded by biosynthesis genes (*mcy*) arranged in a cluster, which in *Microcystis* spans 55 kb (Tillett *et al.*, 2000). Twelve *mcy* genes have been characterized, but in the *mcy* gene clusters characterized to date, a maximum of 10 genes has been included at one time (Table 1) (Dittmann *et al.*, 1997; Tillett *et al.*, 2000; Christiansen *et al.*, 2003; Rouhiainen *et al.*, 2004; Fewer *et al.*, 2007; Fewer *et al.*, 2013; Shih *et al.*, 2013). The number of genes and their arrangement in open reading frames (ORF) depends on the genus of the toxin-producing cyanobacterium, and in *Anabaena* and *Microcystis*, the genes constitute two operons, and are expressed as two polycistronic transcripts (Kaebernick *et al.*, 2002). In *Planktothrix*, all genes except *mcyT* are included in one unidirectional ORF (Christiansen *et al.*, 2003). Non-ribosomal peptide synthesis and polyketide synthesis in prokaryotes often follow the so-called co-linearity rule (Kleinkauf and Von Döhren, 1996), *i.e.*, the order of genes dictates the order in which the building blocks of the product are joined together. However, only the *Anabaena mcy* gene cluster seems to strictly follow this rule, while others, including those in *Microcystis* and *Planktothrix*, do not (Dittmann *et al.*, 2013).

The *mcy* genes or gene domains can be classified into four groups according to their functions: non-ribosomal peptide synthetases (NRPS), polyketide synthases (PKS), modifying enzymes, and transporters, of which there is only one (Table 1). Microcystin biosynthesis is initiated by the multidomain enzyme McyG; a phenylpropanoid starter unit, most likely 3-phenyllactate, is activated by the NRPS adenylation domain (Nishizawa *et al.*, 2000; Hicks *et al.*, 2006). Subsequent malonyl-CoA elongation, C-methylation, reduction, and dehydration steps are catalyzed by the PKS modules of McyG, McyD, and McyE, which finally converts the polyketide to a β-amino acid (Nishizawa *et al.*, 2000; Tillett *et al.*, 2000). The rest of the six amino acids are incorporated in the microcystin molecule by the NRPS modules of McyG and McyA–C, McyC catalyzing the macrocyclization (Nishizawa *et al.*, 1999, 2000; Tillett *et al.*, 2000). The genes in the *mcy* gene cluster, which encode various tailoring enzymes, have functions that include the addition of functional groups (McyJ, McyL), and modification of precursor molecules to produce non-proteinogenic amino acids, *e.g.*, D-MeAsp (McyI) (Tillett *et al.*, 2000; Christiansen *et al.*, 2003; Pearson *et al.*, 2007; Fewer *et al.*, 2013). The racemase enzyme McyF has been proposed to have two possible functions: originally predicted to be a glutamate racemase (Tillett *et al.*, 2000; Nishizawa *et al.*, 2001), later phylogenetic and substrate specificity characterization has pointed to a role of an aspartate racemase (Sielaff *et al.*, 2003). McyT, only found in the *Planktothrix mcy* gene cluster, is thought to be a proofreading enzyme capable of catalyzing the removal of misprimed precursor amino acids from the Mcy enzymes (Christiansen *et al.*, 2003). The McyH, a probable ATP-binding cassette or ABC transporter (Pearson *et al.*, 2004), may be responsible for the observed localization of the toxin to thylakoids (Shi *et al.*, 1995; Young *et al.*, 2005), or for its possible transport out of the cells. However, since the function of the *mcyH* gene has been assigned through functional

bioinformatic analysis (Pearson *et al.*, 2004), direct evidence of microcystin export is yet to be obtained. The inactivation of *mcyH* abolishes toxin production, despite an ongoing expression of the other *mcy* genes, therefore it has been hypothesized that it may also function as a scaffolding protein for the Mcy multienzyme complex (Pearson *et al.*, 2004). The most recently discovered microcystin biosynthesis gene is the O-acetyltransferase *mcyL* (Fewer *et al.*, 2013). Although currently identified only from the *Nostoc* genome, O-acetylated microcystins have been reported to be produced a *Planktothrix agardhii* strain (Laub *et al.*, 2002), so the gene might in fact be more widely present.

Table 1. The microcystin biosynthesis genes.

Gene	Type	Function	References
mcyA	NRPS, two modules	Addition of Mdha and D-Ala.	Nishizawa *et al.*, 1999; Tillett *et al.*, 2000
mcyB	NRPS, two modules	Addition of a varied L-amino acid and D-MeAsp.	Nishizawa *et al.*, 1999; Tillett *et al.*, 2000
mcyC	NRPS	Addition of a varied L-amino acid and cyclization of the peptide chain.	Nishizawa *et al.*, 1999; Tillett *et al.*, 2000
mcyD	PKS, two modules	Extension of Adda carbon chain.	Nishizawa *et al.*, 2000; Tillett *et al.*, 2000
mcyE	Hybrid PKS/NRPS	Finishing the Adda carbon chain, addition of D-Glu, formation of the precursor of Mdha addition.	Nishizawa *et al.*, 2000; Tillett *et al.*, 2000
mcyF	racemase	Conversion of L-Asp to D-Asp. Not found in the *Planktothrix mcy* gene cluster.	Nishizawa *et al.*, 2001; Christiansen *et al.*, 2003; Sielaff *et al.*, 2003
mcyG	Hybrid PKS/NRPS	Inititation of Adda biosynthesis.	Nishizawa *et al.*, 2000
mcyH	ABC-transporter	Stabilization and/or transport of microcystins across membranes.	Tillett *et al.*, 2000; Pearson *et al.*, 2004
mcyI	2-hydroxyacid dehydrogenase	Biosynthesis of D-MeAsp. Not found in the *Planktothrix mcy* gene cluster.	Christiansen *et al.*, 2003; Pearson *et al.*, 2007
mcyJ	O-methyltransferase	Methylation of Adda. Not found in the *Nostoc mcy* gene cluster.	Tillett *et al.*, 2000; Christiansen *et al.*, 2003; Fewer *et al.*, 2013
mcyL	O-acetyltransferase	O-acetylation of Adda. Present only in the *Nostoc mcy* gene cluster.	Fewer *et al.*, 2013
mcyT	thioesterase	Putatively involved in proofreading during MC biosynthesis. Present only in the *Planktothrix mcy* gene cluster.	Christiansen *et al.*, 2003; Christiansen *et al.*, 2008a

Some steps of microcystin biosynthesis, such as the final stage of D-MeAsp production (Pearson *et al.*, 2007), are not covered by the currently characterized genes, and remain unexplained (Neilan *et al.*, 2008). *Planktothrix* spp. are able to produce microcystin variants that would require the activity of both McyF and McyI, despite the apparent lack of the corresponding biosynthetic genes (Christiansen *et al.*, 2003). The roles of

the required enzymes could be fulfilled by other, similar enzymes, which also function as parts of other biosynthetic pathways, and it was also suggested that the genes might be present elsewhere in the genome (Christiansen *et al.*, 2003). Several *P. agardhii* genomes have since been sequenced (Tooming-Klunderud *et al.*, 2013, Christiansen *et al.*, 2014); despite this the *mcyF* and *mcyI* genes remain unidentified in *Planktothrix*.

Numerous microcystin variants have been characterized in cyanobacterial cultures and environmental samples, and the toxin profile of a single microcystin-producing cyanobacterial strain can consist of several microcystins in varying proportions (e.g., Sivonen, 1992; Luukkainen *et al.*, 1993). As the *mcy* genes form a single-copy cluster (Kaneko *et al.*, 2007; Frangeul *et al.*, 2008), it arouses a question as to how these variants are produced with a fixed number of biosynthetic genes. Since multiple microcystin variants can be produced simultaneously, some degree of relaxation regarding the amino acid to be incorporated is likely to exist (Dittmann *et al.*, 1997; Nishizawa *et al.*, 1999; Nishizawa *et al.*, 2000; Tillett *et al.*, 2000; Kurmayer *et al.*, 2002; Mikalsen *et al.*, 2003). However, substrate specificities can be predicted on the basis of the microcystin synthetase amino acid sequences, and they have been found to agree with the observed toxin profiles (Mikalsen *et al.*, 2003). Relatively few, five at the very most, single nucleotide substitutions at critical locations within *mcyC* can result in changed substrate specificity (Christiansen *et al.*, 2008b). The substrate specificity of a NRPS is mainly determined by the adenylation domain sequence, more specifically the amino acids lining the binding pocket (Stachelhaus *et al.*, 1999; Lautru and Challis, 2004). This code, like the ribosomal code, is degenerate (Stachelhaus *et al.*, 1999; Lautru and Challis, 2004). In other words, multiple sequences exist to direct the incorporation of a single amino acid. The two L-amino acids, which are incorporated by the adenylation domains of the NRPS modules of McyB and McyC (Nishizawa *et al.*, 1999; Tillett *et al.*, 2000), are the source of a large part of the structural variety of microcystins (Namikoshi *et al.*, 1998). The gene regions in the corresponding genes encoding these domains are areas where recurrent recombination events involving both smaller sequence segments and the exchange of complete domains occur (Fewer *et al.*, 2007; Christiansen *et al.*, 2008b; Tooming-Klunderud *et al.*, 2008b). These events can involve equivalent modules, *i.e.*, the domains are present in the same position in similar clusters (Tanabe *et al.*, 2004; Tooming-Klunderud *et al.*, 2008b), such as the *mcy* gene clusters of different strains of *Planktothrix* (Kurmayer and Gumpenberger, 2006), or they may originate in different gene clusters (Mikalsen *et al.*, 2003; Kurmayer *et al.*, 2005; Fewer *et al.*, 2007). Recombination between adenylation domains seems to give rise to some of the genetic variation in the *mcy* gene clusters, resulting in the biosynthesis of a host of microcystin congeners (Tooming-Klunderud *et al.*, 2008a). For example, in the majority of cases McyC directs the incorporation of arginine into the microcystin molecule. Consequently, the replacement of the *mcyB* first adenylation domain with a *mcyC*-like adenylation domain leads to the production of microcystin-RR (Mikalsen *et al.*, 2003). However, the adenylation domain sequences do not fully explain the diversity of L-amino acid combinations in microcystins, since microcystin-producing cyanobacteria often produce several variants simultaneously (Sivonen and

Jones, 1999), and two different L-amino acids can be incorporated despite the identical sequences of McyC and first McyB adenylation domains (Fewer *et al.*, 2007).

The genetic characteristics of the microcystin-producing cyanobacterium also affect the methylation status of the toxin produced (Tillett et al., 2000; Christiansen et al., 2003; Pearson et al., 2007). The absence of the methyl group of either Mdha or D-MeAsp, resulting in Dha or D-Asp, respectively, gives rise to the commonly encountered desmethylated microcystins. The absence of both methyl groups results in the didesmethyl variants. The *Planktothrix mcy* gene cluser does not include the gene *mcyI,* which is thought to result in the production of D-Asp varieties of microcystin (Pearson *et al.*, 2007). Point mutations and deletions of the N-methyl transferase (NMT) domain in the *mcyA* gene have been shown to lead to the production of desmethylated microcystin variants; cyanobacteria carrying these mutations incorporate either dehydrobutyrine (Dhb) or Dha instead of Mdha (Kurmayer *et al.*, 2005; Christiansen *et al.*, 2008b; Fewer *et al.*, 2008; Tooming-Klunderud *et al.*, 2008b).

Evolution of the mcy *genes*

The microcystin biosynthesis gene cluster is distributed sporadically among cyanobacteria at all taxonomic levels, and repeated gene loss and horizontal gene transfer (HGT) have been suggested to be the main mechanisms behind the observed distribution (Neilan *et al*., 1999; Tillett *et al*., 2001; Mikalsen *et al*., 203; Rantala *et al*., 2004). Horizontal gene transfer is known to be a major force shaping the cyanobacterial genomes (Palenik *et al.*, 2003). Transposases associated with the *mcy* gene cluster have been identified (Christiansen *et al.*, 2006), and recombination events are thought to maintain the majority of genetic diversity of the *mcy* genes (Tanabe *et al.*, 2004; Tanabe *et al.*, 2009). However, the evidence on HGT events involving the microcystin gene cluster also indicates that it only occurs between closely related strains and not between cyanobacterial genera (Tooming-Klunderud *et al.*, 2008a; Tanabe *et al.*, 2009). Several studies have found that the genomic location of the *mcy* gene cluster is conserved; the two genes flanking the region are always the same (Tillett *et al.*, 2001; Mikalsen *et al.*, 2003; Nishizawa *et al.*, 2007). Thus, frequent HGT events involving the entire *mcy* gene cluster are not likely to have made a major contribution to the current distribution of the genetic capability of microcystin biosynthesis within cyanobacteria.

Phylogenetic analysis of the *mcy* and the related *nda*, or nodularin synthetase, genes has indicated that the capability of microcystin biosynthesis has been present in the distant ancestors of extant cyanobacteria (Moffitt and Neilan, 2004; Rantala *et al.*, 2004; Jungblut and Neilan, 2006). Nodularin, which is structurally and functionally highly similar to microcystin, is thought to have evolved from the latter (Moffitt and Neilan, 2004). The nodularin synthetase cluster, present in some *Nodularia* species, shares an evolutionary history with the *mcy* gene cluster, and has most likely diverged from the ancestral gene cluster at an early stage during its evolution (Moffitt and Neilan, 2004). Changes in substrate specificity, and a major deletion of two NRPS modules, the last of *mcyA* and the first of *mcyB,* resulting in the formation of the fusion gene *ndaA*, have

conferred *Nodularia* spp. the biosynthetic capability to produce the pentapeptide toxin (Moffitt and Neilan, 2004).

The *mcy* genes have been shown to have co-evolved with highly conserved housekeeping genes, indicating that recurrent deletions have given rise to the current distribution pattern (Rantala *et al.*, 2004). Indeed, the inactivation and loss of *mcy* genes seems to be an ongoing process, demonstrated by the description of a number of *mcy* mutants from *Microcystis*, *Planktothrix,* and *Anabaena*, some of which were found already during the initial characterization of the *mcy* gene cluster (Nishizawa *et al.*, 1999; Kaebernick *et al.*, 2001; Mikalsen *et al.*, 2003; Kurmayer *et al.*, 2004; Christiansen *et al.*, 2008a; Christiansen *et al.*, 2008b; Fewer *et al.*, 2011). Point mutations, deletions, or sequence insertions may disrupt a coding sequence and give rise to inactive *mcy* genotypes (Christiansen *et al.*, 2006; Noguchi *et al.*, 2009; Ostermaier and Kurmayer, 2009), which commonly coexist with active microcystin producers in the same environment. Their relative proportions seem to stay stable despite changing environmental conditions (Kurmayer *et al.*, 2002; Kurmayer *et al.*, 2004; Ostermaier and Kurmayer, 2009). Cyanobacteria can spend a considerable amount of energy to produce microcystins, but whether the microcystin biosynthesis offers any benefits is still under debate. Knockout mutants show no immediately obvious phenotypic effects compared to wild type controls (Dittmann *et al.*, 1997; Tanabe *et al.*, 2004), and purifying selection seems to be the only major evolutionary force, if any, that the microcystin gene cluster is subject to (Kurmayer and Gumpenberger, 2006; Tooming-Klunderud *et al.*, 2008a; Tanabe *et al.*, 2009). Several hypotheses of the biological role of microcystins have been suggested, such as possible roles in signaling (Schatz *et al.*, 2007); defense (Rohrlack *et al.*, 2001; Rohrlack *et al.*, 2013); and physiological functions (Neilan *et al.*, 2013), including stress tolerance (Meissner *et al.*, 2015). It appears that no single factor explains microcystin production, and to date, no generally accepted view of the biological incentive for microcystin production exists.

2.2.4 Paralytic shellfish toxins

Paralytic shellfish toxins are a group of alkaloid secondary metabolites produced by some of the members of two unrelated groups of organisms, namely cyanobacteria and dinoflagellates (see section 2.2). Saxitoxin is the parent PST compound, and it was named after the butter clam *Saxidomus giganteus*, from which it was first purified (Schantz *et al.*, 1957). Later the origin of the toxin was traced back to the dinoflagellate *Alexandrium catenella* (Schantz *et al*., 1966). PSTs are alkaloids, heterocyclic nitrogenous compounds, the basic structure of which is based on a 3,4,6-trialkyl tetrahydropurine skeleton (Figure 2), and a total of 57 variants have been identified, including saxitoxin (STX), neosaxitoxin (NeoSTX), gonyautoxins (GTX), C-toxins, and their decarbamoyl variants, among others (Wiese *et al.*, 2010, and references therein).

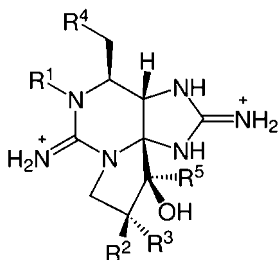

Figure 2. The general structure of paralytic shellfish toxins. The R1–R5 moieties denote various functional groups, including carbamate, sulfate, hydroxyl, hydroxybenzoate, and acetate. In saxitoxin they are H (R1–R3), $CONH_2$ (R4), and OH (R5).

While the majority of PST variants, including STX, NeoSTX, and the GTXs are produced by both cyanobacteria and dinoflagellates, some PST variants have been isolated from freshwater cyanobacteria only, such as the *Lyngbya wollei* toxins (LWT) (Onodera *et al.*, 1997; Lagos *et al.*, 1999). A number of substituent chemical groups give rise to this observed variety in PST structures, including carbamate, sulfate, hydroxyl, hydroxybenzoate, and acetate moieties, which also affect the toxicity of the PST molecule (Llewellyn, 2006). Saxitoxin has a LD_{50} value of 10 µg kg^{-1} upon intraperitoneal administration (Kuiper-Goodman *et al.*, 1999). Generally, the addition of one or more sulfate groups decreases the toxicity; the non-sulfated STX and NeoSTX are the most potent of the PSTs, followed by the gonyautoxins and C-toxins, which contain one or two sulfate groups, respectively (Llewellyn, 2006). At physiological pH, The 1,2,3- and 7,8,9-guanidinium groups are positively charged and partially deprotonated, respectively (Shimizu *et al.*, 1981; Strichartz, 1984). This results in the high solubility of PSTs in water, and insolubility in organic solvents (Schantz *et al.*, 1957). However, some variants, such as the LWTs and GC-toxins (the latter isolated from marine dinoflagellates, namely *G. catenatum*) include hydrophobic side chains (Negri *et al.*, 2003; Negri *et al.*, 2007; Vale, 2008). Due to their small size, hydrophilic characteristics, and the small structural differences between the most common PST variants, the development of analytical methods for the separation and quantification of the toxins has not been without difficulties (McNabb, 2014). Since the current analytical methods are designed to detect highly hydrophilic molecules, the discovery of hydrophobic PST variants with significantly different chemical properties poses further challenges to PST toxin analysis and monitoring (Negri *et al.*, 2007).

Biotransformation of PSTs can take place in some invertebrates feeding on the toxin-producing microalgae, as well as higher up in the food chain in some mammals, including humans. In some cases, this results in variants beyond the biosynthetic capabilities of the original toxin producers (Arakawa *et al.*, 1995; Zaman *et al.*, 1998; Dell'Aversano *et al.*, 2008). The conversion of less potent PST variants into more toxic ones has been observed in the scallop *Placopecten magellanicus*, where enzymatic removal of sulfate groups results in the transformation of GTXs to the more potent STX (Shimizu and Yoshioka, 1981). Biotransformation resulting in the conversion of

carbamoylated PSTs to the less potent decarbamoyl derivatives has also been described to take place in the tissues of the littleneck clam *Prothotheca staminea* (Sullivan *et al.*, 1983). Some of the observed transformations of PSTs may be due to marine bacteria associated with various invertebrates (Smith *et al.*, 2001). In mammals, the exposure of the toxins to gastric juices may be behind the observed transformations; the conversion of GTX5 to STX has been observed to occur in rats, although to a limited degree (Harada *et al.*, 1984). Oxidation of STX, GTX2, and GTX3 into neoSTX, GTX1, and GTX4 has also been reported in human victims of paralytic shellfish poisoning (García *et al.*, 2004).

When ingested, the toxins are readily absorbed in the intestine (Gessner *et al.*, 1997). PST poisoning presents with a range of symptoms, starting with a mild tingling and the numbness of extremities, face and lips, and progressing to a total neuromuscular paralysis, and in severe cases the poisoning can result in respiratory arrest and death if the person affected is not treated (Llewellyn, 2006). Since there is no known antidote, the treatment of PST poisoning is limited to supportive care, *i.e.*, fluid therapy and respiratory aid (Llewellyn, 2006). A full recovery can be expected if the victim survives beyond the first 12 hours after the onset of symptoms (Llewellyn, 2006).

Paralytic shellfish toxins act by selectively and reversibly blocking voltage-gated sodium channels, preventing the transduction of neuronal signals (Llewellyn, 2006). The 7,8,9-guanidinium moiety is responsible for the binding of STX to the sodium channel in a nearly 1:1 ratio (Kao and Walker, 1982; Hartshorne and Catterall, 1984; Lipkind and Fozzard, 1994). The toxin effectively blocks cation influx by lodging itself in the ion conducting pore of the channel. The hydroxyl group on carbon 12 may also participate in the binding (Strichartz, 1984), and the absence of the carbamoyl side chain reduces the affinity of the toxin, as does further structural variation within this side group. In addition to Na-channels, PSTs also affect potassium channels, but the mechanism by which STX binds to potassium channels is different compared to its binding to sodium channels; STX does not block the channel, but instead four or more molecules bind to extracellular sites, modifying the gating of the channel and leading to decreased K^+ ion influx (Kao, 1986; Wang *et al.*, 2003). The binding of STX to calcium channels, the molecule's third target, proceeds *via* a mechanism of action which seems to resemble that of sodium channels, resulting in a partial block on Ca^{2+} ion flow (Su *et al.*, 2004). After the reversal of the binding, the toxin undergoes structural transformation, and is cleared rapidly through purine catabolism and excreted in urine (Gessner *et al.*, 1997; Pomati *et al.*, 2001; García *et al.*, 2009).

2.2.5 *sxt* genes and PST biosynthesis

Paralytic shellfish toxin biosynthesis has been confirmed to take place in two domains of life: in prokaryotic cyanobacteria and in dinoflagellates, the latter of which are classified as eukaryotes, despite the very distinct features and remarkable size (3–215 Gb) of their genomes (Hackett *et al.*, 2004). The paralytic shellfish toxins, however, appear to be produced via the same biosynthetic pathway in both groups of organisms

(Shimizu, 1993). The cyanobacterial *sxt* gene cluster was first characterized in *Cylindrospermopsis raciborskii*, in which it spans 35 kb and includes 31 ORFs (Kellmann *et al.*, 2008a). In addition to *C. raciborskii*, an *sxt* gene cluster has been identified in *Anabaena circinalis*, *Aphanizomenon* sp. (Mihali *et al.*, 2009), *Raphidiopsis brookii* (Stucken *et al.*, 2010), and *Lyngbya wollei* (Mihali *et al.*, 2011). The gene order, composition, and size of the *sxt* gene cluster vary from genus to genus (Mihali *et al.*, 2009). The *R. brookii* genome contains the smallest *sxt* gene cluster identified to date, composed of 24 ORFs spanning only 25.7 kb (Stucken *et al.*, 2010). The size and structure of the dinoflagellate genome poses unique challenges for gene identification, and thus the counterparts to the cyanobacterial *sxt* genes eluded detection for a long time, despite extensive research efforts. The definitive identity of the PST producer was also debated; symbiotic bacteria instead of the dinoflagellate host were suggested as the source of the toxins in several studies (e.g., Kodama *et al.*, 1988; Gallacher and Smith, 1999). Sequence data were first obtained for the *sxtA* gene by screening expressed sequence tag (EST) libraries constructed from *Alexandrium fundyense*, *A. minutum* and *A. tamarense* (Stüken *et al.*, 2011; Hackett *et al.*, 2013). Later, the *sxtG* gene has been sequenced (Orr *et al.*, 2013), but a detailed characterization of other dinoflagellate *sxt* genes is yet to be achieved. Most of the functions of the cyanobacterial *sxt* genes have been predicted by functional bioinformatic analysis, and all characterized *sxt* gene clusters also carry genes with currently unknown functions (D'Agostino *et al.* 2014). The further characterization of these genes is hampered by the fact that methods required for knockout studies of the filamentous cyanobacteria capable of PST production are yet to be developed.

The current understanding is that the biosynthesis of saxitoxin, the parent PST compound, requires a minimum of 13 genes (D'Agostino *et al.*, 2014) (Table 2). This group of genes overlaps with another set of 14 genes conserved in all cyanobacterial *sxt* gene clusters (Murray *et al.*, 2011a). Additionally, a number of tailoring genes, as well as putative exporters and regulatory genes can be included in the cluster (D'Agostino *et al.*, 2014).

Table 2. The current understanding of the functions of PST biosynthesis genes in five genera of cyanobacteria, according to D'Agostino *et al.* (2014) and references therein. Genes essential for STX biosynthesis are indicated in boldtype. Disrupted or truncated genes are excluded.

Gene	Type	Function	No. of *sxt* clusters including the gene
sxtA	PKS	Initiates PST biosynthesis, involving loading of ACP, methylation, Claisen condensation	5
sxtB	Cytidine deaminase	Formation of the first heterocycle through retroaldol condenstation	5
sxtC	Amidino hydrolase	Decarbamoylation	5
sxtD	Sterole desaturase	Double bond formation between C1 and C5	5
sxtE	Unknown	Unknown	5
sxtF	MATE protein	PST export	2
sxtG	Amidinotransferase	Transfer of an amidino group to PST precursor	5
sxtH	Phenylpropionate dehydrogenase	Hydroxylation of the C12 carbon	5
sxtI	O-carbamoyl-transferase	Transfer of a carbamoyl group to the hydroxymethyl side chain of STX	4
sxtJ	Unknown	Unknown	4
sxtK	Unknown	Unknown	4
sxtL	GDSL lipase	Decarbamoylation	4
sxtM	MATE protein	PST export	4*
sxtN	N-sulfo-transferase	Biosynthesis of GTX5–6	4**
sxtO	Adenylylsulfate kinase	3'-Phosphoadenosine-5'-phosphosulfate (PAPS) biosynthesis	3
sxtP	Unknown	Unknown	5
sxtQ	Unknown	Unknown	5
sxtR	Transferase	Acetyl coenzyme A N-acyltransferase	5
sxtS	Phytanoyl dioxygenase	Formation of the second and third heterocycles	5
sxtT	Phenylpropionate dehydrogenase	Hydroxylation of the C12 carbon	5
sxtU	Alcohol dehydrogenase	Reduction of the terminal aldehyde group of the PST precursor	5
sxtV	Reductase/ dehydrogenase	Extraction of an electron pair from succinate to form fumarate	3
sxtW	Ferredoxin	Electron carrier	3
sxtY	Phosphate uptake regulator	Putative regulatory function	1
sxtZ	Histidine kinase	Putative regulatory function	1
sxtX	Cephalosporin hydroxylase	N-1-hydroxylation	3
sxtACT	Acetylase	Acetylation of C13	1
sxtDIOX	Phenylpropionate dioxygenase	Hydroxylation of C11	2
sxtPER	Permease	PST export	3
sxtSUL	O-sulfotransferase	Biosynthesis of GTX1–4	3

**sxtM1–3* in *L. wollei*, ***sxtN1–2* in *L. wollei*, ACP, acyl carrier protein; MATE, multidrug and toxic compound extrusion protein; GDSL, consensus amino acid sequence of Gly, Asp, Ser, and Leu around the active site Ser in a lipase enzyme.

The PST molecule is built out of arginine, acetate, and methionine via a complex sequence of enzymatic reactions (Shimizu, 1993). Many of these reactions, including a Claisen condensation of an amino acid to a carboxylic acid, amidino transfer, unconventional heterocyclization, and O-carbamoylation are rare in microalgal metabolite biosynthesis (Shimizu *et al.*, 1984; Shimizu, 1993). In both cyanobacteria and dinoflagellates, PST biosynthesis is initiated by the four-domain SxtA (Kellmann *et al.*, 2008a; Stüken *et al.*, 2011). The synthesis is continued by the formation of the second guanidine group by SxtG, and the first heterocycle by SxtB, followed by double-bond formation by SxtD (Kellmann *et al.*, 2008a, Mihali *et al.*, 2009). The two remaining heterocycles are formed through bi-cyclization via epoxidation and aldehyde formation by SxtS (Kellmann *et al.*, 2008a, Mihali *et al.*, 2009). Together, SxtU, SxtH, and SxtT perform reduction and hydroxylation to form dcSTX (Kellmann *et al.*, 2008a, Mihali *et al.*, 2009). The regeneration of the dioxygenases SxtH and SxtT requires oxidation after each catalytic cycle, possibly performed by SxtV and SxtW, which might form the necessary electron transport chain (Kellmann *et al.*, 2008a). In *Aphanizomenon* sp., and *A. circinalis* the corresponding genes *sxtV* and *sxtW* are missing or fragmented, possibly indicating other sources for the required oxygenase reductase (Mihali *et al.*, 2009). Finally, the product is carbamoylated by SxtI, possibly together with SxtJ and SxtK, to form STX (Kellmann *et al.*, 2008a, Mihali *et al.*, 2009). The *sxtJ* and *sxtK* genes are located adjacent to each other in the *sxt* cluster, similarly to their homologs in other organisms (Kellmann *et al.*, 2008a). These homologs have not, however, been structurally characterized, and their exact functions remain unknown.

The cyanobacterial PST profiles are governed by the composition of the *sxt* gene clusters; changes in light, nutrient, or temperature regimens have not had an effect on the suite of toxins produced (Soto-Liebe *et al.*, 2010). Decarbamoylated PSTs are produced, if the molecule does not undergo the reaction catalyzed by SxtI, demonstrated by the presence of a truncated *sxtI* gene in *L. wollei*, which produces exclusively decarbamoylated toxin variants (Onodera *et al.*, 1997; Kellmann *et al.*, 2008b; Mihali *et al.*, 2011). Another possibility is that either SxtL or SxtC removes the already incorporated carbamoyl group (D'Agostino *et al.*, 2014). The gene *sxtL* is also missing from the *L. wollei sxt* cluster, lending support to this hypothesis (Mihali *et al.*, 2011). *L. wollei* possesses a unique suite of PSTs (Carmichael *et al.*, 1997; Lajeunesse *et al.*, 2012), and its *sxt* gene cluster includes genes not found in other cyanobacteria. A synthesis pathway for the LWTs, involving the genes *sxtSUL*, *sxtACT*, and *sxtDIOX* has been proposed by Mihali *et al.* (2011).

The biosynthesis of N-1-hydroxylated PST variants, including NeoSTX, putatively requires the action of SxtX. The corresponding gene is not included in the *A. circinalis* and *R. brookii sxt* clusters, rendering them unable to produce N-1-hydroxylated PSTs such as NeoSTX (Kellmann *et al.*, 2008a). GTXs are produced via either O-sulfation or N-sulfation, catalyzed by two separate 3'-phosphate-5'-phosphosulfate-dependent sulfotransferases, characterized in the dinoflagellate *G. catenatum* (Sako *et al.*, 2001; Yoshida *et al.*, 2002). *Aphanizomenon* sp. strain NH-5 has not been shown to produce sulfated PST variants (Mahmood and Carmichael, 1986). It also lacks the gene *sxtO*,

the product of which is a homolog of adenylylsulfate kinases, necessary for the function of sulfotranferases, indicating that it might be required for the production of sulfated PSTs (Mihali *et al.*, 2009). SxtN and SxtSUL are likely to be responsible for the N- and O-sulfation of PSTs, respectively (Soto-Liebe *et al.*, 2010; Stucken *et al.*, 2010; Mihali *et al.*, 2011). In *A. circinalis*, *sxtSUL* is not located within the gene cluster, but elsewhere in the genome, as demonstrated by the generation of a specific amplification product (Soto-Liebe *et al.*, 2010).

PSTs may be actively exported by the cyanobacterial cells in response to environmental stimuli (Castro *et al.*, 2004; Soto-Liebe *et al.*, 2012). A multidrug and toxic compound extrusion (MATE) protein is encoded by the gene *sxtM*, included in all cyanobacterial *sxt* clusters, including the three copies found in *L. wollei* (Kellmann *et al.*, 2008a; Mihali *et al.*, 2009; Stucken *et al.*, 2010; Mihali *et al.*, 2011). Other possible exporter genes are the MATE-type *sxtF*, present in *C. raciborskii* and *D. brookii*, and *sxtPER* which may have replaced *sxtF* in *A. circinalis*, *Aphanizomenon* sp. and *L. wollei* (Mihali *et al.*, 2009).

Evolution of sxt *genes in cyanobacteria and dinoflagellates*

There is considerable variation in the organization of the cyanobacterial *sxt* gene clusters. The sporadic distribution of the genes within each identified cluster points to the conclusion that the forms in which it exists today arose via several independent HGT events in an ancestor of extant cyanobacteria (Kellmann *et al.*, 2008b; Mihali *et al.*, 2009; Moustafa *et al.*, 2009). The phylogenetic relationships of the *sxt* genes are similar to those of the PST-producing genera, supporting the hypothesis that the capability to produce PSTs arose early in the evolution of ancestral cyanobacteria (Murray *et al.*, 2011a). The loss of *sxt* genes through excision events has contributed to the appearance of non-toxic strains that are closely related to those capable of PST biosynthesis (Mihali *et al.*, 2009; D'Agostino *et al.*, 2014). Rearrangements, recombination, and positive selection are also thought to have had a major impact on the formation of the current arrangement of *sxt* clusters (Murray *et al.*, 2011a). The *sxt* clusters seem to be composed of genes of cyanobacterial origin, some common to both toxic and non-toxic species, some exclusive to PST-producing cyanobacteria, as well as genes transferred from other bacterial sources, including proteobacteria (Moustafa *et al.*, 2009). The latter group includes many of the key genes involved in PST biosynthesis, such as *sxtA*, *sxtG*, and *sxtB*. Interestingly, the gene *sxtA* seems to be chimeric, appearing to be composed of two parts with different origins (Moustafa *et al.*, 2009).

The bioinformatic analysis of the recently discovered dinoflagellate *sxt* genes suggests that the genera *Alexandrium* and *Pyrodinium* could have acquired the genes from a prokaryotic source via HGT (Stüken *et al.*, 2011; Orr *et al.*, 2012; Orr *et al.*, 2013). Although at first the presence of *sxt* genes in the more distantly related PST-producing species *G. catenatum* was suggested to be the result of a similar event between dinoflagellates (Stüken *et al.*, 2011), a more recent analysis of *sxtA4* sequences points to the conclusion that the gene was present in a common ancestor of all PST-producing

dinoflagellates (Murray *et al.*, 2015). The cyanobacterial and dinoflagellate *sxtA*, *sxtG*, and *sxtB* genes are similar, but have likely been independently acquired from a common source, since a phylogenetic analysis has not supported direct transfer between these two groups of organisms (Hackett *et al.*, 2013). Like in cyanobacteria, the distribution of PST biosynthesis within genera is sporadic, and probably results from multiple independent losses of the biosynthetic genes (Orr *et al.*, 2011; Orr *et al.*, 2013). The *sxt* genes have been extensively adapted to fit the dinoflagellate genomes: transcripts contain typical dinoflagellate spliced leader sequences and poly-A tails, and the GC-content is similar to other dinoflagellate genes, *i.e.*, it is much higher than in cyanobacteria, and introns are also present (Stüken *et al.*, 2011; Orr *et al.*, 2013). There is no indication that dinoflagellate *sxt* genes are arranged in a single genomic location, like their prokaryotic counterparts. It may even be that genes encoding the domains of multidomain toxin biosynthetic enzymes, such as SxtA, are present at different genomic locations; the failure to amplify the complete *sxtA* suggests that the four domains may not be located directly adjacent to each other, the other possibility being the presence of long intron sequences (Stüken *et al.*, 2011). The dinoflagellate *sxtA1* and *sxtA4* genes have undergone a number of gene duplications during their evolution, resulting in multiple genomic copies of both genes, and the formation of pseudogenes, and in the case of *sxtA1*, also paralogs (Murray *et al.*, 2015). Species of *Alexandrium* have also been shown to actively transcribe the different *sxtA4* copies (Stüken *et al.*, 2011; Wiese *et al.*, 2014). It has been postulated that the presence of a number of slightly different copies of the same gene may confer some kind of an adaptive plasticity (Wiese *et al.*, 2014).

2.3 Nucleic acid-based detection and quantification of toxic microalgae

As the awareness of the risks presented by HABs increases, so does the need for efficient monitoring methods. Microscopy is a classical, gold standard method, and still the recommended primary means of identification and enumeration of harmful species. It can, however, be very labor intensive, and furthermore, it has been shown that toxic and non-toxic species and strains coexist in natural populations, and are often very difficult, if not impossible to distinguish using morphological criteria (Dittmann *et al.*, 2013). Thus, microscopic analysis does not yield enough information to make conclusions on the probability of toxin production in a given cyanobacterial or dinoflagellate population. A harmful microalgal species may also be present only as a minor component of the community, which will increase the time and effort necessary for its reliable identification and enumeration.

Unequivocal evidence of the toxins produced during an HAB event can be obtained by using chemical and biochemical identification and quantification of the suspected toxins. These methods include chromatography, immunoassays, and measuring the specific binding between toxins and certain non-antibody target molecules, such as the protein phosphatases and microcystin (Rivasseau *et al.*, 1999), and the saxiphilin–saxitoxin interaction (Llewellyn and Doyle, 2001). The analysis of microcystins is

mostly carried out using chromatographic methods, enzyme-linked immunosorbent assay, (ELISA), and protein phosphatase inhibition assay (McElhiney and Lawton, 2005). The chromatographic methods for microcystin separation and quantification are reliable, but require expensive instrumentation and specialized skills from the operator. Immunoassays, such as ELISA, allow for specific detection of the toxins. However, the antibodies raised against microcystins do not bind to all variants with the same affinity (Metcalf and Codd, 2003), and erroneous estimation of the concentrations of *e.g.*, hydrophobic microcystins has been reported (Rapala *et al.*, 2002). Commercial ELISA assays have also been shown to be affected by the sample matrix (Metcalf *et al.*, 2000).

The highly polar nature and small size of the most commonly encountered PST variants set high requirements for the chromatographic methods used to detect the toxins. Structural differences between different PST variants are significant from the biological point of view, but a separation of the different toxins can be challenging to achieve and may require several separate runs, as in the method described by Oshima (1995). The availability of certified standard materials may also be an issue, since to obtain quantitative results, each PST variant needs a reference for calibration (McNabb, 2014). Out of the 57 known variants, 13 are available as standards from the Canadian Institute for Marine Biosciences. Immunoassays targeted at PSTs have been developed, but their ability to detect different toxin variants greatly differs, and they are generally recommended to be used mainly for sample screening (Humpage *et al.*, 2010). Mouse bioassay has been, and continues to be used in PST analysis, but there are significant drawbacks associated with its use; namely performance issues (barely adequate sensitivity, sample matrix interference, poor recoveries), as well as ethical concerns regarding testing toxin samples on live animals (Hungerford, 2014).

The use of nucleic acids in the detection and quantification of harmful cyanobacteria and dinoflagellates has many potential benefits (Anderson, 1995). Sample preparation and reaction setup is relatively simple once the assay has been developed, and the sensitivity allows an early detection of developing blooms; the theoretical limit of detection of a qPCR assay is three copies of the target gene per reaction (Wittwer *et al.*, 1997). Nucleic acid-based methods are characterized by their specificity, but also their adaptability for both low- and high-throughput, as well as automated applications. The polymerase chain reaction (PCR), quantitative PCR (qPCR), reverse-transcription PCR (RT-PCR), and quantitative reverse-transcription PCR (qRT-PCR) have all been successfully used in the detection of toxic cyanobacteria and dinoflagellates (see sections 2.4.1–2.4.2). Different multiplex-PCR applications and microarrays enable the simultaneous detection of a number of potentially toxic genera or species (e.g., Sipari *et al.*, 2010; Al-Tebrineh *et al*, 2012). Species-specific identification can be carried out on the basis of the sequences of conserved housekeeping genes, such as those encoding the ribosomal RNA (*e.g.*, Nagai, 2011). These genes have also been used, with varying success, as targets in attempts to develop detection and quantification methods for toxin-producing species and strains (Galluzzi *et al.*, 2004; Hosoi-Tanabe and Sako, 2005; Dyhrman *et al.*, 2006; Touzet *et al.*, 2009; Dyhrman *et al.*, 2010). The discovery of toxin biosynthetic genes has made it possible to develop toxin-specific gene-based assays,

both for cyanobacteria and dinoflagellates. These methods are reviewed in the following sections. With each new assay it has been necessary to establish whether the targeted genetic element truly reflects the amount of toxin produced, and whether it can then be used as a predictive monitoring tool.

2.3.1 Microcystin-producing cyanobacteria

The first nucleic acid-based methods aimed to differentiate microcystin-producing and non-microcystin-producing cyanobacteria on the basis of genetic factors were made using rRNA-encoding genes and the phycocyanin intergenic spacer region (PC-IGS) as targets. The rRNA genes and PC-IGS sequences allow for the specific identification of cyanobacterial genera and species and the investigation of evolutionary relationships, but genotypes based on 16S rRNA, or PC-IGS were often found not to correlate with microcystin production (Neilan *et al.*, 1995; Neilan *et al.*, 1997; Neilan *et al.*, 1997; Otsuka *et al.*, 1999; Lyra *et al.*, 2001; Tillett *et al.*, 2001), although contradicting findings have also been published (Janse *et al.*, 2004). However, the microcystin synthetase genes (see section 2.2.2) have later provided more toxin-specific alternatives for the detection of microcystin-producing cyanobacteria, and have largely replaced other target genes used in nucleic acid-based assays.

Qualitative detection of microcystin-producing cyanobacteria

Qualitative methods aim to establish if the presence of a certain genetic element is positively indicative of microcystin production in a cyanobacterial population. The first applications used hybridization methods to detect *mcy* genes (Neilan *et al.*, 1999; Nishizawa *et al.*, 1999; Nishizawa *et al.*, 2000). In this section, PCR-based methods combined with various methods of post-amplification analysis, *e.g.*, gel electrophoresis, restriction fragment length polymorphism profiling, denaturing gradient gel electrophoresis, and sequencing, for the determination of reaction outcome, are reviewed. Several microcystin synthetase genes, as well as regions spanning areas which include sequences from two adjacent genes and intergenic regions have been targeted by qualitative PCR methods.

Shortly after the discovery of the *mcy* genes, Tillett *et al.* (2001) designed a *Microcystis*-specific assay targeted at a 1.3 kb *mcyA* sequence. It was found to be 95–100% specific for microcystin production, depending on the selection of cyanobacterial strains tested (Tillett *et al.*, 2001; Ouahid *et al.*, 2005; Saker *et al.*, 2005). Using another set of primers for the same gene, Hisbergues *et al.* (2003) reported 100% specificity, *i.e.*, the *sxtA* gene was amplified only in microcystin-producing cyanobacteria. The results applied to all cyanobacterial genera tested, including *Anabaena* and *Planktothrix* (Hisbergues *et al.*, 2003), while Via-Ordorika *et al.* (2004) found a specificity of 95.3% in *Microcystis* colonies. Analysis of environmental samples for the presence of *mcyA* and microcystins has yielded similar results: a specificity of 94–100% (Baker *et al.*, 2002; Yoshida *et al.*, 2005; Saker *et al.*, 2007). The *mcyA* gene can, however, be amplified in cyanobacteria

not capable of microcystin production, especially in the genus *Planktothrix* (Kurmayer *et al*., 2004).

The *mcyB* gene encodes an enzyme that catalyzes the incorporation of a variable L-amino acid and D-MeAsp to the microcystin molecule, and has been frequently used as a target for PCR assays. The correlation between microcystin production and the presence of *mcyB* is generally similar to that of *mcyA*, both in cultured cyanobacteria and in the natural environment (Neilan *et al*., 1999; Pan *et al*., 2002; Bittencourt-Oliveira, 2003). A nested-PCR approach was used by Nonnemann and Zimba (2002), who reported all samples containing over 0.25 µg L^{-1} microcystins positive for *mcyB*. Kurmayer *et al*. (2003) observed that large *Microcystis* colonies contain the largest proportions of *mcyB* genotypes, and the colony size also directly correlates with microcystin production in the environment.

The *mcyE* gene has been considered a good proxy for microcystin production-specific PCR assays (*e.g.*, Vaitomaa *et al*., 2003) due to its high sequence conservation, and since it encodes an enzyme that catalyzes Adda biosynthesis (Nishizawa *et al*., 2000; Tillett *et al*., 2000). The specificity has been supported by the experimental assessment of *mcyE* co-occurrence with microcystin production (Jungblut and Neilan, 2006; Mankiewicz-Boczek *et al*., 2011), although false-positives and false-negatives can occur in environmental samples (Rantala *et al*., 2006). Mbedi *et al*. (2005) aimed to identify the most unambiguous target for microcystin-producing *Planktothrix* spp. They found that the assay specificity for *mcyE* was the same as for *mcyB*, as well as intergenic regions between the genes *mcyC* and *mcyJ*, and genes *mcyH* and *mcyA*, and concluded that the first would be ideal since it is the most conserved of the tested genetic regions.

Despite the fact that the copy number of all *mcy* genes within a single cyanobacterial cell is expected to be the same, dissimilar detection frequencies have been observed. Using primers by Kurmayer *et al*. (2002), the *mcyB* gene was detected in only 62.5% of microcystin-containing samples, compared to *mcyA* found in 95.3% of them (Via-Ordorika *et al*., 2004). Hotto *et al*. (2005) reported a 88% prevalence of *mcyB* compared to 79% of *mcyD* in a North American lake, and similar differences have been observed in Polish lakes as well (Mankiewicz-Boczek *et al*., 2006). These studies highlight the importance of primer design; an amplicon too long (1.3 kb), and sequence mismatches were suggested as the likely reasons for the variation in detection frequency in the above-mentioned studies. Due to the fact that *mcy* genes can be present in non-toxic cyanobacteria, a multi-gene approach might provide added specificity to the detection of potential microcystin producers, since it could help in the detection of *mcy* gene deletion mutants. Primers targeted at two to four *mcy* sequences have already been used in a single reaction (Ouahid *et al*., 2005; Ouellette *et al*., 2006; Baron-Sola *et al*., 2012).

A single assay deviating from the use of PCR, a loop-mediated isothermal amplification (LAMP) method for the detection of *mcyE* has been described (Zhu *et al*., 2014). LAMP relies on auto-cycling strand displacement DNA synthesis at a constant temperature (Notomi *et al*., 2000). The outcome of the LAMP reaction was either determined by

measuring turbidity in real-time, or by visual inspection using the fluorescent dye calcein. The target *mcyE* sequence was detected in three out of seven samples, one of which was positive for microcystins (Zhu *et al*., 2014).

qPCR assays

Quantitative PCR has been widely used in the study of *mcy* genotypes in cyanobacterial populations (*e.g*., Yoshida *et al*., 2007; Briand *et al*., 2009; Savichtcheva *et al*., 2011). This literature review concentrates on the assays and applications directly aimed at the study of the relationship between *mcy* gene copy numbers and microcystin production (Table 3). The principal detection chemistries in these assays have been signal generation by the DNA-intercalating dye SYBR® Green (Wittwer *et al*., 1997), or the Taq nuclease assay (TNA), which uses the enzymatic degradation of dual-labeled probes to monitor the accumulation of the amplification product (Holland *et al*., 1991).

In addition to the qPCR assays listed in Table 3, methods that were originally qualitative have been adapted to a quantitative format by subsequent studies. Using *mcyA* primers by Hisbergues *et al*. (2003) and SYBR® Green detection chemistry, a positive correlation between *mcyA* copy numbers and microcystin concentrations in the environment has been reported (Martins *et al*., 2011; Srivastava *et al*., 2012; Sabart *et al*., 2015; Singh *et al*., 2015). Similarly, the *mcyB* primers originally described by Nonnemann and Zimba (2002) have been adapted to quantitative assays. The results from the subsequent analyses of environmental samples have shown a positive relationship between *Microcystis mcyB* copy numbers and microcystin concentrations (Martins *et al*., 2011; Singh *et al*., 2015), supported also by the *mcyB* copy number and microcystin measurements conducted in Uganda using the assay by Kurmayer and Kutzenberger (2003) (Okello *et al*., 2010). However, the predictive capabilities of both *mcyA* and *mcyB* quantification have also been questioned, as correlations between gene copy numbers and microcystin concentrations have in other instances not been observed (Guedes *et al*., 2014; Beversdorf *et al*., 2015).

Table 3. qPCR assays targeted at *mcy* genes.

Gene	Detection chemistry	Target genera	Main findings	Reference
mcyA	TNA	*Microcystis*	Discrimination of toxic and non-toxic *Microcystis* was achieved.	Foulds *et al*., 2002
mcyB	TNA	*Microcystis*	Positive correlation between *Microcystis* cell counts, *mcyB* copy numbers, and MC concentrations.	Kurmayer and Kutzenberger, 2003
mcyE	Intercalating dye	*Anabaena*, *Microcystis*	Two separate assays for two genera. A statistically significant positive correlation between *mcyE* copy numbers and MC concentrations with a few discrepancies observed.	Vaitomaa *et al*., 2003
mcyD	TNA	*Microcystis*	A newly designed probe combined with primers developed by Kaebernick *et al*. (2000). An analysis of environmental samples noted discrepancies between the presence of MCs and *mcy* genes, possibly due to other MC-producing cyanobacteria.	Rinta-Kanto *et al*., 2005
mcyA	Intercalating dye	*Microcystis*	Assay combines a new 3’ primer to a 5’ primer by Tillett *et al*. (2001).	Furukawa *et al*., 2006
mcyA	TNA	*Planktothrix*	Multiplex assay targeted at *mcyA* and PC-IGS. Statistically significant positive correlation between *mcyA* copy numbers and MC concentrations observed; predictive value poor, however. Cellular MC quota inversely correlated to the proportion of *mcyA* genotypes in the population.	Briand *et al*., 2008
mcyD	Intercalating dye	*Microcystis*	Two targets within the same gene. Both gene copy numbers were shown to be able to predict MC concentrations over time.	Fortin *et al*., 2010
mcyJ	TNA	*Microcystis*	Specificity to cultured, MC-producing *Microcystis* 100%. Statistically significant positive correlation between MC concentrations and *mcyJ* copy numbers in environmental samples.	Joung *et al*., 2011
mcyB, *mcyE*	Intercalating dye	*Microcystis*	Statistically significant positive correlation observed between *mcyB* and *mcyE* copy numbers and MC cocentrations.	Conradie and Barnard, 2012
mcyG	Intercalating dye	*Microcystis*	The *mcyG* assay was used in parallel with the *mcyA* and *mcyE* qPCR assays of Furukawa *et al*. (2006) and Vaitomaa *et al*. (2003). Highest MC concentrations corresponded to highest mcy gene copy numbers.	Ngwa *et al*., 2014b
mcyE	TNA	*Microcystis*, *Anabaena*, *Planktothrix*	Multiplex detection using genus-specific primers and probes. *Microcystis mcyE* copy numbers correlated positively with environmental MC-concentrations.	Ngwa *et al*., 2014a

TNA, Taq nuclease assay; MC, microcystin

The results obtained from the application of the first *mcyD* and *mcyE* qPCR assays indicating a positive correlation between gene copy number abundance and toxin production (Vaitomaa *et al*., 2003; Rinta-Kanto *et al*., 2005) have been supported by later studies (Hotto *et al*., 2008; Rinta-Kanto *et al*., 2009; Te and Gin, 2011). Baxa *et al*. (2010), however, reported no correlation in their study on *mcyD*, but did not account for other possible microcystin producers.

Microcystin biosynthesis genes, as the indicators of the most commonly occurring cyanobacterial toxin, are typically included in multiplex assays, such as the qPCR assay by Al-Tebrineh *et al*. (2012), in which the detection of *mcyE*, and the related nodularin synthetase gene *ndaF*, in four cyanobacterial genera with a qPCR assay using a single primer pair was demonstrated. The same target, combined with *sxtA* for PSTs, *cyrA* for cylindrospermopsin, and a 16S rDNA target have been used in a multiplex TNA-assay (Al-Tebrineh *et al*., 2012). The application of the assay for environmental sample analysis revealed the presence of low *mcyE* copy numbers, with no detectable toxin production (Al-Tebrineh *et al*., 2012).

Baker *et al*. (2013) described another multiplex approach to the analysis of potentially toxic cyanobacteria using automated multiplex tandem PCR (MT-PCR), which proceeds in a multiplex fashion for the first 10–20 cycles, after which the individual targets are amplified in separate, parallel reactions. MT-PCR amplification is monitored in real-time using a DNA-intercalating dye, and the analysis is aided by the generation of melting curves. Using bloom samples with a cyanobacterial cell density of >100 cells mL^{-1}, the specificity of the assay for *mcyE* was calculated to be 100%, while the sensitivity was 99.3% (Baker *et al*., 2013).

The majority of quantitative studies on the relationship between *mcy* gene copy numbers and microcystin concentrations have reported a positive correlation between the two variables, but in some cases, poor correlations have been reported, contradicting these findings, as discussed above. Variable proportions of the *mcy* gene-carrying cyanobacteria in a given population have been observed to be inactive mutants (Mikalsen *et al*., 2003; Kurmayer *et al*., 2004; Christiansen *et al*., 2006), which may cause false-positive qPCR results. False negatives may be caused by unanticipated sequence differences between natural populations and reference strains (Via-Ordorika *et al*., 2004; Mbedi *et al*., 2005). Given the number of potentially microcystin-producing cyanobacterial genera, concentrating on a single genus, most often *Microcystis*, and not thoroughly considering other microcystin sources, may have an effect on the observed correlation.

DNA chip

The rationale behind multiplex assays is the ability to assess the composition of the potentially toxic cyanobacterial population in more detail, thus making a more accurate toxicity estimation. A DNA chip, or a DNA microarray can easily surpass the multiplexing abilities of qPCR, and it has been used to qualitatively characterize

cyanobacterial populations (Castiglioni *et al*., 2004). The method described by Castiglioni *et al*., (2004) utilizes zip-coding; two probes, the first of which carrying a fluorescent dye, the second a complementary zip-code, are used to capture the PCR-amplified target. In the event of positive identification, the probes, hybridized to the target adjacent to each other, are ligated. For detection, the resulting fluorescently labeled single stranded DNA molecule is subsequently hybridized to a sequence complementary to the zip-code bound to the solid surface of the array. An application designed to detect microcystin-producing cyanobacteria has been shown to be able to specifically discriminate between *Anabaena*, *Microcystis*, *Planktothrix*, *Nostoc*, and *Nodularia mcyE* or *ndaF* sequences, and have the same detection rate as a corresponding qPCR analysis (Rantala *et al*., 2008). The chip has also been used to characterize *Microcystis* and *Anabaena mcyE* expression by utilizing cDNA that has been reverse-transcribed and amplified from RNA extracted from environmental samples (Sipari *et al*., 2010). It was found to be applicable to expression analysis, although the sensitivity was slightly below that of the qPCR assay used for comparison (Sipari *et al*., 2010). Microcystin concentrations started to increase only after the expression of the targeted *mcyE* gene was detectable, which was in accordance with the qualitative assessment of *mcyE* expression and microcystin production in a North American lake (Gobler *et al*., 2007). Both Sipari *et al*. (2010) and Gobler *et al*. (2007) noted that the use of universal primers might lead to the non-detection of the less abundant targets, as they may be outcompeted by more common targets during amplification.

2.3.2 Paralytic shellfish toxin -producing cyanobacteria

The gene composition of the cyanobacterial *sxt* gene clusters varies from one genus to another (see section 2.2.4). A set of 14 genes, *sxtA–sxtI*, *sxtP–sxtR*, *sxtS*, and *sxtU* (Murray *et al*., 2011a), has been identified from all known PST producers, and the conserved nature of these genes makes them well-suited targets for nucleic acid-based detection methods. Seven of these genes are thought to be directly involved in the biosynthesis of saxitoxin, the parent PST compound (D'Agostino *et al*., 2014). Qualitative PCR has been the approach of choice in the majority of *sxt* gene studies. The screening of cultured cyanobacteria has been mainly targeted at *sxtA*, and a large number of environmental isolates and strains maintained in culture collections have been confirmed to carry the gene (Ballot *et al*., 2010; Ledreux *et al*., 2010; Foss *et al*., 2012; Hoff-Risseti *et al*., 2013; Cires *et al*., 2014). To a lesser extent, other *sxt* genes, including *sxtG*, *sxtB*, *sxtI*, and *sxtX*, have been used as targets in PCR detection (Foss *et al*., 2012; Hoff-Risseti *et al*., 2013; Casero *et al*., 2014; Gkelis and Zaoutsos, 2014). The presence of complete or partial *sxtA* genes in *Anabaena*, *Anabaenopsis*, and *Aphanizomenon* strains, which lack measurable PST biosynthesis, has been observed in several studies (Ballot *et al*., 2010; Ledreux *et al*., 2010; Casero *et al*., 2014; Cires *et al*., 2014), and similar results have been obtained for the other *sxt* genes as well. A common quality to all of the *sxt* genes investigated in the abovementioned studies is the fact that none of them have been shown to be exclusively specific to PST production.

Few studies have investigated the predictability of cyanobacterial PST production based on *sxt* gene detection without first isolating the suspected producers from samples confirmed to contain these neurotoxins. Bowling *et al*. (2013) reported the presence of *sxtA* in all environmental samples collected for genetic study, but PST concentrations remained below the detection limits of the analytical methods used. Similar results have been obtained in a study of Greek lakes, in which the amplification of *sxtI* was twice as common as PST detection (Gkelis and Zaoutsos, 2014). Quantitative analysis of potentially PST-producing cyanobacterial blooms has been carried out using an *sxtA*-targeted primer pair and SYBR® Green-based detection in Australia (Al-Tebrineh *et al*., 2010). Based on qPCR-derived *Anabaena circinalis* cell counts, the study reported a successful estimation of PST concentrations although the actual toxin concentrations measured with a HPLC method fell below the predicted values, resulting in six of the eleven *sxtA*-positive samples being PST-negative (Al-Tebrineh *et al*., 2010). Later, the *sxtA* qPCR assay was incorporated into a multiplex, TNA-based qPCR method targeted at *sxtA*, *cyrA* for cylindrospermopsin, *mcyE* for microcystin biosynthesis, and 16S rRNA gene for the general detection of cyanobacteria (Al-Tebrineh *et al*., 2012). Originally the limit of detection of the *sxtA* qPCR assay was 5.4 copies of *sxtA* per reaction, but multiplexing resulted in the increase of the detection limit by one order of magnitude (Al-Tebrineh *et al*., 2012). The multiplex assay was later used in the analysis of an exceptionally large cyanobacterial bloom in the Murray River. Over time, gradually increasing *sxtA* copy numbers at three sampling sites were reported; low PST concentrations were measured only at two of the sites, however (Al-Tebrineh *et al*., 2012). In the MT-PCR assay described above in section 2.3.1, the *sxtI* gene was used as the target for PST-producing cyanobacteria. At a detection limit of one cell per reaction, the authors reported the assay specificity to be 100%, and sensitivity 97.7% for *sxtI* (Baker *et al*., 2013).

2.3.3 Paralytic shellfish toxin -producing dinoflagellates

The monitoring of toxic dinoflagellates is required by many countries to ensure the safety of fish and seafood intended for human consumption (Hungerford, 2014). Microscopic identification and enumeration of dinoflagellate species can, however, be very tedious and time-consuming. PST-producing *Alexandrium* spp. can be particularly difficult in this respect, due to the morphological similarities, and the presence of cryptic species (Penna and Galluzzi, 2012; John *et al*., 2014). To avoid these problems, numerous tools based on the detection of species and toxin-specific nucleic acids, including qualitative and quantitative PCR, as well as hybridization-based methods, have therefore been under development. Comparisons between methods for *Alexandrium* cell density determination have indicated that molecular methods have the required sensitivity and species discrimination capabilities for environmental monitoring use (Godhe *et al*., 2007).

The various ribosomal RNA-encoding genes are present in high copy numbers in the dinoflagellate genome, varying from 65 copies (18S rRNA gene) per cell in *Prorocentrum nux* to an average of 462 900 copies (5.8 rRNA gene) per cell in *A.*

catenella, and up to three orders of magnitude within a single genus (Zhu *et al*., 2005; Galluzzi *et al*., 2010). Highly species-specific sequence information on these genes is widely available, and using a high-copy-number gene as a target decreases the number of cells needed for a positive detection. With a qPCR assay, sensitivities as low as 10 cells per sample can be achieved (Dyhrman *et al*., 2006). This is ideal for dinoflagellates, many of which may not achieve high cell concentrations in the environment, but which can elicit harmful effects despite the low cell densities; *Alexandrium* blooms of no more than 200 cells L^{-1} have been known to lead to toxin uptake in shellfish (Murray *et al*., 2011b). The simplest way to use rDNA genes for the detection of potentially toxic dinoflagellates is to use qualitative PCR. With low-cost instrumentation, six *Alexandrium* species can be differentiated on the basis of their rDNA sequences in a multiplex assay (Nagai, 2011).

Species identification on the basis of fluorescence *in situ* hybridization (FISH) targeted at rRNA has been widely used for the detection of harmful dinoflagellates, including PST-producing *Alexandrium* spp. (Anderson *et al*., 2005; Hosoi-Tanabe and Sako, 2006; Touzet *et al*., 2008; Touzet *et al*., 2009; Tang *et al*., 2012; Toebe *et al*., 2013a). FISH is based on the binding of sequence-specific fluorescently labeled oligonucleotide probes to the target nucleic acid in intact dinoflagellate cells, and the subsequent enumeration of cells on the basis of the fluorescent signal using an epifluorescence microscope (Toebe, 2013a). Automated counting by flow cytometry can be used to improve the sample throughput (*e.g*., Eckford-Soper *et al*., 2013). The prediction of PST occurrence based on FISH relies principally on the identification of the potential producer organism, and FISH-based detection and PST measurements have rarely been compared. The detection of HAB species can also be carried out by other types of hybridization methods, such as the sandwich hybridization assay (SHA), as well as different microarrays. The SHA is carried out using sample lysates in microtiter wells; rRNA-targeted capture and detection probes are used, and the signal indicating detection is obtained via the colorimetric detection of an enzymatic reaction (Scholin *et al*., 1999). The assay has been used, *e.g*., in the detection of toxic *A. minutum* (Diercks *et al*., 2008a), and *G. catenatum* (Diercks *et al*., 2008b). Microarrays provide a possibility to characterize populations of eukaryotic microalgae, and many include probes specific to PST-producing dinoflagellate species (Ki and Han, 2006; Gescher *et al*., 2008; Galluzzi *et al*., 2011; Kegel *et al*., 2013). In the abovementioned SHA and microarray methods both RNA and DNA targets have been used, and optionally amplified by PCR prior to the actual assay to increase sensitivity.

Several qPCR methods targeted at the various rDNA genes have been developed for the detection of PST-producing *Alexandrium* species, and for their differentiation from non-toxic species of the same genus. A good positive correlation between qPCR-based and microscopic cell density estimates in environmental samples can be achieved, provided that the cellular rDNA content of the target species has been determined beforehand using an appropriate reference strain or sample (Galluzzi *et al*., 2004; Hosoi-Tanabe and Sako, 2005; Dyhrman *et al*., 2006; Touzet *et al*., 2009; Dyhrman *et al*., 2010). In many areas of the world where recurrent toxic *Alexandrium* blooms take place, cyst beds may

be studied to gain information on past events and to attempt to predict future blooms. Quantitative PCR can be applied also for this purpose, to replace other methods of dinoflagellate cyst enumeration, *e.g.*, primulin staining and microscopy, but again, cyst-specific rDNA copy numbers need to be determined for accurate results (Kamikawa *et al.*, 2007; Erdner *et al.*, 2010). Although the high cellular rDNA copy numbers are helpful in the development of very sensitive assays, their extreme variability can be problematic. Besides the need to construct standard curves for each target species individually, result interpretation can be further complicated by the presence of rDNA pseudogenes (Yeung *et al.*, 1996), and the fact that gene copy numbers can vary even between growth stages (Galluzzi *et al.*, 2010).

Good cell density estimates can be obtained with careful assay design (*e.g.*, Galluzzi *et al.*, 2004). The presence of a potentially toxin-producing species can indicate PST uptake in shellfish; but does not necessarily do so, however (Dyhrman *et al.*, 2010). Phytoplankton communities often consist of a number of species, and species-specific rDNA-targeted detection will usually not cover all the possible toxin-producing dinoflagellates. Some species are exclusively toxic, or may be so at certain geographical locations, but it is common for non-toxic strains to coexist in a population (Toebe *et al.*, 2013b). The cellular rDNA copy numbers are not directly linked to toxin production, which results in a variable and often poor correlation between estimated cell numbers and PST concentrations in water (Garneau *et al.*, 2011), or in shellfish (Dyhrman *et al.*, 2010). Even when targeting up to five potentially PST-producing *Alexandrium* species, the correlation remained poor (Toebe *et al.*, 2013b).

The design of toxin-specific molecular assays for dinoflagellates was made possible by the elucidation of the first *sxt* genes on the basis of expressed sequence tag (EST) data, and subsequent direct sequencing (Stüken *et al.*, 2011; Hackett *et al.*, 2013). Qualitative screening for the presence of *sxtA1*, *sxtA4*, and *sxtG* genes in cultured dinoflagellate strains has revealed that they differ in terms of specificity to PST production, and the results indicate that out of the three target genes used, the presence of the *sxtA4* gene is the best predictor of toxin production (Murray *et al.*, 2011b; Stüken *et al.*, 2011; Suikkanen *et al.*, 2013; John *et al.*, 2014; Murray *et al.*, 2015). Currently, there are five primer pairs available for qPCR and qRT-PCR assays targeted at the dinoflagellate genes *sxtA1*, *sxtA4*, and *sxtG* in *Alexandrium* spp. and *G. catenatum*, and one locked nucleic acid (LNA)-based hydrolysis probe (Table 4). While the *sxtG* qPCR assay was 100% specific, a longer *sxtG* amplification product was obtained from four non-PST-producing *Alexandrium* strains (Orr *et al.*, 2013). In the expression study by Perini *et al.* (2014), only four *A. minutum* strains were analyzed, and assay specificity was not extensively addressed. The sensitivities of the different *sxt* assays cannot be directly compared, as they depend on the targeted nucleic acid, and the method of standard curve construction (Table 4).

Like the rDNA genes, the *sxtA4* gene copy numbers have been found to vary between species. Despite their similar capability of toxin production, *A. pacificum* and *A.minutum* carry significantly different numbers of *sxtA4* copies in their genomes; *A.*

pacificum carries up to 240 copies (Murray *et al*., 2011b; Stüken *et al*., 2011), while *A. minutum* has fewer than 15 (Stüken *et al*., 2015). The copy number variability and the presence of non-identical *sxtA4* copies in a single cell (Wiese *et al*., 2014) complicate the estimation of cell numbers, and needs then to be taken into account when designing qPCR assays. The cellular gene copy numbers have major effect on the sensitivity of an assay, in terms of the minimum number of cells detected, and as a result, the detection limits for a given sample volume will differ between *Alexandrium* species.

Table 4. qPCR and qRT-PCR assays targeted at dinoflagellate *sxt* genes.

Target	Detection chemistry	Detection range	Specificity	Reference
sxtA4, gDNA	EvaGreen	30–2600 *A. pacificum* cells reaction^{-1}, 4.1×10^{5}–4.1×10^{10} copies reaction^{-1}	94%	Murray *et al*., 2011b
sxtA4, cDNA	LNA hydrolysis probe	160–1.6×10^{6} copies reaction^{-1}	100%	Stüken *et al*., 2013
sxtA1, cDNA	SYBR® Green	2 copies reaction^{-1}, 6 orders of magnitude	100%	Perini *et al*., 2014
sxtG, gDNA	SYBR® Green	0.63–10 ng *A. pacificum* gDNA reaction^{-1}	100%	Orr *et al*., 2013
sxtG, cDNA	SYBR® Green	2 copies reaction^{-1}, 6 orders of magnitude	50%	Perini *et al*., 2014

The *sxtA4* is the only target gene used in environmental studies to date (Murray *et al*., 2011b, Stüken *et al*., 2013). A positive correlation has been observed between total genomic *sxtA4* copy numbers and PST concentrations in the environment, the sensitivity of the assay surpassing the Sedgewick-Rafter microscopic counting method by an order of magnitude (Murray *et al*., 2011b). Stüken *et al*. (2013) studied the *sxtA4* transcript abundance in *Alexandrium* spp., as well as *G. catenatum* blooms, and found a positive relationship with PST concentrations. However, false-negative qRT-PCR results were also observed, which were attributed to the different stability of the targets of the qRT-PCR and immunoassays; mRNA has a rapid turnaround time, while PSTs may persist in the water after the bloom has dissociated (Stüken *et al*., 2013). Both studies concluded that qPCR methods have potential use as monitoring and early-warning tools. The utility of *sxt* gene expression in the prediction of toxin production has been challenged by expression studies conducted in culture; both Perini *et al*. (2014) and Wiese *et al*. (2014) reported that the expression levels of *sxt* genes showed no significant positive correlation to the amounts of PSTs produced. However, culture and environmental conditions are difficult to compare, and measuring *sxt* gene expression may be a useful at the population level.

2.3.4 Recent developments and outlook

During the last 15 years PCR as a method has demonstrated its utility in the study of toxin biosynthesis genes in harmful cyanobacteria and dinoflagellates, as well as in the quantitative analysis of cell densities and species identification. Quantitative PCR has been deemed the most cost-effective, sensitive, and rapid method for the enumeration of dinoflagellates in an environmental sample (Ebenezer *et al*., 2012). Intercalibration experiments have demonstrated that it is possible to use a qPCR method in several laboratories by a number of operators, and obtain comparable results (Schober *et al*., 2007). Some assays, such as a series of species-specific qPCR assays designed to detect harmful dinoflagellates of the family *Gymnodiniaceae*, are already used in routine monitoring (Smith *et al*., 2014).

Phytoplankton community composition and dynamics in the natural environment can be very complex. Several species and strains capable of producing the same toxins may be present, and several types of toxins can be found at a single location. To assess toxicity comprehensively, these factors need to be taken into account during nucleic acid assay design. Multiplexing may provide an answer to these issues, and methods have already been described for both cyanobacterial and dinoflagellate targets (*e.g*., Al-Tebrineh *et al*., 2012; Eckford-Soper and Daugbjerg, 2015). However, multiplex assays may not be as sensitive as singleplex assays (Al-Tebrineh *et al*., 2010, 2012). Melting curve analysis is routinely used for post-amplification analysis in qPCR experiments. In the future, high resolution melting may provide improved opportunities for species identification and the investigation of the genetic variability of toxin biosynthesis genes. Microarrays and array-in-well-type assays provide other alternatives for highly multiplexed assays.

Digital PCR techniques have not yet seen widespread use in the detection of harmful microalgae, but it is a promising alternative method for the quantitative analysis of nucleic acids. In standard qPCR, both absolute and relative quantification must be well-controlled, and the standards used need to be chosen with care. The digital PCR approach to absolute quantification has been advocated as more accurate than standard curve-based qPCR, as the quantification does not require external standards. Recently, a duplex assay for the detection of potentially toxic *Microcystis* and *Cylindrospermopsis* using droplet digital PCR (ddPCR) has been described (Te *et al*., 2015). A comparison of the ddPCR assay with a corresponding qPCR method revealed that although the former was slightly less sensitive, the detection of low copy numbers was more precise.

The biological roles of the different cyanobacterial and dinoflagellate toxins still remain largely unknown. Studies on the evolution of microcystin and PST biosynthesis have revealed that both have originated in the ancient ancestors of the toxin-producing organisms, raising even further questions on the nature of the factors driving toxin biosynthesis today. Keeping in mind the increasing trends in HAB prevalence and severity, these questions will likely become even more pressing. Numerous qualitative and quantitative detection methods based on the amplification of toxin biosynthesis

genes in cyanobacteria and dinoflagellates have been developed. Promising results demonstrating positive correlation between the presence and/or quantity of genetic factors and toxin production have been obtained, however, contradictory findings have also been reported every now and then.Therefore, the continued research to understand the genetics and regulation of toxin production is important, as it will help in developing better strategies for HAB monitoring, including the design of even more reliable and comprehensive nucleic acid-based approaches.

3 AIMS OF THE STUDY

The purpose of this doctoral study was to develop PCR-based methods for the detection and quantification of potentially toxin-producing cyanobacteria and dinoflagellates for environmental monitoring. The applicability of the developed methods for the prediction of harmful algal bloom toxicity was assessed by investigating the nature of the correlation between copy numbers of the target genes and observed toxin production.

More specifically, the aims were:

I To develop a quantitative real-time PCR method for the detection of potentially microcystin-producing *Anabaena*, *Microcystis*, and *Planktothrix*, as well as to establish a supporting fast sample preparation method for cyanobacterial samples to provide increased DNA yields.

II To introduce the developed *mcyB* qPCR assay into a dry chemistry format, to assess its performance in water sample analysis, and to determine the predictability of microcystin concentrations in the environment on the basis of the genus-specific detection and quantification of the target gene.

III To develop methods for the detection and quantification of cyanobacterial paralytic shellfish toxin biosynthesis (*sxt*) genes, as well as to use these methods to study the prevalence of toxin biosynthesis potential and also the actual toxin production in freshwater lakes.

IV To establish a qPCR assay capable of detecting PST-toxin producing dinoflagellates of the genus *Alexandrium*, and to use the method to study *sxtA4* gene copy numbers and toxin production in the Baltic Sea *A. ostenfeldii*.

4 SUMMARY OF MATERIALS AND METHODS

The detailed descriptions of the materials and methods used in this study are presented in the original publications (**I**–**IV**). A brief summary is provided here.

4.1 Samples

4.1.1 Cultured cyanobacteria and dinoflagellates

Cyanobacterial and dinoflagellate strains used in this doctoral study are listed in Table 5. All NIVA strains and NIES-107 were cultured in Z8 (Staub, 1961; Kótai, 1972; NIVA, 1976), and PCC strains in either BG11, nitrate-omitted $BG11_0$, or $BG11_0$ with added $NaNO_3$ and $NaHCO_3$, according to PCC formulations. *Anabaena lapponica* 966 and *Anabaena* sp. 90 were cultured in modified Z8 with no added source of combined nitrogen. All cyanobacterial cultures were maintained at 23 °C under continuous illumination (ca. 10 µmol photons m^{-2} s^{-1}). Dinoflagellate strains were grown in f/2-Si medium (Guillard and Ryther, 1962) adjusted to native salinities, *i.e.*, except for brackish water *A. ostenfeldii* which grows at native salinities of 6-8 psu, all tested dinoflagellates originate from marine environments (28-34 psu) (**IV**), and maintained at 16 ºC on a 12:12 light–dark cycle (100 µmol photons m^{-2} s^{-1}).

4.1.2 Environmental samples

Environmental samples for microcystin and *mcy* gene PCR analysis (**I**, **II**) were collected on Åland Islands, mainland Southwestern Finland, Southern Finland and Northern Estonia during April–June 2008 and June–November 2009. The sampling locations varied in size and trophic status and included 34 freshwater lakes, two rivers and three drinking water reservoirs. For PST and cyanobacterial PST biosynthetic gene analysis, freshwater lakes and brackish coastal waters on Åland Islands were sampled annually in August 2010–2012 (**III**). Sampling was conducted at 27 locations each year, and additional five locations were included in 2010 and 2011. At all locations, excluding the drinking water reservoirs, sampling was conducted near the shoreline, at the depth of 10–20 cm. Possible accumulations of decayed cyanobacterial biomass collected at the shore were avoided. The drinking water reservoirs were sampled similarly at water intake pipes (depths of 0.75–1 m). The samples were representative of 0–2 m depth. Samples were filtered onto binder-free glass fiber filters (Whatman GF/C, 47 mm or 25 mm in diameter) under vacuum, or using a syringe-driven filter holder system. The filtered volume was recorded for quantification purposes. The filters were rinsed with deionized water (if intended for PCR and qPCR analysis) and any excess water was removed. The samples were immediately processed further, or stored and transported frozen (-20 °C).

Table 5. Cyanobacterial and dinoflagellate strains used in this study. For toxin data on the strains, refer to Tables 10 and 12, and original publications **I**, **III** and **IV**.

Species	Strain	Species	Strain
Cyanobacteria		***Cyanobacteria***	
Anabaena sp.	90[1]	*Planktothrix agardhii*	NIVA-CYA 15
Anabaena circinalis	CS-337/01		NIVA-CYA 59/1
	CS-530/05		NIVA-CYA 299
	CS-533/12		NIVA-CYA 12
	CS-537/13		NIVA-CYA 21
Anabaena cylindrica	PCC 73105		NIVA-CYA 116
	PCC 7938		PCC 7805
Anabaena flos-aquae	NIVA-CYA 267/4		PCC 7811
	NIVA-CYA 499	***Dinoflagellates***	
Anabaena lapponica	966[2]	*Alexandrium andersonii*	CCMP 2222
Anabaena lemmermannii var. minor	NIVA-CYA 83/1	*Alexandrium fundyense*	CCMP 1911
	NIVA-CYA 266/1	*Alexandrium fundyense*	CCMP 1979
Cylindrospermopsis raciborskii	CS-505	*Alexandrium minutum*	CCMP 113
	CS-506	*Alexandrium ostenfeldii*[3]	AOTVPL25
	CS-510		AOTVA4
Microcystis aeruginosa	NIES 107		AOF 0924
	NIVA-CYA 57		AOF 0927
	NIVA-CYA 140		AOF 0933
	NIVA-CYA 228/1		AOF 0935
	NIVA-CYA 495		AOF 0957
	NIVA-CYA 22		AOKAL 0902
	NIVA-CYA 464		AOPL 0914
	PCC 7806		AOVA 30
	PCC 7820		S06/013/01
	PCC 7941		NCH 85
	PCC 7005		LS A06
Nodularia sphaerocarpa	PCC 7804		CCAP 1119/45
Nostoc sp.	PCC 6310	*Alexandrium tamarense*	SCCAP K1471
	PCC 7422	*Gymnodinium catenatum*	CCMP 1937
		Lingulodinium polyedrum	SCCAP K0982
			CCMP 1933

PCC, Pasteur Culture Collection (Paris, France); NIVA, Norwegian Institute for Water Research Cyanobacterial Culture Collection (Oslo, Norway); NIES, National Institute of Environmental Studies (Tsukuba, Japan); CCMP, National Center for Marine Algae and Microbiota (East Boothbay, Maine, USA); CS, CSIRO/Australian National Algae Culture Collection (Hobart, Tasmania, Australia); CCAP, Culture Collection of Algae and Protozoa (Oban, Scotland, UK); SCCAP, Scandinavian Culture Collection of Algae and Protozoa (Copenhagen, Denmark). [1]University of Helsinki, Prof. Kaarina Sivonen. [2]Finnish Environment Institute, Dr. Jarkko Rapala. [3]For further description on *A. ostenfeldii* strains, refer to Tahvanainen *et al*. (2012) and Kremp *et al*. (2014).

The sampling of dinoflagellates for *sxtA4* and LSU gene and PST analyses was carried out at Föglö, Åland Islands in August 2014 (**IV**). Samples were collected manually at the depth of 0–0.5 m and subsequently filtered onto glass fiber filters as described above. If necessary, samples were concentrated with a 25 µm plankton net prior to filtration. All filters were stored in a cooled container until moved to a freezer (-20 °C).

4.1.3 Microscopic analysis

The counting of cyanobacterial cells was carried out for sample preparation studies from cultures and environmental samples (**I**), and cyanobacterial genera present in environmental samples (**III**) were qualitatively determined using light microscopy. Identification of genera and species were carried out according to Komárek and Anagnostidis (1998, 2005) and morphological criteria described in Tikkanen (1986). Counting was carried out from transects of samples fixed with Lugol's iodine, using Olympus CK-2 and Nikon TE 200 inverted microscopes at 20× or 10× and 40× objectives, respectively. The latter microscope was also used for the qualitative identification of cyanobacteria at 200× and 400× magnification. Species identification and the determination of *A. ostenfeldii* (according to Balech and Tangen, 1985) cell concentrations in samples preserved in Lugol's iodine (**IV**) was carried out using Sedgewick-Rafter chambers and a Leica DMI 3000B inverted microscope, or sedimentation chambers (Utermöhl, 1958) and a Zeiss PrimoVert inverted microscope (100× magnification), for culture and environmental samples, respectively.

4.2 Quantification of toxins

4.2.1 Toxin extraction

To extract microcystins (**I**, **II**), cultured cyanobacterial cells or environmental samples collected on filters were freeze-dried, extracted with 75% methanol and sonicated for a total of 16 min to release cell-bound toxins. After centrifugation the supernatants were divided into aliquots and evaporated to dryness with argon at 40 °C. Extracts intended for chromatographic analysis were resuspended in 75% methanol while those aimed for ELISA were suspended in water.

To extract PSTs (**III**, **IV**), freeze-dried samples were dissolved in 80% acetonitrile (ACN) containing 0.1% formic acid and sonicated while kept on ice. After centrifugation the supernatants were filtered, an aliquot was separated for LC-MS/MS analysis (see section 4.2.3 for details), and placed in a freezer (-20°C) where the extracts were allowed to separate into the upper organic and the lower aqueous phase (Sayfritz *et al*., 2008). The PSTs dissolved in the aqueous phase were analyzed by HPLC-FLD with post-column oxidation (see section 4.2.3). Alternatively, freeze-dried samples were extracted in 0.03 M acetic acid, filtered (Millex, Ø 13 mm, 0.45 µm), and volume-adjusted to 1.5 mL (**IV**).

4.2.2 Microcystin analysis (I, II)

Chromatographic analysis

Two chromatographic methods were used in the identification and quantification of microcystins in the original publications **I** and **II**; high performance liquid chromatography coupled to diode-array UV-detection (HPLC-DAD) and liquid chromatography–mass spectrometry (LC-MS) were utilized.

For the HPLC-DAD, samples were analyzed using an Agilent 1100 series HPLC system and a diode-array detector operated at 200–300 nm (quantification at 238 nm) with an Ascentis RP-Amide column (3 µm particles, 100 mm × 4.6 mm) as the stationary phase. The mobile phase consisted of ACN (solvent B) – MQ-H_2O (solvent A), both solvents containing 0.05% trifluoroacetic acid. The following linear gradient program was used: 0 min 25% B, 7 min 70% B, 10 min 70% B, 10.1 min 25% B; stop time 15 min; flow-rate 1 mL min^{-1}. Injection volumes were 10 µL. An additional sample analysis was carried out on a Merck Purospher STAR RP-18e column according to (Spoof *et al*., 2005). Individual laboratory-purified microcystins and reference samples were used for the identification of microcystins (Spoof *et al*., 2003).

The LC-MS analyses were carried out on an Agilent 1200 Rapid Resolution LC system coupled to a Bruker Daltonics HCT Ultra ion trap mass spectrometer with an electrospray ion (ESI) source according to Spoof *et al*. (2003).

Enzyme-linked immunosorbent assay (ELISA)

In addition to chromatographic analysis, ELISA was used for microcystin quantification (Envirologix Quantiplate Microcystin kit) (**I**, **II**). Prior to analysis, sample extracts were diluted with water using the results obtained from the HPLC-DAD to adjust the microcystin concentrations to fit within the dynamic range of the immunoassay. After the adjustment, analysis was carried out according to the protocol provided by the manufacturer.

4.2.3 Paralytic shellfish toxins (III, IV)

Reference materials

Certified reference materials of paralytic shellfish toxins, including C-toxins (C1–2), gonyautoxins (GTX1–4, GTX2–3, GTX5), decarbamoyl gonyautoxins (dcGTX2–3), saxitoxin (STX), decarbamoyl saxitoxin (dcSTX), and neosaxitoxin (NeoSTX) were acquired from the Canadian National Research Council Institute for Marine Biotechnology (NRC-IMB).

Ion-pair HPLC with post-column oxidation and fluorescence detection (HPLC-FLD)

The HPLC-FLD methods according to Oshima (1995) and Diener *et al*. (2006), with some modifications, were used to quantify PSTs from the cyanobacterial sample extracts (**III**) and laboratory-cultured dinoflagellates (**IV**). A Merck Hitachi LaChrom HPLC-system with a Waters XBridge C18 column (3.5µm particles, 150 mm × 3 mm, column at 40 °C) was used. The mobile phase consisted of eluent A, (6 mM octanesulfonic acid, 6 mM heptanesulfonic acid, 40 mM ammonium phosphate, 20% phosphoric acid, pH 7)–tetrahydrofuran (THF), 99.25:0.75 (v/v); and eluent B, (7 mM octanesulfonic acid, 7 mM heptanesulfonic acid, 48 mM ammonium phosphate, 20% phosphoric acid, pH 7)–THF–ACN, 89:1:10 (v/v/v). The following gradient was used: 0 min 100% A, 6 min 100% A, 7.5 min 100% B, 32 min 100% B, 33 min 100% A, 45 min 100% A, flow rate 0.55 mL min^{-1}. The post-column oxidation was performed with 5 mM periodic acid and 275 mM ammonia in water at 60 °C in a PTFE reaction coil, flow rate 0.3 mL min^{-1}. The pH was lowered using 0.38 M nitric acid (0.4 mL min^{-1}). A Hewlett-Packard Series 1100 fluorescence detector (λ_{Ex} 330 nm, λ_{Em} 395 nm) was used to detect the oxidized toxins.

The Föglö sample extracts (**IV**) were analyzed using a method described by Hakanen *et al*. (2012) with slight modifications, using an Agilent 1100 series HPLC and a Waters XBridge C18 column coupled to a Waters XBridge C18 precolumn (3.5 µm, 3 × 20 mm). The injection volume was 5 µL and the column oven temperature 30 °C. Eluent A consisted of 6 mM octanesulfonic acid, 6 mM heptanesulfonic acid, 40 mM diammonium hydrogen phosphate and 0.5% (v/v) THF in MQ-H_2O. Eluent B consisted of 7 mM octanesulfonic acid, 7 mM heptanesulfonic acid, 48 mM diammonium hydrogen phosphate, 1.0% (v/v) THF, and 10% ACN in MQ-H_2O. Both eluents were adjusted to pH 6.8 with 85% phosphoric acid. The gradient was A 100% 6 min, A 100–0% 6–7.5 min, B 100% 7.5–22 min, B 100%–0% 22–23 min, and A 100% 23–36 min. The flow rate was 0.55 mL min^{-1}. Post-column oxidation was carried out in a coulometric cell (ESA 5011A) and controlled with an ESA Coulochem II 5200A electrochemical detector. The analytes were detected with an Agilent 1100 series fluorescence detector G1321A (λ_{Ex} 335 nm, λ_{Em} 396 nm). The quantitative results were measured against standard solutions within the linear range of 2–200 ng mL^{-1}.

Liquid chromatography–tandem mass spectrometry (LC-MS/MS)

LC-MS/MS experiments were carried out to characterize the toxin profiles of cultured dinoflagellates, and to identify and quantify PSTs from the Föglö sample extracts (**IV**). Dinoflagellate strain extracts were analyzed with an Agilent Technologies 1200 Rapid Resolution (RR) LC coupled to a Bruker Daltonics HCT Ultra Ion trap MS with a positive mode electrospray ion (ESI) source. The separation of the toxins was achieved on a Merck SeQuant hydrophilic interaction chromatography ZIC-HILIC column (3.5 µm, 150 mm × 2.1 mm) at 30°C, with mobile phase solvents A (2 mM ammonium formate and 3.6 mM formic acid in water) and B (2 mM ammonium formate and 3.6 mM formic acid in 5:95 v/v water–acetonitrile), using the following linear gradient: 0

min 50% B, 10 min 15 % B, 18 min 15% B, 18.2 min 50% B, stop time 22 min; flow-rate 0.3 mL min^{-1}. Injection volumes were 3 µL. A post-column split 1:9 was employed.

The LC–MS/MS method used for the identification of STX, GTX2, and GTX3 in the Föglö samples was based on the method validated for algal samples (Halme *et al.*, 2012), with slight modifications. A Thermo Scientific Finnigan LXQ linear ion trap mass spectrometer with Accela LC system and a positive mode electrospray ionization (ESI) source was used. Toxins were separated with a TOSOH Bioscience HILIC TSK-gel Amide-80® column (3 µm, 150 mm × 4.6 mm) under isocratic elution with 4 mM ammonium formate in 40:60 (v/v) H_2O–acetonitrile. The pH was adjusted to 3.5 with formic acid. The injection volume was 15 µL, and the flow rate was 1000 µL min^{-1}. A 1:20 post-column split was employed.

4.3 DNA extraction and sample preparation

4.3.1 Extraction and purification of genomic DNA

Two extraction and purification methods were used to obtain genomic DNA from cultured cyanobacteria. A commercial reagent kit (Macherey-Nagel NucleoSpin Plant II) was used according to the manufacturer's instructions (**I**–**III**). For the purpose of comparing DNA yields obtained by different sample preparation methods in publication **I**, conventional phenol–chloroform extraction was carried out as described by Kurmayer and Kutzenberger (2003). The phenol–chloroform method was used on filtered environmental samples and exponentially growing cultured cells. For extraction by the commercial kit the culture-grown cells were harvested by centrifugation (4 °C, 3220 g, 20 min, Eppendorf 5810R), since the presence of solid filter material clogged the silica columns used to capture DNA in the commercial kit.

For the extraction of genomic DNA from cultured dinoflagellates (**IV**), cells were collected by a 2-step centrifugation: 10 min, 3220 g at 20°C followed by 10 min, 16 000 g at room temperature (Eppendorf 5810R, Eppendorf 5415D). The pelleted cells were homogenized mechanically using a motorized pestle before the extraction of gDNA using the Qiagen Plant Mini reagent kit according to the manufacturer's instructions for maximum DNA yield. Alternatively, a Macherey-Nagel NucleoSpin Tissue reagent kit was used, according to the manufacturer's instructions.

The quality and concentration of the purified genomic DNA was estimated spectrophotometrically (ND-1000, NanoDrop Technologies). For samples where the amount of DNA added to the qPCR reactions was used as the basis of copy number calculation, DNA concentrations were also determined using the QuantIt PicoGreen dsDNA kit (Invitrogen Molecular Probes) according to the manufacturer's instructions. The DNA intercalating dye used in the PicoGreen kit is minimally influenced by short single stranded DNA molecules and degraded DNA possibly present in the sample, thus resulting in a more accurate determination of intact double stranded DNA (Ahn *et al.*, 1996, Li *et al.*, 2014). All purified gDNA was stored at -20 °C until analysis.

4.3.2 Preparation of cell and environmental sample lysates

A cell lysis method for cyanobacterial samples, either cultured or environmental, was developed with the aim of minimizing DNA loss during the sample preparation. Cyanobacterial samples were lysed by suspending entire filters or filter pieces of known dimensions in sterile deionized water, and then heating them at 80 °C. In the publication **I**, a volume of 100 µL for filter pieces was used together with a 5 min incubation time. In the original publications **II** and **III**, after the addition of internal amplification control (see section 4.3.3), filters were suspended whole in a 1.5 mL volume and heated for 10 min. Dinoflagellate samples (**IV**) were lysed essentially as described by Garneau *et al*. (2011). Briefly, the filters were suspended in 1.5 mL of lysis buffer (100 mM Tris, 40 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0, 100 mM NaCl, 1% v/v sodium dodecyl sulphate and subjected to three subsequent rounds of mechanical stress and heating (70 °C for 5 min). Lysis was facilitated with 0.5 mm diameter zirconia–silica beads (BioSpec Products) added to each sample tube, and the lysates clarified with a final centrifugation step (10 min, 16 000 g, RT, Eppendorf 5415D). All obtained lysates were frozen (-20 °C) until further PCR or qPCR analysis. Due to the PCR-inhibiting qualities of the lysis buffer used in the preparation of dinoflagellate samples, the lysates were diluted 1:100 in water. DNA concentrations were not measured from sample lysates, since they were either too dilute to be measured spectrophotometrically, or contained material contributing to the absorbance spectrum, making it impossible to estimate the DNA content.

4.3.3 Internal amplification control (II, III)

Bacillus subtilis strain ATCC 6633 was used as an exogenous, internal amplification control (IAC) in the qPCR analysis of cyanobacterial toxin biosynthesis genes, with the purpose of identifying samples in which amplification was substantially hampered by PCR inhibition. *B. subtilis* cells were grown overnight (37 °C, 300 rpm), harvested by centrifugation, and stored in small aliquots at -20 °C until use. A fixed amount of 1×10^5 cells, enumerated using the qPCR method described in section 4.5.3, was added to the environmental samples to act as a control for both the sample preparation and qPCR analysis steps.

4.4 Qualitative detection of paralytic shellfish toxin biosynthesis genes

Qualitative PCRs were used to analyze environmental samples and cyanobacterial strains for the cyanobacterial saxitoxin biosynthesis genes *sxtA* and *sxtG* (**III**) as well as dinoflagellate strains for the presence of gene domains *sxtA1* and *sxtA4* (**IV**). All primers used in the experiments were synthesized by biomers.net GmbH, and are listed in Table 6.

Table 6. Primers used for the detection of cyanobacterial *sxtA* and *sxtG* genes (**III**), and dinoflagellate *sxtA1* and *sxtA4* genes (**IV**).

Oligonucleotide	**Sequence (5'-3')**	**Target**	**Reference**
sxtA855_F	GACTCGGCTTGTTGCTTCCCC	*sxtA*,	
sxtA1480_R	GCCAAACTCGCAACAGGAGAAGG	cyanobacteria	**III**
sxtG432_F	AATGGCAGATCGCAACCGCTAT	*sxtG*,	
sxtG928_R	ACATTCAACCCTGCCCATTCACT	cyanobacteria	
sxt001	TGCAGCGMTGCTACTCCTACTAC	*sxtA1*,	Stüken *et al*., 2011
sxt002	GGTCGTGGTCYAGGAAGGAG	dinoflagellates	
sxt007	ATGCTCAACATGGGAGTCATCC	*sxtA4*,	
sxt008	GGGTCCAGTAGATGTTGACGATG	dinoflagellates	

Qualitative PCR reactions were run either on a PTC-200 Thermal cycler (MJ Research) or a C1000 Touch Thermal Cycler (Bio-Rad). All reactions (20 µL) contained 0.4 µL Phire II HotStart polymerase and 1X Phire Reaction buffer (Thermo Scientific), 0.2 mM dNTPs (Finnzymes or Bio-Rad), and 500 nM 5'- and 3'-primers. Additionally, reactions for the detection of *sxtA1* and *sxtA4* sequences contained 1% v/v dimethylsulfoxide (DMSO, Finnzymes). The template was either 1 µL of sample lysate or 1 ng of gDNA. The thermal cycling for *sxtA* and *sxtG* was carried out as follows: 98 °C, 30 s; 35 cycles of 98 °C, 5 s; 62 °C, 5 s; 72 °C, 10 s; and finally 72 °C, 1 min. In the analysis of dinoflagellate *sxtA1* and *sxtA4*, a gradual decrease of annealing temperatures was employed according to Stüken *et al*., (2011) and Suikkanen *et al*. (2013). Reaction outcomes were determined using agarose gel electrophoresis and visualized using the Bio-Rad GelDoc XR system.

4.5 Quantitative real-time PCR

4.5.1 Detection principles

The real-time quantitative PCR assays targeted at toxin biosynthesis genes in cyanobacteria (**I–III**) and dinoflagellates (**IV**), as well as the internal amplification control (**II**, **III**) were based on the assay principle originally published by Nurmi *et al*. (2002) (Figure 3).

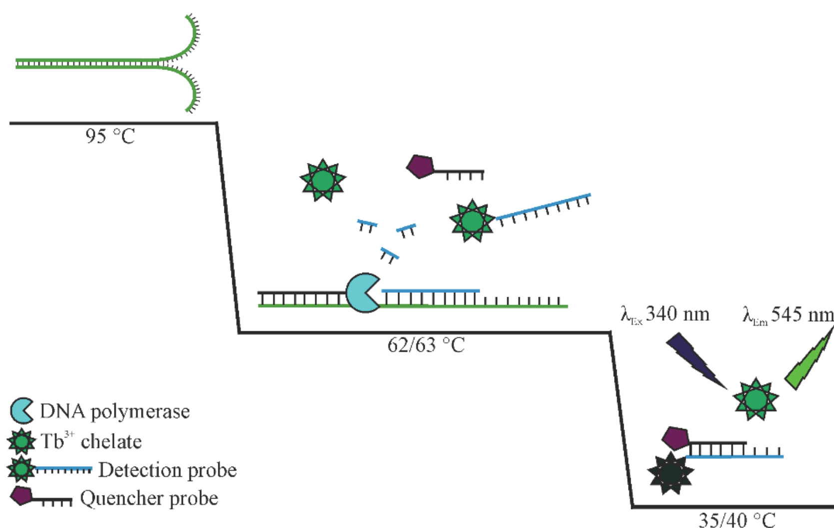

Figure 3. The principle of the probe-based real-time quantitative PCR assays used in the original publications **I**–**IV**. As the thermal cycling proceeds, each probe bound to a template strand is degraded by the 5'→3' exonuclease activity of the DNA polymerase, freeing the intrinsically fluorescent label (here Tb^{3+} chelate) originally attached to the 5' end of the probe in the solution. Before each measurement step, fluorescence from the remaining intact probes is quenched as the decreased temperature allows them to hybridize to the short quencher probes. Thus, the accumulation of the amplification product is reflected by the amount of free label in the reaction solution.

An intercalating dye, SYBR® Green I, was utilized in the quantification of the *A. ostenfeldii* large ribosomal subunit (LSU) gene (**IV**). The dye binds to all double-stranded DNA, resulting in a significant increase in its fluorescence level, enabling the monitoring of amplicon accumulation (Wittwer *et al.*, 1997). The binding is not target-specific, and the final identification of the amplification product or products is carried out by melting curve analysis.

4.5.2 DNA standards for quantification

The DNA standards for absolute quantification in the qPCR assays were produced by amplifying the target regions in *Anabaena* sp. 90, *M. aeruginosa* PCC 7806, *P. agardhii* NIVA-CYA 299 (*mcyB*), *A. circinalis* CS-537/13 (*sxtB*), *B. subtilis* ATCC 6633 (IAC), and *A. ostenfeldii* AOF0933 (*sxtA4* and LSU). The resulting amplicons were purified and quantified with the Qiagen Qiaquick PCR Purification kit and the QuantIt PicoGreen dsDNA kit, respectively, according to the instructions provided by the manufacturers. Dilution series of the obtained standards were then prepared; 5 and 10–10^7 copies per reaction for the toxin biosynthesis genes, 10–10^5 copies per reaction for the IAC, and 10–10^8 copies per reaction for the LSU were used.

4.5.3 Quantitative PCR reaction setup and thermal cycling

The sequences of all oligonucleotides (manufactured by biomers.net) used are listed in Table 8. All detection probes contained a phosphate group at the 3' end to prevent elongation by the DNA polymerase, and an amino-C6-modification at the 5' end to allow labeling with an intrinsically fluorescent Eu^{3+} or Tb^{3+} chelate; the procedures were carried out according to Nurmi *et al*. (2002). The quencher probes were labeled with either Black Hole Quencher® 1 or 2 by the oligonucleotide manufacturer, their absorption wavelengths corresponding to the emission maxima of Tb and Eu, respectively.

The qPCR reagents and their concentrations used in the probe-based assays are listed in Table 7. The 20 µL reactions contained 4 or 8 µL of sample lysate or 1-10 ng of DNA in the same volume. DyNAzyme II HotStart (Finnzymes) and GoTaq HotStart (Promega) DNA polymerases, with the designated buffers, were used in *mcyB*, *sxtB*, and IAC; or *sxtA4* qPCR assays, respectively. The LSU qPCR utilized a ready-made master mix (SsoAdvanced SYBR® Green Supermix, Bio-Rad) and 300 nM primers in a 25 µL volume, including a template in a 4 µL volume as above.

Table 7. The reagent concentrations used in the probe-based qPCR assays.

Target	Primers nM	Detection probe nM	Quencher probe nM	DNA polymerase U $reaction^{-1}$	dNTP mM	$MgCl_2$ mM added	DMSO % (v/v)
mcyB	300, 100*	12.5	125, 150**	0.2	0.2	-	-
sxtB	400	3	24	0.2	0.2	-	-
IAC	300	15	150	0.2	0.2	-	-
sxtA4	500	15	240	0.8	0.4	3	5

*The 3' primer was used in the former, each of the genus-specific 5' primers in the latter concentration

**The mcyB-mQ and mcyB-pQ probes were used in the former, the mcyB-aQ probe in the latter concentration

The thermal profiles of the 96-well plate format assays followed the principle shown in Figure 3, and cycling was carried out in a MJ Research PTC-200 or a Bio-Rad C-1000 Touch thermal cycler. The cycling parameters are shown in Table 9. The initial denaturation steps at the beginning of each assay were carried out for 5 min at the denaturation temperatures listed in Table 9, except for the LSU assay, in which the hold time was 2 min.

Table 8. Oligonucleotides used in real-time quantitative PCR assays (I-IV).

Oligonucleotide	Sequence (5'-3')	Target	Reference
mcyBHF03A, 5' primer	GCTTTAATCCACAAGAAGCTTTATTAGC	*mcyB*, *Anabaena*	I, II
mcyBHF03M, 5' primer	AGATTTTAATCCACAAGAAGCTTTATTAGC	*mcyB*, *Microcystis*	
mcyBHF03P, 5' primer	GGTTTAATCAACAAGAGGCTTTATTAGC	*mcyB*, *Planktothrix*	
mcyBHR04, 3' primer	CTGTTGCCTCCTAGTTCAAAAAATGACT	*mcyB*	
mcyB-aP, detection probe	ACTGAATTATTGGAGGTAGAGGTGAGTGATAC	*mcyB*, *Anabaena*	
mcyB-aQ, quencher probe	CCTCTACCTCCAATAATTCA		
mcyB-mP, detection probe	GGGTGAGTTATTAGAAGCAGAAGTTAGTAACAG	*mcyB*, *Microcystis*	
mcyB-mQ, quencher probe	TTCTGCTTCTAATAACTCACC		
mcyB-pP, detection probe	GGGGTGAATTATTAGAAATAGAAGTAAGTGACAA	*mcyB*, *Planktothrix*	
mcyB-pQ, quencher probe	TTACTTCTATTTCTAATAATTCACC		
sxtB_F2, 5' primer	TGTTGTGCTTGCTGCTCTATCAG	*sxtB,* cyanobacteria	III
sxtB_R2, 3' primer	CAGCGTTTTCAGCGTAYCGAC		
sxtB_P, detection probe	CAATCAAAGTTATGCTCCCTATACGA		
sxtB_Q, quencher probe	GGGAGCATAACTTTGATTG		
sxtA4_220F, 5' primer	GCGGTGCCAAGATACTCGTAGACG	*sxtA4,* dinoflagellates	IV
sxtA4_348R, 3' primer	ATGTAGACGATGTTGTTCTCCGC		
sxtA4_P_245_26, detection probe	CTCGCATGGCTGCGGCGTTCTTGGCC		
sxtA4_Q, quencher probe	CGCAGCCATGCGAG		
AO-Q-LSU1, 5' primer	TGAGATTGTTGCGTCCAC	LSU, *Alexandrium ostenfeldii*	IV
AO-Q-LSU2, 3' primer	TTCCAATGCCCACAGG		
Bs5', 5' primer	GCGGAGCAAGCTTCGTACCTTCT	*Bacillus subtilis*	Korpimäki *et al*., 2007
Bs3', 3' primer	CTAACGCCCAGAACACCGATTGAGT		
BsP, detection probe	CCATACCAGGACGGCAGTTCTCAGC		
BsQ, quencher probe	CTGCCGTCCTGGTATGG		

In the probe-based assays, the first measurement of time-resolved Eu or Tb fluorescence was done after 8 cycles, and then repeated after every second cycle using either a Victor^2 Multilabel Counter or a Victor X4 Multilabel Reader (PerkinElmer). The prompt fluorescence measurements in the LSU qPCR assay were carried out after each cycle, in a Bio-Rad CFX96 Touch Real-Time PCR system, which was also used for the thermal cycling. To generate melting profiles, a gradual increase of temperature in 0.5 °C increments from 65 °C to 95 °C was employed, followed by fluorescence measurements after a 5 s hold at each temperature.

Table 9. Thermal cycling parameters in the plate-based qPCR assays.

Target	Denaturation	Annealing and extension	Measurement	Number of cycles
mcyB	95 °C, 30 s	62 °C, 60 s	35 °C, 15 s	40
sxtB	95 °C, 30 s	62 °C, 60 s	35 °C, 15 s	40
IAC	95 °C, 30 s	62 °C, 60 s	35 °C, 15 s	40
sxtA4	95 °C, 30 s	63 °C, 60 s	40 °C, 24 s	46
LSU	98 °C, 5 s	58 °C, 30 s	-	40

4.5.4 Quantification of *mcyB* genes on dry chemistry chips (II)

The 96-well plate-based *mcyB* qPCR assay was also introduced into a dry chemistry chip format. Three different types of *mcyB* detection chips, specific to the genera *Anabaena*, *Microcystis*, or *Planktothrix*, and IAC chips were prepared. The reagents, including 14 nmol dNTPs, 2 U DyNAzyme II DNA polymerase (Finnzymes), a buffer (35 mg bovine serum albumin (BSA), 192.5 nmol $MgCl_2$, 350 nmol Tris, 1.75 mmol KCl, pH 8.3), primers (mcyBHF03A, mcyBHF03M, and mcyBHF03P; 5.85 pmol each; and mcyBHR04, 17.5 pmol; or Bs5' and Bs3', 17.5 pmol each), detection probes (525 fmol, corresponding to the chip type), and quencher probes (5.25 pmol corresponding to the chip type, or 6.3 pmol mcyB-aQ), were dispensed in two separate droplets onto the empty chips (Abacus Diagnostica). The first droplet contained all other reagents except the BSA and the DNA polymerase, which were included in the second droplet as described by von Lode *et al*. (2007). The chips were then dried under vacuum, sealed, and stored at room temperature (von Lode *et al*., 2007). The sample volume was 35 µL, and an automated nucleic acid analyzer platform was used to run the assays (GenomEra, as described in 2008 by Hagren *et al*.). The thermal cycling principle, which is based on the transfer of the reaction vessel between thermal blocks maintained at a constant temperature, as well as the schematic layout of the optics of the instrument have been described previously (Hagren *et al*. 2008) Thermal cycling was carried out as follows: 100 °C, 2 min; 10 cycles of 62 °C, 16 s; 100 °C, 4.5 s; and 18 cycles of 40 °C, 20 s (measurement); 62 °C, 25 s; 100 °C, 4.5 s; 62 °C, 16 s; 100 °C, 4.5 s.

4.6 Data analysis

4.6.1 Quantitative PCR

The raw fluorescence data obtained from *mcyB*, *sxtB*, and *sxtA4* qPCR experiments were analyzed; signal-to-background ratios were calculated for all reactions individually, with the mean signal from the first two measurements from each well used as the background. The resulting ratios were plotted against their respective cycle numbers to produce the amplification curves from which quantification cycles (C_q) were determined visually (**I**–**IV**). The data from the LSU qPCR experiments were analyzed and C_q-values determined using the CFX Manager Software version 3.1 (Bio-Rad) (**VI**). For all assays, C_q-values were considered as outliers and excluded from further analysis if they deviated from the other replicates by more than one cycle. Amplification in a sample was considered to be inhibited at a non-detection of the IAC, or if it's recovery was below 10% (**I**–**III**). For standard curves and sample dilution series, amplification efficiencies were determined using the equation $E = (10^{(-1/slope)}-1) \times 100\%$ (Higuchi *et al*., 1993, Kubista *et al*., 2006).

4.6.2 Statistical analysis

All statistical testing was carried out using the R Statistical package versions 2.15.1 and 3.2.0 (R Core Team, 2015). To enable correspondence analysis, an additional package, "ca", was installed (Greenacre and Nenadic, 2013). The Spearman rank correlation test was used to study the relationship between gene copy numbers and toxin concentrations in the environmental samples (**I**–**IV**). A non-parametric test was chosen due to the non-normal distribution of numeric results. Copy number differences between sample groups allocated according to total microcystin concentration were analyzed with pair-wise Mann-Whitney U tests (**II**). Correspondence analysis was used to study the co-occurrence of microcystin variants and genus-specific *mcyB* genotypes (**II**).

5 SUMMARY OF RESULTS AND DISCUSSION

In this doctoral work, several candidate genes were studied for their potential as quantitative targets for the estimation of toxicity of cyanobacterial and dinoflagellate blooms, using both conventional, plate-based qPCR as well as a dry chemistry approach. Furthermore, sample preparation methods were evaluated together with the developed assays to achieve high template yields and accurate quantification results. A summary of the results and discussion is presented here.

5.1 Sample preparation and qPCR controls

5.1.1 Comparison of DNA extraction and cell lysis methods

Sample preparation is a crucial part of the process of nucleic acid quantification, the accurate copy number determination being not only reliant on the performance of the qPCR method, but also on the yield of the target nucleic acid in the sample. Multi-step DNA extraction and purification methods easily suffer from DNA loss during the process, and commonly used commercial reagent kits utilizing the adsorption of DNA onto silica membranes can experience both extraction bias and yield issues.

In the original publication **I**, template yield from microcystin-producing strains of the genera *Anabaena*, *Microcystis*, and *Planktothrix* was investigated using known numbers of cells as starting material for three types of sample preparation methods: DNA extraction by a conventional phenol–chloroform method (Kurmayer and Kutzenberger, 2003), a commercial reagent kit, and cell lysis by heat-treatment (Figure 4). Heat-treatment consistently yielded the highest *mcyB* copy numbers per cell in *M. aeruginosa* NIVA-CYA 140, *A.* cf. *flos-aquae* NIVA-CYA 267/4, and *P. agardhii* NIVA-CYA 299, and differences of up to two orders of magnitude were observed between the methods used. The results agree with previous findings, which show that DNA yields from phenol-chloroform extraction are higher compared to commercial silica column kits (Schober and Kurmayer, 2006), which in turn are comparable to cell lysis carried out in a detergent solution (Rasmussen *et al*., 2008). Gene copy numbers exceeding the number of cells were also observed, although *Microcystis* genome sequencing has revealed that the target, *mcyB*, is a single-copy gene (Kaneko *et al*., 2007; Frangeul *et al*., 2008). This phenomenon was the most pronounced in the case of *Microcystis*, and has been observed in the natural environment as well (Vaitomaa *et al*., 2003; Yoshida *et al*., 2008). Up to 10-fold overestimations of gene copy number-based cell counts are known to occur in *M. aeruginosa* during the transition between growth phases (Kurmayer and Kutzenberger, 2003). The reasonable explanation for copy number-based overestimation is therefore the presence of multiple genome copies in one cell, which has been shown to occur in cyanobacteria (Labarre *et al*., 1989; Becker *et al*., 2002). The results obtained using cultured cyanobacteria were confirmed with environmental samples (Figure 5). The *Planktothrix* cell concentrations were not directly comparable to *mcyB* copy numbers in the environmental samples, as the

population probably consisted of both toxic and non-toxic strains, a situation commonly encountered in nature (Dittmann and Wiegand, 2006).

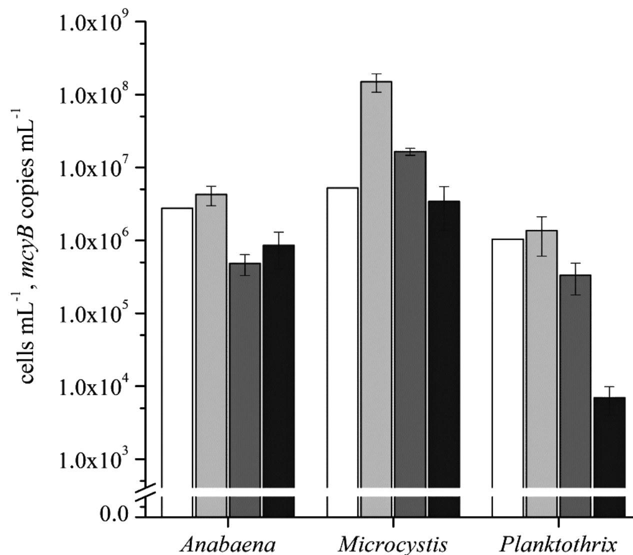

Figure 4. Comparison of copy number yields of sample preparation methods for cyanobacteria. Microscopically determined cell amounts (white) were compared to detected *mcyB* gene copies in heat-treated templates (light grey), and genomic DNA extracted with the phenol-chloroform method (medium grey), or a commercially available kit (dark grey).

Extraction of DNA from armored dinoflagellates, including *Alexandrium* spp., can prove difficult. The cells are covered in tough cellulose plates, called theca, and cell lysis is more difficult to achieve compared to cyanobacteria. In the original publication **IV**, a method based on the combination of heat, a detergent solution, and mechanical disruption was employed in the preparation of dinoflagellate strains and environmental samples according to Garneau *et al*. (2011). The direct use of the obtained lysate as a qPCR template yielded 2–2.5 times as high LSU copy numbers per dinoflagellate cell compared to the use of genomic DNA extracted with a commercial kit (Figure 6). Similar results have been obtained previously by Erdner *et al*. (2010). However, possible PCR inhibitors present in the original sample are not removed during the processing into crude lysate, which may be problematic. Also some reagents, such as phenol in phenol–chloroform extraction, or detergents, as in the method employed in the preparation of dinoflagellate samples, can completely inhibit amplification (Wilson, 1997). Dilution is an effective means to eliminate inhibitory effects caused by environmental agents or detergent solutions (Schrader *et al*., 2012), but to maintain a desired detection limit, the dilution factor needs to be taken into account when sampling volumes are decided.

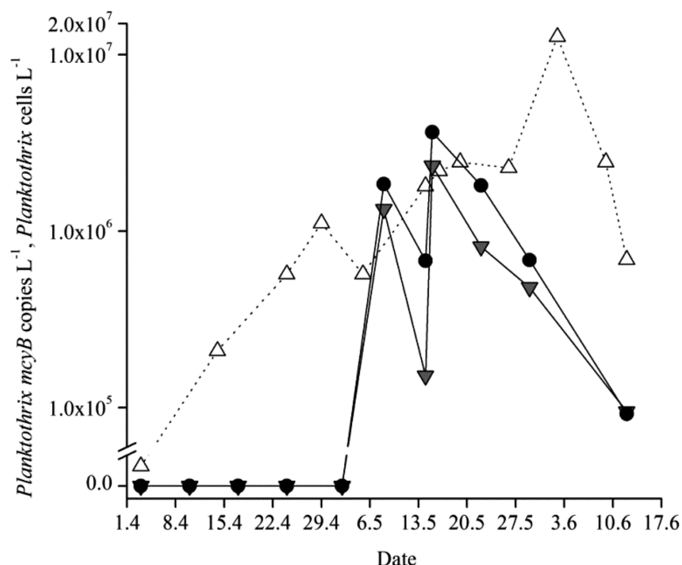

Figure 5. The *mcyB* copy numbers detected in a series of samples collected from Hauninen drinking water reservoir in Raisio, Finland in April–June 2008. Target gene copy numbers are depicted as black circles (sample preparation by heat treatment) and grey triangles (phenol–chloroform DNA extraction). Cell counts are shown in white triangles.

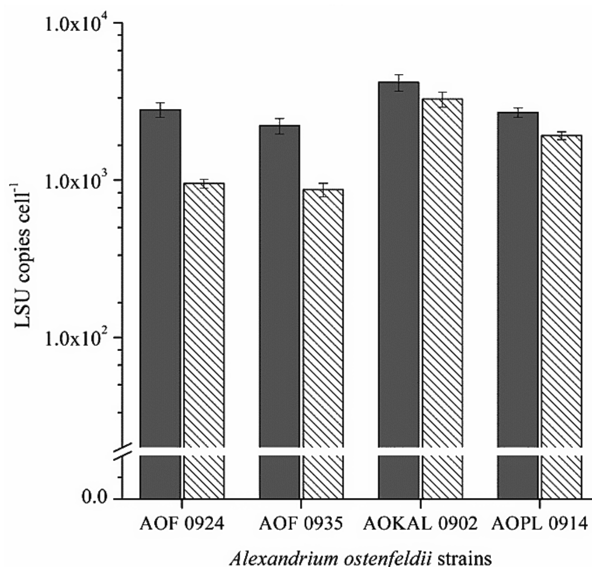

Figure 6. The LSU copy numbers calculated for four *Alexandrium ostenfeldii* strains originating in the Baltic Sea. The copy numbers derived from cell lysates (grey) are consistently higher indicating sample loss during a conventional DNA extraction and purification process (pattern).

5.1.2 Amplification controls

Environmental samples often contain substances inhibiting PCR amplification, such as humic acids and large amounts of non-target DNA, and PCR inhibition can lead to the underestimation of target copy numbers in a sample, or even false negative results (*e.g.*, Wilson, 1997, and references therein). One way to overcome this problem is to introduce a control into the sample or the PCR reaction, the first option accounting for both the sample preparation step and the actual reaction where the target amplification takes place (Schrader *et al*., 2012). In original publications **II** and **III**, laboratory-grown *Bacillus subtilis* cells were introduced to the samples to serve as an exogenous internal amplification control (IAC) for sample preparation and amplification. In the absence of environmental sample material, 84% (sd 18%) of the expected number of IAC copies was observed in the qPCR analysis. The effect of a more complex sample matrix on the outcome was apparent, the mean recovery decreasing and variation increasing (63%, sd 48%). Significant inhibition of IAC amplification (<10% recovery) was not observed in the 2009 sample panel (**II**), but inefficient amplification was observed in three samples collected in 2010 and 2011 (**III**). Upon a 10-fold dilution of the affected samples, the amplification of the IAC was restored as expected. Since the target *sxt* genes remained undetected despite the treatment, the samples were confirmed to be true negatives.

Employing a corresponding control strategy for the dinoflagellate samples was not feasible, as an organism with a similar response to the chosen cell lysis method was not available. In addition to the fact that it was difficult to match the structural characteristics of dinoflagellate cells, the typical 60–70% GC-content of the genome (Hackett *et al*., 2004) would have complicated the choice of a suitable control. Therefore, in the analysis of environmental dinoflagellate samples, the amplification of an LSU sequence with *Alexandrium ostenfeldii*-specific primers in a separate PCR reaction was used to confirm successful sample preparation and the absence of inhibitory substances in the lysate (**IV**). Housekeeping genes can be used as internal controls, and have been included in in quantitative monitoring of cyanobacterial blooms, for example (Al-Tebrineh *et al*., 2010; Baker *et al*., 2013). The results obtained from serial 10-fold dilutions gave no indication of impaired PCR amplification, the PCR efficiencies remaining over 90%.

5.2 Detection and quantification of toxin biosynthesis genes in culture

Starting with the microcystin biosynthesis genes in the late 1990s and early 2000s, the discovery of the genetic background behind various cyanobacterial and dinoflagellate toxins, such as microcystin (Dittmann *et al*., 1997; Tillett *et al*., 2000; Christiansen *et al*., 2003; Rouhiainen *et al*., 2004; Fewer *et al*., 2007; Fewer *et al*., 2013; Shih *et al*., 2013) and paralytic shellfish toxins (Kellmann *et al*., 2008a; Mihali *et al*., 2009; Stucken *et al*., 2010; Mihali *et al*., 2011, Stüken *et al*., 2011) has made it possible to develop qualitative and quantitative nucleic acid-based methods targeted at these toxin-specific genes. The specificity of a qPCR assay can depend on primers alone, *i.e.*, when non-

specific intercalating dyes are used to detect the accumulating amplicon. Primers together with one or several sequence-specific probes can also be used, and in this case the probe or probes provide added sequence specificity to the assay. The latter approach was utilized in the detection and quantification of toxin-specific genes in original publications **I**–**IV**.

5.2.1 *mcyB* (I–II)

The microcystin biosynthesis genes have their specified functions in the construction or modification of the toxin molecule, as discussed in section 2.2.2. The *mcy* genes encode enzymes that are specific to microcystin biosynthesis (Nishizawa *et al.*, 2000; Tillett *et al.*, 2000). Microcystins contain two variable amino acid residues (Carmichael et al., 1988). Substrate flexibility of the enzymes responsible for the incorporation of these amino acids is reflected in the corresponding gene sequences (Dittmann *et al.*, 1997; Nishizawa *et al.*, 1999; Nishizawa *et al.*, 2000; Tillett *et al.*, 2000; Kurmayer *et al.*, 2002; Mikalsen *et al.*, 2003), which could affect the specificity of assays designed to detect potentially microcystin-producing cyanobacteria via the amplification of these genes. The objective of publication **I** was to develop a qPCR assay that would be able to detect and quantify an *mcy* gene sequence from three common cyanobacterial genera. Therefore the identification of a suitably conserved target sequence was crucial. The *mcyB* qPCR assay was designed to target the conserved second thiolation motif within the peptidyl carrier protein domain encoding region in the *mcyB* gene, which serves an important function; the growing polypeptide chain is detached from McyB and transferred to McyC for further elongation (Nishizawa *et al.*, 1999; Tillett *et al.*, 2000). Therefore the sequence of the thiolation motif is unlikely to be affected by amino acid variation in the toxin, making it a suitable target for a qPCR assay.

The primers were designed to amplify a sequence within the *mcyB* genes of the microcystin-producing cyanobacteria belonging to the genera *Anabaena*, *Microcystis* and *Planktothrix*. Using the primers, the *mcyB* gene was detected in all microcystin-producing cyanobacteria investigated, and additionally a homologous sequence from the *ndaA* gene of *Nodularia harveyana* was amplified (Table 10). Each of the 5'-primers, although originally designed to primarily hybridize with a *mcyB* sequence of a single genus, was able to hybridize to the *mcyB* target sequence across genus boundaries. To ensure efficient amplification, a mixture of 5' primers together with a single 3' primer was used in the final assay. The *mcyB* second condensation, adenylation, and thiolation motifs share sequence similarity with the nodularin biosynthesis gene *ndaA* (Moffitt and Neilan, 2004). Consequently, the *mcyB* primers could be used to amplify a region of the *ndaA* gene corresponding to the target *mcyB* sequence from L-HAr^2-nodularin-producing *Nodularia sphaerocarpa* PCC 7804 (Beattie *et al.*, 2000). The detection probes, however, were very efficient in sequence discrimination.

Table 10. The detection of toxin biosynthesis genes in species of cyanobacteria.

Species	Strain	Toxins produced	*mcyB*	*sxtA*	*sxtG*	*sxtB*
Anabaena sp.	90	MC	+	na	na	na
Anabaena circinalis	CS-337/01	PST	na	+	+	+
	CS-530/05	PST	na	+	+	+
	CS-533/12	-	na	-	-	-
	CS-537/13	-	na	-	-	-
Anabaena cylindrica	PCC 73105	-	-	-	-	-
	PCC 7938	-	-	-	-	-
Anabaena flos-aquae	NIVA-CYA	MC	+	na	na	na
	NIVA-CYA 499	-	-	na	na	na
Anabaena lapponica	966	CYN	-	na	na	na
Anabaena lemmermannii var. minor	NIVA-CYA 83/1	MC	+	-	-	-
	NIVA-CYA	MC	+	-	-	-
Cylindrospermopsis raciborskii	CS-505	CYN	na	-	-	-
	CS-506	CYN	na	-	-	-
	CS-510	-	na	-	-	-
Microcystis aeruginosa	NIES 107	MC	+	na	na	na
	NIVA-CYA 57	MC	+	na	na	na
	NIVA-CYA 140	MC	+	-	-	-
	NIVA-CYA	MC	+	na	na	na
	NIVA-CYA 495	MC	+	na	na	na
	NIVA-CYA 22	-	-	na	na	na
	NIVA-CYA 464	-	-	na	na	na
	PCC 7806	MC	+	-	-	-
	PCC 7820	MC	+	na	na	na
	PCC 7941	MC	+	na	na	na
	PCC 7005	-	-	-	-	-
Nodularia sphaerocarpa	PCC 7804	NOD	*	**	-	-
Nostoc sp.	PCC 6310	-	-	-	-	-
	PCC 7422	-	-	-	-	-
Planktothrix agardhii	NIVA-CYA 15	MC	+	-	-	-
	NIVA-CYA 59/1	MC	+	na	na	na
	NIVA-CYA 299	MC	+	-	-	-
	NIVA-CYA 12	-	-	-	-	-
	NIVA-CYA 21	-	-	na	na	na
	NIVA-CYA 116	-	-	na	na	na
	PCC 7805	-	-	na	na	na
	PCC 7811	-	-	na	na	na

na, data not available; **ndaA*, a homolog of *mcyB*, was detected using the primers described in original publication **I**; **A non-*sxtA* amplification products were obtained with a single primer described in original publication **III**. MC, microcystins; PST, paralytic shellfish toxins; CYN, cylindrospermopsin; NOD, nodularin.

The intended genus-specificity was achieved; *i.e.*, probes specific to *Anabaena*, *Microcystis*, or *Planktothrix* hybridized only to the sequence their design was based on,

and no cross-reactions were observed. Therefore specific detection of three major genera capable of microcystin production was demonstrated using the developed qPCR assay.

Quantification of mcyB on dry chemistry PCR chips (II)

The reaction setup, handling of instrumentation, and analysis of results of a conventional qPCR assay require training and expertise in the field of molecular biology. Due to the sensitivity and exponential nature of PCR amplification, amplicon carry-over contamination in diagnostic applications is a serious, ever-present risk (Borst *et al*., 2004). Automation provides possibilities for the reduction of contamination risk, while ensuring increased reproducibility and comparability of results. It would also make monitoring strategies based on specific amplification of genes available for larger user groups. A possible application area is the monitoring of drinking water sources for potentially toxic cyanobacteria, and in the original publication **II**, the *mcyB* qPCR assay was introduced into a dry chemistry PCR chip format. In this type of assay format, all PCR reagents are predispensed into the reaction vessel, in this case a low-cost polypropylene chip, and dried in place (von Lode *et al*., 2007). The introduction of a sample into the chip dissolves the reagents, and allows the amplification to procede.

The *mcyB* qPCR assay sensitivity and amplification efficiency was improved upon its introduction to the dry chemistry chip format (Table 11); in the dry chip format, signal to background ratios on average 2–2.5 times as high as those that were observed in the 96-well plate assay were found, which contributed to the increase in sensitivity. This increase was due to the reaction vessel and instrumentation choices; the PCR chips are laminated with aluminum foil, allowing very rapid heat transfer, as well as providing a surface capable of reflecting the excitation and emission light, unlike the semi-transparent plastic plate (von Lode *et al*., 2007). In dried form, the PCR reagents take up a negligible percentage of the total reaction volume used in this study. Therefore it was possible to introduce 35 µl of template, the total reaction volume, to the chip, compared to the 4 µL template volume used in publication **I,** in which conventional plates were used. This means that in the *mcyB* chip qPCR assay the same limit of detection in respect to the original water sample could be achieved with the use of template volumes 8.75 times as high compared to the conventional plate-based assay. This makes the detection of low target copy numbers more reliable, since the increase of the absolute number of target sequences in a reaction decreases the effect of stochastic processes during amplification (Weusten and Herbergs, 2012). The slightly increased risk of inhibitor presence due to an increased sample volume can be controlled with the inclusion of an amplification control.

Table 11. Comparisons of the characteristics of the 96-well plate and dry chemistry chip formats of the *mcyB* qPCR assay.

	96-well plate	Dry chemistry chip
Linear dynamic range, *mcyB* copies reaction^{-1}	1×10^{1}–1×10^{7}	5×10^{0}–1×10^{7}
Amplification efficiency, %	91.7–95.3	99.6–103.1
Sample volume, µL	4	35

5.2.2 Cyanobacterial *sxt* genes (III)

The cyanobacterial *sxt* gene cluster consists of 24–32 genes, depending on the genus of the PST-producing cyanobacterium (Kellmann *et al*., 2008a; Mihali *et al*., 2009; Stucken *et al*., 2010; Mihali *et al*., 2011). Evolutionary studies have identified a suite of 14 genes conserved in all of the abovementioned genera (Murray *et al*., 2011a). All PCR-based detection methods have been based on these genes, and *sxtA* in particular has been a commonly used target (see section 2.3.2 for references). The biosynthetic enzymes encoded by the genes *sxtA*, *sxtG*, and *sxtB* are needed to initiate the PST biosynthesis (Kellmann *et al*., 2008a), and the genes were found to be exclusively present in PST-producing cyanobacterial strains tested (Table 10). This is, however, not always the case; the *sxtA* gene has been found also in strains that lack detectable toxin production (Ballot *et al*., 2010; Ledreux *et al*., 2010; Casero *et al*., 2014; Cires *et al*., 2014).

5.2.3 Dinoflagellate *sxtA* gene (IV)

The paralytic shellfish toxin biosynthesis genes in cyanobacteria and dinoflagellates share common features (Stüken *et al*., 2011; Hackett *et al*., 2013; Zhang *et al*., 2014). The organization of the *sxt* genes in the genomes of these organisms seems to vary greatly; while the *sxt* genes are arranged in a cluster in cyanobacteria (Kellmann *et al*., 2008a; Mihali *et al*., 2009; Stucken *et al*., 2010; Mihali *et al*., 2011) currently there is no evidence of a gene cluster in dinoflagellates. Most of the dinoflagellate *sxt* gene suite remains uncharacterized to date (see section 2.2.4), the available sequence information increasing one gene at a time. The *sxtA* was the first to be identified (Stüken *et al*., 2011), and was also the focus in this doctoral study.

According to current knowledge, the presence of the *sxtA4* gene, which encodes the fourth domain of the SxtA protein, correlates positively with PST production, which is not the case with *e.g.*, *sxtA1* (Stüken *et al*., 2011; Hackett *et al*., 2013; Orr *et al*., 2013; Suikkanen *et al*., 2013). In agreement with these studies, toxin production was found to correlate with the detection of *sxtA4* in all the tested dinoflagellate strains with available toxin data (Table 12), while the *sxtA1* sequence, although amplified in all *sxtA4*-positive strains, has also been observed in non-PST-producing strains of *A. ostenfeldii* (Suikkanen *et al*., 2013). Variability in the *sxtA4* sequences can occur within a single genome (Wiese *et al*., 2014, Murray *et al*., 2015), which can make the identification of

a qPCR target challenging. In this study, 95% specificity was achieved with the *sxtA4* qPCR assay, including amplification of *sxtA4* in four *Alexandrium* sp., and *Gymnodinium catenatum*. The short *sxA4* qPCR amplicon from *A. minutum* CCMP 113 remained undetected due to two single nucleotide mismatches within the probe binding site (Table 12).

Table 12. The detection of *sxtA1* and *sxtA4* sequences and PST production in dinoflagellate strains.

Species	Strain	*sxtA1*[1] (550 bp)	*sxtA4*[1] (750 bp)	*sxtA4* (145 bp) *sxtA4* primers	*sxtA4* (145 bp) *sxtA4* probe	PSTs detected, HPLC-FLD
Alexandrium andersonii	CCMP 2222	nd	nd	nd	nd	nd
Alexandrium fundyense	CCMP 1911	+	+	+	+	+
Alexandrium fundyense	CCMP 1979	+	+	+	+	+
Alexandrium minutum	CCMP 113	+	+	+	nd	+
Alexandrium ostenfeldii	AOTVPL25	+	+	+	+	+
Alexandrium ostenfeldii	AOTVA4	+	+	+	+	+
Alexandrium ostenfeldii	AOF 0924	+	+	+	+	+
Alexandrium ostenfeldii	AOF 0927	+[2]	+[2]	+	+	+
Alexandrium ostenfeldii	AOF 0933	+	+	+	+	+
Alexandrium ostenfeldii	AOF 0935	+	+	+	+	+
Alexandrium ostenfeldii	AOF 0957	+	+	+	+	+
Alexandrium ostenfeldii	AOKAL 0902	+	+	+	+	na
Alexandrium ostenfeldii	AOPL 0914	+	+	+	+	na
Alexandrium ostenfeldii	AOVA 30	+[2]	+[2]	+	+	+
Alexandrium ostenfeldii	S06/013/01	+[2]	nd[2]	nd	nd	nd
Alexandrium ostenfeldii	NCH 85	+[2]	nd[2]	nd	nd	nd
Alexandrium ostenfeldii	LS A06	nd[2]	nd[2]	nd	nd	nd
Alexandrium ostenfeldii	CCAP 1119/45	nd	nd	nd	nd	nd
Alexandrium tamarense	SCCAP K1471	nd	nd	nd	nd	nd
Gymnodinium catenatum	CCMP 1937	+	+	+	+	+
Lingulodinium polyedrum	SCCAP K0982	nd	nd	nd	nd	nd
Lingulodinium polyedrum	CCMP 1933	nd	nd	nd	nd	nd

nd, not detected; na, data not available; [1]primers by Stüken *et al.*, 2011; [2]data from Suikkanen *et al.*, 2013.

The genomic sxtA4 copy numbers in A. ostenfeldii

Prior to the discovery of the first paralytic shellfish toxin biosynthesis genes, the localization of the genes in dinoflagellates was a matter of some debate. Although studies on axenic cultures and inheritance pointed to a nuclear location (Sako *et al.*, 1992; Hold *et al.*, 2001), even symbiotic bacteria were suggested as the source of the toxins (See Piel, 2004, and references therein). With the sequencing of the *sxtA* gene, the dinoflagellates were confirmed to possess the ability to synthesize the toxins, and the *sxtA* gene was shown to be encoded in the dinoflagellate genome (Stüken *et al.*,

2011). Since then, multiple studies have demonstrated the specific amplification of *sxtA1*, *sxtA4*, and *sxtG* from dinoflagellate genomic DNA (Orr *et al*., 2013; Suikkanen *et al*., 2013; Stüken *et al*., 2015; Murray *et al*., 2015). There has been shown to be large variability in the genomic copy numbers of *sxtA4* in *Alexandrium* species (Murray *et al*., 2011, Stüken *et al*., 2015). In this study, the genomic copy numbers of the *sxtA4* gene were determined in four *A. ostenfeldii* strains isolated in the Baltic Sea (Table 13) to shed further light onto the relationship between *sxtA4* copy numbers and PST production.

The *sxtA4* copy numbers in the studied *A. ostenfeldii* strains were found to be similar to those in *A. minutum* (Stüken *et al*., 2015), a species with a considerably smaller-sized genome, whereas in *A. pacificum*, with a genome size resembling that in *A. ostenfeldii*, the *sxtA4* copy numbers can be up to two orders of magnitude higher (Figueroa *et al*., 2010; Murray *et al*., 2011b; Stüken *et al*., 2011; Murray *et al*., 2012). Similarly to the low copy numbers of the *sxtA4* gene, *A. ostenfeldii* LSU copy numbers were observed to be in the lower end of the greatly variable range of rDNA content in dinoflagellate genomes (Godhe *et al*., 2008; Galluzzi *et al*., 2010). In accordance with Stüken *et al*. (2015), the results obtained in this study support the hypothesis that genome size does not determine the *sxtA4* copy number in different *Alexandrium* species. An analysis of environmental samples also indicated that at least in *A. ostenfeldii* the copy number might not determine the amount of PSTs produced by an individual cell (see section 5.3.3), as suggested by Murray *et al*. (2011b), but merely confers the ability to produce the toxins.

Table 13. The genomic copy numbers of the LSU and *sxtA4* genes in *Alexandrium ostenfeldii strains* in the Baltic Sea, determined from cell lysates as well as extracted and purified gDNA.

Strain[4]	***sxtA4* copies cell^{-1}**			**LSU copies cell^{-1}**			**Mean**
	Cell lysate	**Extracted gDNA**[1]	**Extracted gDNA**[2]	**Cell lysate**	**Extracted gDNA**[1]	**Extracted gDNA**[2]	***sxtA4*: LSU ratio**[3]
AOF 0924	3–9	1–2	6-7	2 480–3 060	890–1 000	3 240–3 580	1:468
AOF 0935	5–11	4–5	4–5	2 010–2 500	820–970	930–1 100	1:287
AOKAL 0902	4–8	1–2	2–3	3 730–4 730	3 060–3 690	4 010–4 830	1:838
AOPL 0914	3–6	2–3	4–5	2 480–2 820	1 800–2 030	3 290–3 540	1:450

[1]Copy number calculated on the basis total yield of extracted DNA from a fixed number of cells. [2]Copy number calculated on the basis of the amount of DNA put in the qPCR reaction and *A. ostenfeldii* genome size (115 pg cell^{-1}; Figueroa *et al*., 2010). [3]Calculated from the results obtained from cell lysates. [4]For further description on *A. ostenfeldii* strains, refer to Tahvanainen *et al*. (2012) and Kremp *et al*. (2014).

5.3 Toxin production and gene copy numbers in the environment

5.3.1 Microcystins and *mcyB* (I, II)

Microcystins are among the most studied of the cyanobacterial toxins, and biosynthesis has been confirmed in free-living planktonic and benthic, as well as symbiotic genera (Sivonen *et al*., 1990a; Prinsep *et al*., 1992; Luukkainen *et al*., 1993; Oksanen *et al*., 2004; Izaguirre *et al*., 2007; Kaasalainen *et al*., 2009; Fiore *et al*., 2009). The main bloom-forming, potentially toxic genera around the world are *Anabaena*, *Microcystis*, and *Planktothrix*, which were also the targets of the *mcyB* qPCR assay developed in this doctoral study. The correlation between the total number of *mcyB* copies and total microcystin concentrations in the environment was studied in the original publications **I** and **II**.

During a study conducted in the mid-1980s, the prevalence of hepatotoxic cyanobacteria in Finnish lakes was estimated to be 30% (Sivonen *et al*., 1990b). In this study, microcystins were very commonly found, being present in 90% or 71% of the samples collected in 2009, depending on the analysis method, ELISA or LC-MS, respectively. These figures are similar to those found in a more recent survey in Finland (Rantala *et al*., 2006), as well as elsewhere in Europe (Henriksen and Moestrup, 1997; Fastner *et al*., 1999b; Utkilen *et al*., 2001). However, rather than reflecting an increase in toxin prevalence in the last 20 years, the rise in the detection frequency has most likely been due to development in the analytical techniques. Microcystin concentrations in this study ranged from 0 (not detected) to 30.4 (mean 2.3) and 0 to 40.9 (mean 2.0) $\mu g\ L^{-1}$ (n=98, ELISA and LC-MS, respectively), and the most commonly found variants were MC-LR and MC-RR, as well as their respective desmethylated forms (Table 14).

Table 14. The prevalence of microcystin variants in environmental samples collected in 2009 (LC-MS, n=98).

Microcystin variant	Variant present in the sample, no. of samples	Variant dominant in the sample, no. of samples
dmMC-RR	65 (66 %)	40 (41 %)
MC-RR	43 (44 %)	23 (24 %)
dmMC-LR	36 (37 %)	-
MC-LR	33 (34 %)	2 (2 %)
MC-YR	17 (17 %)	1 (1 %)
dmMC-YR	14 (14 %)	-
didmMC-RR	3 (3 %)	-
didmMC-LR	2 (2 %)	-
MC-LF	1 (1 %)	-
MC-LW	1 (1 %)	-
MC-LY	1 (1 %)	-

One of the advantages of a genus-specific qPCR assay is the possibility to study the co-occurrence of the target genera and the different microcystin variants (Figure 7). Some variants, the common MC-RR, MC-LR, and MC-YR, were more likely to be found with *Microcystis* and *Anabaena*, while dmMC-LR and dmMC-YR most often co-occurred with *Microcystis* and *Planktothrix*. Fastner *et al*. (1999a) reported the association of MC-RR, MC-LR, and MC-YR and *Microcystis*-dominated environmental samples, and *Planktothrix* spp. typically produce desmethylated microcystins (Luukkainen *et al*., 1993; Fastner *et al*., 1999a). In agreement with these findings, an association between the occurrence of dmMC-LR and dmMC-RR and *mcyB*-positive *Planktothrix* was observed in the Hauninen drinking water reservoir also during the spring bloom in 2008 (**I**). An association of didesmethyl microcystins with *Anabaena* spp. was observed in this study. The methylation degree of the microcystin molecule can sometimes be traced to certain genetic traits in the microcystin-producing cyanobacterium; the biosynthesis of didesmethylated microcystin variants by *Anabaena* spp. has been documented by Sivonen *et al*. (1992), and it can stem from a deletion in the *mcyA* gene, which directs the incorporation of dehydroalanine (Dha) instead of Mdha during microcystin biosynthesis (Fewer *et al*., 2008).

Bloom observations are the primary method used to monitor and assess the risks associated with potentially toxic cyanobacteria. The prevalence of cyanobacterial mass-occurrences in Finnish freshwaters has been estimated to be 50% (Lindholm *et al*., 2003). However, the genetic potential for microcystin production seems to be far more common, and reflects the frequency of recent toxin findings; 93% of the samples collected in this study were positive for *mcyB*, which is in line with the observations of Rantala *et al*. (2006). Statistically significant positive correlation was observed between *mcyB* copy numbers and microcystin concentrations, both overall (Table 15) and in individual lakes, where the development of the toxic cyanobacterial population was followed over time (Table 16). Similar positive correlations between *mcy* genes in a single target genus and microcystin concentrations have been reported in Europe and North America (Rinta-Kanto *et al*., 2009; Fortin *et al*., 2010; Ostermaier and Kurmayer, 2010; Martins *et al*., 2011). However, the co-occurrence of more than one potentially microcystin-producing genus was very common; a majority, 53%, of the samples collected for this study contained at least two of the targeted *mcyB*-positive genera. Importantly, the total *mcyB* copy numbers showed stronger positive correlation with the total toxin concentrations than any genus separately, underlining the significance of not concentrating on a single target, but instead, taking into account the possible contribution of several potentially harmful cyanobacterial genera.

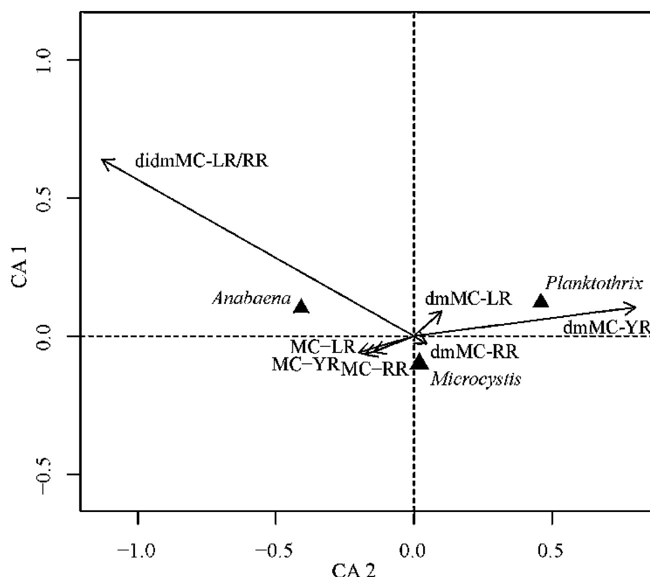

Figure 7. Co-occurrence of specified microcystin variants and genus-specific *mcyB* genotypes in the environmental samples collected in Finland and Estonia in 2009 (n=98). The arrows depict the different microcystin variants, their direction and length corresponding to the strength of the correlation with the different *mcyB* genotypes.

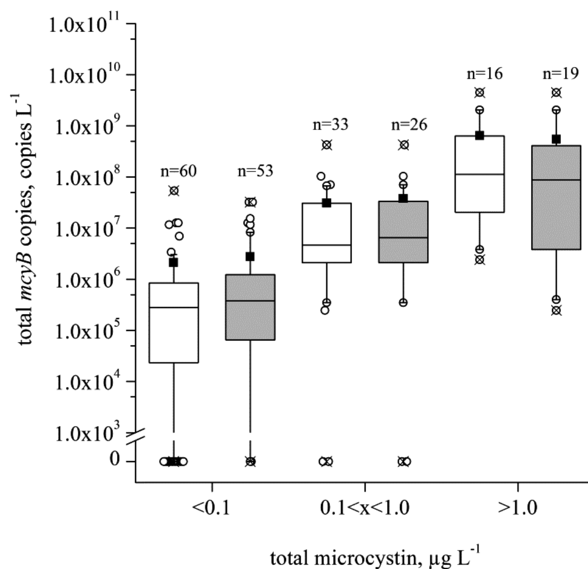

Figure 8. The total *mcyB* copy numbers in environmental samples collected in 2008 and 2009, grouped according to sample microcystin content measured by either LC-MS (white) or ELISA (grey). The boxes range from the 25th to 75th percentile, whiskers ranging from the 10th to the 90th percentile. Minimum and maximum values (×), means (■), medians (line crossing each box) and outliers (○) are indicated.

The World Health Organization currently recommends that the concentration of microcystin-LR in drinking water should not exceed 1 µg L^{-1} (WHO, 2011). On average, water samples with more than 1 µg L^{-1} total microcystin also contained significantly higher amounts of potentially microcystin-producing cyanobacteria compared to samples with less microcystin ($p<0.001$). Importantly, none of the samples with more than 1 µg L^{-1} total microcystin were *mcyB*-negative (Figure 8). Since there are indications that long-term low-level exposure to microcystins may be harmful (Yu, 1995; Svirčev *et al.*, 2009), a statistically significant difference observed between samples with some toxin (0.1–1 µg L^{-1}) and those with none or with very small concentrations (<0.1 µg L^{-1}) ($p<0.001$) might become important from a monitoring point of view.

Table 15. Correlation between *mcyB* copy numbers and total microcystin in environmental samples collected 2008–2009.

Microcystin analysis method	Source of *mcyB* copies	Spearman's ρ	p
ELISA (n=98)	All target genera	0.68	<0.001
	Microcystis	0.50	<0.001
	Planktothrix	0.38	<0.001
	Anabaena	0.17	0.087
LC-MS (n=109)	All target genera	0.69	<0.001
	Microcystis	0.49	<0.001
	Planktothrix	0.33	<0.001
	Anabaena	0.21	0.027

Table 16. The correlation between total microcystin concentrations measured with two different analytical methods and *mcyB* copy numbers in individual lake samples over time, as illustrated by the Spearman rank correlation coefficients (ρ) and the corresponding p-values.

Sampling location	Year	ELISA	LC-MS
Lake Littoistenjärvi, Kaarina, Finland	2009	ρ=0.700, p=0.043	ρ=0.733, p=0.031
Lake Tuusulanjärvi, Tuusula, Finland	2009	ρ=0.737, p=0.023	ρ=0.302, p=0.428
Hauninen Reservoir, Raisio, Finland	2008	na	ρ=0.894, p<0.001
	2009	ρ=0.508, p=0.037	ρ=0.481, p=0.051

Nutrient availability, light intensity, and temperature have been shown to affect gene expression and toxin biosynthesis rates (Kaebernick *et al.*, 2000; Vezie *et al.*, 2002; Downing *et al.*, 2005; Tonk *et al.*, 2005; Straub *et al.*, 2011). The probable effect of these factors on environmental toxin concentrations was indirectly observed also in this study; amplification of genomic *mcyB* was observed in a number of samples that contained no microcystin, or only trace amounts of it. This could have indicated downregulation of *mcyB* expression and resulted in microcystin not being produced at all, or at levels below the detection limits of the analytical methods. Inactive microcystin biosynthesis genes have also been described (Kurmayer *et al.*, 2004; Tanabe *et al.*, 2007;

Ostermaier and Kurmayer, 2009), and the contribution of possible deletion mutants to the populations of apparently *mcy*-positive cyanobacteria cannot be excluded.

5.3.2 Cyanobacterial *sxt* genes and PST production (III)

PCR and qPCR-based methods have been increasingly used to screen environmental samples for PST biosynthesis genes in the recent years, and the results have shown that the potential for toxin production is widespread (see section 2.3.2). Neurotoxicity was first described in Finnish freshwaters in the early 1990s (Sivonen *et al*., 1990b), and PST production was confirmed in 2005 by Rapala *et al*. (2005). In this doctoral study, PCR and qPCR methods were developed and applied to the investigation of the prevalence of cyanobacterial paralytic shellfish toxin biosynthesis genes in combination with PST production in Finnish freshwater lakes for the first time. Three target genes, connected to the crucial first steps of PST synthesis (Kellmann *et al*., 2008a; Murray *et al*., 2011a), were chosen for this study: *sxtA*, *sxtG*, and *sxtB*.

A total of 90 samples were collected at 32 locations including freshwater lakes and brackish coastal waters on Åland Islands during the three-year monitoring period. A third of the samples, 31% on average, were positive for all three *sxt* genes analyzed. The detected *sxtB* copy numbers varied within four orders of magnitude (5.24×10^{4}–5.31×10^{8}, median 1.86×10^{6} copies L^{-1}). The *sxt*-positive samples originated in a total of 14 lakes; 10 of them were *sxt*-positive at least in two successive years, indicating that the potentially neurotoxic cyanobacterial populations were relatively stable, and that they formed blooms on a regular basis. PSTs were detected in 13%, 28%, and 59% of the samples in 2010, 2011, and 2012, respectively, but concentrations remained low; the median was 0.031 $\mu g\ L^{-1}$ and the mean 0.29 $\mu g\ L^{-1}$. Two PST variants were found: saxitoxin and decarbamoyl saxitoxin, in 86% and 16% of the samples, respectively. The majority of the sampling locations were classified as eutrophic or hypertrophic (78%). A limited availability of organic nitrogen has been associated with increased toxin production and release (Dias *et al*., 2002; Casero *et al*., 2014), which might explain the fact that PST concentrations, as well as *sxtB* copy numbers well above the median values were observed in some of the least nutrient-laden lakes studied. However, freshwater environments may not favor PST biosynthesis; increasing salinity has also been shown to lead to PST accumulation in *Cylindrospermopsis raciborskii* (Pomati *et al*., 2004) and release in *Raphidiopsis brookii* (Soto-Liebe *et al*., 2012). Thus, while a cyanobacterial subpopulation in a given lake can possess the genetic capability of PST biosynthesis, the prevailing environmental conditions may restrict toxin production.

Positive correlation between *sxtB* copy numbers and PST concentrations was indicated in the samples when the toxin concentrations rose above the quantification limit of the analytical method (Spearman's $\rho=0.53$, $p=0.012$). A similar trend between *sxtA* copy numbers and PST concentrations has been reported previously (Al-Tebrineh *et al*., 2010). However, when the samples were grouped according to PST content, despite the difference between the mean *sxtB* copy numbers of the two groups (Mann-Whitney U test, $P=0.002$), the overlap between the groups was apparent (Figure 9). Therefore,

neither the presence of the *sxt* genes nor their copy number could be considered a reliable predictor of PST production or quantity. In addition to the potential downregulating effects of zero or low water salinity at the sampling sites (maximum 6-7 psu), deletions in the *sxt* gene cluster may give rise to situations where the target genes will be detected but PSTs are not produced. Such a phenomenon is well-known, and non-functional deletion mutants of *e.g.*, microcystin biosynthesis genes have been characterized (Ostermaier and Kurmayer, 2009), and the detection of cylindrospermopsin as well as PST genes without apparent toxin production has been described (Ballot *et al.*, 2010; Ledreux *et al.*, 2010; Kokocinski *et al.*, 2013; Casero *et al.*, 2014; Cires *et al.*, 2014). Therefore it could be possible that the samples in which *sxt* genes were amplified but PSTs were not detected could have contained *sxt* gene clusters inactivated by mutations. PSTs were also observed in samples which were negative for the targeted *sxt* genes. PCR inhibition was ruled out with the use of an amplification control, but although the *sxt* genes selected for this study, *sxtA*, *sxtB*, and *sxtG* show a high level of sequence conservation (Murray *et al.*, 2011a), the possibility of the presence of unknown PST producers cannot be excluded. Since the sampling sites included coastal locations, PST-producing dinoflagellates, known to bloom in the Åland archipelago (Kremp *et al.*, 2009), may have contributed to the toxin findings at these sites.

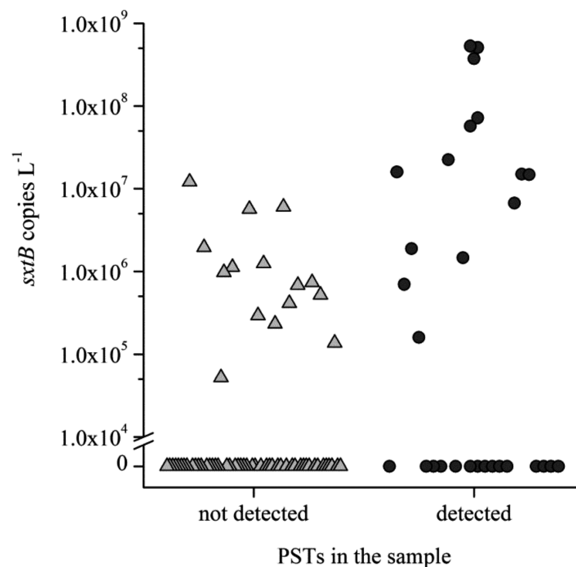

Figure 9. The distribution of *sxtB* copy numbers within two sample groups: 1) PSTs not detected (n=62, 76% *sxtB* negative samples) and 2) PSTs detected (n=29, including trace detections, 52% *sxtB* negative samples).

Identification of the main cyanobacterial PST producer

In the central and south European freshwaters the cyanobacterial PST producers are typically members of the genus *Aphanizomenon*, *e.g.*, *A. gracile* (Pereira *et al.*, 2000; Ferreira *et al.*, 2001; Ballot *et al.*, 2010; Ledreux *et al.*, 2010). However, in the Nordic countries, *Anabaena,* especially *A. lemmermannii*, has been more often connected to the occurrence of PSTs (Kaas and Henriksen, 2000; Rapala *et al.*, 2005). In the original publication **III**, one of the aims was to identify the cyanobacterial genus or genera responsible for PST production in freshwater lakes and coastal waters in Åland Islands. A total of 19 main cyanobacterial genera were identified with microscopic analyses. To study the co-occurrence of cyanobacterial genera and PST production and/or *sxt* gene presence, the number of samples in which each specified genus was observed was counted, and then this group of samples was divided into categories based on *sxt* gene and PST presence (Figure 10).

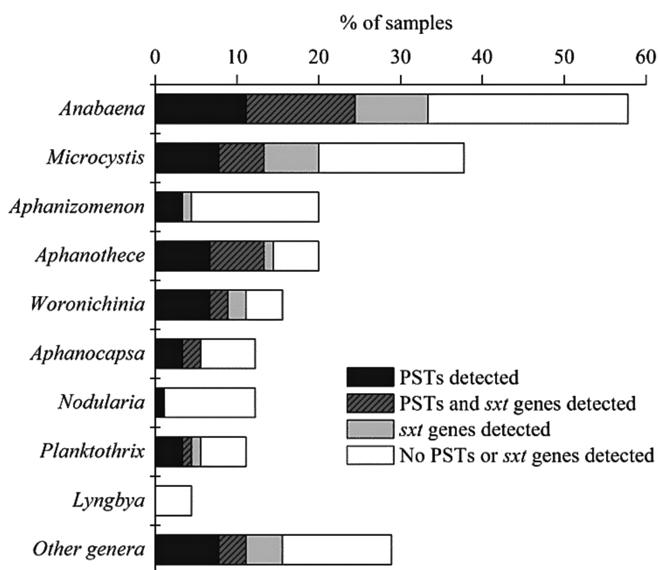

Figure 10. Prevalence of cyanobacterial genera in the samples collected in Åland Islands in 2010–2012 (n=90). The number of samples in which each specified genus was observed was counted, after which the group of samples obtained this way was divided into categories based on *sxt* gene and PST presence. The process was repeated for each genus independently. The cumulative sum of these categories indicates the total percentage of samples in which a cyanobacterial genus was observed. Other genera include *Achroonema*, *Chroococcus*, *Cyanodictyon*, *Gloeotrichia*, *Merismopedia*, *Nostoc*, *Oscillatoria*, *Phormidium*, *Pseudanabaena*, and *Snowella*.

In agreement with previous observations from Finland and Denmark (Kaas and Henriksen, 2000; Rapala *et al.*, 2005), *Anabaena* was most often associated with both the genes and the toxins; the genus was present in 72% of the *sxt*-positive and 71% of the PST-positive samples. Within the genus *Anabaena*, the presence of *A.*

lemmermannii was emphasized in the PST and *sxt*-containing samples, in line with the observations of Rapala *et al*. (2005), who also reported that the only PST variant found was STX, which also dominated in the PST-positive Åland Islands samples. *Aphanizomenon*, however, was present only in a few *sxt*- and toxin-containing samples (n=1 and n=4, respectively), indicating that it may have had an insignificant role or no part at all in PST production in the studied bodies of water.

The partial *sxtA* genes amplified from the environmental samples shared significant nucleotide identity with both *Anabaena circinalis* and *Aphanizomenon flos-aquae* sequences: 98.5–99.7% and 98.8%–99.5% identical bases, respectively. Slightly lower levels of identity were observed between the environmental *sxtA* sequences and *Cylindrospermopsis raciborskii* or *Lyngbya wollei sxtA* genes, 93.6–94.3% and 92.3–93.0%, respectively. Although one exact match with the *A. flos-aquae* strain NH-5 *sxtA* sequence was found, *Anabaena* was one of the main cyanobacterial genera present in that sample, while *Aphanizomenon* was not observed. Overall, despite the slightly higher nucleotide identity between the Åland sequences and *A. flos-aquae* compared to *A. circinalis*, the results from the microscopic analysis, the toxin profile, as well as the pronounced co-occurrence of *Anabaena* with both the investigated *sxt* genes and PST lead to the likely conclusion that the majority of cyanobacterial PST producers in Åland Islands belonged to the genus *Anabaena*.

5.3.3 Dinoflagellate *sxtA* and PST production (IV)

Recurrent blooms of *Alexandrium ostenfeldii* are known to occur in the Baltic Sea (see section 2.3), including certain areas in the Åland archipelago in Finland (Kremp *et al*., 2009). In the original publication **IV**, the intracellular PST concentrations, *A. ostenfeldii* cell density, and the presence and quantity of a PST biosynthesis gene, *sxtA4*, were studied using samples collected at Föglö, Åland Islands.

Cells of *A. ostenfeldii* were present at all three sampling stations (Figure 11). At two of the stations (denoted 3 and 4, see Kremp *et al*., 2009), the cell densities and toxin concentrations resembled those previously recorded in the area (Hakanen *et al*., 2012). The third sampling station, Lillängö, was located west of station 3, between stations 1 and 2 as described by Kremp *et al*., (2009). At Lillängö, the bloom was exceptionally dense, and clearly visible in the water as a reddish brown discoloration. At night, strong bioluminescence characteristic of *A. ostenfeldii* was observed. Microscopic enumeration confirmed that the cell density peaked at 6.3×10^6 cells L^{-1}, which is the highest recorded for the species to date, exceeding previous observations at Föglö (Kremp *et al*., 2009), and in the Netherlands (Burson *et al*., 2014).

The toxin variant composition was typical for *A. ostenfeldii* strains isolated in the Baltic Sea (Kremp *et al*., 2014). All samples contained GTX3 (66–74%), GTX2 (8–14%), and STX (18–20%), confirmed by both HPLC-FLD and LC-MS (Figure 11). Likewise, dinoflagellate *sxtA4* and *A. ostenfeldii*-specific LSU sequences were amplified in all samples. The highest total PST concentrations coincided with the high cell densities at

Lillängö, reaching 70 µg L^{-1}. The copy numbers of the target genes *sxtA4* and LSU ranged between 2.5×10^4–8.0×10^4 and 1.0×10^7–2.5×10^7 copies L^{-1}, respectively. The corresponding copy numbers at Lillängö were on average two orders of magnitude higher: 7.7×10^5–9.4×10^6 and 3.0×10^8–3.0×10^9 cells L^{-1}, respectively, reflecting the differences between cell densities at the different sampling stations. Cellular toxin quotas varied between 4–41 pg $cell^{-1}$, and were similar to previous measurements from the same area (Hakanen *et al*., 2012).

The presence of *sxtA4* gene copies predicted PST production at Föglö without exception; this was in agreement with previous studies which have linked both the total quantity of *sxtA4* copies and expression of the same gene to PST production in the natural environment (Murray *et al*., 2011b; Stüken *et al*., 2013). Positive correlation was observed between *sxtA4* and LSU copy numbers, as well as between *A. ostenfeldii* cell density and total PST concentrations (Table 17). The correlation was statistically significant at the 0.05 level between PST concentrations and LSU copy numbers or cell densities. Highly significant positive correlation between *sxtA4* and LSU copy numbers in the Föglö samples was observed ($\rho=0.96$, $p<0.01$), and the average *sxtA4*–LSU copy number ratio, 1:327, was similar to ratios determined for cultured Baltic Sea *A. ostenfeldii*. The constant *sxtA4*–LSU ratio indicates that the whole *A. ostenfeldii* population was able to produce PSTs. Despite this, the absolute cellular copy numbers of both genes were lower than in the cultured strains (see section 5.2). The reason for this difference is not clear, although several factors could have affected the results, including loss of sample material during collection and preparation, local cell density differences, and the possible presence of DNA-sequestering compounds in the samples (Wilson, 1997). PCR inhibition was not observed during sample analysis, but the presence of factors capable of binding DNA may not show as decreased amplification efficiency, which is typically caused by many other substances, such as humic and fulvic acids, which interfere with the reaction (Ijzerman *et al*., 1997).

The intracellular toxin quotas measured for the Föglö samples did not correlate with either of the two target gene copy numbers or the cell densities (Table 17), which indicates that although *sxtA4* is required for PST production, the amount of produced toxin does not depend on its copy number in the genome. Candidate homologs of cyanobacterial *sxt* genes have been identified in dinoflagellate EST libraries (Stüken *et al*., 2011; Hackett *et al*., 2013; Zhang *et al*., 2014); it remains to be established, whether the genomic copy numbers of these genes have an effect on cellular PST quotas. Regulatory mechanisms operating at the transcriptional and translational level may also affect PST production, and studies have presented evidence both for and against the possible role of transcriptional regulation in PST biosynthesis. Stüken *et al*. (2013) found a positive correlation between *sxtA4* expression and PST concentrations in the environment, whereas others have found that none of the studied transcripts, *sxtA4*, *sxtA1*, or *sxtG* did correlate with cellular PST concentrations, suggesting translational rather than transcriptional regulation (Orr *et al*., 2013; Perini *et al*., 2014; Wiese *et al*., 2014). In this study, a positive correlation between *sxtA4* copy numbers and PST concentrations was therefore observed at the population, but not at the cellular level.

According to the data, the best quantitative predictor of PST concentrations in an *A. ostenfeldii* bloom was the cell density. Thus, the positive correlation observed between the *sxtA4* gene and total intracellular PSTs arises from the fact that all *A. ostenfeldii* cells in the studied populations had the biosynthetic capability to produce the toxins.

Table 17. Spearman rank correlation calculated for each pair of variables measured in the Föglö samples (n=9, measurement means from biological duplicates were used for the calculations). Statistically significant correlations are presented in boldtype.

	***sxtA4*, copies L^{-1}**	**LSU, copies L^{-1}**	**PSTs, µg L^{-1}**	**toxin quota, pg $cell^{-1}$**
Cells, L^{-1}	ρ=0.57, p=0.12	**ρ=0.68, p=0.050**	**ρ=0.93, p<0.01**	ρ=0.17, p=0.67
***sxtA4*, copies L^{-1}**		**ρ=0.92, p<0.01**	ρ=0.6, p=0.097	ρ=0.26, p=0.49
LSU, copies L^{-1}			**ρ=0.75, p=0.025**	ρ=0.43, p=0.24

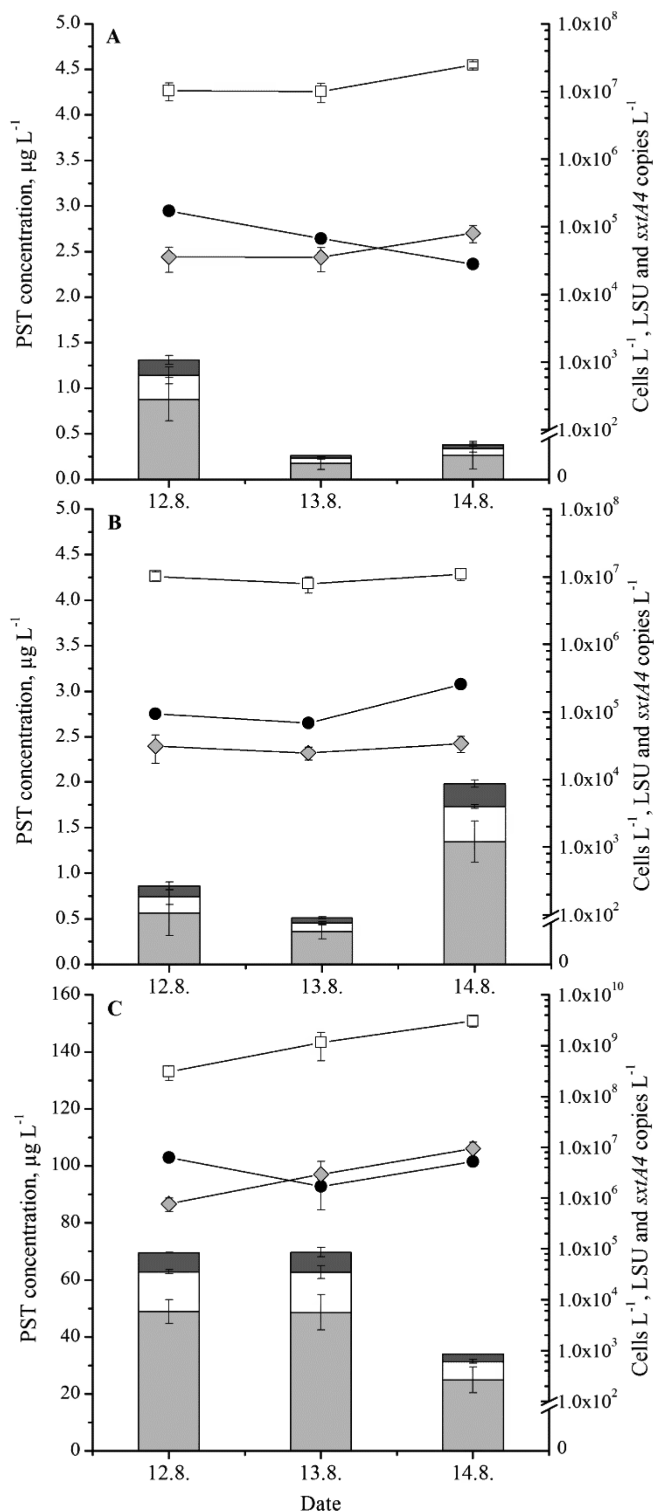

Figure 11. *A. ostenfeldii* cell densities, LSU, and *sxtA4* copy numbers (line and symbol), and total PST concentrations (column) at three Föglö sampling stations: A) Station 3, B) Station 4 and C) Lillängö sampling station. LSU copy numbers are shown as white squares, cell concentrations as black circles and *sxtA4* copy numbers as grey diamonds. The columns depicting total PST concentrations consist of GTX3 (light grey), STX (white), and GTX2 (dark grey). Note the scale differences in C) compared to A) and B).

6 CONCLUSIONS

In this doctoral study, PCR-based methods for the quantification of potentially microcystin and PST-producing cyanobacteria and dinoflagellates were developed. The intrinsic characteristics of qPCR, high sensitivity and specificity, provided an excellent basis for the development of methods for the identification and quantification of harmful cyanobacteria and dinoflagellates in complex natural populations.

The main conclusions based on the original publications are:

I The sample treatment method used in this study, based on a simple heat-treatment of cyanobacterial cells, increased template yields compared to conventional DNA extraction techniques. The accuracy of nucleic acid-based quantification was improved with minimized sample loss during the preparation step. The chosen target region of the qPCR method, the second thiolation motif of *mcyB*, permitted the genus-specific detection of microcystin-producing cyanobacteria belonging to three major bloom-forming genera, *Microcystis*, *Anabaena*, and *Planktothrix*, with good sensitivity and over a broad quantification range. All assay characteristics demonstrated here are important for a monitoring application, which needs to be able to respond to variable cyanobacterial population compositions and cell densities in the environment.

II The dry chemistry PCR chip format was well-suited for the analysis of environmental samples. A strong, statistically significant positive correlation was observed between the combined *mcyB* copy numbers of the three cyanobacterial genera investigated and the total microcystin concentrations across a range of freshwater sampling sites. All samples with microcystin concentrations above 1 µg L^{-1} contained at least 10^5 *mcyB* copies L^{-1}. The introduction of an exogenous internal amplification control, used for the first time to aid the detection of potentially toxic cyanobacteria, improved the assay reliability by ensuring that false negative results caused by inhibitory substances in the sample lysates would be detected. The assay is fast, taking approximately 1.5 hours from the start of sample preparation to results, and due to its semi-automated nature no specialized expertise is required of the operator. The results indicate that measuring genetic factors connected to toxin biosynthesis can be very useful in the monitoring of potentially microcystin-producing cyanobacteria.

III The presence of cyanobacterial PST biosynthetic genes in fresh and brackish waters was not a reliable indicator of toxin production, despite the moderate positive correlation between *sxtB* copy numbers and PST concentrations in samples where toxins were quantifiable. Possible reasons for this outcome could include incomplete *sxt* gene clusters, the downregulation of toxin production, or the presence of unknown toxin producers. Further research is

needed to understand the causes underlying the discrepancy between the observed presence of *sxt* genes and PST production. A microscopic examination of the phytoplankton populations and a comparative analysis of the environmental *sxtA* sequences provided strong evidence that *Anabaena* spp. were the cyanobacterial PST producers in the investigated lakes and coastal waters.

IV The presence of the dinoflagellate *sxtA4* gene was a good predictor of PST production in *A. ostenfeldii* blooms. At the population level, the copy numbers of the *sxtA4* gene were positively correlated with cell densities and toxin concentrations, which indicated that quantifying *sxtA4* is a useful method in estimating the potential PST-neurotoxicity of dinoflagellate mass occurrences. The low cellular copy numbers of *sxtA4* in *A. ostenfeldii* were not correlated with cellular toxin quotas, which indicates that PST production may be regulated by transcriptional or translational factors, rather than being dependent on the genomic copy numbers of the gene.

Generally, the potential utility of nucleic acid-based detection methods in the monitoring of potentially toxic cyanobacteria and dinoflagellates has been established by numerous studies conducted around the world. More specifically, they can be most useful in high-throughput sample screening, early-warning-type applications, and the temporal monitoring of well-characterized harmful microalgal populations. In the event of positive detection of a nucleic acid target, further confirmatory analysis of toxins can be carried out if necessary. According to the results obtained of this doctoral study, a positive correlation between specific genes and toxin concentrations, or the lack thereof, is highly dependent on the genetic target and the toxin-producing organism, and certain targets, such as the cyanobacterial *mcyB* and the dinoflagellate *sxtA4* could be used to estimate and predict the production of microcystin and PSTs, respectively. Therefore, during assay development, each toxin biosynthetic gene and sequence needs to be individually and carefully evaluated for specificity in terms of observed toxin production. To estimate environmental toxin concentrations as accurately as possible, it was shown that it is clearly beneficial to utilize either universal or genus-specific assays to determine the copy numbers in several potentially toxic genera simultaneously. Therefore it is likely that future research will increasingly concentrate on multiplexed quantitative assays.

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8 REFERENCES

Ahn, S.J., Costa, J., Emanuel, J.R., 1996. PicoGreen quantitation of DNA: effective evaluation of samples pre- or post-PCR. Nucleic Acids Res. 24, 2623-2625.

Al-Tebrineh, J., Merrick, C., Ryan, D., Humpage, A., Bowling, L., Neilan, B.A., 2012. Community composition, toxigenicity, and environmental conditions during a cyanobacterial bloom occurring along 1,100 Kilometers of the Murray River. Appl. Environ. Microbiol. 78, 263-272.

Al-Tebrineh, J., Mihali, T.K., Pomati, F., Neilan, B.A., 2010. Quantitative PCR detection of saxitoxin-producing cyanobacteria and *Anabaena circinalis* in environmental water blooms. Appl. Environ. Microbiol. 76, 7836-7842.

Al-Tebrineh, J., Pearson, L.A., Yasar, S.A., Neilan, B.A., 2012. A multiplex qPCR targeting hepato- and neurotoxigenic cyanobacteria of global significance. Harmful Algae 15, 19-25.

Anderson, D.M., Alpermann, T.J., Cembella, A.D., Collos, Y., Masseret, E., Montresor, M., 2012. The globally distributed genus *Alexandrium*: multifaceted roles in marine ecosystems and impacts on human health. Harmful Algae 14, 10-35.

Anderson, D., 1995. Identification of harmful algal species using molecular probes: an emerging perspective, in Lassus, P., Arzul, G., Erard, E., Gentien, P., Marcaillou, C. (Eds.), Harmful Marine Algal Blooms. Technique et Documentation - Lavoisier, Intercept Ltd, Paris, France, pp. 3-13.

Anderson, D.M., Glibert, P.M., Burkholder, J.M., 2002. Harmful algal blooms and eutrophication: nutrient sources, composition, and consequences. Estuaries 25, 704-726.

Anderson, D.M., Kulis, D.M., Keafer, B.A., Gribble, K.E., Marin, R., Scholin, C.A., 2005. Identification and enumeration of *Alexandrium* spp. from the Gulf of Maine using molecular probes. Deep Sea Research Part II: Topical Studies in Oceanography 52, 2467-2490.

Arakawa, O., Nishio, S., Noguchi, T., Shida, Y., Onoue, Y., 1995. A new saxitoxin analogue from a xanthid crab *Atergatis floridus*. Toxicon 33, 1577-1584.

Bagu, J.R., Sykes, B.D., Craig, M.M., Holmes, C.F., 1997. A molecular basis for different interactions of marine toxins with protein phosphatase-1. Molecular models for bound motuporin, microcystins, okadaic acid, and calyculin A. J. Biol. Chem. 272, 5087-5097.

Baker, J.A., Entsch, B., Neilan, B.A., McKay, D.B., 2002. Monitoring changing toxigenicity of a cyanobacterial bloom by molecular methods. Appl. Environ. Microbiol. 68, 6070-6076.

Baker, L., Sendall, B.C., Gasser, R.B., Menjivar, T., Neilan, B.A., Jex, A.R., 2013. Rapid, multiplex-tandem PCR assay for automated detection and differentiation of toxigenic cyanobacterial blooms. Mol. Cell. Probes 27, 208-214.

Balech, E. & Tangen, K. 1985. Morphology and taxonomy of toxic species in the tamarensis group (*Dinophyceae*): *Alexandrium excavatum* (Braarud) comb. nov. and *Alexandrium ostenfeldii* (Paulsen) comb. nov. Sarsia 70:333–43.

Ballot, A., Fastner, J., Wiedner, C., 2010. Paralytic shellfish poisoning toxin-producing cyanobacterium *Aphanizomenon gracile* in northeast Germany. Appl. Environ. Microbiol. 76, 1173-1180.

Baron-Sola, A., Ouahid, Y., del Campo, F.F., 2012. Detection of potentially producing cylindrospermopsin and microcystin strains in mixed populations of cyanobacteria by simultaneous amplification of cylindrospermopsin and microcystin gene regions. Ecotoxicol. Environ. Saf. 75, 102-108.

Bartram, J., Carmichael, W.W., Chorus, I., Jones, G., Skulberg, O.M., 1999. Introduction, in Bartram, J., Chorus, I. (Eds.), Toxic Cyanobacteria in Water: A Guide to their Health Consequences, Monitoring and Management. Spon Press, UK, pp. 1-13.

Baxa, D.V., Kurobe, T., Ger, K.A., Lehman, P.W., Teh, S.J., 2010. Estimating the abundance of toxic *Microcystis* in the San Francisco Estuary using quantitative real-time PCR. Harmful Algae 9, 342-349.

Beattie, K.A., Kaya, K., Codd, G.A., 2000. The cyanobacterium *Nodularia* PCC 7804, of freshwater origin, produces [L-Har2]nodularin. Phytochemistry 54, 57-61.

Becker, S., Fahrbach, M., Boger, P., Ernst, A., 2002. Quantitative tracing, by Taq nuclease assays, of a *Synechococcus* ecotype in a highly diversified natural population. Appl. Environ. Microbiol. 68, 4486-4494.

Beversdorf, L.J., Chaston, S.D., Miller, T.R., McMahon, K.D., 2015. Microcystin *mcyA* and *mcyE* gene abundances are not appropriate indicators of microcystin oncentrations in lakes. PLoS One 10, e0125353.

Bianchi, T.S., Engelhaupt, E., Westman, P., Andrén, T., Rolff, C., Elmgren, R., 2000. Cyanobacterial blooms in the Baltic Sea: Natural or human-induced? Limnol. Oceanogr. 45, 716-726.

Bialojan, C., Takai, A., 1988. Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. Specificity and kinetics. Biochem. J. 256, 283-290.

Bittencourt-Oliveira, M.d.C., 2003. Detection of potential microcystin-producing cyanobacteria in Brazilian reservoirs with a *mcyB* molecular marker. Harmful Algae 2, 51-60.

Bøe, R., Gjertsen, B.T., Vintermyr, O.K., Houge, G., Lanotte, M., Døskeland, S.O., 1991. The protein phosphatase inhibitor okadaic acid induces morphological changes typical of apoptosis in mammalian cells. Exp. Cell Res. 195, 237-246.

Borst, A., Box, A.T., Fluit, A.C., 2004. False-positive results and contamination in nucleic acid amplification assays: suggestions for a prevent and destroy strategy Eur. J. Clin. Microbiol. Infect. Dis. 23, 289-299.

Botes, D., Tuinman, A., Wessels, P., Viljoen, C., Kruger, H., Williams, D., Santikarn, S., Smith, R., Hammond, S., 1984. The structure of cyanoginosin-LA, a cyclic heptapeptide toxin from the cyanobacterium *Microcystis aeruginosa*. J. Chem. Soc. Perkin. Trans. 1, 2311-2318.

Bourke, A.T.C., Hawes, R.B., Neilson, A., Stallman, N.D., 1983. An outbreak of hepato-enteritis (the Palm Island mystery disease) possibly caused by algal intoxication. Toxicon 21, 45-48.

Bourne, D.G., Jones, G.J., Blakeley, R.L., Jones, A., Negri, A.P., Riddles, P., 1996. Enzymatic pathway for the bacterial degradation of the cyanobacterial cyclic peptide toxin microcystin LR Appl. Environ. Microbiol. 62, 4086-4094.

Bowling, L.C., Merrick, C., Swann, J., Green, D., Smith, G., Neilan, B.A., 2013. Effects of hydrology and river management on the distribution, abundance and persistence of cyanobacterial blooms in the Murray River, Australia. Harmful Algae 30, 27-36.

Briand, E., Escoffier, N., Straub, C., Sabart, M., Quiblier, C., Humbert, J.F., 2009. Spatiotemporal changes in the genetic diversity of a bloom-forming *Microcystis aeruginosa* (*Cyanobacteria*) population. ISME J 3, 419-429.

Briand, E., Gugger, M., Francois, J.C., Bernard, C., Humbert, J.F., Quiblier, C., 2008. Temporal variations in the dynamics of potentially microcystin-producing strains in a bloom-forming *Planktothrix agardhii* (*Cyanobacteria*) population. Appl. Environ. Microbiol. 74, 3839-3848.

Burja, A.M., Banaigs, B., Abou-Mansour, E., Grant Burgess, J., Wright, P.C., 2001. Marine cyanobacteria—a prolific source of natural products. Tetrahedron 57, 9347-9377.

Burson, A., Matthijs, H.C.P., de Bruijne, W., Talens, R., Hoogenboom, R., Gerssen, A., Visser, P.M., Stomp, M., Steur, K., van Scheppingen, Y., Huisman, J., 2014. Termination of a toxic *Alexandrium* bloom with hydrogen peroxide. Harmful Algae 31, 125-135.

Carmichael, W.W., Evans, W.R., Yin, Q.Q., Bell, P., Moczydlowski, E., 1997. Evidence for paralytic shellfish poisons in the freshwater cyanobacterium *Lyngbya wollei* (Farlow ex Gomont) comb. nov. Appl. Environ. Microbiol. 63, 3104-3110.

Carmichael, W.W., Beasley, V., Bunner, D.L., Eloff, J.N., Falconer, I., Gorham, P., Harada, K., Krishnamurthy, T., Min-Juan, Y., Moore, R.E., Rinehart, K., Runnegar, M., Skulberg, O.M., Watanabe, M., 1988. Naming of cyclic heptapeptide toxins of cyanobacteria (blue-green algae). Toxicon 26, 971-973.

Caron, D.A., Garneau, M.E., Seubert, E., Howard, M.D., Darjany, L., Schnetzer, A., Cetinic, I., Filteau, G., Lauri, P., Jones, B., Trussell, S., 2010. Harmful algae and their potential impacts on desalination operations off southern California. Water Res. 44, 385-416.

Casero, M.C., Ballot, A., Agha, R., Quesada, A., Cirés, S., 2014. Characterization of saxitoxin production and release and phylogeny of *sxt* genes in paralytic shellfish poisoning toxin-producing *Aphanizomenon gracile*. Harmful Algae 37, 28-37.

Castiglioni, B., Rizzi, E., Frosini, A., Sivonen, K., Rajaniemi, P., Rantala, A., Mugnai, M.A., Ventura, S., Wilmotte, A., Boutte, C., Grubisic, S., Balthasart, P., Consolandi, C., Bordoni, R., Mezzelani, A., Battaglia, C., De Bellis, G., 2004. Development of a universal microarray based on the ligation detection reaction and 16S rRNA gene polymorphism to target diversity of cyanobacteria. Appl. Environ. Microbiol. 70, 7161-7172.

Castro, D., Vera, D., Lagos, N., García, C., Vásquez, M., 2004. The effect of temperature on growth and production of paralytic shellfish poisoning toxins by the cyanobacterium *Cylindrospermopsis raciborskii* C10. Toxicon 44, 483-489.

Cembella, A.D., Lewis, I.D., Quilliam, N.A., 2000. The marine dinoflagellate *Alexandrium ostenfeldii* (*Dinophyceae*) as the causative organism of spirolide shellfish toxins. Phycologia 39, 67-74.

Choi, B.W., Namikoshi, M., Sun, F., Rinehart, K.L., Carmichael, W.W., Kaup, A.M., Evans, W.R., Beasley, V.R., 1993. Isolation of linear peptides related to the hepatotoxins nodularin and microcystins. Tetrahedron Lett. 34, 7881-7884.

Christiansen, G., Fastner, J., Erhard, M., Börner, T., Dittmann, E., 2003. Microcystin biosynthesis in *Planktothrix*: genes, evolution, and manipulation. J. Bacteriol. 185, 564-572.

Christiansen, G., Kurmayer, R., Liu, Q., Börner, T., 2006. Transposons inactivate biosynthesis of the nonribosomal peptide microcystin in naturally occurring *Planktothrix spp*. Appl. Environ. Microbiol. 72, 117-123.

Christiansen, G., Molitor, C., Philmus, B., Kurmayer, R., 2008a. Nontoxic strains of cyanobacteria are the result of major gene deletion events induced by a transposable element. Mol. Biol. Evol. 25, 1695-1704.

Christiansen, G., Yoshida, W.Y., Blom, J.F., Portmann, C., Gademann, K., Hemscheidt, T., Kurmayer, R., 2008b. Isolation and structure determination of two microcystins and sequence comparison of the *mcy*ABC adenylation domains in *Planktothrix* species. J. Nat. Prod. 71, 1881-1886.

Christiansen, G., Goesmann, A., Kurmayer, R., 2014. Elucidation of insertion elements carried on plasmids and *in vitro* construction of shuttle vectors from the toxic cyanobacterium *Planktothrix*. Appl. Environ. Microbiol. 80, 4887-4897.

Cires, S., Wormer, L., Ballot, A., Agha, R., Wiedner, C., Velazquez, D., Casero, M.C., Quesada, A., 2014. Phylogeography of cylindrospermopsin and paralytic shellfish toxin-producing *Nostocales* cyanobacteria from mediterranean Europe (Spain). Appl. Environ. Microbiol. 80, 1359-1370.

Codd, G.A., Metcalf, J.S., Beattie, K.A., 1999. Retention of *Microcystis aeruginosa* and microcystin by salad lettuce (*Lactuca sativa*) after spray irrigation with water containing cyanobacteria. Toxicon 37, 1181-1185.

Conradie, K.R., Barnard, S., 2012. The dynamics of toxic *Microcystis* strains and microcystin production in two hypertrofic South African reservoirs. Harmful Algae 20, 1-10.

Craig, M., Luu, H.A., McCready, T.L., Williams, D., Andersen, R.J., Holmes, C.F., 1996. Molecular mechanisms underlying he interaction of motuporin and microcystins with type-1 and type-2A protein phosphatases. Biochem. Cell Biol. 74, 569-578.

D'Agostino, P.M., Moffitt, M.C., Neilan, B.A., 2014. Current knowledge of paralytic shellfish toxin biosynthesis, molecular detection and evolution, in Rossini, G.P. (Ed.), Toxins and Biologically Active Compounds from Microalgae, Volume 1. CRC Press, pp. 251-280.

Deeds, J.R., Landsberg, J.H., Etheridge, S.M., Pitcher, G.C., Longan, S.W., 2008. Non-traditional vectors for paralytic shellfish poisoning. Mar. Drugs 6, 308-348.

Dell'Aversano, C., Walter, J.A., Burton, I.W., Stirling, D.J., Fattorusso, E., Quilliam, M.A., 2008. Isolation and structure elucidation of new and unusual saxitoxin analogues from mussels. J. Nat. Prod. 71, 1518-1523.

Dias, E., Pereira, P., Franca, S., 2002. Production of paralytic shellfish toxins by *Aphanizomenon* sp. LMECYA 31 (*Cyanobacteria*). J. Phycol. 38, 705-712.

Diener, M., Erler, K., Hiller, S., Christian, B., Luckas, B., 2006. Determination of paralytic shellfish poisoning (PSP) toxins in dietary supplements by application of a new HPLC/FD method. European Food Research and Technology 224, 147-151.

Diercks, S., Medlin, L.K., Metfies, K., 2008a. Colorimetric detection of the toxic dinoflagellate *Alexandrium minutum* using sandwich hybridization in a microtiter plate assay. Harmful Algae 7, 137-145.

Diercks, S., Metfies, K., Medlin, L.K., 2008b. Molecular probe sets for the detection of toxic algae for use in sandwich hybridization formats. Journal of Plankton Research 30, 439-448.

Dillenberg, H.O., Dehnel, M.K., 1960. Toxic waterbloom in Saskatchewan, 1959. Can. Med. Assoc. J. 83, 1151-1154.

Dittmann, E., Neilan, B.A., Erhard, M., von Döhren, H., Börner, T., 1997. Insertional mutagenesis of a peptide synthetase gene that is responsible for hepatotoxin production in the cyanobacterium *Microcystis aeruginosa* PCC 7806. Mol. Microbiol. 26, 779-787.

Dittmann, E., Wiegand, C., 2006. Cyanobacterial toxins--occurrence, biosynthesis and impact on human affairs. Mol Nutr Food Res 50, 7-17.

Dittmann, E., Fewer, D.P., Neilan, B.A., 2013. Cyanobacterial toxins: biosynthetic routes and evolutionary roots. FEMS Microbiol. Rev. 37, 23-43.

Doblin, M.A., Coyne, K.J., Rinta-Kanto, J.M., Wilhelm, S.W., Dobbs, F.C., 2007. Dynamics and short-term survival of toxic cyanobacteria species in ballast water from NOBOB vessels transiting the Great Lakes—implications for HAB invasions. Harmful Algae 6, 519-530.

Downing, T.G., Sember, C.S., Gehringer, M.M., Leukes, W., 2005. Medium N:P ratios and specific growth rate comodulate microcystin and protein content in *Microcystis aeruginosa* PCC7806 and *M. aeruginosa* UV027. Microb. Ecol. 49, 468-473.

Dyhrman, S.T., Haley, S.T., Borchert, J.A., Lona, B., Kollars, N., Erdner, D.L., 2010. Parallel analyses of *Alexandrium catenella* cell concentrations and shellfish toxicity in the Puget Sound. Appl. Environ. Microbiol. 76, 4647-4654.

Dyhrman, S.T., Erdner, D., Du, J.L., Galac, M., Anderson, D.M., 2006. Molecular quantification of toxic *Alexandrium fundyense* in the Gulf of Maine using real-time PCR. Harmful Algae 5, 242-250.

Ebenezer, V., Medlin, L.K., Ki, J.S., 2012. Molecular detection, quantification, and diversity evaluation of microalgae. Mar. Biotechnol. (NY) 14, 129-142.

Eckford-Soper, L.K., Daugbjerg, N., 2015. Development of a multiplex real-time qPCR assay for simultaneous enumeration of up to four marine toxic bloom-forming microalgal species. Harmful Algae 48, 37-43.

Eckford-Soper, L.K., Davidson, K., Bresnan, E., 2013. Identification and quantification of toxic and nontoxic strains of the harmful dinoflagellate *Alexandrium tamarense* using fluorescence *in situ* hybridization and flow cytometry. Limnol Oceanogr: Methods 11, 540-548.

Erdner, D.L., Percy, L., Keafer, B., Lewis, J., Anderson, D.M., 2010. A quantitative real-time PCR assay for the identification and enumeration of *Alexandrium* cysts in marine sediments. Deep-Sea Res Pt II 57, 279-287.

Eriksson, J.E., Toivola, D., Meriluoto, J.A., Karaki, H., Han, Y.G., Hartshorne, D., 1990. Hepatocyte deformation induced by cyanobacterial toxins reflects inhibition of protein phosphatases. Biochem. Biophys. Res. Commun. 173, 1347-1353.

Falconer, I.R., Beresford, A.M., Runnegar, M.T., 1983. Evidence of liver damage by toxin from a bloom of the blue-green alga, *Microcystis aeruginosa*. Med. J. Aust. 1, 511-514.

Falconer, I.R., Yeung, D.S., 1992. Cytoskeletal changes in hepatocytes induced by *Microcystis* toxins and their relation to hyperphosphorylation of cell proteins. Chem. Biol. Interact. 81, 181-196.

Fastner, J., Erhard, M., Carmichael, W.W., Sun, F., Rinehart, K.L., Ronicke, H., Chorus, I., 1999a. Characterization and diversity of microcystins in natural blooms and strains of the genera *Microcystis* and *Planktothrix* from German freshwaters. Arch. Hydrobiol. 45, 147-163.

Fastner, J., Neumann, U., Wirsing, B., Weckesser, J., Wiedner, C., Nixdorf, B., Chorus, I., 1999b. Microcystins (hepatotoxic heptapeptides) in German fresh water bodies. Environ. Toxicol. 14, 13-22.

Ferreira, F.M., Franco Soler, J.M., Fidalgo, M.L., Fernandez-Vila, P., 2001. PSP toxins from *Aphanizomenon flos-aquae* (cyanobacteria) collected in the Crestuma-Lever reservoir (Douro river, northern Portugal). Toxicon 39, 757-761.

Fewer, D.P., Halinen, K., Sipari, H., Bernardova, K., Manttari, M., Eronen, E., Sivonen, K., 2011. Non-autonomous transposable elements associated with inactivation of microcystin gene clusters in strains of the genus *Anabaena* isolated from the Baltic Sea. Environ. Microbiol. Rep. 3, 189-194.

Fewer, D.P., Köykkä, M., Halinen, K., Jokela, J., Lyra, C., Sivonen, K., 2009. Culture-independent evidence for the persistent presence and genetic diversity of microcystin-producing Anabaena (Cyanobacteria) in the Gulf of Finland. Environ. Microbiol. 11, 855-866.

Fewer, D.P., Rouhiainen, L., Jokela, J., Wahlsten, M., Laakso, K., Wang, H., Sivonen, K., 2007. Recurrent adenylation domain replacement in the microcystin synthetase gene cluster. BMC Evol. Biol. 7, 183.

Fewer, D.P., Tooming-Klunderud, A., Jokela, J., Wahlsten, M., Rouhiainen, L., Kristensen, T., Rohrlack, T., Jakobsen, K.S., Sivonen, K., 2008. Natural occurrence of microcystin synthetase deletion mutants

capable of producing microcystins in strains of the genus *Anabaena* (*Cyanobacteria*). Microbiology 154, 1007-1014.

Fewer, D., Wahlsten, M., Österholm, J., Jokela, J., Rouhiainen, L., Kaasalainen, U., Rikkinen, J., Sivonen, K., 2013. The genetic basis for O-acetylation of the microcystin toxin in cyanobacteria. Chem. Biol. 20, 861-869.

Figueroa, R.I., Garcés, E., Bravo, I., 2010. The use of flow cytometry for species identification and life-cycle studies in dinoflagellates. Deep-Sea Res. Pt II 57, 301-307.

Finni, T., Kononen, K., Olsonen, R., Wallström, K., 2001. The history of cyanobacterial blooms in the Baltic Sea. Ambio 30, 172-178.

Fiore, M.F., Genuario, D.B., da Silva, C.S., Shishido, T.K., Moraes, L.A., Cantusio Neto, R., Silva-Stenico, M.E., 2009. Microcystin production by a freshwater spring cyanobacterium of the genus *Fischerella*. Toxicon 53, 754-761.

Fischer, W.J., Altheimer, S., Cattori, V., Meier, P.J., Dietrich, D.R., Hagenbuch, B., 2005. Organic anion transporting polypeptides expressed in liver and brain mediate uptake of microcystin. Toxicol. Appl. Pharmacol. 203, 257-263.

Fjellså, A., Nordberg, K., 1997. Toxic dinoflagellate blooms in the Kattegat, North Sea, during the Holocene. Oceanograph. Lit. Rev. 5, 459.

Fortin, N., Aranda-Rodriguez, R., Jing, H., Pick, F., Bird, D., Greer, C.W., 2010. Detection of microcystin-producing cyanobacteria in Missisquoi Bay, Quebec, Canada, using quantitative PCR. Appl. Environ. Microbiol. 76, 5105-5112.

Foss, A.J., Phlips, E.J., Yilmaz, M., Chapman, A., 2012. Characterization of paralytic shellfish toxins from *Lyngbya wollei* dominated mats collected from two Florida springs. Harmful Algae 16, 98-107.

Foulds, I.V., Granacki, A., Xiao, C., Krull, U., Castle, A., Horgen, P.A., 2002. Quantification of microcystin-producing cyanobacteria and *E. coli* in water by 5'-nuclease PCR. J. Appl. Microbiol. 93, 825-834.

Francis, G., 1878. Poisonous Australian lake. Nature 18, 11-12.

Frangeul, L., Quillardet, P., Castets, A.M., Humbert, J.F., Matthijs, H.C., Cortez, D., Tolonen, A., Zhang, C.C., Gribaldo, S., Kehr, J.C., Zilliges, Y., Ziemert, N., Becker, S., Talla, E., Latifi, A., Billault, A., Lepelletier, A., Dittmann, E., Bouchier, C., Tandeau de Marsac, N., 2008. Highly plastic genome of *Microcystis aeruginosa* PCC7806, a ubiquitous toxic freshwater cyanobacterium. BMC Genomics 9, 274.

Furukawa, K., Noda, N., Tsuneda, S., Saito, T., Itayama, T., Inamori, Y., 2006. Highly sensitive real-time PCR assay for quantification of toxic cyanobacteria based on microcystin synthetase A gene. J Biosci Bioeng 102, 90-96.

Gallacher, S., Smith, E.A., 1999. Bacteria and paralytic shellfish toxins. Protist 150, 245-255.

Galluzzi, L., Bertozzini, E., Penna, A., Perini, F., Garcés, E., Magnani, M., 2010. Analysis of rRNA gene content in the Mediterranean dinoflagellate *Alexandrium catenella* and *Alexandrium taylori*: implications for the quantitative real-time PCR-based monitoring methods. J. Appl. Phycol. 22, 1-9.

Galluzzi, L., Cegna, A., Casabianca, S., Penna, A., Saunders, N., Magnani, M., 2011. Development of an oligonucleotide microarray for the detection and monitoring of marine dinoflagellates. J. Microbiol. Methods 84, 234-242.

Galluzzi, L., Penna, A., Bertozzini, E., Vila, M., Garcés, E., Magnani, M., 2004. Development of a real-time PCR assay for rapid detection and quantification of *Alexandrium minutum* (a Dinoflagellate). Appl. Environ. Microbiol. 70, 1199-1206.

García, C., del Carmen Bravo, M., Lagos, M., Lagos, N., 2004. Paralytic shellfish poisoning: post-mortem analysis of tissue and body fluid samples from human victims in the Patagonia fjords. Toxicon 43, 149-158.

García, C., Rodriguez-Navarro, A., Díaz, J.C., Torres, R., Lagos, N., 2009. Evidence of *in vitro* glucuronidation and enzymatic transformation of paralytic shellfish toxins by healthy human liver microsomes fraction. Toxicon 53, 206-213.

Garneau, M.E., Schnetzer, A., Countway, P.D., Jones, A.C., Seubert, E.L., Caron, D.A., 2011. Examination of the seasonal dynamics of the toxic dinoflagellate *Alexandrium catenella* at Redondo Beach, California, by quantitative PCR. Appl. Environ. Microbiol. 77, 7669-7680.

Gescher, C., Metfies, K., Medlin, L.K., 2008. The ALEX CHIP—Development of a DNA chip for identification and monitoring of *Alexandrium*. Harmful Algae 7, 485-494.

Gessner, B.D., Bell, P., Doucette, G.J., Moczydlowski, E., Poli, M.A., Van Dolah, F., Hall, S., 1997. Hypertension and identification of toxin in human urine and serum following a cluster of mussel-associated paralytic shellfish poisoning outbreaks. Toxicon 35, 711-722.

Gkelis, S., Zaoutsos, N., 2014. Cyanotoxin occurrence and potentially toxin producing cyanobacteria in freshwaters of Greece: A multi-disciplinary approach. Toxicon 78, 1-9.

Glibert, P.M., Burkholder, J.M., 2006. The complex relationships between increases in fertilization of the earth, coastal eutrophication and proliferation of harmful algal blooms, in Granéli, E., Turner, J. (Eds.), Ecology of Harmful Algae. Springer Berlin Heidelberg, pp. 341-354.

Gobler, C.J., Davis, T.W., Coyne, K.J., Boyer, G.L., 2007. Interactive influences of nutrient loading, zooplankton grazing, and microcystin synthetase gene expression on cyanobacterial bloom dynamics in a eutrophic New York lake. Harmful Algae 6, 119-133.

Godhe, A., Asplund, M.E., Harnstrom, K., Saravanan, V., Tyagi, A., Karunasagar, I., 2008. Quantification of diatom and dinoflagellate biomasses in coastal marine seawater samples by real-time PCR Appl. Environ. Microbiol. 74, 7174-7182.

Godhe, A., Cusack, C., Pedersen, J., Andersen, P., Anderson, D.M., Bresnan, E., Cembella, A., Dahl, E., Diercks, S., Elbrächter, M., Edler, L., Galluzzi, L., Gescher, C., Gladstone, M., Karlson, B., Kulis, D., LeGresley, M., Lindahl, O., Marin, R., McDermott, G., Medlin, L.K., Naustvoll, L., Penna, A., Töbe, K., 2007. Intercalibration of classical and molecular techniques for identification of *Alexandrium fundyense* (*Dinophyceae*) and estimation of cell densities. Harmful Algae 6, 56-72.

Greenacre, M., Nenadic, O., 2013. Package 'ca'. A package for computation and visualization of simple, multiple and joint correspondence analysis.

Guedes, I.A., da Costa Leite, D.M., Manhães, L.A., Bisch, P.M., Azevedo, S.M.F.O.e., Pacheco, A.B.F., 2014. Fluctuations in microcystin concentrations, potentially toxic *Microcystis* and genotype diversity in a cyanobacterial community from a tropical reservoir. Harmful Algae 39, 303-309.

Guillard, R.R., Ryther, J.H., 1962. Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt, and *Detonula confervacea* (Cleve) Gran. Can. J. Microbiol. 8, 229-239.

Gulledge, B.M., Aggen, J.B., Huang, H.B., Nairn, A.C., Chamberlin, A.R., 2002. The microcystins and nodularins: cyclic polypeptide inhibitors of PP1 and PP2A. Curr. Med. Chem. 9, 1991-2003.

Hackett, J.D., Anderson, D.M., Erdner, D.L., Bhattacharya, D., 2004. Dinoflagellates: a remarkable evolutionary experiment. Am. J. Bot. 91, 1523-1534.

Hackett, J.D., Wisecaver, J.H., Brosnahan, M.L., Kulis, D.M., Anderson, D.M., Bhattacharya, D., Plumley, F.G., Erdner, D.L., 2013. Evolution of saxitoxin synthesis in cyanobacteria and dinoflagellates. Mol. Biol. Evol. 30, 70-78.

Hagenbuch, B., Meier, P.J., 2003. The superfamily of organic anion transporting polypeptides. Biochim. Biophys. Acta - Biomembranes 1609, 1-18.

Hagren, V., von Lode, P., Syrjälä, A., Soukka, T., Lövgren, T., Kojola, H., Nurmi, J., 2008. An automated PCR platform with homogeneous time-resolved fluorescence detection and dry chemistry assay kits. Anal. Biochem. 374, 411-416.

Hakanen, P., Suikkanen, S., Franzén, J., Franzén, H., Kankaanpää, H., Kremp, A., 2012. Bloom and toxin dynamics of *Alexandrium ostenfeldii* in a shallow embayment at the SW coast of Finland, northern Baltic Sea. Harmful Algae 15, 91-99.

Halinen, K., Jokela, J., Fewer, D.P., Wahlsten, M., Sivonen, K., 2007. Direct evidence for production of microcystins by *Anabaena* strains from the Baltic Sea. Appl. Environ. Microbiol. 73, 6543-6550.

Hallegraeff, G.M., 2003. Harmful algal blooms: a global overview, in Hallegraeff, G.M., Anderson, D.M., Cembella, A.D. (Eds.), Manual on harmful marine microalgae. UNESCO, Paris, France, pp. 25-49.

Hallegraeff, G.M., 1993. A review of harmful algal blooms and their apparent global increase. Phycologia 32, 79-99.

Hallegraeff, G.M., 2014. Harmful algae and their toxins: progress, paradoxes and paradigm shifts, in Rossini, G.P. (Ed.), Toxins and Biologically Active Compounds from Microalgae, Volume 1. CRC Press, pp. 3-20.

Halme, M., Rapinoja, M., Karjalainen, M., Vanninen, P., 2012. Verification and quantification of saxitoxin from algal samples using fast and validated hydrophilic interaction liquid chromatography–tandem mass spectrometry method. J. Chromatogr. B 880, 50-57.

Harada, K., Matsuura, K., Suzuki, M., Watanabe, M.F., Oishi, S., Dahlem, A.M., Beasley, V.R., Carmichael, W.W., 1990a. Isolation and characterization of the minor components associated with microcystins LR and RR in the cyanobacterium (blue-green algae). Toxicon 28, 55-64.

Harada, K., Ogawa, K., Matsuura, K., Murata, H., Suzuki, M., Watanabe, M.F., Itezono, Y., Nakayama, N., 1990b. Structural determination of geometrical isomers of microcystins LR and RR from cyanobacteria by two-dimensional NMR spectroscopic techniques. Chem. Res. Toxicol. 3, 473-481.

Harada, T., Oshima, Y., Yasumoto, T., 1984. Assessment of potential activation of gonyautoxin V in the stomach of mice and rats. Toxicon 22, 476-478.

Hartshorne, R.P., Catterall, W.A., 1984. The sodium channel from rat brain. Purification and subunit composition J. Biol. Chem. 259, 1667-1675.

Heisler, J., Glibert, P.M., Burkholder, J.M., Anderson, D.M., Cochlan, W., Dennison, W.C., Dortch, Q., Gobler, C.J., Heil, C.A., Humphries, E., Lewitus, A., Magnien, R., Marshall, H.G., Sellner, K., Stockwell, D.A., Stoecker, D.K., Suddleson, M., 2008. Eutrophication and harmful algal blooms: A scientific consensus. Harmful Algae 8, 3-13.

Henriksen, P., Moestrup, Ø., 1997. Seasonal variations in microcystin contents of Danish cyanobacteria. Nat. Toxins 5, 99-106.

Hereman, T.C., Bittencourt-Oliveira, M.d.C., 2012. Bioaccumulation of microcystins in lettuce. J. Phycol. 48, 1535-1537.

Hicks, L.M., Moffitt, M.C., Beer, L.L., Moore, B.S., Kelleher, N.L., 2006. Structural characterization of *in vitro* and *in vivo* intermediates on the loading module of microcystin synthetase. ACS Chem Biol 1, 93-102.

Higuchi, R., Fockler, C., Dollinger, G., Watson, R., 1993. Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Biotechnology (N. Y) 11, 1026-1030.

Hisbergues, M., Christiansen, G., Rouhiainen, L., Sivonen, K., Börner, T., 2003. PCR-based identification of microcystin-producing genotypes of different cyanobacterial genera. Arch. Microbiol. 180, 402-410.

Hoagland, P., Scatasta, S., 2006. The economic effects of harmful algal blooms, in Granéli, E., Turner, J. (Eds.), Springer Berlin Heidelberg, pp. 391-402.

Hoff-Risseti, C., Dorr, F.A., Schaker, P.D., Pinto, E., Werner, V.R., Fiore, M.F., 2013. Cylindrospermopsin and saxitoxin synthetase genes in *Cylindrospermopsis raciborskii* strains from Brazilian freshwater. PLoS One 8, e74238.

Hold, G.L., Smith, E.A., Birkbeck, T.H., Gallacher, S., 2001. Comparison of paralytic shellfish toxin (PST) production by the dinoflagellates *Alexandrium lusitanicum* NEPCC 253 and *Alexandrium tamarense* NEPCC 407 in the presence and absence of bacteria FEMS Microbiol. Ecol. 36, 223-234.

Holland, P.M., Abramson, R.D., Watson, R., Gelfand, D.H., 1991. Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of *Thermus aquaticus* DNA polymerase. Proc. Natl. Acad. Sci. U. S. A. 88, 7276-7280.

Honkanen, R.E., Dukelow, M., Zwiller, J., Moore, R.E., Khatra, B.S., Boynton, A.L., 1991. Cyanobacterial nodularin is a potent inhibitor of type 1 and type 2A protein phosphatases. Mol. Pharmacol. 40, 577-583.

Hosoi-Tanabe, S., Sako, Y., 2006. Development and application of fluorescence in situ hybridization (FISH) method for simple and rapid identification of the toxic dinoflagellates *Alexandrium tamarense* and *Alexandrium catenella* in cultured and natural seawater. Fisheries Sci. 72, 77-82.

Hosoi-Tanabe, S., Sako, Y., 2005. Species-specific detection and quantification of toxic marine dinoflagellates *Alexandrium tamarense* and *A. catenella* by Real-time PCR assay. Mar. Biotechnol. (NY) 7, 506-514.

Hotto, A., Satchwell, M., Boyer, G., 2005. Seasonal production and molecular characterization of microcystins in Oneida Lake, New York, USA. Environ. Toxicol. 20, 243-248.

Hotto, A.M., Satchwell, M.F., Berry, D.L., Gobler, C.J., Boyer, G.L., 2008. Spatial and temporal diversity of microcystins and microcystin-producing genotypes in Oneida Lake, NY. Harmful Algae 7, 671-681.

Humpage, A.R., Magalhaes, V.F., Froscio, S.M., 2010. Comparison of analytical tools and biological assays for detection of paralytic shellfish poisoning toxins. Anal. Bioanal. Chem. 397, 1655-1671.

Hungerford, J., 2014. Marine toxin detection methods in regulation - from validation to implementation, in Rossini, G.P. (Ed.), Toxins and Biologically Active Compounds from Microalgae, Volume 1. CRC Press, pp. 487-502.

IARC, 2010. Working group on the evaluation of carcinogenic risks to humans; ingested nitrate and nitrite, and cyanobacterial peptide toxins. IARC Monogr. Eval. Carcinog. Risks. Hum. 94, 327-357.

Ijzerman, M.M., Dahling, D.R., Fout, G.S., 1997. A method to remove environmental inhibitors prior to the detection of waterborne enteric viruses by reverse transcription-polymerase chain reaction J. Virol. Methods 63, 145-153.

Izaguirre, G., Jungblut, A.D., Neilan, B.A., 2007. Benthic cyanobacteria (*Oscillatoriaceae*) that produce microcystin-LR, isolated from four reservoirs in southern California. Water Res. 41, 492-498.

Janse, I., Kardinaal, W.E., Meima, M., Fastner, J., Visser, P.M., Zwart, G., 2004. Toxic and nontoxic *Microcystis* colonies in natural populations can be differentiated on the basis of rRNA gene internal transcribed spacer diversity. Appl. Environ. Microbiol. 70, 3979-3987.

John, U., Litaker, R.W., Montresor, M., Murray, S., Brosnahan, M.L., Anderson, D.M., 2014. Formal revision of the *Alexandrium tamarense* species complex (*Dinophyceae*) taxonomy: The introduction of five species with emphasis on molecular-based (rDNA) classification. Protist 165, 779-804.

Joung, S., Oh, H., Ko, S., Ahn, C., 2011. Correlations between environmental factors and toxic and non-toxic *Microcystis* dynamics during bloom in Daechung Reservoir, Korea. Harmful Algae 10, 188-193.

Jungblut, A.D., Neilan, B.A., 2006. Molecular identification and evolution of the cyclic peptide hepatotoxins, microcystin and nodularin, synthetase genes in three orders of cyanobacteria. Arch. Microbiol. 185, 107-114.

Kaas, H., Henriksen, P., 2000. Saxitoxins (PSP toxins) in Danish lakes. Water Res. 34, 2089-2097.

Kaasalainen, U., Jokela, J., Fewer, D.P., Sivonen, K., Rikkinen, J., 2009. Microcystin production in the tripartite cyanolichen *Peltigera leucophlebia*. Mol. Plant Microbe Interact. 22, 695-702.

Kaasalainen, U., Fewer, D.P., Jokela, J., Wahlsten, M., Sivonen, K., Rikkinen, J., 2012. Cyanobacteria produce a high variety of hepatotoxic peptides in lichen symbiosis Proc. Natl. Acad. Sci. U. S. A. 109, 5886-5891.

Kaebernick, M., Dittmann, E., Börner, T., Neilan, B.A., 2002. Multiple alternate transcripts direct the biosynthesis of microcystin, a cyanobacterial nonribosomal peptide. Appl. Environ. Microbiol. 68, 449-455.

Kaebernick, M., Neilan, B.A., Börner, T., Dittmann, E., 2000. Light and the transcriptional response of the microcystin biosynthesis gene cluster. Appl. Environ. Microbiol. 66, 3387-3392.

Kaebernick, M., Rohrlack, T., Christoffersen, K., Neilan, B.A., 2001. A spontaneous mutant of microcystin biosynthesis: genetic characterization and effect on *Daphnia*. Environ. Microbiol. 3, 669-679.

Kahru, M., Savchuk, O., Elmgren, R., 2007. Satellite measurements of cyanobacterial bloom frequency in the Baltic Sea: interannual and spatial variability. Mar. Ecol. Prog. Ser. 343, 15-23.

Kalaitzis, J.A., Chau, R., Kohli, G.S., Murray, S.A., Neilan, B.A., 2010. Biosynthesis of toxic naturally-occurring seafood contaminants. Toxicon 56, 244-258.

Kamikawa, R., Nagai, S., Hosoi-Tanabe, S., Itakura, S., Yamaguchi, M., Uchida, Y., Baba, T., Sako, Y., 2007. Application of real-time PCR assay for detection and quantification of *Alexandrium tamarense* and *Alexandrium catenella* cysts from marine sediments. Harmful Algae 6, 413-420.

Kaneko, T., Nakajima, N., Okamoto, S., Suzuki, I., Tanabe, Y., Tamaoki, M., Nakamura, Y., Kasai, F., Watanabe, A., Kawashima, K., Kishida, Y., Ono, A., Shimizu, Y., Takahashi, C., Minami, C., Fujishiro, T., Kohara, M., Katoh, M., Nakazaki, N., Nakayama, S., Yamada, M., Tabata, S., Watanabe, M.M., 2007. Complete genomic structure of the bloom-forming toxic cyanobacterium *Microcystis aeruginosa* NIES-843. DNA Res. 14, 247-256.

Kao, C.Y., 1986. Structure-activity relations of tetrodotoxin, saxitoxin, and analogues. Ann. N. Y. Acad. Sci. 479, 52-67.

Kao, C.Y., Walker, S.E., 1982. Active groups of saxitoxin and tetrodotoxin as deduced from actions of saxitoxin analogues on frog muscle and squid axon J. Physiol. 323, 619-637.

Karlsson, K.M., Kankaanpää, H., Huttunen, M., Meriluoto, J., 2005. First observation of microcystin-LR in pelagic cyanobacterial blooms in the northern Baltic Sea. Harmful Algae 4, 163-166.

Kegel, J.U., Del Amo, Y., Medlin, L.K., 2013. Introduction to project MIDTAL: its methods and samples from Arcachon Bay, France. Environ. Sci. Pollut. Res. Int. 20, 6690-6704.

Kellmann, R., Mihali, T.K., Jeon, Y.J., Pickford, R., Pomati, F., Neilan, B.A., 2008a. Biosynthetic intermediate analysis and functional homology reveal a saxitoxin gene cluster in cyanobacteria. Appl. Environ. Microbiol. 74, 4044-4053.

Kellmann, R., Mihali, T.K., Neilan, B.A., 2008b. Identification of a saxitoxin biosynthesis gene with a history of frequent horizontal gene transfers. J. Mol. Evol. 67, 526-538.

Kellmann, R., Stüken, A., Orr, R.J., Svendsen, H.M., Jakobsen, K.S., 2010. Biosynthesis and molecular genetics of polyketides in marine dinoflagellates. Mar. Drugs 8, 1011-1048.

Ki, J., Han, M., 2006. A low-density oligonucleotide array study for parallel detection of harmful algal species using hybridization of consensus PCR products of LSU rDNA D2 domain. Biosens. Bioelectron. 21, 1812-1821.

Kita, M., Ohno, O., Han, C., Uemura, D., 2010. Bioactive secondary metabolites from symbiotic marine dinoflagellates: symbiodinolide and durinskiols. Chem. Rec. 10, 57-69.

Klais, R., Tamminen, T., Kremp, A., Spilling, K., Olli, K., 2011. Decadal-scale changes of dinoflagellates and diatoms in the anomalous Baltic Sea spring bloom. PLoS ONE 6, e21567.

Kleinkauf, H., Von Döhren, H., 1996. A nonribosomal system of peptide biosynthesis. Eur. J. Biochem. 236, 335-351.

Kodama, M., Ogata, T., Sato, S., 1988. Bacterial production of saxitoxin. Agric. Biol. Chem. 52, 1075-1077.

Kokocinski, M., Mankiewicz-Boczek, J., Jurczak, T., Spoof, L., Meriluoto, J., Rejmonczyk, E., Hautala, H., Vehniäinen, M., Pawelczyk, J., Soininen, J., 2013. *Aphanizomenon gracile* (*Nostocales*), a cylindrospermopsin-producing cyanobacterium in Polish lakes. Environ. Sci. Pollut. Res. Int. 20, 5243-5264.

Korpimäki, T., Nurmi, J., Lövgren, T., 2007. Method for Stabilizing Assay Reagents, Reagent Container with Stabilized Assay Reagents and Use Thereof. United States Patent Application 11/628354.

Komárek J., Anagnostidis K., 1998, Cyanoprokaryota 1. Teil: Chroococcales, in Ettl, H., Gärtner G., Heynig H., Mollenhauer D. (Eds): Süsswasserflora von Mitteleuropa 19/1, Gustav Fischer, Jena-Stuttgart-Lübeck-Ulm, 548 pp.

Komárek J., Anagnostidis K., 2005, Cyanoprokaryota 2. Teil/ 2nd Part: Oscillatoriales, in Büdel B., Krienitz L., Gärtner G., Schagerl, M. (Eds): Süsswasserflora von Mitteleuropa 19/2, Elsevier/Spektrum, Heidelberg, 759 pp.

Kótai, J., 1972. Instructions for preparation of modified nutrient solution Z8 for algae. NIVA B-11/69.

Kremp, A., Lindholm, T., Dreßler, N., Erler, K., Gerdts, G., Eirtovaara, S., Leskinen, E., 2009. Bloom forming *Alexandrium ostenfeldii* (*Dinophyceae*) in shallow waters of the Åland Archipelago, Northern Baltic Sea. Harmful Algae 8, 318-328.

Kremp, A., Tahvanainen, P., Litaker, W., Krock, B., Suikkanen, S., Leaw, C.P., Tomas, C., 2014. Phylogenetic relationships, morphological variation, and toxin patterns in the *Alexandrium ostenfeldii* (*Dinophyceae*) complex: implications for species boundaries and identities. J. Phycol. 50, 81-100.

Kubista, M., Andrade, J.M., Bengtsson, M., Forootan, A., Jonák, J., Lind, K., Sindelka, R., Sjöback, R., Sjögreen, B., Strömbom, L., Ståhlberg, A., Zoric, N., 2006. The real-time polymerase chain reaction. Mol. Aspects Med. 27, 95-125.

Kuiper-Goodman, T., Falconer, I.R., Fitzgerald, J., 1999. Human health aspects, in Bartram, J., Chorus, I. (Eds.), Toxic Cyanobacteria in Water: A Guide to their Health Consequences, Monitoring and Management. Spon Press, UK, pp. 112-147.

Kurmayer, R., Christiansen, G., Chorus, I., 2003. The abundance of microcystin-producing genotypes correlates positively with colony size in *Microcystis* sp. and determines its microcystin net production in Lake Wannsee. Appl. Environ. Microbiol. 69, 787-795.

Kurmayer, R., Christiansen, G., Fastner, J., Börner, T., 2004. Abundance of active and inactive microcystin genotypes in populations of the toxic cyanobacterium *Planktothrix spp*. Environ. Microbiol. 6, 831-841.

Kurmayer, R., Christiansen, G., Gumpenberger, M., Fastner, J., 2005. Genetic identification of microcystin ecotypes in toxic cyanobacteria of the genus *Planktothrix*. Microbiology 151, 1525-1533.

Kurmayer, R., Dittmann, E., Fastner, J., Chorus, I., 2002. Diversity of microcystin genes within a population of the toxic cyanobacterium *Microcystis* spp. in Lake Wannsee (Berlin, Germany). Microb. Ecol. 43, 107-118.

Kurmayer, R., Gumpenberger, M., 2006. Diversity of microcystin genotypes among populations of the filamentous cyanobacteria *Planktothrix rubescens* and *Planktothrix agardhii*. Mol. Ecol. 15, 3849-3861.

Kurmayer, R., Kutzenberger, T., 2003. Application of real-time PCR for quantification of microcystin genotypes in a population of the toxic cyanobacterium *Microcystis* sp. Appl. Environ. Microbiol. 69, 6723-6730.

Kuuppo, P., Uronen, P., Petermann, A., Tamminen, T., Granéli, E., 2006. Pectenotoxin-2 and dinophysistoxin-1 in suspended and sedimenting organic matter in the Baltic Sea. Limnol. Oceanogr. 51, 2300-2307.

Labarre, J., Chauvat, F., Thuriaux, P., 1989. Insertional mutagenesis by random cloning of antibiotic resistance genes into the genome of the cyanobacterium *Synechocystis* strain PCC 6803. J. Bacteriol. 171, 3449-3457.

Lagos, N., Onodera, H., Zagatto, P.A., Andrinolo, D., Azevedo, S.M., Oshima, Y., 1999. The first evidence of paralytic shellfish toxins in the fresh water cyanobacterium *Cylindrospermopsis raciborskii*, isolated from Brazil. Toxicon 37, 1359-1373.

Lajeunesse, A., Segura, P.A., Gélinas, M., Hudon, C., Thomas, K., Quilliam, M.A., Gagnon, C., 2012. Detection and confirmation of saxitoxin analogues in freshwater benthic *Lyngbya wollei* algae collected in the St. Lawrence River (Canada) by liquid chromatography–tandem mass spectrometry. Journal of Chromatography A 1219, 93-103.

Lanaras, T., Cook, C.M., Eriksson, J.E., Meriluoto, J.A., Hotokka, M., 1991. Computer modelling of the 3-dimensional structures of the cyanobacterial hepatotoxins microcystin-LR and nodularin. Toxicon 29, 901-906.

Laub, J., Henriksen, P., Brittain, S.M., Wang, J., Carmichael, W.W., Rinehart, K.L., Moestrup, Ø., 2002. [ADMAdda^{5}]-microcystins in *Planktothrix agardhii* strain PH-123 (cyanobacteria) - importance for monitoring of microcystins in the environment. Environ. Toxicol. 17, 351-357.

Lautru, S., Challis, G.L., 2004. Substrate recognition by nonribosomal peptide synthetase multi-enzymes. Microbiology 150, 1629-1636.

Ledreux, A., Thomazeau, S., Catherine, A., Duval, C., Yéprémian, C., Marie, A., Bernard, C., 2010. Evidence for saxitoxins production by the cyanobacterium *Aphanizomenon gracile* in a French recreational water body. Harmful Algae 10, 88-97.

Li, X., Wu, Y., Zhang, L., Cao, Y., Li, Y., Li, J., Zhu, L., Wu, G., 2014. Comparison of three common DNA concentration measurement methods. Anal. Biochem. 451, 18-24.

Lindholm, T., Vesterkvist, P., Spoof, L., Lundberg-Niinistö, C., Meriluoto, J., 2003. Microcystin occurrence in lakes in Åland, SW Finland. Hydrobiologia 505, 129-138.

Lipkind, G.M., Fozzard, H.A., 1994. A structural model of the tetrodotoxin and saxitoxin binding site of the Na+ channel. Biophys. J. 66, 1-13.

Llewellyn, L.E., 2006. Saxitoxin, a toxic marine natural product that targets a multitude of receptors. Nat. Prod. Rep. 23, 200-222.

Llewellyn, L.E., Doyle, J., 2001. Microtitre plate assay for paralytic shellfish toxins using saxiphilin: gauging the effects of shellfish extract matrices, salts and pH upon assay performance. Toxicon 39, 217-224.

Llewellyn, L.E., Negri, A.P., Doyle, J., Baker, P.D., Beltran, E.C., Neilan, B.A., 2001. Radioreceptor assays for sensitive detection and quantitation of saxitoxin and its analogues from strains of the freshwater cyanobacterium, *Anabaena circinalis*. Environ. Sci. Technol. 35, 1445-1451.

Luukkainen, R., Sivonen, K., Namikoshi, M., Fardig, M., Rinehart, K.L., Niemela, S.I., 1993. Isolation and identification of eight microcystins from thirteen *Oscillatoria agardhii* strains and structure of a new microcystin. Appl. Environ. Microbiol. 59, 2204-2209.

Lyra, C., Suomalainen, S., Gugger, M., Vezie, C., Sundman, P., Paulin, L., Sivonen, K., 2001. Molecular characterization of planktic cyanobacteria of *Anabaena*, *Aphanizomenon*, *Microcystis* and *Planktothrix* genera. Int. J. Syst. Evol. Microbiol. 51, 513-526.

MacKintosh, C., Beattie, K.A., Klumpp, S., Cohen, P., Codd, G.A., 1990. Cyanobacterial microcystin-LR is a potent and specific inhibitor of protein phosphatases 1 and 2A from both mammals and higher plants. FEBS Lett. 264, 187-192.

MacKintosh, R.W., Dalby, K.N., Campbell, D.G., Cohen, P.T.W., Cohen, P., MacKintosh, C., 1995. The cyanobacterial toxin microcystin binds covalently to cysteine-273 on protein phosphatase 1. FEBS Lett. 371, 236-240.

Mahmood, N.A., Carmichael, W.W., 1986. Paralytic shellfish poisons produced by the freshwater cyanobacterium *Aphanizomenon flos-aquae* NH-5. Toxicon 24, 175-186.

Mankiewicz-Boczek, J., G?ga?a, I., Kokoci?ski, M., Jurczak, T., Stefaniak, K., 2011. Perennial toxigenic *Planktothrix agardhii* bloom in selected lakes of Western Poland. Environ. Toxicol. 26, 10-20.

Mankiewicz-Boczek, J., Izydorczyk, K., Romanowska-Duda, Z., Jurczak, T., Stefaniak, K., Kokocinski, M., 2006. Detection and monitoring toxigenicity of cyanobacteria by application of molecular methods. Environ. Toxicol. 21, 380-387.

Martins, A., Moreira, C., Vale, M., Freitas, M., Regueiras, A., Antunes, A., Vasconcelos, V., 2011. Seasonal dynamics of *Microcystis* spp. and their toxigenicity as assessed by qPCR in a temperate reservoir. Mar. Drugs 9, 1715-1730.

Mbedi, S., Welker, M., Fastner, J., Wiedner, C., 2005. Variability of the microcystin synthetase gene cluster in the genus *Planktothrix* (*Oscillatoriales*, *Cyanobacteria*). FEMS Microbiol. Lett. 245, 299-306.

McElhiney, J., Lawton, L.A., 2005. Detection of the cyanobacterial hepatotoxins microcystins. Toxicol. Appl. Pharmacol. 203, 219-230.

McMinn, A., 1989. Late pleistocene dinoflagellate cysts from Botany Bay, New South Wales, Australia. Micropaleontology 35, 1-9.

McMinn, A., Hallegraeff, G.M., Thomson, P., Jenkinson, A., Heijnis, H., 1997. Cyst and radionucleotide evidence for the recent introduction of the toxic dinoflagellate *Gymnodinium catenatum* into Tasmanian waters. Mar. Ecol. Prog. Ser. 161, 165-172.

McNabb, P., 2014. Instrumental methods for determination of marine microalgal toxins, in Rossini, G.P. (Ed.), Toxins and Biologically Active Compounds from Microalgae, Volume 1. CRC Press, pp. 413-448.

Meissner, S., Steinhauser, D., Dittmann, E., 2015. Metabolomic analysis indicates a pivotal role of the hepatotoxin microcystin in high light adaptation of *Microcystis*. Environ. Microbiol. 17, 1497-1509.

Metcalf, J.S., Hyenstrand, P., Beattie, K.A., Codd, G.A., 2000. Effects of physicochemical variables and cyanobacterial extracts on the immunoassay of microcystin-LR by two ELISA kits. J. Appl. Microbiol. 89, 532-538.

Metcalf, J.S., Codd, G.A., 2003. Analysis of cyanobacterial toxins by immunological methods. Chem. Res. Toxicol. 16, 103-112.

Mihali, T.K., Carmichael, W.W., Neilan, B.A., 2011. A putative gene cluster from a *Lyngbya wollei* bloom that encodes paralytic shellfish toxin biosynthesis. PLoS One 6, e14657.

Mihali, T.K., Kellmann, R., Neilan, B.A., 2009. Characterisation of the paralytic shellfish toxin biosynthesis gene clusters in *Anabaena circinalis* AWQC131C and *Aphanizomenon* sp. NH-5. BMC Biochem. 10, 8.

Mikalsen, B., Boison, G., Skulberg, O.M., Fastner, J., Davies, W., Gabrielsen, T.M., Rudi, K., Jakobsen, K.S., 2003. Natural variation in the microcystin synthetase operon *mcyABC* and impact on microcystin production in *Microcystis* strains. J. Bacteriol. 185, 2774-2785.

Moffitt, M.C., Neilan, B.A., 2004. Characterization of the nodularin synthetase gene cluster and proposed theory of the evolution of cyanobacterial hepatotoxins. Appl. Environ. Microbiol. 70, 6353-6362.

Moustafa, A., Loram, J.E., Hackett, J.D., Anderson, D.M., Plumley, F.G., Bhattacharya, D., 2009. Origin of saxitoxin biosynthetic genes in cyanobacteria. PLoS One 4, e5758.

Murray, S.A., Mihali, T.K., Neilan, B.A., 2011a. Extraordinary conservation, gene loss, and positive selection in the evolution of an ancient neurotoxin. Mol. Biol. Evol. 28, 1173-1182.

Murray, S.A., Wiese, M., Stüken, A., Brett, S., Kellmann, R., Hallegraeff, G., Neilan, B.A., 2011b. A quantitative molecular assay based on the gene *sxtA* to identify saxitoxin-producing harmful algal blooms in marine waters. Appl. Environ. Microbiol. 77, 7050-7057.

Murray, S.A., Diwan, R., Orr, R.J.S., Kohli, G.S., John, U., 2015. Gene duplication, loss and selection in the evolution of saxitoxin biosynthesis in alveolates. Mol. Phylogenet. Evol. 92, 165-180.

Murray, S.A., Wiese, M., Neilan, B.A., Orr, R.J.S., de Salas, M., Brett, S., Hallegraeff, G., 2012. A reinvestigation of saxitoxin production and *sxtA* in the 'non-toxic' *Alexandrium tamarense* Group V clade. Harmful Algae 18, 96-104.

Myhre, O., Utkilen, H., Duale, N., Brunborg, G., Hofer, T., 2013. Metal dyshomeostasis and inflammation in Alzheimer's and Parkinson's diseases: possible impact of environmental exposures Oxid Med. Cell. Longev. 2013, 726954.

Nagai, S., 2011. Development of a multiplex PCR assay for simultaneous detection of six *Alexandrium* species (*Dinophyceae*). J. Phycol. 47, 703-708.

Namikoshi, M., Yuan, M., Sivonen, K., Carmichael, W.W., Rinehart, K.L., Rouhiainen, L., Sun, F., Brittain, S., Otsuki, A., 1998. Seven new microcystins possessing two L-glutamic acid units, isolated from *Anabaena* sp. strain 186. Chem. Res. Toxicol. 11, 143-149.

Negri, A., Stirling, D., Quilliam, M., Blackburn, S., Bolch, C., Burton, I., Eaglesham, G., Thomas, K., Walter, J., Willis, R., 2003. Three novel hydroxybenzoate saxitoxin analogues isolated from the dinoflagellate *Gymnodinium catenatum*. Chem. Res. Toxicol. 16, 1029-1033.

Negri, A.P., Bolch, C.J.S., Geier, S., Green, D.H., Park, T., Blackburn, S.I., 2007. Widespread presence of hydrophobic paralytic shellfish toxins in *Gymnodinium catenatum*. Harmful Algae 6, 774-780.

Neilan, B.A., Dittmann, E., Rouhiainen, L., Bass, R.A., Schaub, V., Sivonen, K., Börner, T., 1999. Nonribosomal peptide synthesis and toxigenicity of cyanobacteria. J. Bacteriol. 181, 4089-4097.

Neilan, B.A., Jacobs, D., Del Dot, T., Blackall, L.L., Hawkins, P.R., Cox, P.T., Goodman, A.E., 1997. rRNA sequences and evolutionary relationships among toxic and nontoxic cyanobacteria of the genus *Microcystis*. Int. J. Syst. Bacteriol. 47, 693-697.

Neilan, B.A., Jacobs, D., Goodman, A.E., 1995. Genetic diversity and phylogeny of toxic cyanobacteria determined by DNA polymorphisms within the phycocyanin locus Appl. Environ. Microbiol. 61, 3875-3883.

Neilan, B.A., Pearson, L.A., Moffitt, M.C., Mihali, K.T., Kaebernick, M., Kellmann, R., Pomati, F., 2008. The genetics and genomics of cyanobacterial toxicity. Adv. Exp. Med. Biol. 619, 417-452.

Neilan, B.A., Pearson, L.A., Muenchhoff, J., Moffitt, M.C., Dittmann, E., 2013. Environmental conditions that influence toxin biosynthesis in cyanobacteria. Environ. Microbiol. 15, 1239-1253.

Neilan, B.A., Stuart, J.L., Goodman, A.E., Cox, P.T., Hawkins, P.R., 1997. Specific amplification and restriction polymorphisms of the cyanobacterial rRNA operon spacer region. Syst. Appl. Microbiol. 20, 612-621.

Ngwa, F.F., Madramootoo, C.A., Jabaji, S., 2014a. Development and application of a multiplex qPCR technique to detect multiple microcystin-producing cyanobacterial genera in a Canadian freshwater lake J. Appl. Phycol. 26, 1675-1687.

Ngwa, F., Madramootoo, C., Jabaji, S., 2014b. Monitoring toxigenic *Microcystis* strains in the Missisquoi bay, Quebec, by PCR targeting multiple toxic gene loci. Environ. Toxicol. 29, 440-451.

Nishiwaki-Matsushima, R., Ohta, T., Nishiwaki, S., Suganuma, M., Kohyama, K., Ishikawa, T., Carmichael, W.W., Fujiki, H., 1992. Liver tumor promotion by the cyanobacterial cyclic peptide toxin microcystin-LR. J. Cancer Res. Clin. Oncol. 118, 420-424.

Nishizawa, T., Asayama, M., Fujii, K., Harada, K., Shirai, M., 1999. Genetic analysis of the peptide synthetase genes for a cyclic heptapeptide microcystin in *Microcystis* spp. J Biochem 126, 520-529.

Nishizawa, T., Asayama, M., Shirai, M., 2001. Cyclic heptapeptide microcystin biosynthesis requires the glutamate racemase gene. Microbiology 147, 1235-1241.

Nishizawa, T., Nishizawa, A., Asayama, M., Harada, K., Shirai, M., 2007. Diversity within the microcystin biosynthetic gene clusters among the genus *Microcystis*. Microb. Environ. 22, 380-390.

Nishizawa, T., Ueda, A., Asayama, M., Fujii, K., Harada, K., Ochi, K., Shirai, M., 2000. Polyketide synthase gene coupled to the peptide synthetase module involved in the biosynthesis of the cyclic heptapeptide microcystin. J Biochem 127, 779-789.

NIVA, 1976. Estimation of algal growth potential. Norwegian Inst. for Water Research, Publ. D2-25 .

Noguchi, T., Shinohara, A., Nishizawa, A., Asayama, M., Nakano, T., Hasegawa, M., Harada, K., Nishizawa, T., Shirai, M., 2009. Genetic analysis of the microcystin biosynthesis gene cluster in Microcystis strains from four bodies of eutrophic water in Japan J. Gen. Appl. Microbiol. 55, 111-123.

Nonnemann, D., Zimba, P.V., 2002. A PCR-based test to assess the potential for microcystin occurrence in channel catfish production ponds. J. Phycol. 38, 230-233.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., Hase, T., 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res. 28, E63.

Nurmi, J., Wikman, T., Karp, M., Lövgren, T., 2002. High-performance real-time quantitative RT-PCR using lanthanide probes and a dual-temperature hybridization assay. Anal. Chem. 74, 3525-3532.

Okello, W., Ostermaier, V., Portmann, C., Gademann, K., Kurmayer, R., 2010. Spatial isolation favours the divergence in microcystin net production by Microcystis in *Ugandan* freshwater lakes. Water Res. 44, 2803-2814.

Oksanen, I., Jokela, J., Fewer, D.P., Wahlsten, M., Rikkinen, J., Sivonen, K., 2004. Discovery of rare and highly toxic microcystins from lichen-associated cyanobacterium *Nostoc* sp. strain IO-102-I. Appl. Environ. Microbiol. 70, 5756-5763.

Onodera, H., Satake, M., Oshima, Y., Yasumoto, T., Carmichael, W.W., 1997. New saxitoxin analogues from the freshwater filamentous cyanobacterium *Lyngbya wollei*. Nat. Toxins 5, 146-151.

Orr, R.J., Murray, S.A., Stuken, A., Rhodes, L., Jakobsen, K.S., 2012. When naked became armored: an eight-gene phylogeny reveals monophyletic origin of theca in dinoflagellates. PLoS One 7, e50004.

Orr, R.J., Stüken, A., Murray, S.A., Jakobsen, K.S., 2013. Evolutionary acquisition and loss of saxitoxin biosynthesis in dinoflagellates: the second "core" gene, *sxtG*. Appl. Environ. Microbiol. 79, 2128-2136.

Orr, R.J.S., Stüken, A., Rundberget, T., Eikrem, W., Jakobsen, K.S., 2011. Improved phylogenetic resolution of toxic and non-toxic *Alexandrium* strains using a concatenated rDNA approach. Harmful Algae 10, 676-688.

Oshima, Y., 1995. Post-column derivatization liquid-chromatographic method for paralytic shellfish toxins. J. AOAC Int. 78, 528-532.

Oshima, Y., Blackburn, S.I., Hallegraeff, G.M., 1993. Comparative study on paralytic shellfish toxin profiles of the dinoflagellate *Gymnodinium catenatum* from three different countries. Mar. Biol. 116, 471-476.

Ostenfeld, C.H., 1913, De danske farvandes plankton i årene 1898–1901. K. Danske Vidensk. Selsk. Skr. 7. raekke, Afd., 9 (2), 117-478.

Ostermaier, V., Kurmayer, R., 2010. Application of real-time PCR to estimate toxin production by the cyanobacterium *Planktothrix* sp. Appl. Environ. Microbiol. 76, 3495-3502.

Ostermaier, V., Kurmayer, R., 2009. Distribution and abundance of nontoxic mutants of cyanobacteria in lakes of the Alps. Microb. Ecol. 58, 323-333.

Otsuka, S., Suda, S., Li, R., Watanabe, M., Oyaizu, H., Matsumoto, S., Watanabe, M.M., 1999. Phylogenetic relationships between toxic and non-toxic strains of the genus *Microcystis* based on 16S to 23S internal transcribed spacer sequence. FEMS Microbiol. Lett. 172, 15-21.

Ouahid, Y., Perez-Silva, G., del Campo, F.F., 2005. Identification of potentially toxic environmental *Microcystis* by individual and multiple PCR amplification of specific microcystin synthetase gene regions. Environ. Toxicol. 20, 235-242.

Ouellette, A.J., Handy, S.M., Wilhelm, S.W., 2006. Toxic *Microcystis* is widespread in Lake Erie: PCR detection of toxin genes and molecular characterization of associated cyanobacterial communities. Microb. Ecol. 51, 154-165.

Paerl, H.W., Paul, V.J., 2012. Climate change: Links to global expansion of harmful cyanobacteria. Water Res. 46, 1349-1363.

Palenik, B., Brahamsha, B., Larimer, F.W., Land, M., Hauser, L., Chain, P., Lamerdin, J., Regala, W., Allen, E.E., McCarren, J., Paulsen, I., Dufresne, A., Partensky, F., Webb, E.A., Waterbury, J., 2003. The genome of a motile marine *Synechococcus*. Nature 424, 1037-1042.

Pan, H., Song, L., Liu, Y., Börner, T., 2002. Detection of hepatotoxic *Microcystis* strains by PCR with intact cells from both culture and environmental samples. Arch. Microbiol. 178, 421-427.

Pearson, L.A., Barrow, K.D., Neilan, B.A., 2007. Characterization of the 2-hydroxy-acid dehydrogenase McyI, encoded within the microcystin biosynthesis gene cluster of *Microcystis aeruginosa* PCC7806. J. Biol. Chem. 282, 4681-4692.

Pearson, L.A., Hisbergues, M., Börner, T., Dittmann, E., Neilan, B.A., 2004. Inactivation of an ABC transporter gene, *mcyH*, results in loss of microcystin production in the cyanobacterium *Microcystis aeruginosa* PCC 7806. Appl. Environ. Microbiol. 70, 6370-6378.

Penna, A., Galluzzi, L., 2012. The quantitative real-time PCR applications in the monitoring of marine harmful algal bloom (HAB) species. Environ. Sci. Pollut. Res. Int. 20, 6851-6862.

Pereira, P., Onodera, H., Andrinolo, D., Franca, S., Araujo, F., Lagos, N., Oshima, Y., 2000. Paralytic shellfish toxins in the freshwater cyanobacterium *Aphanizomenon flos-aquae*, isolated from Montargil reservoir, Portugal. Toxicon 38, 1689-1702.

Perini, F., Galluzzi, L., Dell'Aversano, C., Dello Iacovo, E., Tartaglione, L., Ricci, F., Forino, M., Ciminiello, P., Penna, A., 2014. *SxtA* and *sxtG* gene expression and toxin production in the Mediterranean *Alexandrium minutum* (*Dinophyceae*). Mar. Drugs 12, 5258-5276.

Piel, J., 2004. Metabolites from symbiotic bacteria. Nat. Prod. Rep. 21, 519-538.

Pilotto, L.S., Douglas, R.M., Burch, M.D., Cameron, S., Beers, M., Rouch, G.J., Robinson, P., Kirk, M., Cowie, C.T., Hardiman, S., Moore, C., Attewell, R.G., 1997. Health effects of exposure to cyanobacteria (blue-green algae) during recreational water-related activities. Aust. N. Zealand J. Public Health 21, 562-566.

Pomati, F., Rossetti, C., Manarolla, G., Burns, B.P., Neilan, B.A., 2004. Interactions between intracellular Na+ levels and saxitoxin production in *Cylindrospermopsis raciborskii* T3. Microbiology 150, 455-461.

Pomati, F., Manarolla, G., Rossi, O., Vigetti, D., Rossetti, C., 2001. The purine degradation pathway: Possible role in paralytic shellfish toxin metabolism in the cyanobacterium *Planktothrix* sp. FP1. Environ. Int. 27, 463-470.

Pomati, F., Sacchi, S., Rossetti, C., Giovannardi, S., Onodera, H., Oshima, Y., Neilan, B.A., 2000. The freshwater cyanobacterium *Planktothrix* sp. FP1: Molecular identification and detection of paralytic shellfish poisoning toxins. J. Phycol. 36, 553-562.

Pouria, S., de Andrade, A., Barbosa, J., Cavalcanti, R.L., Barreto, V.T., Ward, C.J., Preiser, W., Poon, G.K., Neild, G.H., Codd, G.A., 1998. Fatal microcystin intoxication in haemodialysis unit in Caruaru, Brazil. Lancet 352, 21-26.

Poutanen, E., Nikkilä, K., 2001. Carotenoid pigments as tracers of cyanobacterial blooms in recent and post-glacial sediments of the Baltic Sea. Ambio 30, 179-183.

Prinsep, M., Caplan, F., Moore, R., Patterson, G., Honkanen, R., Boynton, A., 1992. Microcystin-LA from a blue-green alga belonging to the *Stigonematales*. Phytochemistry 31, 1247-1248.

R Core Team, 2015. R: A Language and Environment for Statistical Computing, http://www.R-Project.Org. R Foundation for Statistical Computing, Vienna, Austria.

Rantala, A., Fewer, D.P., Hisbergues, M., Rouhiainen, L., Vaitomaa, J., Börner, T., Sivonen, K., 2004. Phylogenetic evidence for the early evolution of microcystin synthesis. Proc. Natl. Acad. Sci. U. S. A. 101, 568-573.

Rantala, A., Rajaniemi-Wacklin, P., Lyra, C., Lepisto, L., Rintala, J., Mankiewicz-Boczek, J., Sivonen, K., 2006. Detection of microcystin-producing cyanobacteria in Finnish lakes with genus-specific microcystin synthetase gene E (*mcyE*) PCR and associations with environmental factors. Appl. Environ. Microbiol. 72, 6101-6110.

Rantala-Ylinen, A., Kana, S., Wang, H., Rouhiainen, L., Wahlsten, M., Rizzi, E., Berg, K., Gugger, M., Sivonen, K., 2011. Anatoxin-a synthetase gene cluster of the cyanobacterium *Anabaena* sp. Strain 37 and molecular methods to detect potential producers. Appl. Environ. Microbiol. 77, 7271-7278.

Rantala, A., Rizzi, E., Castiglioni, B., de Bellis, G., Sivonen, K., 2008. Identification of hepatotoxin-producing cyanobacteria by DNA-chip. Environ. Microbiol. 10, 653-664.

Rapala, J., Robertson, A., Negri, A.P., Berg, K.A., Tuomi, P., Lyra, C., Erkomaa, K., Lahti, K., Hoppu, K., Lepistö, L., 2005. First report of saxitoxin in Finnish lakes and possible associated effects on human health. Environ. Toxicol. 20, 331-340.

Rapala, J., Erkomaa, K., Kukkonen, J., Sivonen, K., Lahti, K., 2002. Detection of microcystins with protein phosphatase inhibition assay, high-performance liquid chromatography–UV detection and enzyme-linked immunosorbent assay: Comparison of methods. Anal. Chim. Acta 466, 213-231.

Rasmussen, J.P., Barbez, P.H., Burgoyne, L.A., Saint, C.P., 2008. Rapid preparation of cyanobacterial DNA for real-time PCR analysis. Lett. Appl. Microbiol. 46, 14-19.

Rein, K.S., Snyder, R.V., 2006. The biosynthesis of polyketide metabolites by dinoflagellates. Adv. Appl. Microbiol. 59, 93-125.

Rinehart, K.L., Harada, K.I., Namikoshi, M., Chen, C., Harvis, C., Munro, M., Blunt, J., Mulligan, P., Beasley, V., Dahlem, A.M., Carmichael, W.W., 1988. Nodularin, microcystin and the configuration of Adda. J. Am. Chem. Soc. 110, 8557-8558.

Rinehart, K., Namikoshi, M., Choi, B., 1994. Structure and biosynthesis of toxins from blue-green algae (cyanobacteria). J. Appl. Phycol. 6, 159-176.

Rinta-Kanto, J.M., Ouellette, A.J., Boyer, G.L., Twiss, M.R., Bridgeman, T.B., Wilhelm, S.W., 2005. Quantification of toxic *Microcystis* spp. during the 2003 and 2004 blooms in western Lake Erie using quantitative real-time PCR. Environ. Sci. Technol. 39, 4198-4205.

Rinta-Kanto, J.M., Konopko, E.A., DeBruyn, J.M., Bourbonniere, R.A., Boyer, G.L., Wilhelm, S.W., 2009. Lake Erie *Microcystis*: Relationship between microcystin production, dynamics of genotypes and environmental parameters in a large lake. Harmful Algae 8, 665-673.

Rivasseau, C., Racaud, P., Deguin, A., Hennion, M., 1999. Development of a bioanalytical phosphatase inhibition test for the monitoring of microcystins in environmental water samples. Anal. Chim. Acta 394, 243-257.

Rohrlack, T., Christiansen, G., Kurmayer, R., 2013. Putative antiparasite defensive system involving ribosomal and nonribosomal oligopeptides in cyanobacteria of the genus *Planktothrix*. Appl. Environ. Microbiol. 79, 2642-2647.

Rohrlack, T., Dittmann, E., Borner, T., Christoffersen, K., 2001. Effects of cell-bound microcystins on survival and feeding of *Daphnia* spp. Appl. Environ. Microbiol. 67, 3523-3529.

Rouhiainen, L., Vakkilainen, T., Siemer, B.L., Buikema, W., Haselkorn, R., Sivonen, K., 2004. Genes coding for hepatotoxic heptapeptides (microcystins) in the cyanobacterium *Anabaena* strain 90. Appl. Environ. Microbiol. 70, 686-692.

Runnegar, M., Berndt, N., Kong, S.M., Lee, E.Y., Zhang, L., 1995. *In vivo* and *in vitro* binding of microcystin to protein phosphatases 1 and 2A. Biochem. Biophys. Res. Commun. 216, 162-169.

Runnegar, M.T., Gerdes, R.G., Falconer, I.R., 1991. The uptake of the cyanobacterial hepatotoxin microcystin by isolated rat hepatocytes. Toxicon 29, 43-51.

Sabart, M., Crenn, K., Perrière, F., Abila, A., Leremboure, M., Colombet, J., Jousse, C., Latour, D., 2015. Co-occurrence of microcystin and anatoxin-a in the freshwater lake Aydat (France): Analytical and molecular approaches during a three-year survey. Harmful Algae 48, 12-20.

Saker, M., Vale, M., Kramer, D., Vasconcelos, V., 2007. Molecular techniques for the early warning of toxic cyanobacteria blooms in freshwater lakes and rivers. Appl. Microbiol. Biotechnol. 75, 441-449.

Saker, M.L., Fastner, J., Dittmann, E., Christiansen, G., Vasconcelos, V.M., 2005. Variation between strains of the cyanobacterium *Microcystis aeruginosa* isolated from a Portuguese river. J. Appl. Microbiol. 99, 749-757.

Sako, Y., Kim, C., Ishida, Y., 1992. Mendelian inheritance of paralytic shellfish poisoning toxin in the marine dinoflagellate *Alexandrium catenella*. Biosci. Biotechnol. Biochem. 56, 692-694.

Sako, Y., Yoshida, T., Uchida, A., Arakawa, O., Noguchi, T., Ishida, Y., 2001. Purification and characterization of a sulfotransferase specific to N-21 of saxitoxin and gonyautoxin 2+3 from the toxic dinoflagellate *Gymnodinium catenatum* (*Dinophyceae*). J. Phycol. 37, 1044-1051.

Sant'Anna, C.L., de Carvalho, L.R., Fiore, M.F., Silva-Stenico, M.E., Lorenzi, A.S., Rios, F.R., Konno, K., Garcia, C., Lagos, N., 2010. Highly toxic *Microcystis aeruginosa* strain, isolated from Sao Paulo-Brazil, produce hepatotoxins and paralytic shellfish poison neurotoxins. Neurotox. Res. 19, 389-402.

Satake, M., Ofuji, K., Naoki, H., James, K.J., Furey, A., McMahon, T., Silke, J., Yasumoto, T., 1998. Azaspiracid, a new marine toxin having unique spiro ring assemblies, isolated from Irish mussels, *Mytilus edulis*. J. Am. Chem. Soc. 120, 9967-9968.

Savichtcheva, O., Debroas, D., Kurmayer, R., Villar, C., Jenny, J.P., Arnaud, F., Perga, M.E., Domaizon, I., 2011. Quantitative PCR enumeration of total/toxic *Planktothrix* rubescens and total cyanobacteria in preserved DNA isolated from lake sediments. Appl. Environ. Microbiol. 77, 8744-8753.

Sayfritz, S.J., Aasen, J.A., Aune, T., 2008. Determination of paralytic shellfish poisoning toxins in Norwegian shellfish by liquid chromatography with fluorescense and tandem mass spectrometry. Toxicon , 330-340.

Schantz, E.J., Mold, J.D., Stanger, D.W., Shavel, J., Riel, F.J., Bowden, J.P., Lynch, J.M., Wyler, R.S., Riegel, B., Sommer, H., 1957. Paralytic shellfish poison. VI. A procedure for the isolation and purification of the poison from toxic clam and mussel tissues. J. Am. Chem. Soc. 79, 5230-5235.

Schantz, E.J., Lynch, J.M., Vayvada, G., Matsumoto, K., Rapoport, H., 1966. The purification and characterization of the poison produced by *Gonyaulax catenella* in axenic culture. Biochemistry (N. Y.) 5, 1191-1195.

Schatz, D., Keren, Y., Vardi, A., Sukenik, A., Carmeli, S., Börner, T., Dittmann, E., Kaplan, A., 2007. Towards clarification of the biological role of microcystins, a family of cyanobacterial toxins. Environ. Microbiol. 9, 965-970.

Schober, E., Kurmayer, R., 2006. Evaluation of different DNA sampling techniques for the application of the real-time PCR method for the quantification of cyanobacteria in water. Lett. Appl. Microbiol. 42, 412-417.

Schober, E., Werndl, M., Laakso, K., Korschineck, I., Sivonen, K., Kurmayer, R., 2007. Interlaboratory comparison of Taq Nuclease Assays for the quantification of the toxic cyanobacteria *Microcystis* sp. J. Microbiol. Methods 69, 122-128.

Scholin, C., Hallegraeff, G.M., Anderson, D.M., 1995. Molecular evolution of the *Alexandrium tamarense* 'species complex' (*Dinophyceae*): dispersal in the North American and West Pacific regions. Phycologia 34, 472-485.

Scholin, C.A., Marin, R., Miller, P.E., Doucette, G.J., Powell, C.L., Haydock, P., Howard, J., Ray, J., 1999. DNA probes and a receptor-binding assay for detection of *Pseudo-nitzscia* (*Bacillariophyceae*) species and domoid acid activity in cultured and natural samples. J. Phycol. 35, 1356-1367.

Schrader, C., Schielke, A., Ellerbroek, L., Johne, R., 2012. PCR inhibitors - occurrence, properties and removal. J. Appl. Microbiol. 113, 1014-1026.

Schwinghamer, P., Hawryluk, M., Powell, C., MacKenzie, C.H., 1994. Resuspended hypnozygotes of *Alexandrium fundyense* associated with winter occurrence of PSP in inshore Newfoundland waters. Aquaculture 122, 171-179.

Seki, T., Satake, M., Mackenzie, L., Kaspar, H.F., Yasumoto, T., 1995. Gymnodimine, a new marine toxin of unprecedented structure isolated from New Zealand oysters and the dinoflagellate, *Gymnodinium* sp. Tetrahedron Lett. 36, 7093-7096.

Shi, L., Carmichael, W.W., Miller, I., 1995. Immuno-gold localization of hepatotoxins in cyanobacterial cells. Arch. Microbiol. 163, 7-15.

Shih, P.M., Wu, D., Latifi, A., Axen, S.D., Fewer, D.P., Talla, E., Calteau, A., Cai, F., Tandeau de Marsac, N., Rippka, R., Herdman, M., Sivonen, K., Coursin, T., Laurent, T., Goodwin, L., Nolan, M., Davenport, K.W., Han, C.S., Rubin, E.M., Eisen, J.A., Woyke, T., Gugger, M., Kerfeld, C.A., 2013. Improving the coverage of the cyanobacterial phylum using diversity-driven genome sequencing. Proc. Natl. Acad. Sci. U. S. A. 110, 1053-1058.

Shimizu, Y., Yoshioka, M., 1981. Transformation of paralytic shellfish toxins as demonstrated in scallop homogenates Science 212, 547-549.

Shimizu, Y., 1993. Microalgal metabolites. Chem. Rev. 93, 1685-1698.

Shimizu, Y., Hsu, C., Genenah, A., 1981. Structure of saxitoxin in solutions and stereochemistry of dihydrosaxitoxins. J. Am. Chem. Soc. 103, 605-609.

Shimizu, Y., Norte, M., Hori, A., Genenah, A., Kobayashi, M., 1984. Biosynthesis of saxitoxin analogs: the unexpected pathway. J. Am. Chem. Soc. 106, 6433-6434.

Shumway, S.E., 1990. A review of the effects of algal blooms on shellfish and aquaculture. J. World Aquacult. Soc. 21, 65-104.

Sielaff, H., Dittmann, E., Tandeau De Marsac, N., Bouchier, C., Von Döhren, H., Börner, T., Schwecke, T., 2003. The *mcyF* gene of the microcystin biosynthetic gene cluster from *Microcystis aeruginosa* encodes an aspartate racemase. Biochem. J. 373, 909-916.

Singh, S., Rai, P.K., Chau, R., Ravi, A.K., Neilan, B.A., Asthana, R.K., 2015. Temporal variations in microcystin-producing cells and microcystin concentrations in two fresh water ponds. Water Res. 69, 131-142.

Sipari, H., Rantala-Ylinen, A., Jokela, J., Oksanen, I., Sivonen, K., 2010. Development of a chip assay and quantitative PCR for detecting microcystin synthetase E gene expression. Appl. Environ. Microbiol. 76, 3797-3805.

Sivonen, K., Himberg, K., Luukkainen, R., Niemelä, S.I., Poon, G.K., Codd, G.A., 1989a. Preliminary characterization of neurotoxic cyanobacteria blooms and strains from Finland. Toxic. Assess. 4, 339-352.

Sivonen, K., Kononen, K., Carmichael, W.W., Dahlem, A.M., Rinehart, K.L., Kiviranta, J., Niemela, S.I., 1989b. Occurrence of the hepatotoxic cyanobacterium *Nodularia spumigena* in the Baltic Sea and structure of the toxin. Appl. Environ. Microbiol. 55, 1990-1995.

Sivonen, K., Carmichael, W.W., Namikoshi, M., Rinehart, K.L., Dahlem, A.M., Niemela, S.I., 1990a. Isolation and characterization of hepatotoxic microcystin homologs from the filamentous freshwater cyanobacterium *Nostoc* sp. strain 152. Appl. Environ. Microbiol. 56, 2650-2657.

Sivonen, K., Jones, G., 1999. Cyanobacterial toxins, in Bartram, J., Chorus, I. (Eds.), Toxic Cyanobacteria in Water: A Guide to their Health Consequences, Monitoring and Management. Spon Press, UK, pp. 41-111.

Sivonen, K., Namikoshi, M., Evans, W.R., Carmichael, W.W., Sun, F., Rouhiainen, L., Luukkainen, R., Rinehart, K.L., 1992. Isolation and characterization of a variety of microcystins from seven strains of the cyanobacterial genus *Anabaena*. Appl. Environ. Microbiol. 58, 2495-2500.

Sivonen, K., Niemelä, S., Niemi, R., Lepistö, L., Luoma, T., Räsänen, L., 1990b. Toxic cyanobacteria (blue-green algae) in Finnish fresh and coastal waters. Hydrobiologia 190, 267-275.

Sivonen, K., 2009. Cyanobacterial toxins, in Schaechter, M. (Ed.), Encyclopedia of Microbiology (Third Edition). Academic Press, Oxford, pp. 290-307.

Smith, E.A., Grant, F., Ferguson, C.M., Gallacher, S., 2001. Biotransformations of paralytic shellfish toxins by bacteria isolated from bivalve molluscs. Appl. Environ. Microbiol. 67, 2345-2353.

Smith, F.M., Wood, S.A., van Ginkel, R., Broady, P.A., Gaw, S., 2011. First report of saxitoxin production by a species of the freshwater benthic cyanobacterium, *Scytonema* Agardh. Toxicon 57, 566-573.

Smith, K., de Salas, M., Adamson, J., Rhodes, L., 2014. Rapid and accurate identification by real-time PCR of biotoxin-producing dinoflagellates from the family *Gymnodiniaceae*. Mar. Drugs 12, 1361-1376.

Soto-Liebe, K., Murillo, A.A., Krock, B., Stucken, K., Fuentes-Valdes, J.J., Trefault, N., Cembella, A., Vasquez, M., 2010. Reassessment of the toxin profile of *Cylindrospermopsis raciborskii* T3 and function of putative sulfotransferases in synthesis of sulfated and sulfonated PSP toxins. Toxicon 56, 1350-1361.

Soto-Liebe, K., Méndez, M.A., Fuenzalida, L., Krock, B., Cembella, A., Vásquez, M., 2012. PSP toxin release from the cyanobacterium *Raphidiopsis brookii* D9 (*Nostocales*) can be induced by sodium and potassium ions. Toxicon 60, 1324-1334.

Spoof, L., Vesterkvist, P., Lindholm, T., Meriluoto, J., 2003. Screening for cyanobacterial hepatotoxins, microcystins and nodularin in environmental water samples by reversed-phase liquid chromatography-electrospray ionisation mass spectrometry. J. Chromatogr. A 1020, 105-119.

Spoof, L., Meriluoto, J., 2005. Analysis of microcystins by high-performance liquid chromatography with photodiode-array detection., in Meriluoto, J., Codd, G.A. (Eds.), TOXIC: Cyanobacterial Monitoring and Cyanotoxin Analysis. Åbo Akademi University Press, Turku, Finland, pp. 77-84.

Srivastava, A., Choi, G., Ahn, C., Oh, H., Ravi, A.K., Asthana, R.K., 2012. Dynamics of microcystin production and quantification of potentially toxigenic *Microcystis* sp. using real-time PCR. Water Res. 46, 817-827.

Stachelhaus, T., Mootz, H.D., Marahiel, M.A., 1999. The specificity-conferring code of adenylation domains in nonribosomal peptide synthetases. Chem. Biol. 6, 493-505.

Staub, R., 1961. Ernährungsphysiologisch-autökologische Untersuchungen an der Planktischen Blaualge *Oscillatoria rubescens* DC. Schweitz. Z. Hydrol. 23, 82-198.

Stewart, I., Seawright, A.A., Shaw, G.R., 2008. Cyanobacterial poisoning in livestock, wild mammals and birds–an overview. Adv. Exp. Med. Biol. 619, 613-637.

Straub, C., Quillardet, P., Vergalli, J., de Marsac, N.T., Humbert, J.F., 2011. A day in the life of *Microcystis aeruginosa* strain PCC 7806 as revealed by a transcriptomic analysis. PLoS One 6, e16208.

Strichartz, G., 1984. Structural determinants of the affinity of saxitoxin for neuronal sodium channels. Electrophysiological studies on frog peripheral nerve. J. Gen. Physiol. 84, 281-305.

Stucken, K., John, U., Cembella, A., Murillo, A.A., Soto-Liebe, K., Fuentes-Valdes, J.J., Friedel, M., Plominsky, A.M., Vasquez, M., Glockner, G., 2010. The smallest known genomes of multicellular and toxic cyanobacteria: comparison, minimal gene sets for linked traits and the evolutionary implications. PLoS One 5, e9235.

Stüken, A., Orr, R.J., Kellmann, R., Murray, S.A., Neilan, B.A., Jakobsen, K.S., 2011. Discovery of nuclear-encoded genes for the neurotoxin saxitoxin in dinoflagellates. PLoS One 6, e20096.

Stüken, A., Dittami, S.M., Eikrem, W., McNamee, S., Campbell, K., Jakobsen, K.S., Edvardsen, B., 2013. Novel hydrolysis-probe based qPCR assay to detect saxitoxin transcripts of dinoflagellates in environmental samples. Harmful Algae 28, 108-117.

Stüken, A., Riobó, P., Franco, J., Jakobsen, K.S., Guillou, L., Figueroa, R.I., 2015. Paralytic shellfish toxin content is related to genomic *sxtA4* copy number in *Alexandrium minutum* strains. Front. Microbiol. 6, Article 404.

Su, Z., Sheets, M., Ishida, H., Li, F., Barry, W.H., 2004. Saxitoxin blocks L-type I_{Ca}. J. Pharmacol. Exp. Ther. 308, 324-329.

Suikkanen, S., Kremp, A., Hautala, H., Krock, B., 2013. Paralytic shellfish toxins or spirolides? The role of environmental and genetic factors in toxin production of the *Alexandrium ostenfeldii* complex. Harmful Algae 26, 52-59.

Sullivan, J.J., Iwaoka, W.T., Liston, J., 1983. Enzymatic transformation of PSP toxins in the littleneck clam (*Protothacastaminea*). Biochem. Biophys. Res. Commun. 114, 465-472.

Svirčev, Z., Krstič, S., Miladinov-Mikov, M., Baltič, V., Vidovič, M., 2009. Freshwater cyanobacterial blooms and primary liver cancer epidemiological studies in Serbia. J. Environ. Sci. Health. C. Environ. Carcinog. Ecotoxicol. Rev. 27, 36-55.

Tahvanainen, P., Alpermann, T.J., Figueroa, R.I., John, U., Hakanen, P., Nagai, S., Blomster, J., Kremp, A., 2012. Patterns of post-glacial genetic differentiation in marginal populations of a marine microalga. PLoS One 7, e53602.

Tanabe, Y., Kasai, F., Watanabe, M.M., 2007. Multilocus sequence typing (MLST) reveals high genetic diversity and clonal population structure of the toxic cyanobacterium *Microcystis aeruginosa*. Microbiology 153, 3695-3703.

Tanabe, Y., Kaya, K., Watanabe, M.M., 2004. Evidence for recombination in the microcystin synthetase (*mcy*) genes of toxic cyanobacteria *Microcystis* spp. J. Mol. Evol. 58, 633-641.

Tanabe, Y., Sano, T., Kasai, F., Watanabe, M.M., 2009. Recombination, cryptic clades and neutral molecular divergence of the microcystin synthetase (*mcy*) genes of toxic cyanobacterium *Microcystis aeruginosa*. BMC Evol. Biol. 9, 115-2148-9-115.

Tang, X., Yu, R., Zhou, M., Yu, Z., 2012. Application of rRNA probes and fluorescence in situ hybridization for rapid detection of the toxic dinoflagellate *Alexandrium minutum*. Chin. J. Oceanol. Limnol. 30, 256-263.

Te, S.H., Chen, E.Y., Gin, K.Y., Schaffner, D.W., 2015. Comparison of quantitative PCR and droplet digital PCR multiplex assays for two genera of bloom-forming cyanobacteria, *Cylindrospermopsis* and *Microcystis*. Appl. Environ. Microbiol. 81, 5203-5211.

Te, S.H., Gin, K.Y., 2011. The dynamics of cyanobacteria and microcystin production in a tropical reservoir of Singapore. Harmful Algae 10, 319-329.

Teixeira Mda, G., Costa Mda, C., de Carvalho, V.L., Pereira Mdos, S., Hage, E., 1993. Gastroenteritis epidemic in the area of the Itaparica Dam, Bahia, Brazil Bull. Pan Am. Health Organ. 27, 244-253.

Tikkanen, T., 1986, Kasviplanktonopas. Suomen luonnonsuojeluliitto, Helsinki, 279 pp.

Tillett, D., Dittmann, E., Erhard, M., von Döhren, H., Börner, T., Neilan, B.A., 2000. Structural organization of microcystin biosynthesis in *Microcystis aeruginosa* PCC7806: an integrated peptide-polyketide synthetase system. Chem. Biol. 7, 753-764.

Tillett, D., Parker, D.L., Neilan, B.A., 2001. Detection of toxigenicity by a probe for the microcystin synthetase A gene (*mcyA*) of the cyanobacterial genus *Microcystis*: comparison of toxicities with 16S rRNA and phycocyanin operon (Phycocyanin intergenic spacer) phylogenies. Appl. Environ. Microbiol. 67, 2810-2818.

Toebe, K., 2013a. Whole cell hybridisation for monitoring harmful marine microalgae. Environ. Sci. Pollut. Res. 20, 6816-6823.

Toebe, K., Alpermann, T.J., Tillmann, U., Krock, B., Cembella, A., John, U., 2013b. Molecular discrimination of toxic and non-toxic *Alexandrium* species (*Dinophyta*) in natural phytoplankton assemblages from the Scottish coast of the North Sea. Eur. J. Phycol. 48, 12-26.

Tonk, L., Visser, P.M., Christiansen, G., Dittmann, E., Snelder, E.O., Wiedner, C., Mur, L.R., Huisman, J., 2005. The microcystin composition of the cyanobacterium *Planktothrix agardhii* changes toward a more toxic variant with increasing light intensity. Appl. Environ. Microbiol. 71, 5177-5181.

Tooming-Klunderud, A., Fewer, D.P., Rohrlack, T., Jokela, J., Rouhiainen, L., Sivonen, K., Kristensen, T., Jakobsen, K.S., 2008a. Evidence for positive selection acting on microcystin synthetase adenylation domains in three cyanobacterial genera. BMC Evol. Biol. 8, 256.

Tooming-Klunderud, A., Mikalsen, B., Kristensen, T., Jakobsen, K.S., 2008b. The mosaic structure of the *mcyABC* operon in *Microcystis*. Microbiology 154, 1886-1899.

Tooming-Klunderud, A., Sogge, H., Rounge, T.B., Nederbragt, A.J., Lagesen, K., Glockner, G., Hayes, P.K., Rohrlack, T., Jakobsen, K.S., 2013. From green to red: horizontal gene transfer of the phycoerythrin gene cluster between *Planktothrix* strains. Appl. Environ. Microbiol. 79, 6803-6812.

Touzet, N., Franco, J., Raine, R., 2008. PSP toxin analysis and discrimination of the naturally co-occurring *Alexandrium tamarense* and *A. minutum* (*Dinophyceae*) in Cork Harbour, Ireland. Aquat. Microb. Ecol. 51, 285-299.

Touzet, N., Keady, E., Raine, R., Maher, M., 2009. Evaluation of taxa-specific real-time PCR, whole-cell FISH and morphotaxonomy analyses for the detection and quantification of the toxic microalgae *Alexandrium minutum* (*Dinophyceae*), Global Clade ribotype. FEMS Microbiol. Ecol. 67, 329-341.

Turner, P.C., Gammie, A.J., Hollinrake, K., Codd, G.A., 1990. Pneumonia associated with contact with cyanobacteria. BMJ 300, 1440-1441.

Usup, G., Kulis, D.M., Anderson, D.M., 1994. Growth and toxin production of the toxic dinoflagellate *Pyrodinium bahamense* var. *compressum* in laboratory cultures. Nat. Toxins 2, 254-262.

Utermöhl, H., 1958. Zur Vervollkommnung der quantitativen Phytoplankton-Methodik. Mitt. Int. Verein. Limnol. 9, 1-38.

Utkilen, H., Skulberg, O.M., Skulberg, R., Gjølme, N., Underdal, B., 2001. Toxic cyanobacterial blooms of inland waters in southern Norway, in Chorus, I. (Ed.), Cyanotoxins: Occurrence, Causes, Consequences. Springer-Verlag, Germany, pp. 46-49.

Vaitomaa, J., Rantala, A., Halinen, K., Rouhiainen, L., Tallberg, P., Mokelke, L., Sivonen, K., 2003. Quantitative real-time PCR for determination of microcystin synthetase E copy numbers for *Microcystis* and *Anabaena* in lakes. Appl. Environ. Microbiol. 69, 7289-7297.

Vale, P., 2008. Complex profiles of hydrophobic paralytic shellfish poisoning compounds in *Gymnodinium catenatum* identified by liquid chromatography with fluorescence detection and mass spectrometry. J. Chromatogr. A 1195, 85-93.

van Apeldoorn, M.E., van Egmond, H.P., Speijers, G.J., Bakker, G.J., 2007. Toxins of cyanobacteria. Mol. Nutr. Food Res. 51, 7-60.

Vezie, C., Rapala, J., Vaitomaa, J., Seitsonen, J., Sivonen, K., 2002. Effect of nitrogen and phosphorus on growth of toxic and nontoxic *Microcystis* strains and on intracellular microcystin concentrations. Microb. Ecol. 43, 443-454.

Via-Ordorika, L., Fastner, J., Kurmayer, R., Hisbergues, M., Dittmann, E., Komarek, J., Erhard, M., Chorus, I., 2004. Distribution of microcystin-producing and non-microcystin-producing *Microcystis* sp. in European freshwater bodies: detection of microcystins and microcystin genes in individual colonies. Syst. Appl. Microbiol. 27, 592-602.

von Lode, P., Syrjälä, A., Hagren, V., Kojola, H., Soukka, T., Lövgren, T., Nurmi, J., 2007. Fully automated, homogeneous nucleic acid detection technology based on dry-reagent assay chemistry and time-resolved fluorometry. Clin. Chem. 53, 2014-2017.

Wang, J., Salata, J.J., Bennett, P.B., 2003. Saxitoxin is a gating modifier of HERG K+ channels J. Gen. Physiol. 121, 583-598.

Weusten, J., Herbergs, J., 2012. A stochastic model of the processes in PCR based amplification of STR DNA in forensic applications. Forensic. Sci. Int. Genet. 6, 17-25.

WHO, 2011. Guidelines for Drinking-Water Quality, 4th ed. World Health Organization.

Wiese, M., D'Agostino, P.M., Mihali, T.K., Moffitt, M.C., Neilan, B.A., 2010. Neurotoxic alkaloids: saxitoxin and its analogs. Mar. Drugs 8, 2185-2211.

Wiese, M., Murray, S.A., Alvin, A., Neilan, B.A., 2014. Gene expression and molecular evolution of *sxtA4* in a saxitoxin producing dinoflagellate *Alexandrium catenella*. Toxicon 92, 102-112.

Wilson, I.G., 1997. Inhibition and facilitation of nucleic acid amplification. Appl. Environ. Microbiol. 63, 3741-3751.

Wittwer, C.T., Herrmann, M.G., Moss, A.A., Rasmussen, R.P., 1997. Continuous fluorescence monitoring of rapid cycle DNA amplification. BioTechniques 22, 130-1, 134-8.

Yeung, P.K., Kong, K.F., Wong, F.T., Wong, J.T., 1996. Sequence data for two large-subunit rRNA genes from an Asian strain of *Alexandrium catenella*. Appl. Environ. Microbiol. 62, 4199-4201.

Yoshida, M., Yoshida, T., Kashima, A., Takashima, Y., Hosoda, N., Nagasaki, K., Hiroishi, S., 2008. Ecological dynamics of the toxic bloom-forming cyanobacterium *Microcystis aeruginosa* and its cyanophages in freshwater. Appl. Environ. Microbiol. 74, 3269-3273.

Yoshida, M., Yoshida, T., Takashima, Y., Hosoda, N., Hiroishi, S., 2007. Dynamics of microcystin-producing and non-microcystin-producing *Microcystis* populations is correlated with nitrate concentration in a Japanese lake. FEMS Microbiol. Lett. 266, 49-53.

Yoshida, M., Yoshida, T., Takashima, Y., Kondo, R., Hiroishi, S., 2005. Genetic diversity of the toxic cyanobacterium *Microcystis* in Lake Mikata. Environ. Toxicol. 20, 229-234.

Yoshida, T., Sako, Y., Uchida, A., Kakutani, T., Arakawa, O., Noguchi, T., Ishida, Y., 2002. Purification and characterization of sulfotransferase specific to O-22 of 11-hydroxy saxitoxin from the toxic dinoflagellate *Gymnodinium catenatum* (*Dinophyceae*). Fisheries Sci. 68, 634-642.

Young, F.M., Thomson, C., Metcalf, J.S., Lucocq, J.M., Codd, G.A., 2005. Immunogold localisation of microcystins in cryosectioned cells of *Microcystis*. J. Struct. Biol. 151, 208-214.

Yu, S.Z., 1995. Primary prevention of hepatocellular carcinoma. J. Gastroenterol. Hepatol. 10, 674-682.

Yunes, J.S., De La Rocha, S., Giroldo, D., Silveira, S.B.d., Comin, R., Bicho, M.d.S., Melcher, S.S., Sant'anna, C.L., Vieira, A.A.H., 2009. Release of carbohydrates and proteins by a subtropical strain of *Raphidiopsis brookii* (*Cyanobacteria*) able to produce saxitoxin at three nitrate concentration. J. Phycol. 45, 585-591.

Zaman, L., Arakawa, O., Shimosu, A., Shida, Y., Onoue, Y., 1998. Occurrence of a methyl derivative of saxitoxin in Bangladeshi freshwater puffers. Toxicon 36, 627-630.

Zhang, Y., Zhang, S.F., Lin, L., Wang, D.Z., 2014. Comparative transcriptome analysis of a toxin-producing dinoflagellate *Alexandrium catenella* and its non-toxic mutant. Mar. Drugs 12, 5698-5718.

Zhu, F., Massana, R., Not, F., Marie, D., Vaulot, D., 2005. Mapping of picoeucaryotes in marine ecosystems with quantitative PCR of the 18S rRNA gene. FEMS Microbiol. Ecol. 52, 79-92.

Zhu, P., Zhang, B., Wu, J., Dang, C., Lv, Y., Fan, J., Yan, X., 2014. Sensitive and rapid detection of microcystin synthetase E Gene (*mcyE*) by loop-mediated isothermal amplification: A new assay for detecting the potential microcystin-producing *Microcystis* in the aquatic ecosystem. Harmful Algae 37, 8-16.