Uptake and depuration of cyanotoxins in the common blue mussel *Mytilus edulis*

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Declaration

I declare that the work presented in this thesis is my own, except where otherwise acknowledged and has not been submitted in any form for another degree or qualification at any other academic institution.

Information derived from published or unpublished work of others has been acknowledged in the text and a list of references is given.

Julia Waack

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List of Abbreviations

afdw	Ash free dry weight
BEH	Ethylene Bridged Hybrid
bw	Body weight
CCAP	Culture collection of algae and protozoa
cGST	cytosolic glutathione-S-transferase
CR	Clearance rate
CYN	Cylindrospermopsin
Cys	Cysteine
DA	Domoic acid
dm	demethylated
dw	Dry weight
EDI	Estimated daily intake
EFSA	European Food Standard Agency
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionisation
FID	Flame ionisation detector
FR	Filtration rate
fdt	freeze dried tissue
GSH	Glutathione
GST	Glutathione-S-transferase
IOS	Instant Ocean Salt
i.p.	Intraperitoneal

IPA	Isopropyl alcohol
KAC	Kalmar Algae Collection
LNOD	Linear nodularin
LOD	Limit of detection
LOQ	Limit of quantification
LRM	Laboratory reference material
m	methylated
MC	Microcystin
MC-LReq	Microcystin-LR equivalent
Mdha	Methyldehydroalanine
Mdhb	Methyldehydrobutyrine
MMPB	2-methyl-3-methoxy-4-phenylbutyric acid
NA	Not applicable
NIVA CCA	Norwegian Institute for Water Research Culture Collection of Algae
NIES	National Institute for Environmental Studies, Japan
NOD	Nodularin
NSD	No significant difference
PCC	Pasteur Culture collection of Cyanobacteria
PPases	Protein phosphatases
PPIA	Protein phosphatase inhibition assay
PSP	Paralytic shellfish poisoning
PST	Paralytic shellfish toxin
PVC	Polyvinyl chloride

RRF	Relative response factor
SPE	solid phase extraction
SRM	Selective Reaction Monitoring
STX	Saxitoxin
TDI	Tolerable daily intake
TIC	Total ion count
UTEX	Culture Collection of Algae at The University of Texas at Austin
UV	Ultraviolet
WHO	World Health Organisation
ww	wet weight
w/v	weight per volume

Abstract

Cyanobacteria produce a variety of secondary metabolites which possess amongst others antifungal, antibacterial, and antiviral properties. Being primary producers they are also a vital component within the food web. However, certain strains also produce toxic metabolites such as the hepatotoxins microcystin (MC) and nodularin (NOD). Their toxicity in combination with the increasing global occurrence has resulted in a drinking water guideline limit of 1 µg L^{-1} being issued by the World Health Organisation (WHO). However, these toxins are not only present in water, but can be accumulated by fish and shellfish. Currently, no regulations regarding cyanotoxin contaminated seafood has been established despite similar toxicity to routinely monitored marine toxins such as domoic acid (DA). To facilitate regular monitoring, a high performance liquid chromatography photo diode array (HPLC-PDA) analysis method for the detection of DA was optimised to enable the simultaneous detection of DA and nine cyanotoxins. This method was then utilised to determine cyanotoxin concentration in laboratory cyanobacteria strains.

To assess the accumulation and depuration of cyanotoxins in the common blue mussel *Mytilus edulis*, three feeding trials were performed. During these, mussels were exposed to two cyanobacteria strains, *Nodularia spumigena* KAC66, *Microcystis aeruginosa* PCC 7813, both individually and simultaneously. A rapid dose dependent accumulation of cyanotoxins was observed with maximum concentration of 3.4 -17 µg g^{-1} ww accumulated by *M. edulis*, which was followed by a much slower depuration observed. During the final feeding trial, with *N. spumigena* KAC 66 and *M. aeruginosa* PCC7813, cyanotoxins were still detectable following 27 days of depuration. Mortality in all studies was 7% or less indicating that most mussels were unaffected by the maximum dose of 480 µg L^{-1} NOD (feeding study 1), 390 µg L^{-1} MC (feeding study 2), or 130 µg L^{-1} total cyanotoxins (feeding trial 3), respectively. Mortality in negative control tanks was lower throughout all three feeding trials (<1 - 2.6%). Consumption of a typical portion size (20 mussels) would result in ingestion of cyanotoxins at levels significantly higher than the WHO recommended tolerable daily intake (TDI) of 2.4 µg NOD and/or MCs for a 60 kg adult. This value was exceeded not only during the exposure period (maximum levels 270 - 1370 µg cyanotoxins per 20 mussels), but also at the end of the depuration period 39-600 µg

cyanotoxins per 20 mussels. These results illustrated that cyanotoxin monitoring of seafood should be considered not only during, but also following bloom events. In an attempt to investigate the cyanotoxin budget of the experimental system, not only mussels, but cyanobacteria cultures, the tank water, and the mussel faeces were also analysed for their cyanotoxin content. Results showed that large quantities of MCs and NOD were unaccounted for during all exposure trials. The combined effect of cyanotoxin metabolism in *M. edulis*, biotic and/or abiotic degradation, protein binding, and losses during the extraction and analysis were thought to have contributed to the unaccounted cyanotoxin fraction. Mussel flesh was analysed for the presence of glutathione or cysteine conjugates, however, there was no evidence of their occurrence in the samples tested. Due to these discrepancies in the toxin budget of the system, the introduction of correction factors for the analysis of cyanotoxins in *M. edulis* was suggested in order to protect the general public.

Keywords: Cyanobacteria, cyanotoxins, microcystin, nodularin, *Mytilus edulis*, *Microcystis aeruginosa*, *Nodularia spumigena*

Chapter 1

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1.1 Cyanobacteria

Cyanobacteria are a group of aerobic photoautotroph bacteria, which have been around for 3.5 billion years (Gibbons and Murray 1979; Schopf and Packer 1987). They are thought to have played a major role in the oxygenation of the atmosphere, providing an essential component for the evolution of aerobic organisms (Paerl and Huisman, 2009). In addition to being a source of a vast number of bioactive compounds with promising antialgal, antibacterial, antifungal, antiviral, antibiotic, and anticancer properties, cyanobacteria also produce toxic metabolites (cyanotoxins; Harke *et al.,* 2016; Carmichael 1992; Volk and Furkert 2006; Schaeffer and Krylov 2000; Nunnery *et al*., 2010: Gademann and Portman 2008).

A large number of these important primary producers thrive in freshwater, but they can also inhabit the marine (Kalaitzidou *et al*., 2015; Guidi-Rontani *et al*., 2014; Banack *et al*., 2007; Golubic *et al*., 2010) and terrestrial environment (Vijayan and Ray 2015). In addition, cyanobacteria have been isolated from thermal springs (Beattie *et al*., 2000), sulfidic springs (Klatt *et al.*, 2016), arctic freshwater (Kleinteich *et al*., 2013), Antarctic microbial matts (Hitzfeld *et al*., 2000) and deserts (Pringault and Garcia-Pichel 2004) illustrating their ability to adapt to extreme habitats. Similar to the variety of environments cyanobacteria inhabit, they also show a diversity in appearance, which is mainly divided into unicellular or filamentous strains. Present in filamentous strains, heterocysts are specialised cells able to fix nitrogen (Gibbon and Murray 1979).

Factors influencing cyanobacteria growth include light availability, water temperature, phosphate and nitrate availability and salinity (Howard and Easthope 2002; Miller *et al*., 2010). Increased phosphate and nitrate concentrations are often the result of industrial and agricultural sewage run-offs (Hunter *et al*., 2012). Once the combined effect of these factors reach species dependent optimums, mass growth (so called blooms) are formed in water bodies all around the globe (Table 1. 1; Paerl 1988; Dokulil and Teubner 2000). Toxin production by blooms is not the only threat to the ecosystem as increased pH and ammonia concentration as well as oxygen depletion are often a result of blooms. In combination, these changes can reduce the diversity, survival, and growth rate of zooplankton species (Tyler *et al*., 2009, Li *et al*., 2007; Sotton *et*

al., 2014). Nonetheless, toxin production remains a major hazard associated with cyanobacterial blooms (Sotton *et al*., 2011). The most common cyanobacterial taxa forming toxic blooms are *Microcystis, Anabaena, Dolichospermum, Aphanizomenon, Oscillatoria, Nodularia, Cylindrospermopsis,* and *Planktothrix* (Howard and Easthope 2002, Dyble *et al*., 2011; Oehrle *et al*., 2010; Kinnear 2010; Wacklin *et al*., 2009).

Table 1. 1 Reported MCs and NOD levels detected in water bodies all around the world

Water body	Country	Toxin	C_{max} µg L^{-1}	Reference
Lake Marathonas	Greece	MC-YR†	0.717	Kaloudis *et al*. (2013)
		MC-LR†	0.451	
		MC-RR†	0.174	
Amvrakikos Gulf	Greece	MC-LR*	0.0198	Vareli *et al*. (2012)
Lake Suwa	Japan	MC-LR + MC-RR*	184	Park *et al.* (1998)
Zemborzycki dam reservoir	Poland	MCs*	22.2	Pawlik-Skowrońska *et al.* (2013)
Grand-Lieu Lake (Capitaine)	France	MC*	7.16	Lance *et al.* (2010)
Lake Taihu	China	MC-LR*	15.58	Li *et al.* (2007)
		MCs*	5.7	Chen and Xie (2007)
		MC-LR + MC-RR*	1.37	Zhang *et al.* (2009b)
Lake Erie	USA	MCs*	4.284	Wilson *et al.* (2008)
Lake Occhito	Italy	Total MCs (ELISA)*†	298	De Pace *et al*. (2014)
Asriatic Sea (Apulia region)			0.38	
Funil Reservoir	Brazil	MCs*	2.168	Deblois *et al.* (2008)
Furnas reservoir			0.941	
Gulf of Finland	Finland	NOD†	2.6	Kankaanpää *et al.* (2001)
Sound and Køge Bay, Baltic Sea	Denmark	NOD*	565	Henriksen (2005)
Lake Alexandria	Australia	NOD*†[1]	~0.9	Heresztyn *et al.* (1997)
Lake Albert			~1.0	
Narrung Channel			~1.7	

c_{max} - maximum concentration reported, ***** - intracellular, **†** - extracellular, **1 -** estimated from graph

Table 1. 1 continued

Water body	Country	Toxin	c_{max} µg L^{-1}	Reference
Jacarepaguá Lagoon	Brazil	MCs*	1.7	Freitas de Magalhães, *et al*. (2001)
Lake Lac des Allemands	United States of America	Total MCs (ELISA)*	1.42	Garcia *et al*. (2010)
Lake Ijsselmeer	Netherlands	MC*	17	Ibelings *et al*. (2005)
Fish pond Novoveský	Czech Republic	MCs†	9.51	Kopp *et al*. (2013)
Pinto Lake	United States of America	MCs*	2.9	Miller *et al*. (2010)
Guadiana River	Spain	Total MC (ELISA)*†	6.4	Moreno *et al*. (2005)
Grand Lake St. Marys	United States of America	Total MCs (ELISA)*†	>100	Schmidt *et al*. (2013)
Murchison Bay, Lake Victoria	Uganda	MC-LR + MC-YR + MC-RR*	1.11	Semyalo *et al*. (2011)
Chesapeake Bay	United States of America	Total MCs (ELISA)*	658	Tango and Butler (2008)
Lake Hallwil	Switzerland	MC-LR + MC-RR*	6.36	Sotton *et al.* (2014)
Gulf of Gdańsk	Poland	NOD	25852* 95†	Mazur-Marzec *et al.* (2006a)
Tai Lake	Taiwan	MCs*†	3.38	Yen *et al*. (2011)

c_{max} - maximum concentration reported, *** -** intracellular, **† -** extracellular

Table 1. 1 continued

Water body	Country	Toxin	C_{max} µg L^{-1}	Reference
Sestroretskij Razliv Lake	Russia	MCs	41.37† 380*	Chernova *et al*. (2016)
Lower Suzdal Lake		MCs	2.89† 234*	
		ATX-a	0.54† 30*	
Lake Karczemne	Poland	MCs	305.4*	Mazur-Marzec *et al*. (2008)
Lake Iznik	Turkey	CYN	4.92*	Akcaalan *et al.* (2014)
Aquaculture pond	Australia	CYN	589 *†	Saker and Eaglesham (1999)
Monteleone (reservoir)	Italy	MCs	226.16†	Messineo *et al*. (2009)
Albano Lake		CYN	2126†	
Mulgaria Lake		ATX-a	100 µg g^{-1} in scums	
Anzali wetland	Iran	MC-LR	3.02† 1.13*	Rezaitabar *et al.* (2017)
Lake Prästträsket	Finland	dmMC-RR, MC-RR, MC-LR, MC-YR	42	Spoof *et al*. (2003)

C_{max} - maximum concentration reported, *** -** intracellular, **† -** extracellular

Microcystis thrive in stable water columns (Tonk *et al.,* 2007). Increased temperatures favour their bloom formation, as higher growth rates have, for example, been observed at 32 °C in comparison to 18 or 25 °C (Watanabe and Oishi 1985). In addition nitrogen has been suggested to be crucial for the growth of *M. aeruginosa*. Reduction of nitrogen by a twentieth in growth medium has resulted in retarded growth. Similar changes to phosphate content did not affect the growth of *M. aeruginosa* (Watanabe and Oishi 1985). Meanwhile, an interactive effect of both nitrogen and phosphorus availability on the other hand has been reported for *Microcystis* spp. when grown in medium of varying nitrogen and phosphorus concentration (Vézie *et al*. 2002). Due to its global occurrence in freshwater combination with the production of the toxic metabolite group of microcystins, *Microcystis* was chosen as an experimental strain. Metcalf *et al*. (2008) investigated cyanotoxin presence in 11 freshwater bodies in England, Scotland, and Wales. Seven of these water bodies contained MCs at concentrations ranging from 0.002-780 μg g^{-1} dw.

Nodularia sp. have adapted to the brackish water environment. Optimum growth has been reported at varying salinities: 0-5 practical salinity units (psu; Lehtimaki *et al*., 1997) and 7-18 psu (Mazur-Marzec *et al.,* 2005). In agreement with these reports Moisander *et al.* (2002) showed that *N. spumigena* FL2f did not change growth rates when grown at salinities of 2, 6, 10, 15, and 20 psu. Similarly, Blackburn *et al.* (1996) reported no significantly different growth rates for *N. spumigena* grown at salinities of 12, 24, 35 psu. Due to this ability to grow in the brackish water environment, *Nodularia* was chosen as one of the experimental strains. The abundancy of *Nodularia* within the Baltic Sea is restricted to the warmer summer month and increased from North to South in accordance with increasing salinities (Lehtimaki *et al*., 1997). Despite this, Galanti *et al*. (2013) reported NOD concentrations of up to 0.2 μg L^{-1} in freshwater (San Roque Reservoir; Argentina), however, the cyanobacteria producing NOD was not identified. In the UK a *N. spumigena* strain (UD15) has been isolated from Lincolnshire (Twist and Codd 1997). In addition, NOD was detected in brackish water in England (950 μg g^{-1}; Metcalf *et al*., 2008).

1.2 Toxins produced by cyanobacteria

Cyanobacteria are known to produce multiple toxin groups. Frequently occurring are the hepatotoxins. Other substances encountered include neurotoxins, dermatotoxins and lipopolysaccharides (Tyler *et al*., 2009; Zimba *et al*., 2006; Lance *et al*., 2014). Most of these toxins are confined within the cyanobacterial cell and only become available after lysis. However, these toxins can also occur extracellularly (Adamovský *et al*., 2007). Based on their toxic mechanism these can be classified into the following groups: hepatotoxins, neurotoxins, cytotoxins, and dermatotoxins and irritants (Table 1. 2).

Table 1. 2 Key groups of cyanotoxins and their toxicity

Toxin group	Toxin	LD_{50}	Reference
Hepatotoxins	Microcystins (peptide)	50-600[a] μg kg^{-1}	Gunn (1992); An and Carmichael (1994); Codd and Roberts (1991)
	Nodularin (peptide)	50[a] μg kg^{-1}	Codd *et al*. (1999); An and Carmichael (1994)
	Cylindrospermopsin (alkaloid)	200[a,b] μg kg^{-1}	Foss and Aubel, (2013); Seifert *et al*. (2007)
Neurotoxins	Kalkitoxin (lipopeptide)	3.86 nM	Paerl and Huisman, 2009, Wu *et al.*, 2000; Choi *et al*. (2012);
	Antillatoxin (lipopeptide)	180[d] nM	Paerl and Huisman (2009); Li *et al*. (2004); Choi *et al*. (2012)
	Beta-N-methylamino-L-alanine (amino acid)	1[e] nM	Brand *et al.* (2010); Weiss *et al*. (1989)
	Anatoxin-a (alkaloid)	250[a, f] μg kg^{-1}	Kuiper-Goodman *et al*. (1999); Devlin *et al.* (1977)
	Homoanatoxin-a (alkaloid)	0.228-5.781[a,g] μg kg^{-1}	Edwards *et al*. (1996); Lileheil *et al*. (1997)
	Saxitoxins	8-10[a] μg kg^{-1}	Codd and Roberts (1991); Llewellyn (2006)
	Anatoxin-a(s)	40[a] μg kg^{-1}	Mahmood and Carmichael 1987
Dermatotoxins	Aplysiatoxins (polyketide)	300[a] μg kg^{-1}	Paerl and Huisman (2009); Yadav *et al.* (2011)
	Lyngbyatoxin (terpenoids)	250[a,h] μg kg^{-1}	Choi *et al*. (2012)
Irritants	Lipopolysaccharides	0.045-0.19[a] μg kg^{-1}	Stewart *et al*. (2006); Yadav *et al.* (2011)

LD_{50} dose that leads to the death of 50% of the tested population
a intraperitoneal (i.p.) in mouse **b** after 27 hours **c** cell culture of rat neurons **d** in mouse neuro 2a cell line **e** given as concentration which caused destruction of 50% of mouse cortical neurons **f** given as minimum lethal dose **g** survival time was 4 min **h** young mice

1.2.1 Hepatotoxins

Hepatotoxins produced by cyanobacteria include microcystins (MCs), nodularins (NODs) and cylindrospermopsin (CYN). MCs are actively transported via organic anion transporting polypeptides in liver and in the brain (Fischer *et al*., 2005). The effect on the liver is generally most severe (Cazenave *et al*., 2005; Bury *et al.*, 2005). However, intestines, kidneys, and muscle tissue of fish can also contain these toxins (Mekebri *et al*., 2009, Li *et al*., 2007; Cazenave *et al*., 2005; Papadimitriou *et al*., 2013; Papadimitriou *et al*., 2010).

MCs are cyclic heptapeptides with the general structure cyclo(-D-Ala^{1}-L-**X**$^{\mathbf{2}}$-erhtro-β-methyl-D-isoAsp^{3}-L-**Z**^{4}-Adda^{5}-D-isoGlu^{6}-N-methyldehydro-Ala^{7}; Dyble *et al*. 2011; Cazenave *et al*. 2005; de Figueiredo *et al*., 2004). Adda is a hydrophobic amino acid with the following structure: 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Hyenstrand *et al*., 2001, Neffling *et al*., 2010). Due to their cyclic structure MCs are chemically very stable (Sivonen and Jones 1999) and can withstand temperatures of up to 100°C (Kos et al., 1995). As a result of amino acid variation (at positions **X** and **Z**; Figure 1. 1), variations in methylation status of amino acids, modifications of Adda, and methyl esterification of glutamic acid, to date nearly 250 MC variants have been reported (Sotton *et al*., 2011; Zurawell *et al*., 2005, Catherine *et al*., 2016; Figure 1. 1; Table 1. 3). Kuiper-Goodman *et al*. (1999) reported toxicity levels for various MC congeners which had been determined after i.p. injection in mice. Reported LD_{50} concentrations, the dose causing death of half of the mice, ranged from 50 to >1200 μg kg^{-1}. The Adda moiety of the molecule has been suggested to be essential for MC toxicity as its isomerisation rendered MCs non-toxic (Mekebri *et al.,* 2009). Due to its toxicity and global occurrence the variant MC-LR, which incorporates the amino acids leucine (L, at position X) and arginine (R, at position Z) has been studied extensively. Other commonly encountered variants include MC-RR and MC-YR (de Figueiredo *et al*., 2004; Ríos *et al*., 2013). Demethylated variants of MC-LR and MC-RR are produced by for example *P. agardhii* and *P. rubescens* (Lance *et al*., 2010b). MCs are produced by species such as: *Microcystis, Planktothrix (Oscillatoria), Anabaena, Phormidium* and *Nostoc* (Freitas *et al*., 2014; de Figueiredo *et al*., 2004; Oehrle *et al*., 2010).

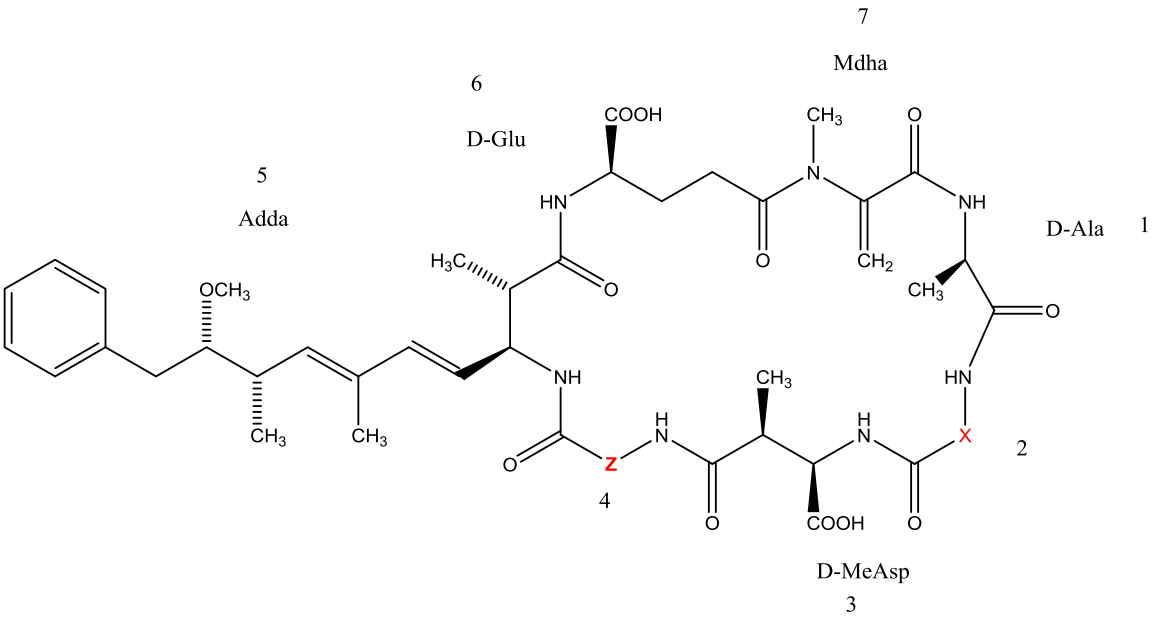

Figure 1. 1 General structure of MCs (adapted from An and Carmichael 1994). Position Z and X are those where the variable amino acids are located.

Table 1. 3 Example of amino acid composition of common MCs and reported toxicity (adapted from Zurawell *et al*., 2005; Chorus and Bartram 1999)

Variant	Position X	Position Z	Molecular weight	Toxicity LD_{50} i.p. in mice (μg kg^{-1})
MC-RR	Arginine	Arginine	1037	600
MC-LR	Leucine	Arginine	994	50
MC-LA	Leucine	Alanine	909	50
MC-LY	Leucine	Tyrosine	1001	90
MC-LW	Leucine	Tryptophan	1024	NR
MC-LF	Leucine	Phenylalanine	985	NR

LD_{50} – dose causing death of 50% of experimental animals, NR – not reported

NOD (Figure 1. 2), structurally similar to MCs, consist of a five membered ring (pentapeptide): cyclo(-D-*erythro*-β-methy-1Asp[1](iso-linkage)-L-Arg[2]-Adda[3]-D-Glu[4](iso-linkage)-*N*-methyldehydrobutyrine[5]; Namikoshi *et al.,* 1994). The smaller size compared to MCs is associated with higher tumour promoting activity due to easier access to liver cells (Kankaanpää *et al*., 2002; Karlsson *et al*. 2003). Due to variations in demethylation state, methylation, and isomerisation of Adda eight NOD variants have been reported (Mazur-Marzec *et al*., 2006c). Its toxicity has been assessed in mouse bioassays and the LD_{50} was determined to be 50-70 μg kg^{-1} (Carmichael *et al*., 1988; Runnegar *et al*., 1998; Namikoshi *et al*., 1993).

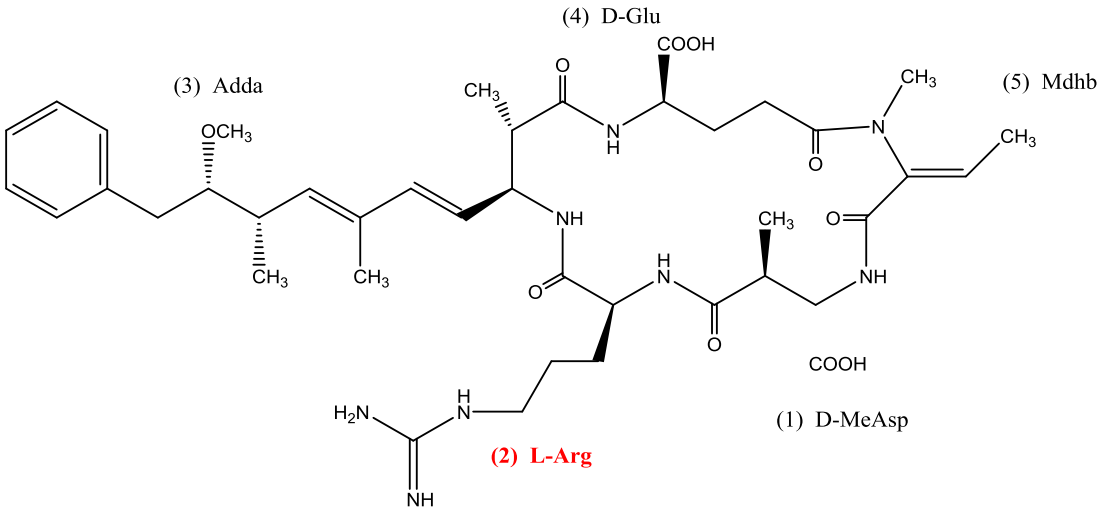

Figure 1. 2 Molecular structure of nodularin-R (adapted from Namikoshi *et al.,* 1994).

The linear congener of NOD (LNOD), which has been reported as existing in three isomeric forms, was non-toxic in mice bioassays and was suggested to be a biogenic precursor of NOD (Choi *et al*., 1993; Mazur-Marzec *et al*., 2008). Similarly to MCs, isomerisation at the Adda moiety in NOD results in loss of toxicity (Agrawal and Gopla 2013). NODs are mainly produced by *Nodularia spumigena* in brackish waters (Mekebri *et al.*, 2009, Sipiä *et al.,* 2002). The Baltic Sea in particular, supports toxic blooms predominately containing NOD (Kankaanpää *et al.,* 2007, Sipiä *et al.*, 2002, Lehtonen *et al.*, 2003, Karlsson *et al.*, 2003, Karlson and Mozūraitis 2011). Despite the initial belief that *N. spumigena* was the only species capable of NOD production, the endosymbiosis of *Nostoc* and cycad plants produces NOD and [L-har2] NOD-R (Wood *et al*., 2012).

Both MCs and NOD inhibit the serine/threonine protein phosphatases (PPases) (1, 2A, 4, and 5; Yoshizawa *et al*., 1990; El Ghazali *et al*., 2010, Neffling *et al*., 2010). The degree of this binding can be reversible via hydrogen bond formation between the Adda moiety of both MCs and NOD with the catalytic subunit of the enzyme (Smith *et al*. 2010). The Adda moiety, present in both NOD and MCs, moves into the hydrophobic grove of PPases' and consequently blocks the active site of the PPases which is located at the head of this groove (Goldberg *et al*., 1995; Kelker *et al*., 2009). In addition to this interaction, MCs are also able to form a permanent bond between the Mdha moiety of MCs and a cysteine residue within the PPases' active site (Smith *et al.*, 2010). NOD on the other hand

contains methyldehydrobutyrine (Mdhb) instead of Mdha and only binds non-covalently to PPases (Bagu *et al*., 1997).

Smith and Boyer (2009) estimated that the mammalian liver is able to bind about 1 µg of MC-LR per gram liver (wet weight, ww). PPase inhibition causes the disintegration of the liver cytoskeleton resulting in liver haemorrhage, liver necrosis, and ultimately death of the organism (Lance *et al*., 2014; Lehtonen *et al*., 2003; Dyble *et al*., 2011). Chronic exposure to MCs and NODs has further been associated with increased occurrence of liver cancer (Mekebri *et al*., 2009). Ueno *et al.* (1996) reported close correlation of primary liver cancer and the consumption of water from ponds and ditches in Haimen, China. During their water survey in 1993-1995, the authors detected MCs mainly during the summer months. Symptoms that have been described in humans following exposure to MCs and NOD are vomiting, diarrhoea, abdominal cramping, the formation of blisters at the mouth, muscle tremors, general weakness, and cold extremities (Dyble *et al*., 2011; Garcia *et al*., 2010; Kankaanpää *et al*., 2002).

An additional hepatotoxin produced by cyanobacteria is cylindrospermopsin (CYN), a zwitterionic alkaloid consisting of a tricyclic guanidine moiety, a sulphate ester functionality, and a dioxypyrimidine (uracil) moiety (Gutiérrez-Praena *et al*., 2012; White *et al*., 2006; Elliott *et al*., 2013). The uracil moiety is required for the toxicity of CYN (Banker *et al*., 2001). Two tautomers have been reported, the enol and the keto tautomer (Figure 1. 3). CYN is less toxic in mouse bioassay compared to MCs and NOD, but toxicity was shown to be time dependent. The LD_{50} in mouse after i.p. injection of lysed *C. raciborskii* cells was 286 µg kg^{-1} after 24 h. However, the LD_{50} reported over 7 days was 176 µg kg^{-1} (Hawkins *et al*., 1997). Furthermore, an epimere (7-epiCYN; Banker *et al*., 2000) and a variant in which the hydroxyl group at position 7 is replaced by hydrogen (deoxyCYN) exist (Norris *et al*., 1999). According to mouse bioassays 7-epiCYN does have a similar toxicity compared to CYN whereas deoxyCYN did not show a toxic effect, but does have the potential to inhibit protein synthesis (Runnegar *et al*., 2002).

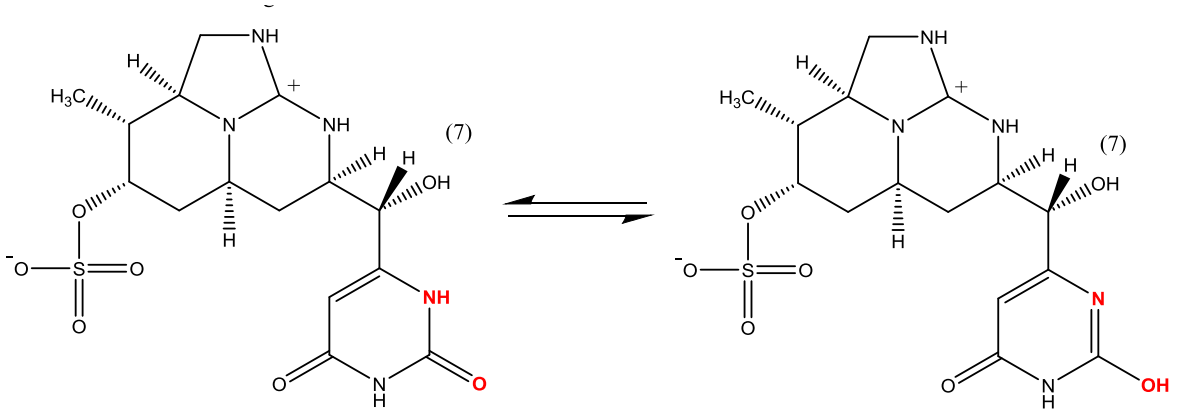

Figure 1. 3 Chemical structure of the CYN tautomers (adapted from Elliott *et al*. 2013)

CYNs are produced by amongst others *Cylindrospermopsis raciborskii*, *Anabaena* spp., *Lyngbya wollei,* and *Aphanizomenon ovalisporum* (White *et al.,* 2007; Seifert *et al.,* 2007; White *et al*., 2006). Similar to MCs, CYN is also very stable when exposed to extreme temperature and pH. It was reported in Australia after 148 people suffered from hepatoenteritis in 1979 (Foss and Aubel 2013). This incident is also known as Palm Island mystery disease. Treatment of drinking-water with copper sulphate caused *C. raciborskii* cells to lyse which led to the release of CYN into the water (Hawkins *et al.*, 1985). CYN is known to mainly target the liver (Foss and Aubel 2013). However, lungs, kidneys, intestinal tract, heart, spleen, thymus, and reproductive organs have also been affected (Foss and Aubel 2013; Elliott *et al*., 2013). Reportedly uracil is the moiety associated with the toxicity of CYN which is able to inhibit both glutathione and protein synthesis (Gutiérrez-Praena *et al*. 2012; White *et al*., 2007).

1.2.2 Neurotoxins

Anatoxin-a (ATX-a) is another alkaloid produced, amongst others, by strains of the *Anabaena* genus with the following structure: 2-acetyl-9-azabicyclo[4.2.1]non-2-ene (Devlin *et al.,* 1977; Figure 1. 4 A). This neurotoxin mimics the neurotransmitter acetylcholine, however, it cannot be degraded by acetylcholinesterase which would enable both receptor and muscle to return to the resting state. Therefore, ATX-a initiated overstimulation results in exhaustion of respiratory muscles which finally cease to function causing death through suffocation (Carmichael 1994). Another molecule isolated from

Anabaena flos-aquae was termed ATX-a(s) due to its similar neurotoxic effect (Figure 1. 4 C; Mahmood and Carmichael 1986). In contrast to ATX-a, this naturally occurring organophosphate inhibits acetylcholine esterase which would normally degrade the neurotransmitter acetylcholine in respiratory muscles (Carmichael 1994). Consequently the muscle is overstimulated leading to exhaustion and finally cessation of their function causing death through suffocation, similar to ATX-a. An actual methylene analogue of ATX-a, homoanatoxin-a, which blocks neuromuscular signal transmission has been purified from *Oscillatoria formosa* NIVA-CYA 92 (Figure 1. 4 B; Skulberg *et al*., 1992; Lilheil *et al*., 1997).

In addition to ATX-a the neurotoxicity of the more hydrophobic cyanotoxins, MC variants MC-LF and MC-LW has recently emerged. Rozman *et al.* (2017) reported dose-dependent toxicity of these two congeners to rat astrocytes (star shaped cells providing support for neurons in brain and spinal column). Therefore, potential neurotoxic effects in humans following the exposure to MC-LW and MC-LF need to be considered.

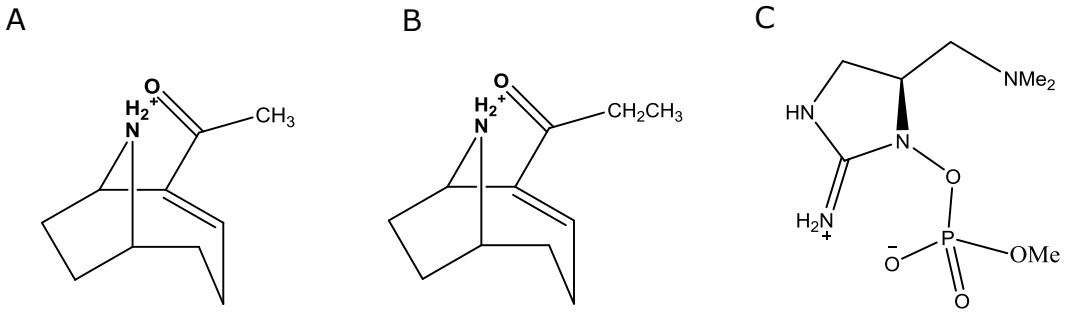

Figure 1. 4 Molecular structure of anatoxin-a (A), homoanatoxin (B), and anatoxin-s(s) (C) (adapted from Skulberg *et al*., 1992; Matsunaga *et al*., 1989)

Other neurotoxins produced by cyanobacteria are the group of saxitoxins (STX; Wiese *et al*., 2010; Figure 1. 5). This group of toxins, also called paralytic shellfish toxins (PST), has been termed to be the most toxic naturally occurring compound (Wiese *et al*., 2010). STXs were first associated to marine dinoflagellates (genus *Alexandrium;* Anderson *et al.,* 1990) and to date 57 analogues have been reported (Cirés and Ballot 2016; Wiese *et al*., 2010). STXs are alkaloids that causes paralytic shellfish poisoning (PSP), which act by blocking sodium channels with great affinity (Strichartz *et al*., 1987). STX is able to block these channels in muscles causing paralysis (Kao and Nishiyama 1965). This group of toxins has been detected in *Dolichospermum circinale,*

Aphanizomenon gracile, *Cuspidothrix issatschenkoi, C. raciborskii*, and *Phormidium uncinatum* (Cirés and Ballot 2016).

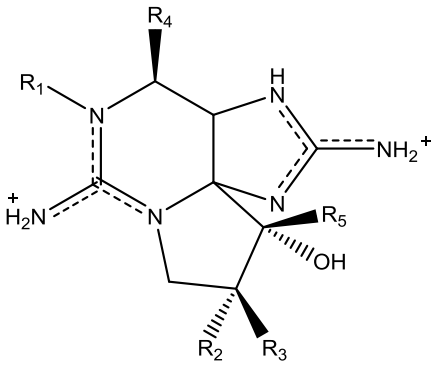

Figure 1. 5 General structure of saxitoxins (adapted from Wiese *et al*., 2010)

1.3 Analysis of cyanotoxins

To determine cyanotoxin content of blooms, visual examination is not sufficient (Mekebri *et al*., 2009). Therefore, various analytical techniques have been developed, the most common techniques being enzyme linked immunosorbent assays (ELISA), protein phosphatase (PPase) inhibition assays (PPIA), and liquid chromatography (LC) based separation methods. ELISA analysis relies on antibody interactions specifically designed for the respective analyte hence resulting in good sensitivity and fast analysis (de Figueiredo *et al*., 2004; Kankaanpää *et al*., 2002). In addition, commercial ELISA kits are available enabling fast screening of field samples. Excellent applicability of ELISA was demonstrated for the detection of MCs in snail organs with negligible matrix effects, LODs of 0.1 ng g^{-1} dry weight (dw) and excellent analyte recoveries (>88%; Zhang *et al*., 2012). Variability was low with relative standard deviations (%RSD) <5%.

However, ELISAs are susceptible to specificity issues as the antibodies are not specific for a certain variant but interact with the Adda moiety of MCs, NOD, and their less toxic conjugates (Mekebri *et al.,* 2009; de Figueiredo *et al*., 2004; Lance *et al*., 2010b; Sotton *et al*., 2012; Kankaanpää *et al*., 2005). In addition, during an interlaboratory comparison experiment, ELISA analysis of an MC-LR standard showed significantly lower precision, as higher repeatability values compared to HPLC-PDA analysis were encountered (Fastner *et al*., 2002). Other challenges encountered were false positive and negatives and varying affinities

towards MC variants (Moreno *et al*., 2004; Sotton *et al*., 2014). The specificity issues of ELISA were illustrated by Dyble *et al.* (2011) who detected MC-LR in liver tissue of control fish which had not been fed with food pellets containing MC-LR. However, this could be a result of previous exposure to MCs as fish were only acclimatised for a maximum of 28h. Nonetheless, discrepancies between ELISA and LC mass spectrometry (LC-MS) results have been reported. Kankaanpää *et al*. (2007) investigated NOD presence in digestive glands of the common blue mussel *Mytilus edulis.* Compared to LC-MS analysis ELISA detected only 12% of NOD in mussel tissue. Lower MC quantities were also detected in water samples analysed by ELISA when compared to high performance LC combined with both ultraviolet (UV) and MS detection (Spoof *et al*., 2003). Similarly, when determining the MC content in snails following 5 weeks of exposure to dissolved MC-LR, the results varied based on the detection method applied (Lance *et al*., 2010b). No MC-LR was detected in snail tissue utilising LC tandem MS (MS/MS) analysis, whereas ELISA analysis resulted in 0.07 and 0.26 μg g^{-1} MC-LR being detected in snails exposed to 33 and 100 μg L^{-1} MC-LR, respectively. On the other hand, no significant differences were determined between results obtained for ELISA and LC-MS/MS methods utilised to determine free, unbound MC concentrations in fish tissue (Lance *et al.,* 2014). Similarly, agreeable results within a MC concentration range of 0.5-35 μg g^{-1}, were obtained when utilising both ELISA and LC-MS/MS analysis for the quantification of MCs in blue-green algae products (Lawrence *et al*., 2001). Overall therefore there are significant concerns about the specificity and applicability of ELISA detection methods for cyanotoxins in some matrices.

PPIAs utilise the ability of both MCs and NOD to inhibit PPases (section 1.2.1). Similar to ELISA this assay cannot distinguish between different hepatotoxins and their conjugates, but, unlike ELISAs, results show an overview of the potential total toxicity. Furthermore, PPIA was prone to overestimations due to varying specificity towards different MC variants and NODs (Engström-Öst *et al*., 2002; de Figueiredo *et al.,* 2004; McElhiney and Lawton 2005). Other substances that lead to positive results were for example calyculin, cantharidin, okadaic acid, tautomycin, and anabenapeptins also produced by cyanobacteria (de Figueiredo *et al*., 2004; Engström-Öst *et al*., 2002; McElhiney and Lawton 2005).

Given the specificity issues of both ELISA and PPIA, these techniques are often utilised for screening purposes followed by LC analysis for confirmatory purposes. LC is able to separate individual MC variants based on their polarities (Figure 1. 6). Most commonly HPLC and ultra-high performance liquid chromatography (UHPLC) have been applied for the detection of different cyanotoxins in multiple matrices. However, without pre-treatment the analysis is limited to the detection of free analytes.

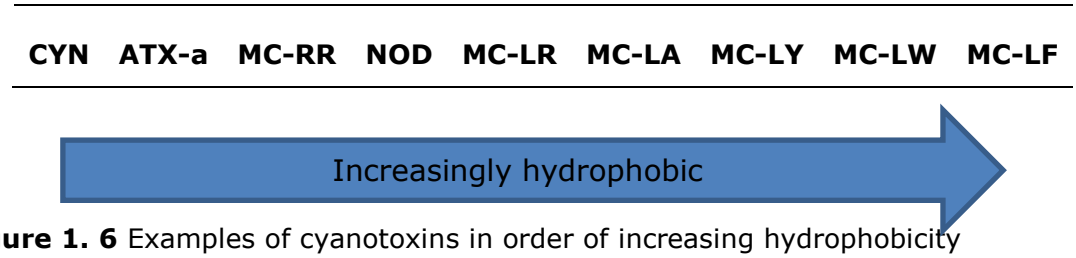

Figure 1. 6 Examples of cyanotoxins in order of increasing hydrophobicity

In combination with MS detection, excellent specificity and sensitivities of up to 100 times greater compared to UV detection can be accomplished (Meriluoto and Codd 2005). LODs were found in the range of 0.01-0.05 µg L^{-1} for six MC variants (Mekebri *et al.*, 2009; Ríos *et al*., 2013). Furthermore, MS detection helps to determine the structure of different variants and can be applied in tandem (MS/MS) to further enhance the sensitivity (Ríos *et al*., 2013). The observed fragments are used as means of identification (Table 1. 4).

Table 1. 4 Example of MS/MS fragmentation of some cyanotoxins (adapted from Zhang *et al*., 2009a; Oehrle *et al*., 2010; Neffling *et al*., 2010; Mekebri *et al*., 2009)

Cyanotoxin	Molecular weight g mol^{-1}	Parent molecular ion m/z	Daughter fragments observed m/z
CYN	415	416	194, 176
MC-RR	1038	520	213, 135
MC-YR	1045	1046	135
MC-LR	995	996	977, 599, 135
MC-LA	910	911	135
MC–LY	1002	1003	135
MC-LW	1025	1026	135
MC-LF	986	987	135
NOD	825	826	135, 70

UV detectors have also been used in combination with LC analysis. Challenges associated with high backgrounds especially in biological samples can be overcome when utilising photo diode array (PDA) detectors (Neffling *et al*.,

2010). The maximum UV absorption for MC in general is 238 nm, however, variants containing tryptophan (W) have a maximum absorbance at 222 nm (Lawton *et al*, 1994). Cazenave *et al.* (2005) applied both UV detection and electrospray ionisation time of flight MS (ESI-ToF-MS) in order to detect MC-RR in fish muscle and brain. LC-ESI-ToF-MS detected MC-RR in both tissue types, whereas HPLC-UV analysis only detected quantifiable concentrations in fish muscle.

In comparison to both ELISA and PPIA, LC based methods are generally more expensive, method optimisation and development are time consuming and require experienced analysts to not only prepare the samples, but also to evaluate the results. Furthermore, certified reference materials are required for method validation and not routine application which are not always available especially for specific MC variants.

To date, the accurate detection of covalently bound MCs remains a challenge. The main technique used for the detection of this fraction is the Lemieux oxidation of the Adda moiety of the molecule to form 2-methyl-3-methox-4-phenylbutiric acid (MMPB) which was detected by GC and HPLC analysis in combination with flame ionisation and fluorescent detection, respectively (Sano *et al.*, 1992). In short, MC-RR and MC-LR were dissolved in 90% acetic acid and reacted with sodium metaperiodate and potassium permanganate. Following the addition of 20% sodium bisulfit soulution and sulfuric acid MMPB was extracted with ethyl acetate and treated with 14 % trifuoroborate methanol to produce the methyl ester of MMPB (Sano *et al*., 1992). In more recent studies MMPB has also been detected utilising LC-MS/MS (Neffling *et al*., 2010)

Similar to ELISA and PPIA analysis Lemineux oxidation only determines the total concentration of Adda containing molecules (Neffling *et al*., 2010, McElhiney and Lawton 2005). Furthermore, the additional analysis steps increases overall cost and duration of the procedure for which, the reported recoveries were low: in snail tissue 22.4-38.4% and in serum 28.9-39.5% (Neffling *et al*., 2010). Pires *et al.* (2004) reported average recoveries for MC-LR of 50% (%RSD 4.2) from zebra mussels.

CYN is typically analysed by LC-MS (Foss and Aubel 2013; White *et al*., 2007; White *et al*., 2006). Reported LODs were 0.5 µg L^{-1} for the analysis of extracts

from the fresh water gastropod *Melanoides tuberculata* (White *et al*., 2006) and 0.3 µg L^{-1} when analysing *Bufo marinus* (cane toad; White *et al*., 2007). Foss and Aubel (2013) were able to obtain LODs of 0.50 µg L^{-1} and 0.25 µg L^{-1} in human urine and serum, respectively. Alternatively, Elliott *et al.* (2013) synthesised CYN antibodies in order to enable the development of ELISA methods, which are beneficial for the rapid screening of field samples.

1.4 Transfer of nodularin and microcystins in the food web

Cyanotoxins can be accumulated by marine and freshwater organisms, especially phytoplanktivorous fish and filter feeding organisms such as bivalves (Ibelings *et al*., 2005). Despite accumulation of high levels, cyanotoxins rarely induce mortality in bivalves. The only adverse effect that has been reported was the production of mucous pseudofaeces (Juhel *et al.,* 2006). Hence, mussels can act as a cyanotoxin vector and represent a route of exposure for higher predators including humans. The risk of cyanotoxin transfer is generally greater in mussels as the whole organism is consumed.

NOD accumulation hardly affected survival of bivalves (Table 1. 5). Following the exposure of *M. edulis* to *N. spumigena* bloom extracts at final tank water concentrations of 70-110 µg L^{-1} NOD, no mortalities were reported even though up to 1.1 µg g^{-1} NOD were detected in the mussels' hepatopancreas (Kankaanpää *et al*., 2007; Table 1. 5).

Table 1. 5 NOD accumulation and mortality rates in bivalves reported in the literature

Organism	Exposure	Concentration in exposure tanks (µg L^{-1} unless specified)	Concentration µg g^{-1} (sampled organ)	Mortality	Reference
Mussel *M. edulis*	bloom crude extract containing *N. spumigena*	70-110	0.4-1.10 dw (hepatopancreas) 0.2 dw (Soft tissue)	0%	Kankaanpää *et al.* (2007)
Clam *Macoma balthica*	Purified toxin	73 ± 14	<LOD - 30# dw (whole organism)	4-5%	Lehtonen *et al*. (2003)
	N. spumigena AV1	20 (intracellular)	<LOD - 1.4# dw (whole organism)	8%	
Clam *Macoma balthica*	Bloom suspension dominated by *N. spumigena*	178 µg g^{-1} (dw; in bloom used for challenge)	~ 0.06# dw (whole organism)	NSD compared to control	Karlson and Mozūraitis (2011)
Mussel	*N. spumigena*	NR (field samples); cell count up to 40,000 cells mL^{-1}	0.04-2.5 (whole organism)	Not quantified (field samples)	Van Buynder *et al*. (2001)
Mussel *M. edulis*	*N. spumigena*	1.6	1.18 ± 1.09† dw (whole organism)	0%	Strogyloudi *et al*. (2006)
		15.6	13.8 ± 5.26† dw (whole organism)		
Zebra mussel *D. polymorpha*	Lake containing *P. agardhii* and *M. aeruginosa* blooms	0.21-10	0-30 (whole organism)	NR	Ibelings *et al*. (2005)

dw - dry weight; LOD – limit of detection # - values estimated from graphs; NSD - No significant difference; † average ± standard deviation (n=6)

Low mortalities rates of 8% were reported in the clam *Macoma balthica* exposed to *N. spumigena* AV1 containing 20 µg NOD L^{-1} (Lehtonen *et al*., 2003). When exposed to dissolved NOD at approximately 70 µg L^{-1} the mortality rate was halved (4-5%). Due to their filter feeding nature, cell associated NOD will most likely be more easily ingested compared to dissolved toxins as reported for the snail *L. stagnalis* following the exposure to both whole cells of *P. agardhii* (33 µg MC-LReq L^{-1}) and to dissolved MC-LR (100 µg L^{-1}; Lance et al., 2010b). Snails accumulated ~ 69.9 µg g^{-1} total MC-LR when exposed to cells compared to 0.26 µg g^{-1} (total MC-LR) following the exposure to dissolved MC-LR. It was worth noting, that no bound MC-LR was detected in snails exposed to dissolved MC-LR compared to up to 66.7% bound MC-LR when exposed to *P. agardhii* cells.

Similar to NOD, mussels can tolerate and survive the accumulation of MCs rendering them dangerous toxin vectors (Table 1. 6) as shown in fishermen at Lake Chaohu (China). Their serum contained MCs as a consequence of the combined exposure to MC contaminated fish, shellfish, and drinking water (Chen *et al.,* 2009). The estimated total MC-LReq fishermen could have been exposed to per day was 2.2-3.9 µg MC-LReq, which could exceed the WHO recommended TDI of 2.4 µg MC-LR per day (for a 60 kg adult).

Table 1. 6 Reported MC accumulation and mortality rates in bivalves

Organism	Cyanobacteria	Concentration in exposure tanks	Concentration µg g^{-1} (sampled organ)	Mortality	Reference
Clam *Mesodesma mactroides*	*M. aeruginosa* RST9501 (cells)	0.4-0.7 µg L^{-1}	5.27 ± 0.23 dw (hepatopancreas)	13%[a]	Leão *et al*. (2010)
Mussel *Mytilus galloprovincialis*	*M. aeruginosa* IZANCYA2 (cells)	10^8 cells L^{-1}	<LOD-10#	6%	Vasconcelos (1995)
Clam *Diplodon chilensis patagonicus*	*M. aeruginosa* NPJB1 (cells)	0.625 µg MC-LR per g clam	0.0062 ± 0.0031 - 0.0469 ± 0.0134 (hepatopancreas)	0%	Sabatini *et al*. (2011)
Zebra mussel *Dreissena polumorpha*	*Microcystis aeruginosa* NIVA-CYA 140 cells	2 mg C L^{-1} (dissolved organic carbon)	6.2-10.8 dw (whole organism)	0%	Pires *et al.* (2004)
Bivalve *Unio douglasiae*	*M. ichthyoblabe (TAC 95)*	27 ± 4.27 µg L^{-1} (15°C water temperature)	<LOD - 130 dw (hepatopancreas)#	~3%	Yokoyama and Park (2003)
		50 ± 7.52 µg L^{-1} (25°C water temperature)	<LOD - 300 dw (hepatopancreas)#	0%	

dw - dry weight, # - values estimated from graphs, a - total mortality in all treatment groups including the control, LOD- limit of detection

Table 1. 6 continued

Organism	Cyanobacteria	Concentration in exposure tanks	Concentration µg g^{-1} (sampled organ)	Mortality	Reference
Clam *Corbicula leana*	*M. aeruginosa* NIES-1086	~ 100 µg L^{-1}	12.7 ± 2.5 dw (whole organism)	0%	Pham *et al.* (2015)
Mussel *M. californianus*	*M. aeruginosa* bloom	5.6 µg L^{-1} (intracellular toxin)	0.006 (whole organism)#	0%	Gibble *et al*. (2016)
		26.65 µg L^{-1}(intracellular toxin)	0.030 (whole organism)#		
		7.73 µg L^{-1} (dissolved toxin)	0.013 (whole organism)#		
Golden mussel *Limnoperna fortunei*	*M. aeruginosa* (NPLJ-4)	1.8-2.6 µg L^{-1}	NR	0%	Gazulha *et al*. (2012)
Mussel *M. galloprovincialis*	*M. aeruginosa* (M6 isolate)	1.5 x 10^5 cells mL^{-1}	<LOD - 0.38 dw (whole)	5%	Fernandes *et al*, (2009)
Mussel *Hyridella menziesi*	*Anabaena planktonica* and *Microcystis aeruginosa*	<LOD - 21 µg L^{-1}#*	<LOD – 0.065 (whole)#	NR	Wood *et al.* (2006)

dw - dry weight, # - values estimated from graphs, LOD – limit of detection, NR- not reported, * - smoothed surface water levels

In large fish, on the other hand, the NOD exposure risk is reduced as generally only the muscle and not the liver, which was reported as main organ of NOD accumulation, is consumed (Table 1. 7; Kankaanpää *et al*., 2002; Sipiä *et al.*, 2002). However, this can vary in different geographical regions as other people do consume fish livers and whole small fish in their diet.

Following exposure to a *N. spumigena* bloom, finfish accumulated up to 0.003 µg g^{-1} NOD in their muscle (Van Buynder *et al*., 2001), whereas values of up to 0.124 µg g^{-1} dw NOD were reported in sea trout (Kankaanpää *et al*., 2002). Field samples from the Baltic Sea reached comparable levels of 0.1 and 0.2 µg g^{-1} in muscle of flounders and roaches, respectively. Concentrations in liver were generally higher (Table 1. 7; Sipiä *et al*., 2006). Despite high NOD concentrations in flounder liver (maximum 0.41 µg g^{-1}), NOD levels in muscle tissue were below LOD (Sipiä *et al*., 2002). Another study has reported NOD concentrations of up to 0.057 µg g^{-1} in mullet muscle following a significant bloom of *N. spumigena* (Stewart *et al*., 2012), however, these values were obtained from dead mullets.

Table 1. 7 NOD concentrations reported in fish.

Organism	Analysis method	Cyanobacteria	Toxin concentration µg g^{-1} (sampled organ)	Reference
Finfish	HPLC-MS/MS	*N. spumigena*	0.7-2.5 x 10^{-3} (muscle)	Van Buynder *et al*. (2001)
Roach *Rutilus rutilus* Flounder *Platichthys flesus* L.	HPLC-MS	Field samples from region with annual *N. spumigena* blooms	0.003-0.9 dw (liver) 0.002-0.2 dw (muscle) 0-1.1 dw (liver) 0.005-0.1 dw (muscle)	Sipiä *et al*. (2006)
Flounder *P. flesus*	ELISA	Field samples from region with annual *N. spumigena* blooms	0.473 ± 0.015 dw (liver) 0.8 – 1.3 10^{-3} dw (muscle)	Mazur-Marzec *et al*. (2007)
Flounder *P. flesus*	HPLC-MS	Field samples from region with annual *N. spumigena* blooms	<LOD - 0.294 dw (liver)	Karlsson *et al*. (2003)
Sea trout (*Salmo trutta m. trutta* L.)	ELISA	*N. spumigena* (oral dose)	1.2 dw (max. 1.6; liver)* 0.125 dw (muscle)	Kankaanpää *et al.* (2002)
Flounder *P. flesus*	ELISA	Bloom containing *Nodularia*, *Anabaena*, and *Aphanizomenon*	0.399 ± 0.005 dw (liver) <LOD (muscle)	Sipiä *et al*. (2001)
Mullet (dead specimen) *Mugil cephalus*	HPLC-MS/MS	*Nodularia* bloom	40.8-47.8 dw (liver) 0.032 - 0.057 dw (muscle)	Stewart *et al*. (2012)
Shortfin eel *Anguilla australis*	ELISA	Field samples from lake with *N. spumigena* bloom	<LOD - 0.147 ww (liver) <LOD - 0.029 ww (muscle)	Dolamore *et al*. (2016)

dw - dry weight, LOD - limit of detection, NR - not reported, * - average of five

Similarly, MCs concentrations in fish were highest in liver compared to fish muscle tissue (Table 1. 8). Following force feeding of dissolved MC-LR, concentrations in muscle tissue were approximately three times lower compared to concentrations in liver (Sotton *et al*., 2012). Similarly, carp exposed to *M. viridis* cells also accumulated the least amount of MCs in their muscle, 1.77 µg g^{-1} dw compared to 49.7 and 17.8 µg g^{-1} dw determined in blood and liver, respectively (Xie *et al*., 2004; Table 1. 8). Field samples of 16 freshwater fish also supported these observations, since 351 muscle samples contained no detectable MCs, whereas 53 of the 291 livers sampled contained MCs (maximum 50.3 µg kg^{-1} fresh weight, fw; Kopp *et a*l., 2013).

In contrast to these reports, field samples of silver carp and pike from the Anzali wetland in Iran contained equal levels of MCs in both fish liver muscle (Table 1. 8). In addition, Peng *et al*. (2010) determined estimated daily intake (EDI) of MC-LReq (ELISA) in fish and shellfish from three Chinese lakes (Lake Taihu, Lake Chaohu, and Lake Dianchi) during fishing season (September-December 2008). This was determined based on a 300 g portion of eatable fish and shellfish organs consumed by a 60 kg adult. In all of these lakes bloom samples were dominated by *M. aeruginosa*. Average EDI determined in fish and shellfish from Lake Taihu were all above the TDI of 0.04 µg kg^{-1}, as recommended by the WHO (WHO 2011). In various economically important aquatic organisms caught in Lake Taihu the EDI was 4-148 times higher than the TDI deeming the fish unsafe for human consumption. Similarly, aquatic organism caught in Lake Chaohu contained MC-LReq resulting in EDIs 2-50 times the TDI. Four fish species caught in Lake Dianchi (mid August until end of September) reached MC-LReq in their muscle 0.06-0.17 µg kg^{-1} (Peng *et al*., 2010).

Carnivorous fish generally do not directly feed on cyanobacteria, but can ingest cyanotoxins via contaminated prey (Smith *et al*., 2010). Sotton *et al*. (2014) reported MCs transfer to whitefish (*Coregonus suidteri*) preying on zooplankton such as *Chaoborus* (glassworm) larvae; *Daphnia*, and *Bosmina* taxa.

Table 1. 8 MC accumulation and mortality in fish reported in the literature.

Organism	Analysis method	Cyanobacteria	Toxin	Toxin concentration µg g^{-1} (sampled organ)	Mortality	Reference
Silver carp *Hypophthalmichthys molitrix*	HPLC-MS	*M. viridis* (cells)	MC-RR	41.5 - 99.5 (intestine) 0.34 - 49.7 (blood) ~2* - 17.8 (liver) 0.5 - 1.77 (muscle)	0%	Xie *et al*. (2004)
			MC-LR	6.9-15.8 (intestine) <LOD (blood) <LOD-2.8 (liver) <LOD (muscle)		
Redbreast tilapia *Tilapia rendalli*	HPLC-PDA ELISA	*Microcystis* sp. (bloom)	MCs	<LOD - 31.1 (liver) 0.003 - 0.026 (muscle)	0%	Freitas de Magalhães *et al*. (2001)
Whitefish *Coregonus lavaretus* (juvenile)	ELISA	Purified toxin	MC-LReq	0.03 - 0.120 fw (liver) 0.015 - 0.040 fw (muscle)	NSD compared to control	Sotton *et al.* (2012)
Silver carp *H. molitrix*	HPLC-MS	Bloom containing *M. aeruginosa* (Lake Taihu, China)	Total MC (MC-LR, -RR, -YR)	<LOD- 6.84 dw (liver) 0.004-4.88 dw (kidney)	0%	Li *et al.* (2007)
Silver carp *H. molitrix*	HPLC-UV	*Anabaena* dominated field samples	MC-LR	<LOD-0.044 ww (liver)# 0.01-0.041 ww (muscle)#	NR	Rezaitabar *et al.* (2017)
Northern Pike *Esox lucius*				<LOD-0.052 ww (liver)# 0.016-0.035 ww (muscle)#		

* - estimated from graph, LOD- limit of detection, fw – fresh weight, dw - dry weight, ww –wet weight, NSD - no significant difference, NR - not reported, MC-LReq – MC-LR equivalent, # - mean values (n=3)

Table 1. 8 continued

Organism	Analysis method	Cyanobacteria	Toxin	Toxin concentration µg g^{-1} (sampled organ)†	Mortality	Reference
Stickleback *Gasterosteus aculeatus*	HPLC-MS/MS	Fed on bloom exposed *L. stagnalis*	MC-LReq	0.3 ± 0.01 - 3.9 ± 0.1 dw (liver)	NR	Lance *et al.* (2014)
Common carp larvae *Cyprinus carpio*	ELISA	Fed on *M. aeruginosa* bloom exposed brine shrimp *Artemia salina nauplii*	MCs	0.022 ± 0.004 - 0.055 ± 0.003 fw (whole)	NSD compared to control	El Ghazali *et al.* (2010)
Nile tilapia *Oreochromis miloticus* L.	HPLC-MS	Bloom containing *M. aeruginosa* and *M. ichthyoblabe*	Total MC (MC-RR, -LR, -YR)	0.004*-0.35 fw (hepatopancreas) <LOD - 0.015 fw (muscle)	NR	Palikova *et al.* (2011)
Common carp *C. carpio*	ELISA	Bloom dominated by *M. aeruginosa, M. ichthyoblabe*, and *A. flos-aquae*	MCs	<LOD - 0.098 ± 0.006 fw (muscle) <LOD - 0.132 ± 0.06 fw (liver)	0%	Adamovský *et al.* (2007)
Silver carp *H. molitrix*				<LOD - 0.01 ± 0.01 fw (muscle) <LOD 0.124 ± 0.056 fw (liver)		
Common carp *C. carpio*	HPLC-MS/MS	Pure toxin bloom dominated by *M. aeruginosa* (pond)	MC-RR, MC-LR, MC-YR	0.008 fw (muscle) 0.909 ± 0.375 fw (hepatopancreas) < LOD (muscle) 0.014-0.123 fw (hepatopancreas)	NR 0%	Kohoutek *et al.* (2010)

MC-LReq - MC-LR equivalents, fw - fresh weight, dw - dry weight, * - estimated from graph, NSD - no significant difference, LOD - limit of detection, NR - not reported, † - If not specified concentrations (mean ± standard deviation as far as available) are given for whole organisms

Table 1. 8 continued

Organism	Analysis method	Cyanobacteria	Toxin	Toxin concentration µg g^{-1} (sampled organ)†	Mortality	Reference
Yellow perch *Perca flavescens*	ELISA	*M. aeruginosa*	MCs	0.017 - 1.18 dw (liver) 0.12 – 4.02 x 10^{-3} dw (muscle)	NR	Wilson *et al*. (2008)
Tiger fish *Hoplias malabaricus*	HPLC-UV	*Radiocystis fernandoi* R28	MC-LR, MC-RR	136 ± 17 (liver) < LOD (muscle)	0%	Paulino *et al*. (2017)
Perch *Perca fluviatilis*	HPLC-PDA	Lake containing *P. agardhii* and *M. aeruginosa* blooms	MCs	17 - 51 afdw (liver)	NR	Ibelings *et al*. (2005)
Ruffe *Gymnocephalus cerna*				9 - 194 afdw (liver)		
Smelt *Osmerus eperlanus*				59 - 874 afdw (liver)		

NR – not reported, LOD – limit of detection, afdw – ash-free dry weight

Crustacean seem to be more susceptible to cyanotoxin exposure showing higher mortality rates compared to both fish and bivalves (Table 1. 8; Table 1. 9). The main organs of NOD accumulation in prawns were hepatopancreas, heart, and brain which are generally not consumed within the UK (Kankaanpää *et al*., 2005). The risk of cyanotoxin exposure via contaminated crustacean should be lower compared to mussels due to their higher susceptibility to these toxins in combination with consumption habits. Generally, the prawn tail, which evidently contained lower concentrations of NOD, will be consumed within the UK. However, the effect of cooking methods on cyanotoxin availability in seafood is currently understudied. Even though, the whole organism might not generally be consumed, the remaining parts of both prawns and fish are often utilised in the preparation of soups and/or sauces, so the risk cannot be discounted.

Table 1. 9 Cyanotoxin accumulation and mortality in crustacean.

Organism	Cyanobacteria	Toxin	Toxin concentration µg g^{-1} (affected organ)†	Mortality	Reference
Prawn *Penaeus monodon*	Blooms containing *Microcystis, Aphanocapsa, Oscillatoria, Pseudoanabaena, Romeria*	MCs and/ or NOD (ELISA)	0.006-0.0816 dw (hepatopancreas)	43-60.5%	Kankaanpää *et al.* (2005)
	Pure toxin	NOD	0.830 dw (hepatopancreas) <0.005 (muscle)	NR	
Crayfish *Procambarcus clarkii*	*M. aeruginosa* IZANCY A2 (fresh for larvae otherwise freeze-dried)	MCs (ELISA)	NR (larvae) NR (juvenile) 0.4# - 2.9 dw (adults)	0-35%* larvae NSD compared to control juvenile NR adults	Vasconcelos *et al*. (2001)
Shrimp *Palaemonetes argentinus*	Purified toxin	MC-LR	<LOD - 0.7 ± 0.2 (after 3 days at 50 µg L^{-1})	100% after 7 days (50 µg L^{-1})	Galanti *et al.* (2013)
	Cyanobacteria bloom	NOD	<LOD - 0.09 ± 0.02	NR	
Shrimp *Litopenaeus vannamei*	*M. aeruginosa* and *Anabaena* sp. Containing bloom	MC-LR	55 (hepatopancreas)	Dead specimen	Zimba *et al*. (2006)
Crayfish *P. clarkii*	*M. aeruginosa* and *A. spiroides* containing bloom	MC-LR	2.3 - 18.1	NR	Ríos *et al*. (2013)
		MC-RR	1.4 - 7.8		

dw - dry weight, NR - not reported, NSD - no significant difference, LOD - limit of detection, ***** controls showed up to 10% mortality
† If not specified concentrations (mean ± standard deviation were available) are given for whole organisms, # - estimated from graph

Within the foodweb, transfer of toxins can have a concentrating effect (biomagnification), or levels can be reduced mainly due to metabolism processes (biodilution; Ibelings *et al*., 2005; Gutiérrez-Praena *et al*., 2013; Lance *et al*., 2014). The meta-analysis of various laboratory and field studies showed that generally, biodilution was evident for the majority of primary consumers. However, biomagnification potential of MCs was seen for zooplankton and zooplanktivorous fish (Kozlowsky-Suzuki *et al*., 2012). In laboratory studies NOD transfer was reported from copepods to shrimp and sticklebacks (Engström-Öst *et al*., 2002), however, results had been determined utilising ELISA and PPIA. Consequently, the contribution of potential NOD metabolism products that might have been formed by shrimp and/or fish could not be considered as these cannot be distinguished from the parent compound utilising these techniques.

There is clearly evidence regarding cyanotoxin accumulation in seafood. The risk of cyanotoxin transfer to humans via their food source depends not only on the individual cyanobacteria and seafood species, but also on consumer specific diets. Therefore, the assessment of risks associated with cyanotoxin contaminated seafood is highly complex process and needs to be performed for specific circumstances individually.

1.5 Effect of cooking on microcystin content in seafood

Currently, the majority of analytical methods detect free, unbound MCs only and not the fraction that is covalently bound (section 1.3). However, the potential release of bound MC following passage through the gastro intestinal (GI) tract has been investigated as an important aspect helping to evaluate the risks associated with MC contaminated seafood. Smith *et al*. (2010) reported that MC-LR and MC-RR were not digested by the endopeptidases pepsin, chymotrypsin, and trypsin following 6 h incubation. Similarly, free MCs containing peptide bonds which links the *D* amino acids were resistant to hydrolytic enzymes and will therefore not be digested in the GI tract (Dyble *et al*., 2011). However, it was hypothesised that an amino acid in close proximity to the covalent link formed between MCs and PPases could be cleaved during GI

passage. This could potentially release MCs containing a peptide residue at the Mdha moiety (Smith *et al*., 2010). Based on the cleavage sites of pepsin and chymotrypsin Smith *et al.* (2010) predicted and synthesised PPase 1 peptides resulting from the enzymatic digestion of their catalytic subunits. The resulting peptides were conjugated to MC-LR which showed up to 58% of MC-LR's PPase inhibition potential, indicating that digestion products of bound MC-LR need to be further investigated in order to accurately assess potential health risks.

Different cooking methods have also been investigated regarding their potential MC release. Increases in detectable MC-LR concentration in carp and clam (muscle) were observed following boiling for 5 and 15 min (Zhang *et al*., 2010; Freitas *et al*., 2014; Figure 1. 7). Decreasing MC concentration on the other hand were reported in mussels and fish when boiled for 5 min and 30 min, respectively (Guzmán-Guillén *et al.*, 2011; Morais *et al*., 2007; Figure 1. 7). These reports illustrate that the effect of boiling could be species dependent. Consequently, risk assessments regarding MC contaminated seafood should consider individual species encountered, but also consumption habits within different countries.

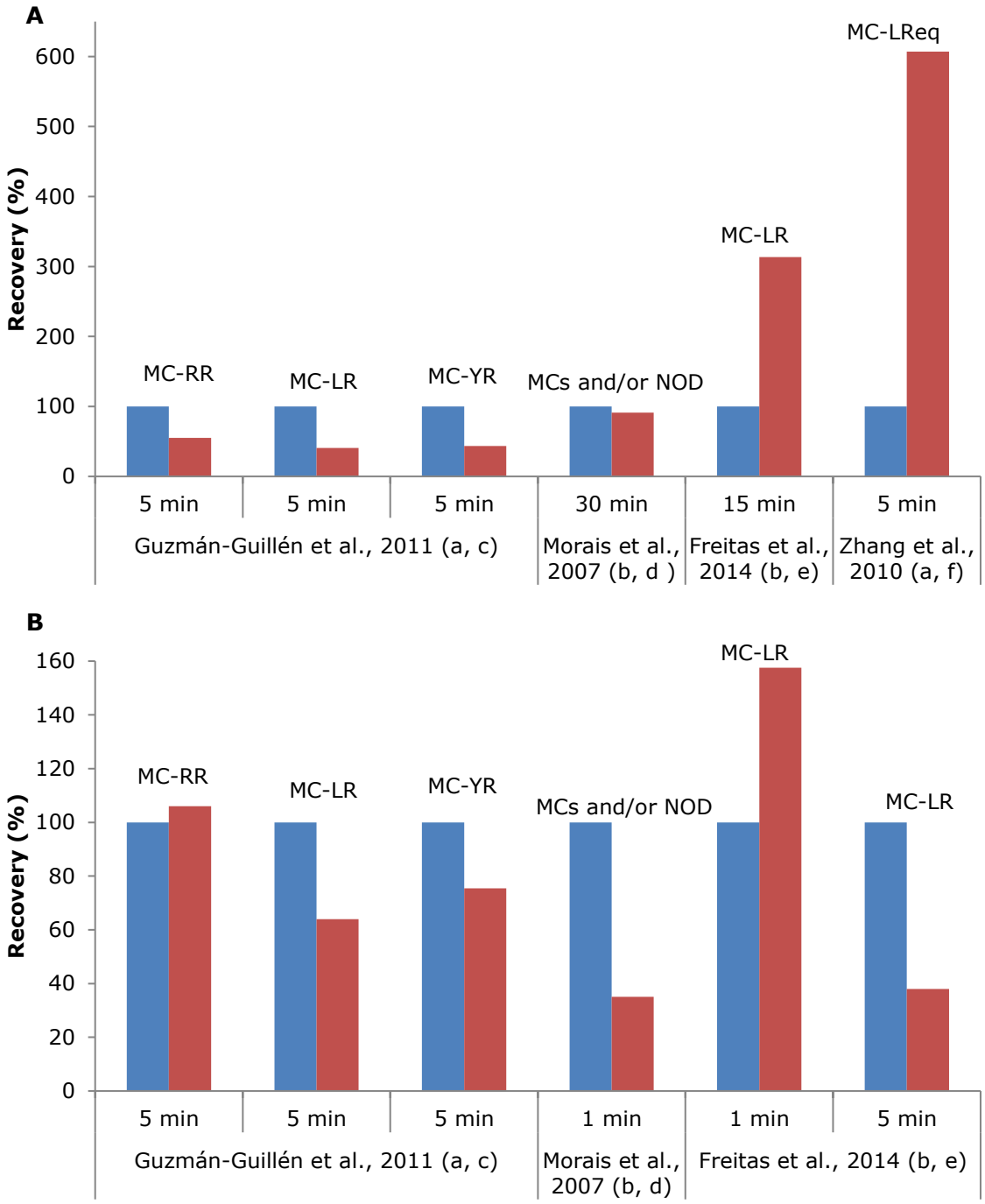

Figure 1. 7 Effect of boiling (A) and microwaving (B) on the recovery of MCs from fish (a) and shellfish (b). Toxin exposure: muscle samples were spiked with toxin standards (c), fed on *M. aeruginosa* (c), fed on *M. aeruginosa* (LEGE 91094) (e), or intra peritoneal injection (f). Blue – control, red - after boiling

Not only the seafood itself, but also the water it was cooked in can become a source of MCs. Transfer of MCs to water was reported when cooking fish and mussels (Guzmán-Guillén *et al.*, 2011; Freitas *et al.,* 2014; Zhang *et al.,* 2010). Freitas *et al.*, (2014) did not report the concentrations of MCs in clams and boiling water separately, but combined. Hence, it was not clear if the concentration in muscle tissue decreased or increased. Zhang *et al.*, (2010) performed their experiment three and six hours after the carp had been injected with 500 µg kg^{-1} bw MC-LReq. The reported increase of MC-LReq detected after boiling for 5 min could be explained by the denaturation of proteins and consequently the possible release of MCs from PPs. Similarly to MCs, NOD redistribution was observed following the cooking of prawns (Van Buynder *et al*., 2001; Table 1. 10). However, looking more closely at the reported values nearly half the NOD concentration was not detected after boiling compared to total levels prior to cooking. The reason for this discrepancy could potentially be toxin degradation and/or poor recovery of NOD from the matrix. As tandem MS analysis was utilised for toxin detection it was not possible to draw conclusions regarding the formation of potential degradation products as this method only detects parent to daughter ion transitions that had been set up during method development, rather than scanning for available fragments.

Table 1. 10 Changes in NOD concentration (µg kg^{-1}) in prawn tissue following boiling (adapted from Van Buynder *et al*., 2001)

	prior to cooking	**after cooking**
viscera	1,640	415
water	0	53
flesh	10.3	460
Total	1,650	928

Similarly to boiling, microwaving showed both increasing and decreasing effects on MC concentration (Figure 1. 7). Increases in MC concentrations could again be associated with the denaturation of PPase and the release of bound toxin. The reduction in MC could be associated to molecular vibrations caused by the radiation which led to cleavage of peptide bonds. Reichelt *et al.* (1999), for example, observed complete cleavage of peptide bonds in NOD and MCs after microwave hydrolysis (160°C, 10 min). The temperature itself should not affect the availability of MCs during either boiling as they have been reported to be heat stable at 100°C for 60 min (Kos *et al.*, 1995). Based on the contradictory reports concerning the effects of boiling and microwaving on cyanotoxin concentration in seafood, more research is needed to further understand the mechanisms involved. This will be vital for the investigation of health risks associated with cyanotoxin contaminated fish and shellfish.

With increased awareness of multiple exposure routes to cyanotoxins and the health risks associated with them the World Health Organisation (WHO) has released guidelines for cyanotoxin exposure. The concentration of MC-LR in drinking water should not exceed 1 µg L^{-1}, which has been adopted globally in several countries (Ibelings 2012). Furthermore, the lifetime tolerable daily intake (TDI) has been set to 0.04 µg kg^{-1} bw (WHO 2011). Ibelings and Chorus (2007) have also derived acute and seasonal tolerable intakes of 190 and 30 µg (75 kg adult), respectively. These tolerable intake levels take into account that generally shellfish are not eaten throughout the whole year and acute and seasonal exposure could be more representative of the dangers associated to cyanotoxin contaminated seafood (Ibelings and Chorus 2007). The toxin guidelines for maximum concentration of CYN in drinking water have been set at 1 µg L^{-1} in Brazil, Australia and New Zealand whereas only 0.3 µg L^{-1} are recommended in France (Elliott *et al*. 2013). In addition, the European Food Safety Authority (EFSA) has recognised emerging health risks associated with cyanotoxin contaminated seafood (Testai *et al*., 2016), however, no regulatory guidelines have yet been established. Saker *et al*. (2004) have proposed health alert levels for CYN in seafood based on no observed adverse effect levels of 30 µg kg^{-1} d^{-1} observed during oral exposure of mice (Humpage and Falconer, 2003; Table 1. 11). Similarly, Van Buydner *et al*. (2001) and Mulvenna *et al*. (2012) derived health alerts for NOD and MCs in seafood (Table 1. 11). The derived

NOD guideline level has been adopted for seafood monitoring in Victoria, Australia (Van Buydner *et al*., 2001). However, it needs to be stated that currently there are no legislations regarding cyanotoxin concentration in seafood anywhere in the world.

Table 1. 11 Health alert levels (µg kg^{-1} ww) for cyanotoxins in seafood as recommended by Saker *et al*. (2004), Van Buydner *et al*. (2001), and Mulvenna *et al*. (2012)

Cyanotoxin	Mussels	Fish	Prawn
CYN	933	158	720
NOD	1500	250	1100
MCs	83	39	39

1.6 The common blue mussel *Mytilus edulis*

The common blue mussel *M. edulis* (Figure 1. 8) can be found globally in marine coastal areas (Seed 1969; Winter 1973; Riisgård *et al.,* 2008; Jones *et al*, 2010). In addition, at salinities >6 psu they are a vital component of the Baltic Sea foodweb (Kankaanpää *et al*., 2007). Utilising their byssus threats these mussels form attachments to rocks and each other withstanding tidal movements in coastal areas (Winter 1973). Despite the surface waters, *M. edulis* can be found at depth of up to 30 m in the Baltic Sea (Kautsky 1982). In Britain *M. edulis* was found in open coastal areas, but also in more protected estuaries all around the country (Seed 1969). Even in sub-Arctic fjords in Greenland, *M. edulis* can thrive and an estimated age of 19 years can be reached (Blicher *et al*., 2013). Contrastingly, high temperatures of up to 32 °C were associated with the increased mortality of *M. edulis* along the US Atlantic coast (Jones *et al*., 2010).

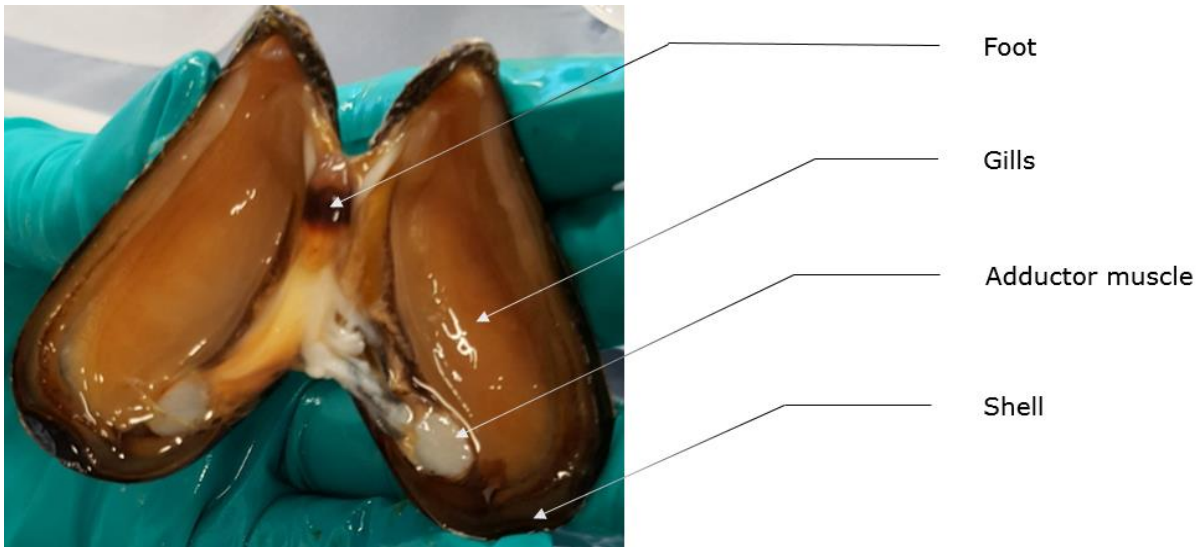

Figure 1. 8 Common blue mussel *M. edulis* maintained in UV sterilised seawater (16 ± 1°C).

Equivalent to extracellular fluid in for example mammals, the inner cavity between mussel shell and mantle is filed with extrapallial fluid (Crenshaw 1972). To feed *M. edulis* utilise their gills to filter particles from the water that is being pumped through their mantle cavity (Gosling 2003). Filtration rates of up to 9.13 L h^{-1} were reported for *M. edulis* fed *Phaeodactylum tricornutum*, *Dunaliella marina*, and *Tetraselmis suesica* (Table 1. 12; Møhlenberg and Riisgård 1979). Particle concentration, temperature, and salinity were among factors reported to affect filtration rates in mussels (Gosling 2003). Bayne *et al*. (1987) investigated the gut passage time of *M. edulis* following exposure to diets containing different percent organic matter (low, medium, and high), which varied from 3.28 ± 0.3 to 15.34 ± 1.6 h. However, no statistical difference in gut passage time was determined with regards to diet type (Bayne *et al*., 1987). Particles which are not ingested but rejected by mussels are called pseudofaeces (Widdows *et al*., 1979). The particle concentration which causes pseudofaeces production was size dependent and increased with increasing shell length. For mussels larger than 50 mm concentrations of 4.5-5.0 mg L^{-1} caused pseudofaeces production (Widdows *et al.*, 1979). In addition to particle concentration cell diameters can affect pseudofaeces production. Following the exposure to silicon dioxide particles of varying diameter (5-37 µm), *M. edulis* selective rejection particles greater than 7.5 µm (Defossez and Hawkins 1997).

Table 1. 12 Published filtration rates of *M. edulis*

Filtration rate per individual (L h^{-1})	Temperature (^{0}C)	Salinity (psu)	Reference
0.06-1.52	10-13	30	Møhlenberg and Riisgård 1979
1.3-2.8	12	25	Winter 1973
2.9 3.5-4.3 4.2 1.2	8 8.4 10 18	Seawater*	Riisgård *et al*. (2003)
0.21-0.25	15	27	Riisgård and Møhlenberg (1979)

*14-22 psu according to Riisgård *et al.* (2008)

Mussels are a vital component of the aquatic food web. They provide a food source for organisms such as crabs, fish, and birds (Farrell and Nelson 2013; Chen and Xie 2007), but are also consumed globally by humans. They are generally either harvested from wild mussel beds or grown in commercial farms (Figure 1. 9). The shellfish industry represents a part of the British economy valued at £33.2 million in 2012 when 27,360 t of shellfish (95 % mussels) were harvested (Ellis 2015). It is therefore vital to ensure that the shellfish produced are safe for human consumption.

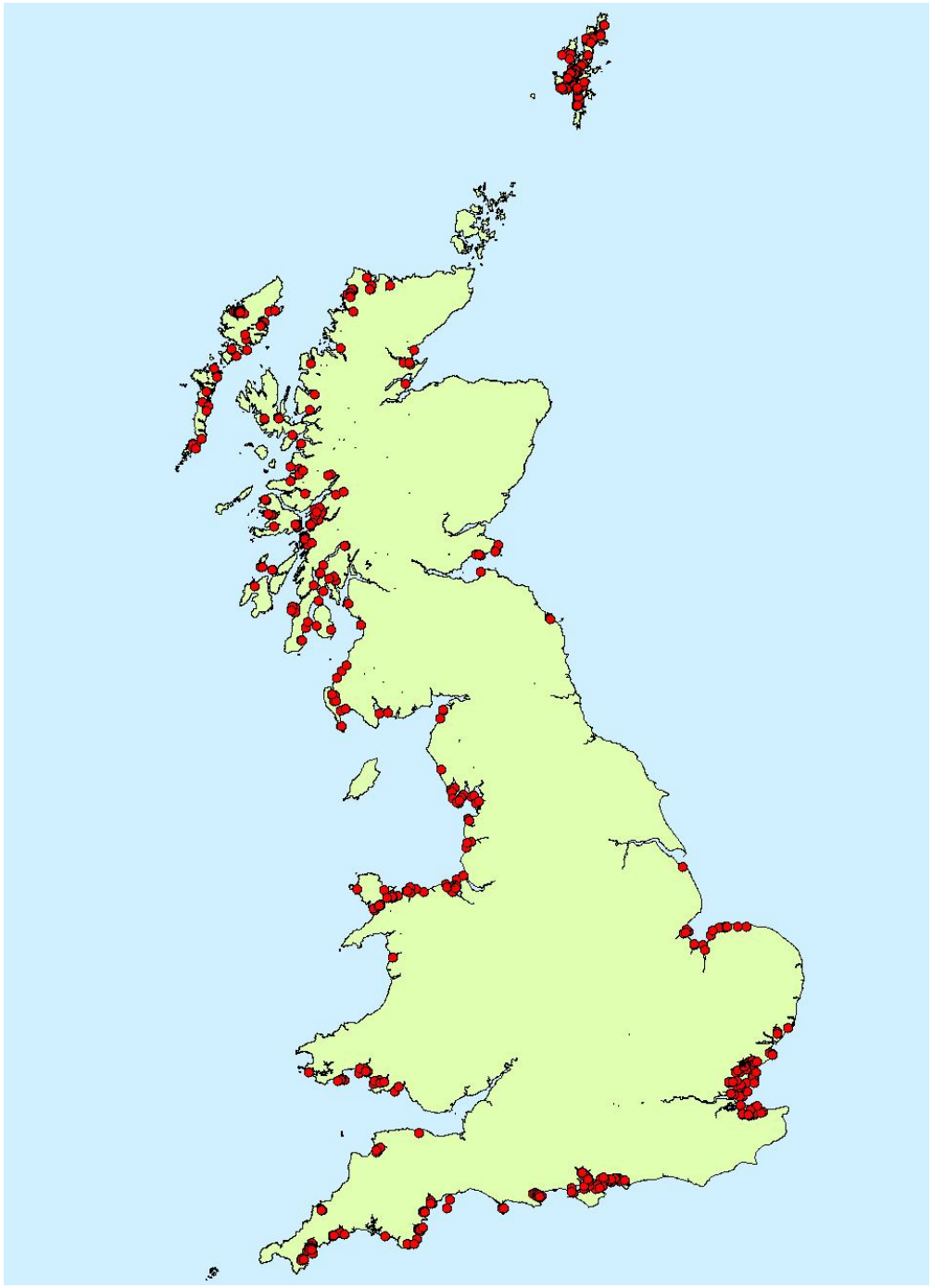

Figure 1. 9 Shellfish production sites in England, Wales, and Scotland (Cefas, 2016)

1.7 Aims and objectives

It is clear there is evidence that freshwater cyanotoxins such as MCs can be transported from inland waterbodies to coastal and estuary areas (see review by Preece *et al*., 2017). This, along with the occurrence of brackish blooms producing NOD is a cause for concern in shellfish harvesting zones. Consequently, this presents a cyanotoxin exposure route for higher predators including marine mammals and humans. Hence, the aim of this study was to develop a method for the detection of cyanotoxins of varying polarity. For this purpose a method, currently applied by the Centre for Environment, Fisheries and Aquaculture Science (Cefas), for the detection of the domoic acid (DA) in shellfish, was investigated for its potential to be modified to include nine additional cyanotoxins. Cefas, being an Official Control Testing laboratory, contracted to the Food Standards Agencies of England, Wales and Scotland for the analysis of marine toxins in live bivalve molluscs, has access to many shellfish samples which are analysed on a daily basis as part of the European Union regulations (EC Regulation 853/2004; 786/2013) Hence, the additional incorporation of cyanotoxins into screening methodologies would be beneficial from both an economic and food safety perspective. For this purpose the following cyanotoxins were selected to cover a range of different polarities including commonly occurring MCs: CYN, ATX-a, NOD and the MC variants RR, LR, LA, LY, LF, and LW.

Despite cyanotoxins showing similar toxicity compared to currently monitored marine toxins, no legal regulations regarding their concentration in seafood have been established. The present study therefore further aimed to assess the accumulation and depuration of cyanotoxins in the blue mussel *M. edulis*, which is a common part of the human diet. Results will provide vital data for the assessment of risks to the general public associated with cyanotoxin contaminated mussels. Key objectives determined were:

1. Development of a multi-toxin HPLC-PDA method for the detection of DA and nine cyanotoxins.
2. Large scale culturing of *N. spumigena* KAC66 and *M. aeruginosa* PCC 7813 for *M. edulis* feeding trials.

3. Determination of salinity tolerance of *M. aeruginosa* PCC 7813 to establish suitability for feeding trials.
4. Design of cyanobacteria feeding trial utilising *M. edulis*.
5. Performance of single and mixed cyanobacteria feeding trials to assess NOD and/or MCs accumulation and depuration in *M. edulis*.
6. Determination of the cyanotoxin budget of the experimental system.

CHAPTER 2

Optimisation of a HPLC-PDA method for the simultaneous detection of domoic acid and nine cyanotoxins

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2.1 Introduction

The dangers cyanotoxins pose to both marine and human life have long been recognised (section 1.3). To allow the appropriate assessment of risks to human health associated with cyanotoxin exposure it is essential to enable their accurate and precise detection in various matrices. With increasing understanding and reports of cyanotoxin transfer to organisms such as filter feeding bivalve molluscs and phytoplanktivorous fish it is no longer sufficient to detect the toxins only in water or cell extracts. The analytical focus has shifted to developing appropriate extraction and detection methods for more complex matrices. Also, the discovery of a large number of different cyanotoxins represents another challenge during the analysis due to the varying polarities of the congeners involved. Three of the most commonly applied methods are the enzyme linked immune sorbent assays (ELISA), protein phosphatase inhibition assays (PPIA), and high performance liquid chromatography (HPLC) in combination with photodiode array (PDA) and/or mass spectrometry (MS) detection. ELISA enables the rapid analysis of cyanotoxins with commercially available detection kits. Furthermore, portable tubes and dipsticks are available for onsite testing which can provide a critical tool for fast screening purposes. Some ELISA kits are based on the detection of the Adda side chain found in both MCs and NOD (Chu *et al*., 1989). It is not possible, however, to distinguish between NOD, MC variants or their conjugates when utilising ELISA. Both MCs and NOD are PPase inhibitors (de Figueiredo *et al.,* 2004; Sotton *et al*., 2011, Lance *et al*., 2010). Similar to ELISA, PPase inhibition assays indicate the overall potential of the sample extract to inhibit PPase and therefore cannot distinguish MC variants nor NOD, and their associated metabolites. PPIA is known to overestimate MC and NOD concentrations (de Figueiredo *et al.,* 2004; Engström-Öst *et al*., 2002). This is due to co-extractive substances that lead to positive PPIA results, for example calyculin, okadaic acid, tautomycin, and anabenapeptins produced by cyanobacteria (Engström-Öst *et al*., 2002; McElhiney and Lawton 2005). Nonetheless, an overview of the total toxicity is gained, which can provide useful information for the purpose of cyanotoxin risk assessments. To obtain more information regarding the identity of specific cyanotoxins present, an analysis method capable of separating compounds of varying polarity such as

HPLC is required. Generally, mobile phases used for HPLC analysis of different cyanotoxins are comprised of varying mixtures of water and methanol (MeOH) or water and acetonitrile (MeCN). Commonly, these contain ion pairing agents such as trifluoroacetic, formic or acetic acid which also suppress the ionisation of the silanol groups forming the stationary phase of C18 columns which are commonly used for the separation of cyanotoxins (Amé *et al*., 2003; Lawton *et al*., 1994). They therefore aid retention by minimising secondary interactions between the silanol groups of the stationary phase and the analytes, which otherwise could cause peak broadening and/or tailing (Cai and Li 1999). Other mobile phases utilised for HPLC analysis are aqueous buffer solutions of ammonium acetates or phosphates adjusted to a pH of 2.7-4 in combination with MeCN or MeOH (Vasconcelos 1995; Harada *et al*., 1988; Saker and Eaglesham 1999). Controlling the pH enables the manipulation of the degree of ionisation of acids and bases to improve retention (Snyder and Kirkland 1979). With the different modifiers enhancing the shape of the separated peaks, higher sample loads are enabled (Johnson *et al*., 2013). Utilising a mobile phase gradient in which the composition of inorganic and organic solvent is altered enables the separation of compounds of varying polarity such as cyanotoxins.

HPLC-UV analysis is a common technique applied to a wide range of different analytes from a variety of sample matrices, including the detection of domoic acid in bivalve mollusc shellfish such as mussels, oysters, scallops and clams. Domoic acid (DA, Figure 2. 1) is a neuroexcitatory amino acid produced by a range of marine algae including the red algae *Chondria armata* (Ohfune and Tomita 1982) and the diatoms *Nitzschia pungens* (Bates *et al*., 1989) and *Pseudonitzschia australis* (Garrison *et al*., 1992). It was first associated with human poisoning following the consumption of *M. edulis* from the Prince Edward Island (Johnson *et al*., 2013; Quilliam *et al*., 1989; Wright *et al*., 1989). Symptoms reported were vomiting, abdominal cramps, diarrhoea, headache, and short-term memory loss. Out of the reported 107 cases 19 patients were hospitalised of which three died (Perl *et al*., 1990). The causative agent was later identified as DA. Ultraviolet (UV) spectroscopy showed an absorbance maximum λ_{max} of 242 nm enabling the analysis via HPLC-PDA methods (Quilliam *et al*., 1989). MCs and NOD are also suited for PDA detection due to

the presence of the Adda moiety which is the main chromophore of these molecules in combination with α, β-unsaturated carbonyl groups in the Mdha residue of MCs and Mdhb residue of NOD, respectively. Together, these structures result in a UV absorbance maximum at 238 nm (MCs) and 237.8 nm (NOD). Slightly altered absorbance spectra are observed when the amino acids tyrosine (Y) or tryptophan (W) are encountered in MCs (Figure 2. 2). With current regulatory testing of DA in UK shellfish being conducted using HPLC-UV, there is the potential to optimise the existing analysis method to incorporate MCs.

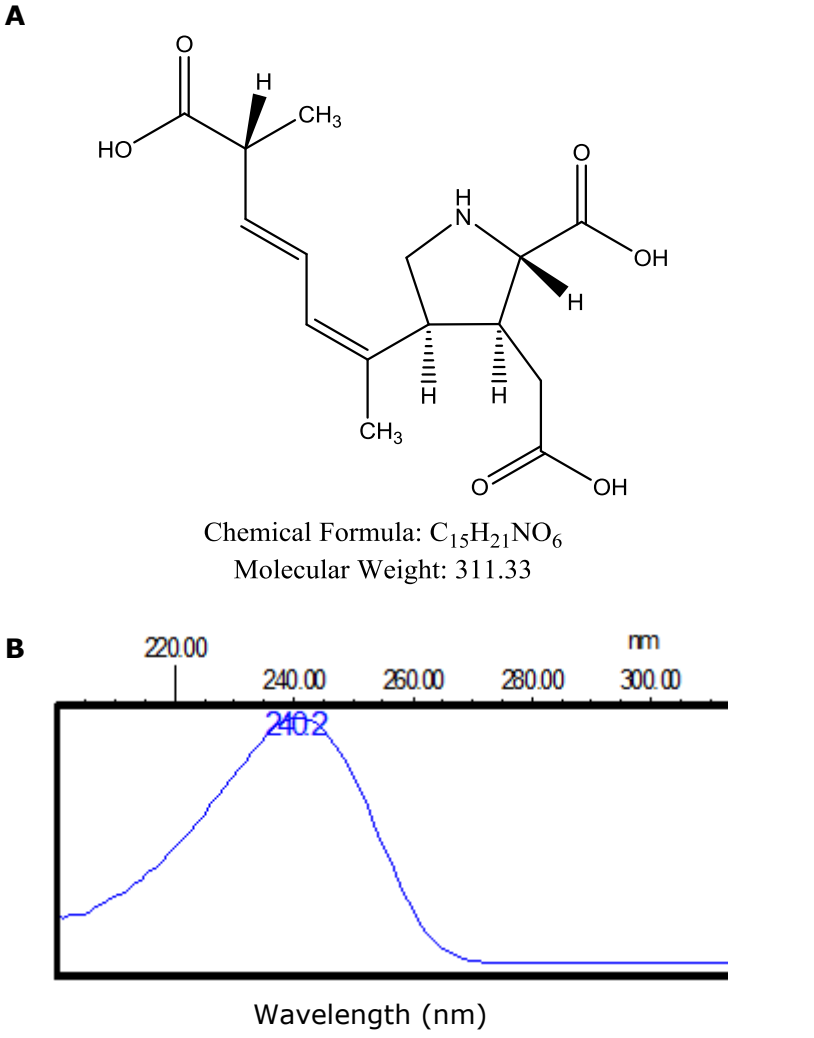

Figure 2. 1 Molecular structure (A) and UV spectrum (B) of domoic acid

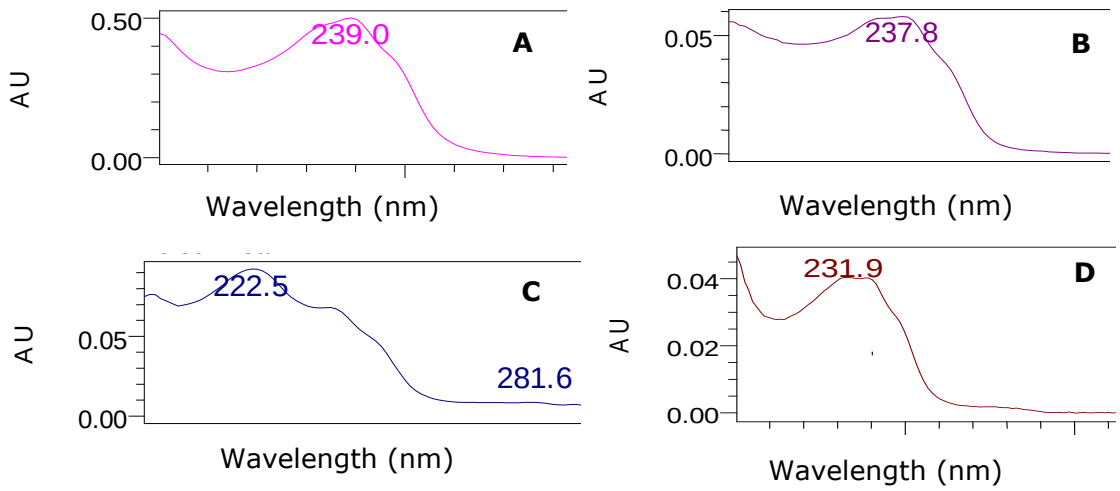

Figure 2. 2 UV absorbance spectra (nm) of MC-LR (A), NOD (B), MC-LW (C), and MC-LY (D)

To date, the majority of reported HPLC-PDA methods have focused on the analysis of MC-RR, MC-LR, and NOD (Table 2. 1). Similarly mass spectrometry (MS, Table 2. 2) and tandem MS (MS/MS, Table 2. 3) based detection in combination with either HPLC or UHPLC have generally been tuned for up to three different cyanotoxins. In addition, multi-toxin methods have also been developed (Table 2. 4).

Table 2. 1 Examples of HPLC-UV methods of up to three MC variants, CYN and NOD in a range of sample matrices.

Cyanotoxin	Matrix	Reference
MC-LR	Mussel	Vasconcelos *et al*. (1995)
MC-LR, MC-LR-Cys, MC-LR-GSH	Plant, invertebrate, fish, fish egg	Pflugmacher *et a*l. (1998)
NOD	Trout	Kankaanpää *et al*. (2002)
NOD, MC-LR, MC-HtyR	Brine shrimp *Artemia salina*	Beattie *et al*. (2003)
NOD	Clam	Lehtonen *et al*. (2003)
MC-RR, MC-LR	Snail, clam	Ozawa *et al*. (2003)
CYN	Mussel	Saker *et al*. (2004)
MC-LR, MC-RR	Fish	Xie *et al*. (2004)
MC-RR, NOD, MC-YR, MC-LR	Prawn	Kankaanpää *et al*. (2005)
NOD	*N. spumigena*	Mazur-Marzec *et al*. (2005)
MC-RR, dmMC-LR	*P. agardhii*	Lance *et al*. (2006)
MC-LR, MC-RR, MC-YR	Fish	Kopp *et al.* (2013)

Table 2. 2 Examples of mass spectrometric methods reporting the analysis of up to three MC variants and NOD in a variety of sample matrices.

Cyanotoxin	Method	Matrix	Reference
CYN	HPLC-MS	Crayfish	Saker and Eaglesham (1999)
MC-RR, MC-YR, NOD, MC-LR	HPLC-MS	Blue-green algae products	Lawrence *et al.* (2001)
NOD	MALDI-TOF-MS	Mussel	Sipiä *et al.* (2002)
NOD, dmNOD	HPLC-MS	Mussel, Fish	Karlsson *et al.* (2003)
MC-RR	HPLC-MS	Fish	Cazenave *et al*. (2005)
MC-RR, MC-LR	HPLC-MS	Shrimp, crayfish	Chen and Xie (2005)
NOD	HPLC-MS	Mussel	Kankaanpää *et al*. (2007)
MC-LR, MC-YR	HPLC-MS	Fish	Li *et al*. (2007)
MC-RR, MC-LR, MC-LA	HPLC-MS	Fish	Smith and Boyer *et al.* (2009)
MC-LR, [Dha7]-MC-LR	HPLC-MS	Water	Yu *et al*. (2009)
MC-LR, MC-LR-Cys, MC-LR-GSH	HPLC-MS	Snail, shrimp, fish	Zhang *et al*. (2009a)
MC-RR, MC-RR-Cys, MC-RR-GSH	HPLC-MS	Fish	Wu *et al.* (2010)
NOD	HPLC-MS	Amphipods, bivalve	Karlson and Mozūraitis (2011)
MC-LR, MC-RR, MC-YR	HPLC-MS	Fish	Palikova *et al*. (2011)
NOD	UPLC-MS	Crayfish	Wood *et al.* (2012)
MC-RR, MC-LR, MC-YR	HPLC-MS	Fish, crayfish	Ríos *et al.* (2013)
MC-LR	HPLC-MS	Clam	Freitas *et al*.(2014)

Table 2. 3 Examples of tandem mass spectrometry based detection methods reported for the analysis of up to three MC variants, CYN, and NOD in a range of sample matrices.

Cyanotoxin	Method	Matrix	Reference
NOD	HPLC-MS/MS	Mussel	Sipiä *et al*. (2002)
CYN	HPLC-MS/MS	*Aphanizomenon flos-aquae*	Preußel *et al.* (2006)
MC-RR, MC-LR, MC-YR	HPLC-MS/MS	Carp tissue	Chen *et al.* (2007)
MC-LR, MC-RR	HPLC-MS/MS	Rat	Wang *et al*. (2008)
MC-RR, MC-LR, MC-YR	HPLC-MS/MS	Human serum	Chen *et al*. (2009)
CYN	HPLC-MS/MS	Water Lake Averno	Gallo *et al*. (2009)
MC-LR, MC-RR, MC-YR	HPLC-MS/MS	Water	Amé *et al*. (2010)
MC-LR, MC-RR, MC-YR	HPLC-MS/MS	Carb tissue	Kohoutek *et al*. (2010)
MCs*	HPLC-MS/MS	Snail	Lance *et al*. (2010b)
MCs*	HPLC-MS/MS	Snail	Neffling *et al*. (2010)
MC-RR, MC-LR, MC-YR	UHPLC-MS/MS	Fish	Nyakairu *et al*. (2010)
MC-RR, MC-LR	HPLC-MS/MS	Fish	Zhang *et al.* (2010)
MC-RR, MC-LR, MC-YR	HPLC-MS/MS	Fish	Guzmán-Guillén *et al*. (2011)
MC-LR	HPLC-MS/MS	Fish	Sotton *et a*l. (2011)

*Lemieux oxidation method for which only total MC can be determined

Table 2. 4 Examples of reported multi-toxin detection methods for more than three microcystin variants and nodularin in a range of sample matrices.

Cyanotoxins	Method	Matrix	Reference
NOD, MC-RR, MC-LR, MC-YR, MC-LA, MC-LW	HPLC-MS/MS	Fish	Bogialli *et al*. (2005)
MC-RR, NOD, MC-YR, MC-LR	HPLC-UV	Prawn	Kankaanpää *et al*. (2005)
MC-RR, MC-LR, MC-YR, MC-LA, MC-LW, MC-LF	HPLC-UV	Fish	Deblois *et al.* (2008)
NOD, MC-RR, MC-YR, MC-LR, MC-LA, MC-LW, MC-LF	UPLC-TOF-MS	Algae supplements	Ortelli *et al*. (2008)
MC-RR, MC-LR, MC-YR, MC-LA, MC-LW, MC-LF, dmMC-RR, dmMC-LR	HPLC-MS	Mussels, Rainbow trout	Mekebri *et al*. (2009)
dmMC-RR, MC-RR, dmMC-LR, MC-LR, MC-YR, MC-LA, MC-LW, MC-LF, NOD, dmNOD, LNOD	UPLC-UV	35% aqueous MeOH	Spoof *et al*. (2009)
NOD, dmMC-RR, MC-RR, MC-LR, MC-YR, MC-LY, MC-LW, MC-LF	HPLC-UV	Standards	Purdie *et al*. (2009)
MC-RR, MC-LR, MC-LA, MC-LY, MC-LW, MC-LF	HPLC-MS/MS	Clam	Yang *et al*. (2009)
CYN, ATX-a, MC-RR, MC-LR, MC-LF	UPLC-MS/MS	Water	Oehrle *et al*. (2010)
31 MCs*	HPLC-MS/MS	Bloom	Miles *et al.* (2013)
dmMC-RR, MC-RR, MC-YR, Dem-MC-LR, MC-LR, MC-LA, MC-LY, MC-LW, MC-LF	HPLC-MC/MS	*M. aeruginosa* PCC 7806	Stefanelli *et al.* (2014)
MC-RR, MC-LR, MC-LY, MC-LW, MC-LF, NOD	HPLC-UV	Cyanobacteria cells and water	Lawton *et al*. (1994)
MC-RR, NOD, MC-YR, MC-LR, MC-LA, MC-LW, MC-LF	HPLC-MS	Phytoplankton	Dahlmann *et al*. (2003)#

* identity of 9 MCs was confirmed by standards. For remaining MCs no standards were available, but retention time and fragmentation was consistent with proposed structure. # also included saxitoxin, ATX-a, DA, okadaic acid (OA) and dinophysistoxin-1 (DTX1)

Based on the current reports of the transfer of freshwater cyanotoxins to the marine environment (Tango and Butler 2008; Miller *et al.,* 2010; Garcia *et al*., 2010; Mazur and Pliński, 2003) and the severe consequences of exposure to cyanotoxins, this investigation aimed to include nine additional cyanotoxins in a method currently deployed for the analysis of DA in seafood. Developing a robust and reliable method for the accurate and precise detection of multiple cyanotoxins of varying polarity is a vital first step towards monitoring their occurrence. Ultimately this will help to prevent cyanotoxin exposure to both humans. In order to achieve this aim a method, currently applied by the Centre for Environment, Fisheries and Aquaculture Science (Cefas), for the detection of DA in shellfish, was optimised to include nine additional cyanotoxins of varying polarity. Cefas, being an Official Control testing laboratory, conducting routine monitoring on behalf of the UK Competent Authorities, the Food Standards Agency and Food Standards Scotland, has access to many shellfish samples which are analysed routinely basis as part of the European Union regulations (EC Regulation 853/2004 and 786/2013). According to these regulations PSTs are monitored in shellfish from designated shellfish harvesting areas within England, Scotland and Wales. The regulatory limit for PST in live bivalve molluscs is 800 µg STX equivalent kg^{-1} (EC Regulation 853/2004). For official control purposes HPLC in combination with fluorescent detection is utilised (Turner *et al.*, 2009). Briefly, shellfish homogenates are extracted with 1% acetic acid, prior to clean up utilising C18 solid phase extraction (SPE). A pre-column oxidation derivatisation is performed using a periodate reagent prior to preliminary screening of samples to determine toxin presence in a semi-quantitative assessment of toxicity. Positive samples above 400 µg STX equivalent kg^{-1} are subjected to full quantitation following a secondary clean up utilising ion exchange SPE, further pre-column oxidation derivatisation using peroxide and periodate, prior to HPLC with fluorescence detection. Chromatographic separation is subsequently performed utilising a 5 µm fused-core column (Hartfield *et al*., 2016).

The aim of the present study was to develop a method for the simultaneous detection of DA and nine additional cyanotoxins, which would be highly beneficial both from an economic and food safety perspective. For this purpose the following cyanotoxins were selected as some of the most important globally

occurring cyanotoxins in combination with DA: CYN, ATX-a, NOD and the MC variants MC-RR, MC-LR, MC-LA, MC-LY, MC-LW, and MC-LF.

2.2 Materials and Methods

2.2.1 Chemicals

Methanol (MeOH) and acetonitrile (MeCN) were of HPLC gradient grade (Sigma-Aldrich, Dorset, UK). Water was purified utilising a PURELAB® flex (18.2 MΩ, Elga Veolia, UK). Domoic acid, Trifluoroacetic acid (TFA), and formic acid (FA) were obtained from Sigma-Aldrich (Dorset, UK). Domoic acid (1 mg) was dissolved in 2 mL 5% aqueous MeCN and 20 µL aliquots were transferred to HPLC glass autosampler vials. Anatoxin-a (ATX-a, 1 mg) was provided by Tocris (Bristol, UK) and dissolved in 1 mL of ultrapure water. Aliquots of 100 µL were transferred to HPLC glass vials. Both DA and ATX-a were dried separately utilising centrifugal evaporation at 45°C (Genevac EZ2.3; SP Scientific, Ipswich, UK). Dried DA and ATX-a vials were stored at -20°C until analysis. MC congeners and NOD were purified from laboratory batch cultures (Lawton *et al*., 1999; Liu *et al*., 2005; Diehnelt *et al*., 2006; Barco *et al*., 2005; Table 2. 5). Cylindrospermopsin (CYN) was provided by Enzo Life Sciences (Exeter, UK). Dried toxins were dissolved in ultrapure water and utilised for the preparation of the multi-toxin stock solution. A total of 50 µg of each toxin was added following which the stock was dried utilising the Genevac (Table 2. 6).

Table 2. 5 Extraction of cyanotoxins from different cyanobacteria

Cyanobacteria strain	Cyanotoxin	Reference
M. aeruginosa PCC 7813	MC-LR, MC-LY, MC-LW, MC-LF	Lawton *et al*. (1999)
N. spumigena KAC 66	NOD	Liu *et al*. (2005)
M. aeruginosa UTEX B2666	MC-LA	Diehnelt *et al*. (2006)
M. aeruginosa SCIENTO	MC-RR	Barco *et al*. (2005)

Table 2. 6 Toxin stock (50 µg mL^{-1}) preparation for the optimisation of a multi-toxin HPLC-PDA method.

Toxin	Quantity (µg)	Volume added (µL)	Volume used for stock (µL)
CYN, ATX-a, MC-RR, NOD, MC-LR, MC-LA, MC-LF	100	1000	500
MC-LY, MC-LW	25 (2x)*	250 (2x)*	250 (2x)*
DA	10 (5x)*	100 (5x)*	100 (5x)*

* multiple dried vials of these toxins were required to obtain the desired quantities. Number in parenthesis states the number of vials used

2.2.2 Method transfer

The current method for the detection of DA in seafood (Table 2. 7) was transferred to the Alliance 2695 separation module coupled to a 2996 PDA detector (both Waters, Elstree, UK). For data acquisition and processing purposes the Empower v2 software was employed. Quantitation of DA was conducted against a four-point calibration of working standards containing DA concentrations dissolved in 5% aqueous MeCN between 0.2-7.2 µg mL^{-1}. This method was taken as a starting point and refined as required to allow the simultaneous separation of DA and nine cyanotoxins. The linearity of the calibration was assessed based on correlation coefficients (R^2) obtained when plotting the detected area against the toxin concentration, and found to be acceptable ($R^2 > 0.990$).

Table 2. 7 Cefas standard method for the detection of DA in seafood

Instrument system	HPLC-UV (Agilent)
Column	Luna PFP(2)
Column length (mm)	150
Column diameter (mm)	4.6
Particle size (µm)	3
Pore size (Å)	100
Guard column	SecurityGuard™ cartridges PFP (4x 3 mm)
Mobile phase	32.5% aqueous MeOH with 0.1% TFA
Column temperature (°C)	30
Flow rate (mL min^{-1})	1
Injection volume (µL)	20
Run time (min)	13

2.2.2.1 Mobile phase and solvent optimisation

The use of a MeCN based mobile phase was investigated based on lower UV absorbance, higher elution strength and decreased system pressure compared to MeOH. To achieve the separation of DA and nine additional toxins of varying polarity a gradient separation was introduced. In addition, to allow the incorporation of CYN in the analytical method the applicability of water as toxin stock solvent was investigated.

2.2.3 Toxin calibration

The dried multi-toxin stock (Table 2. 6) was dissolved in 1 mL of Elga water and diluted to prepare a calibration of the following concentrations: 0.5, 1, 5, 10, 20, and 50 µg mL^{-1}. Each calibrant solution (100 µL) was transferred to an HPLC vial with low volume inserts. Intra-day precision was investigated following the injection of five consecutive multi-toxin standards. Inter-day precision was investigated by comparing the quantification of multi-toxin standards on three different days. In addition, a low range calibration (0.1-0.45 µg mL^{-1}) was prepared to investigate the practical limits of quantification (LOQ) for each toxin. This was defined as the concentration at which reproducible detection was achieved with variations (%RSD) below or equal to 10% (Taverniers *et al*., 2004). The theoretical LOD and LOQ were calculated based on signals equal to three and ten times the standard deviation of the blank, respectively. Linearity of the calibrations was assessed (section 2.2.2).

2.2.4 Investigation of solvent suitability for toxin re-suspension

A stock solution (10 µg mL^{-1}) of the following toxins was prepared: CYN, ATX-a, DA, MC-RR, MC-LR, MC-LA, MC-LY, MC-LW, and MC-LF from dried standards stored at -20°C. Aliquots of 100 µL were transferred to HPLC vials. Three of these standard solutions were analysed immediately using the refined HPLC method (section 2.2.2). The remaining vials were dried using centrifugal evaporation (Genevac, max. 45°C). The dried toxin standards were subsequently re-suspended (in triplicate) in a range of solvents: water, aqueous MeOH (50%), acetonitrile, and aqueous MeCN (50%), respectively.

Re-suspended standards were vortexed and analysed utilising the refined HPLC-PDA method. Toxin concentrations detected before and after drying were compared and reported as percent recovery.

2.2.5 Analysis of cyanobacteria culture extracts by HPLC-UV and UPLC-QToF-MS

2.2.5.1 Extraction of cyanotoxins from laboratory cultures

Water was selected as the most suitable diluent for multi-toxin analysis. Therefore, to test the method's applicability for the detection of cyanotoxins, 22 cyanobacteria cultures (Table 2. 8) maintained within the laboratory (20-22°C, continuous light intensity of 10-12 µmol m^{-2} s^{-1}), were analysed. A 2 mL sample was taken from each culture (in triplicate), centrifuged (20 min, 15,493x g). The supernatant was discarded and the pellet extracted with 80% aqueous MeOH for one hour, with vortexing for ~ 10 s every 15 min. Following extraction samples were centrifuged (20 min, 15,493 x g), the supernatants was pipetted into HPLC vials and dried (Genevac ≤45°C). Samples were re-suspended in 200 µL of water and analysed utilising the optimised HPLC-PDA method (section 2.2.2, Figure 2. 3).

Table 2. 8 Cyanobacteria strains utilised for determination of cyanotoxin content by HPLC-PDA and UPLC-QToF-MS.

Culture	strain
Anabaena flos-aquae	CCAP 1403/13A NIES-2095, PCC 7938, UTEX 1444
Aphanizomenon flos-aquae	PCC 7905
C. raciborskii	CYL, NIES-930
M. botrys	NIVA CYA 161/1
M. aeruginosa	B2666 UTEX
M. aeruginosa	CCAP 1450/3
M. aeruginosa	CCAP 1450/10
M. aeruginosa	NIES 1071
M. aeruginosa	NIES 1099
M. aeruginosa	PCC 7820
M. aeruginosa	PCC 7813
M. aeruginosa	SCIENTO
M. flos-aquae	SAT
Nodularia sphaerocarpa	PCC 7804
N. spumigena	KAC66
Oscillatoria formosa	NIVA CYA 92, PCC 10111
Phormidium autumnale	CCAP 1462/6
Phormidium	CCPA 1462/8
Planktothrix agardhii	CCAP 1459/37, NIVA CYA 18 PCC 7821
P. agardhii	NIVA CYA 29
P. rubescens	PCC 10608
Colombo Lake sample	N/A

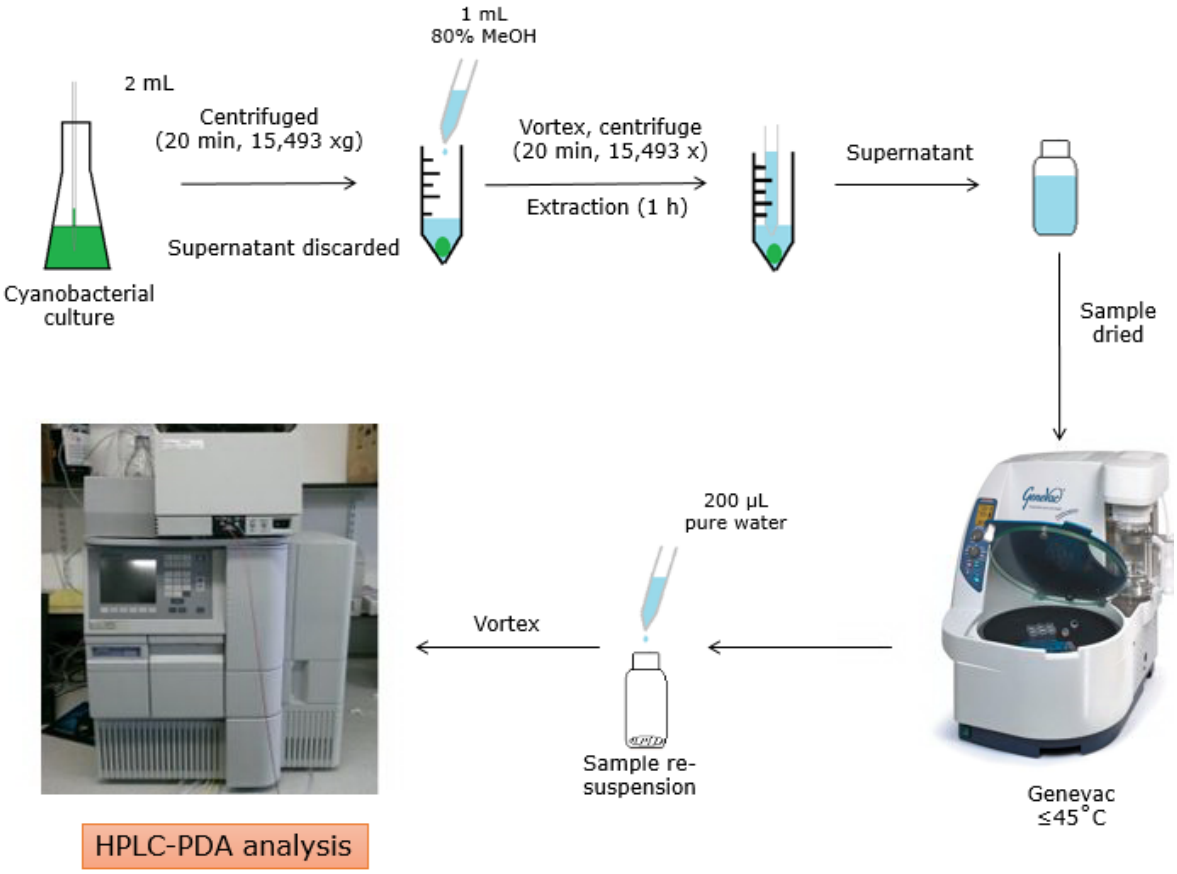

Figure 2. 3 Cyanotoxin extraction from laboratory cultures (2 mL) which were centrifuged (20 min, 15,493 xg) and extracted with 80% aqueous MeOH. The supernatant was dried and the cyanotoxin content determined utilising the optimised HPLC-PDA method (section 2.2.2).

2.2.5.2 UPLC-QToF-MS confirmatory analysis of laboratory cyanobacteria cultures

In order to confirm toxin identification (section 2.2.5.1) cyanobacterial culture extracts were also analysed by UPLC in combination with a quadrupole time of flight mass detector (UPLC-QToF-MS; Table 2. 9). During mass spectrometry ions are detected based on their mass to charge ratio (m/z). For this purpose the sample needs to be ionised which can be achieved amongst other via electrospray ionisation (ESI). At atmospheric pressure the HPLC or UPLC effluent is pumped through a capillary needle, at which a high potential difference is present (in the region of kilovolts). This results in a charged spray being ejected which is passed through a desolvation capillary where the solvent evaporates causing the droplet size to decrease. This continues until the repulsion forces between same charge ions exceeds the surface tension of the droplets. Consequently the droplets disintegrate and a gas is created. This

technique is considered a soft ionisation method as little fragmentation occurs and the molecular ion is conserved (Skoog *et al*., 1998). Common mass analysers utilised following ESI are quadrupole time of flight (QToF) or triple quadrupole (TQ) analysers. A quadrupole consists of four cylindrical rods which are electrically connected, one pair being the positive side of a direct current, whereas the other pair acts as negative side. Furthermore, a radiofrequency (180 degrees out of phase) is also applied to the two rod pairs. This allows the selection of ions of certain m/z ratios to pass through the quadrupole (Skoog *et al*., 1998). In a QToF mass analyser the quadruple is connected to a ToF detector where samples of different charge and mass travel at different speed. During TQ analysis three quadrupoles are arranged in sequence and enable MS/MS analysis. The first quadrupole filters the ions at the m/z ratio of interest, the parent ion, from the remaining ions present. The second quadrupole acts as a collision cell in which the ions with the selected m/z are fragmented into so called daughter ions. Finally the third quadrupole enables the selection of the fragments, also known as daughter ions, of interest (Skoog *et al.*, 1998).

During the present study extracts were analysed utilising an ACQUITY UPLC system equipped with a quadrupole time of flight (Xevo QToF) mass spectrometer (all Waters, Elstree, UK). A Waters Acquity BEH C18 column (2.1 x 100 mm, 1.7 µm, 40 °C) was utilised for separation. Samples (5 µL injection volume) were analysed using a mobile phase gradient of 0.1 % formic acid in water (A) and 0.1 % formic acid in acetonitrile (B). The gradient increased from 20% to 70% B over 10 min at a flow rate of 0.2 mL min^{-1} followed by a ramp up to 100% B held for 1 min. The column was washed with 100% B for 1 min and allowed to re-equilibrate over the next 3 min. Mass spectrometry analysis was performed in positive ion electrospray mode, scanning from m/z 50 to 2000 Da with a scan time of 1s. Instrumental control, data acquisition and processing were achieved using Masslynx v4.1 software. The molecular ion $[M+H]^+$ was monitored for all cyanotoxins except for MC-RR for which the double charged molecular ion $[M+2H]^{2+}$ was monitored.

Table 2. 9 Mass spectrometric conditions applied for the analysis of cyanobacteria cell extracts

Parameter	**1st & 2nd batches (30-05-2014)**	**3rd batches (01-08-2016)**
Scan range m/z	50-2000	50-1500
Capillary voltage (kV)	3.0	3.3
Sampling cone	25	25
Extraction Cone	4	3
Source temperature (°C)	80	80
Desolvation temperature (°C)	300	350
Cone gas flow (L hr^{-1})	50	50
Desolvation gas flow (L hr^{-1})	500	400
Collision energy (eV)	15	15
Scan time (s)	1	1

2.3 Results and Discussion

2.3.1 Method transfer and optimisation

Transfer of the existing DA method to the HPLC-PDA instrument was successful with a single peak of DA at a retention time of ~6.5 min eluting from the column (Figure 2. 4). In addition, the DA standard calibration showed excellent linearity (R^2=1.0000; Figure 2. 5) Therefore, the transferred HPLC-PDA method could be optimised to achieve the simultaneous detection of DA and nine cyanotoxins.

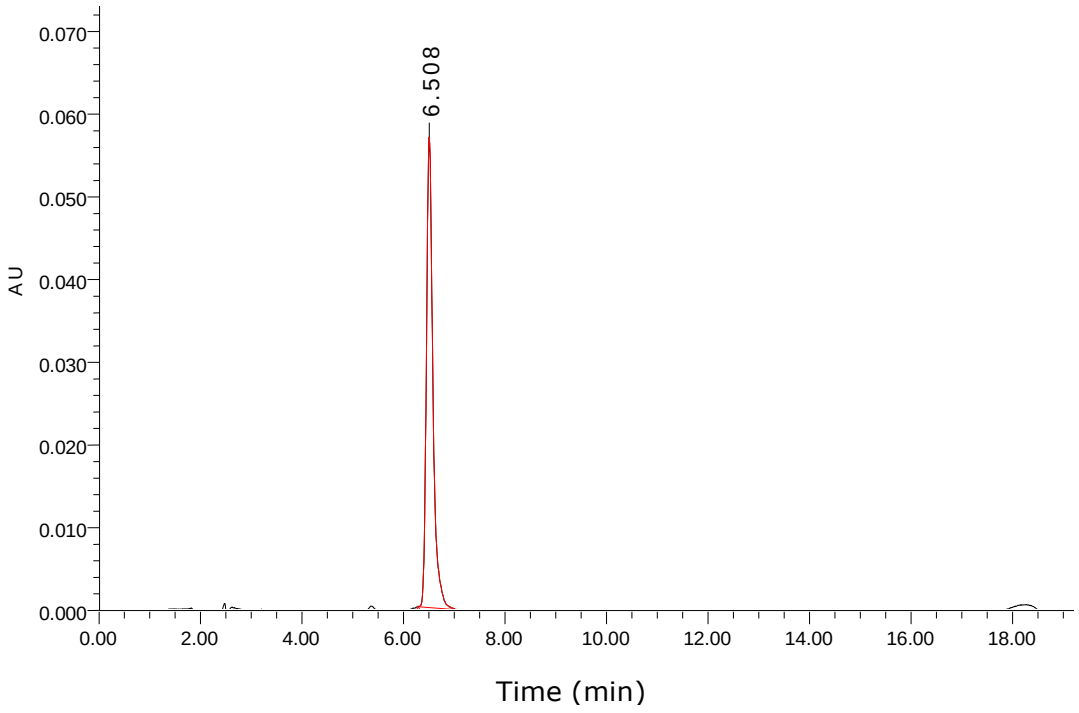

Figure 2. 4 HPLC-PDA chromatogram of DA (10 µg mL^{-1}) in 5 % aqueous MeCN monitored at 240 nm (V_{inj}= 10 µL).

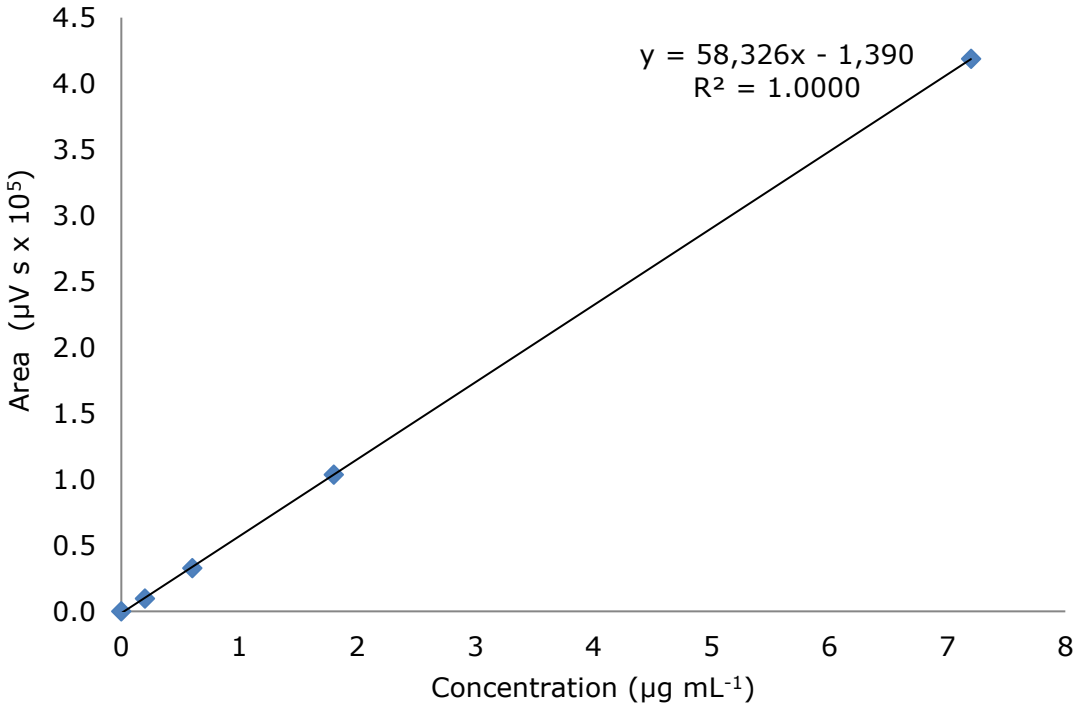

Figure 2. 5 Calibration of DA in 5% aqueous MeCN obtained by HPLC-PDA monitored at 240 nm

2.3.1.1 Solvent optimisation

In order to develop a multi-toxin method that accurately detected toxins of varying polarity the solvent composition was investigated. The main factor for this was the incorporation of CYN which is the most polar of the toxins to be included in the method. Signal losses for CYN have been reported when utilising either >50% aqueous MeOH or >30% aqueous MeCN as diluent for the HPLC-PDA analysis (Metcalf *et al*., 2002). Therefore, the possibility of utilising water as a diluent for DA was investigated. Linearity of the obtained calibration for DA in water remained excellent (Figure 2. 6). A small change in slope was observed compared to that detected for DA in 5% aqueous MeCN (Figure 2. 5). However, results obtained were within 5% variation which allowed the use of water for sample preparation.

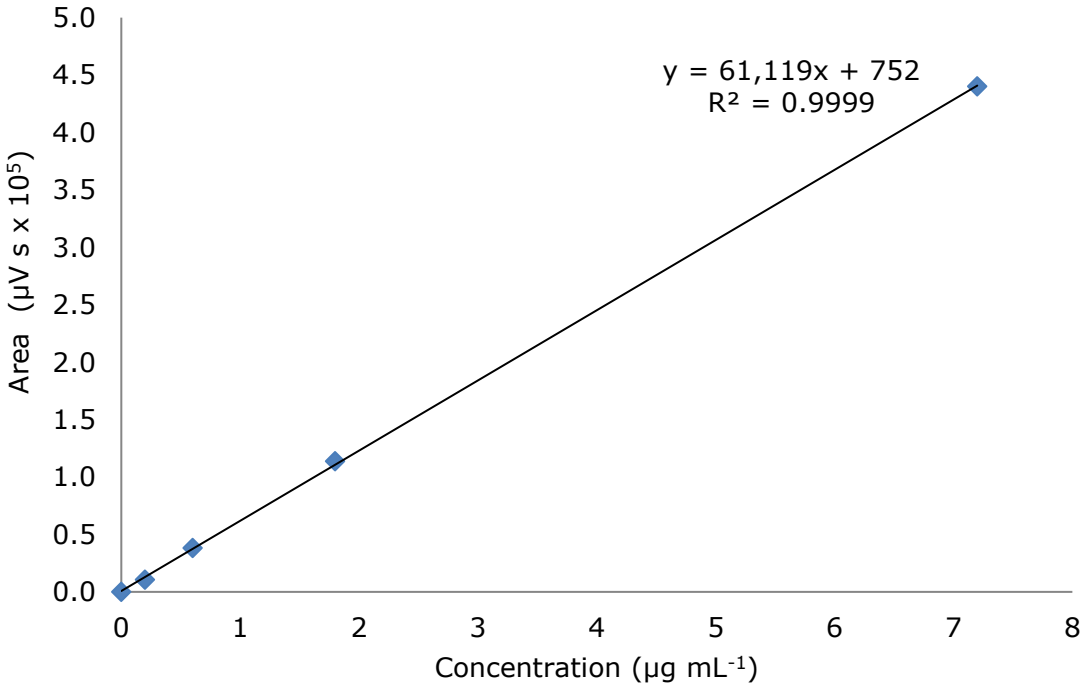

Figure 2. 6 Calibration of DA using water as the sample solvent obtained by HPLC-PDA (240 nm)

2.3.1.2 Method optimisation

To adapt the isocratic protocol for the DA detection to the analysis of additional cyanotoxins a gradient elution was introduced. The two mobile phases utilised were 0.1% TFA in water (A) and 0.1% TFA in MeOH (B). The first gradient (Table 2. 10) which included the initial Cefas isocratic stage followed by a 30 min gradient form 32.5 to 100% MeOH showed good separation between all toxins (Figure 2. 7). However, a baseline shift was observed as increasing MeOH interfered with the UV absorbance.

Table 2. 10 Initial Cefas isocratic method followed by a 30 min gradient from 32.5 to 100% MeOH utilised for the separation of DA and six cyanotoxins

Time in min	0.1 % TFA in water	0.1% TFA in MeOH
0	67.5	32.5
10	67.5	32.5
40	0	100

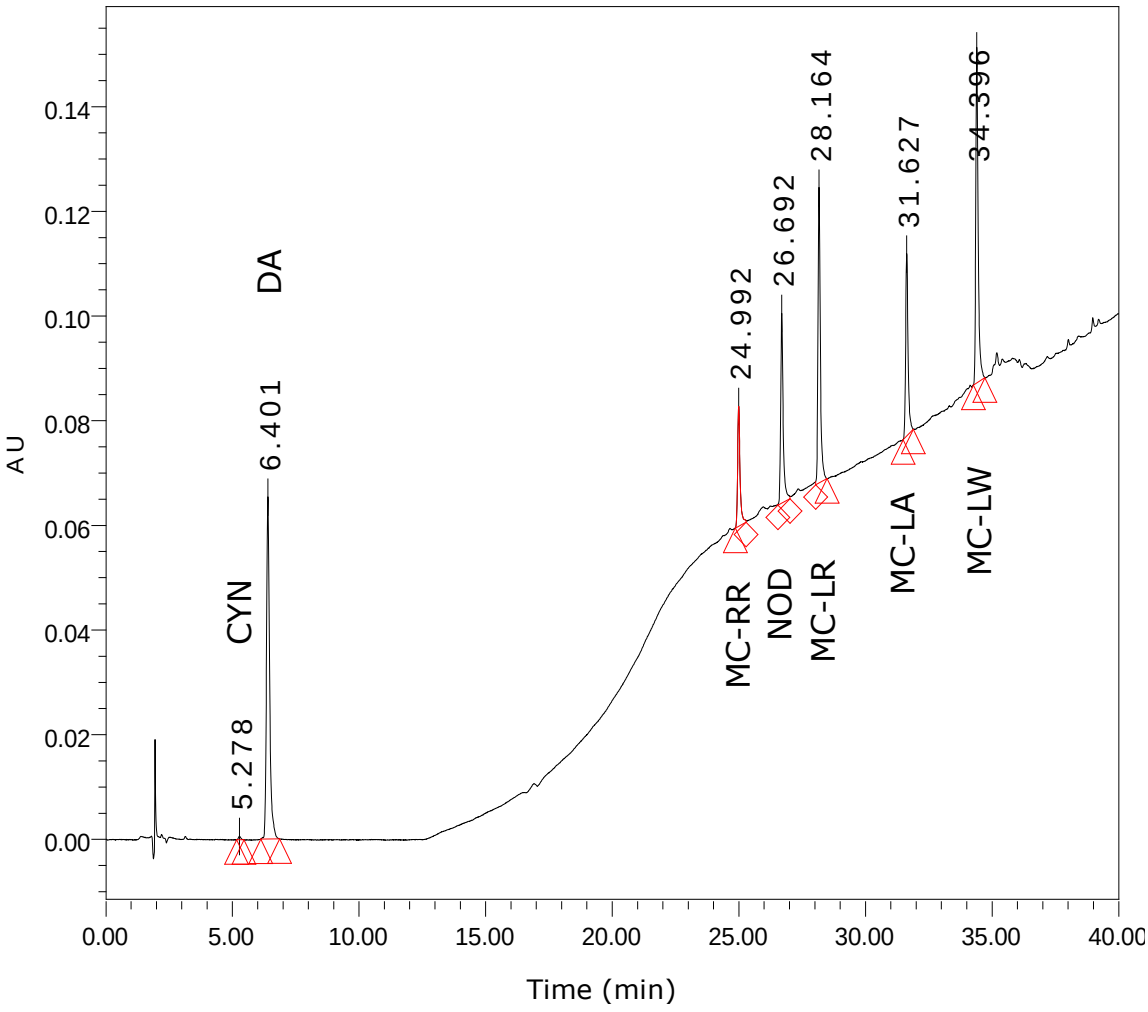

Figure 2. 7 HPLC-PDA analysis of DA and six cyanotoxins (monitored at 240 nm) using initial isocratic 10 min DA method (Cefas) followed by a 30 min gradient from 32.5% to 100% MeOH.

In order to achieve better separation between DA and CYN the starting conditions were refined to 2% MeOH. The MeOH content of the mobile phase increased to 26.4% over the next eight minutes. This was followed by a one minute increase to reach 32.5% MeOH, which was maintained for six minutes. The 30 min gradient from 32.5 to 100% MeOH was unchanged and followed by a five minute re-equilibration to reach the starting conditions (Table 2. 11, Figure 2. 8).

Table 2. 11 Adjusted gradient for the HPLC separation of DA and six cyanotoxins

Time in min	0.1 % TFA in water	0.1% TFA in MeOH
0	98	2
8	73.6	26.4
9	67.5	32.5
15	67.5	32.5
45	0	100
46	98	2
51	98	2

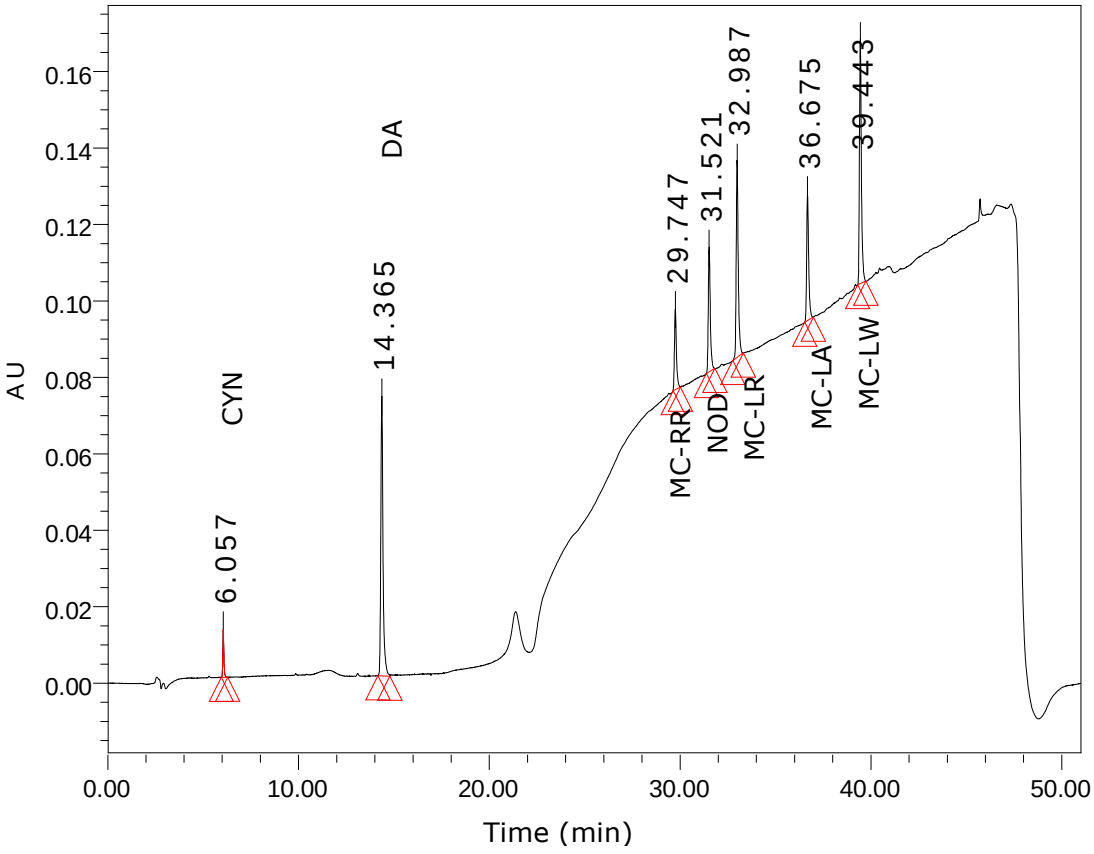

Figure 2. 8 Improved HPLC-PDA separation of DA and six cyanotoxins with adjusted gradient (240 nm)

It was again observed that the increasing MeOH content resulted in a baseline drift due to UV absorbance of MeOH (Figure 2. 7, Figure 2. 8). Hence MeOH was substituted with MeCN to alleviate this. This resulted in an improved baseline (Figure 2. 9). Due to the different elution strength of MeOH and MeCN the gradient was re-adjusted to start at 99.5% A which showed excellent results for CYN retention. In addition, a short isocratic step at 15% B for six minutes showed good elution for DA. With this adjusted gradient it was possible to also include ATX-a and two additional MC variants: MC-LY and MC-LF in the

analysis (Table 2. 12). To achieve optimum sensitivity CYN and ATX-a were monitored at their λ_{max} 262 and 228 nm, respectively (Figure 2. 9). The maximum absorbance for MCs, NOD, and DA was very similar (238-240 nm) allowing them to be monitored in one channel at 238 nm.

Table 2. 12 Final optimised gradient for the HPLC-PDA separation of DA and nine cyanotoxins

Time in min	0.1 % TFA in water	0.1% TFA in MeCN
0	99.5	0.5
8	90	10
9	85	15
15	85	15
42.5	30	70
44	0	100
46	0	100
51	99.5	0.5
56	99.5	0.5

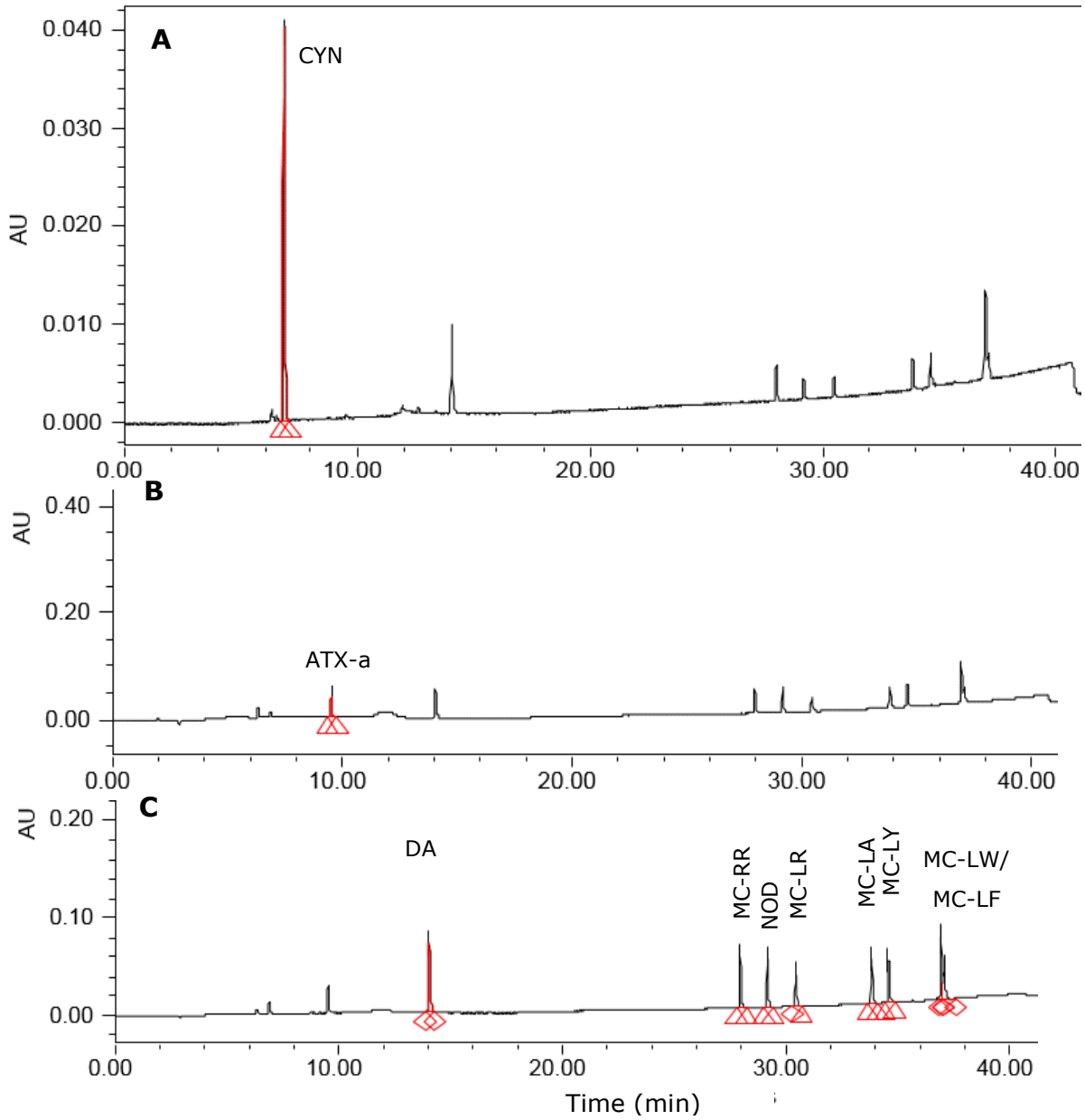

Figure 2. 9 Separation of DA and nine cyanotoxins utilising HPLC-PDA gradient separation (Table 2. 12). CYN monitored at 262 nm (A) ATX-a monitored at 228 nm (B) DA, MCs, and NOD monitored at 238 nm (C)

Applying the finalised method gradient to a stock solution of all 10 toxins (10 µg mL^{-1}) showed sufficient separation (resolution R_s>1.5) between all peaks except for MC-LW and MC-LF (Figure 2. 10). Despite the lack of resolution (R_s≈ 0.25), reproducible peak areas (%RSD <5%) were achieved. This incomplete resolution was accepted to allow the simultaneous analysis of 10 toxins of varying polarity.

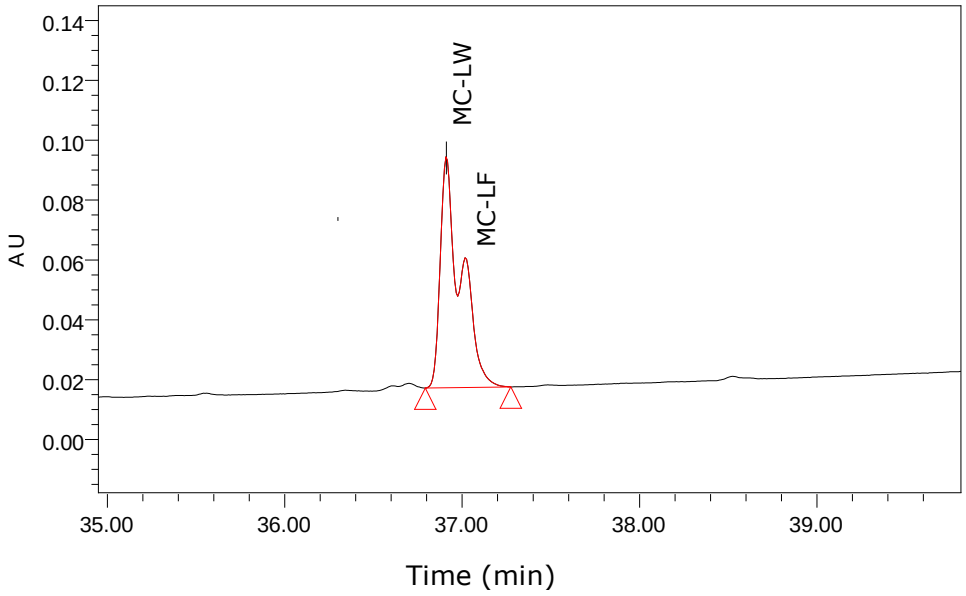

Figure 2. 10 HPLC-PDA chromatogram of incomplete MC-LW and MC-LF (10 µg mL^{-1}) monitored at 238 nm utilising the optimised gradient for the separation of DA and nine cyanotoxins

2.3.2 Toxin calibrations

Utilising multi-toxin stock solutions a calibration was obtained for each of the ten toxins (Table 2. 13). Excellent linearity was achieved for all toxins from 0.5-50 µg mL^{-1} with all toxins showing R^2 > 0.999 except for ATX-a. Investigating linearity at toxin concentrations below 0.5 µg mL^{-1} revealed that only CYN, DA, and NOD showed excellent linearity down to concentrations of 0.1 µg mL^{-1} (DA, CYN) and 0.25 µg mL^{-1} (NOD), respectively (Table 2. 13).

The practical LOQ determined for each of the ten toxins ranged from 0.1-5 µg mL^{-1} (Table 2. 14). CYN, DA, MC-RR, and MC-LA showed excellent reproducibility with %RSD below 10% at the lowest concentration investigated (0.1 µg mL^{-1}). For the more polar cyanotoxin ATX-a the practical LOD was not found in the lower concentration range, but at 5 µg mL^{-1}.

Table 2. 13 Toxin detection and calibration utilising the finalised gradient HPLC-PDA method (Table 2. 12).

Toxin	Λ nm	Linear regression equation	Linear range in µg mL^{-1}	R^2
CYN[a]	262	y= 15,786x + 497	0.1-50	1.0000
ATX-a[b]	228	y= 17,783x – 2,750	5-50	0.9999
DA[c]	238	y= 38,893x – 307	0.1-50	1.0000
MC-RR[c]	238	y= 27,098x – 10,133	0.5-50	0.9996
NOD[c]	238	y= 26,810x -1,475	0.25-50	1.0000
MC-LR[c]	238	y= 16,395x - 3,572	0.5-50	0.9999
MC-LA[c]	238	y= 26,100x - 2,834	0.5-50	1.0000
MC-LY[c]	238	y= 24,217x – 7,038	0.5-50	0.9998
MC-LW[c]	238	y= 36,722x – 17,335	0.5-50	0.9995
MC-LF[c]	238	y= 22,014x - 10,706	0.5-50	0.9996

Monitored at: a - 262 nm, b - 228 nm, c - 238 nm
%RSD - relative standard deviation

Table 2. 14 Practical concentration reliably detected (c_p) utilising HPLC-PDA and theoretically calculated LODs and LOQs based on signal to noise ratios of 3:1 and 10:1, respectively.

Toxin	c_p in µg mL^{-1}	%RSD	LOD in µg mL^{-1}	LOQ in µg mL^{-1}
CYN[a]	0.10	3.64	0.16	0.54
ATX-a[b]	5.00	0.83	0.49	1.63
DA[c]	0.10	2.32	0.11	0.36
MC-RR[c]	0.10	6.24	0.23	4.09
NOD[c]	0.20	0.85	0.41	1.36
MC-LR[c]	0.15	6.29	0.50	1.68
MC-LA[c]	0.10	7.84	0.24	0.79
MC-LY[c]	0.50	6.55	0.82	2.73
MC-LW[c]	0.35	9.97	0.96	3.19
MC-LF[c]	0.20	7.64	1.19	3.97

Monitored at: a - 262 nm, b - 228 nm, c - 238 nm
%RSD - relative standard deviation

The theoretically determined LOQ values calculated from the signal to noise ratios of the blanks (10:1) were higher than the practically determined ones (Table 2. 14). The theoretical LODs and LOQs generally increased with increasing polarity. Only ATX-a showed a practical LOQ that was approximately three times higher than the calculated value. The determined LODs from the present study did agree with some reports within the literature (Spoof *et al*., 2009; Barco *et al*., 2002). However, as expected, much lower LODs were found for tandem mass applications due to their higher specificity and sensitivity

enabling the analysis of sub ng mL^{-1} concentrations (Table 2. 15; Amé *et al*., 2010; Ortelli *et al*., 2008; Preußel *et al.,* 2006). In addition, LODs as low as 2.5 x 10^{-5} µg mL^{-1} have been reported for HPLC-PDA detection of CYN, ATX-a, MC-RR, MC-LR, and MC-LW (Szlag *et al.*, 2015 Table 2. 15). This illustrated that the developed method lacked sensitivity in comparison to other methods. Potentially, the reported methods could be amended to include DA and additional cyanotoxins to enable detection at significantly lower levels.

Table 2. 15 Limits of detection reported in published literature for the detection of cyanotoxins utilising different analysis methods

Cyanotoxin	Analysis method	LOD in water µg mL^{-1}	Reference
MC-RR, MC-LR, MC-YR	HPLC-MS/MS[a]	1.5×10^{-3}	Amé *et al.* (2010)
MC-[Dha^{7}]-LR, MC-LR	HPLC-ESI-MS[b]	0.015	Yu *et al*. (2009)
CYN	UPLC-MS/MS[c]	1.7×10^{-4}	Oehrle *et al.* (2010)
ATX-a		1.3×10^{-4}	
MC-RR		2.1×10^{-4}	
MC-LR		1.0×10^{-4}	
MC-LF		2.0×10^{-4}	
MC	HPLC-UV[d]	0.001	Lance *et al.* (2010a)
MC-RR, NOD, MC-YR, MC-LR, MC-LA, MC-LW, MC-LF	UPLC-TOF-MS[e]	1×10^{-4}	Ortelli *et al.* (2008)
dmMC-RR, MC-RR, LNOD, dmNOD, NOD, dmMC-LR, MC-LR, MC-LY, MC-YR, MC-LA, MC-LW, MC-LF	UPLC-UV	0.015-0.025	Spoof *et al*. (2009)
CYN	HPLC-MS/MS	$< 1 \times 10^{-4}$	Preußel *et al.* (2006)
MC-RR	HPLC-ESI-MS	0.014 - 0.138	Barco *et al*. (2002)
NOD		0.002 - 0.016	
MC-YR		0.004 - 0.420	
MC-LR		0.002 - 0.036	
MC-RR, MC-YR, MC-LR, MC-LA	HPLC-PDA	2.5×10^{-4}	Triantis *et al*. (2010)
CYN, ATX-a, MC-RR, MC-LR, MC-LW	HPLC-PDA	2.5×10^{-5}	Szlag *et al*. (2015)

a - analysis of standards, b - analysis of dried and re-suspended standards, c - standard addition to freeze-thawed water samples, d - after solid phase extraction e - filtered water samples with added internal standards

2.3.2.1 Intra-day and inter-day precision

Intra-day precision of the developed method was excellent since quantified toxin concentrations varied by <5% for all ten analytes (Table 2. 16). The inter-day precision as tested by the analysis of five replicates of the multi-toxin standard on three separate days also showed excellent results with variations of less than 5% when comparing the quantified concentration of all ten toxins (Table 2. 17). The developed method was therefore proven to be precise.

Table 2. 16 Cyanotoxin and DA concentrations (µg mL^{-1}) of a multi-toxin standard (~10 µg mL^{-1}) determined using the finalised gradient HPLC-PDA method (Table 2. 12)

Toxin	I	II	III	IV	V	mean	SD	%RSD
CYN[a]	8.64	8.32	8.46	8.53	8.06	8.40	0.23	2.69
ATX-a[b]	9.08	8.23	9.13	9.01	8.43	8.78	0.42	4.75
DA[c]	6.60	6.16	6.57	6.39	5.86	6.32	0.31	4.92
MC-RR[c]	9.04	8.65	8.94	8.94	8.41	8.80	0.26	2.93
NOD[c]	9.64	9.16	9.42	9.39	8.76	9.27	0.33	3.58
MC-LR[c]	14.64	13.85	14.50	14.20	13.35	14.11	0.52	3.70
MC-LA[c]	9.42	8.85	9.26	9.15	8.78	9.09	0.27	3.01
MC-LY[c]	10.91	10.24	10.56	10.52	9.96	10.44	0.36	3.41
MC-LW[c]	2.64	2.45	2.61	2.64	2.49	2.57	0.09	3.50
MC-LF[c]	10.29	9.41	9.99	9.94	9.33	9.79	0.41	4.19

Monitored at: a - 262 nm, b - 228 nm, c - 238 nm
SD **-** standard deviation, %RSD - relative standard deviation

Table 2. 17 Cyanotoxin and DA concentration (µg mL) of a multi-toxin standard determined using HPLC-PDA on three separate days over a period of time > 2 weeks

Toxin	23/10/14	03/11/14	06/11/14	mean	SD	%RSD
CYN[a]	8.40	8.73	8.49	8.54	0.17	1.97
ATX-a[b]	8.78	8.99	8.55	8.79	0.22	2.50
DA[c]	6.32	6.19	5.91	6.13	0.21	3.39
MC-RR[c]	8.80	8.90	9.04	8.91	0.12	1.38
NOD[c]	9.27	9.45	9.27	9.36	0.11	1.13
MC-LR[c]	14.11	14.51	14.19	14.27	0.21	1.47
MC-LA[c]	9.09	9.38	9.14	9.20	0.15	1.68
MC-LY[c]	10.44	10.73	10.63	10.60	0.15	1.39
MC-LW[c]	2.57	2.57	2.60	2.58	0.02	0.76
MC-LF[c]	9.79	9.89	10.03	9.90	0.12	1.23

Monitored at: a - 262 nm, b - 228 nm, c - 238 nm
SD **-** standard deviation, %RSD - relative standard deviation

2.3.3 Investigation of solvent suitability for toxin re-suspension

Prior to drying the toxin concentration had been determined in triplicate (Table 2. 18). These values where utilised to determine the effect of drying on the detectable toxin concentration of the ten toxins by comparing them to concentrations following the re-suspension in a range of solvents.

Table 2. 18 Concentration of multi-toxin stock prior to drying (n=3).

Toxin	concentration in µg mL^{-1}	Standard deviation	%RSD
CYN[a]	11.01	0.09	0.83
ATX-a[b]	11.35	0.05	0.45
DA[c]	9.88	0.16	1.61
MC-RR[c]	7.46	0.04	0.53
NOD[c]	11.12	0.11	1.02
MC-LR[c]	15.97	0.27	1.66
MC-LA[c]	11.28	0.10	0.92
MC-LY[c]	8.17	0.03	0.39
MC-LW[c]	10.37	0.15	1.40
MC-LF[c]	12.16	0.14	1.12

Monitored at: a - 262 nm, b - 228 nm, c - 238 nm

Table 2. 19 Percentage toxin compared to starting amount following re-suspension in selected solvents compared to values obtained prior to drying. The actual concentrations detected can be found in the appendix (section 2.5.2)

Toxin	water	MeOH	50% MeOH	MeCN	50% MeCN
CYN[a]	100.16	106.09	100.97	0.00	104.47
ATX-a[b]	97.47	92.78	98.26	76.75	95.24
DA[c]	94.19	95.47	92.62	0.00	89.82
MC-RR[c]	84.11	96.65	96.59	0.00	94.74
NOD[c]	97.34	98.82	97.79	0.00	95.84
MC-LR[c]	94.33	100.94	97.49	4.47	96.31
MC-LA[c]	96.13	99.48	96.02	86.32	95.20
MC-LY[c]	88.94	96.61	87.58	67.21	92.21
MC-LW[c]	89.72	100.05	97.79	82.70	98.08
MC-LF[c]	88.99	102.13	99.38	89.47	96.49

Monitored at: a - 262 nm, b - 228 nm, c - 238 nm

Comparing toxin concentrations before and after drying showed that generally water, MeOH, and 50% aqueous MeOH resulted in good recoveries for the range of toxins (Table 2. 19). MeCN was eliminated as potential solvent choice due to the effect it had on the more polar analytes: the CYN signal was lost and low recovery of ATX-a as well as some of the MC variants were observed. It was noticed that when re-suspended in both MeCN and 50% aqueous MeCN a double peak was observed for ATX-a (Figure 2. 11). This double peak can be explained chromatographically by the interaction of the less polar MeCN with ATX-a at the beginning of the gradient. With increasing water content (50% aqueous MeCN) this effect was less dominant, but still observed.

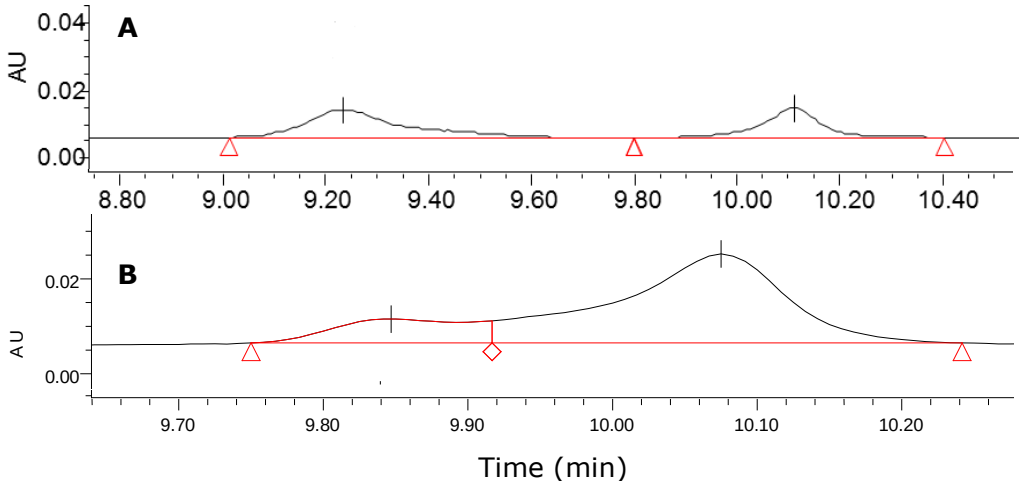

Figure 2. 11 ATX-a (10 µg mL^{-1}) after re-suspension in MeCN (A) and 50% aqueous MeCN (B) monitored at 228 nm.

A split peak was also observed for CYN when re-suspended in 50% aqueous MeCN caused by the zwitterionic nature of CYN. This also led to a significant shoulder when re-suspended in MeOH (Figure 2. 12). Consequently, 50% aqueous MeCN was eliminated as a potential solvent choice.

Generally, precise results were obtained with variations of approximately 5% (n=3) except when using MeCN containing solvents (Table 2. 20). Signal losses for CYN have been reported when utilising either >50% MeOH or >30% MeCN for the HPLC-PDA analysis (Metcalf *et al*., 2002). In the current study this was only observed when re-suspended in MeCN but not for the other organic solvents.

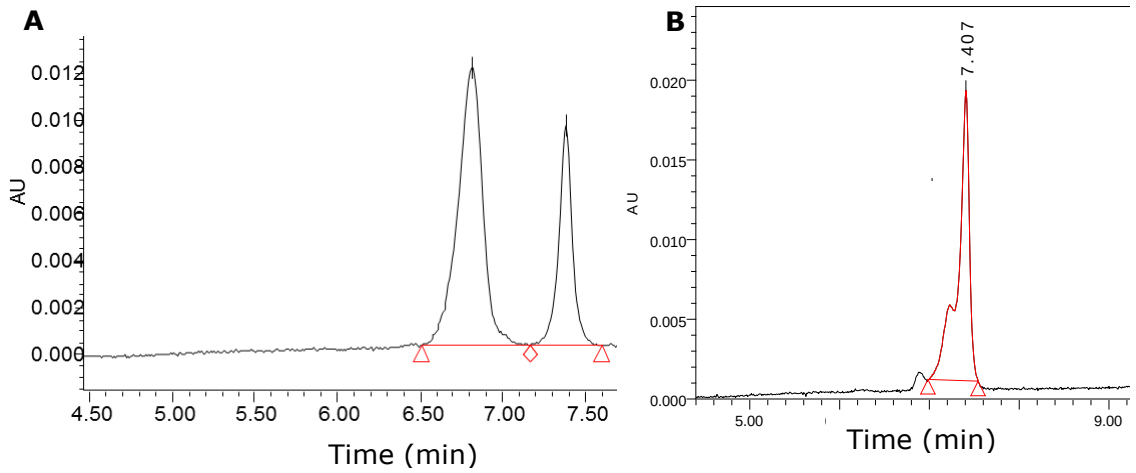

Figure 2. 12 CYN (10 µg mL^{-1}) after re-suspension in 50 % aqueous MeCN (A) and 100% MeOH (B) monitored at 262 nm.

Table 2. 20 Relative standard deviation (n=3) for concentrations of toxins re-suspended in different solvents utilising HPLC-PDA detection.

Toxin	water	MeOH	50% aqueous MeOH	MeCN	50% aqueous MeCN
CYN[a]	1.65	0.12	0.48	NA	0.93
ATX-a[b]	0.56	3.60	1.41	0.92	3.06
DA[c]	0.39	3.81	0.46	NA	2.36
MC-RR[c]	6.04	2.25	1.15	NA	1.39
NOD[c]	0.51	1.06	0.70	NA	1.03
MC-LR[c]	0.79	1.36	1.48	29.08	0.73
MC-LA[c]	0.84	2.39	0.93	7.07	0.87
MC-LY[c]	1.54	2.28	1.17	3.58	4.61
MC-LW[c]	1.78	3.21	1.98	5.25	3.38
MC-LF[c]	4.16	2.25	1.01	4.74	1.93

Monitored at: a - 262 nm, b - 228 nm, c - 238 nm
NA - not applicable as toxin was not detected

Overall 100% MeOH showed best recovery and reproducibility compared to all other solvents. However, it led to poor peak shape when analysing CYN. The second best recoveries were found for water and 50% aqueous MeOH. Due to lower price, preparation effort and the less hazardous nature of water compared to 50% aqueous MeOH water is recommended for the re-suspension of the mixed toxin standards.

2.3.4 Analysis of cyanobacteria culture extracts by HPLC-UV and UPLC-QToF-MS

To assess the applicability of the developed method for the analysis of environmental samples, 22 cyanobacteria laboratory cultures were analysed for the presence of cyanotoxins. Cyanobacteria produce an array of different metabolites, which allows the evaluation of potential matrix effects on the separation of the cyanotoxins. To further support the identification of cyanotoxins within cultures, extracts were also analysed by UPLC-QToF-MS (Table 2. 21). All chromatograms and MS spectra for each cyanobacteria strain analysed can be found in the appendix (section 2.5.4).

CYN was the most polar cyanotoxin included in the analysis. Its presence was confirmed in extracts of *C. raciborskii* CYL (NIES-930) by both HPLC and UPLC analysis. The HPLC analysis of *A. flos-aquae* PCC 7905 suggested the presence of CYN which was in agreement with reports by Nováková *et al.* (2011). This was confirmed by UPLC-QToF-MS analysis as a peak corresponding to m/z 416 was detected in the solvent front. In addition, it showed the fragments at m/z 176 and 194 characteristic for CYN when analysed by UPLC-QToF-MS (Figure 2. 13). The HPLC-PDA analysis of *A. flos-aquae* PCC 7905 had also suggested the presence of a polar MC at a retention time of 12.74 min. However, UPLC-QToF-MS analysis could not confirm this as being associated with a molecule containing the fragment characteristic of the Adda moiety (m/z 135; Figure 2. 13).

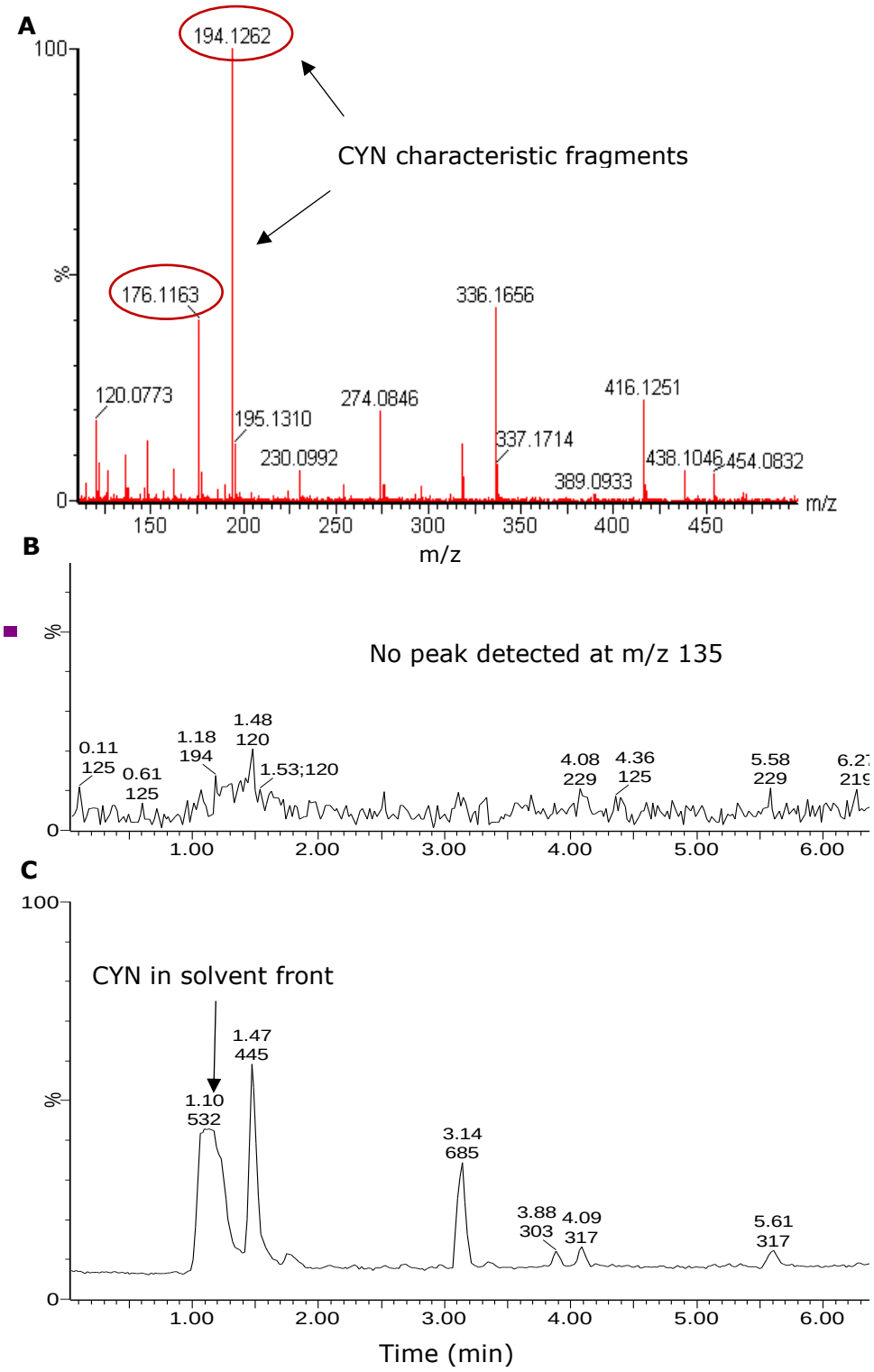

Figure 2. 13 UPLC-QToF-MS analysis of *A. flos-aquae* PCC 7905 extracts. Extracted mass spectrum at t_R= 1.27 showing the CYN characteristic fragments at m/z 176 and 194 (A) M^E chromatogram extracted at m/z 135 which could not confirm the suspected presence of a polar MC (B). TIC chromatogram (C)

Table 2. 21 Cyanotoxins detected in laboratory cultures following extraction with 80% aqueous MeOH

Culture	Strain	HPLC-PDA	UPLC-QToF-MS	Toxin according to literature	Detection method	Reference
Anabaena flos-aquae	CCAP 1403/13A NIES-2095 PCC 7938 UTEX 1444	ND	ND	No MCs or CYN	HPLC-PDA; HPLC-MS/MS	Nováková *et al*. (2011)
Aphanizomenon flos-aquae	PCC 7905	CYN, polar MC^{t}	CYN	CYN	HPLC-MS/MS	Nováková *et al*. (2011)
C. raciborskii	CYL NIES-930	CYN	CYN in solvent front	CYN, deoxy CYN	HPLC-PDA	Norris *et al*. (1999)
M. botrys	NIVA CYA 161/1	dmMC-RR^{t}, MC-RR, MC-LR	dmMC-RR, MC-RR, MC-LR	Contains MCs	HPLC-PDA, Immunoassay	Lawton *et al.* (2010)
M. aeruginosa	B2666 UTEX	dmMC-LR^{t}, MC-LR, dmMC-LA^{t}, MC-LA, MC-L(Aba)$^{\#}$, MC-LF, MC-LL$^{\#*1}$	MC-LR, dmMC-LA, MC-LA, [D Glu(OCH$_3$)^6, D-Asp^3] MC-L(Aba), dmMC-LL, MC-LF, MC-LL	ddmMC-LR, dmMC-LR, MeSer^7-MC-LR, MC-LR, dmMC-LA, MC-LA, [D Glu(OCH$_3$)^6, D-Asp^3] MC-L(Aba), MC-L(Aba), dmMC-LL, MC-LF, MC-LL	HPLC-MS/MS	Diehnelt *et al*. (2006); Del Campo and Quahid (2010)
M. aeruginosa	CCAP 1450/3	ND	ND	Non-toxic	Immunoassay	Devlin *et al.* (2013)
M. aeruginosa	CCAP 1450/10	dmMC-LR^{t}, MC-LR, mMC-LR?, MC-LY, dmMC-LF?, MC-LF^{*1}	MC-LR, mMC-LR, MC-LY, dmMC-LF, MC-LF	MC-LR, MC-LF	HPLC-MS/MS*	Juhel *et al*. (2006)

ND - not detected CAIA - Colorimetric acetylcholinesterase inhibition assay, dm – demethylated, ddm - desdimethyl
t - tentative identification as detected peak at that retention time could correspond to respective congener, but no standard was available
* only studied MC-LR, MC-RR, MC-YR, and MC-LF
*1 additional peaks with UV absorbance at 238 nm were recorded, however due to the lack of standards these could not be identified

Table 2. 21 continued

Culture	Strain	HPLC-PDA	UPLC-QToF-MS	Toxin according to literature	Detection method	Reference
M. aeruginosa	NIES 1071	dmMC-RR?, MC-RR, dmMC-LR?, MC-LR*1	dmMC-RR, MC-RR, MC-LR	dmMC-RR, MC-RR, MC-LR	HPLC-MS	Anas *et al*. (2015)
M. aeruginosa	NIES 1099	dmMC-RR?, MC-YR?, dmMC-LR? MC-RR MC-LR*1	dmMC-RR, MC-RR, MC-YR, MC-LR	Toxic, contains MCs	HPLC-PDA, Immunoassay	Lawton *et al*. (2010)
M. aeruginosa	PCC 7820	dmMC-LR?, MC-LR, dmMC-LF?*1	MC-LR, mMC-LR, MC-LY, dmMC-LF, MC-LW, MC-LF	dmMC-LR, MC-LR, mMC-LR, MC-LL, MC-LY, MC-LM, dmMC-LW, MC-LW, dmMC-LF, MC-LF	HPLC-MS	Robillot *et al.* (2000), Rios *et al*. (2014)
M. aeruginosa	PCC 7813	dmMC-LR?, MC-LR, MC-LW, MC-LF*1	MC-LR, MC-LY, MC-LW, MC-LF	dmMC-LR, MC-LR, mMC-LR, MC-LY, MC-LM, MC-LW, MC-LF,	HPLC-MS	Lawton *et al*. (1995); Fastner *et al.* (2001)
M. aeruginosa	SCIENTO	dmMC-RR?, MC-RR, MC-YR, dmMC-LR?, MC-LR*1	dmMC-RR, MC-RR, MC-YR, MC-LR	MC-RR, MC-LR, MC-LY	HPLC-PDA	Barco *et al*. (2005)
M. flos-aquae	SAT	MC-RR	dmMC-RR, MC-RR	MCs	HPLC-PDA	Lawton *et al.* (2010)

ND - not detected, NR - not reported, dm –demthylated, m - methylated
? - tentative identification as detected peak at that retention time could correspond to respective congener, but no standard available
*1 additional peaks with UV absorbance at 238 nm were recorded, however due to the lack of standards these could not be identified

Table 2. 21 continued

Culture	Strain	HPLC-PDA	UPLC-QToF-MS	Toxin according to literature	Detection method	Reference
N. spumigena	KAC 66	NOD	dmNOD, NOD	NOD	HPLC-PDA-MS	Liu *et al*, (2005)
Nodularia sphaerocarpa	PCC 7804	NOD	dmNOD, NOD	NOD, [L-homoarginine[2]]NOD	HPLC-MS	Beattie *et al*. (2000)
Oscillatoria formosa	NIVA CYA 92 PCC 10111	homoATX-a?	homoATX-a	homoATX-a	HPLC-MS	Skulberg *et al*. (1992)
Phormidium autumnale	CCAP 1462/6	ND	ND	NR	N/A	NR
Phormidium	CCPA 1462/8	ND	ND	NR	N/A	NR
Planktothrix agardhii	CCAP 1459/37 NIVA CYA 18 PCC 7821	MC-RR	dmMC-RR	Contains mcyE gene	PCR	Głowacka *et al*. (2011)
P. agardhii	NIVA CYA 29	ND	ND	No MCs	HPLC-PDA	Lawton *et al.* (2010)
P. rubescence	PCC 10608	ND	ND	homoATX-a	GC-MS	Cadel-Six *et al.* (2007)
Colombo Lake sample	N/A	ND	ND	NR	N/A	NR

ND - not detected, N/A - not applicable, NR - not reported
? - tentative identification as detected peak at that retention time could correspond to respective congener, but no standard available

All *Microcystis* species analysed showed the presence of cyanotoxins using the HPLC-PDA method except for the strain *M. aeruginosa* CCAP 1450/3 which has been reported to be non-toxic based on immunoassay tests (Devlin *et al*., 2013). For the remaining strains the cyanotoxins detected were in good agreement with the reported literature (Table 2. 21) using a variety of techniques. When utilising the HPLC-PDA method for the analysis of *M. botrys* NIVA CYA 161/1 and the *M. aeruginosa* strains UTEX B2666, CCAP 1450/10, NIES 1071, NIES 1099, PCC 7820, PCC 7813, SCIENTO, and SAT the results suggested the presence of demethylated variants of MCs. The identification of demethylated or methylated variants of the MCs included in the HPLC-PDA method was done tentatively based on shifts observed in retention times that correlated with the respective changes in polarities. Methylated variants eluted later whereas the demethylated variants eluted earlier than the unchanged MCs. For example, in extracts of *M. aeruginosa* UTEX B2666 three peaks were detected by HPLC-PDA (238 nm) that could not be assigned to any of the toxin standards available (Figure 2. 14). At t_R = 30.549 the peak was too early to be MC-LR, at t_R = 33.320 the peak was too early to be MC-LA and at t_R = 36.532 the peak was too early to be either MC-LW of MC-LF. Hence, these peaks could be associated to demethylated MCs of MC-LR, MC-LA, and MC-LW/MC-LF, respectively. UPLC-QToF-MS analysis confirmed the presence of dmMC-LA (Figure 2. 14).

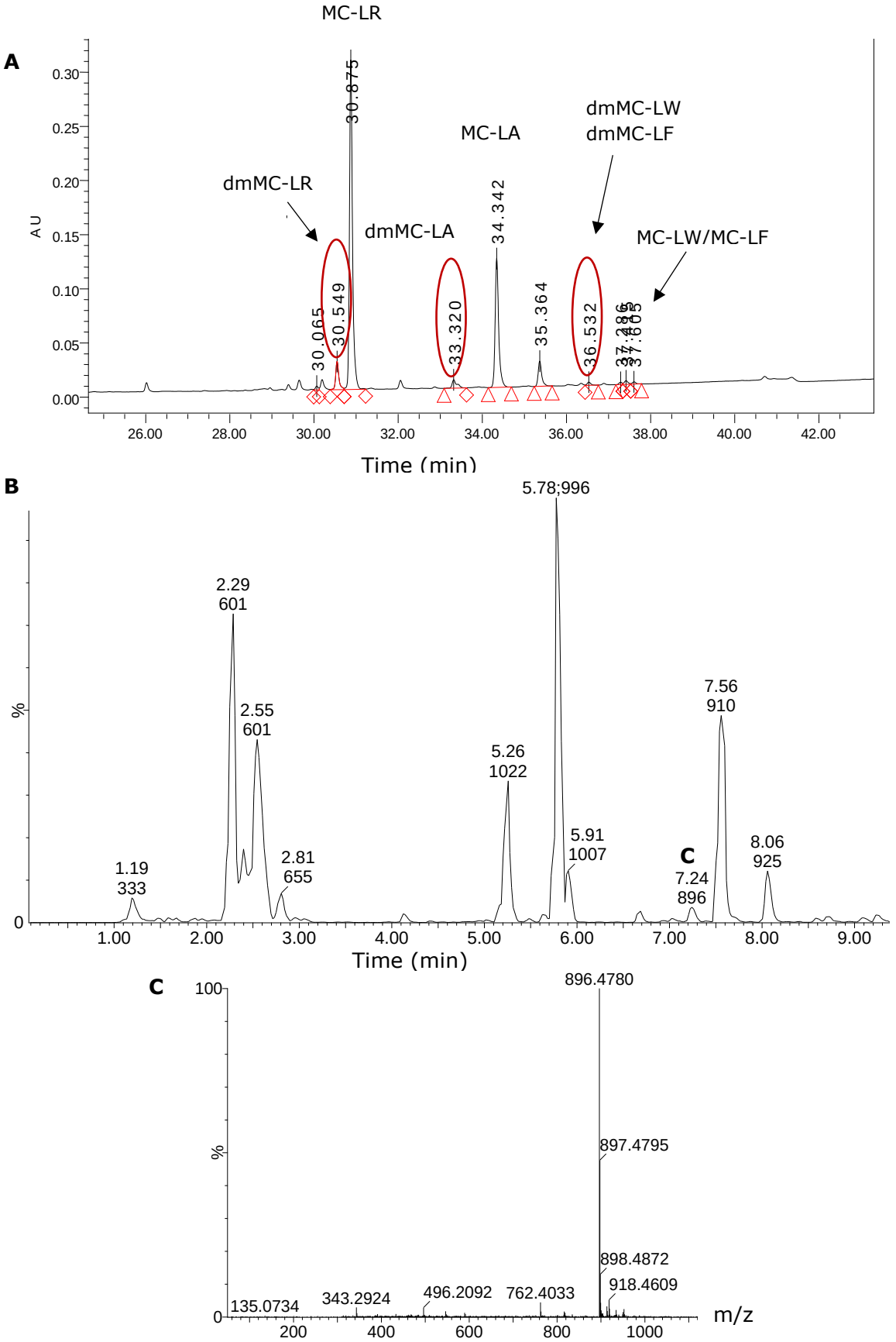

Figure 2. 14 *M. aeruginosa* UTEX B2666 analysed utilising HPLC-PDA monitored at 238 nm (A) and UPLC-QToF-MS (B). Extracted mass spectrum at t_R=7.24 showing the presence of dmMC-LA (C). No standards were available for dmMCs during HPLC-PDA analysis. Their presence was suspected due to available literature and t_R of additional peaks observed (red circles).

Generally, the MC variants showing changes in methylation status detected utilising confirmatory UPLC-QToF-MS analysis were dmMC-RR, mMC-LR, dmMC-LA, dmMC-LL, and dmMC-LF (Table 2. 21). UPLC-QToF-MS confirmatory analysis did not detect peaks corresponding to demethylated MC-LR (dmMC-LR) in the *M. aeruginosa* strains UTEX B2666, NIES 1071 and 1099, PCC 7820 and PCC 7813 as suggested by the HPLC-PDA results. In addition, dmMC-LR has been reported in the *M. aeruginosa* strains PCC7820 (Robillot *et al*., 2000; Ríos et al., 2014), PCC7813 (Fastner *et al*., 2001), and UTEX B2666 (DelCampo and Quahid 2010).

Potentially, due to shortened analysis times of UPLC-QToF-MS analysis, dmMC-LR could have co-eluted with MC-LR as reported by Bateman *et al*. (1995) compared to being detected following HPLC-PDA analysis. Closer observation of the mass spectral peak corresponding to MC-LR (m/z 995.5580) showed a greater proportion of the fragment corresponding to dmMC-LR (m/z 981) when compared to pure MC-LR standards (Figure 2. 15). This supported the suggestion that dmMC-LR co-eluted with MC-LR in the *M. aeruginosa* strains CCAP 1450/10, NIES 1071 and 1099, PCC 7813 and 7820 following UPLC-QToF-MS analysis. *M. aeruginosa* CCAP 1450/10 also contained methylated MC-LR (mMC-LR, m/z 1010; Figure 2. 16).

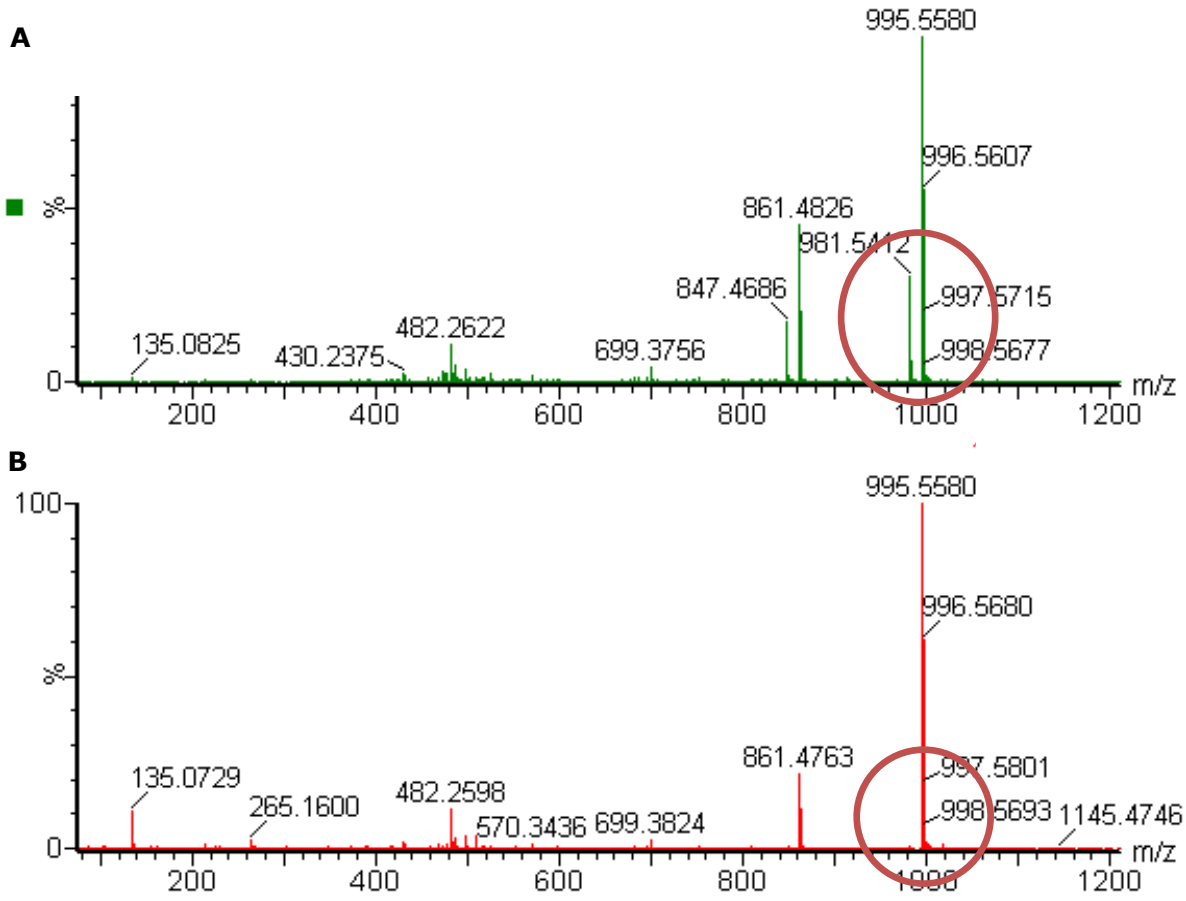

Figure 2. 15 Mass spectrum of MC-LR detected in *M. aeruginosa* NIES 1071 (A) and a 10 µg mL^{-1} standard (B) illustrating the different intensities of the 981 fragment indicating the presence of dmMC-LR in the cyanobacterial extract.

Juhel *et al*. (2006) reported only two MC variants for *M. aeruginosa* CCAP 1450/10: MC-LR and MC-LF. Both HPLC-PDA and UPLC-QToF-MS analysis performed in the present study indicated the presence of additional MC variants. For their analysis Juhel *et al*. (2006) utilised MS/MS detection, during which transitions of parent ions fragmenting to daughter ions were scanned. This technique provides a highly specific method of analysis for the respective target toxins. However, only the toxins for which transitions have been optimised will be detected. Juhel *et al*. (2006) monitored MC-LR, MC-RR, MC-YR and MC-LF only. In the present study, when conducting full scan analysis over a wide range of m/z values, utilising the UPLC-QToF-MS method, additional MCs were detected: methylated MC-LR (mMC-LR), MC-LY, and dmMC-LF.

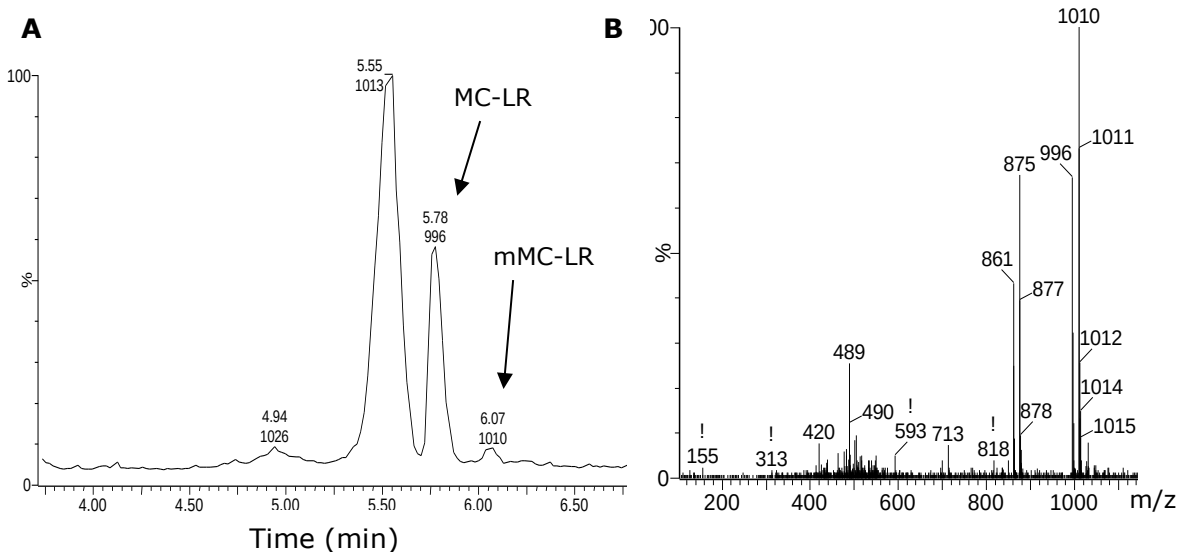

Figure 2. 16 TIC chromatogram of *M. aeruginosa* CCAP 1450/10 (A). Mass spectrum extracted at t_R= 6.07 min corresponding to mMC-LR (B).

Additional peaks detected at 238 nm besides those caused by changes in methylation status were observed for the *M. aeruginosa* strains UTEX B2666, CCAP 1450/10, NIES 1099, and SCIENTO. These peaks were thought to correspond to other MC variants present that had not been included in the HPLC-PDA method. The UPLC-QToF-MS analysis of *M. aeruginosa* B2666 showed the presence of two additional MC variants: [D Glu$(OCH_3)^6$, D-Asp^3] MC-L(Aba) and MC-LL which was in agreement with literature reports of Del Campo and Quahid (2010) and Diehnelt *et al.* (2006), respectively. Another MC variant reported in *M. aeruginosa* UTEX B2666 was desdimethyl MC-LR (Del Campo and Quahid 2010), however its presence could not be confirmed during the present study. In addition, it was not possible to identify MeSer^7-MC-LR in this strain (Diehnelt *et al*., 2006). Both *M. aeruginosa* strains NIES 1099 (Figure 2. 17) and SCIENTO showed the presence of a peak corresponding to the m/z 1046 of MC-YR.

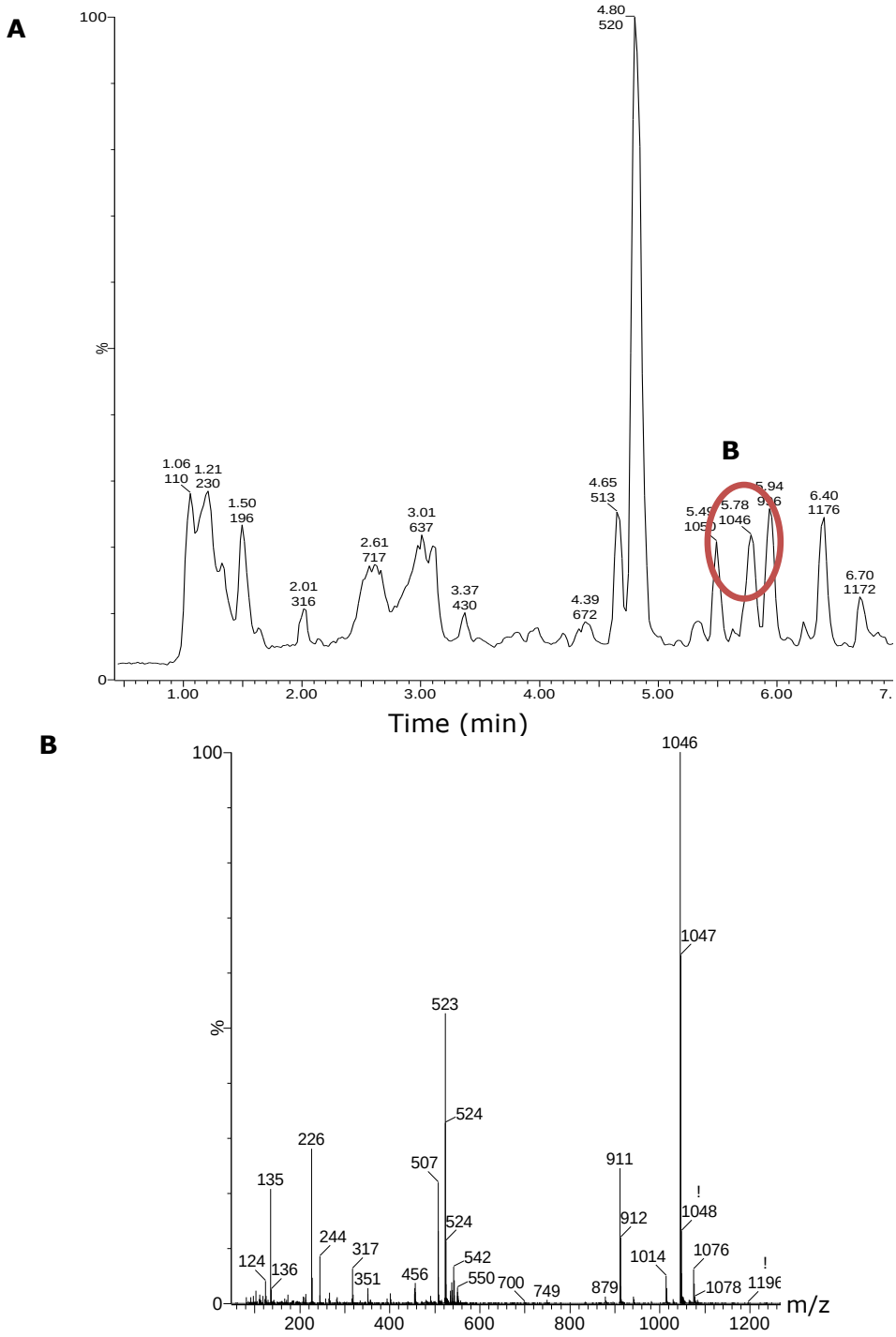

Figure 2. 17 TIC UPLC-QToF-MS chromatogram of *M. aeruginosa* NIES 1099 (A). Mass spectrum corresponding to MC-YR extracted at t_R= 5.78 min (B).

The detected additional MC variants (MC-YR, MC-LL, [D Glu(OCH_3)^6, D-Asp^3] MC-L(Aba), and MeSer^7-MC-LR) had not been incorporated into the developed HPLC method due to the lack of analytical standards. Therefore, they could only be identified by UPLC-QToF-MS. However, whenever these variants were detected by UPLC-QToF-MS analysis, additional peaks with a UV absorbance at 238 nm were also observed during the HPLC-PDA analysis (for example Figure 2. 18). This showed that the developed method could potentially include even more than the currently detected nine cyanotoxins and DA.

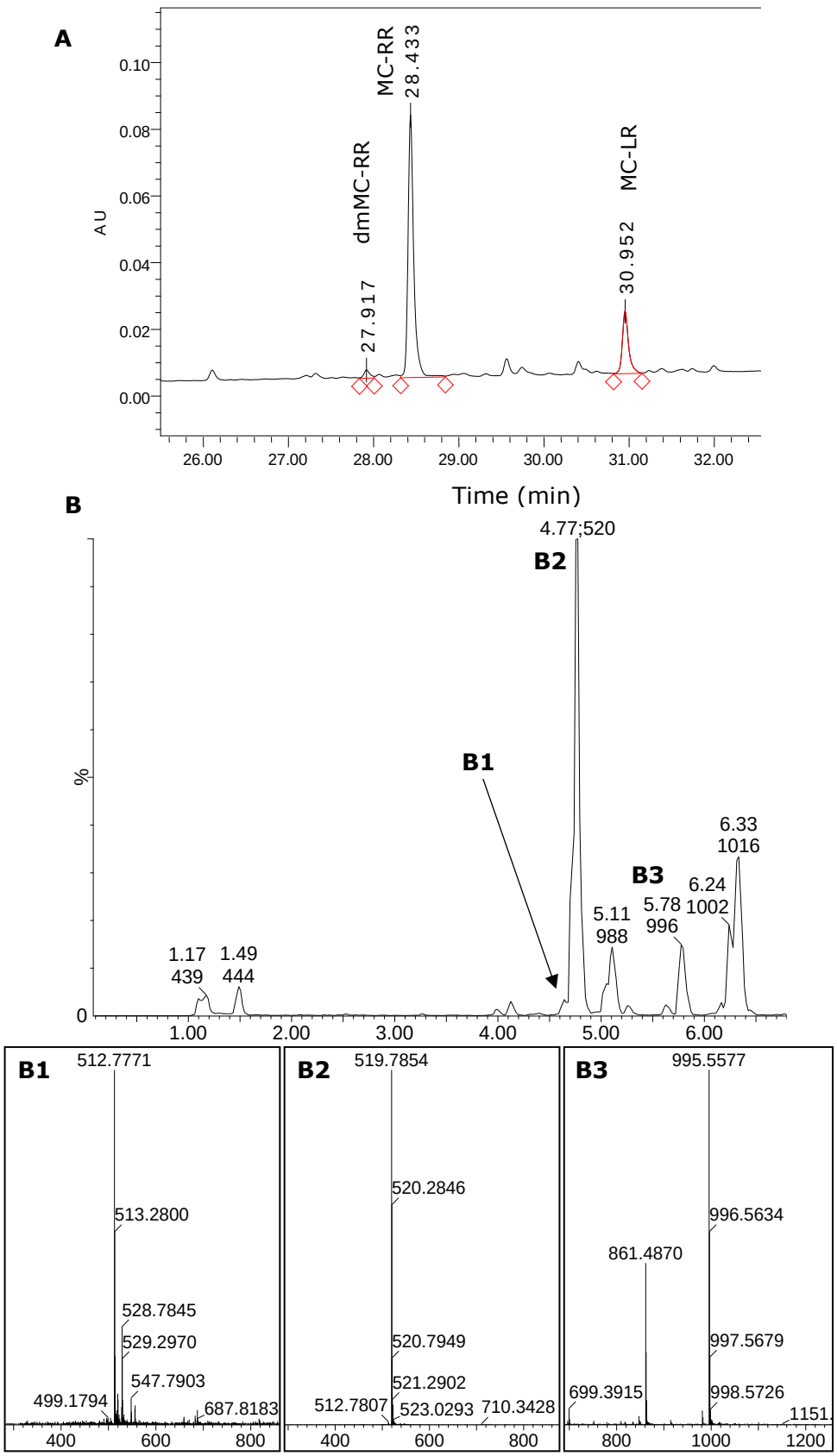

Figure 2. 18 HPLC-PDA chromatogram monitored at 238 nm (dmMC-RR was identified tentatively based on retention time shift; A). TIC UPLC-QToF-MS chromatogram and MS spectrum (base peak) of dmMC-RR (B1), MC-RR (B2), and MC-LR (B3) detected in *M. botrys* NIVA CYA 161/1 (B)

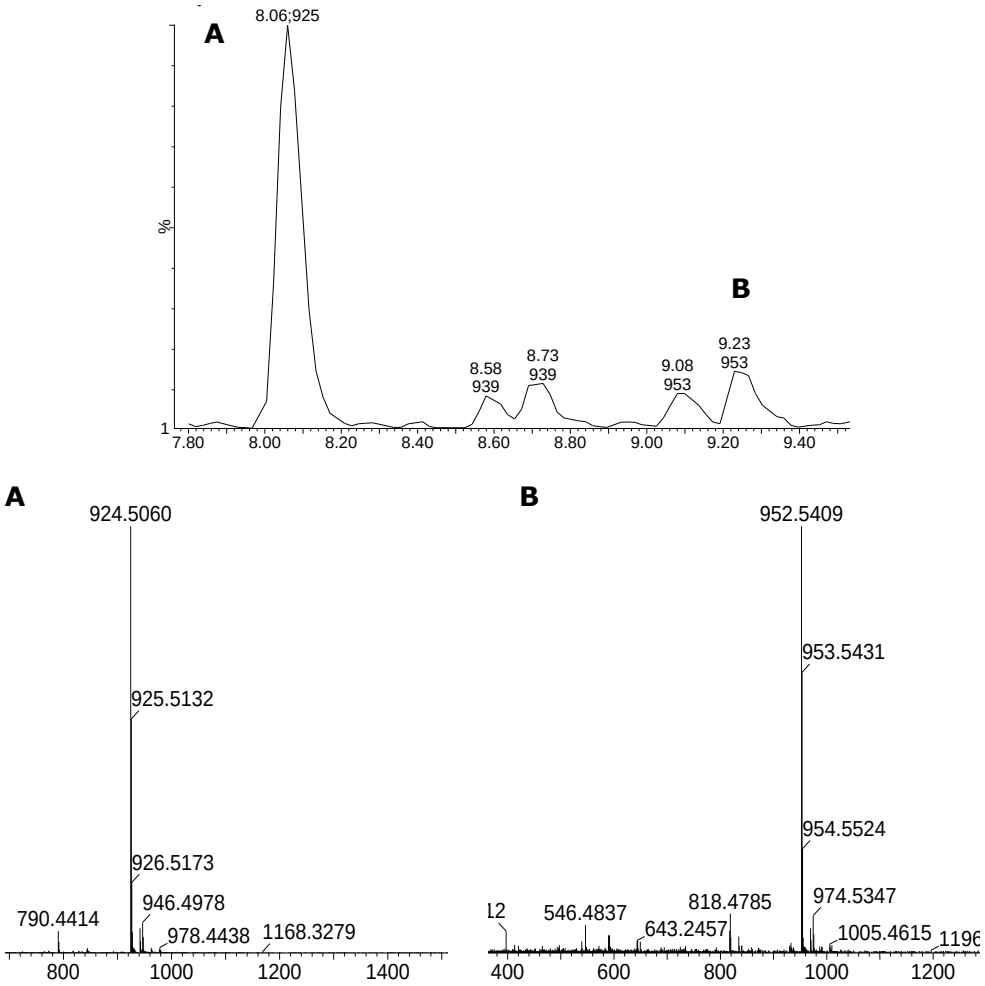

Figure 2. 19 UPLC-ToF MS chromatogram and MS spectrum (base peak) of [D Glu(OCH_3)^6,D-Asp^3]MC-L(Aba) (A), and MC-LL (B) detected in *M. aeruginosa* UTEX B2666

In a few cases it was not possible to detect MC variants that had been reported in the literature for the respective strains. This was the case for *M. aeruginosa* UTEX B2666 in which the presence of MeSer^7-MC-LR could not be confirmed following UPLC-QToF-MS analysis. In addition, MC-LL and dmMC-LW could not be detected in *M. aeruginosa* PCC 7820. Potentially, this could have been due to the small volume extracted. Minor metabolites could therefore be present at quantities below LOD or also due to changes in MC production based on different growth conditions.

The presence of NOD was detected in both *Nodularia* strains analysed by HPLC-PDA. This was confirmed by UPLC-QToF-MS along with the presence of additional NOD variants. The strain *N. sphaerocarpa* PCC 804 showed two peaks at m/z 825 at retention times 5.32 and 5.44 minutes, respectively (Figure 2. 20). This could indicate that two isomer forms of NOD were present as reported

by Mazur-Marzec *et al*. (2006b). Moreover, a peak at m/z 839 was observed which suggests the presence of methylated NOD, which has been reported in *N spumigena* NSGG-1 (Mazur-Marzec *et al*., 2006b). Similarly, NOD and dmNOD were detected in extracts of *N. spumigena* KAC66 (Mazur-Marzec *et al*., 2006b).

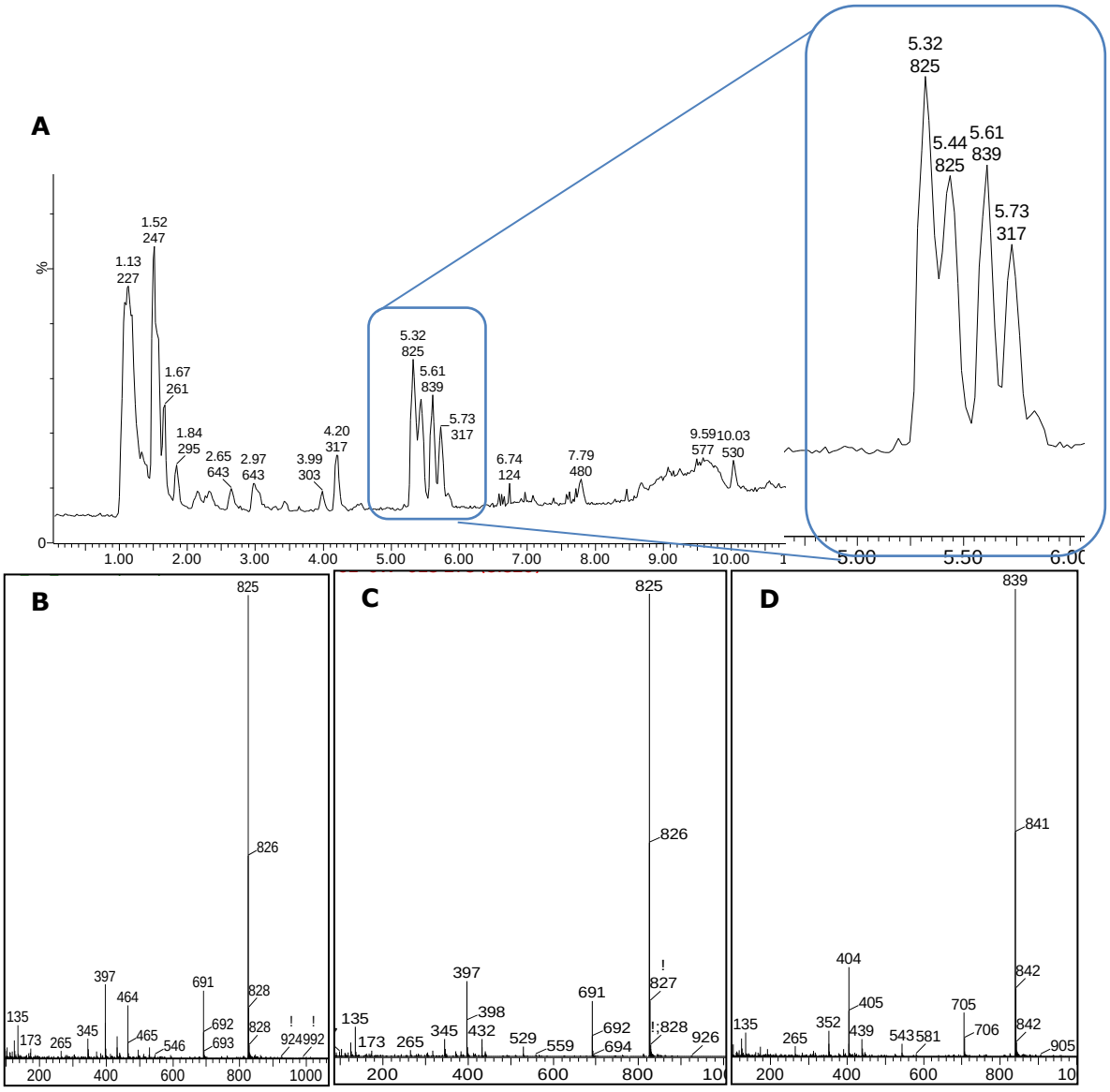

Figure 2. 20 TIC UPLC-QToF-MS chromatogram of N. *sphaerocarpa* PCC 7804 (A) and mass spectrum (base peak) of peaks at t_R 5.32 min (B), 5.44 min (C), and 5.61 min (D)

The presence of homoATX-a has been reported for two of the strains tested: *Oscillatoria formosa* NIVA CYA 92 (PCC 10111; Skulberg *et al*., 1992) and *P. rubescence* PCC 10608 (Cadel-Six *et al*., 2007). Utilising both HPLC-PDA and UPLC-QToF-MS analysis, homoATX-a was only detected in *O. formosa* NIVA CYA 92 (PCC 10111). This was contradictory to reports by Cadel-Six et *al*. (2007) who detected homoATX-a in *P. rubescence* PCC 10608 following gas chromatography MS analysis. Potentially, other interfering components extracted from *P. rubescence* PCC 10608 hindered the detection of homoATX-a under the current conditions. *P. agardhii* NIVA CYA 29, *Phormidium autumnale* CCAP 1462/6, and *Phormidium sp.* CCAP 1462 did not show the presence of cyanotoxins utilising both the developed HPLC-PDA method and UPLC-QToF-MS analysis. The sample obtained from Colombo Lake (Sri Lanka) tested negative for cyanotoxins when analysed by both HPLC-PDA and UPLC-QToF-MS, despite reports of toxic *Microcystis* blooms containing approximately 0.7 μg L^{-1} MCs (Jayatissa *et al*., 2006).

2.4 Conclusion

A method for the simultaneous detection of DA and nine cyanotoxins has been successfully developed. The method was accurate and was able to detect quantities as low as 0.1 µg mL^{-1} (CYN). Intra-day and inter-day precision were excellent illustrating the precision of the developed method. Analysis of cyanobacteria cultures showed good results generally agreeing with reported literature. Moreover, results were promising that additional MC variants could be included in the developed HPLC-PDA method once appropriate standards become available.

A full method validation was not carried out due to the current runtime of the method. To overcome this issue the method could be transferred to the UPLC system which will significantly lower the analysis time. In addition, volumes of solvents and additives required for the analysis would be lowered resulting in the reduction in cost of sample analysis.

Initially, an existing method for the detection of DA was intended to be optimised to include nine cyanotoxins. In retrospect it might have been more beneficial to optimise a published sensitive multi-cyanotoxin method to include DA. For the purpose of cyanotoxin analysis during the three feeding trials during which *M. edulis* will be exposed to cyanobacteria it was decided to utilise a sensitive LC-MS/MS method which had been validated for the use in different matrices such as algal cells, mussel tissue, and water.

2.5 Appendix

2.5.1 Toxin calibration

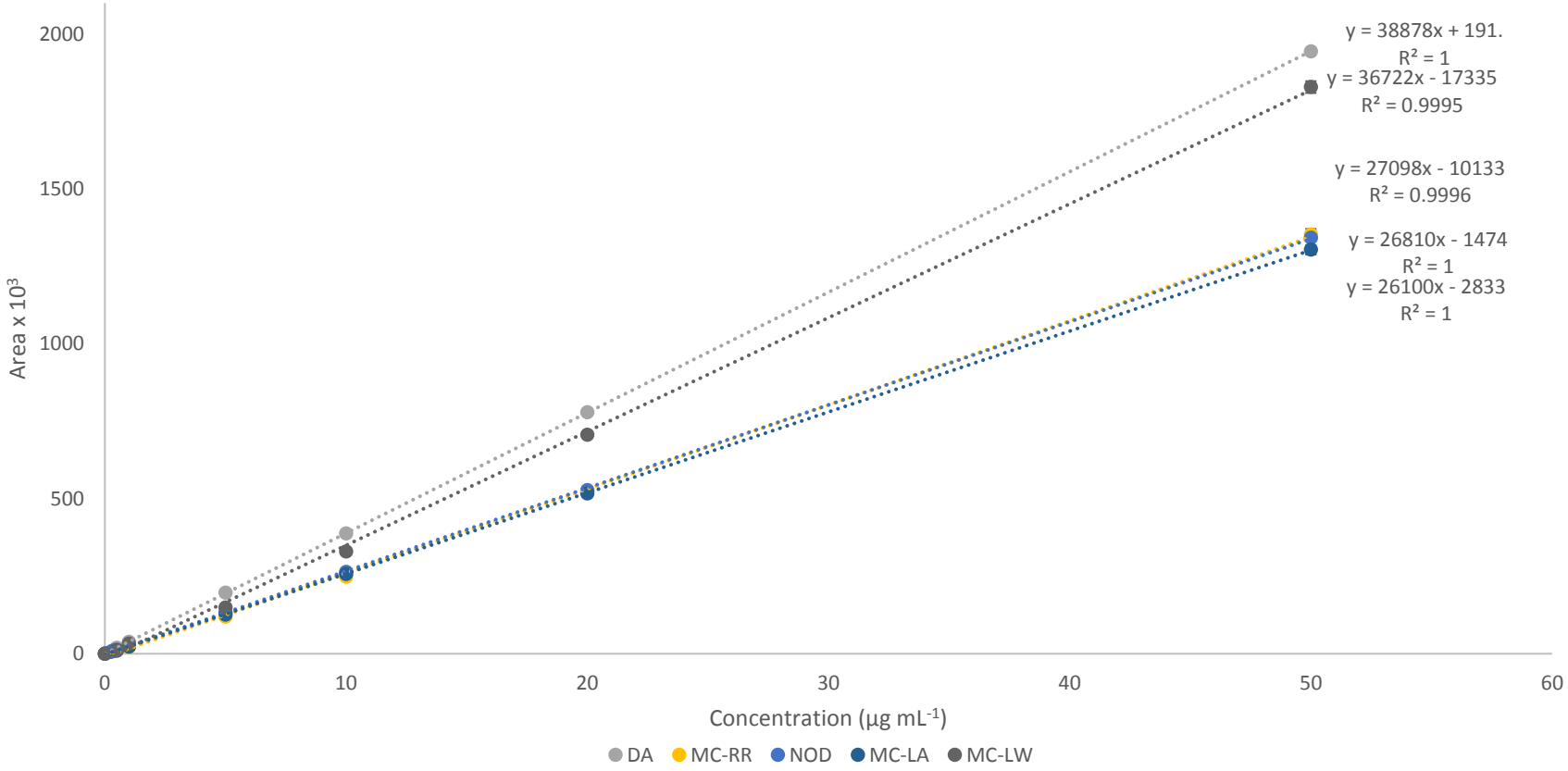

Figure 2. 21 Cyanotoxin calibration obtained after HPLC-PDA analysis monitored at 238 nm.

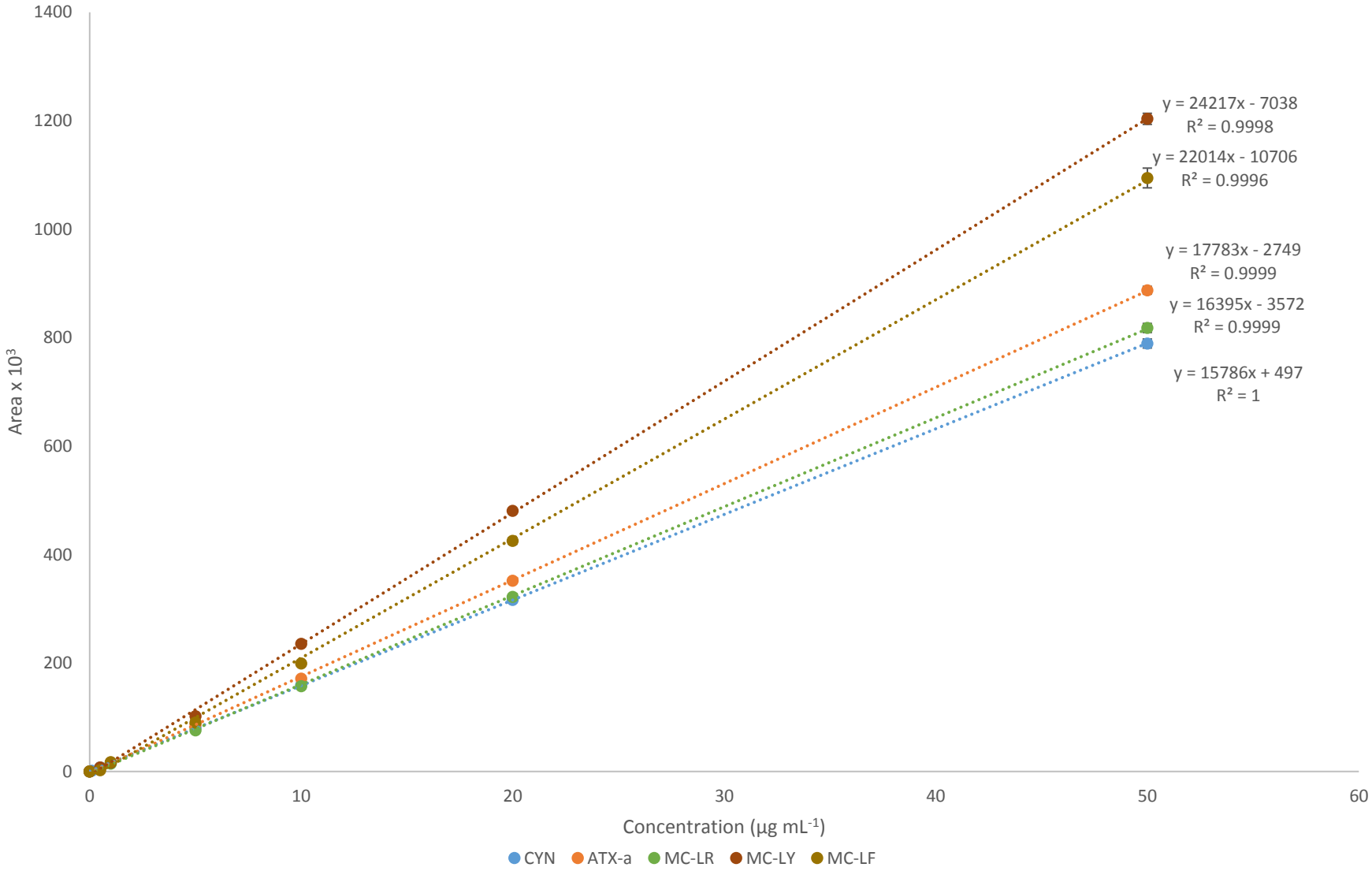

Figure 2. 22 Cyanotoxin calibration obtained after HPLC-PDA analysis monitored at 262 nm (CYN), 228 nm (ATX), and 238 nm (MCs).

2.5.2 Investigation of solvent suitability for toxin re-suspension

Table 2. 22 Average concentration detected after drying and re-suspending a mixed standard of DA and nine cyanotoxins. Analysis was performed utilising HPLC-PDA analysis (n=3)

Toxin	Prior to drying	water	MeOH	50% aqueous MeOH	MeCN	50% aqueous MeCN
CYN	11.01	11.03	11.68	11.12	0.00	11.50
ATX-a	11.35	11.06	10.53	11.15	8.71	10.81
DA	9.89	9.31	9.44	9.16	0.00	8.88
MC-RR	7.46	6.28	7.21	7.21	0.00	7.07
NOD	11.09	10.79	10.96	10.84	0.00	10.63
MC-LR	15.97	15.07	16.12	15.57	0.71	15.38
MC-LA	11.28	10.84	11.22	10.83	9.73	10.73
MC-LY	8.17	7.26	7.89	7.15	5.49	7.53
MC-LW	10.37	9.31	10.38	10.14	8.58	10.17
MC-LF	12.16	10.82	12.42	12.08	10.88	11.73

2.5.3 Analysis of cyanobacteria culture extracts by HPLC-UV and UPLC-QToF-MS

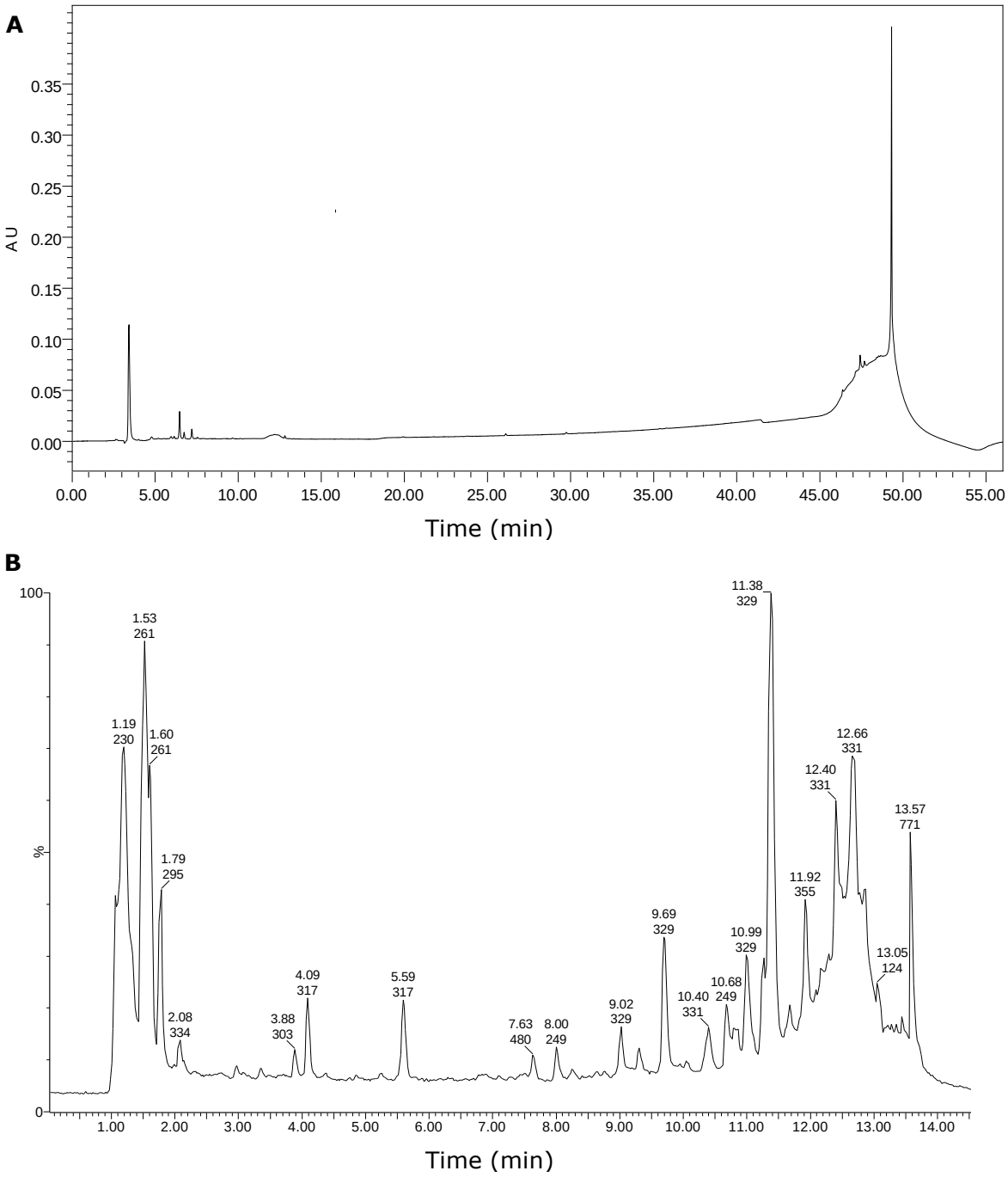

Figure 2. 23 HPLC-PDA chromatogram monitored at 238 nm (A), TIC UPLC-QToF-MS chromatogram of *A. flos-aquae* UTEX 1444 (B).

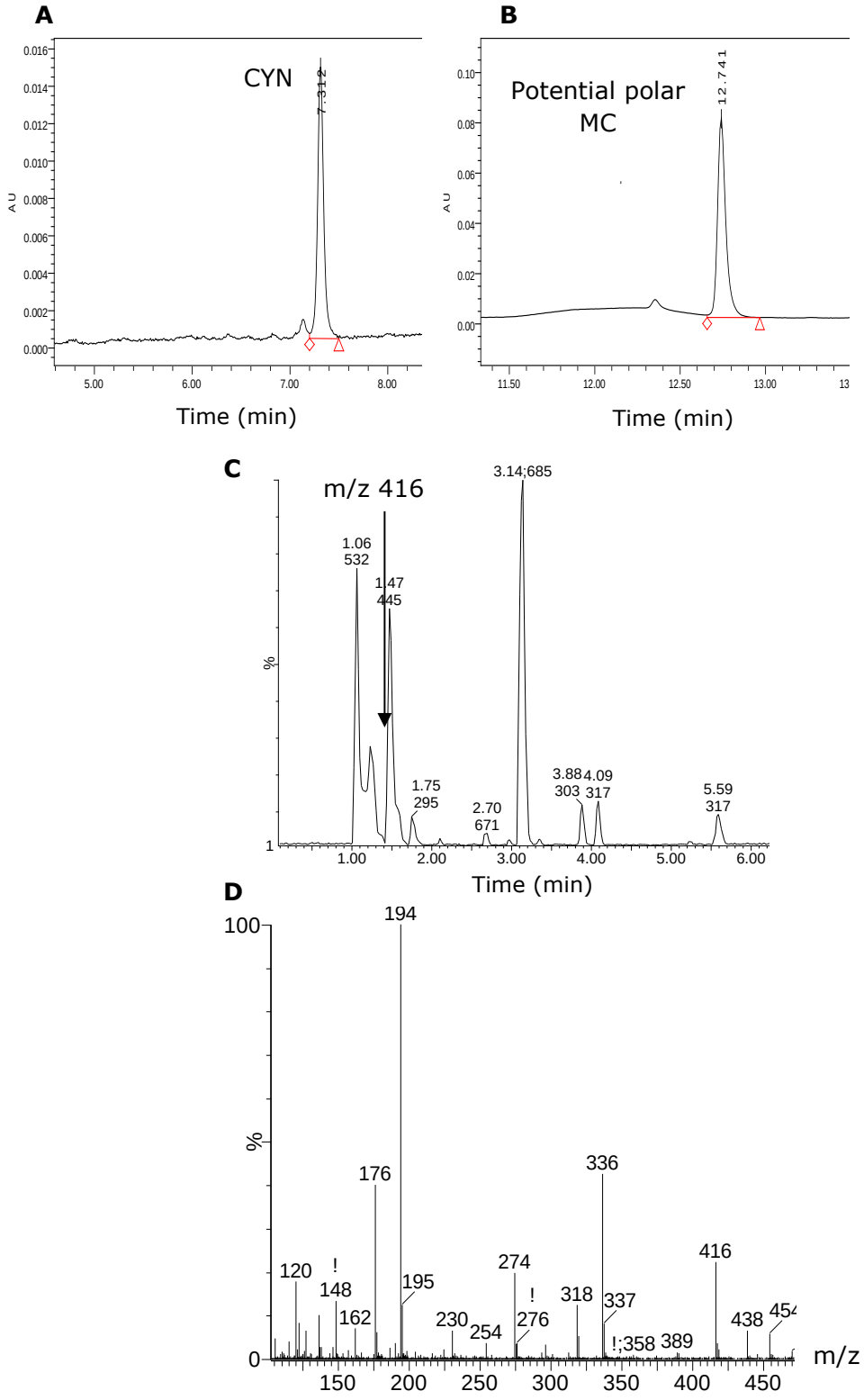

Figure 2. 24 HPLC-PDA chromatogram monitored at 262 nm (A), HPLC-PDA chromatogram monitored at 238 nm (B), TIC UPLC-QToF-MS chromatogram (C), Mass spectrum at t_R=1.23 min detected in *A. flos aquae* PCC 7905 obtained at high energy (D). The presence of a polar MC as suggested by HPLC-PDA analysis could not be confirmed following UPLC-QToF-MS analysis

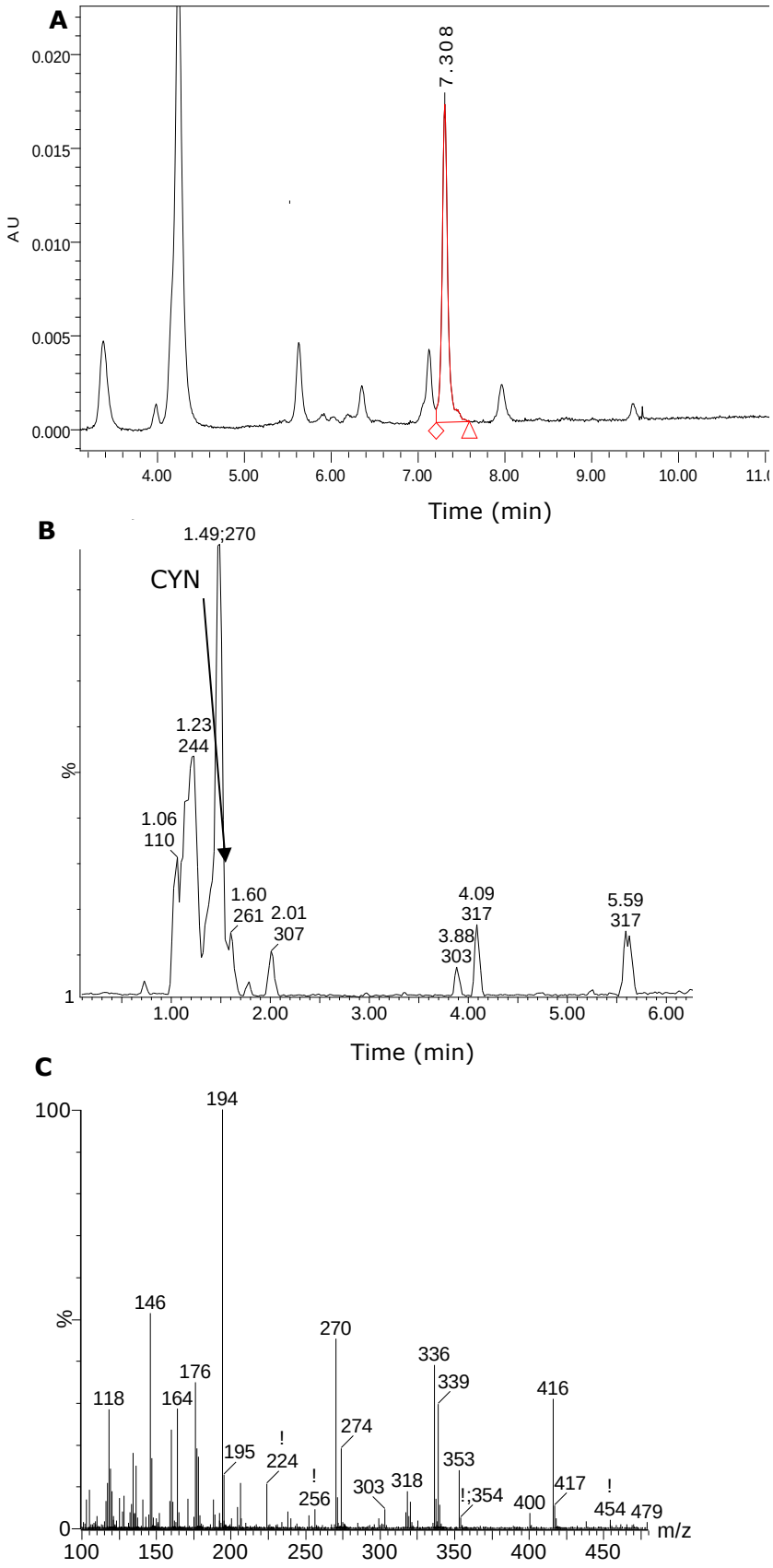

Figure 2. 25 HPLC-PDA chromatogram monitored at 262 nm (A). TIC UPLC-QToF-MS chromatogram (B) Mass spectrum (high energy) of CYN detected in *C. raciborskii* CYN (NIES-930) extract (C).

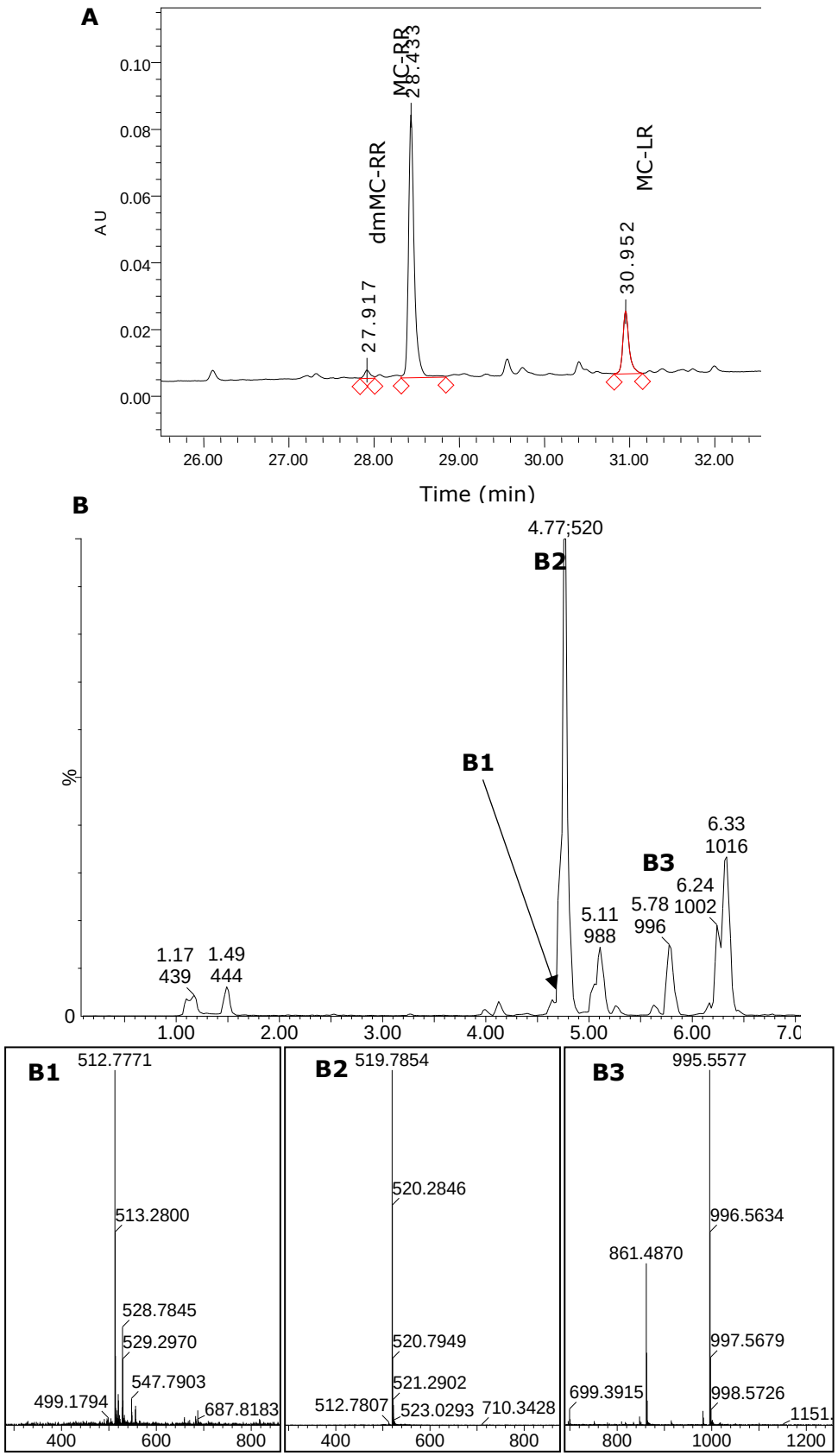

Figure 2. 26 HPLC-PDA chromatogram monitored at 238 nm (A). TIC UPLC-QToF-MS chromatogram (B) and Mass spectrum (base peak) of dmMC-RR (B1), MC-RR (B2), and MC-LR (B3) detected in *M. botrys* NIVA CYA 161/1.

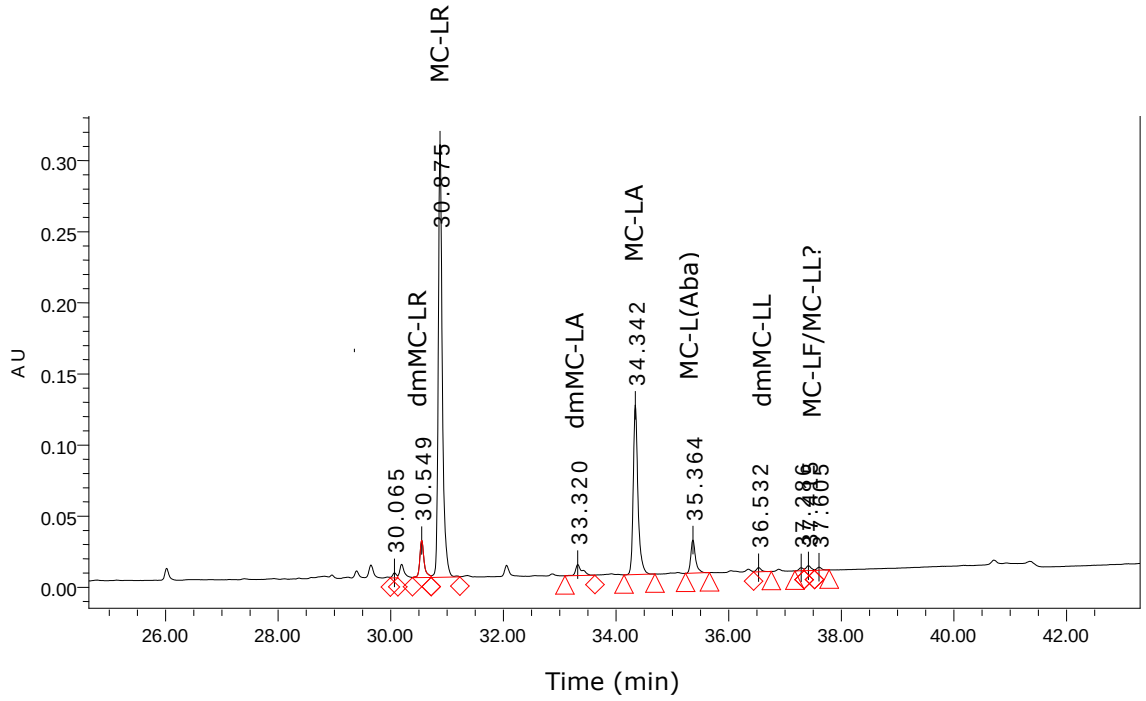

Figure 2. 27 HPLC-PDA chromatogram of *M. aeruginosa* UTEX B2666 monitored at 238 nm. The presence of demethylated MCs and MC-L(Aba) was tentatively suggested based on variants reported in the literature and shifts in t_R observed.

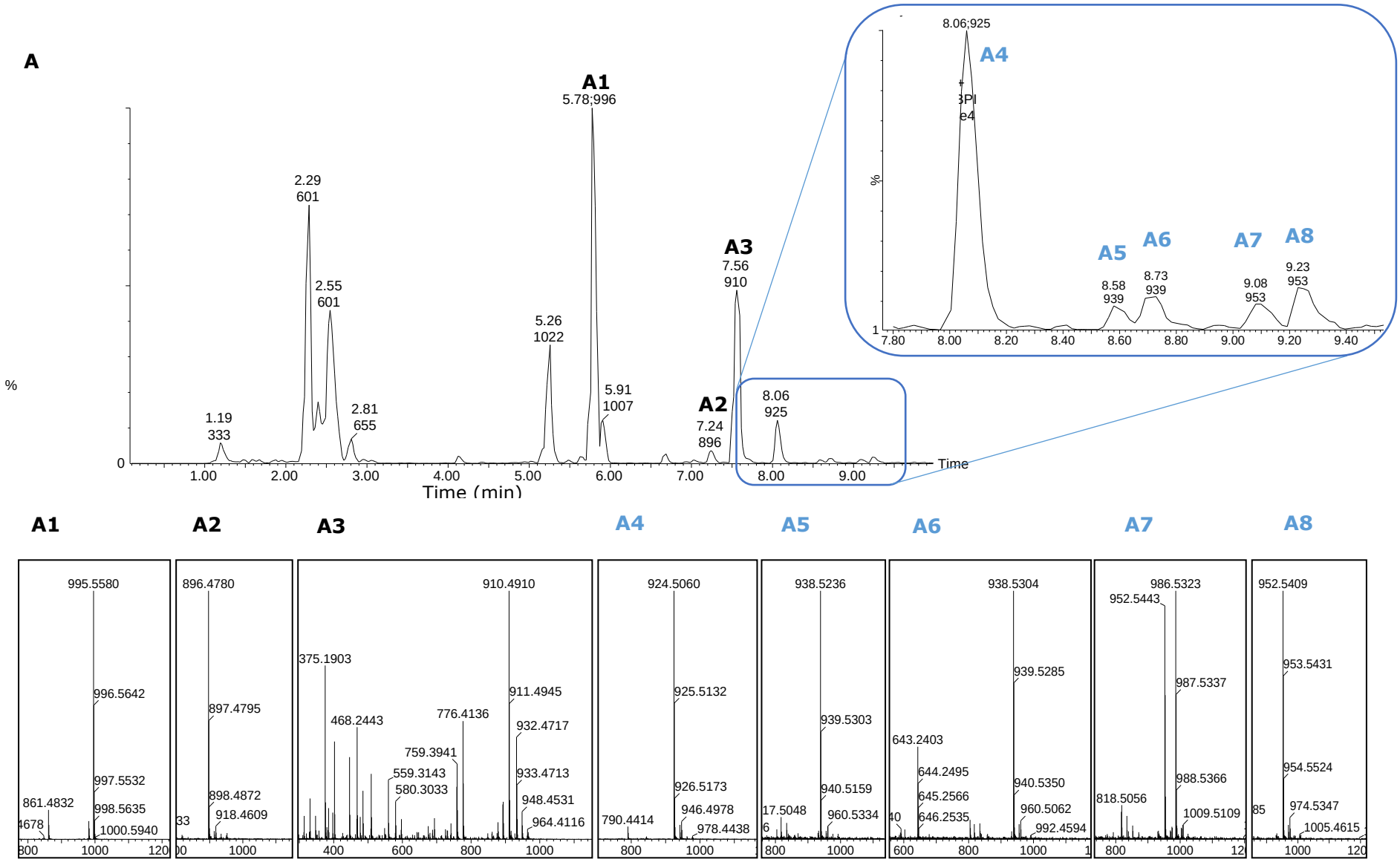

Figure 2. 28 TIC UPLC-QToF-MS chromatogram (A) and mass spectrum (base peaks) of MC-LR (A1), dmMC-LA (A2), MC-LA (A3),[D-Glu(OCH_3)^6, D-Asp^3] MC-L(Aba) (A4), dmMC-LL (A5, A6), co-eluting MC-LL and MC-LF (A7), and MC-LL (A8) detected in *M. aeruginosa* UTEX B2666.

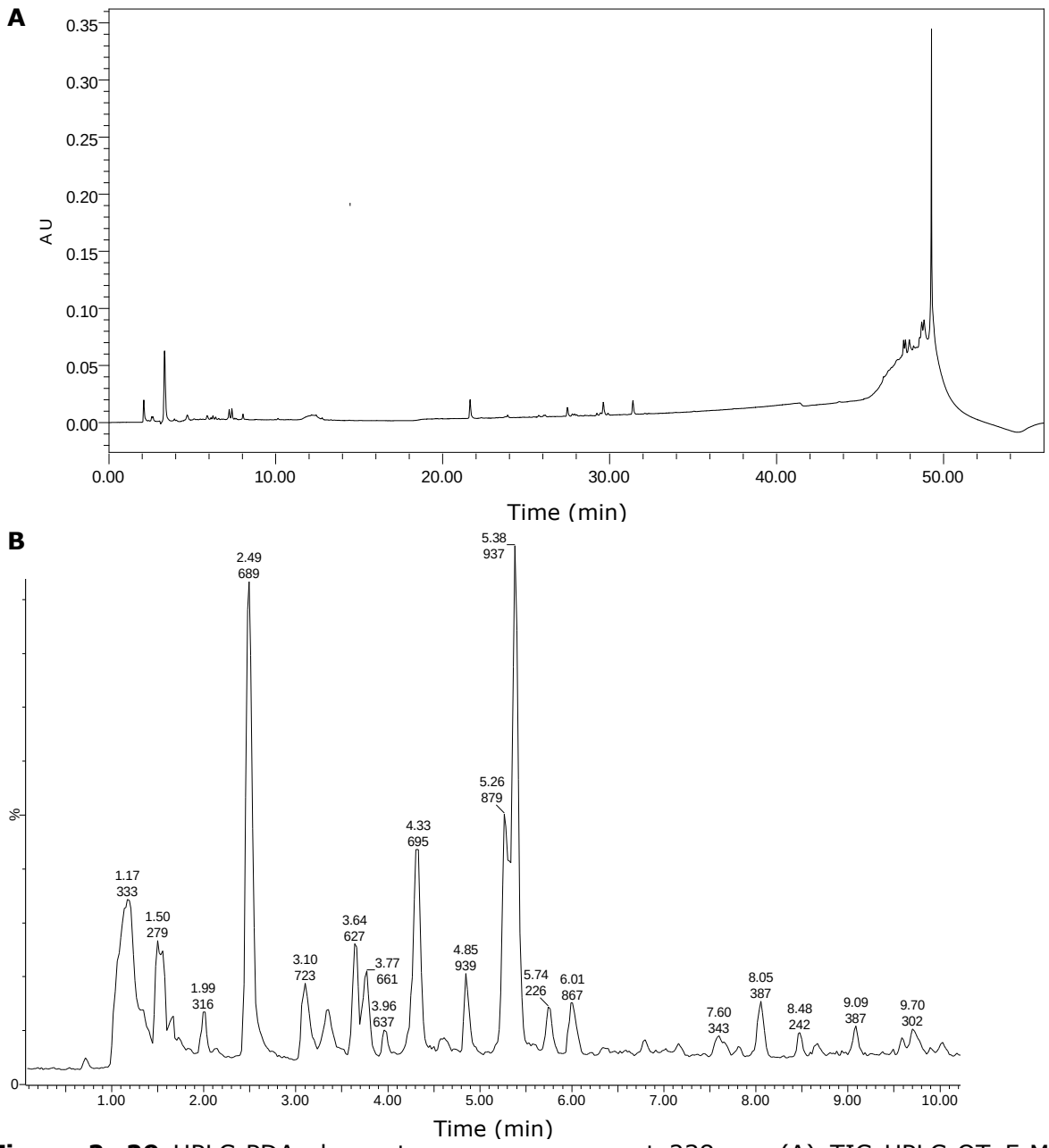

Figure 2. 29 HPLC-PDA chromatogram monitored at 238 nm (A) TIC UPLC-QToF-MS chromatogram of *M. aeruginosa* CCAP 1450/3 (B).

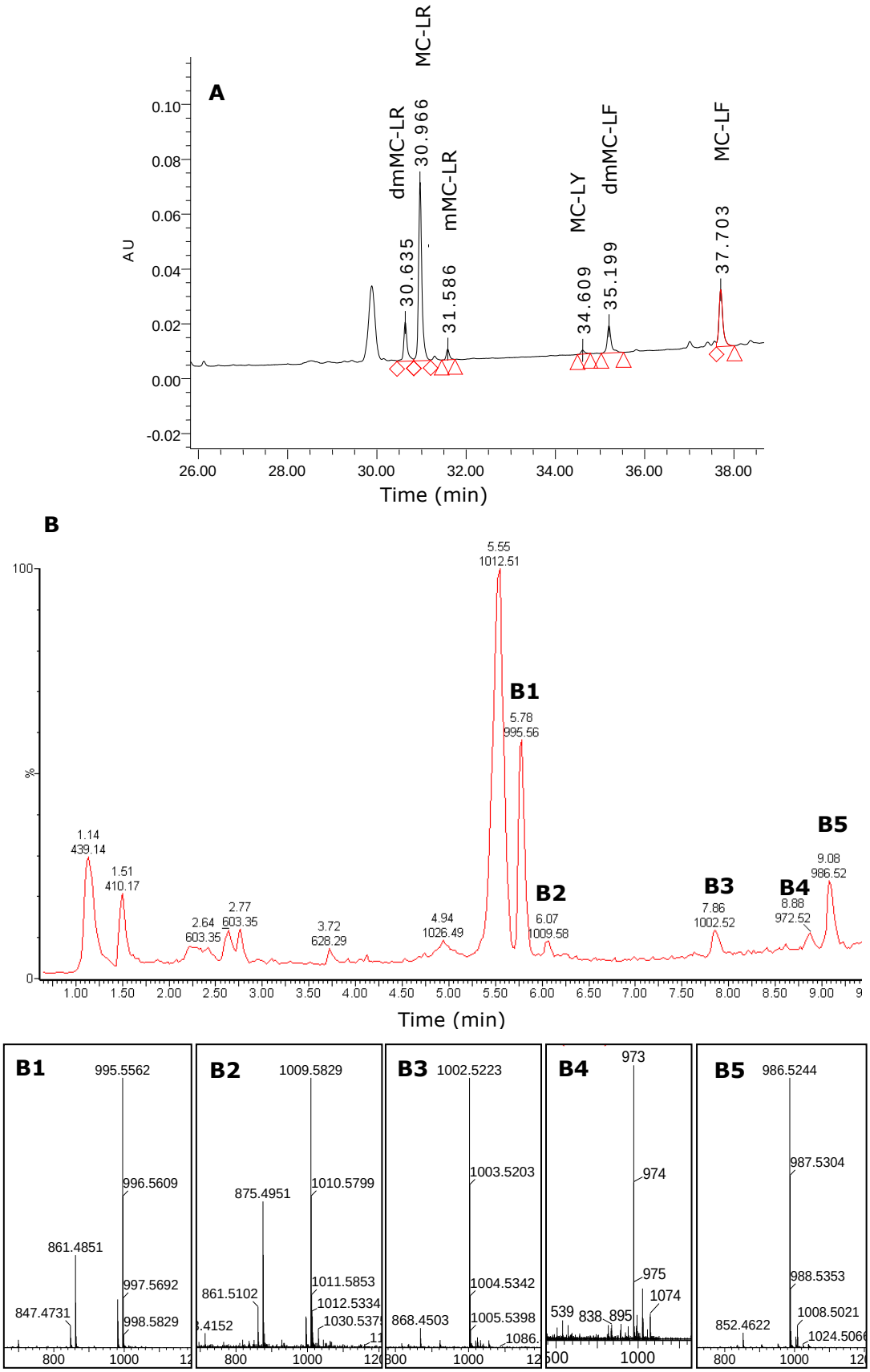

Figure 2. 30 HPLC-PDA chromatogram monitored at 238 nm A). TIC UPLC-QTOF-MS chromatogram (B) and mass spectrum (base peak) of MC-LR (B1), mMC-LR (B2), MC-LY (B3), dmMC-LF (B4) and MC-LF (B5) detected in *M. aeruginosa* CCAP 1450/10 extracts.

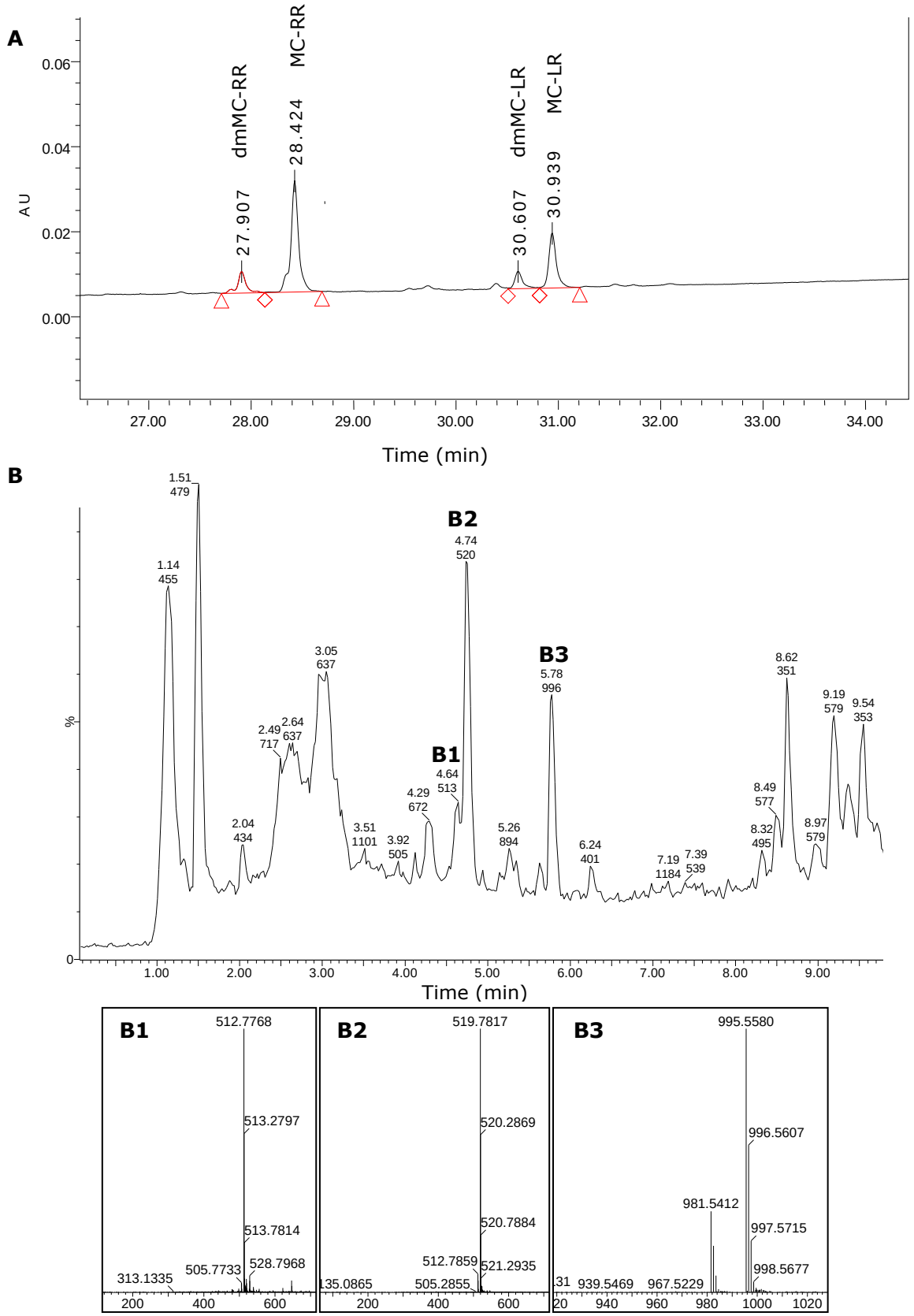

Figure 2. 31 HPLC-PDA chromatogram monitored at 238 nm (A) TIC UPLC-QToF-MS chromatogram (B) and mass spectrum (base peak) of dmMC-RR (B1), MC-RR (B2), and MC-LR (B3) detected *in M. aeruginosa* NIES 1071.

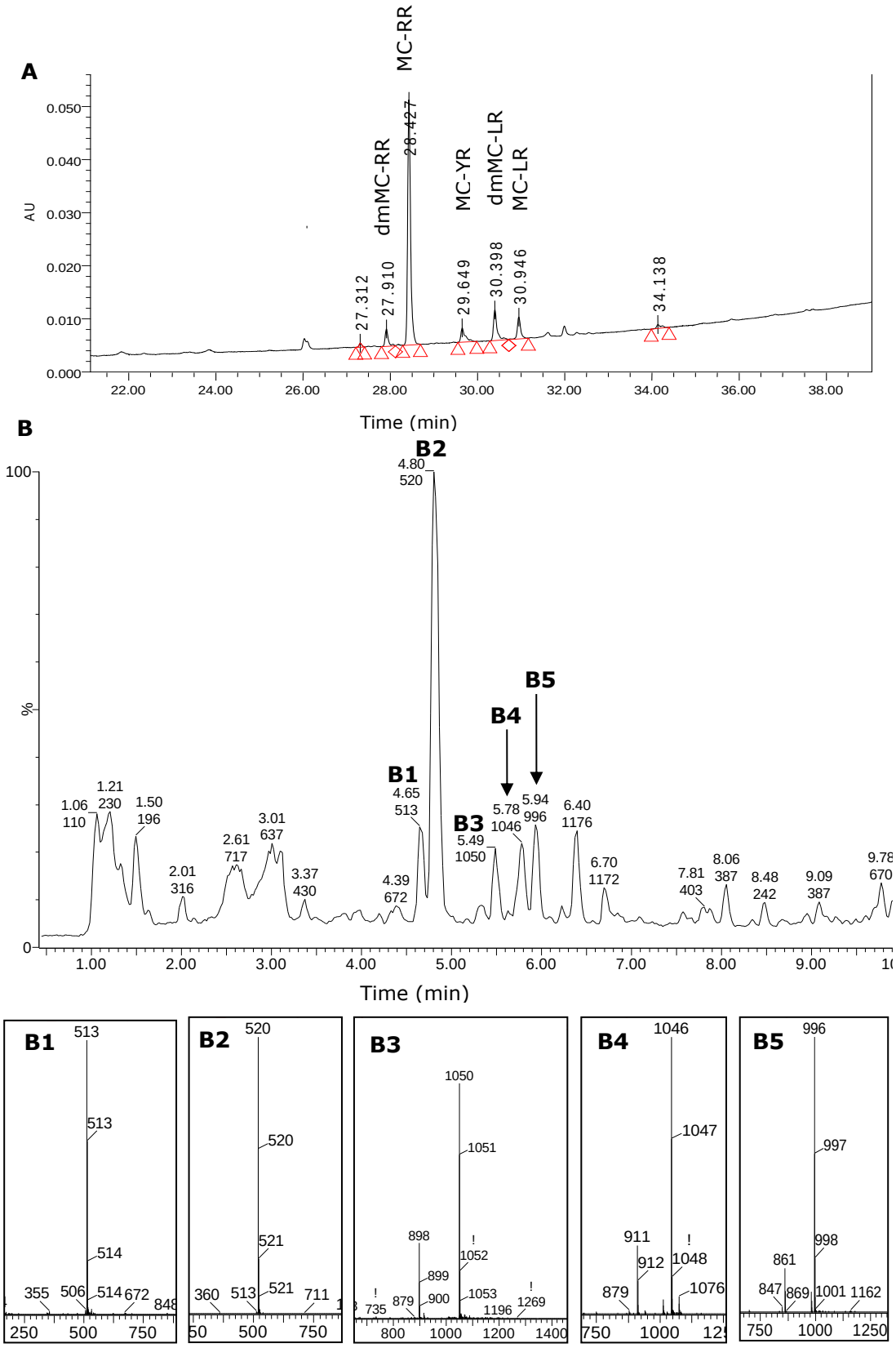

Figure 2. 32 HPLC-PDA chromatogram monitored at 238 nm (A) TIC UPLC-QToF-MS chromatogram (B) and mass spectra (base peak) of dmMC-RR (B1), MC-RR (B2), MC-YR (B3), and MC-LR (B4) detected *in M. aeruginosa* NIES 1099.

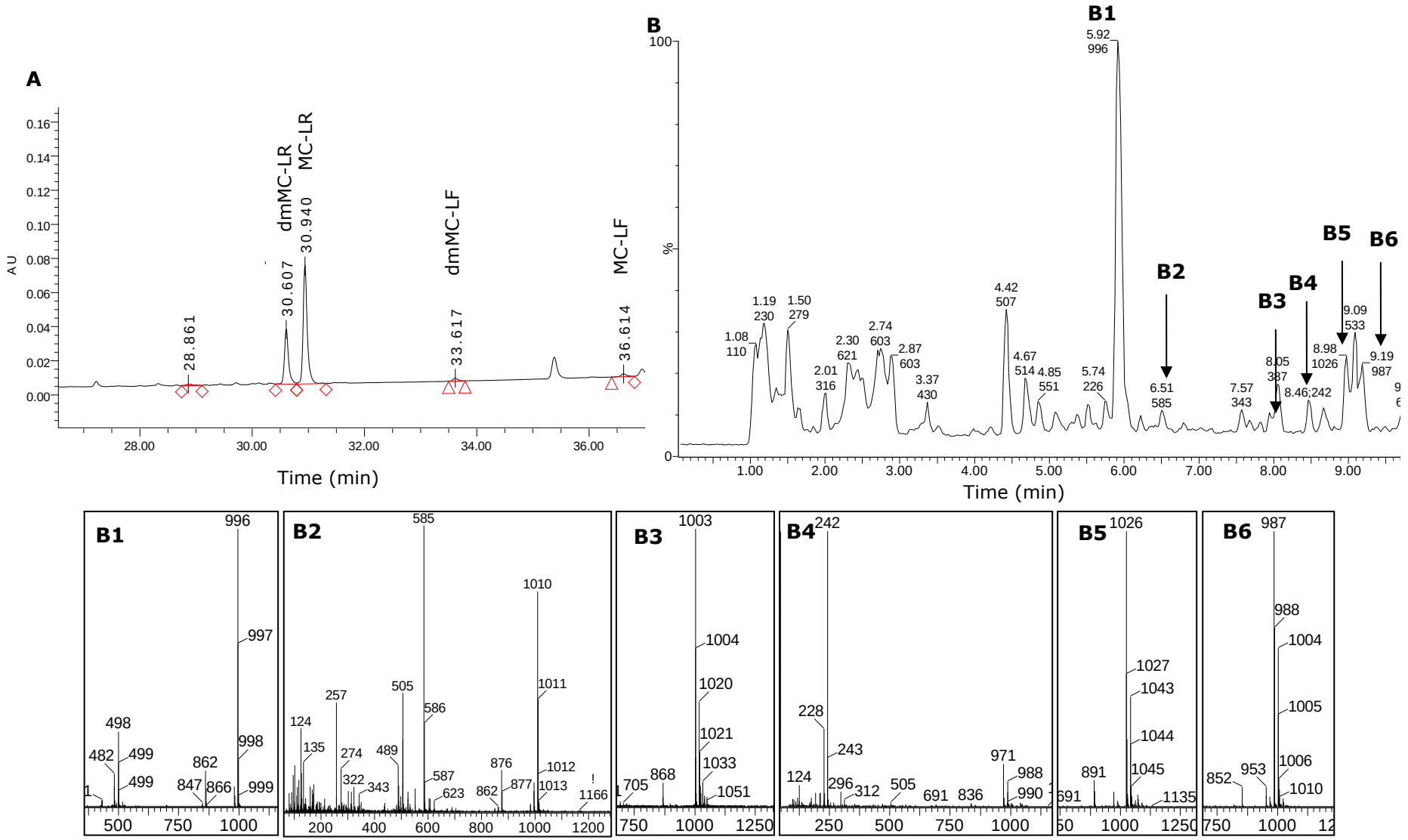

Figure 2. 33 HPLC-PDA chromatogram monitored at 238 nm (A) TIC UPLC-QToF-MS chromatogram (B) and mass spectra (base peak) of MC-LR (B1), mMC-LR (B2), MC-LY (B3), dmMC-LF (B4), MC-LW (B5) and MC-LF (B6) detected in *M. aeruginosa* PCC 7820.

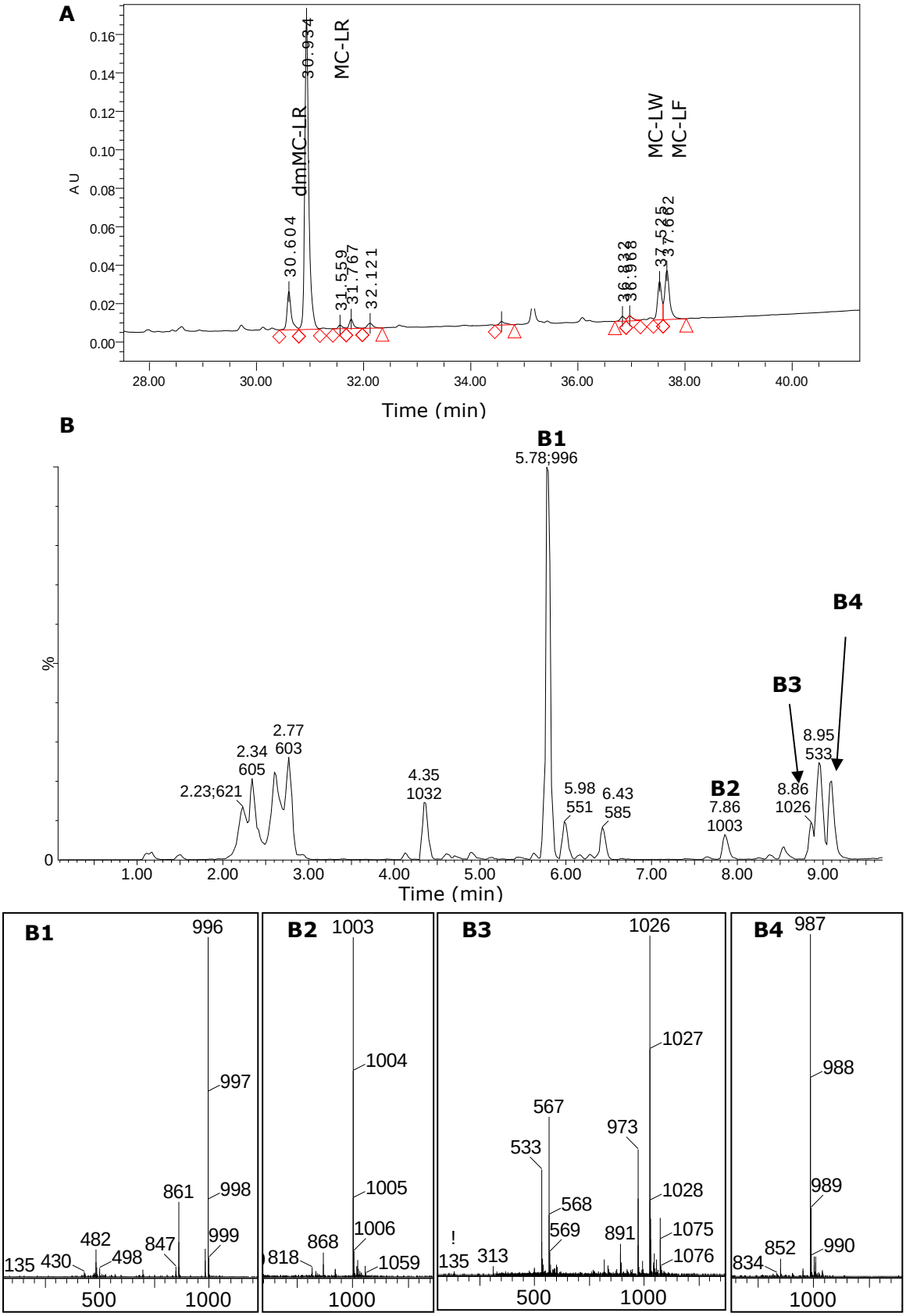

Figure 2. 34 HPLC-PDA chromatogram monitored at 238 nm (A), TIC UPLC-QToF-MS chromatogram B) and mass spectra (base peak) of MC-LR (B1), MC-LY (B2), MC-LW (B3) and MC-LF (B4) detected in *M. aeruginosa* PCC 7813.

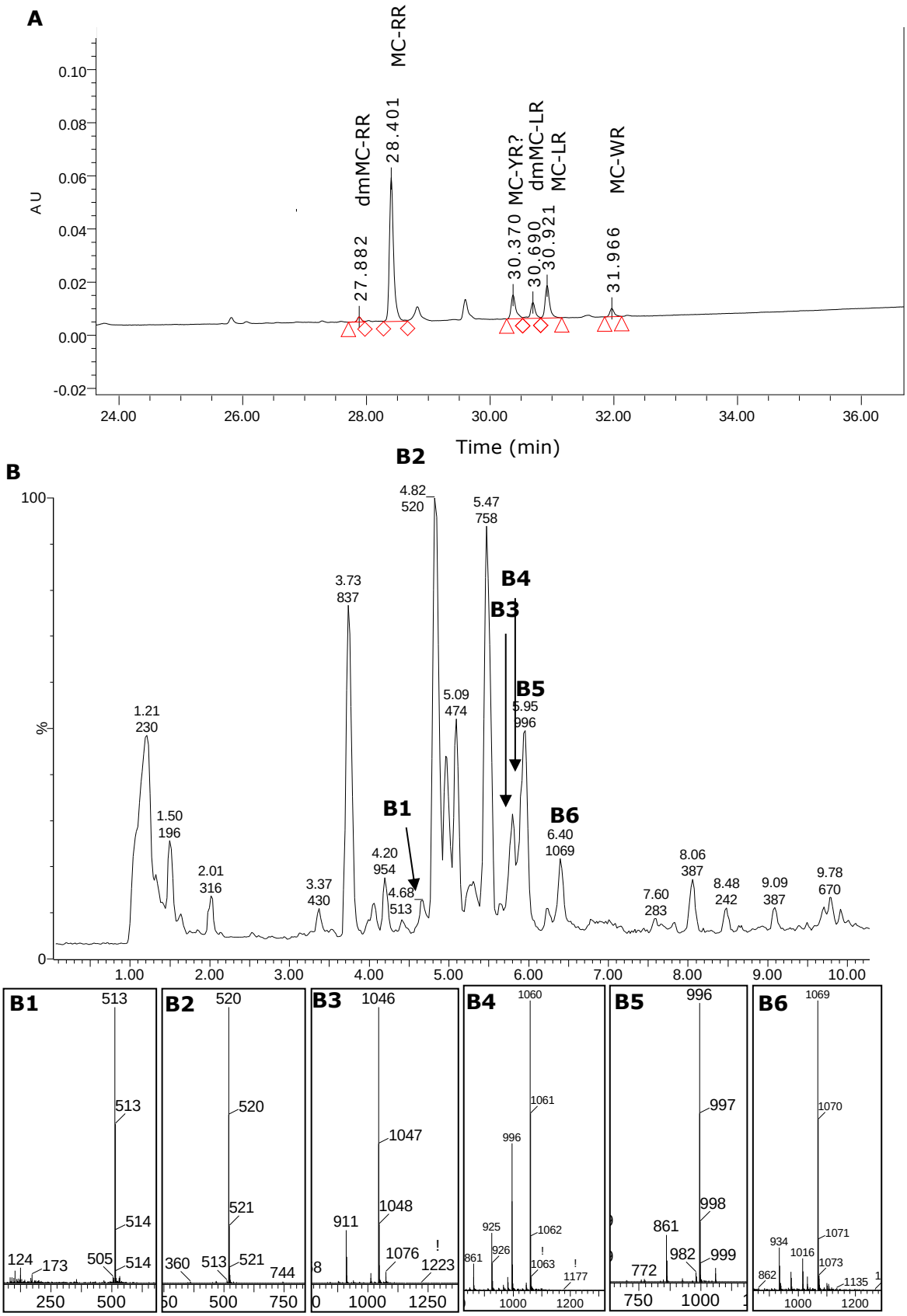

Figure 2. 35 HPLC-PDA chromatogram monitored at 238 nm (A) TIC UPLC-QToF-MS chromatogram (B) and mass spectrum (base peak) corresponding to dmMC-RR (B1), MC-RR (B2), MC-YR (B3), MC-HtyR (B4)MC-LR (B5), and MC-WR (B6) detected in *M. aeruginosa* SCIENTO.

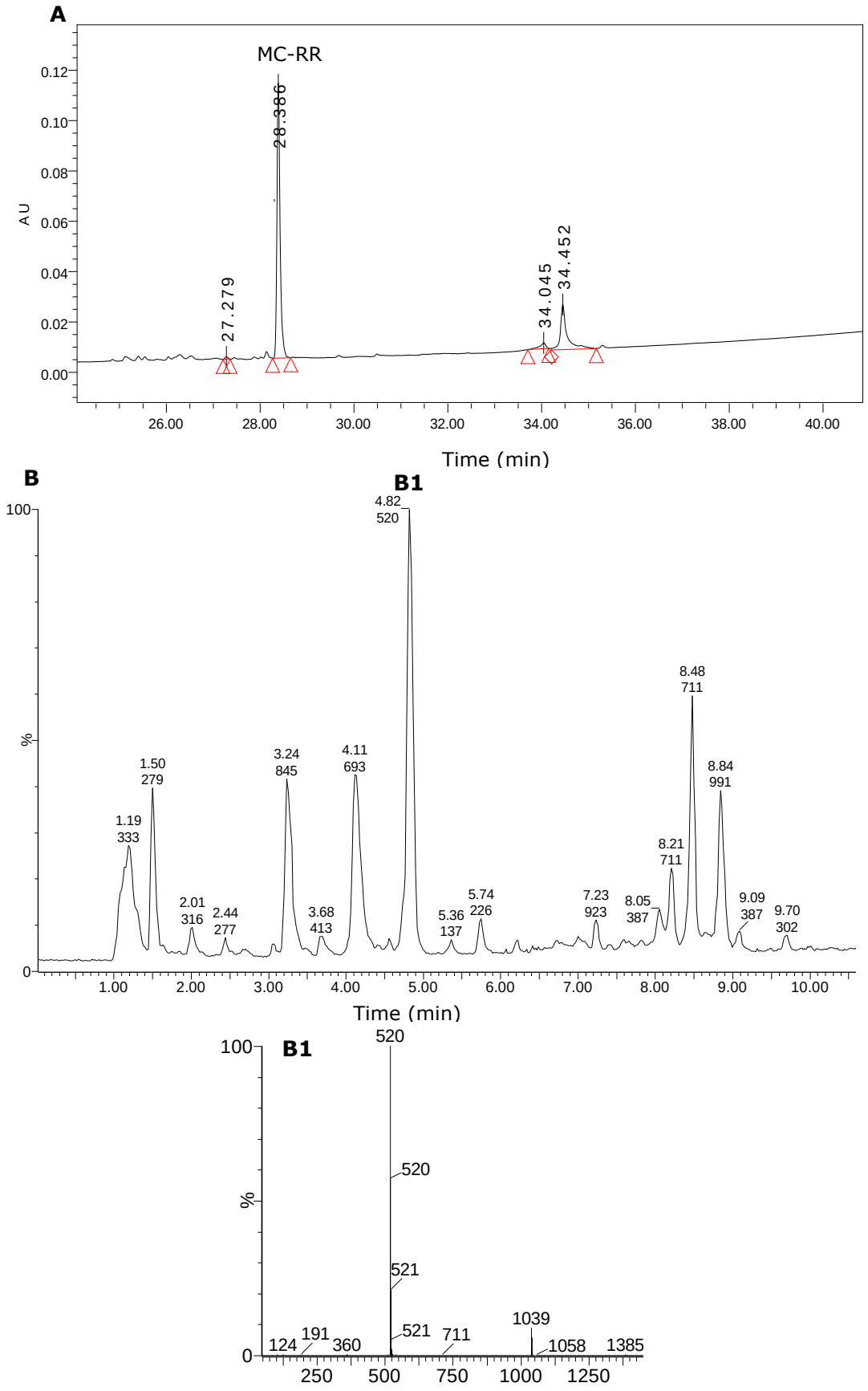

Figure 2. 36 HPLC-PDA chromatogram monitored at 238 nm (A) TIC UPLC-QToF-MS chromatogram (B) and mass spectrum corresponding to MC-RR (B1) detected in *M. aeruginosa* SAT.

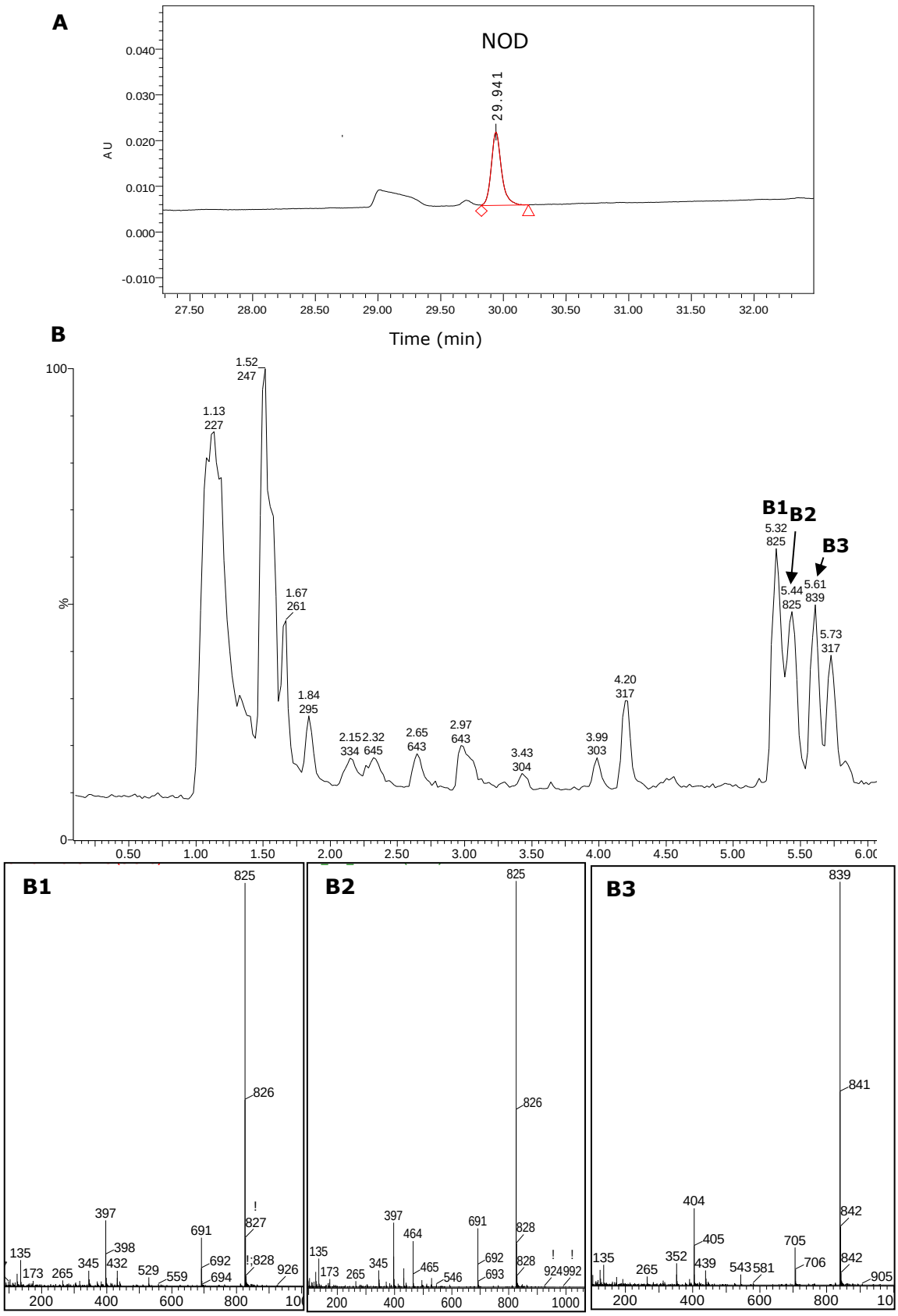

Figure 2. 37 HPLC-PDA chromatogram monitored at 238 nm (A) TIC UPLC-QToF-MS chromatogram (B) and mass spectrum corresponding to NOD (B1), NOD (B2), and mNOD (B3) detected in *N. sphaerocarpa* PCC 7804.

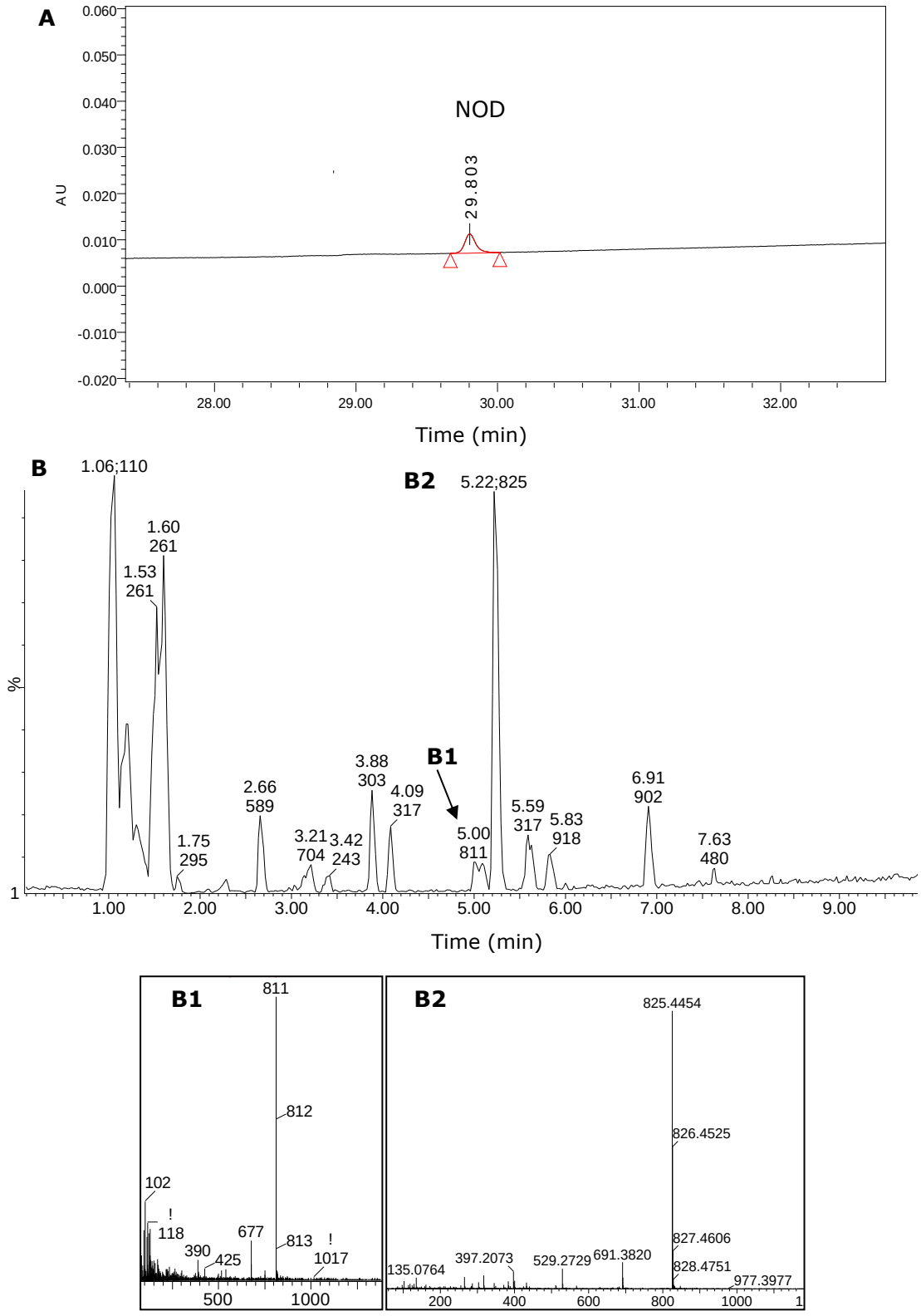

Figure 2. 38 HPLC-PDA chromatogram monitored at 238 nm (A) TIC UPLC-QToF-MS chromatogram (B) and mass spectrum corresponding to dmNOD (B1) and NOD (B2) *in N. spumigena* KAC66.

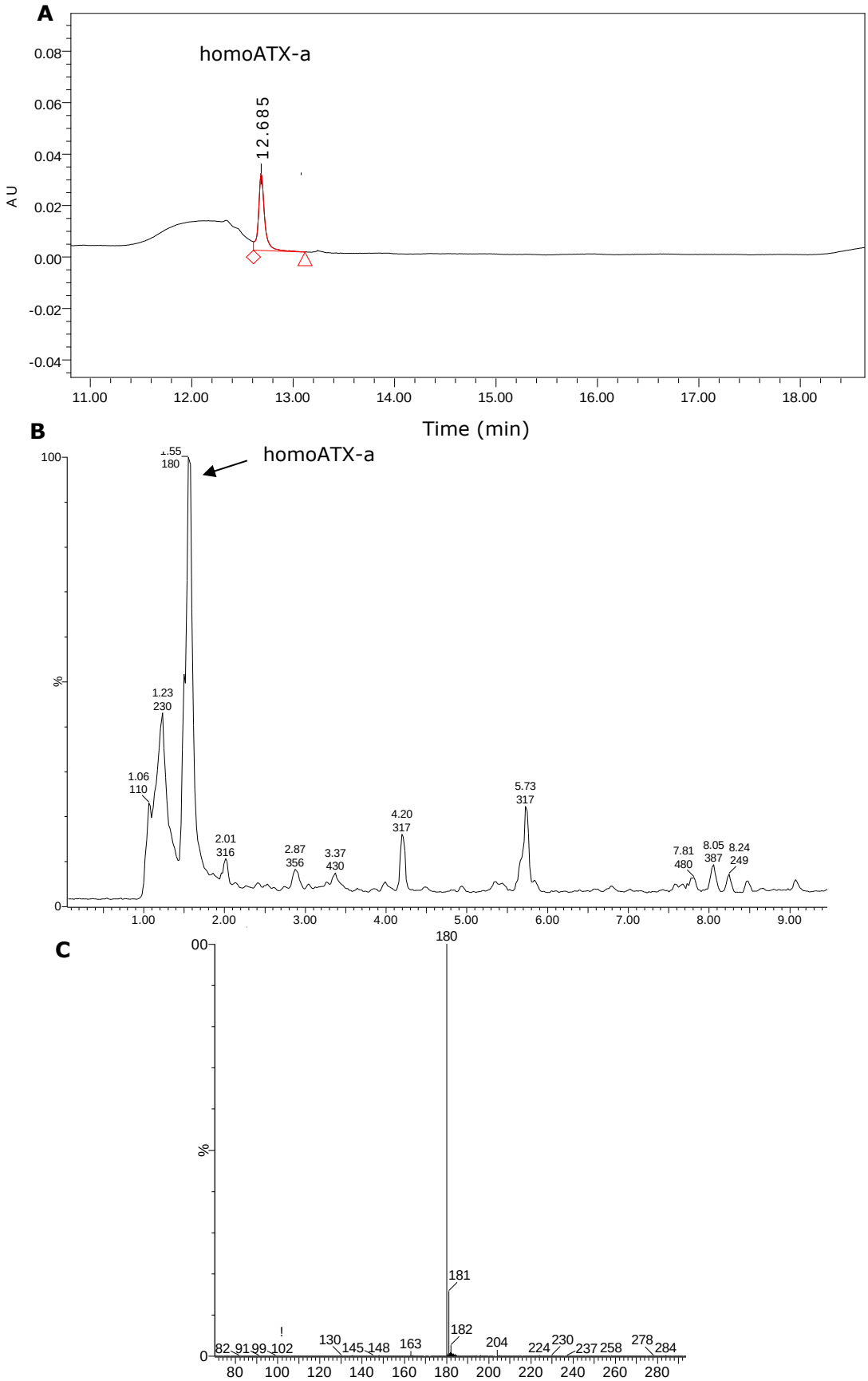

Figure 2. 39 HPLC-PDA chromatogram monitored at 228 nm (A) TIC UPLC-QToF-MS chromatogram (B) and mass spectrum corresponding to homoATX-a detected in *O. formosa* NIVA CYA 92 (PCC 10111; C).

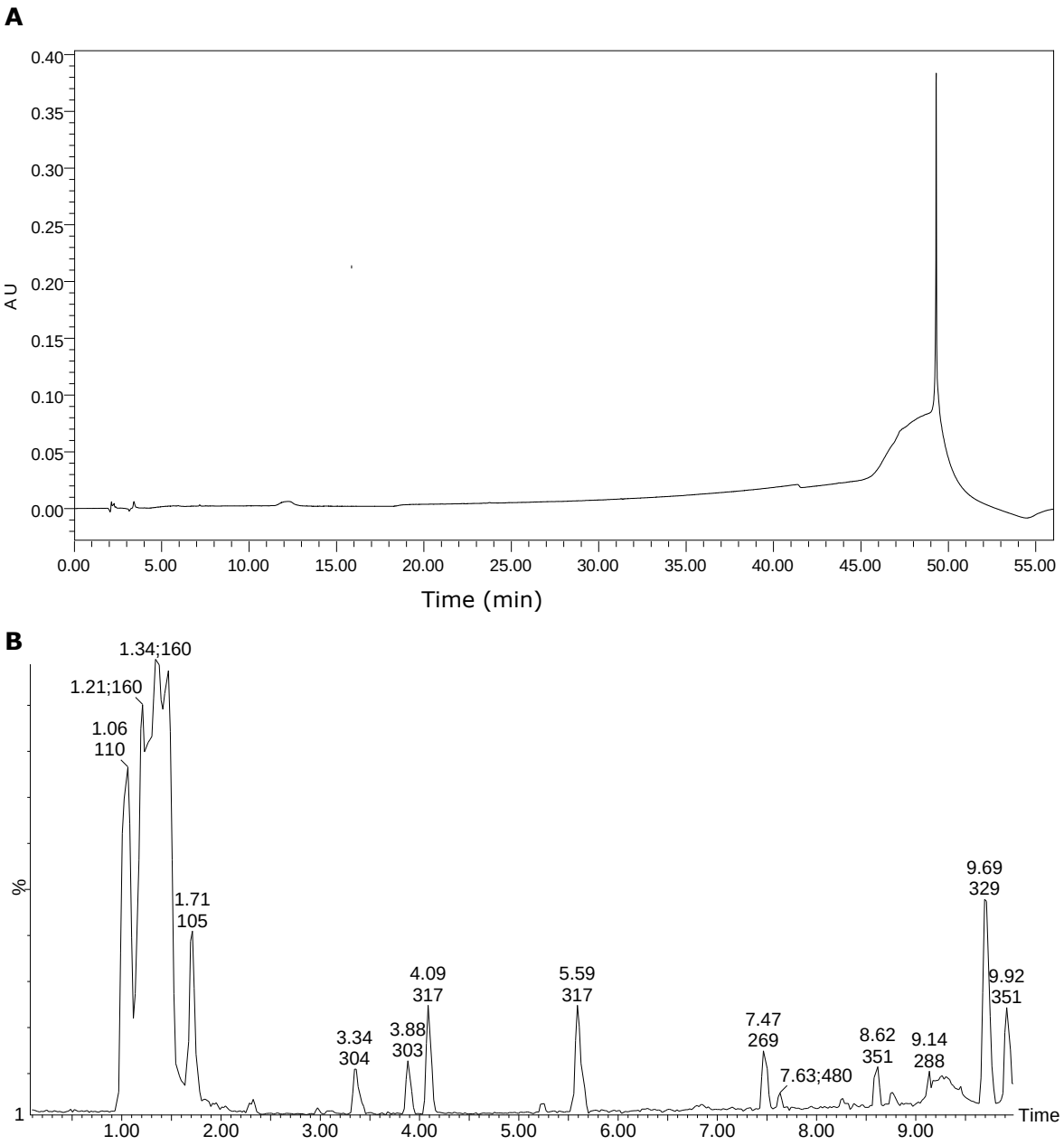

Figure 2. 40 HPLC-PDA chromatogram monitored at 238 nm (A) TIC UPLC-QToF-MS chromatogram of *P. autumnale* CCAP 1462/6 (B).

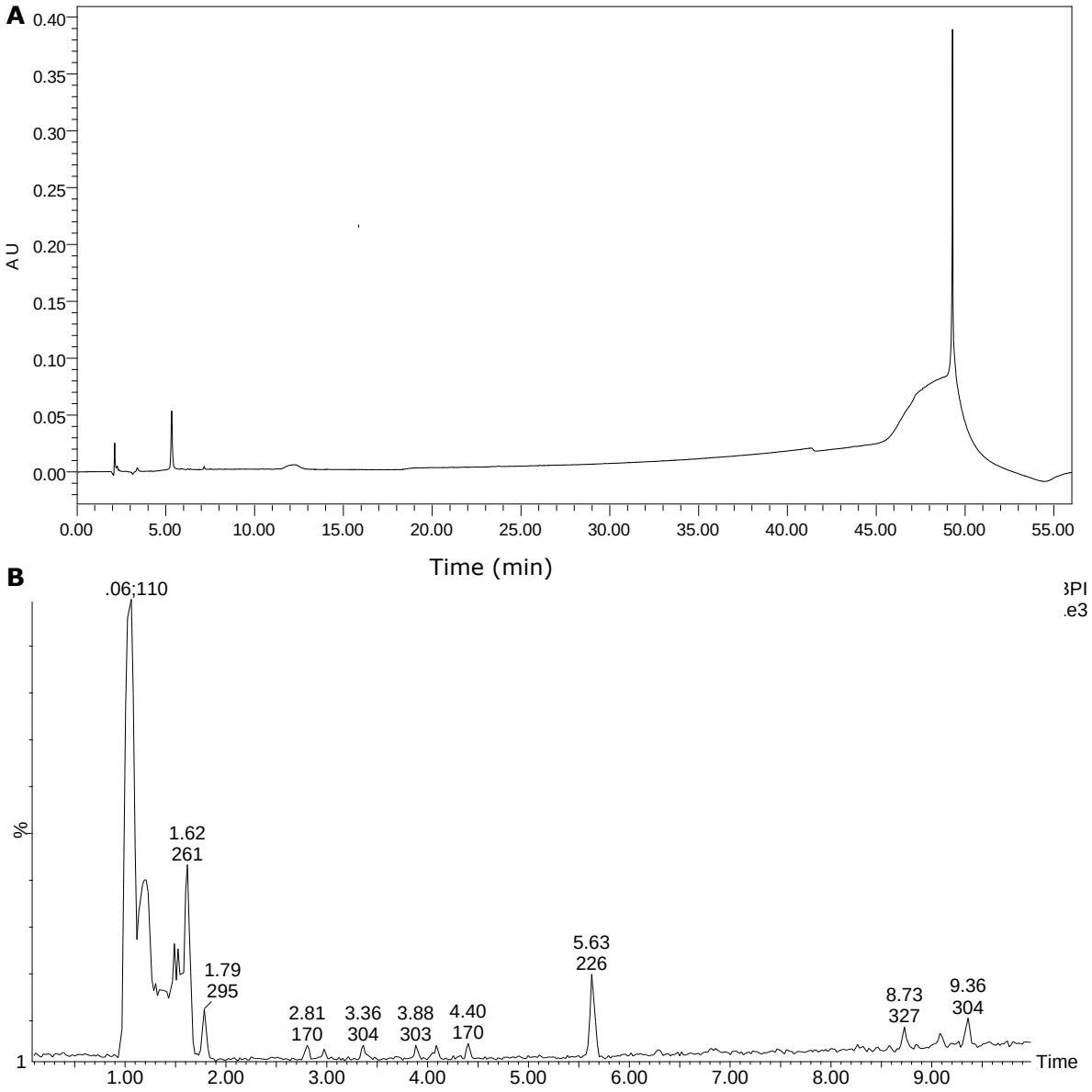

Figure 2. 41 HPLC-PDA chromatogram monitored at 238 nm (A) TIC UPLC-QToF-MS chromatogram of *P. autumnale* CCAP 1462/8 (B).

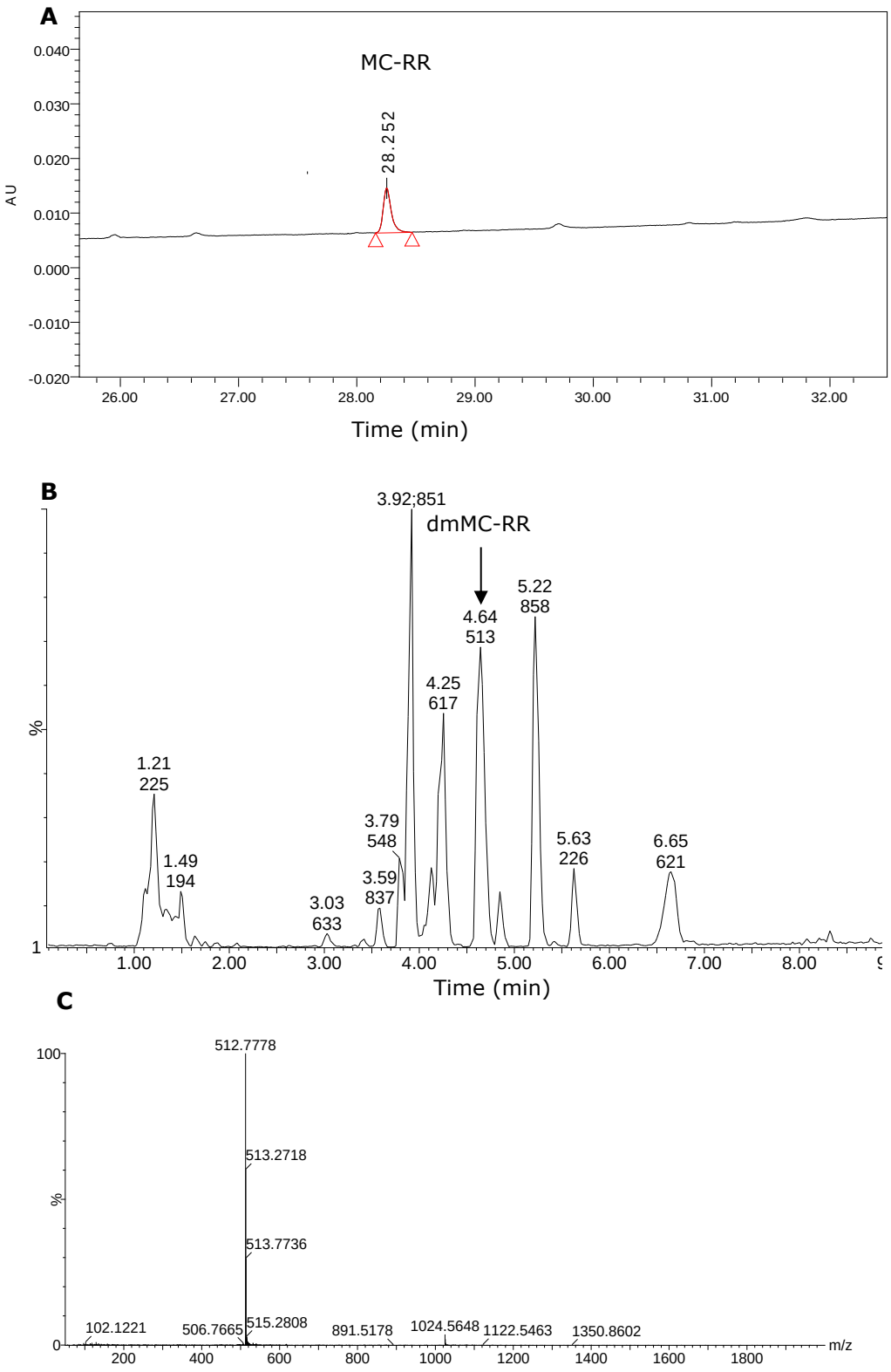

Figure 2. 42 HPLC-PDA chromatogram monitored at 238 nm (A) TIC UPLC-QToF-MS chromatogram (B) and mass spectrum corresponding to dmMC-RR detected *in P. agardhii* CCAP 1459/37 (NIVA CYA 18, PCC 7821; C).

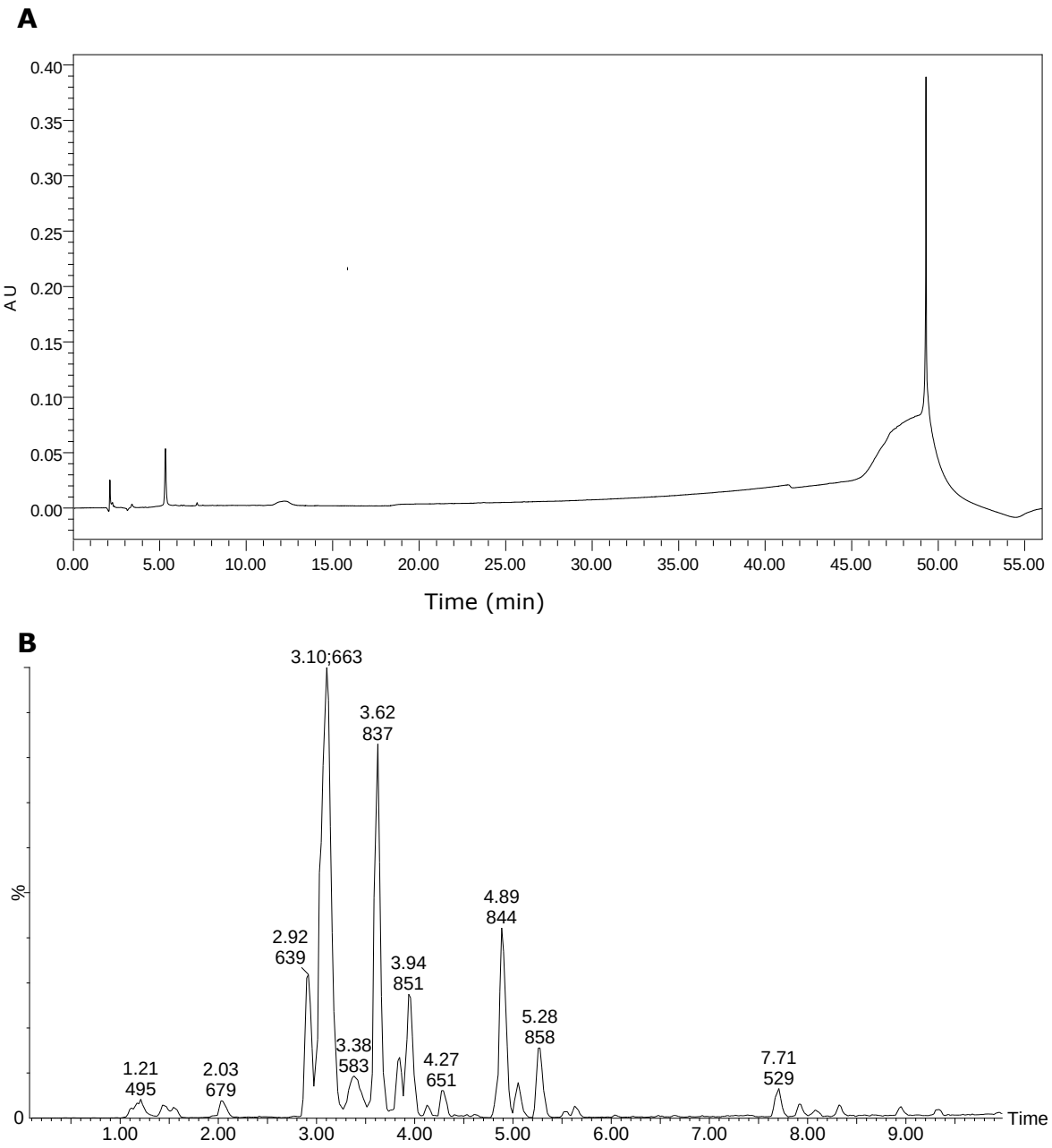

Figure 2. 43 HPLC-PDA chromatogram monitored at 238 nm (A) TIC UPLC-QToF-MS chromatogram of *P. agardhii* NIVA CYA 29.

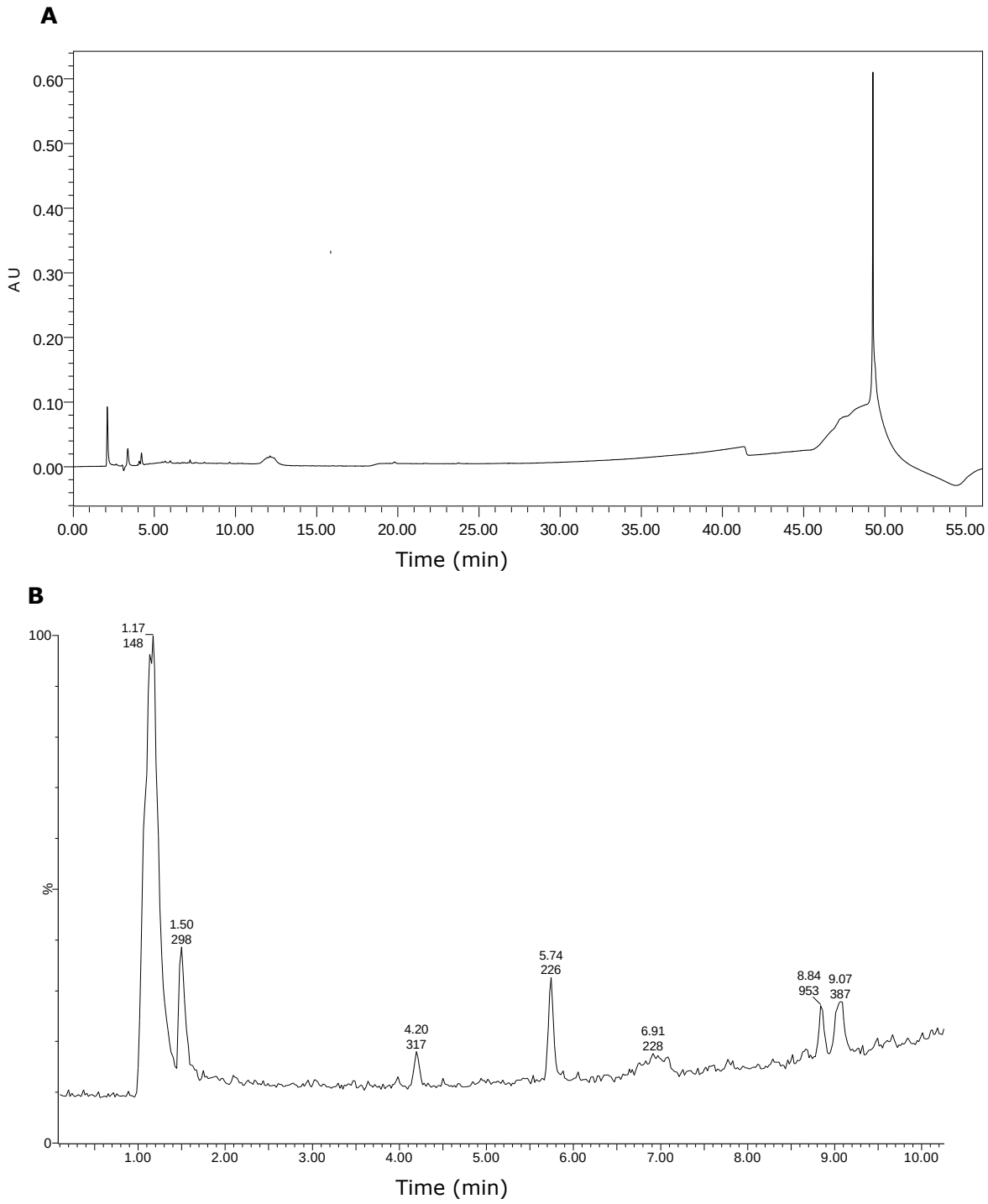

Figure 2. 44 HPLC-PDA chromatogram monitored at 238 nm (A) TIC UPLC-QToF-MS chromatogram of *P. rubescence* PCC 10608 (B).

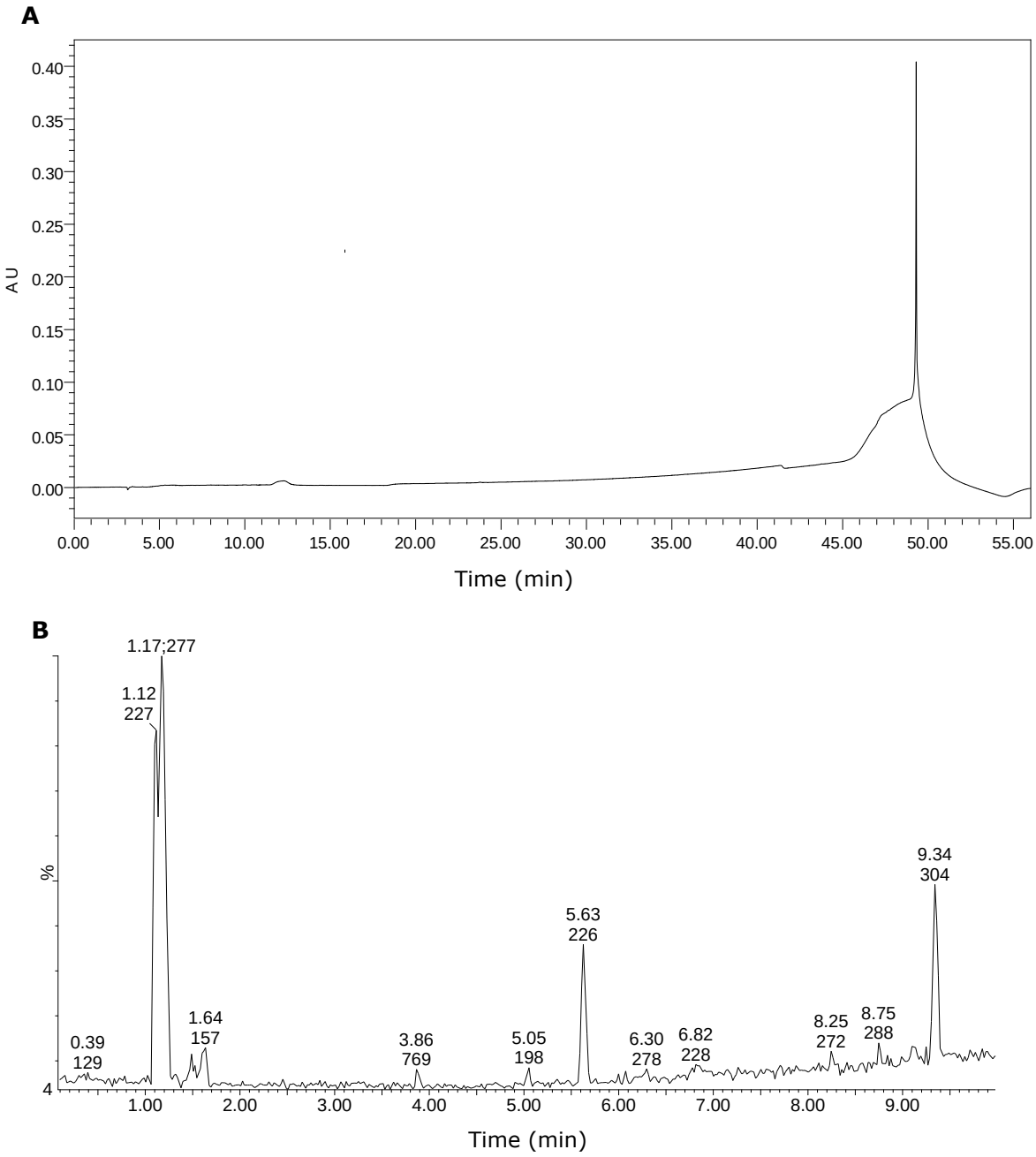

Figure 2. 45 HPLC-PDA chromatogram monitored at 238 nm (A) TIC UPLC-QToF-MS chromatogram of the Colombo Lake samples (B).

CHAPTER 3

The accumulation and depuration of nodularins in the edible blue mussel *Mytilus edulis* after seven days exposure to *Nodularia spumigena* KAC66

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3.1 Introduction

The toxic potential of *Nodularia* has long been recognised. The first report of animal poisoning caused by this cyanobacterium dates back to 1878 (Francis 1878). A scum formation was observed on Lake Alexandrina (Australia) which has since been attributed to the cyanotoxin NOD. Manubolu *et al.* (2014) investigated potential NOD exposure of cattle grazing in coastal areas of the Baltic Sea. During the grazing period (May to October) NOD concentration in water ranged from 0.24 – 0.64 (highest mean 0.92 ± 0.19 µg MC-LReq) and 0.12 – 7.45 µg MC-LReq based on PPIA and ELISA analysis, respectively. However, presence of NOD was only confirmed for one of the four sample sites following LC-MS analysis (Manubolu *et al.,* 2014). In addition, cattle serum from two sampling sites were positive following ELISA analysis: 0.14 – 0.125 µg MC-LReq. However, LC-MS analysis could not confirm the presence of neither MC-LR, MC-RR, MC-YR, nor NOD, which suggested that other Adda containing molecules, potentially metabolites of NOD and/or MCs, were present in blood samples (Manubolu *et al.,* 2014).

As previously detailed bivalves can survive the accumulation of significant NOD levels in their tissue (section 1.4), hence the indirect exposure to NOD and other cyanotoxins via food sources can represent a significant route of intoxication. High accumulation of NOD has been reported in bivalves associated to their filter feeding nature and as they are part of the human diet they could become significant cyanotoxin vectors (Kankaanpää *et al*., 2007; Strogyloudi *et al*., 2006; Svensen *et al*., 2005; Lehtonen *et al*., 2003; Karlson and Mozūraitis 2011; Van Buynder *et al*., 2001). The toxicity of extracts of *M. edulis* during *N. spumigena* blooms in an Australian estuary was demonstrated after i.p. injection in mice (Falconer *et al.,* 1992). Gut contents of mussels were analysed and *N. spumigena* filaments were identified indicating that the cyanobacterium was the causative agent of the observed toxicity. In addition, carbon and nitrogen labelled *N. spumigena* was utilised to demonstrate ingestion by mussels and clams (Karlson and Mozuraitis 2011). Similarly, Wood *et al.* (2012) demonstrated the ingestion of ^{13}C labelled benthic cyanobacteria by crayfish.

The trophic transfer of NOD to higher predators has been demonstrated in mysid shrimp (*Mysis relicta*) and sticklebacks (*Gasterosteus aculeatus; E*ngström-Öst

et al., 2002). Copepods (*Eurytemora affinis*) fed on *N. spumigena* AV1 were utilised as a food source for shrimp and sticklebacks. Based on ELISA analysis up to ~ 7 µg g^{-1} and 1.5 µg g^{-1} NOD (estimated from graph) was found in shrimp and sticklebacks, respectively. These results therefore showed that shrimp accumulated more than four times higher NOD levels compared to sticklebacks, which illustrated the varying uptake dynamics in different organisms.

In addition to mussel flesh itself the excreted faeces can also represent a cyanotoxin exposure route, especially for benthic organisms. Excretion of cyanotoxins within faeces has been demonstrated in *M. edulis* (Svensen *et al*., 2005), in *M. galloprovincialis* (Amorim and Vasconcelos 1999), and in silver carp (Xie *et al*., 2004). Moreover, mussels re-ingested the toxin when exposed to faeces containing NOD (Svensen *et al*., 2005).

For the present study the edible blue mussel species *M. edulis* was chosen as it is part of the human diet and the most commonly-consumed bivalve mollusc. During 2012 26,000 tonnes of mussels were harvested within the UK, representing £27 million assigned value of production (Ellis *et al*., 2015). Currently, in accordance with EU regulations 853/2004 and 786/2013, monitoring is undertaken to ensure the safety of seafood produced within the UK of which mussels account for 65-70% (private communication Dr Andrew Turner). However, this monitoring only includes regulated marine toxins and not those produced by cyanobacteria, except for STXs (section 2.1). Therefore, the aim of the present study was to assess the accumulation and depuration of NOD in *M. edulis* after seven day exposure to *N. spumigena* KAC66, which was chosen due to its ability to tolerate the salt water environment in which the mussels are found. In addition, based on the NOD exposure concentrations, the recovery within mussel flesh and faeces was determined to provide vital information for the assessment of NOD exposure risks for other organisms including humans.

3.2 Materials and Methods

3.2.1 Chemicals

MeCN, MeOH, and formic acid were all HPLC gradient-grade (ThermoFisher, UK). Water, purified to 18 MΩ (Purelab flex, Veolia Water Technologies, UK), was utilised for mobile phase preparation and sample processing. Standards of NOD and LNOD were purified from *N. spumigena* KAC66 cultures (Liu *et al*., 2005). Chemicals for BG-11 preparation were supplied by FisherScientific (Leicestershire, UK).

3.2.2 UPLC-QToF-MS/PDA analysis and optimisation

The UPLC-QToF-MS analysis was performed as described in Chapter 2 (section 2.2.5.2). In addition, a photodiode array (ACQUITY UPLC PDA) detector was utilised to monitor the UPLC eluent from 200-400 nm with a resolution of 1.2 nm.

3.2.2.1 Quantitation of nodularin and linear nodularin

A NOD standard (10 µg mL^{-1}) was infused directly into the QToF-MS system to optimise MS parameters for NOD detection at a mass to charge ration (m/z 825). No collision energy was used. A combined calibration of NOD and LNOD was prepared in triplicate by applying appropriate dilutions to stock solutions (10 µg mL^{-1}) prepared from dried standards. The regression curve and the respective correlation coefficient (R^2) were obtained by plotting concentrations against peak areas determined by UPLC-MS utilising Excel (Microsoft, Redmond, USA). LNOD was monitored at a m/z of 692. The PDA signal was monitored at 238 nm. The practical limit of detection was set as the lowest reliably detectable concentration with a variation (%RSD) of less than 10% between triplicates.

3.2.2.2 Optimisation of mass spectrometer cone cleaning interval for the analysis of mussel flesh and faecal material samples

To prevent signal losses caused by matrix deposition on the MS cone the cleaning interval was investigated for mussel flesh and faecal pellet samples. In addition to the MS signal, the eluent was monitored utilising a PDA detector (200-400 nm) with a resolution of 1.2 nm. The ratio of MS to PDA signal (MS:PDA) was determined to illustrate changes in MS signal. A negative control mussel flesh sample was filtered (0.2 µm, nylon) and injected 100 times into the UPLC-TOF-MS/PDA system. A NOD standard (10 µg mL^{-1}) was analysed after every tenth injection of mussel flesh extract. Similarly, negative control faecal pellet extracts were filtered and injected into the system. Based on results obtained for mussel flesh samples, NOD signal stability following the injection of 60 faecal samples was investigated. Prior to each investigation the MS cone had been sonicated in 50% aqueous MeOH for 10 min and dried with nitrogen gas.

3.2.2.3 Determination of matrix effects of mussel flesh and faecal material samples on NOD and LNOD signals

Calibrations of NOD and LNOD were prepared in triplicate utilising negative control mussel flesh and faecal pellet extracts, respectively (Karlsson *et al*., 2005). Mixed stock solutions (10 µg mL^{-1}) were prepared from dried standards and appropriate dilutions were applied to obtain concentration ranges of 0.001-1 µg mL^{-1} and 0.01-5 µg mL^{-1} for mussel flesh and faecal pellet calibrations, respectively. Similarly, a mixed calibration was prepared utilising 50% aqueous MeOH to match the concentration ranges for the two matrices.

3.2.3 Media preparation and culturing of *N. spumigena* KAC66

Stock solutions were prepared in water (Table 3. 1). For BG-11 media preparation 5 mL of the sodium nitrate and 1 mL of the remaining stocks were added per one litre of water. Furthermore, 1 mL of a trace element solution was

also added per litre of medium (Table 3. 2). The salinity was adjusted by adding 6.6 g of instant ocean salt (IOS; ~20% seawater) per litre of media (Aquarium Systems, Sarrebourg, France). Following preparation all media were autoclaved (121 °C, 50 min; Astell, Kent, UK). *N. spumigena* KAC66 was obtained from the Kalmar Culture Collection (Kalmar, Sweden). Cultures were maintained by monthly sub-culturing in BG-11 + 0.66 % wv IOS at 20-23°C with continuous illumination (10-15 µmol m^{-2} s^{-1}). In addition, gentle aeration was provided for volumes >100 mL (0.22 µm, Millipore, UK). A homogenous feed stock culture of *N. spumigena* KAC66 for the exposure trial of *M. edulis* was prepared by mixing ~120 L of individual cultures. During the experiment the feed stock cultures were maintained in 10 L opaque carboys in a temperature controlled room (17 ± 1°C). Mild aeration was provided via 6 mm polyvinyl chloride (PVC) tubing. Light conditions were set to 17 hours light and 7 hours dark cycles (~24 µmol m^{-2} s^{-1}).

Table 3. 1 Stock solutions utilised for BG-11 media preparation (adapted from Stanier *et al*., 1971)

Salt	**Concentration (g L^{-1})**
Sodium nitrate ($NaNO_3$)	150
Dipotassium phosphate (K_2HPO_4)	40
Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)	75
Calcium chloride dihydrate ($CaCl_2.2H_2O$)	36
Sodium carbonate (Na_2CO_3)	20
Citric acid ($C_6H_8O_7$)	6
Iron sulphate heptahydrate($FeSo_4.7H_2O$)	6
EDTA (Disodium)	1

Table 3. 2 Components of trace metal stock solution utilised for BG-11 preparation (Stanier *et al.,* 1971)

Trace element	**Concentration (g L^{-1})**
Boric acid(H_3BO_3)	2.860
Manganese chloride tetrahydrate ($MnCl_2.4H_2O$)	1.810
Zinc sulphate septahydrate ($ZnSO_4.7H_2O$)	0.222
Sodium molybdate dihydrate ($Na_2MoO_4.2H_2O$)	0.390
Copper sulphate pentahydrate ($CuSO_4.5H_2O$)	0.079
Cobalt nitrate hexahydrate($Co(NO_3)_2.6H_2O$)	0.049

3.2.4 Accumulation and depuration of nodularins in *M. edulis*

M. edulis (shell length 55.8 ± 8.7 mm) originating from Scotland were acclimatised to the laboratory conditions for a minimum of five days and were cleaned of barnacles and other debris prior to the experiment. Seawater (~35 psu) was filtered (50 µm) and pumped into a 300 L tank equipped with ultraviolet (UV) sterilisers (class 1 IP64, twin UV 24 W, 240 V, 50 Hz, Tropical Marine Centre, UK). Mussels (70) were placed in mesh baskets into 10 L of UV sterilised seawater (n = 12) to which aeration was provided via air lines (internal bore 4 mm, PVC; Figure 3. 1). Experimental tanks were housed in two additional large temperature controlled tanks (16 ± 1 °C) filled with approximately 150 L of filtered seawater containing six experimental tanks, each. Three of these were exposure and three were control tanks. (Figure 3. 1). Additional mussels were maintained in separate seawater tanks. These mussels ('stunt doubles', SD) were used to replace those taken for sampling purposes in order to maintain a constant ratio of available *N. spumigena* KAC66 per mussel.

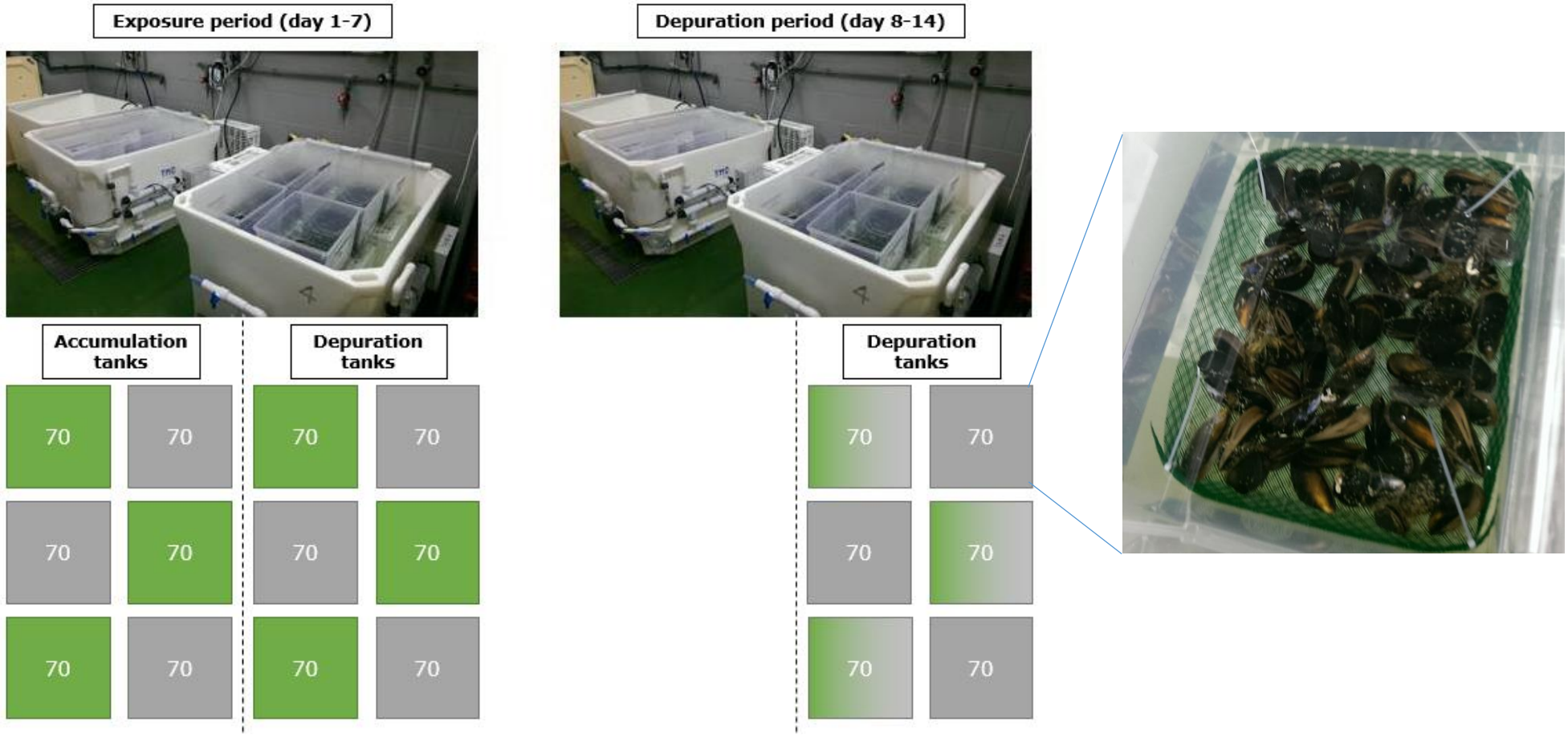

Figure 3. 1 Experimental tanks containing *M. edulis* (70) placed in mesh baskets in 10 L of UV sterilised seawater at 16 ± 1 °C with aeration. Mussels were exposed to *N. spumigena* KAC66 (green) and commercial shellfish diet (negative control; grey). Following the seven day exposure the three remaining exposure tanks were treated like negative controls (gradient green to grey).

The six exposure tanks were fed 2.5 L of *N. spumigena* KAC66 (final concentration ~2.7 x 10^6 cells per litre), containing 100-480 µg L^{-1} nodularins (NODs). To encourage feeding 500 mL of 20% seawater containing 1 mL of commercial shellfish diet (final concentration 5.7 x 10^{10} cells per litre, shellfish diet 1800, ReedMariculture Inc., US) was also added to all exposure tanks (Figure 3. 1). Control tanks were fed 5.7 mL of shellfish diet, consisting of a mixture of algae (*Isochrysis sp., Tetraselmis sp., Pavlova lutheri*, *I. galbana, Nanochloropsis*) and diatoms (*Chaetoceros gracilis, C. calcitrans, Skeletonema costatum*), diluted into 3 L of 20% seawater (final concentration ~3.2 x 10^{11} cells per litre). Each day a full water exchange of all twelve tanks was performed prior to feeding.

Regular water samples (~ 100 mL) from each tank were taken following feeding (approximately 2 pm) and prior to daily tank cleaning (~ 9 am on the following day). The water sample was utilised to determine intracellular and extracellular NODs. Furthermore, mussels (n=10) were sampled once daily prior to the water exchange and faecal material collected. During the first week these were obtained from only six of the tanks to assess accumulation of NODs (three exposure, 3 negative controls). Similarly, faecal material was obtained only from those six tanks that mussels had been sampled from. Mussels in the remaining six tanks were only sampled during the second week of the feeding study to assess depuration. To obtain the faecal material the tank water was filtered through a mesh sieve (pore size 80 µm) and the collected material washed into 50 mL centrifuge tubes using 20% seawater. Faecal material was then centrifuged at 2279x g for 20 min (Sorvall, ST 40R, ThermoScientific, USA). The supernatant was discarded and the pellet frozen prior to analysis (Figure 3. 2).

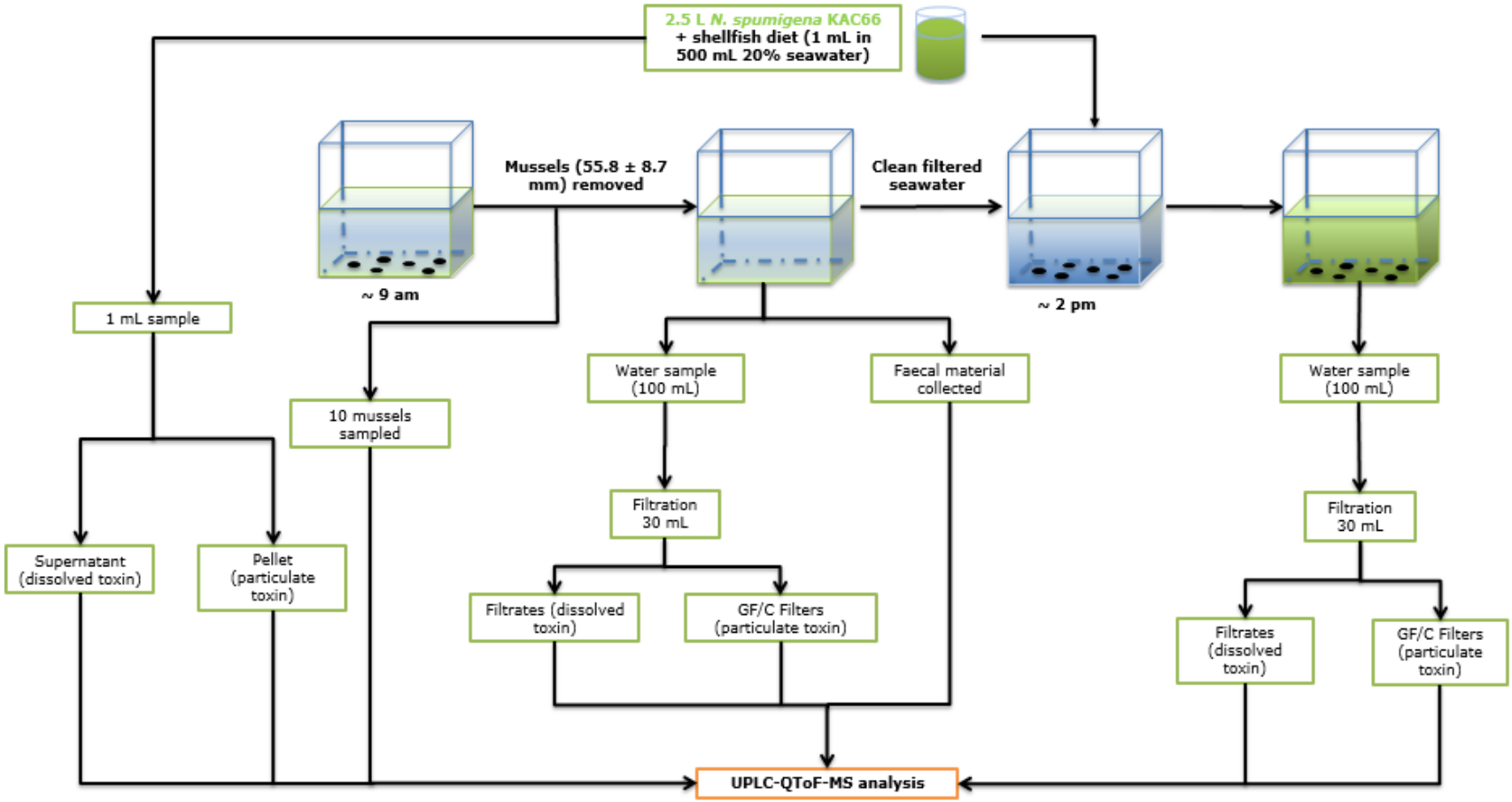

Figure 3. 2 Daily sampling of exposure tanks. Post-feeding of *M. edulis* with 2.5 L *N. spumigena* KAC66, of which 1 mL were sampled to assess NODs content, a water sample (100 mL) was taken to quantify dissolved and particulate NODs. Approximately 19 h following feeding and before performing the water exchange mussels (10), water (100 mL), and faecal material (total) were sampled and analysed for their NODs content. Negative control tanks were treated identically except that 5.7 mL shellfish diet diluted into 3 L of 20% seawater was utilised as food source. Sample analysis was performed utilising UPLC-QToF-MS (n=3). During depuration all tanks were treated like negative controls.

3.2.5 Sample processing

3.2.5.1 Extraction of intracellular nodularins in *N. spumigena* KAC66 culture

Prior to use 1 mL was removed from each culture used for feeding (1.5 mL centrifuge tube; Figure 3. 2). These samples were centrifuged for 10 min at 12,470 x g (SIGMA 1-14 K, Germany) and the supernatant discarded. The cell pellet was extracted with 1 mL 80% aqueous MeOH for 1 hour during which it was vortexed for ~10 s every 15 min. After extraction, samples were centrifuged for 10 min at 12,470 x g and the supernatant transferred into glass LC-MS vials, which were stored at -20°C until analysed. Due to the conversion of NOD to LNOD the combined concentration of these congeners will be reported as nodularins (NODs).

3.2.5.2 Influence of *N. spumigena* KAC66 handling on NOD to LNOD conversion

When investigating the potential conversion of NOD to LNOD no significant increase in LNOD content of *N. spumigena* KAC66 was observed (Figure 3. 3). The initial hypothesis that the storage conditions could affect the structure of NOD could not be proven as NOD remained the clearly dominant congener. Potentially, mechanical stress or the vibrations experienced during the 12 hour transport could have affected the concentration of NOD and LNOD within the cultures, however, further studies are required to elucidate this. These could include investigation the effect of even lower temperatures (~1°C) and the effect of mechanical stress such as continuous shaking on the presence of LNOD within *N. spumigena* KAC66.

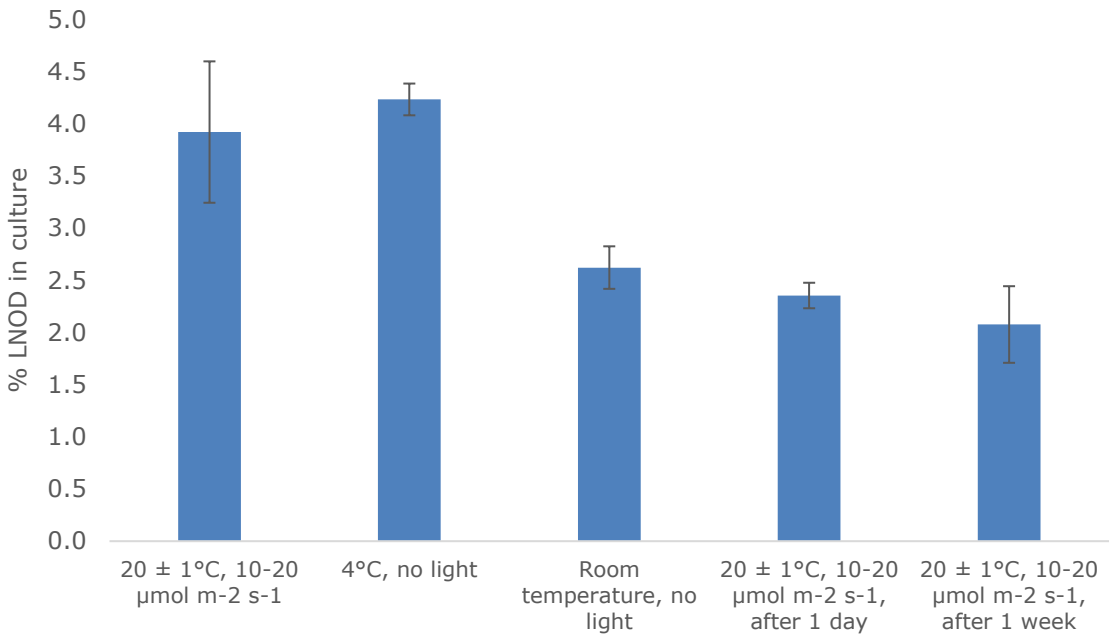

Figure 3. 3 Percentage of combined intracellular and extracellular LNOD (m/z 692) compared to NOD (m/z 825) detected following sequential storage of *N. spumigena* KAC66 at varying storage conditions. Values are shown ± standard deviation (n=2) as determined after UPLC-QToF-MS analysis.

3.2.5.3 Extraction of intracellular and extracellular nodularins in tank water

All water samples (30 mL) were filtered through 47 mm glass microfiber filters (GF/C, GE Health Care, Little Chalfont, Buckinghamshire, UK) into 50 mL centrifuge tubes (filtrate; dissolved NODs). Filter papers (particulate NODs) were transferred to 15 mL centrifuge tubes. Both filtrate and filters were stored at -20°C. Frozen filtrates were freeze dried, extracted with 1 mL 80% aqueous MeOH, and vortexed for approximately 10s. This was repeated another four times (total extraction volume 5 mL). Pooled, suspended samples were allowed to extract for 15 min. Frozen filter samples were extracted with 5 mL 80% aqueous MeOH and vortexed every 15 min for 1 hour. A 1 mL subsample of both filter and filtrate extracts was centrifuged (10 min at 10,433x g) and supernatants transferred to LC-MS vials. The remaining filtrate and filter extracts were stored at -20°C.

3.2.5.4 Extraction of nodularins from *M. edulis* flesh

From each sampling tank 10 specimens (batch) were randomly selected. This batch of mussels was shucked (n=3) and their flesh collected in a sieve to drain excess fluid (Figure 3. 4). The drained tissue was pulse blended for 15 s (Waring Commercial, USA) which was repeated three times with 5 s breaks between each pulse. Triplicates of the homogenate were weighed (4 g ± 0.005 g) into 50 mL centrifuge tubes and extracted with 8 mL of 50% aqueous MeOH. Samples were vortexed for 2 min and centrifuged (2,279 x g, 20 minutes; Storvall ST 40R, Thermo Scientific, UK). The supernatant was transferred into a 20 mL volumetric flask. Samples were re-extracted and supernatants combined. Finally the flask was made up to 20 mL using 50% aqueous MeOH. The extract were stored at -20°C until analysis (Figure 3. 4). All samples were filtered (0.22 µm nylon syringe filters, Millex 33 mm Durapore, Merck Millipore, UK) prior to UPLC-QToF-MS analysis.

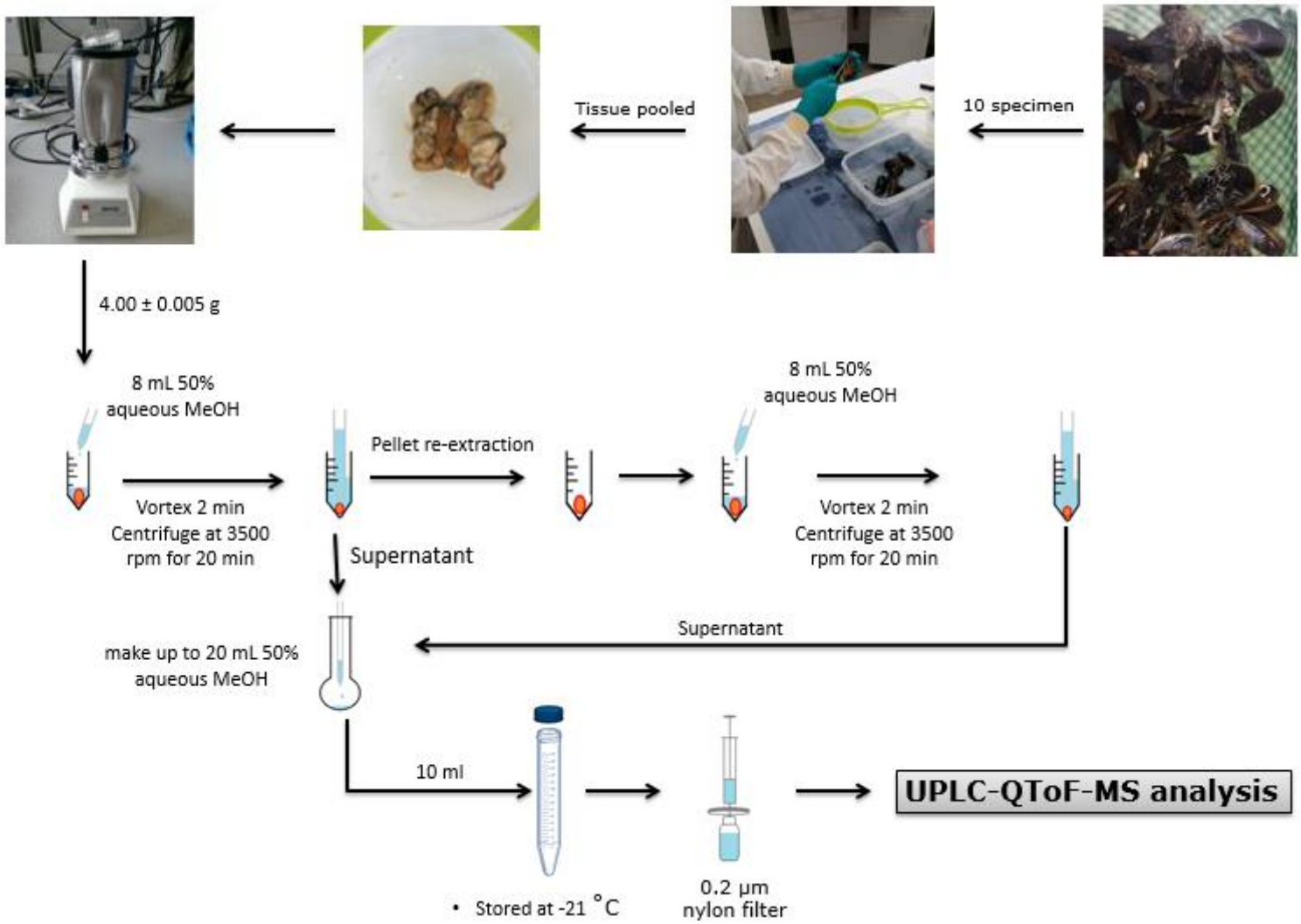

Figure 3. 4 Double 50% aqueous MeOH extraction of NODs from *M. edulis* exposed to *N. spumigena* KAC66 or shellfish diet. Mussels (n=10) had been shuck, pooled, and pulse blended. All samples were analysed utilising UPLC-QToF-MS (n=3)

3.2.5.5 Extraction of faecal material

Prior to analysis the frozen faecal pellet was extracted with 5 mL of 80% aqueous MeOH for 30 min during which samples were vortexed every 15 min for approximately 10 s. Samples (1 mL) were centrifuged (10 min at 10,433 x g) and the supernatant transferred into UPLC vials prior to UPLC-QToF-MS analysis.

3.2.6 UPLC-QToF-MS analysis of samples obtained during the *N. spumigena* KAC66 exposure trial of *M. edulis*

Samples were analysed utilising the UPLC-QToF conditions described in section 3.2.2. The remaining MS parameters had been optimised following the infusion of a NOD standard (3.3.1 Preliminary UPLC-QToF-MS analysis and optimisation).

3.2.7 Influence of *N. spumigena* KAC66 handling on NOD to LNOD conversion

Following ~10 h transport of the prepared *N. spumigena* KAC66 feed stock cultures to Cefas (Weymouth, UK), were the feeding trials were performed, an increase in LNOD concentration was observed. Prior to the transport, cultures had been stored in a car overnight during February 2015 without aeration. That night temperatures of 1°C were encountered. In addition, all cultures had been covered and hence experienced a paucity of light for approximately 24 h. To simulate these changes in storage conditions experienced prior and during the transport of *N. spumigena* KAC66, cultures previously stored within a temperature controlled room (20 ± 1°C), were maintained in a fridge (4-8°C) for approximately 13 h (Figure 3. 5). This was followed by 10 h storage in the dark (at room temperature) and transferral back to the original conditions of the culture room.

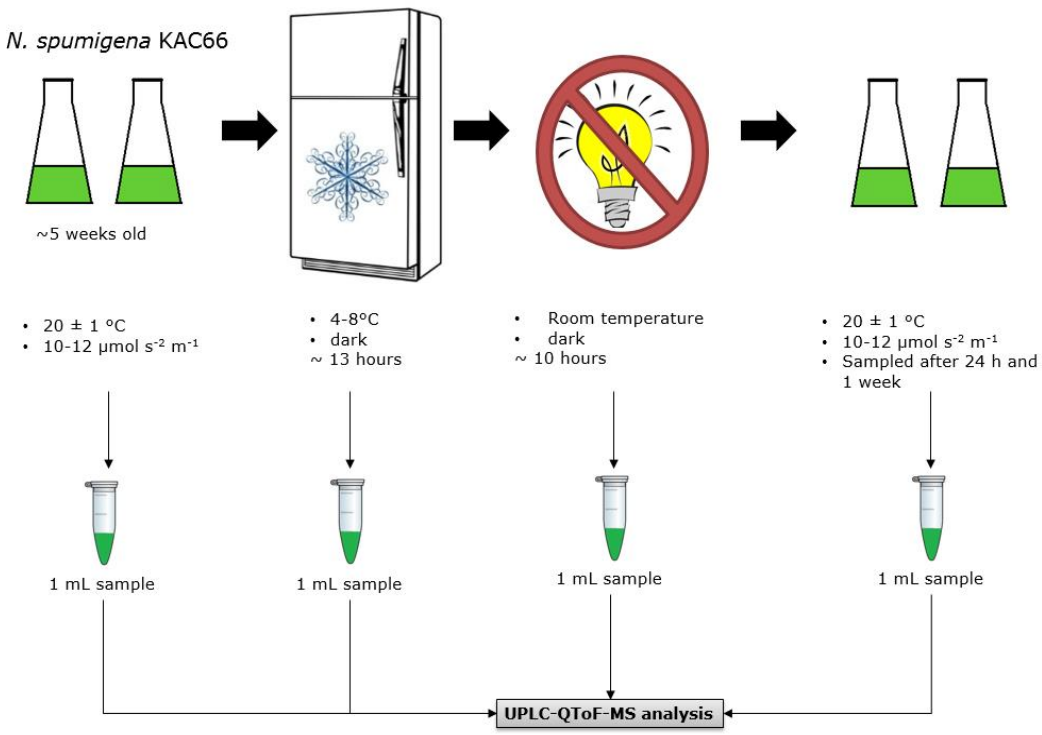

Figure 3. 5 Sampling to investigate the influence of storage condition (at low temperatures followed by paucity of light) on conversion of NOD to LNOD in *N. spumigena* KAC66. Samples were analysed by UPLC-QToF-MS analysis

Samples (1 mL) of the culture were taken at the following time points: before and after storage in the fridge, following storage in the dark, and one day and one week after cultures were transferred back to the original conditions. The one day and one week sampling points were chosen to assess potential effect of change back to the original conditions as following transport cultures were placed in a temperature and light controlled culture room and maintained there for duration of the one week exposure period. Samples were centrifuged (10 min, 10,433 x g) and supernatants transferred to 1.5 mL tubes, frozen, freeze-dried (Freeze Dryer Modulyo; Edwards, UK), and stored at -20°C until analysis. The cell pellets were frozen prior to analysis. Pellets and freeze-dried supernatants were extracted with 1 mL 80% aqueous MeOH and vortexed (~10 s) every 15 min for 1 hour. Following this, samples were centrifuged (10 min, 10,433 xg) and supernatants were transferred to LC-MS vials and analysed utilising UPLC-QToF-MS.

3.3 Results and Discussion

3.3.1 Preliminary UPLC-QToF-MS analysis and optimisation

3.3.1.1 Quantitation of nodularin and linear nodularin in 80% aqueous methanol

Following the infusion of a NOD standards (10 µg mL^{-1}) optimum mass spectrometer conditions were determined (Table 3. 3). The LOQ of NOD in 80% aqueous MeOH was determined based on the lowest detectable value with relative standard deviations (%RSD) of less than ten percent which was found at 0.05 µg mL^{-1}. Linearity was excellent over the calibration range of 0.01-10 µg mL^{-1} with a correlation coefficient of R^2=0.9996 (Figure 3. 6).

Table 3. 3 Mass spectrometer conditions optimised following the infusion of NOD (10 µg mL^{-1}) in 45.7% aqueous acetonitrile containing 0. % formic acid.

Parameter	Value
Sampling cone (kV)	40
Source capillary	3.3
Extraction cone	3.0
Source temperature (°C)	120
Desolvation temperature (°C)	400
Cone gas flow (L h^{-1})	20
Desolvation gas flow (L h^{-1})	600
Scan time (s)	1

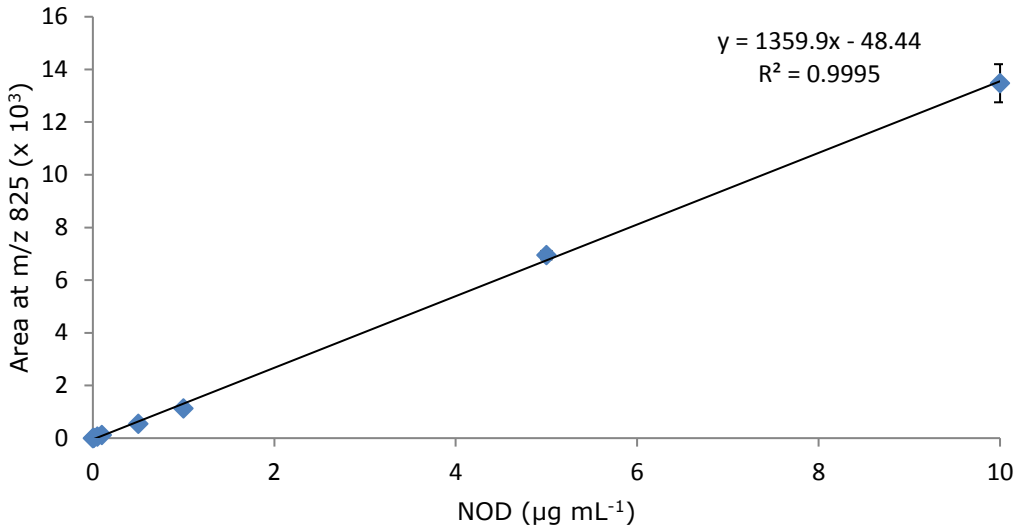

Figure 3. 6 Linear calibration graph for NOD in 80% aqueous MeOH by UPLC-QToF-MS (m/z 825). Error bars represent standard deviations (n=3)

Due to the presence of LNOD in *N. spumigena* KAC66, a calibration in 80% aqueous MeOH was prepared. The variability between replicates was slightly higher compared to NOD. Nonetheless, reproducibility at 0.05 µg mL^{-1} was acceptable (%RSD 7.51) so that LOQs were identical for both congeners. Linearity was excellent with R^2=0.9998 (Figure 3. 7). The molecular weight of LNOD is 843, however, under the given conditions the dominant fragment observed was the one associated with $[M+H-NH_2-135]^+$ (Mazur-Marzec *et al*., 2006b; Figure 3. 8), which was monitored during future analysis.

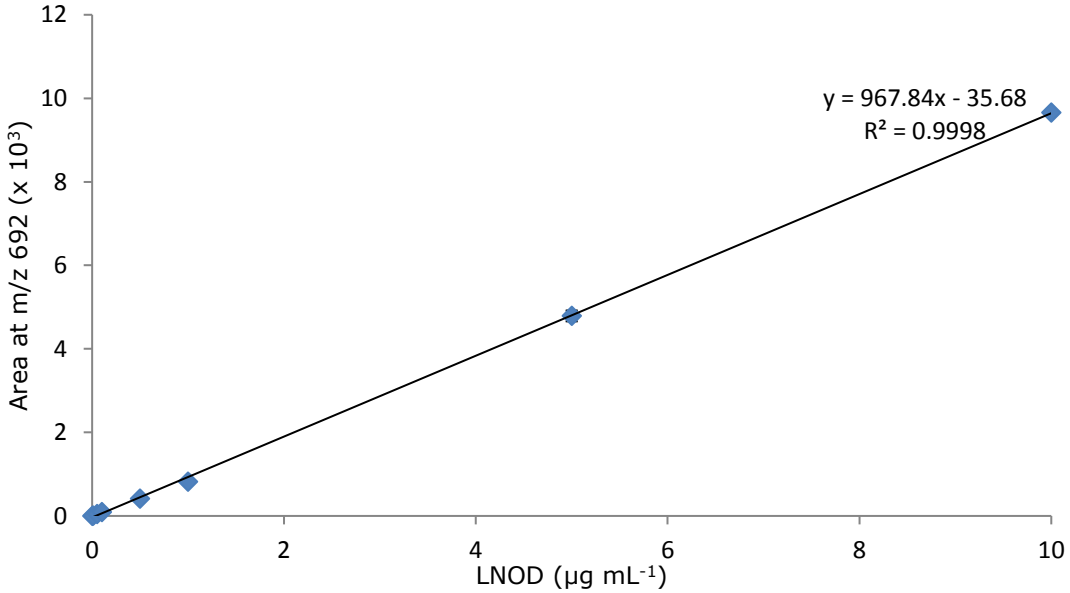

Figure 3. 7 Calibration of LNOD in 80 % aqueous MeOH utilising UPLC-QToF-MS at m/z 692. Error bars represent standard deviation (n=3).

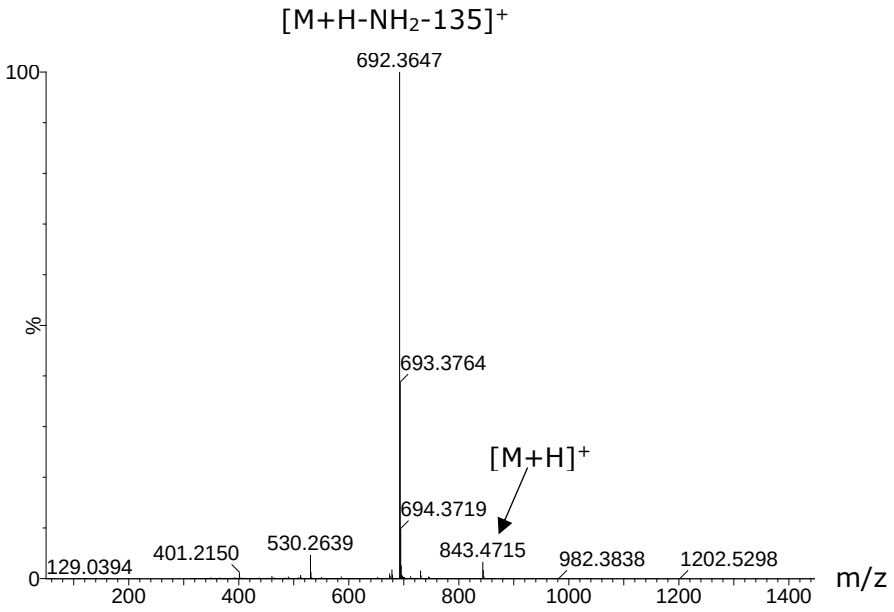

Figure 3. 8 Mass spectrum of LNOD detected at t_R = 4.459 min utilising UPLC-QToF-MS analysis.

3.3.1.2 Optimisation of mass spectrometer cone cleaning interval for the analysis of mussel flesh and faecal material samples

Losses in MS signal were observed after the injection of 50 mussel flesh extracts (Figure 3. 9) whereas the PDA signal remained consistent even after 100 injections (%RSD=5.44). A change in MS:PDA was observed following 40 injections of the mussel matrix. Therefore, a cone cleaning interval of no more than 40 injections of mussel flesh samples was implemented.

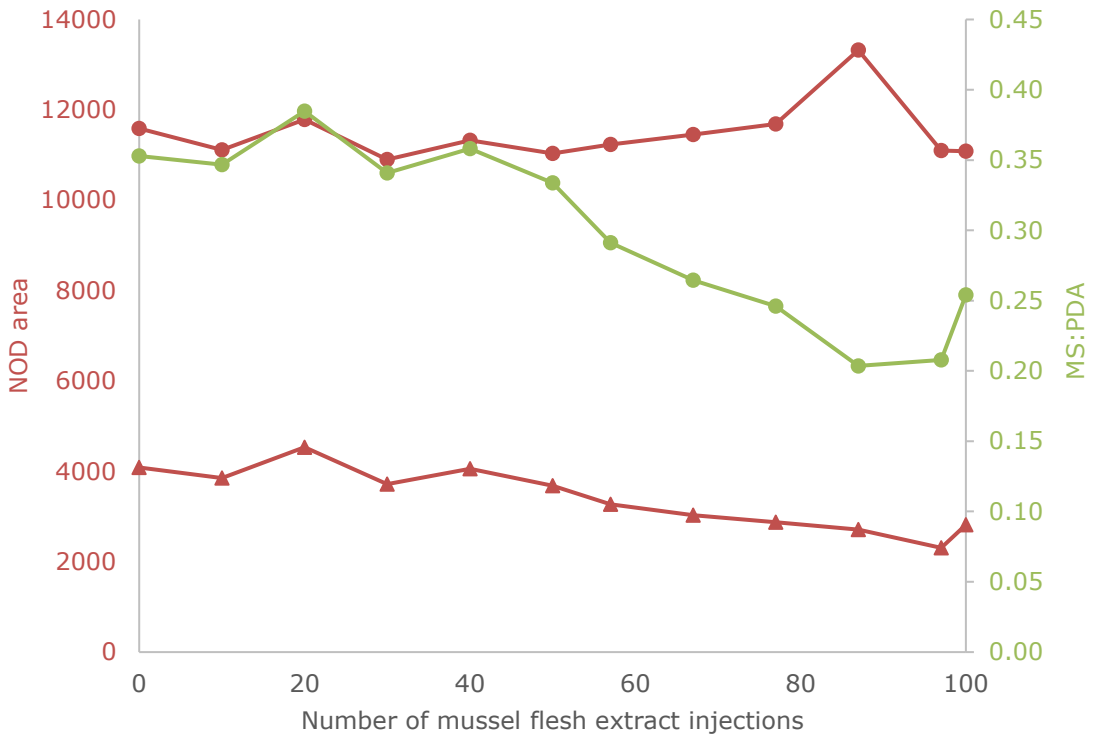

Figure 3. 9 Change in NOD (10 µg mL^{-1}) signal after repeated injecting of negative control mussel flesh extracts. Analysis was performed utilising UPLC followed by PDA (238 nm; red circles) and QToF-MS (m/z 825; red triangles) detection. In addition, the ratio of MS to UV signal was determined (green circles).

Similarly, the injection of faecal pellet extracts caused signal losses for NOD following MS detection. Results were comparable to those obtained for mussel flesh extracts. A reduction in MS:PDA ratio was observed following the injection of 50 negative control faecal pellet extracts: 1.44 compared to previously 1.52-1.63 (Figure 3. 10). General reproducibility between injections was excellent when utilising PDA detection as %RSD was below one. Mass spectrometry detection showed slightly larger variations with %RSD 6.1. Hence, the MS cone was cleaned after injecting 40 faecal pellet samples.

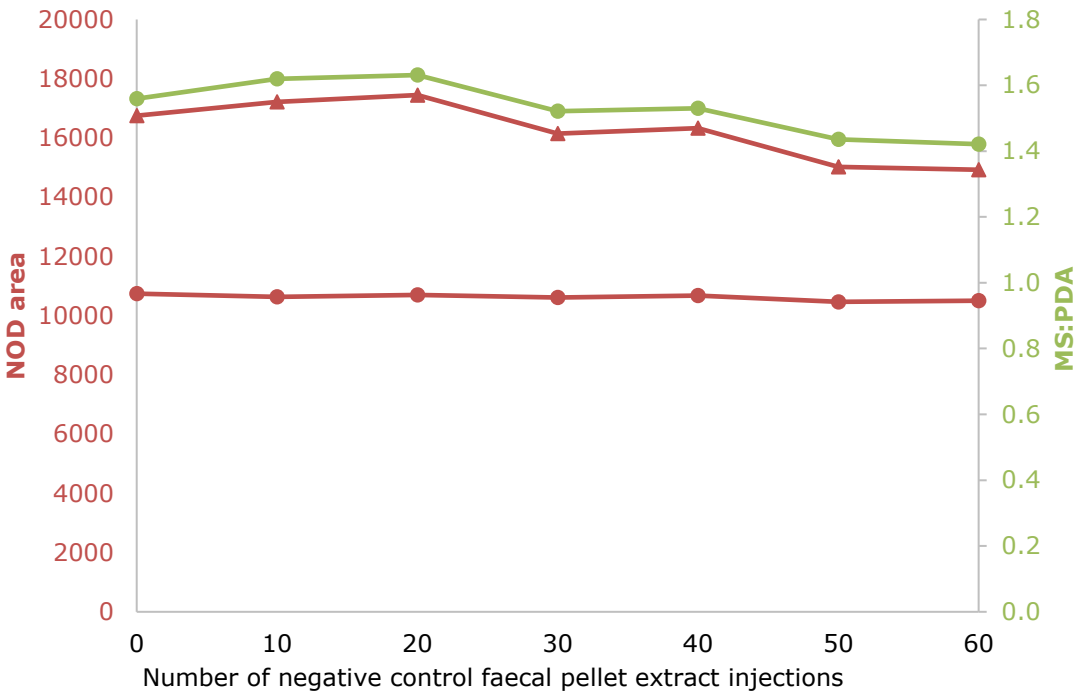

Figure 3. 10 Change in NOD (10 µg mL^{-1}) signal after injecting negative control faecal pellet extracts. Analysis was performed utilising UPLC followed by PDA (238 nm; red circles) and QToF-MS (m/z 825; red triangles) detection. In addition, the ratio of MS to UV signal was determined (green circles).

3.3.1.3 Determination of matrix effects of mussel flesh and faecal material samples on NOD and LNOD signals

Excellent linearity was obtained for NOD prepared in both 50% aqueous MeOH and mussel flesh extracts ($R^2 \geq 0.995$; Figure 3. 11). The LOQ (%RSD < 5) was found at 0.05 µg mL^{-1} (250 pg on column) for both solvents. Matrix suppression effects resulted in signal loss at concentrations of 0.01 µg mL^{-1} (50 pg on column) when prepared in mussel flesh extracts. At higher concentrations no matrix effect was detected as slopes did not differ by more than 5%. NOD peak areas obtained in 50% aqueous MeOH were compared to those obtained in negative control mussel flesh extracts and the response calculated (Equation 3. 1, Table 3. 4).

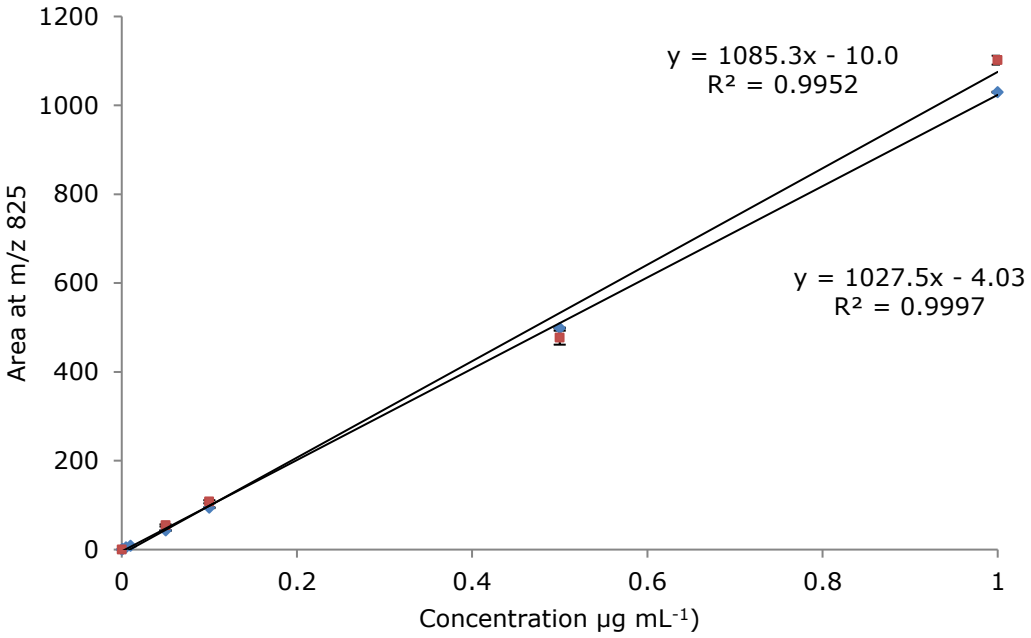

Figure 3. 11 Calibration of NOD (0.001-1 µg mL^{-1}) in 50% aqueous MeOH (blue) and negative mussel extracts (red) utilising UPLC-QToF-MS at m/z 825. Error bars represent standard deviation (n=3).

Equation 3. 1 Calculation of response to determine presence of matrix effects

$$response = \frac{peak\ area\ in\ negative\ control\ mussel\ flesh\ extracts}{peak\ area\ detected\ in\ 50\%\ aqueous\ MeOH} \times 100$$

Comparing the signals obtained in mussel flesh extract to those obtained in 50% aqueous MeOH showed a maximum response of 126% at 0.05 µg mL^{-1}. This was associated with higher variability close to LOD values rather than a matrix effect. Remaining levels were within 15% variation between responses (Table 3. 4).

Table 3. 4 Response (%) of NOD signal in negative mussel flesh extracts compared to values obtained in 50% aqueous MeOH. Samples were analysed by UPLC-QToF-MS at m/z 825 (n=3).

On column pg	c in µg mL^{-1}	50% aqueous MeOH	Negative control mussel flesh extracts	Response (%)
5	0.001	1 ± 0.0	0	0
25	0.005	5 ± 0.6	0	0
50	0.01	8 ± 0.6	0	0
250	0.05	43 ± 1.0	54 ± 2.5	126 ± 0.34
500	0.10	94 ± 3.5	108 ± 3.5	114 ± 0.30
2500	0.50	499 ± 12	477 ± 16	96 ± 0.15
5000	1.00	1030 ± 42	1102 ± 10	107 ± 0.19

c – concentration

The interference of matrix components during electrospray ionisation MS has been well documented within the literature (Trufelli *et al*., 2011; Taylor 2005; King *et al*., 2000; Tang and Kebarle 1993). It was reported that varying analyte ions were affected by increasing concentrations of NH_4^+ during electron spray MS analysis (Tang and Kebarle 1993). During the analysis of NOD and six MC variants in mussels and liver samples (common eider and rainbow trout) both ion suppression and enhancement have been reported (Karlsson *et al.*, 2005). The response varied from 58-134% depending on the sample matrix. Similarly, analysis of eider tissue showed responses of 90-140% (Sipiä *et al*., 2006).

Prepared in faecal pellet extracts, the NOD calibration also showed excellent linearity (R^2=0.9995; Figure 3. 12). However, no signal was obtained for NOD at a concentration of 0.01 µg mL^{-1}. The range of the calibration had been changed due to the observed signal loss in mussel flesh extracts. The lowest concentration reliably detected when prepared in faecal pellet extract was 0.53 µg mL^{-1} (2650 pg on column) which was more than ten times higher compared to the LOQ obtained in 80% aqueous MeOH (0.05 µg mL^{-1}) illustrating a signal suppression effect. In additions, comparing the slope obtained in faecal pellet extract to that obtained in 80% aqueous MeOH showed a difference of 13% further supporting the presence of a slight signal suppression effect (Table 3. 5).

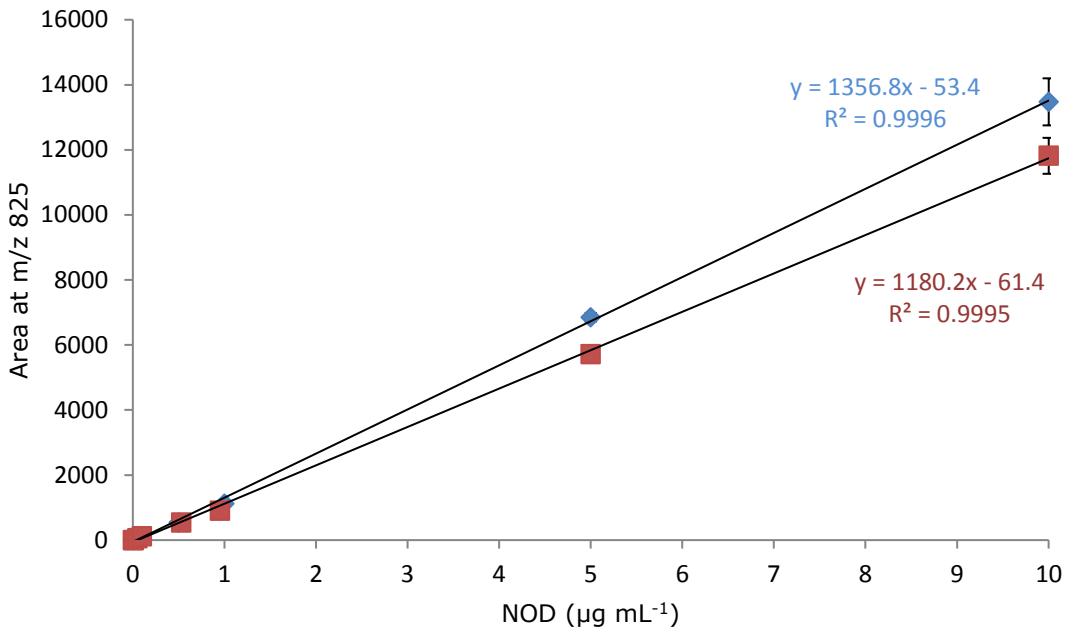

Figure 3. 12 Calibration of NOD in 80% aqueous MeOH (blue) and negative control faecal pellet extracts (red) utilising UPLC-QToF-MS at m/z 825. Error bars represent standard deviation (n=3).

Table 3. 5 Response (%) of NOD signal in negative control faecal pellet extracts compared to signals obtained in 80% aqueous MeOH. Samples were analysed by UPLC-QToF-MS at m/z 825

On column pg	c µg mL^{-1}	80% aqueous MeOH	Negative control faecal pellet extracts	Response (%)
50	0.01	8 ± 6	0	0
250	0.05	56 ± 0.6	58 ± 4.0	104 ± 0.51
500	0.10	115 ± 3.8	112 ± 6.4	97 ± 0.42
2500	0.50	575 ± 23*	542 ± 22	94 ± 0.19
5000	1.00	1069 ± 88*	900 ± 19	84 ± 0.61
25000	5.00	6852 ± 131	5719 ± 85	83 ± 0.05
50000	10.0	13475 ± 724	11816 ± 553	88 ± 0.44

c - concentration * were adjusted for different concentration

The calibration of LNOD in both 50% aqueous MeOH and negative control mussel flesh extracts showed excellent linearity (R^2 ≥0.9999; Figure 3. 13). The LOQ was determined at 250 pg on column (0.05 µg mL^{-1}) when dissolved in 50% aqueous MeOH. Utilising negative mussel flesh extract as solvent resulted in higher LOQs. Acceptable variability was found for all concentrations of 500 pg on column (0.10 µg mL^{-1}) and above. The slopes of the prepared calibrations differed by nearly 30% illustrating a signal suppression effect when analysing LNOD in mussel flesh extracts.

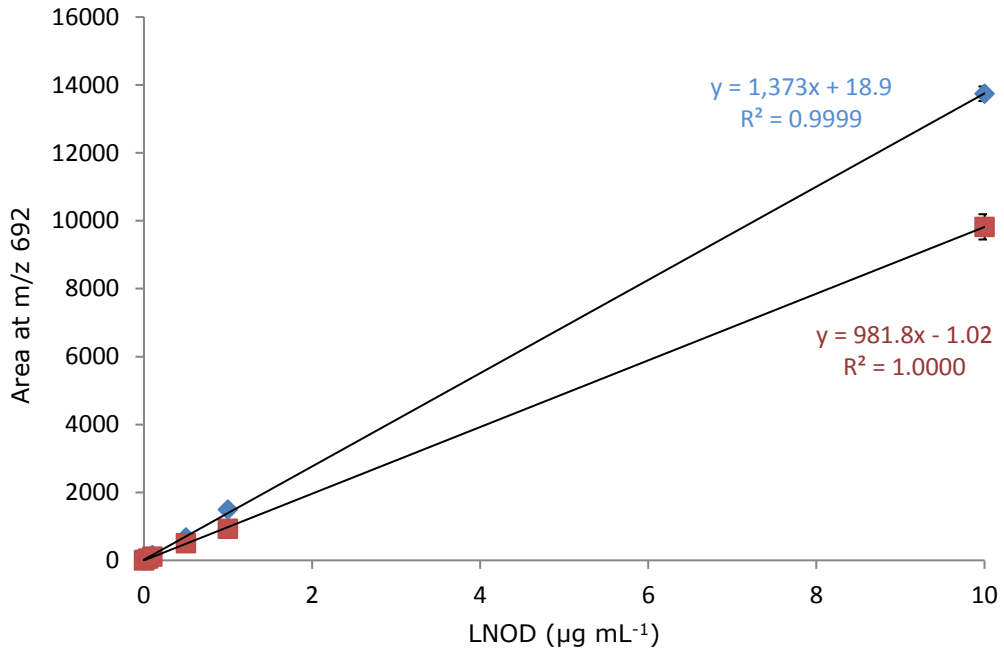

Figure 3. 13 Calibration of LNOD in 50% aqueous MeOH (blue) and negative mussel extracts (red) utilising UPLC-QToF-MS at m/z 692. Error bars represent standard deviation (n=3).

During analysis a shoulder was detected at the LNOD peak (Figure 3. 14). The LNOD used for this experiment had previously been stored in the fridge. Due to its linear structure LNOD is more susceptible to degradation. However, the m/z observed at different times was consistent at m/z 692. Had LNOD been degraded a change in m/z value would have been expected. Furthermore, the UV spectrum at 238 nm also showed a broad peak at the same retention time (Figure 3. 14). This indicates that retention was not optimal for LNOD under the given conditions. Potentially, the LNOD peak could be improved by optimising the chromatographic conditions for future analysis.

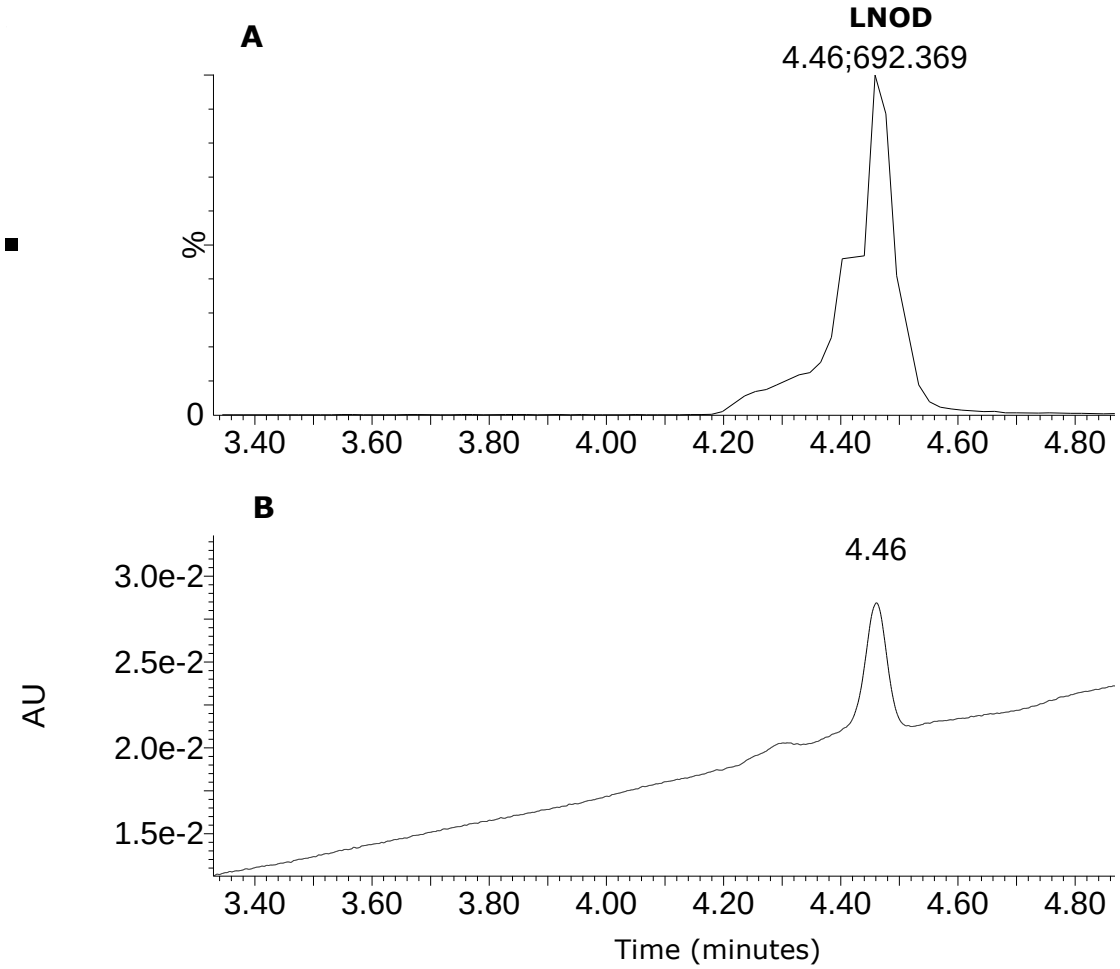

Figure 3. 14 LNOD (0.5 µg mL^{-1}) in 50% aqueous MeOH. UPLC-QToF-MS chromatogram extracted at m/z 692 (A) and UPLC**-**PDA chromatogram extracted at 238 nm (B).

The mussel flesh suppressed the LNOD signal with losses ranging from 8 to 38% (Table 3. 6). For all levels, including and above the LOQ, the discrepancy between the samples prepared in aqueous 50% MeOH and mussel flesh extracts was 29-38%. The same peak shoulder as detected in aqueous 50% MeOH was observed for LNOD in negative control mussel flesh extracts (Figure 3. 15), which further supports the hypothesis that chromatography conditions were responsible rather than the matrix.

Table 3. 6 Response (%) of LNOD signal in negative control mussel flesh extracts compared to values obtained in 50% aqueous MeOH. Samples were analysed by UPLC-QToF-MS at m/z 692 (n=3)

On column pg	c µg mL^{-1}	50% aqueous MeOH	Negative control mussel flesh extracts	% Response
50	0.010	16 ± 1.4	15 ± 2.4	92 ± 3.1
250	0.050	77 ± 2.9	55 ± 4.1	71 ± 0.50
500	0.100	163 ± 4.6	111 ± 3.4	68 ± 0.12
2500	0.50	683 ± 25	509 ± 8.2	74 ± 0.12
5000	1.00	1499 ± 16	929 ± 11	62 ± 0.02
50000	10	13742 ± 215	9821 ± 373	71 ± 0.12

c - concentration

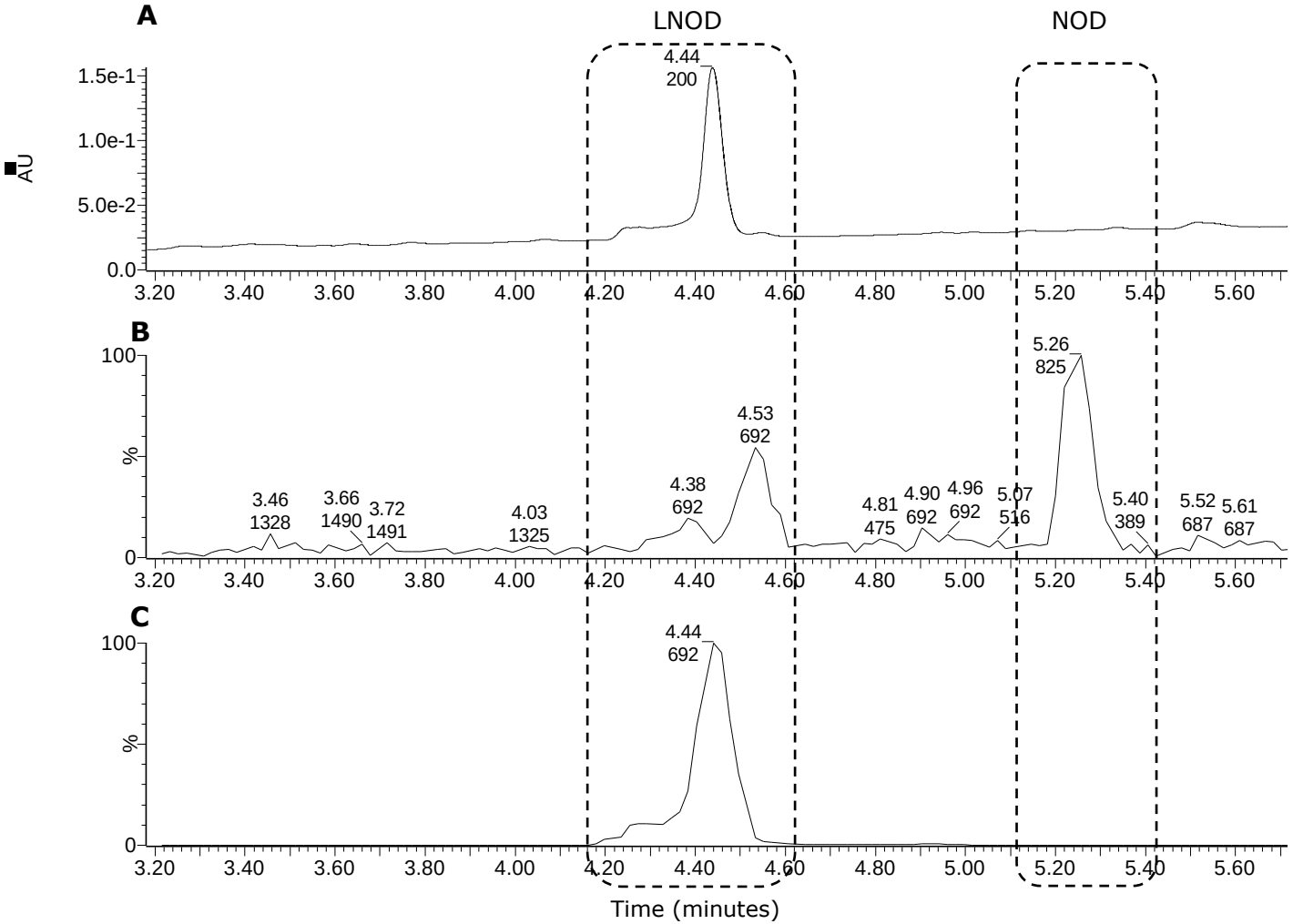

Figure 3. 15 LNOD (10 µg mL^{-1}) in negative control mussel flesh extracts. UPLC**-**PDA chromatogram extracted at 238 nm (A) UPLC-QToF-MS chromatogram extracted at m/z 825 (B) and m/z 692 (C)

Prepared in faecal pellet extracts, LNOD showed good linearity (R^2=0.9952; Figure 3. 16). Limit of quantitation was found at 0.10 µg mL^{-1} (500 pg on column) with a %RSD = 3.75. At a concentration of 0.53 µg mL^{-1} LNOD showed poor reproducibility between triplicates (%RSD 26.45). Consequently this level was excluded from the calibration. In addition, the faecal pellet matrix caused a signal loss of LNOD, similar to observations made for NOD.

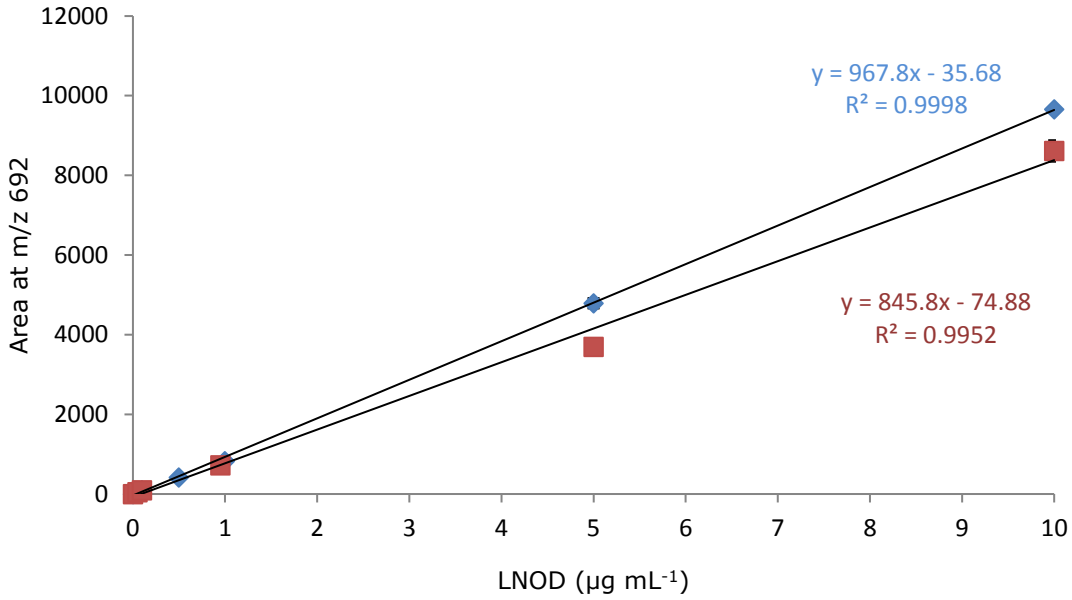

Figure 3. 16 Calibration of LNOD in 80% aqueous MeOH (blue) and negative control faecal pellet extracts (red) utilising UPLC-QToF-MS at m/z 692. Error bars represent standard deviation (n=3)

Comparing the response for the calibration prepared in the two different solvents showed generally that recoveries of LNOD were above 89% (Table 3. 7). The only exception was found at concentrations of 5 µg mL^{-1} where the response was only 77%.

Table 3. 7 Response (%) of LNOD signal in negative control faecal pellet extracts compared to preparation in 80% aqueous MeOH. Samples were analysed by UPLC-QToF-MS at m/z 692

On column pg	c µg mL^{-1}	80% aqueous MeOH	Negative control faecal pellet extracts	Response (%)
250	0.05	48 ± 3.6	49 ± 3.5	101 ± 1.1
500	0.10	93 ± 5.3	94 ± 3.5	101 ± 0.47
4750	0.95	782 ± 45*	720 ± 13	92 ± 0.34
25000	5.00	4789 ± 130	3692 ± 68	77 ± 0.08
50000	10.0	9662 ± 62	8614 ± 270	89 ± 0.09

c - concentration, * was adjusted for different concentration

3.3.2 Quantitation of nodularins in *Nodularia spumigena* KAC66 feed stock culture

Nodularin was present in feed stock cultures, but at levels below LOQ (0.05 µg mL^{-1}). In individual cultures utilised for the feed stock preparation prior to transport of *N. spumigena* KAC66, NOD levels ranged from 0.23-0.77 µg mL^{-1}. Generally, only small or no linearised NOD (LNOD) was detected in these cultures (Figure 3. 17 A). However, daily sampling of the feed stock cultures utilised for the exposure trial showed the linearised congener to be dominant (Figure 3. 17 B). Potentially this conversion could have been caused by the change in storage conditions prior and during transportation of feed stock cultures which had been stored overnight in a car during February 2015 (~ 1°C). This was a substantial change compared to the conditions of the temperature controlled room which cultures had been maintained in previously (20 ± 1°C). In addition, cultures experienced a paucity of light for approximately 24 h in combination with a shaking movement during transport. During the exposure trial cultures were maintained at 17°C. Potentially, these changes to the environment of *N. spumigena* KAC66 could have caused the structural change of NOD via means currently not understood.

Generally, LNOD concentration was stable for the first three days of the experiment: the average concentration of LNOD was 0.565 ± 0.03 µg mL^{-1}. However, with increasing storage time the LNOD concentration decreased considerably (Table 3. 8).

Table 3. 8 LNOD detected in *N. spumigena* KAC66 feed stock cultures utilised for the seven day exposure of *M. edulis*. Samples were analysed utilising UPLC-QToF-MS (m/z 692)

Time days	Feed stock culture	LNOD µg mL^{-1}
1	1	0.624
	2	0.567
2	3	0.569
	4	0.546
3	5	0.526
	6	0.556
4	7	0.557
	8	0.166
5	9	0.369
	10	Below LOQ
6	11	0.392
	12	0.474
7	13	0.153
	14	0.129

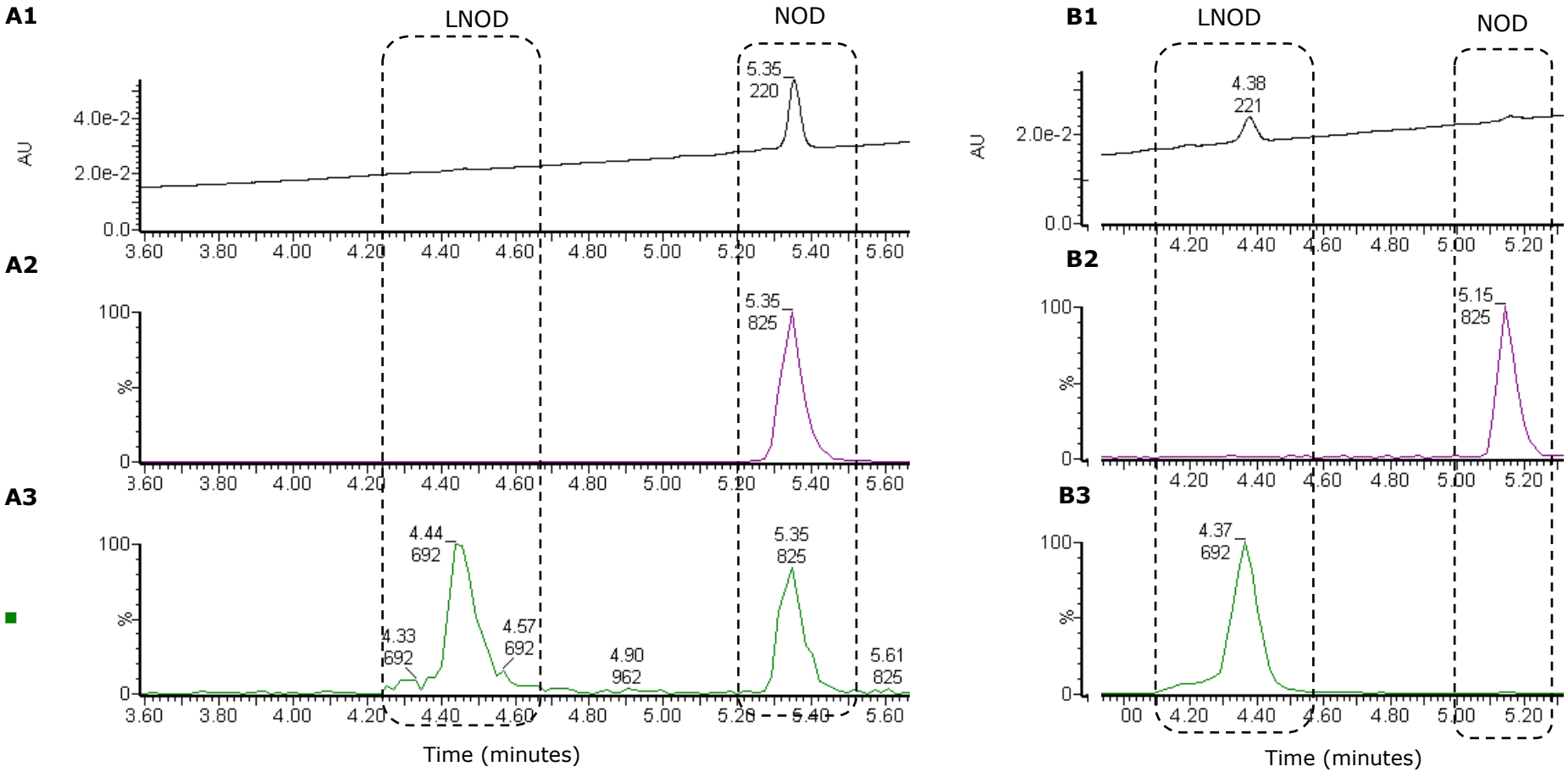

Figure 3. 17 UPLC-UV chromatogram monitored at 238 nm of *N. spumigena* KAC66 prior to feed stock preparation (A1) and after feed stock preparation and transport (B1). UPLC-QToF-MS chromatogram of *N. spumigena* KAC66 recorded at m/z 825 prior to feed stock preparation (A2) and after feed stock preparation and transport (B2). UPLC-QToF-MS chromatogram of *N. spumigena* KAC66 recorded at m/z 692 prior to feed stock preparation (A3) and after feed stock preparation and transport (B3)

3.3.3 Quantification of cyanotoxins in tank water

3.3.3.1 Intracellular nodularins

Analysis of water showed that NODs were detected at concentrations below the LOQ (Figure 3. 18). Based on levels of NODs in feed stock culture, intracellular NODs concentrations of 0.03-0.12 µg mL^{-1} would have been expected in the tank water, but only three of the 252 filter samples contained quantifiable NODs. Those samples were taken post-feeding on day six (0.005 µg mL^{-1}) and in two tanks on day seven (0.003 µg mL^{-1}, 0.004 µg mL^{-1}). Strikingly, despite low NODs quantities detected in the respective culture stock samples for days five and seven, NODs were detected in tanks on day seven and in one depuration tank on day five. The discrepancies could indicate that the culture stock samples had not been as homogenous. In addition, extraction of NODs from the GF/C disks could have been incomplete.

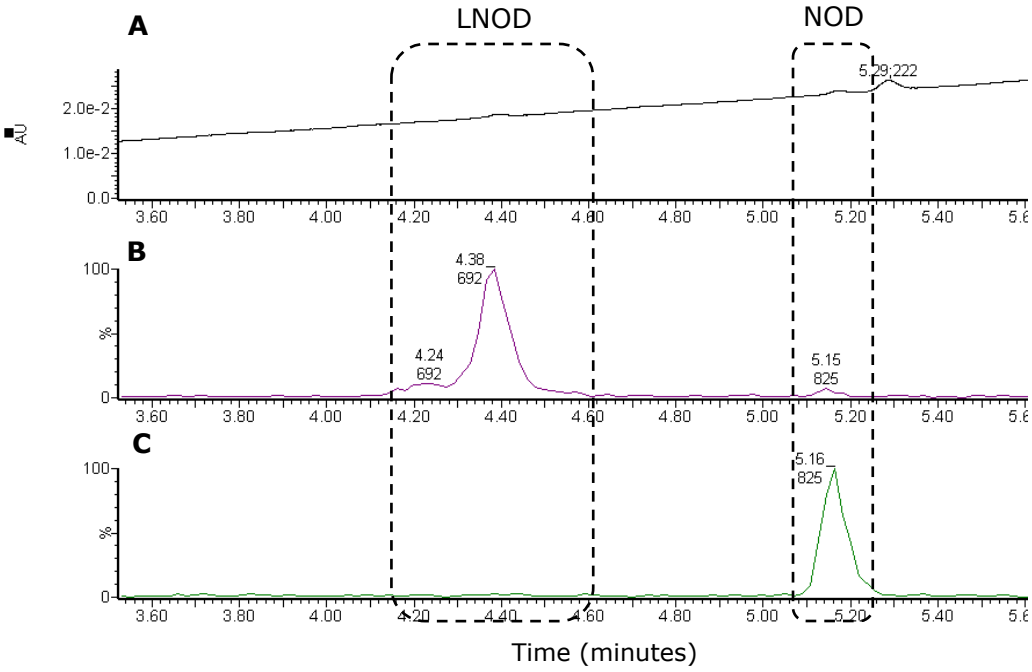

Figure 3. 18 Intracellular NODs detected in water sample following the exposure of *M. edulis* to *N. spumigena* KAC66. Samples were analysed utilising UPLC-PDA (238 nm; A) and UPLC-QToF-MS recorded at m/z 692 (LNOD; B) and m/z 825 (NOD; C)

Alternatively, the difference in salinity of the culture medium (~ 6 psu) and the tank water (~35 psu) could have induced cell lysis and the release of NODs into the water. However, NODs were not detected in extracellular samples (0). Following re-evaluation of the feeding procedure, it was noticed that water

samples were not taken at the actual time of feeding (t_0). The *N. spumigena* KAC66 feeding stock culture was added to tanks containing mussels and the overall feeding procedure lasted ~30 min. Consequently, mussels to which the cyanobacterium was added first had already been feeding for approximately 30 min when water samples were taken. In addition, mussels were able to accumulate NODs which potentially led to intracellular toxin levels below the LOQ. This was supported by pictures taken immediately after addition of *N. spumigena* KAC66 to the exposure tanks and 30 min post feeding (Figure 3. 19). A considerable reduction in green colour representing the presence of the cyanobacterium was observed in combination with a slight scum formation (Figure 3. 19). Reported clearance rates (CR) of *M. edulis* feeding on water enriched with the diatom *S. costatum* ranged from 2.2-11.0 L g^{-1} h^{-1} (Petersen *et al*., 2004). Based on the tank volume of 13 L and the 70 specimens present in each tank, a large proportion of *N. spumigena* KAC66 was likely to be filtered within the feeding time of 30 min. This could explain the observed decrease of NODs in filter samples. Cell aggregation of *N. spumigena* KAC66 on the water surface was observed (Figure 3. 19 B). Potentially, this could be associated with the change in salinity experienced when transferring the culture (~ 6 psu) to the seawater tanks (~35 psu). Care was taken to thoroughly mix the tank water before obtaining water samples, hence the aggregation should not have affected the intracellular toxin results obtained.

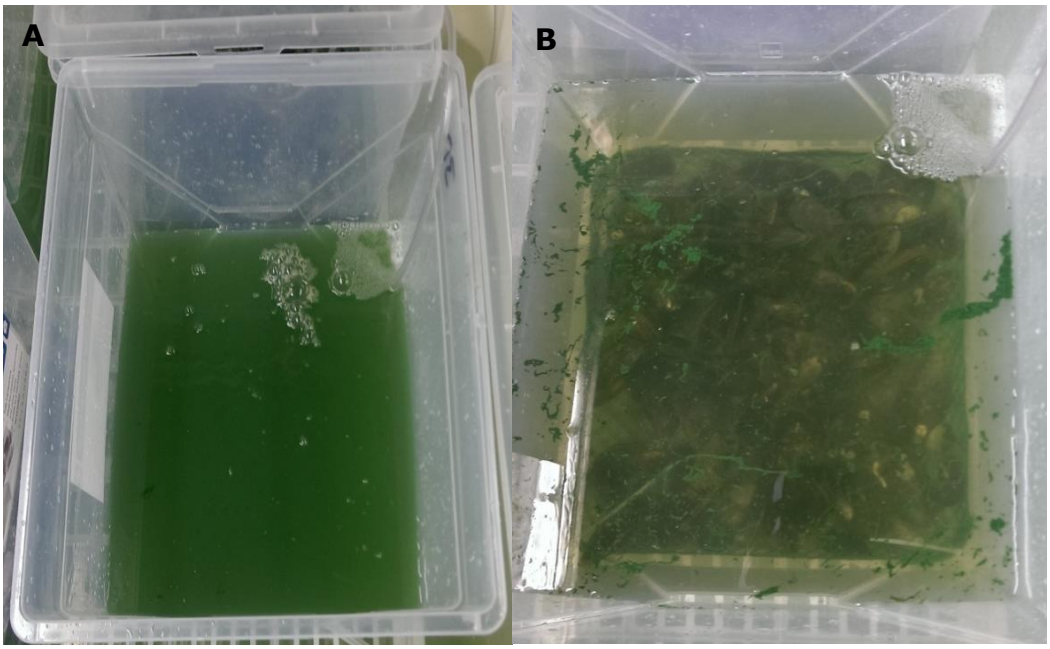

Figure 3. 19 Example exposure tank photographed immediately after feeding (A) and 30 minutes post feeding (B) illustrating the rapid clearance of green colour associated with the presence of *N. spumigena* KAC66.

In the evening of day one small beige coloured particles floating on the water surface were observed. These particles were suspected to be pseudofaeces (particles rejected prior to ingestion) produced by *M. edulis* (Figure 3. 20; Svensen *et al*., 2005; Strogyloudi *et al*., 2006). High cell densities of above 70 x 10^6 cells per litre have been reported to cause the production of pseudofaeces (Schulte 1975). However, lower exposure concentrations of *N. spumigena* KAC66 were utilised in this study (~ 2.7 x 10^6 cells per tank). Despite cell density, the actual particle size can affect the formation of pseudofaeces in *M. edulis* (Defossez and Hawkins, 1997). Particles with a diameter of 7.5 µm and above were preferably rejected. In addition, *M. edulis* selectively rejected the diatoms *S. costatum* (diameter ~9 µm) and *Pavlova lutheri* (diameter ~4 µm; Bougrier *et al*., 1997). Another diatom (*T. suecica*) with a diameter of ~8 µm was preferably ingested, which was associated to its high carbon content compared to the remaining two species.

Being a filamentous cyanobacteria strain *N. spumigena* KAC66 can reach length of up to 400 µm (Figure 3. 21). Therefore, it was likely that filaments formed coils while mussels were filtering the water, reaching overall diameters of 7.5 µm and above. Production of pseudofaeces by *M. edulis* following exposure to high density *N. spumigena* cultures has been demonstrated in laboratory studies

when utilising cultures at final concentrations of 60 µg Chl-a L^{-1} and 5 µg Chl-a L^{-1} (Svensen *et al*., 2005; Strogyloudi *et al*., 2006). The Chl-a concentration in experimental tanks utilised during this study ranged from 273-763 µg L^{-1} in exposure tanks, respectively. Compared to the literature this was considerably higher and agrees with the reported pseudofaeces production at high cell densities. The observed increase in NODs in filter samples of day seven compared to the expected values could have been caused by the pseudofaeces present in the water column (Figure 3. 20). To what extent this might have happened on previous days cannot be stated due to the low concentrations encountered. It could be possible that the selective rejection of *N. spumigena* KAC66 only started on day seven, as a reaction to the toxic effect of NODs. All samples taken approximately 19 h after feeding did not contain NODs at levels above LOD. Both filter and filtrate samples were analysed after the dominance of LNOD was discovered. Hence, only NOD standards had been analysed for quantitation purposes. Linear NOD was quantified retrospectively utilising the calibration data (section 3.2.2.1).

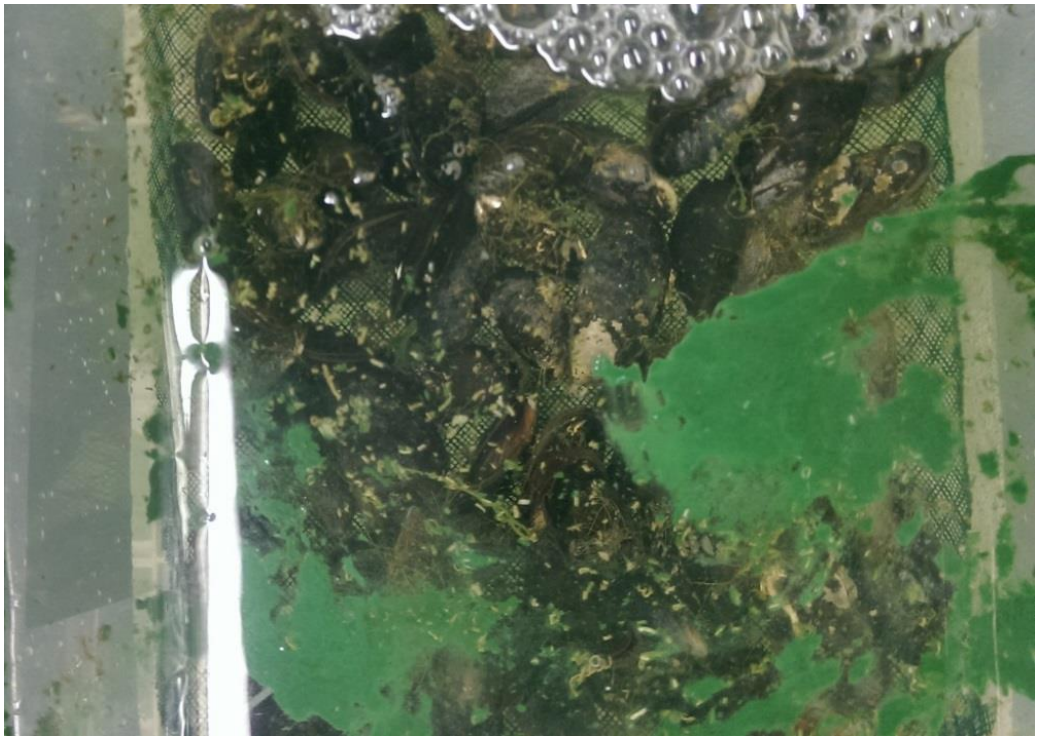

Figure 3. 20 Example of pseudofaeces (beige coloured particles) produced by *M. edulis* in tanks exposed to *N. spumigena* KAC66 on day one

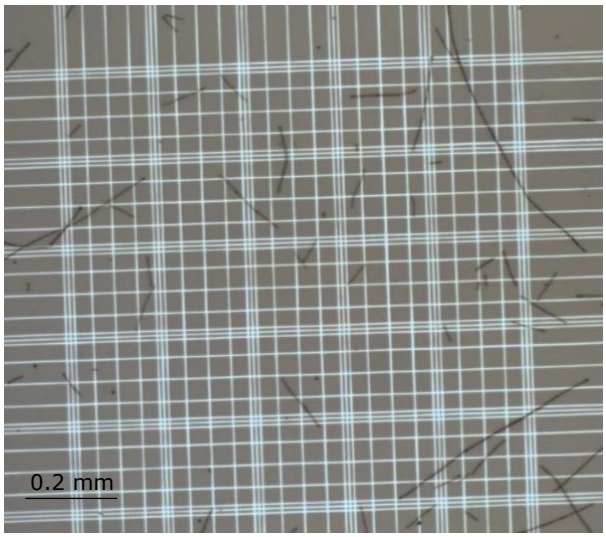

Figure 3. 21 Example photomicrograph of *N. spumigena* KAC66 (40x).

3.3.3.2 Dissolved nodularins

Nodularins were below the LOQ (0.05 µg mL^{-1}) in all filtrate samples during both the accumulation and depuration period (Figure 3. 22). Hence, the majority of detectable NODs were present within *N. spumigena* KAC66 cells, which was in agreement with reports by Møgelhøj *et al*., (2006). They detected extracellular NOD only in the stationary growth phase (after 12 days of inoculation) where it amounted to up to 12% of overall NOD.

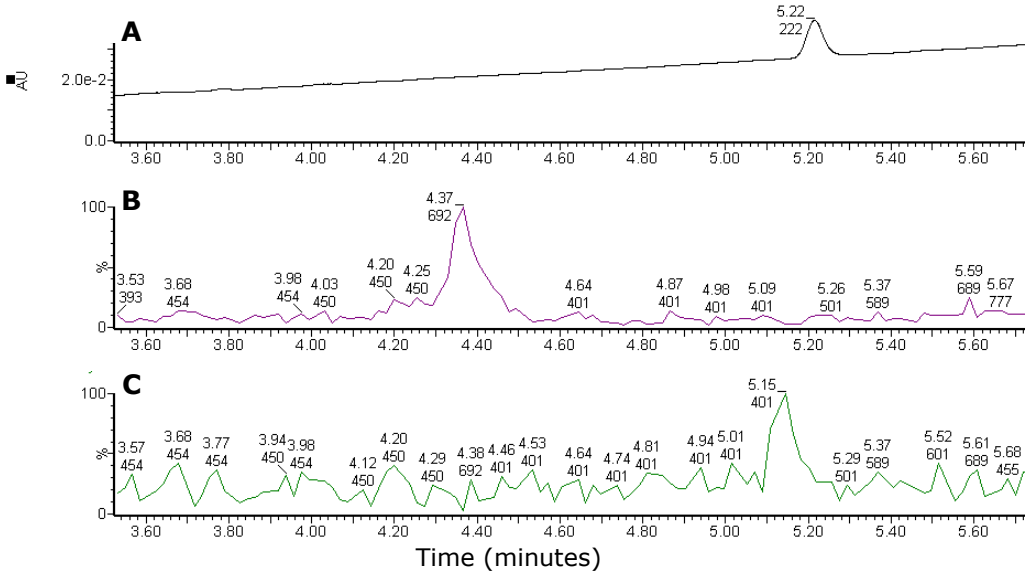

Figure 3. 22 Free NODs detected in water sample following the exposure of *M. edulis* to *N. spumigena* KAC66. Samples were analysed utilising UPLC-PDA (238 nm; A) and UPLC-QToF-MS recorded at m/z 692 (LNOD; B) and m/z 825 (NOD; C)

3.3.4 Quantitation of nodularins in *M. edulis* following the exposure to *N. spumigena* KAC66

Immediately, 24 h post exposure NODs were detected in mussel flesh (3 μg g^{-1}). Throughout the seven day accumulation period a steady increase was observed except following the last feeding on day seven (Figure 3. 23). Potentially the activity of elimination processes of *M. edulis* could have resulted in this reduction. In addition, the cells utilised contained lower NOD levels (Table 3. 8). The maximum concentration was detected on day six with a total cyanotoxin concentration of 17 μg g^{-1} which was followed by an decrease to ~7 μg g^{-1} detected on day 14 (~44% compared to day six). Utilising the logarithmic trend line starting from day seven (y=-8.282ln(x) + 30.591; Figure 3. 23) full depuration of NODs would be expected following 40 day depuration. During the feeding trial mortality was below 1% which was well within the range reported in the literature (Kankaanpää *et al.*, 2007; Lehtonen *et al*., 2003; Strogyloudi *et al*., 2006; Table 1.5).

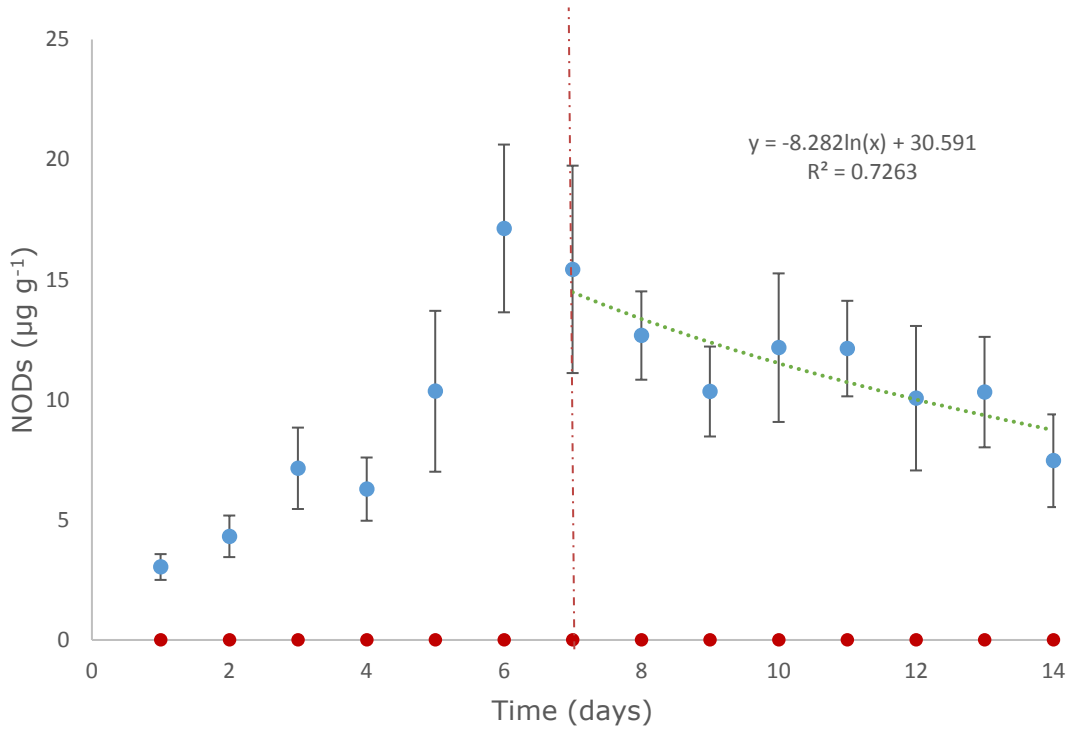

Figure 3. 23 Nodularins detected in *M. edulis* following exposure to *N. spumigena* KAC66 (blue) and shellfish diet (red) for seven days as determined by UPLC-QToF-MS analysis (LNOD m/z 692, NOD m/z 825). Error bars represent one standard deviation. The red dashed line indicates the end of the accumulation period. During the depuration period all mussels were fed shellfish diet. The green dotted line illustrates the logarithmic trend line for nodularin depuration.

Compared to the present study, more rapid accumulation was reported in *M. edulis* following exposure to *N. spumigena* KAC13 at a slightly lower exposure concentration (0.0156 µg mL^{-1}; Strogyloudi *et al*., 2006). Tissue concentrations reached values of 13.8 ± 5.26 µg g^{-1} after 12 h. Comparable values were reached only on day six of the present study. Similarly, rapid NOD accumulation was reported in *Macoma balthica* when exposed to *N spumigena AV1* (0.020 µg mL^{-1}; Lehtonen *et al*., 2003). Up to 16.6 µg g^{-1} NOD were detected in mussel flesh 24 h post exposure based on ELISA analysis. This was more than five times higher compared to levels reached after 24 h exposure in the present study (3.0 µg g^{-1}). Potentially, this difference could be associated to the different organisms utilised, illustrating varying uptake dynamics. Also, potential differences in water temperature could account for varying uptake dynamics, however, no clear temperature values were given in above reports (Lehtonen *et al*., 2003; Strogyloudi *et al*., 2006). In addition, ELISA analysis could have caused

overestimation of NOD due to the assay's positive response to Adda containing metabolites and potential degradation products.

Persson *et al.* (2009) have suggested that NOD can persist in flounder muscle from *Nodularia* blooms of previous years, supporting the slow depuration observed during the present study. Similarly, residual NOD was detected in *M. edulis* collected from the Baltic Sea despite the absence of a *Nodularia* bloom (Kankaanpää *et al.*, 2007). This was also reported for sediment and mussel samples obtained from the Gulf of Gdańsk which contained maximum NOD concentration in mussel of 0.139 μg g^{-1} (Mazur-Marzec *et al*., 2007). These field observations were supported by laboratory experiments in which *M. edulis* were exposed to a single dose of *N. spumigena* (0.07-0.11 μg mL^{-1}). Following the initial rapid NOD decline 24 h post exposure (1.2 to 0.7 μg g^{-1}), NOD concentration remained constant for the next five and a half days at approximately 0.6 μg g^{-1} (Kankaanpää *et al*., 2007).

Contradictory to these reports, rapid NOD depuration was reported in *M. edulis* after a three day exposure to *N. spumigena* KAC13 (NOD 0.2-15.6 x 10^{-3} μg mL^{-1}; Strogyloudi *et al.*, 2006). Following only three days of depuration, the NOD concentration had been reduced by 75%. Compared to the present study, even after seven days of depuration NODs concentration was only reduced to ~7 μg g^{-1} which represents a reduction of ~56 % of the concentration detected at the end of the exposure period.

It was noticed that the ratio of NOD to LNOD within the mussel flesh changed during the time course of the feeding trial. The percentage of NOD compared to total NODs detected in *M. edulis* showed a constant increase (Figure 3. 24). Following the first feeding NOD and LNOD amounted to 35% and 65% respectively, of total cyanotoxin detected. By day three this was nearly reversed and values stayed constant until the end of the experiment (59% NOD, 41% LNOD). Beattie *et al*. (2003) suggested NOD excretion via conjugation with glutathione (GSH) following *in vitro* assays in brine shrimp. However, no change in glutathione-s-transferase activity, neither during a 24 h exposure period nor during the six day depuration period, was reported in *M. edulis* when exposed to *N. spumigena* extracts (Kankaanpää *et al*., 2007). In addition, no GSH conjugates were detected which was interpreted as NOD-GSH being ejected via unspecific pumps within the membrane.

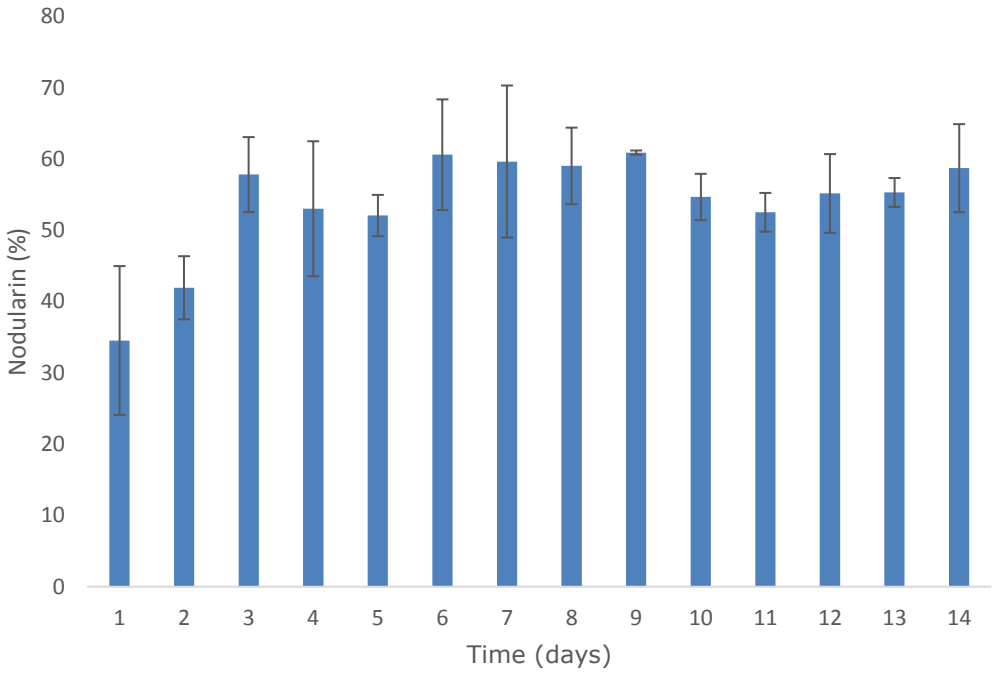

Figure 3. 24 Percentage of NOD detected in *M. edulis* flesh compared to total NODs after seven day exposure to *N. spumigena* KAC66 as determined following UPLC-QToF-MS analysis. Values are mean ± standard deviation (n=3)

Further studies have also shown a lack of NOD-GSH formation when analysing flounders (Mazur-Marzec *et al*., 2007; Karlsson *et al*., 2003; Vuorinen *et al*., 2009) and mussels (Kankaanpää *et al*., 2007; Mazur-Marzec *et al*., 2007; Karlsson *et al*., 2003; Sipiä *et al*., 2002) from areas within the Baltic Sea known to be affected by *Nodularia* blooms. In addition, during the laboratory exposure of the clam *Macoma balthica* to *N. spumigena* no GSH conjugates were detected (Lehtonen *et al*., 2003). Potentially, this lack of detection could have been associated with conjugate instability during MS detection (Karlsson *et al*., 2003). No GSH conjugates were detected in mussel flesh, faecal material, or water samples of the present study. Currently only Sipiä *et al.* (2002) have reported the presence of NOD-GSH (m/z 1131.56) in mussel flesh. However, not all of the above studies investigated the presence of NOD cysteine conjugates (NOD-Cys) which could have been the dominant metabolite as found for MC-LR (Li *et al*., 2014). Karlsson *et al.*, 2005 synthesised amongst others NOD-GSH and NOD-Cys conjugates. Utilising LC-MS four Cys and four GSH conjugates were detected. NOD-GSH especially showed shortened retention times of 1.4-2.4 min compared to 4.3 min for NOD. This could potentially explain the lack of

conjugate detection in this study as they might have been too polar to be retained. However, no evidence for NOD conjugates were found in the solvent front of the analysed samples.

As the feeding trial was not performed in a sterile environment, both biotic and abiotic degradation could have also contributed to the loss of eliminated NOD, LNOD, and/or their metabolites. Heresztyn and Nicholson (1997) reported rapid degradation of NOD after blooms in Australian lakes in which NOD half-lives of 24 h were associated with the presence of microorganisms. Linear NOD has been described as the first intermediate product of this microbial degradation (Mazur-Marzec *et al*., 2009; Edwards *et al*. 2008; Kato *et al*., 2007; Feng *et al*., 2016). Bacteria capable of NOD degradation originated from water or sediment samples. Within 72 h *Sphingopyxis* sp. USTB-05 completely degraded NOD from a 11.6 mg L^{-1} solution (Feng *et al*., 2016). Similarly, cell extract of a bacterial strain isolated from Lake Tsukui in Japan (Imanishi *et al*., 2005) and bacterial communities found in sediments of the Baltic Sea (Mazur-Marzec *et al*., 2009) degraded NOD. This process followed a two-step mechanism: first the hydrolysis of NOD resulting in LNOD via the cleavage of the Arg-Adda bond (Kato *et al*., 2007; Feng *et al*., 2016; Figure 3. 25). The second and final step was the disruption of the Adda-Glu bond of LNOD during which the Adda fragment was released (Feng *et al*., 2016).

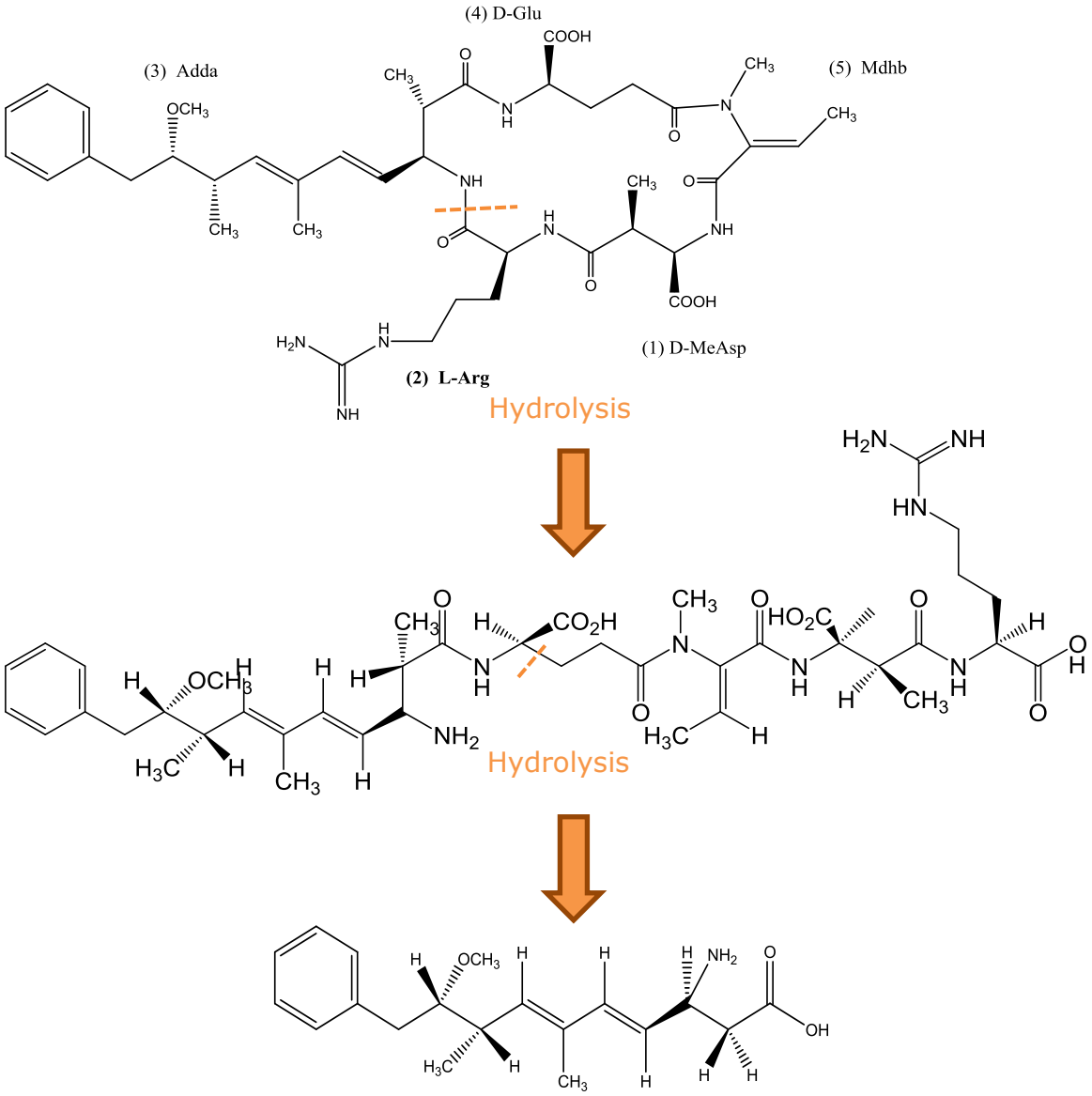

Figure 3. 25 Microbial degradation of NOD (adapted from Edwards *et al*., 2008; Feng *et al*., 2016; Imanishi *et al*., 2005)

The results obtained in this study, combined with the available literature, illustrate the need for further research to fully elucidate the metabolism of cyanotoxins such as NOD in seafood. Generally, the risks cyanotoxin contaminated seafood can pose have been recognised and a provisional tolerable daily intake (TDI) guideline of 0.04 µg MC-LR kg^{-1} body weight has been issued by the WHO (Kuiper-Goodman *et al*., 1999). Due to its similar toxicity, the same guidelines should be utilised for NODs (Kuiper-Goodman *et al*., 1999). For an average 75 kg person this would correspond to a TDI of 3 µg MC-LR. However, shellfish are generally eaten seasonally, hence lifetime TDI guidelines do not consider the acute and seasonal exposure to cyanotoxins via contaminated seafood. Therefore, Ibelings and Chorus (2007) have derived acute (per single exposure) and a seasonal daily tolerable intakes of 190 and 30 µg MC-LR, respectively.

Based on a typical portion size of 20 mussels (~ 4 g each, section 5.3.4) the NODs intake was calculated based on results obtained during the feeding trial (Equation 3. 2). Results showed that eating a portion of mussels at the highest NODs concentration (day six) would result in the ingestion of approximately 1.4 mg which was more approximately 7 and 46 times higher than the recommended acute and seasonal tolerable intake, respectively. In addition, even following one week of depuration, consumption of 20 mussels would still result in exposure to more than 3 and ~20 times the recommended acute and seasonal limits.

Equation 3. 2 Cyanotoxin load expected in a serving of 20 mussels obtained on day six and day 14 of the present study.

On day six

$$m_{LNOD\ and\ NOD} = m_{mussel} \times n_{mussel} \times c_{LNOD\ and\ NOD}$$
$$m_{LNOD\ and\ NOD} = 4\ g \times 20 \times 17.133\ \mu g\ g^{-1}$$
$$m_{LNOD\ and\ NOD} = 1370.64\ \mu g$$

On day 14

$$m_{LNOD\ and\ NOD} = m_{mussel} \times n_{mussel} \times c_{LNOD\ and\ NOD}$$
$$m_{LNOD\ and\ NOD} = 4\ g \times 20 \times 7.462\ \mu g\ g^{-1}$$
$$m_{LNOD\ and\ NOD} = 596.96\ \mu g$$

m = amount in µg n= number of specimen c= concentration in mussel flesh

The calculated human exposure levels were obtained after exposing *M. edulis* to 2.5 L of *N. spumigena* KAC66 containing NODs at concentrations ranging from 0.1 to 0.6 µg mL^{-1} which was well within the values reported in environmental samples (Table 3. 9). However, it needs to be noted that during this experiment mussels were kept within small tanks filled with a volume of 13 L. It is likely, that in real life bloom scenarios the volume would be considerably larger. In addition, *N. spumigena* is known to form scums on the water surface. These would not necessarily be available as food source for mussels. Consequently, toxin loads encountered during this experiment were likely considerably higher compared to real life scenarios.

Table 3. 9 Intracellular NOD concentration reported during global bloom events

Waterbody	Country	c_{max} µg mL^{-1}	Reference
Køge Bay (Baltic Sea)	Denmark	0.565	Henriksen (2005)
Gulf of Gdańsk (Baltic Sea)	Poland	25.85	Mazur-Marzec *et al*. (2006a)
Lake Alexandria	Australia	0.002	Heresztyn and Nicholson (1997)
Baltic Sea	Germany	0.090	Dahlmann *et al*. (2003)
Gulf of Bothnia (Åland)	Finland	0.61	Spoof *et al*. (2003)
Binder Lake	United States	0.19	Graham *et al*. (2010)
Tai Lake	Taiwan	0.004*	Yen *et al*. (2011)
San Roque Reservoir	Argentina	2.4×10^{-4}*	Galanti *et al*. (2013)

c_{max} - Maximum reported concentration * also includes extracellular toxin

Based on the amount of NODs detected in the daily feed stock cultures, the recovery of NODs within mussel flesh was calculated (Table 3. 10). For the purpose of the calculation it was assumed that one mussel weighed 4 g. Best recovery was seen on day six with 83%. For the remaining days recoveries ranged from 38-71%. These values were considerably higher compared to those obtained in clam *M. balthica* after exposure to *N. spumigena.* Only 0.2% of the added dose was recovered in clam flesh (Karlson and Mozūraitis 2011). Similarly, 0.53% of the administered dose was recovered in sea trout liver eight days after being exposed to a single dose of *N. spumigena* AV1 containing slurry. Dose recovery in trout muscle was even lower (0.055%; Kankaanpää *et al*., 2002). Neither NOD nor potential metabolites were detected utilising HPLC-PDA

analysis(Kankaanpää *et al*., 2002). Higher dose recoveries of ~30%, were reported for NOD in black tiger prawns (*Penaeus monodon*) after oral exposure to NOD containing feed (Kankaanpää *et al*., 2005). In addition, NOD recoveries in *M. edulis* were reported to be concentration dependant. Exposed to *N. spumigena* KAC13 at NOD concentrations of 1.6 x 10^{-3} µg mL^{-1} for 12 h resulted in dose recoveries of 9%. This was increased to 12% for the high concentration experiment (15.6 x 10^{-3} µg mL^{-1}; Strogyloudi *et al*., 2006).

Table 3. 10 Recovery (%) of NODs in *M. edulis* after exposure to *N. spumigena* KAC66 compared to the amount of NODs available in feed stock cultures

Time in days	NODs available (µg)*	NODs in mussel (µg)	Recovery (%)
1	1417	850	60
2	2783	1208	43
3	4172	2001	48
4	4587	1758	38
5	4587	2899	63
6	5772	4797	83
7	6094	4320	71

*based on cumulative NODs in feed stock culture samples

Generally, metabolism processes aim to increase the polarity of the compound to achieve effortless elimination of the xenobiotic. This could have resulted in very polar, yet unknown, products which may not have been detected by HPLC. Lehtonen *et al*. (2003) suggested that *M. balthica* was able to metabolise NOD due to higher concentration of a NOD-like compound the authors detected in the water at 96 h post exposure compared to 24 h. The identity of this compound could not be elucidated. However, the presence of NOD isomers and NOD-GSH was excluded. The authors did not investigate the presence of NOD-Cys which has been suggested to be the end product of NOD elimination (Karlsson *et al*., 2005). A small proportion of NOD and/or LNOD could have been lost during the shucking process as the extrapallial fluid that drained from mussels was not collected. As considerable amounts of NODs were accumulated by *M. edulis*, monitoring of these in seafood should be considered. In addition, due to the worst case dose recovery of 38%, a correction factor of 2.6 could be applied when analysing NODs in *M. edulis* to protect human health and wildlife.

3.3.5 Quantitation of nodularins in faecal pellet of *M. edulis* following exposure to *N. spumigena* KAC66

As early as 24 h post exposure, NODs were detected in faecal material collected from the mussel tanks (example chromatogram Figure 3. 26). During the following days the amount of NODs increased until maximum values of 5.64 µg per exposure tank were reached on day seven. (Figure 3. 27). No NODs were detected in faecal material during the depuration period (days eight to 14).

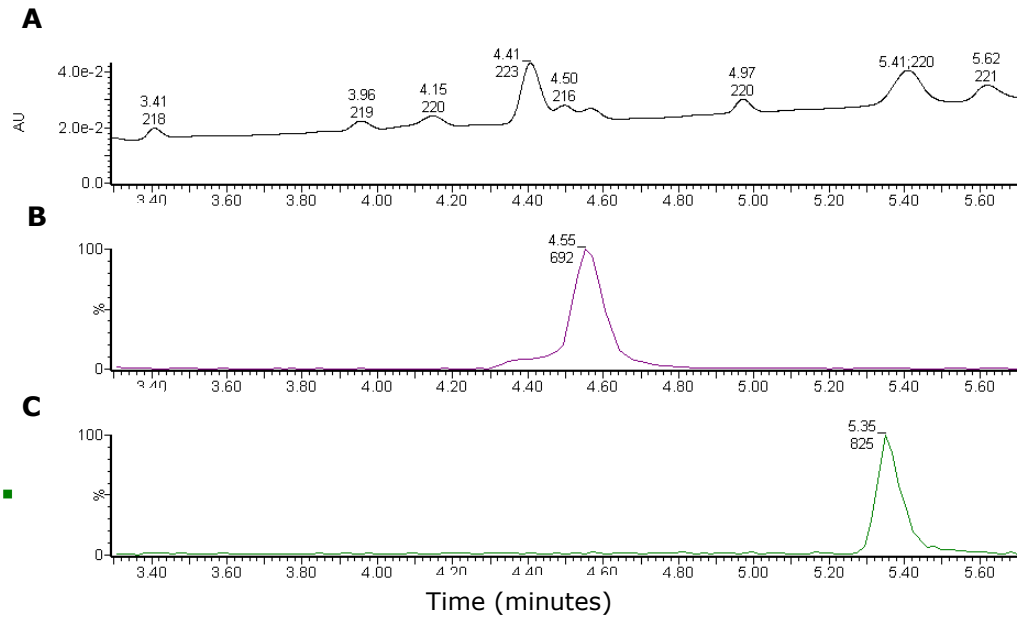

Figure 3. 26 NODs detected in faecal material of *M. edulis* following five day exposure to *N. spumigena* KAC66. Samples were analysed utilising UPLC-PDA (A) and UPLC-QToF-MS analysis recorded at m/z 692 (LNOD; B) and m/z 825 (NOD; C)

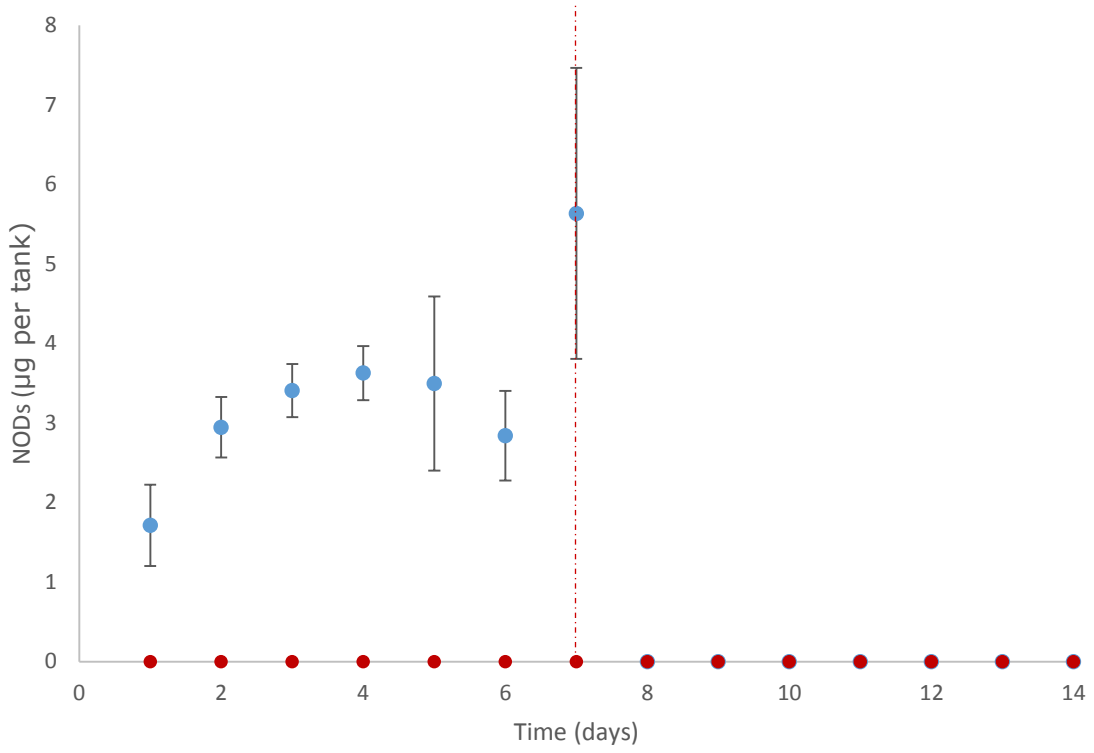

Figure 3. 27 Amount of NODs detected in faecal material of *M. edulis* following seven day exposure to *N. spumigena* KAC66 (blue) and shellfish diet (red). Analysis was performed utilising UPLC-QToF-MS analysis (LNOD m/z 692, NOD m/z 825). Values are mean ± standard deviation (n=3). The red dashed line marks the end of the exposure period.

These results were in agreement with reports of NOD detected in faeces of *M. edulis* (Strogyloudi *et al*., 2006; Svensen *et al*., 2005). Svensen *et al*. (2005) found that when exposed to *N. spumigena* (0.05 µg g^{-1} dw NOD) for 48 h *M. edulis* eliminated ~95 µg g^{-1} dw NOD via their faeces. The overall amount of NOD present in faeces of 60 mussels was 11.21 µg. During the present study the cumulative amount of NODs detected in faecal pellets produced by 70 mussels over the first 48 h was 4.66 µg g^{-1} ww. In order to compare the reported values the amount of cyanotoxin in faecal material of 60 mussels was calculated. For the purpose of this calculation, the average weights of faecal pellets produced by 70 mussels, obtained during a later feeding study (section 5.3.4), were utilised to calculate the expected faecal material production of 60 mussels (Equation 3. 3). Based on those results 60 mussels produced faeces containing 3.83 µg NODs, which was about three times lower compared to the results of Svensen *et al.* (2005).

Equation 3. 3 Calculation of expected NOD content (µg) in faeces of 60 mussels

$$m_{faeces\ of\ 70\ mussels} = 0.96\ g\ ww$$

$$m_{faeces\ 60\ mussels} = \frac{70}{0.96\ g\ ww} = \frac{60}{x}\ \ x = \frac{60\ x\ 0.96\ g\ ww}{70} = 0.82\ g\ ww$$

$$c_{NODs\ after\ 48\ h} = 4.66\ \mu g\ g^{-1}$$

$$m_{NODs\ after\ 48\ h} = c\ x\ m_{faeces\ 60\ mussels} = 0.82\ g\ x\ 4.66\ \mu g\ g^{-1} = 3.83\ \mu g$$

The difference of NODs detected in faecal material could potentially be associated to the different feeding regimes. Svensen *et al*. (2005) exposed *M. edulis* every 12 hours to *N. spumigena* KAC13 (final NOD concentration 16 µg L^{-1}). This was considerably lower compared to exposure levels of the present study (129-624 µg L^{-1}). Once the different feeding regimes were taken into consideration (Table 3. 11), it was determined that at considerably lower exposure doses, *M. edulis* produced faeces containing three times more NOD when exposed to the strain *N. spumigena* KAC13 compared to KAC66. This could potentially be explained due to LNOD being the dominant congener detected in the present study. Due to its higher polarity it could have been eliminated more easily than NOD. However, no increase in extracellular NODs was detected in tank water. In addition, LNOD could have been subjected to abiotic and/or biotic degradation as well as metabolism processes within the mussel (section 3.3.4). Although, the addition of shellfish diet to encourage feeding during the present study could have influenced the results.

Table 3. 11 Comparison of NODs exposure doses utilised in the present study and the NOD concentrations of Svensen *et al*. (2005)

Parameter	**Present study - *N. spumigena* KAC66**	**Svensen *et al*. (2005) – *N. spumigena* KAC13**
Tank volume (L)	13	7
NODs in tank water (µg L^{-1})	129-624*	16
Number of feedings per day	1	2
Available NODs (µ)	1677-8112*	224
Number of mussels	70	15
Available NODs per mussel per day (µ)	24-116*	15

* minimum and maximum values

Compared to the NOD exposure dose Svensen *et al*. (2005) were able to recover approximately 25% in faecal material. In the present study considerably less NODs were recovered in faecal material: only approximately 0.1-0.4% of the dose. This low recovery was similar to values of NOD reported by Strogyloudi *et al*. (2006). In their study, following the twelve hour exposure of *M. edulis* to *N. spumigena* at high (15.6 µg L^{-1}) and low (1.6 µg L^{-1}), recovery was 0.1% and 0.2% for high and low exposure, respectively. These results indicate, that during *N. spumigena* blooms, re-ingestion of faeces represents an additional NOD exposure route. This supports the findings of Svensen *et al*. (2005), who demonstrated that *M. edulis* can accumulate NOD from contaminated faeces. However, given the small amount of faeces produced and the likelihood of cyanotoxin degrading bacteria being present in natural environments, this would be the less dominant route of cyanotoxin exposure.

3.4 Conclusion

A conversion of NOD to LNOD was seen in *N. spumigena* KAC66 cultures following transport. The change in temperature and the paucity of light could not be proven to have caused this conversion. During the exposure trial NODs were rapidly accumulated by *M. edulis* with the maximum concentration detected on day six (~17 µg kg^{-1} ww). Eating a portion of 20 mussels, obtained on this day would result in ingestion of approximately 7 and 46 times the recommended acute and seasonal tolerable intake for NOD. The observed slow depuration of NODs within mussel flesh further illustrated the risk that NODs contaminated seafood can pose to higher predators not only during but also following bloom events. Based on the depuration trend observed, full depuration of NODs would be expected only after 40 days. It is therefore vital to monitor *M. edulis* in areas of potential *Nodularia* blooms to minimise exposure risks to the general public and wild life. In addition to mussel flesh, NODs were excreted via faeces produced by *M. edulis* during the seven day exposure period. Based on quantities encountered faeces represented only a minor NODs exposure route during *N. spumigena* bloom events.

CHAPTER 4

The accumulation and depuration of six microcystins in *Mytilus edulis* after seven day exposure to *Microcystis aeruginosa* PCC 7813

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Table 4. 15 Distribution of individual MC congeners (µg) in the experimental system following the exposure of *M. edulis* to *M. aeruginosa* PCC 7813. 259

4.1 Introduction

The dangers MC ingestion can pose for humans have been recognised by the WHO, who released a guideline maximum MC concentration in drinking water of 1 μg L^{-1}. Humans can be exposed to these hepatotoxins produced by mainly freshwater cyanobacteria via exposure to contaminated water and/or seafood with potentially severe consequences. Generally, there is a large body of evidence of MC causing adverse human health effects (Svirčev *et al*., 2016). Acute exposure caused the death of 72 people as reported in Brazil following dialysis with MCs contaminated water (Jochimsen *et al.,* 1998), and chronic low dose exposure has been linked to higher risks of cancer when MCs detected in river and pond water were positively correlated to increased incidents of colorectal cancer in Haining China (Lun *et al*. 2002). Furthermore, MCs were correlated to incidences of primary liver cancer in Haimen city (China, Ueno *et al*., 1996). In both studies MCs were detected at concentrations in the range of ng L^{-1}, considerably lower than the WHO guideline.

Indirect exposure to MC via the ingestion of contaminated seafood on the other hand, have not yet been thoroughly investigated. An acute and seasonal tolerable intake of 190 and 30 μg for a 75 kg adult has been determined based on calculated no adverse effect levels in humans (Ibelings and Chorus 2007). As MCs are known to accumulate in mussels (Amorim and Vasconcelos 1999; Vasconcelos 1995), fish (Bury *et al.*, 1998; Li *et al*., 2007; Freitas de Magalhães *et al*., 2001; Xie *et a*l., 2004; Sotton *et al*., 2012; Kankaanpää *et al.,* 2002; Kohoutek *et al*., 2010; Lance *et al*., 2014; El Ghazali *et al*., 2010; Palikova *et al*., 2011; Adamovský *et al*., 2007), and plants (Mohamed and Al Shehri 2009; Tyler *et al*., 2009; Pflugmacher *et al*., 2001; Cordeiro-Araújo *et al*., 2016) the risks of MC transfer to higher predators including humans need to be evaluated.

Recent reports of *Microcystis* blooms being transported into estuaries have highlighted the dangers freshwater toxins can pose to marine life. Tango and Butler (2008) reported maximum MCs levels of 658 μg L^{-1} in water samples taken from Chesapeake Bay. They were suggested to have originated from a *Microcystis* bloom in the Sassafras River. In addition, Mazur and Pliński (2003) reported the presence of MC-LR and MC-RR in water samples taken in Gulf of Gdańsk near the mouth of the Vistula River. This was further supported by Vareli

et al. (2012) who detected MCs in the Amvrakikos Gulf (30-34 psu). Even though levels were below the WHO limit of 1 µg L^{-1} (0.003-19.8 ng L^{-1}) field samples of *M. galloprovincialis* contained MCs at concentrations of up to 142 ng g^{-1}.

These reports illustrate that freshwater toxin can contaminate the marine environment and consequently be accumulated by marine fish and shellfish. Evidence for this transfer was found in blue crab, sampled from the lake Lac des Allemands located in an estuary system of South Louisiana, which accumulated up to 105 µg kg^{-1} MC-LR in muscle tissue (Garcia *et al*., 2010). Consequent exposure to MC contaminated fish and shellfish can have fatal effects on higher predators as reported in sea otters (*Enhydra lutris nereis*; Miller *et al*., 2010). Animals died following the ingestion of MC contaminated seafood for which the likely MC source was a *Microcystis* bloom, present in Lake Pinto that had been transported into the coastal area via the Pajaro River (Miller *et al*., 2010). A recent publication by the European Food Standard Authority has pointed out that the potential of estuarine and coastal aquaculture being exposed to cyanotoxins remains a global concern and represents an arising hazard for the general public (Testai *et al*., 2016).

Based on the large body of evidence, freshwater cyanobacteria do pose risks to human consumers not only in freshwater, but also in estuary and marine environments. It was therefore the aim of the present study to first evaluate the short-term (24 h) salinity tolerance of *M. aeruginosa* PCC 7813, a MC producing freshwater cyanobacterium. Furthermore, the accumulation and depuration of six MCs in *M. edulis* following the exposure to *M. aeruginosa* PCC 7813 was assessed and the MC budget of the experimental system determined. Results will provide vital data for the assessment of potential risks to the general public associated with MC contaminated mussels.

4.2 Materials and Methods

4.2.1 Chemicals

Isoton II and Cleanz solution were provided by Meritics (United Kingdom). MeCN and water were of LC-MS-grade (Fisher Optima, ThermoFisher, UK). Formic acid (FA) was of LC-MS reagent grade. MeOH and water used for sample preparation were of HPLC grade (all Fisher, ThermoFisher, UK). Reference toxins of MC-LY, MC-LF, MC-LW, MC-HilR, MC-LR, and Asp^3-MC-LR were obtained from Enzo Life Sciences, Exeter, UK. A certified standard of [Dha^7]-MC-LR was obtained from the Institute of Biotoxin Metrology, National Research Council Canada (NRCC). A mixed toxin stock solution was prepared by opening CRM ampoules and accurately pipetting aliquots into vials, giving a concentration of 327.7 µg L^{-1} for each compound. A seven-level suite of working calibration standards was subsequently prepared through serial dilution of the multi-toxin stock solution using 50% aqueous MeOH as the diluent, resulting in a calibration range between 0.33 µg L^{-1} to 327 µg L^{-1}.

4.2.2 Media preparation and culturing of *Microcystis aeruginosa* PCC 7813

M. aeruginosa PCC 7813 was obtained from the Pasteur Culture collection of Cyanobacteria (Paris, France). Prior to the experiment cultures of *M. aeruginosa* PCC 7813 were maintained in 250 mL Erlenmeyer flasks containing 100 mL of sterilised BG-11. The BG-11 medium was prepared as previously described (section 3.2.3) except that no instant ocean salt was added. These cultures were scaled up to a final volume of 8 L and maintained in 10 L Pyrex glass flasks at 22 ± 1°C with a continuous light intensity of 10-15 µmol m^{-2} s^{-1}. Cultures were sparged with filtered air (0.22 µm filter, Millipore, UK). For the *M. edulis* feeding trial approximately 40 L of concentrated feed stock culture were prepared by terminating the aeration of *M. aeruginosa* PCC 7813 cultures three days prior to transport. This allowed cells to sink to the bottom of the culture vessel enabling easy disposal of the media supernatant. Concentrated cells were divided into four 10 L carboys and maintained during the feeding trial as described in Chapter 3.3.2.

4.2.3 Evaluation of MC-LR content of *M. aeruginosa* PCC 7813 grown in BG-11 containing seawater

During the planned feeding study the freshwater cyanobacteria strain *M. aeruginosa* PCC 7813 will be utilised as food source for *M. edulis* which are marine or brackish water organisms. Therefore, the salinity tolerance of *M. aeruginosa* PCC 7813 was investigated. Control samples were grown in BG-11 medium. For the salt exposure seawater (~ psu 35) was utilised to prepare BG-11 medium rather than deionised water (BG-11 SW). It had been sourced from Stonehaven harbour, Scotland (latitude 56° 58'N, longitude 2° 12' W). For the experiment 200 µL of *M. aeruginosa* PCC 7813 were inoculated into 27 1.5 mL centrifuge tubes containing 1 mL of BG-11 and another 27 tubes containing BG-11 SW. All inoculated samples were stored in a growth chamber (Fitotron, Weiss Gallenkamp, Leicestershire, UK) at 10 µmol m^{-2} s^{-1} and a temperature of 20°C. Intracellular and extracellular MC-LR content was assessed at the following time points (n=3): 0, 1, 2, 3, 6, 8, 10, 12, and 24 h post inoculation. Samples were centrifuged (10,433x g; 10 min) and supernatants were transferred to clean 1.5 mL centrifuge tubes. Both supernatant and pellets were freeze-dried. Following this the supernatant and pellet were re-suspended in 120 µL 80% aqueous MeOH, centrifuged (10,433x g, 10 min). Due to the observed MS signal loss of MC-LR following the analysis of seawater, the UV signal monitored at 238 nm (resolution 1.2 nm) was utilised for quantitation purposes (Figure 4. 1).

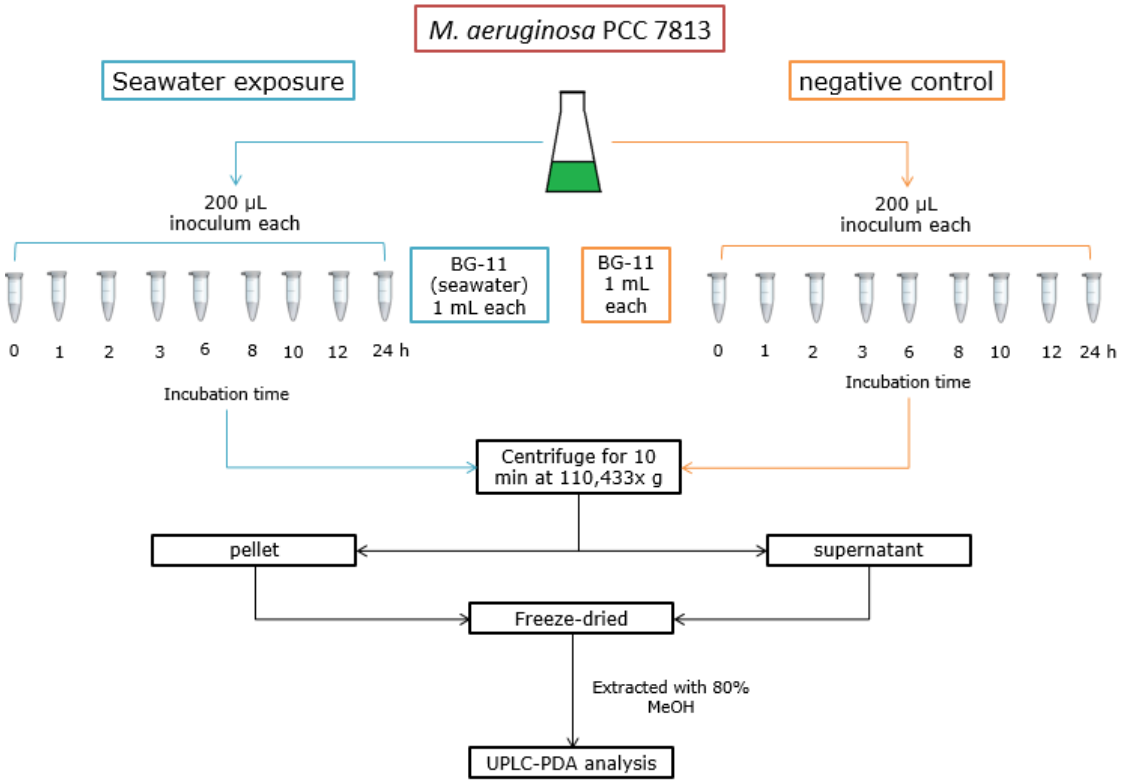

Figure 4. 1 Schematic illustration of *M. aeruginosa* PCC 7813 salinity tolerance experiment. Following inoculation into BG-11 and BG-11 prepared with seawater, the intracellular and extracellular MC content was determined at different time points. Samples were centrifuged and supernatant and cell pellet freeze-dried, extracted with 80% aqueous MeOH and analysed utilising UPLC-PDA.

4.2.4 Accumulation and depuration of microcystins in *M. edulis*

The experimental set up was identical to that described in section 3.2.4, except mussels (57.9 ± 7.4 mm) were exposed to 400 mL *M. aeruginosa* PCC 7813 at a final concentration of approximately 4.1 x 10^9 cells L^{-1} and 240-390 µg L^{-1} MCs. These levels were chosen as they were similar to NOD levels used in the previous study (chapter 3), which did not show any adverse effects on the mussels. In addition, 1.5 mL of shellfish diet was added to the exposure tanks to encourage feeding (final concentration ~106 x 10^9 cells L^{-1}). All negative control tanks were exclusively fed shellfish diet (final concentration 514 x 10^9 cells L^{-1}). Sampling was performed as previously described (section 3.2.4) except for depuration sampling time points of mussels. In order to extend the depuration period samples were collected on the following days: 1-7, 9, 11, 13, 17, 21, 25, and 29. Sample extraction was performed as described in section 3.2.5 except for *M. aeruginosa* PCC 7813 cultures. Following initial centrifugation the supernatant was transferred to a 1.5 mL centrifuge tube and stored at -80°C until analysis. Pellets were extracted with 80% aqueous MeOH (section 3.2.5.1). Removal of *M. aeruginosa* PCC 7813 cells by *M. edulis* was assessed by comparing MC levels in tank water immediately after addition to MC levels obtained approximately 19 h post exposure. To assess the overall MC budget within the experimental system, the amount of toxin found in water (intracellular and dissolved), mussel flesh, and faecal material was compared to the amount of MCs detected in *M. aeruginosa* PCC 7813 used in the feeding study (theoretical maximum amount). This allowed the calculation of the theoretically expected amount of MCs in mussel flesh. A suggested correction factor for the analysis of MCs in mussel flesh was determined based on these values.

4.2.5 UPLC-TQ-MS/MS analysis of samples obtained during the feeding trial

A Waters (Manchester, UK) Xevo TQ tandem quadrupole mass spectrometer (MS/MS) coupled to a Waters Acquity UPLC was used for UPLC-MS/MS analysis. Separation was achieved using a 1.7 µm, 2.1x50 mm Waters Acquity UPLC BEH

C18 column (P/N 186002350, Lot no. 0249343351) with a Waters VanGuard BEH C18 1.7 µm 2.1x5 mm guard cartridge. The column was maintained at 60°C, and the sample manager was set to 4°C. The sample injection volume was 5 µL and the mobile phase flow rate was 0.6 mL min^{-1}. Mobile phase A and B utilised for gradient separation consisted of 0.025% FA in water and MeCN, respectively (Table 4. 1).

Table 4. 1 Mobile phase gradient composition utilised for the UPLC-TQ-MS/MS analysis of all samples

Time in min	0.025 % FA in water (A)	0.025 % FA in MeCN (B)
0.0	98	2
0.5	75	25
4.0	50	50
5.0	98	2
5.5	98	2

The Waters Xevo TQ tune parameters were as follows: 150°C source temperature, 600°C desolvation temperature, 600 L h^{-1} desolvation gas flow, 0.15 mL min^{-1} collision gas flow. Capillary voltage was held at 1.0kV. Selected Reaction Monitoring (SRM) transitions were built into the MS/MS method using positive mode acquisition for each toxin. Parent and daughter ions, as well as cone and collision voltages were optimised following infusion of pure standards into the mass spectrometer in the mobile phase (Table 4. 2). The majority of toxins exhibited unique SRM transitions and chromatographic retention times, resulting in good separation of cyanotoxins over the 5 min run time except for Asp^3-MC-LR and [Dha^7]-MC-LR. These shared the same transitions and could not be completely resolved and were therefore reported together. Each instrumental sequence started with a series of instrumental blanks, followed by toxin calibration standards. Instrumental sequences finished with a water and MeCN flush, first at 60°C and followed by a second at 30°C. All optimised SRMs were utilised simultaneously during analysis (multiple reaction monitoring MRM) and toxins were directly quantified against working standards. Quantitation was performed using external calibration and results calculated in terms of µg L^{-1}. Data acquisition and processing was achieved using Masslynx v4.1 software. LODs and LOQs were determined in different matrices (Table 4. 3; Turner unpublished).

Table 4. 2 SRM transitions in positive mode utilised for detection and quantitation of MCs following UPLC separation.

Analyte	SRM transitions	Cone in V	CE in eV
Asp^3-MC-LR/[Dha^7]-MC-LR	981.5 > 134.9; 106.9	75	70; 80
MC-LF	986.5 > 213.0; 135.0	35	60; 65
MC-LR	995.6 > 135.0; 127.0	60	70; 90
MC-LY	1002.5 > 135.0; 106.9	40	70; 90
MC-HilR	1009.7 > 134.9; 126.9	75	75; 90; 80
MC-LW	1025.5 > 134.9; 126.8	35	65; 90

CE = Collision energy

Table 4. 3 Limits of detection (LOD) and limits of quantification (LOQ) obtained for the UPLC-TQ-MS/MS analysis of MCs in various matrices

Matrix	Analyte	LOD	LOQ
Algal cells (µg L^{-1})	MC-LR	0.0013 ± 0.0011	0.0043 ± 0.0036
	Asp^3-MC-LR/[Dha^7]-MC-LR	0.0020 ± 0.0014	0.0066 ± 0.0048
	MC-HilR	0.0020 ± 0.0019	0.0068 ± 0.0063
	MC-LY	0.0036 ± 0.0024	0.0119 ± 0.0079
	MC-LW	0.0034 ± 0.0010	0.0112 ± 0.0034
	MC-LF	0.0033 ± 0.0010	0.0110 ± 0.0034
Water (µg L^{-1})	MC-LR	0.05 ± 0.03	0.18 ± 0.10
	Asp^3-MC-LR/[Dha^7]-MC-LR	0.19 ± 0.20	0.64 ± 0.65
	MC-HilR	0.14 ± 0.14	0.46 ± 0.46
	MC-LY	0.08 ± 0.04	0.26 ± 0.14
	MC-LW	0.14 ± 0.06	0.46 ± 0.22
	MC-LF	0.13 ± 0.04	0.45 ± 0.13
Mussel flesh (µg kg^{-1})	MC-LR	0.09 ± 0.08	0.31 ± 0.27
	Asp^3-MC-LR/[Dha^7]-MC-LR	0.21 ± 0.17	0.69 ± 0.57
	MC-HilR	0.14 ± 0.08	0.45 ± 0.26
	MC-LY	0.11 ± 0.03	0.37 ± 0.08
	MC-LW	0.10 ± 0.03	0.35 ± 0.12
	MC-LF	0.15 ± 0.08	0.51 ± 0.26

4.2.6 Statistical evaluation

A statistically significant value of p=0.05 was set. To evaluate the effect of seawater containing BG-11 on the intracellular and extracellular MC-LR concentrations in *M. aeruginosa* PCC 7813 two way ANOVA with replicates was utilised (Excel software, Microsoft office). The two factors were incubation time and growth medium composition (BG-11 or BG SW).

4.3 Results and discussion

4.3.1 Evaluation of MC-LR content of *M. aeruginosa* PCC 7813 grown in BG-11 containing seawater

Following inoculation of *M. aeruginosa* PCC 7813 into BG-11 medium consistent levels of intracellular and extracellular MC-LR were observed for the full duration of the experiment (Figure 4. 2A) consistent intracellular MC-LR concentrations with no statistical difference to those in BG-11 were observed until 8 h post inoculation ($p=0.310$). This was followed by a significant decrease of intracellular MC-LR starting at 10 h until the end of the incubation period (Figure 4. 2B; $p=0.044$). Extracellular MC-LR concentration in control samples ranged from 4-10% of total MC-LR detected. On the other hand, during seawater exposure extracellular MC-LR reached concentration of up to 84% of total MC-LR. Starting from 10 h post seawater exposure extracellular MC-LR amounted to 18% of total MC-LR detected, which was nearly twice as high as the largest level obtained in control samples, however no statistical difference was determined ($p = 0.127$). Significantly differences extracellular MC-LR concentrations were observed from 12 h seawater exposure onwards, which amounted to 35% (12 h, $p = 5.0 \times 10^{-7}$) and 84 % (24 h, $p = 2.0 \times 10^{-8}$) of total MC-LR, respectively. In addition to the growth medium, statistically significant differences were observed when evaluating the effect of time and the combined effect of medium and time for both intracellular and extracellular MC-LR concentration (Table 4. 4).

Table 4. 4 Probability values obtained for the statistical evaluation of the effect of time and medium composition on intracellular, extracellular, and total MC-LR concentration of *M. aeruginosa* PCC7813 grown in BG-11 and BG-11 (SW) medium

Factor	Intracellular MC-LR concentration	Extracellular MC-LR concentration	Total MC-LR concentration
Time (h)	8.39×10^{-4}	6.71×10^{-11}	0.237
Medium	9.48×10^{-7}	1.95×10^{-8}	1.13×10^{-4}
Interaction	1.66×10^{-5}	8.27×10^{-11}	7.85×10^{-3}

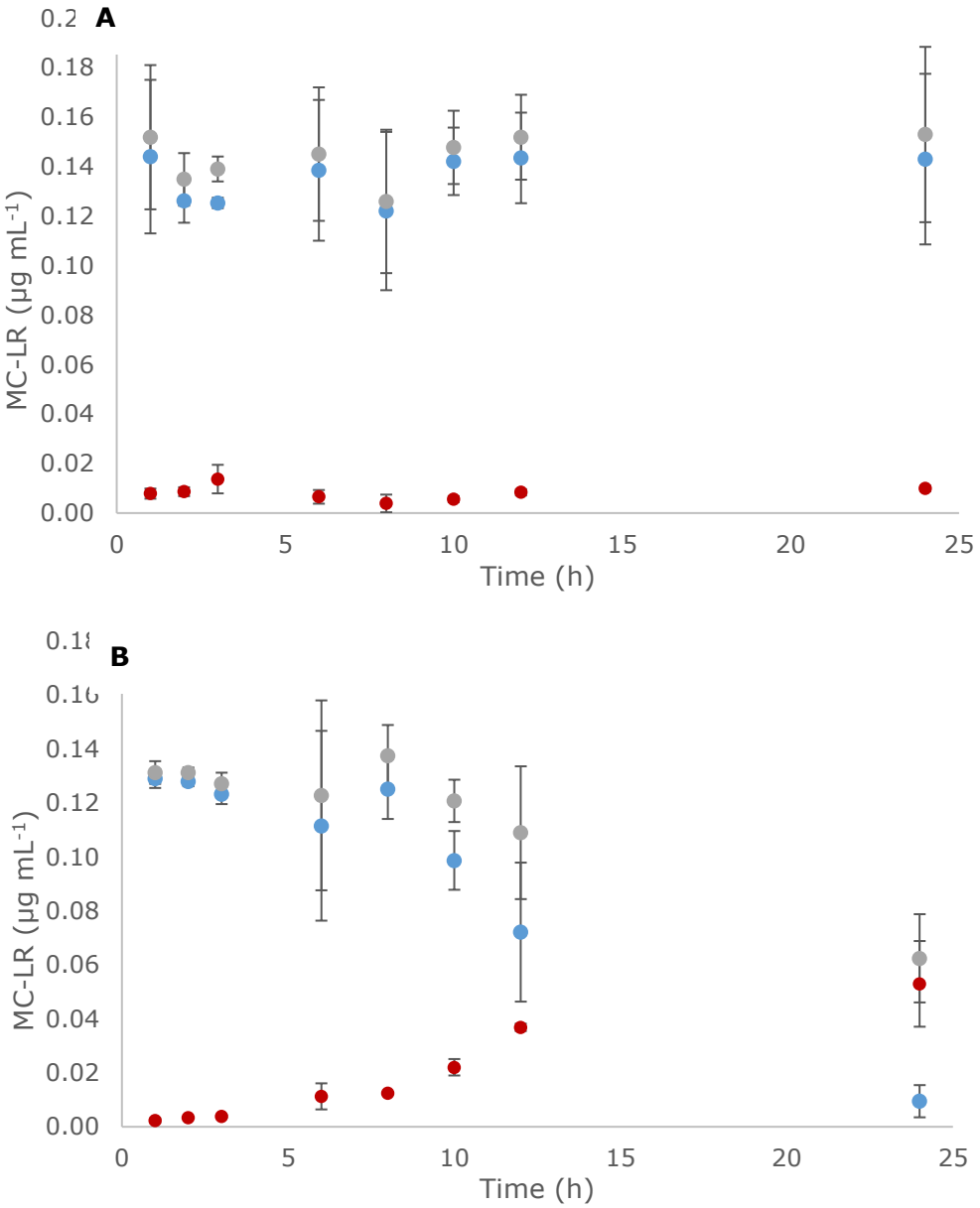

Figure 4. 2 Changes in intracellular (blue), extracellular (red), and total (grey) MC-LR concentration following growth in BG-11 medium (A) and BG-11 medium prepared utilising seawater (B). Values are shown as mean ± standard deviation (n=3).

In agreement with these results, Tonk *et al*. (2007) showed that exposure to salinities >10 psu has been reported to cause increased extracellular MCs. In addition, Tonk *et al*. (2007) also reported that following salt-shock experiments (exposure to 10-17.5 psu) *M. aeruginosa* continued growth for nine days, whereas MC cell quotas increasing for two days. Miller *et al*. (2010) reporting near complete lysis of *M. aeruginosa* cells within 48 h of the exposure to seawater. This was in agreement with results of the present study, in which extracellular MC-LR already accounted for 84% after 24 h seawater exposure.

It was noted, that starting from 10 h post seawater exposure total (extracellular and intracellular) MC-LR concentrations declined compared to total MC-LR at inoculation until only approximately 48% were detected at the end of the experiment. Upon examination of the UPLC-QToF-MS data, no additional peaks were observed that might have indicated a conversion of MC-LR. Potentially, the salt interfered with the MS signal (Ott and Carmichael 2006) and could have contributed to the loss of MC-LR.

The results illustrated that *M. aeruginosa* PCC 7813 cells showed no MC leakage following seawater exposure for the first eight hours. Therefore, this strain can be utilised for the exposure trial of *M. edulis* which already demonstrated good clearance of cyanobacteria within 30 min (section 3.3.3.1) and should therefore clear the majority of available cells within 8 h before lysis becomes an issue. These results also highlight that *M. aeruginosa* can survive in seawater environments long enough to be accumulated by marine organisms. Therefore, *Microcystis* blooms, which can potentially be transported to estuary and coastal areas via rivers, do represent a risk to marine wildlife and higher predators.

4.3.2 Water sample

4.3.3 Quantitation of microcystins in *M. aeruginosa* PCC 7813 feed stock cultures

The *M. aeruginosa* PCC 7813 feed stock culture contained six MCs. Dominant congener was MC-LR. The remaining congeners in order of ascending dominance were: MC-LF, MC-LW, MC-LY, Asp^3-MC-LR/[Dha^7]-MC-LR (Figure 4. 3, Figure 4. 4). Lower total MCs concentrations were detected on days one and five compared to the remaining days (Table 4. 5). Total intracellular MC concentration on these days ranged from 3,782 to 4,893 µg L^{-1} compared to 6,355-7,639 µg L^{-1} on days two, three, four, six, and seven. Extracellular MC concentrations were relatively high compared to total values on all days. This could indicate that cultures had not been mixed well enough and that cells had settled at the bottom of the culture vessels. Generally, in healthy cultures extracellular MCs of 10-20% would be expected (Sivonen 1990; Tonk *et al.*, 2007). Values observed in the present study were higher: ranging from 13-

44%. Before transportation to the study site the feed stock cultures contained intracellular MC-LR at levels of, on average approximately 3 mg L^{-1}. This reinforces the results obtained for stock culture samples taken on days two, three, four, six, and seven. Days one and five meanwhile showed lower than expected concentrations. Potentially, this could have been associated with the initially large volume of culture in the carboy on day one as this could have prevented thorough mixing. However, the reason for the low concentrations detected on day five remain unknown.

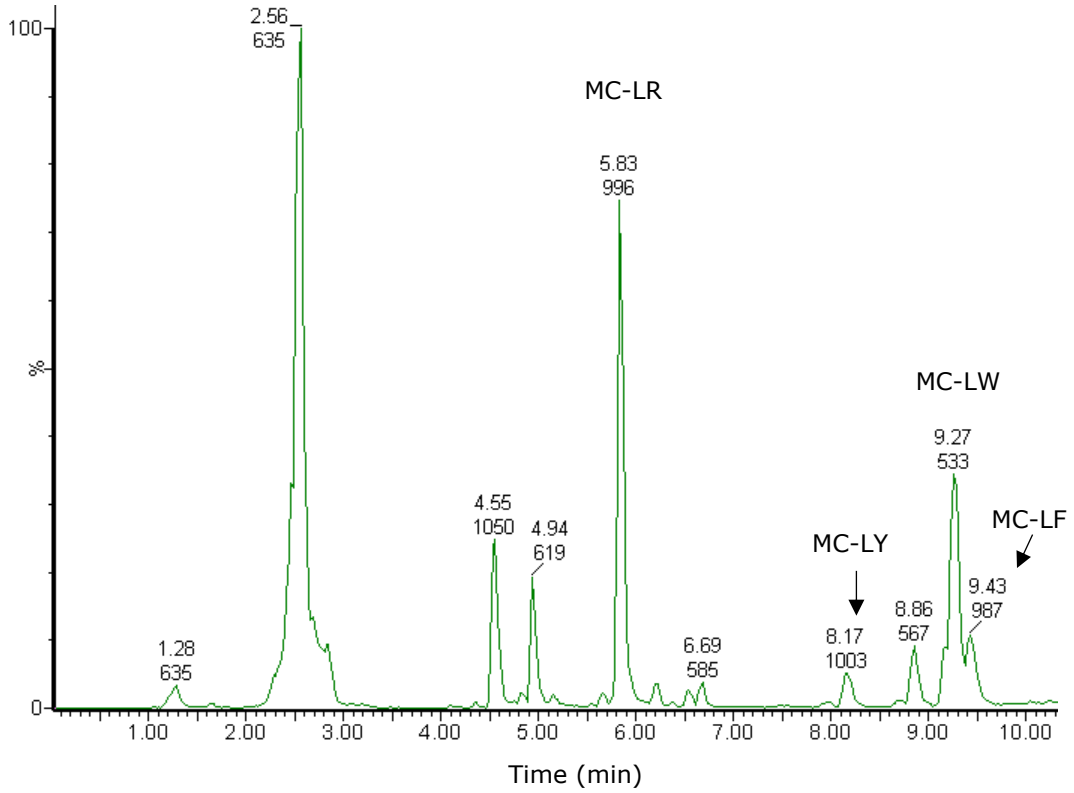

Figure 4. 3 Example UPLC-QToF-MS base peak intensity chromatogram of *M. aeruginosa* PCC 7813 extracts.

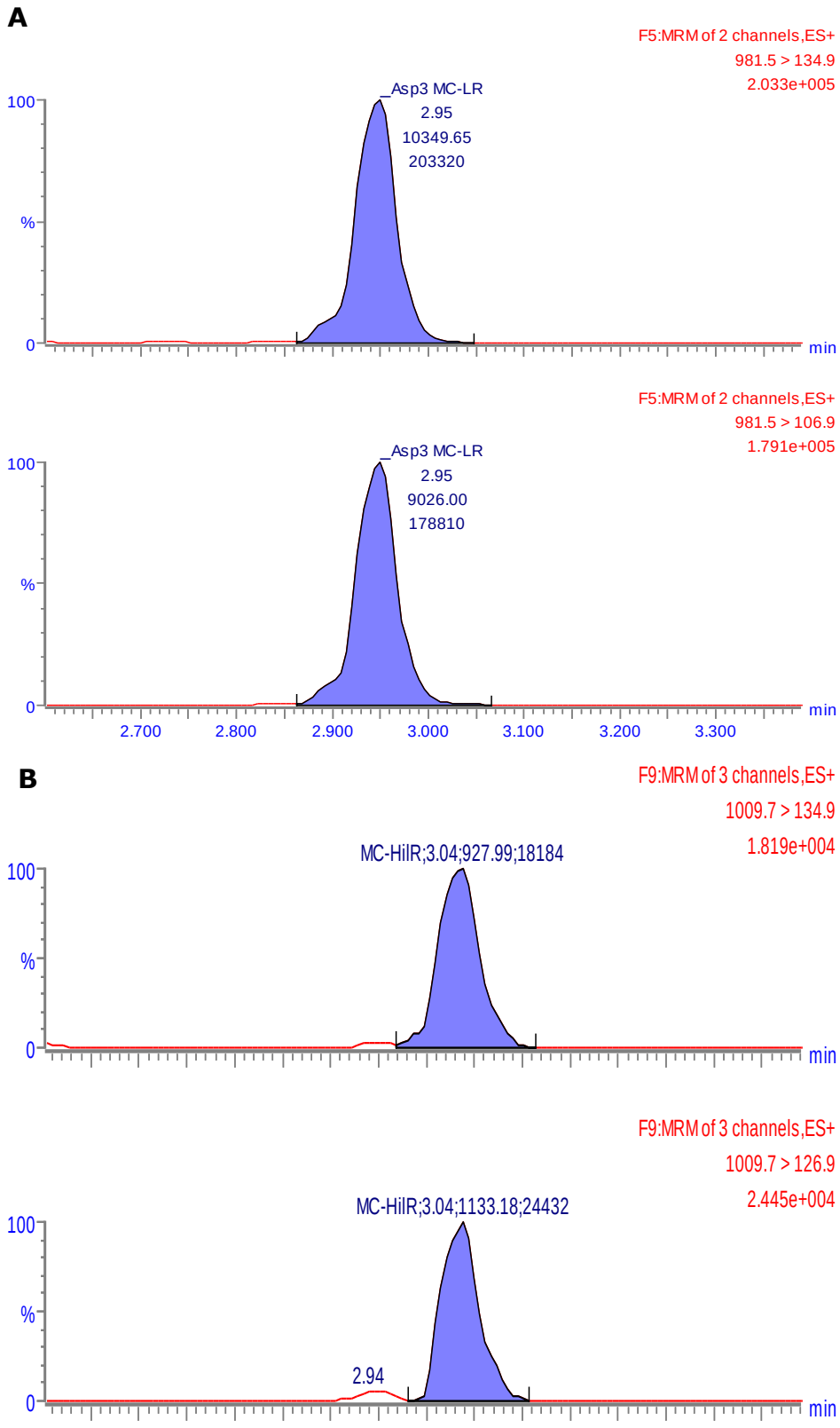

Figure 4. 4 Example UPLC-TQ-MS/MS SRM chromatograms of Asp^3-MC-LR/[Dha^7]-MC-LR (A) and MC-HilR (B) detected *M. aeruginosa* PCC 7813 feed stock culture.

Table 4. 5 Intracellular and extracellular MCs (µg L^{-1}) detected in *M. aeruginosa* PCC 7813 feed stock culture following UPLC-TQ-MS/MS analysis (n=1).

Time in days		1	2	3	4	5	6	7	mean	SD	%RSD
MC-LR	Intracellular	1557	2834	3088	2858	2115	2739	2828	2574	540	20.97
	Extracellular	844	674	781	883	848	845	717	799	78	9.74
Asp^3-MC-LR/[Dha^7]-MC-LR	Intracellular	144	255	275	255	204	257	261	236	46	19.64
	Extracellular	112	114	108	123	155	136	131	126	16	13.08
MC-HilR	Intracellular	23	45	47	43	32	43	43	39	9	22.00
	Extracellular	11	9	10	12	15	12	13	12	2	16.78
MC-LY	Intracellular	335	561	617	569	409	519	509	503	99	19.61
	Extracellular	225	228	231	246	285	273	270	251	25	9.85
MC-LF	Intracellular	1031	1897	2213	1973	1293	1723	1762	1699	407	23.96
	Extracellular	586	400	331	490	753	663	497	531	147	27.71
MC-LW	Intracellular	693	1235	1398	1244	840	1074	1102	1084	245	22.60
	Extracellular	393	368	313	367	507	479	393	403	67	16.74
Total	Intracellular	3782	6827	7639	6943	4893	6355	6505	6135	1333	21.72
	Extracellular	2171	1792	1775	2120	2563	2408	2022	2122	294	13.86

SD - standard deviation, %RSD - relative standard deviation

4.3.3.1 Quantitation of intracellular microcystins in tank water

The analysis of the water samples obtained immediately after addition of *M. aeruginosa* PCC 7813 was consistent for all six exposure tanks. No intracellular MCs were detected in negative control tanks. In agreement with results obtained for the feed stock culture, MC-LR was the dominant variant followed by MC-LF, MC-LW, MC-LY, Asp^3-MC-LR/[Dha^7]-MC-LR, and MC-HilR (Table 4. 6). A comparison between the amount of MCs present in the stock culture and the concentration of MCs detected after dilution in the feeding tanks was carried out. Based on the volume of *M. aeruginosa* PCC 7813 used and the MC concentrations previously determined, the retrieval of MC congeners after feeding was assessed, which ranged from 43-105% (Table 4. 6). Similar to the previous NOD study this was associated with the sampling procedure. Mussels had not been removed from tanks prior to addition of *M. aeruginosa* PCC 7813, hence they were able to accumulate MCs for up to 30 min before the water sample was taken. No MCs were detected in the tank water during the depuration period. In contrast to results obtained during the exposure of *M. edulis* to *N. spumigena* KAC66 neither pseudofaeces production nor cell aggregation was observed.

Table 4. 6 Average recovery (%) of intracellular MCs in tank water following addition of *M. aeruginosa* PCC 7813. Values were calculated based on available MCs in feed stock culture (n=6).

Congener	Time in days	Actual µg L^{-1}	Theoretical µg L^{-1}	% Recovery
MC-LR	1	47.52	73.30	65
	2	62.37	137.65	45
	3	67.07	146.24	46
	4	83.86	134.89	62
	5	56.10	100.45	56
	6	98.97	131.82	75
	7	85.59	135.09	63
Asp^3-MC-LR/[Dha^7]-MC-LR	1	4.43	5.47	81
	2	5.67	9.73	58
	3	6.07	10.48	58
	4	7.17	9.70	74
	5	5.23	7.77	67
	6	9.29	9.78	95
	7	7.60	9.94	76
MC-HilR	1	0.84	0.88	96
	2	1.11	1.71	65
	3	1.34	1.79	75
	4	1.66	1.65	100
	5	1.10	1.22	90
	6	1.70	1.62	105
	7	1.66	1.63	102
MC-LY	1	10.31	12.75	81
	2	13.63	21.38	64
	3	14.40	23.50	61
	4	18.10	21.69	83
	5	12.06	15.57	77
	6	20.21	19.78	102
	7	17.79	19.40	92

Table 4. 6 continued

Congener	Time in days	Actual µg L^{-1}	Theoretical µg L^{-1}	% Recovery
MC-LF	1	27.54	39.26	70
	2	35.63	72.27	49
	3	36.63	84.31	43
	4	45.39	75.18	60
	5	31.19	49.27	63
	6	54.51	65.64	83
	7	46.12	67.12	69
MC-LW	1	20.89	26.40	79
	2	27.21	47.06	58
	3	29.04	53.27	54
	4	35.16	47.38	74
	5	23.99	32.00	75
	6	40.08	40.92	98
	7	35.23	41.98	84

4.3.3.2 Quantitation of extracellular microcystins in tank water

Low levels of MCs were detected in tank water of exposure tanks with no significant difference between the three accumulation and three depuration tanks for all congeners except for MC-HilR (Table 4. 7). No extracellular MCs were detected in negative control tanks. The change in salinity experienced once *M. aeruginosa* PCC 7813 (~7 psu) was added to the *M. edulis* containing tanks (~ 35 psu) should not have affected the content of extracellular MCs. This was based on the results obtained following the salinity exposure experiment which demonstrated increases in extracellular MCs only after 10 h of seawater exposure (section 4.3.1). The recovery of extracellular MCs in tank water based on concentrations in feed stock cultures ranged from 47 to 102% which was likely affected by the feeding regime as previously described. In addition, *M. edulis* accumulated dissolved DA to a lesser extend compared to particulate associated DA (Novaczek *et al*., 1991). Similarly, MC-LR was not accumulated by *A. grandis simpsoniana* when exposed to dissolved MC-LR (51-55 µg L^{-1}; Prepas *et al*., 1997).

Table 4. 7 Average recovery (%) of extracellular MCs in tank water following addition of *M. aeruginosa* PCC 7813. Values were calculated based on available MCs in feed stock culture (n=6).

Microcystin congener	Time in days	Actual concentration in µg L^{-1}	Theoretical concentration in µg L^{-1}	% Recovery
MC-LR	1	22.33	32.14	69
	2	23.75	25.67	92
	3	23.72	29.75	80
	4	27.82	33.64	83
	5	25.58	32.30	79
	6	27.82	32.19	86
	7	27.93	27.31	102
Asp^3-MC-LR/[Dha^7]-MC-LR	1	3.35	4.28	78
	2	3.22	4.32	74
	3	3.02	4.12	73
	4	4.02	4.69	86
	5	4.22	5.90	72
	6	3.95	5.17	76
	7	3.97	4.99	80
MC-LY	1	6.03	8.58	70
	2	6.03	8.68	70
	3	6.15	8.80	70
	4	7.30	9.38	78
	5	6.88	10.87	63
	6	7.12	10.42	68
	7	7.00	10.30	68
MC-LF	1	10.73	22.32	48
	2	9.85	15.22	65
	3	7.38	12.60	58
	4	13.48	18.66	72
	5	14.95	28.69	52
	6	13.93	25.27	55
	7	11.72	18.94	62
MC-LW	1	7.00	14.97	47
	2	7.57	14.03	54
	3	5.97	11.93	50
	4	9.37	13.97	67
	5	9.72	19.30	50
	6	9.33	18.25	51
	7	8.37	14.98	56

During the depuration period no dissolved MCs were detected immediately after feeding. Strikingly, however, on the morning of day nine, approximately 19 h after feeding, the dissolved MC-LR concentration was ~ 4 µg L^{-1}. In addition, three MC congeners (MC-LY, MC-LF, and MC-LW) were also detected at levels above LOD but below LOQ on day nine, even though they had not been detected after feeding on day eight. A similar trend was observed until day ten for these three congeners and until day 17 for MC-LR, respectively. Even though the concentrations could not be quantified, these results suggest that *M. edulis* eliminated a small amount of MCs in dissolved form. This suggestion was supported by Doucette *et al*. (2006) who reported that as well as via faeces, bivalves can eliminate toxins in dissolved form. Specifically mussels (*M. galloprovincialis*) and scallops (*Patinopecten yessoensis*) eliminated dissolved paralytic shellfish poisoning (PSP) toxins when fed *Alexandrium tamarense* (Suzuki *et al*., 2003; Sekiguchi *et al*., 2001). Similarly, the excretion of dissolved ATX-a by *M. galloprovincialis* following the exposure to *Anabaena* sp (ANA 37) has been reported (Oswald *et al*., 2008). Consequently, the results of the present study in combination with the literature suggest that bivalves can re-introduce toxins into the environment via the excretion of dissolved toxins.

4.3.3.3 Clearance of MCs from tank water

Assessing the clearance of MCs from tank water showed that generally 20-46% of the available intracellular MCs remained detectable in water ~ 19 h post exposure. Slightly more intracellular MCs were cleared on days one and three with a maximum of 15% of the dose retrieved in tank water (Table 4. 8). Dissolved MCs on the other hand showed increases of up to 24% for individual MC congeners (Table 4. 8).

As not all of the available cell bound MCs were accumulated by *M. edulis* (Table 4. 8) the prolonged exposure to an increased salinity could have caused cell lysis and consequently the release of MCs as observed during the salinity tolerance investigation from 10 h post exposure onwards (section 4.3.1). Alternatively, the increase in extracellular MC could also be associated with the elimination of dissolved MCs by *M. edulis* as indicated by results of the depuration period (section 4.3.3.2).

Table 4. 8 Average intracellular MCs (%) detected in tank water following the exposure of *M. edulis* to *M. aeruginosa* PCC 7813 over a period of ~ 19 h compared to concentrations after feeding (n=6).

Time in days	MC-LR	Asp^3-MC-LR/[Dha^7]-MC-LR	MC-HilR	MC-LY	MC-LW	MC-LF
1	8.38 ± 3.65	14.8 ± 3.59	0	10.2 ± 3.34	10.7 ± 2.14	12.5 ± 3.13
2	30.9 ± 12.0	33.9 ± 10.7	0	35.0 ± 13.2	35.2 ± 15.6	34.7 ± 14.4
3	9.50 ± 7.40	0	0	11.6 ± 7.52	11.3 ± 6.53	12.0 ± 6.63
4	21.9 ± 30.1	23.3 ± 28.1	0	21.9 ± 28.1	24.2 ± 29.0	25.2 ± 29.7
5	42.4 ± 28.0	45.9 ± 28.7	0	44.9 ± 27.9	45.8 ± 27.9	48.0 ± 28.2
6	25.2 ± 26.9	29.2 ± 29.0	0	29.0 ± 27.6	29.8 ± 28.6	26.8 v 25.0
7	25.0 ±32.1	27.8 ± 31.1	0	27.8 ± 31.4	27.9 ± 32.2	26.6 ± 30.6

Table 4. 9 Average dissolved MCs (%) detected in tank water following the exposure of *M. edulis* to *M. aeruginosa* PCC 7813 over a period of ~ 19 h compared to concentrations after feeding (n=6).

Time in days	MC-LR	Asp^3-MC-LR/[Dha^7]-MC-LR	MC-LY	MC-LW	MC-LF
1	123 ± 30.0	117 ± 35.6	114 ± 25.1	121 ± 38.1	109 ± 31.4
2	111 ± 12.7	111 ± 22.6	120 ± 20.2	92.1 ± 25.3	110 ± 18.4
3	97.4 ± 32.7	104 ± 37.5	97.2 ± 37.7	122 ± 49.4	130 ± 54.1
4	107 ± 6.90	109 ± 9.13	114 ± 9.78	112 ± 22.6	118 ± 11.6
5	128 ± 15.5	108 ± 19.7	123 ± 23.5	134 ± 16.5	124 ± 23.3
6	109 ± 14.7	108 ± 17.9	120 ± 17.6	118 ± 35.0	123 ± 20.5
7	106 ± 11.1	100 ± 15.6	120 ± 7.38	124 ± 20.3	129 ± 14.5

4.3.4 Quantitation of MCs in *M. edulis* following the exposure to *M. aeruginosa* PCC 7813

Rapid accumulation of MCs by *M. edulis* was observed (Figure 4. 5). No MCs were detected in tissue of negative control samples. The first four days showed a linear increase in total MC concentration (R^2=1.0000). The maximum concentration of 8.5 µg g^{-1} was detected on day seven of this study. The depuration period began with a considerable decrease in toxin concentration down to ~6 µg g^{-1} (~70% of maximum concentration) after two days, which continued less quickly until day 13 (3.4 µg g^{-1}). Less rapid depuration was observed over the last 16 days following which the total MC concentration had been reduced to approximately 1.2 µg g^{-1} (~ 14% of maximum concentration; Figure 4. 5). The individual MC congeners showed the same overall trend as described for total MC except that Asp^3MC-LR/[Dha^7]-MC-LR and MC-HilR were not detected on day 29. Utilising the logarithmic trend line starting from day eight (y=-4793ln(x) + 17427; Figure 4. 5) full depuration of MCs would be expected following 38 day depuration.

The presence of cyanobacteria affected the survival of *M. edulis* as increased mortality rates of ~11% where observed in exposure tanks compared to ~2% of negative control tanks. Mortalities of 0-13% have been reported when exposing clam and mussels to *Microcystis* strains (Vasconcelos 1995; Leão *et al*., 2010; Sabatini *et al*., 2011; Pires *et al.,* 2004). Despite the increased mortality, the majority of mussels tolerated the exposure to *M. aeruginosa* indicating that they are a vector of MCs in the food web.

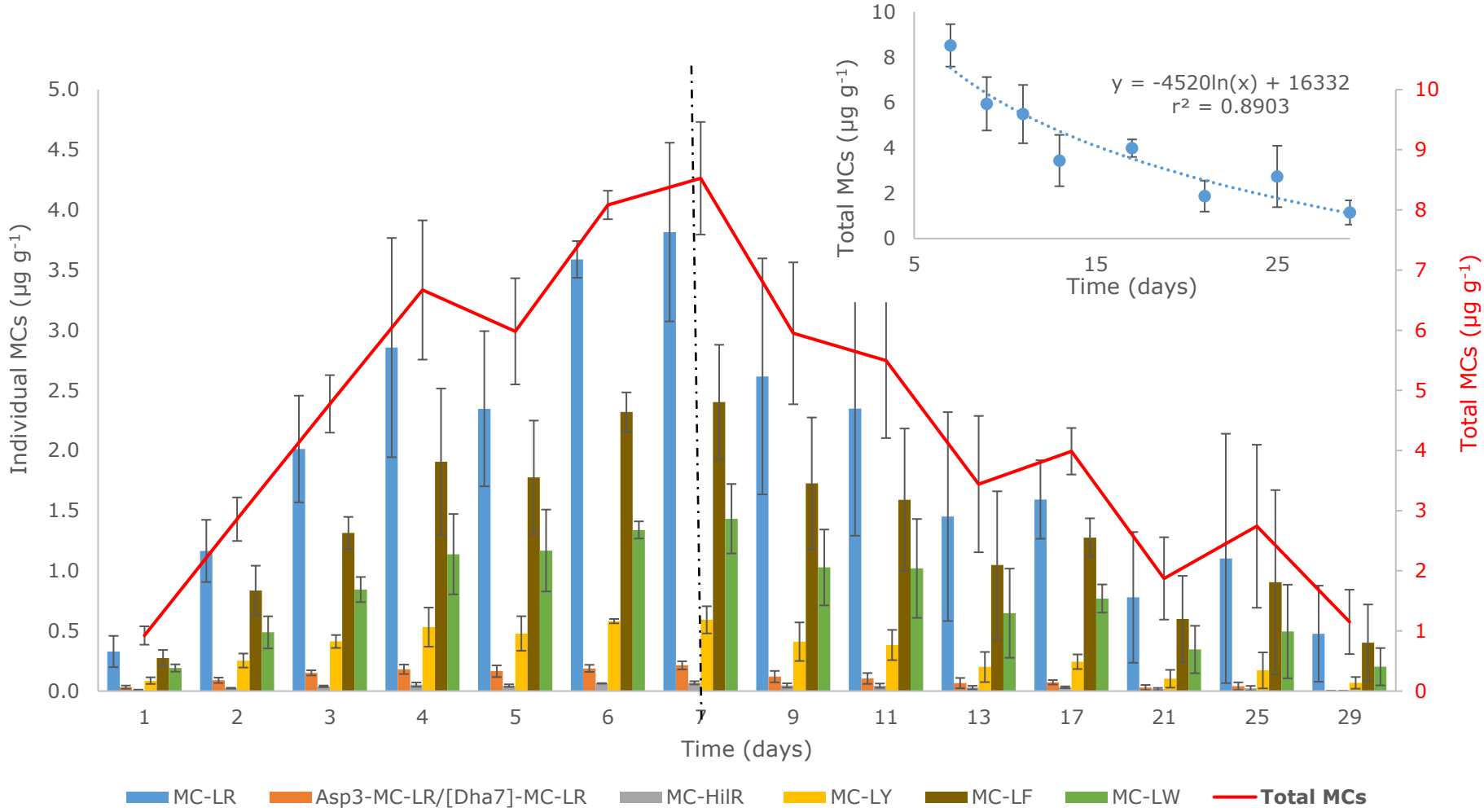

Figure 4. 5 MC concentration in *M. edulis* after seven day exposure to *M. aeruginosa* PCC 7813. The dashed black line shows the end of the exposure period. The analysis was performed utilising UPLC-TQ-MS/MS analysis. Values are shown as mean ± standard deviation (n=3). Insert: Logarithmic decrease of total MCs in *M. edulis* after the seven day exposure to *M. aeruginosa* PCC 7813

In agreement with results obtained during the present study rapid accumulation of MCs was reported in the river mussel *Unio douglasiae* following the exposure to *M. ichthyoblabe* cells at a final MC-LR concentration of 27-50 μg L^{-1} (Yokoyama and Park 2003; Table 4. 10). Mussels accumulated ~50-150 μg g^{-1} (estimated from graph) during the first 24 h of exposure. Similar results were reported following three day exposure of *M. edulis* to freeze-dried cells of *M. aeruginosa* (305 mg MCs 100 g^{-1}; Williams *et al.,* 1997b; Table 4. 10). Mussels contained 0.015 μg g^{-1} ww of free and 3.4 μg g^{-1} covalently bound MCs illustrating that the majority of toxin burden was present in bound form. Increase in MC concentration during the depuration period associated with the release of bound toxin (Vasconcelos 1995; Amorim and Vasconcelos 1999) was not observed in the present study. It has been suggested that mixed food sources can also affect the accumulation of MCs in zebra mussels (*Dreissena polymorpha*; Pires *et al*., 2004). When offered a mixture of *M. aeruginosa* and the microalga *Nannochloropsis limnetica* MC-LR was accumulated to a lesser extent compared to only feeding on *M. aeruginosa* NIVA-CYA 140. In addition, the clearance rate for *Microcystis* was higher compared to that of the microalga (Pires *et al*., 2004).

Table 4. 10 Reported accumulation and depuration of cyanotoxins in mussels following the exposure to cyanobacterial cells.

Cyanobacteria (cyanotoxin)	Exposure concentration µg L^{-1}*	Bivalve	Uptake (d)	Depuration (d)	Maximum toxin concentration µg g^{-1}†	Levels after depuration µg g^{-1}†	Mortality	Reference
M. aeruginosa RST 9501 (MC)	0.4 ± 0.04 0.7 ± 0.08	*Mesodesma mactroides*	12	None	5.27 ± 0.23 dw (hepatopancreas) ~ 3.7 (based on graph)	Not applicable	13%	Leão *et al*. (2010)
M. aeruginosa IZANCYA2 (MC-LR)	~ 7.7 (on average)[a]	*M. gallopro-vincialis*	1	15	10.52 mg g dw (day 10)	not detected on day 13	6%	Vasconcelos (1995)
M. aeruginosa (MCs)	~300[a]	*M. edulis*	3	53	336.9 ± 45.8 ww 0.204 ww	not detected on day 11	Not reported	Williams *et al.* (1997b)
M. aeruginosa (MC-LR)	~34 (10^5 cells mL^{-1})[a]	*M. gallo-provincialis*	4	14	10.7 dw	2.5	0	Amorim and Vasconcelos (1998)
M. aeruginosa NIVA-CYA 140 (MC-LR)	2 mg C L^{-1}	*D. polymorpha*	21	21	10.8 dw	6.2 dw		Pires *et al*. (2004)
O. agardhii (Oscillatoria toxin)	40-60	*A. cygnea*	15	2 month	130 fdt (hepatopancreas)	< LOD	0%	Eriksson *et al*. (1989)

* Unless specified values are shown as µg mL^{-1}

† If not specified concentrations (mean ± standard deviation were available) are given for the whole organisms

dw – dry weight, a – calculated based on experimental details given by authors, fdt – freeze dried tissue

Table 4. 10 continued

Cyanobacteria (cyanotoxin)	Dose µg L^{-1}*	Bivalve	Uptake (d)	Depuration (d)	Maximum toxin concentration µg g^{-1}†	Levels after depuration µg g^{-1}†	Mortality	Reference
M. aeruginosa NIES-1086 (MCs)	100	*C. leana*	10	10	12 ± 2.5 (free) 4.2 ± 0.6 (bound)	< LOD (free) ~1[a]	0%	Pham *et al*. (2015)
M. ichthyoblabe TAC95 (MC-LR)	27 ± 4.27 (15°C)[#] 50 ± 7.52 (25°C)[#]	*U. douglasiae*	15	15	~130 dw[a] 630 dw	17.1 dw 8.4 dw	~3%	Yokoyama and Park (2003)
N. spumigena KAC66 (NOD)	100-480	*M. edulis*	7	7	17.13 ± 3.49 ww	7.46 ± 1.93 ww	<1%	Present work (Chapter 3)
M. aeruginosa PCC 7813 (MCs)	240-390	*M. edulis*	7	22	8.5 ± 0.9 ww	1.2 ± 0.54 ww	7%	Present work (Chapter 4)

* - Unless specified values are shown as µg mL^{-1}

† - If not specified concentrations (mean ± standard deviation were available) are given for the whole organisms

a – estimated from graph, # - exposed every second day, dw - dry weight, ww - wet weight

Contradictory to results obtained in the present study Prepas *et al*. (1997) reported rapid decrease of MC-LReq in clams (*Anodonta grandis*) which had been exposed to MC-LR containing phytoplankton in natural lakes. Following six days of depuration clams of all three lakes had reduced MC-LReq content by 71%. Compared to the present study a similar reduction of total MCs concentration was only seen following 18 days of depuration. The slow depuration of MCs from *M. edulis* corresponded to literature reports. Even following 2 months of depuration *Oscillatoria* toxin were detected in the swan mussel (*A. cygnea*) which had been exposed to live cells of *O. agardhii* at final *Oscillatoria* toxin concentrations of 40-60 µg L^{-1} (Eriksson *et al*., 1989). This illustrated the potential of MC intoxication of higher predators and humans via their food source.

The form (e.g. dissolved or particulate) of MC available was reported to affect toxin depuration in *M. californianus* (Gibble *et al*., 2016). Following exposure to either dissolved (7.73 µg L^{-1}) or particulate associated MCs (5.6 µg L^{-1}), faster depuration was observed in mussels exposed to dissolved MCs when levels were <LOD after 72 h of depuration. In contrast, depuration of particulate associated MCs was incomplete even three weeks post exposure. In addition, a temperature dependent depuration of MC-LR has been suggested in the river mussel *Unio douglasiae* (Yokoyama and Park 2003). Mussels had been exposed to *M. ichthyoblabe* (TAC95) at 15 and 25°C. Applying first order kinetics based on a one-compartment model the authors reported depuration rate constants of 0.142 ± 0.044 day^{-1} and 0.226 ± 0.046 day^{-1} when exposed at 15°C and 25°C, respectively. The authors concluded that, potentially, due to slow depuration in the colder months cyanotoxin could persist until spring which has been reported for NOD in flounder (Persson *et al*., 2009) and mussel (Kankaanpää *et al.*, 2007; Mazur-Marzec *et al*., 2007). However, comparing the actual exposure concentrations of MC-LR showed that the 25°C treatment received nearly twice as much MC-LR compared to the 15°C treatment: 50 ± 7.52 µg L^{-1} compared to 27 ± 4.27 µg L^{-1}. Hence, the observed dynamics might be related to concentration, as well as temperature.

In addition, differences in depuration of free and covalently bound MCs have been reported (Williams *et al*., 1997b; Pham *et al*., 2015). Within 24 h free MC-LReq was reduced by ~50% in clam (*C. leana*) which had been exposed to *M.*

aeruginosa NIES-1086. After seven days depuration MC concentration was below LOD. In contrast, covalently bound MC-LReq remained constant for the first five days of depuration and were still detected on day ten of the depuration period. Contrary to these results, Williams *et al.* (1997b) reported complete elimination of bound MCs following 11 days depuration, whereas free MCs (PPase assay) were still detected following 53 days of depuration. Utilising the PPase assay for MC quantitation does not differentiate between individual MC congeners and/or their metabolites which could explain the prolonged detection of MCs in *M. edulis*. Pham *et al*. (2015) utilised HPLC-PDA analysis which shows greater specificity towards the analytes.

Mussels were exposed to a combined intracellular and extracellular MCs concentration ranging from 240-390 µg L^{-1}. Reports have shown that these levels were representative of concentrations occasionally reported in environmental samples (Table 4. 11). Preece *et al*. (2017) who reviewed MC concentration in various estuaries. In only three of the reviewed studies MC concentrations similar or greater than those used during the feeding trial in the present study were reported. However, for 13 of the 27 reviewed studies no MC concentration had been reported (Preece *et al*., 2017).

Table 4. 11 MC levels reported in water bodies

Water body	**Country**	**Toxin**	**c_{max} µg L^{-1}**	**Reference**
Grand Lake St. Marys	United States of America	Total MCs (ELISA)*†	>100	Schmidt *et al*. (2013)
Chesapeake Bay	United States of America	Total MCs (ELISA)*	658	Tango and Butler (2008)
Lake Occhito	Italy	Total MCs (ELISA)*†	298	De Pace *et al*. (2014)
Lake Suwa	Japan	MC-LR + MC-RR*	184	Park *et al.* (1998)

c_{max} **-** maximum concentration reported ***** - intracellular **†** - extracellular
dm - demethylated

Based on the results of the present study consumption of a serving of 20 mussels (~4 g per mussel, section 5.3.4) harvested on day seven, would result in the ingestion of 680 µg MCs. For an average adult of 75 kg this would be more than 3.5 and ~ 23 times the acute and seasonal tolerable intake of 190

and 30 µg MCs, respectively (Ibelings and Chorus 2007). In addition, following 22 days of depuration MC levels would be below the acute tolerable intake, however they would still exceed the seasonal guideline by a factor of ~3. This showed that exposure to environmentally relevant cyanotoxin concentrations caused a significant accumulation of MCs in *M. edulis*. Hence, monitoring of cyanotoxins in *M. edulis* not only during, but following bloom events is vital in order to protect wildlife and humans from MCs exposure.

Equation 4. 1 Cyanotoxin load expected in a serving of 20 mussels

On day seven

$$m_{MCs} = m_{mussel} \times n_{mussel} \times c_{MCs}$$
$$m_{MCs} = 4\ g \times 20 \times 8.5\ \mu g\ g^{-1}$$
$$m_{MCs} = 680\ \mu g$$

On day 29

$$m_{MCs} = m_{mussel} \times n_{mussel} \times c_{MCs}$$
$$m_{MCs} = 4\ g \times 20 \times 1.2\ \mu g\ g^{-1}$$
$$m_{MCs} = 96\ \mu g$$

m - amount in µg, n - number of specimen, c - concentration in mussel flesh

4.3.5 Quantitation of MCs in faecal material of *M. edulis* following the exposure to *M. aeruginosa* PCC 7813

The first four days showed a steady increase of total MC concentration in faecal material followed by declining levels over the next two days (Figure 4. 6). No MCs were detected in faeces of negative control mussels. An MC increase was observed on days seven and eight. However, due to the large variations observed especially on day seven, the accuracy of values was questionable. From day eight onwards an exponential decrease was observed until an amount of 22 µg was reached on day 29 ($y = 8E+07x^{-4.322}$; $R^2 = 0.9573$). Only the congeners MC-LY, MC-LF, and MC-LW were detected by that time. MC-HilR, Asp^3-MC-LR/[Dha^7]-MC-LR, ad MC-LR had reached levels below LOQ on days ten, 15, and 28, respectively. It was noticed, that despite continued exposure to *M. aeruginosa* PCC 7813 total MC concentration in faecal material started to decrease on day five. Potentially, metabolism processes within *M. edulis* were activated as a response to MC exposure. Unfortunately, due to the lack of appropriate standards, the presence of MC metabolites could not be evaluated in the present study. Pseudofaeces production as reported in zebra mussels and

fresh water clams following the exposure to *M. aeruginosa* was also observed (Pires *et al.*, 2004; Pham *et al.*, 2015).

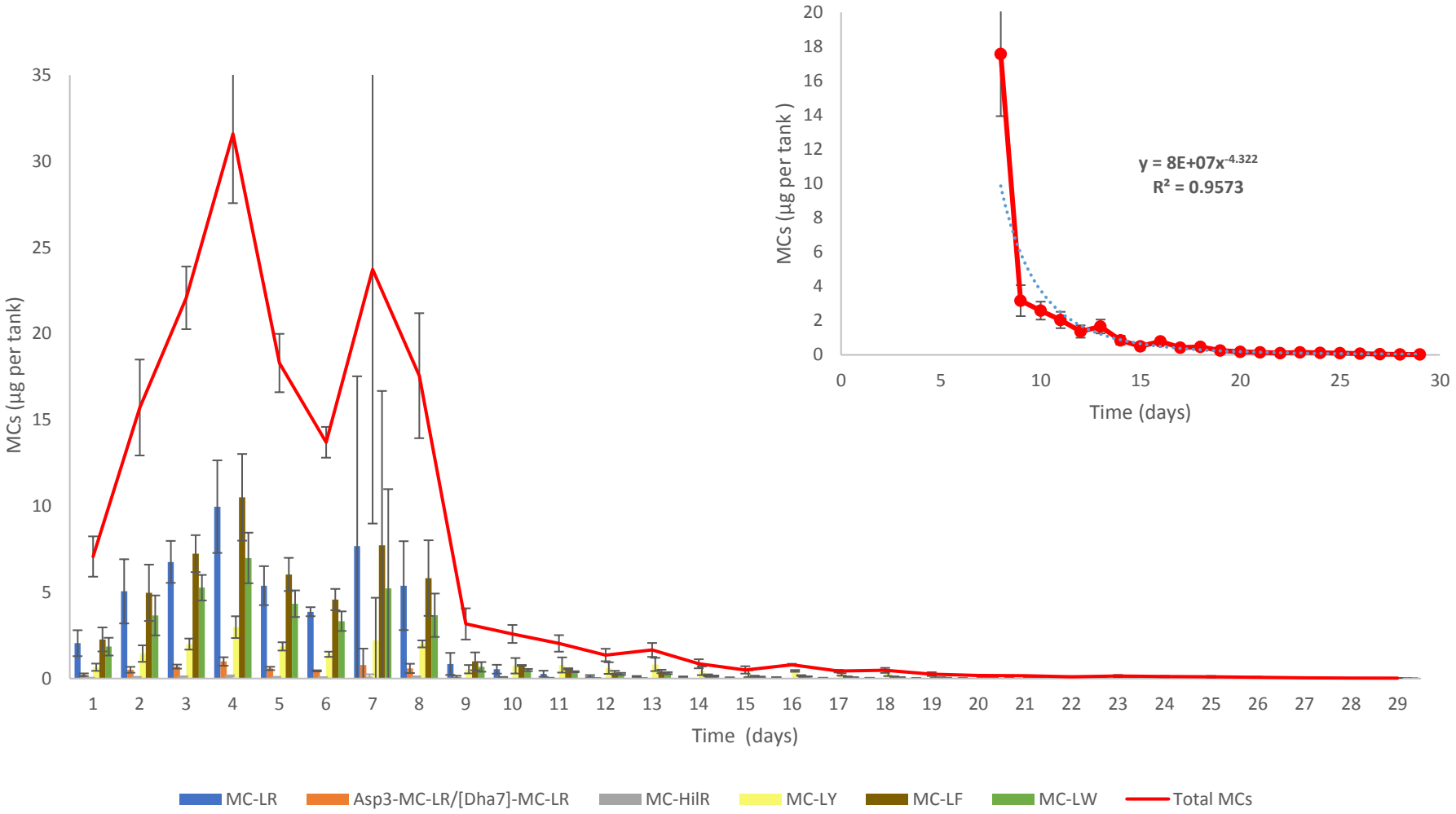

Figure 4. 6 MC variants and total MCs detected in faecal material of *M. edulis* following seven day exposure to *M. aeruginosa* PCC 7813. Analysis was performed utilising UPLC-TQ-MS/MS analysis. Values are mean ± standard deviation (n=3). Insert: Exponential decrease of total MCs in faecal material of *M. edulis* after the seven day exposure to *M. aeruginosa* PCC 7813.

The average weight of faeces produced by 140 mussels during a later feeding study (chapter 5) was ~ 1 g. Therefore, 70 mussels should have produced ~ 0.5 g faeces. Extrapolated based on this weight concentrations in the faeces of the present study ranged from 14-63 µg kg^{-1}, which were lower compared to reported field levels. Poste and Ozersky (2013) detected up to 125 µg kg^{-1} MC in biodeposits, which was defined as the sum of pseudofaeces and faeces, produced by Dreissenid mussels (*D. rostriformis bugensis*). Mussels had been collected from Lake Ontario (Bay of Quinte), in which *M. aeruginosa* blooms are a common nuisance during the summer months. MC levels in water ranged from 0.1-1.1 µg L^{-1} and a *M. aeruginosa* containing scum samples reached MC concentrations of up to 2.3 µg L^{-1}. These were a factor of ~100 lower than exposure values utilised during the present study, but still resulted in faecal MC concentrations approximately twice as high. This suggests that excretion of MCs in faeces is species dependent.

Contrary to the steady decrease of MCs in faecal material during the depuration period, Amorim and Vasconcelos (1999) reported increases in MCs concentration for the first three days of depuration following the exposure of *M. galloprovincialis* to *M. aeruginosa* IZANCY A2. Similar observations were made after 15 days exposure of tilapia (*T. rendalli*) to *M. aeruginosa* (Soares *et al*., 2004). Both of these studies utilised ELISA for cyanotoxin analysis. Hence, it could not be determined if the potential formation MC conjugates and/or degradation products could have contributed to the reported concentrations of MCs. This could explain the increased concentration of MCs reported in the literature compared to the decrease observed in the present study.

4.3.6 Determination of the MC budget within the experimental system

Generally, a large proportion of MCs added over the duration of the study was unaccounted for within the experimental system (40-53%; Figure 4. 7). Detectable MCs were present in mussel flesh (19-34%) followed by dissolved MCs (15-21%) and intracellular MCs (6-10%). Lowest MC concentration was detected in faecal material (0.1-0.3%). The amount of MCs accumulated by stunt doubles (SD; mussels placed into tanks for each sampled mussel) was extrapolated based on the MC concentrations detected in *M. edulis* flesh. MCs

detected in mussel flesh and extrapolated MC amounts of SDs were combined to assess the MC budget within the experimental system. Generally, the distribution of MC within experimental systems has thus far been overlooked in the literature. However, in a small number of studies the recovery of MC dose in bivalve tissue has been determined. Similar dose recoveries have been reported in *M. galloprovincialis* exposed to a toxic *Microcystis* strain (Vasconcelos 1995). Mussels accumulated MCs at levels corresponding to 24-55% of available MCs which showed slightly higher maximum retrieval compared to the 34% determined during the present study. Dose recovery of NODs in *M. edulis* following the exposure to *N. spumigena* KAC66 ranged from 38-83% (section 3.3.4) and was greater compared to the percentage of MCs recovered in mussel flesh (19-34%). Potentially, the observed decrease of MC-LR concentration detected in *M. aeruginosa* PCC 7813 cultured in seawater for an excess of 10 h could have contributed to the observed losses (section 4.3.1).

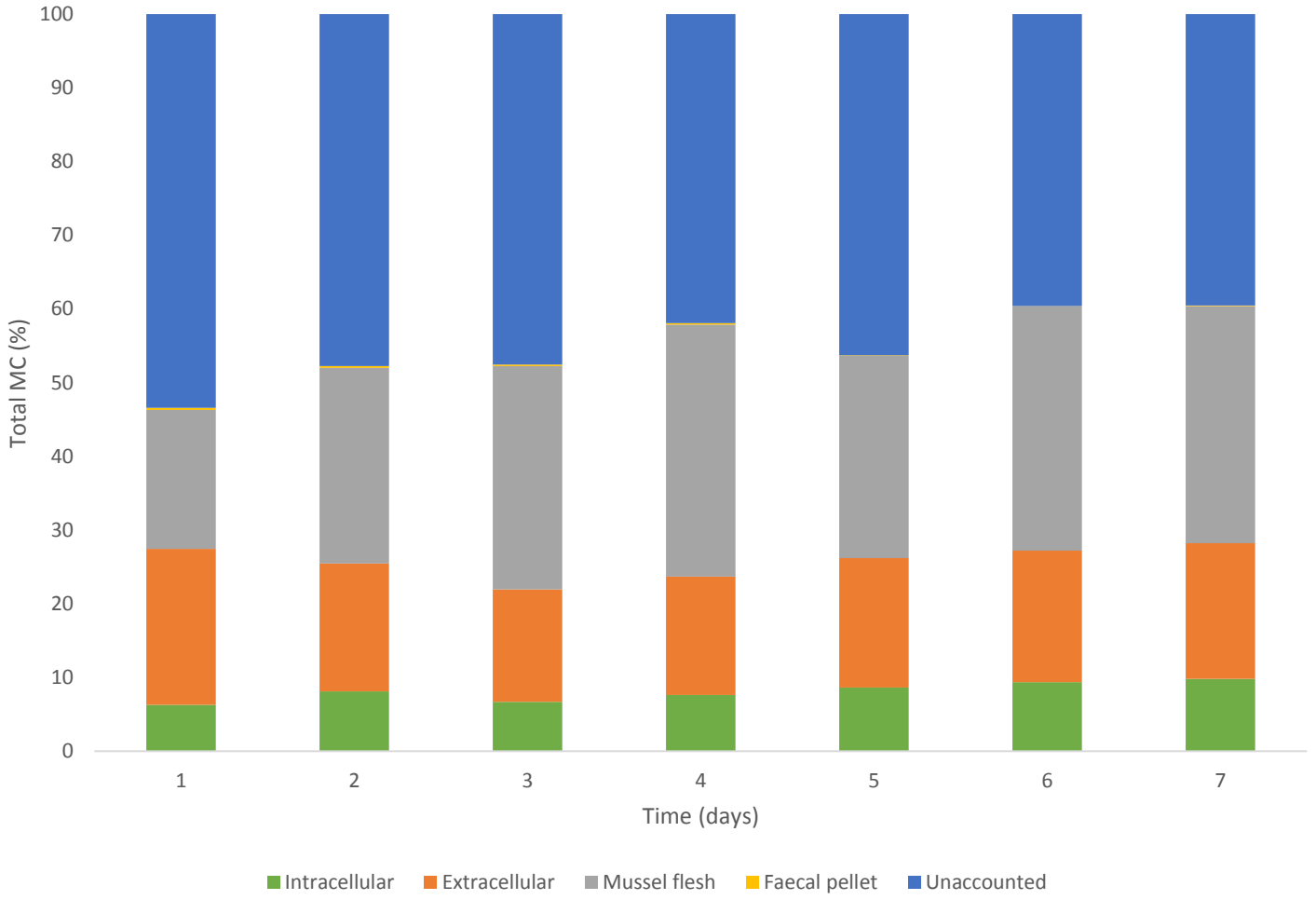

Figure 4. 7 Distribution (%) of total MCs in experimental system following the exposure of *M. edulis* to *M. aeruginosa* PCC 7813. Values for stunt doubles (flesh) were estimated based on results obtained for mussel flesh and added to mussel values.

A shift in the proportions of individual MC variants in feed stock cultures and faecal material compared to MC-LR was observed for MC-LY, MC-LW, and MC-LF (Table 4. 12). MC-LF, which was present in feed stock cultures at amounts approximately half that of MC-LR, was detected in faecal pellet at amounts equal to or even larger than MC-LR (Table 4. 12). This is the first report of differential excretion of MCs in *M. edulis* showing that the three most hydrophobic MC variants studied were selectively excreted. These results contradict the reports of less hydrophobic MCs being more easily excreted, whereas the more hydrophobic variants would be expected to be accumulated (Kozlowsky-Suzuki *et al*., 2012).

Table 4. 12 Percentage of MC variants compared to MC-LR in *M. aeruginosa* PCC 7813 and faecal material of *M. edulis*

	Time in days	Asp^3-MC-LR/ [Dha^7]-MC-LR	MC-HilR	MC-LY	MC-LW	MC-LF
Feed culture stock	1	9	1	20	39	58
	2	9	1	18	37	53
	3	8	1	18	36	53
	4	8	1	18	36	54
	5	9	1	18	36	54
	6	9	1	18	36	53
	7	9	1	18	35	52
Faecal material	1	11	2	32	90	111
	2	10	1	29	72	98
	3	10	2	30	78	107
	4	10	2	30	70	105
	5	11	2	35	80	112
	6	11	2	36	86	118
	7	10	1	29	68	101

The large proportion of unaccounted MCs was likely associated with the combined effect of cyanotoxin metabolism, cyanotoxin degradation (biotic and/or abiotic), covalent binding of MCs and losses during the extraction of samples. An early study has suggested that cyanotoxins were not metabolised in mussels as the purified toxin of *O. agardhii* (CYA-38) and extracts of mussels that had been exposed to live cells of the cyanobacterium showed same effects in mouse bioassays (Eriksson *et al*., 1989). Had mussels metabolised the toxin, it would have been expected to show lower toxicity in the mouse bioassay. More recent studies reported conjugation of MCs to glutathione (GSH) and/or cysteine (Cys) as part of the detoxification process in different animals (He *et al*., 2012; Zhang *et al*., 2012;

Kondo *et al.,* 1996; Zhang *et al*., 2009a; Table 4. 13; Figure 4. 8). Spontaneous conjugation via a Michael type addition of the GSH and Cys thiol groups to the Mdha moiety of MCs has been suggested (Kondo *et al*. 1996; Kondo *et al*., 1992). This hypothesis could not be supported when investigating the reaction of MC-LR with GSH in different pH as results showed no conjugation products being formed (Takenaka 2001). Glutathione-S-transferase (GST) assays on the other hand resulted in MC-LR conjugation indicating the enzyme driven nature of the reaction (Takenaka 2001). This was supported by *in vivo* studies illustrating enzymatic formation of the conjugates via GST (Pflugmacher *et al*., 1998). Beattie *et al.* (2003) showed that soluble GST (sGST), extracted from different developmental stages of *Artemia salina*, were able to conjugate MC-LR and NOD to GSH. Increased activity of microsomal GST (mGST) has also been reported in *P. argentinus* during exposure to dissolved MC-LR (Galanti *et al*., 2013). Furthermore, during the depuration period mGST, cytosolic GST and GSH reductase also showed increased activity which suggested their involvement during MC-LR detoxification (Galanti *et al*., 2013). Contrary to these reports, GST activity determined in clams (*C. leana*) exposed to *M. aeruginosa* NIES-1086 proved no different to that of clams fed the non MC containing *M. aeruginosa* NIES-101 suggesting that GST was not involved in MC elimination (Pham *et al*., 2015).

Zhang *et al*. (2012) exposed bighead carp to 500 µg MC-LR kg^{-1} bw (i.p.) and investigated conjugate formation over twelve hours. The authors detected mainly MC-LR-Cys starting from 60 min post injection. MC-LR-GSH was also detected, although at markedly lower concentrations. Analysis of environmental samples showed the presence of MC-LR-Cys with occasional observation of GSH conjugates. These results support the hypothesis of Li *et al.* (2014) who suggested that MC-RR-GSH was a reactive intermediate further transformed to MC-RR-Cys in the detoxification process of MC-RR in bighead carp. In support of this hypothesis He *et al*. (2012), reported that following i.p. injection of MC-LR and MC-RR only Cys conjugates were detected in liver, kidney, intestine and blood of bighead carp, whereas GSH conjugates were below the LOD. Due to the lack of available standards it was not possible to investigate the presence of potential MC conjugates in the present study.

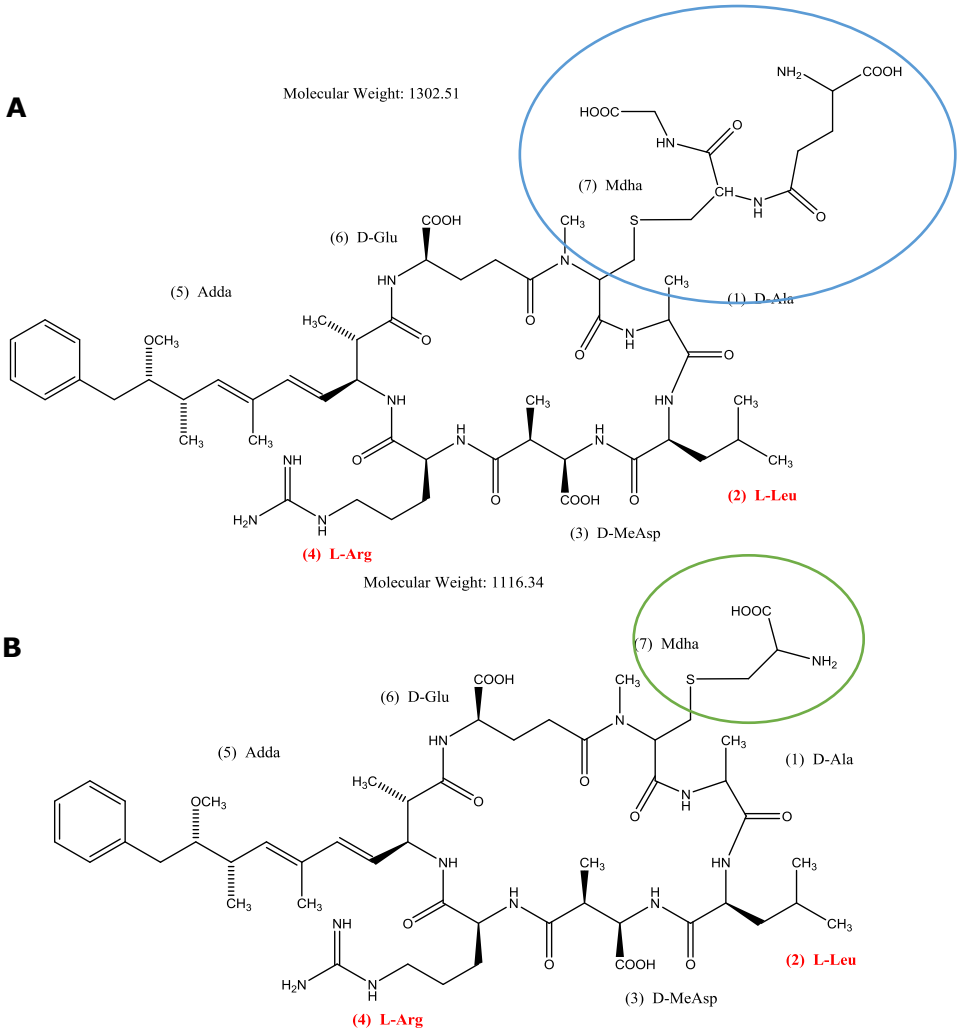

Figure 4. 8 Chemical structure of MC-LR conjugates MC-LR-GSH (A; blue circle) and MC-LR-Cys (B; green circle; adapted from Pflugmacher *et al*., 2001)

Table 4. 13 Detection of MCs conjugates in animals

Cyanotoxin	Form of administration	Experimental animal	Time post exposure	Organ	Conjugate	Reference
MC-LR	i.p. injection	rats	24 h	liver	MC-LR-Cys	Kondo *et al.* (1996)
MC-RR		mice	3 h		MC-RR-GSH	
			24 h		MC-RR-Cys	
MC-LR	i.p. injection	Bighead carp *Aristichthys nobilis*	1h 3 h 5 h 12 h	liver	MC-LR-Cys, MC-LR-GSH	Zhang *et al.* (2012)
	Unknown (field samples)		unknown		mainly MC-LR-Cys, occasionally MC-LR-GSH	
MC-LR	Fed *Anabaena* strain 90	Swan mussels *Anodonta cygnea*	24 h	whole organism	MC-LR-Cys, MC-RR-Cys; no GSH conjugates	Karlsson *et al.* (2005)
MC-LR, MC-HytR	*In vitro* essay	Brine shrimp *Artemia salina*	24 h	whole organism	MC-LR-GSH, MC-HytR-GSH	Beattie *et al.* (2003)
MC-LR	Unknown (field samples)	Silver carp *Hypophthalmichthys molitrix*	Unknown	liver	MC-LR-GSH‡, MC-LR-Cys	Zhang *et al.* (2009a)
				kidney	MC-LR-Cys	
				intestine	MC-LR-GSH, MC-LR-Cys	
MC-LR	Fed *M. aeruginosa* NIVA-CYA 140	Zebra mussel *D. polymorpha*	9 week feeding trial (3 week exposure)	whole organism	None	Pires *et al*. (2004)

i.p. – intraperitoneal, MC-HytR - [D-Asp^3, (Z)-Dhb^7] microcystin HtyR (no Mdha)
‡ Detected in one sample only

The loss in MCs within the experimental system could signal the biotic and/or abiotic degradation of extracellular MCs. Bourne *et al.* (1996), showed that a *Sphingomonas* species was able to cleave the Adda-Arg peptide bond. The enzymatic hydrolysis resulted in linearised MC-LR and a tetrapeptide. Similar results were reported by Imanishi *et al*. (2005) utilising a bacterial strain isolated form Lake Tsukui in Japan. In addition to MC-LR this bacterium also degraded NOD, MC-LF, MC-RR, and geometrical isomers of MC-LR and MC-RR. The authors also suggested a sequential enzymatic hydrolysis initially via the Arg-Adda bond. During the present study the water was not tested for the presence of MCs degrading bacteria. However, the mussels themselves can introduce a significant microbial pollution, which could have contributed to the unaccounted proportion of MCs.

Finally, a portion of the undetected MCs could have been covalently bound to PPases and other proteins. This bond forms between the Mdha moiety of MCs and the sulphur atom of Cys 273 within PPases (Goldberg *et al*., 1995). Consequently, MCs are unavailable for detection. One method to assess the bound MC-LR fraction is based on a Lemieux oxidation (section 1.3). The Adda amino acid of MCs, is oxidised resulting in the formation of 2-methyl-3-methoxy-4-phenylbutyric acid (MMPB) which can be detected by GC in combination with a flame ionisation detector (FID; Sano *et al*., 1992), GC-MS (Williams *et al*., 1997b), or LC-MS/MS (Neffling *et al*., 2010; Cadel-Six *et al*., 2014). However, the oxidation recovery and signal suppression issues have yet to be overcome (Neffling *et al.,* 2010; Cadel-Six *et al*., 2014). Based on the method described by Neffling *et al*. (2010), Lance *et al*. (2010) determined that the bound fraction of MC-LR accounted for up to 66.7% after the exposure of snails to *P. agardhii*. Introducing sodium hydroxide to aid digestion resulted in increased recoveries for oxidation and digestion (70-75%) and solid phase extraction steps (86-103%) for MC-LR (Roy-Lachapelle *et al.,* 2014). Utilising the Lemieux oxidation Williams *et al*. (1997b) compared the amount of free and bound MC present in *M. edulis* following three days exposure to *M. aeruginosa* cells. Results obtained after PPase assays suggested that the extractable fraction of MCs was less than 0.1% of the total amount detected. Pires *et al*. (2004) reported 38% of bound MC-LR in zebra mussel tissue after two weeks exposure to *M. aeruginosa* NIVA-CYA 140. Exposing snails to *P. agardhii* resulted

in 18-67% of bound MCs (Lance *et al*., 2010b). These values were similar to the percentage of unaccounted MCs determined during the present study. In future studies the Lemieux oxidation method could be applied to assess the presence of bound MCs to gain further insights into the distribution of MCs in the experimental system.

Based on the results obtained, the theoretical amount of each MC congener in mussel was calculated and compared to the actual amount detected (Table 4. 14). For all six congeners the amount detected (%) ranged from 22-52% of the amount expected. In order to protect the general public it was therefore suggested to include a correction factor of 2.1-2.8 in the analysis of MCs in mussel flesh. These were calculated by dividing the average amount detected (%) of each of the six congeners (n=7) by 100%. These values compared well to a correction factor of 2.6, obtained for NOD during the first feeding trial (section 3.3.4).

Table 4. 14 Dose recovery (%) of MCs in mussel flesh based on cyanotoxins available in feed stock cultures and suggested correction factors for the analysis of MCs in *M. edulis*.

Congener	Time in days	Added MCs in µg	Added minus amount in water and faecal pellet (Theoretical amount in mussels)	Actual in mussel	% detected	Suggested correction factor
MC-LR	1	1124	789	171	21.67	2.8
	2	2929	2166	691	31.92	
	3	4834	3767	1343	35.66	
	4	6652	5094	2118	41.57	
	5	8189	6095	1915	31.42	
	6	10033	7409	3194	43.10	
	7	11902	8705	3679	42.26	
Asp^3-MC-LR/[Dha^7]-MC-LR	1	102	37	17	46.02	2.1
	2	250	107	53	49.97	
	3	403	195	101	51.90	
	4	554	257	135	52.36	
	5	698	315	135	42.81	
	6	855	366	168	45.85	
	7	1012	431	206	47.89	
MC-HilR	1	14	14	5	34.34	2.3
	2	35	35	14	41.00	
	3	58	58	26	45.32	
	4	80	80	40	50.20	
	5	99	99	37	37.58	
	6	121	121	57	47.16	
	7	143	143	66	46.39	

Table 4. 14 continued

Congener	Time in days	Added MCs in µg	Added minus amount in water and faecal pellet (Theoretical amount in mussels)	Actual in mussel	% detected	Suggested correction factor
MC-LY	1	224	196	45	23	2.6
	2	540	400	151	38	
	3	879	663	275	42	
	4	1205	862	395	46	
	5	1483	1007	391	39	
	6	1800	1186	518	44	
	7	2111	1363	571	42	
MC-LW	1	434	319	99	31	2.4
	2	1076	815	290	36	
	3	1760	1392	564	40	
	4	2405	1843	844	46	
	5	2943	2155	953	44	
	6	3565	2561	1192	47	
	7	4163	2944	1381	47	
MC-LF	1	647	485	142	29	2.3
	2	1565	1227	496	40	
	3	2583	2112	877	42	
	4	3568	2872	1415	49	
	5	4387	3445	1449	42	
	6	5341	4151	2064	50	
	7	6245	4721	2316	49	

4.4 Conclusion

The freshwater cyanobacterium *M. aeruginosa* PCC 7813 did not leech extracellular MCs when grown in BG-11 containing seawater for up to 8 h enabling it to be used in the feeding trial of *M. edulis*. MCs were rapidly accumulated by mussels with maximum concentrations of 8.5 µg g^{-1} detected on day seven. After the initial drop in MC concentration following the start of the depuration period, total MC concentration of 1.2 µg g^{-1} was still detected after 22 days of depuration. Consuming a serving of mussels on both day seven (end of dosing) and after depuration would result in a significant MC intoxication. The acute and seasonal tolerable intake guideline for a 75 kg adult would be exceeded by more than 3.5 and ~23 times on day seven , respectively (Ibelings and Chorus 2007). In addition, even after 22 days of depuration the seasonal tolerable intake would be exceeded by a factor of approximately 3. The distribution of MCs within the experimental system showed that the majority was unaccounted for, likely due to a combined effect of metabolism, biotic and/or abiotic degradation, covalent binding of MCs and losses during sample extraction. Based on the present study the incorporation of a correction factor (2.1-2.8) in the analysis of MCs in mussel flesh was suggested.

4.5 Appendix

Table 4. 15 Distribution of individual MC congeners (µg) in the experimental system following the exposure of *M. edulis* to *M. aeruginosa* PCC 7813.

	Time in days	MC-LR	Asp^3-MC-LR/[Dha^7] -MC-LR	MC-HilR	MC-LY	MC-LW	MC-LF
Feed culture stock (cumulative)	1	1124	102	14	224	434	647
	2	2929	250	35	540	1076	1565
	3	4834	403	58	879	1760	2583
	4	6652	554	80	1205	2405	3568
	5	8189	698	99	1483	2943	4387
	6	10033	855	121	1800	3565	5341
	7	11902	1012	143	2111	4163	6245
Intracellular (cumulative)	1	44	7	0	12	25	38
	2	214	25	0	54	108	145
	3	286	32	0	73	145	195
	4	526	54	0	124	257	342
	5	816	82	0	190	390	521
	6	1075	110	0	250	512	673
	7	1304	133	0	303	632	828
Extracellular (cumulative)	1	283	38	0	11	83	113
	2	534	71	0	17	158	215
	3	808	107	0	19	238	317
	4	1124	153	0	38	366	485
	5	1473	201	0	45	510	681
	6	1818	245	0	57	650	866
	7	2226	288	0	60	769	1113
Mussel flesh	1	171	17	5	45	99	142
	2	691	53	14	151	290	496
	3	1343	101	26	275	564	877
	4	2118	135	40	395	844	1415
	5	1915	135	37	391	953	1449
	6	3194	168	57	518	1192	2064
	7	3679	206	66	571	1381	2316
Faecal material	1	1.99	0.21	0.04	0.64	1.80	2.20
	2	4.91	0.49	0.07	1.41	3.55	4.83
	3	6.57	0.67	0.11	1.94	5.12	7.03
	4	9.68	0.96	0.15	2.89	6.79	10.21
	5	5.23	0.57	0.09	1.81	4.21	5.86
	6	3.76	0.43	0.07	1.37	3.23	4.45
	7	7.46	0.76	0.11	2.14	5.08	7.50

CHAPTER 5

The accumulation and depuration of nodularin and six microcystins in the edible blue mussel *Mytilus edulis* after the exposure to mixed cultures of *Nodularia spumigena* KAC66 and *Microcystis aeruginosa* PCC 7813

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5.1 Introduction

In Scotland no targeted monitoring of cyanotoxins is performed (Krokowski *et al.,* 2012). Only in the case of observed cyanobacteria blooms will these be evaluated for cyanotoxin presence. Genera encountered in 2008-2010 were *Aphanocapsa, Aphanothece, Microcystis, Woronichina, Oscillatoria* (*Planktothrix*), *Anabaena, Aphanizomenon* and *Gloeotrichia* (Krokowski *et al*., 2012). The potential impacts of MCs and NOD exposure on human health have long been recognised. Currently a tolerable daily intake (TDI) level of 0.04 µg kg^{-1} d^{-1} has been issued by the WHO (Kuiper-Goodman *et al*., 1999), however, no legally binding limits are in place. In addition, acute and seasonal tolerable intake guidelines of 190 and 30 µg, respectively have been derived for a 75 kg adult (Ibelings and Chorus 2007). In contrast these recommended values, strict guidelines and monitoring programs for marine toxins in seafood are in place according to the EU legislation 853/2004 (Table 5. 1). Clearly, both MC-LR and NOD show toxicities well within the range of these toxins illustrating the need for monitoring of cyanotoxins in seafood during and after cyanobacteria blooms. In order to protect the general public it is vital to better understand the mechanisms of trophic transfer of cyanotoxins in both freshwater and the marine environments (Carmichael and Boyer 2016).

Table 5. 1 Toxicity of marine toxins and their regulatory limits compared to cyanotoxins.

Toxin	LD_{50} *i.p.* in mice (μg kg^{-1})	Limit per kg seafood (μg)	Reference
Saxitoxin	3-10	800*	Wang (2008)
Okadaic acid	204-225	160*	Aune *et al*. (2007); Tubaro *et al*. (2003)
DA	3,600	20,000*	Grimmelt *et al*. (1990)
Yessotoxins	512	3750†	Tubaro *et al*. (2003)
Azaspiracid	100-700	160*	Wang (2008); Twiner *et al*. (2008)
MC-LR	50-60	2.4 (TDI)‡ 152 (acute tolerable intake)‡ 24 (seasonal tolerable intake)‡	Kuiper-Goodman *et al*. (1999); Ibelings and Chorus 2007
NOD	50-70	2.4 (TDI)‡	Namikoshi *et al*. (1993); Runnegar *et al.* (1988)

*** -** EU regulation 853/2004 † - EU regulation 786/2013 ‡ - for a 60 kg adult
TDI - Tolerable daily intake according to the WHO

The estimation of risks associated with cyanotoxin contaminated seafood is a highly complex process. This process is further hindered by the lack of knowledge regarding the fate of NOD and MCs in seafood. Microcystins covalently bind to PPases (1 and 2A) and other proteins. The covalent bond forms between the methyldehydroalanine (Mdha) residue of MCs and the cysteine of the PPase (MacKintosh *et al.,* 1995). Nodularin on the other hand does not covalently bind to PPase as the Mdha moiety is replaced by methyldehydrobutyrine (Mdhb, Namikoshi *et al.,* 1994). It has been suggested that only free, detectible MCs are relevant for the determination of risks to public health (Freitas *et al.,* 2014). However, the effect of cooking methods and the passage through the gastro intestinal tract could potentially lead to MC release (Smith *et al.,* 2010). Reports describing the effect of cooking methods on MC availability are controversial. Guzmán-Guillén *et al.* (2011), showed that boiling of the fish *Oreochromis nitoticus* (muscle) resulted in a decrease of MC-RR, MC-LR, and MC-YR concentration. Analysis of the water used for boiling revealed the presence of all three MC congeners. Highest concentrations were found for MC-RR followed by MC-LR and MC-YR. Morais *et al.,* (2007), reported a slight decrease of MCs (ELISA analysis) after boiling of MC-contaminated mussels (*Mytilus galloprovincialis*), however, the reduction was not deemed significant.

Contrary to these results Freitas *et al.* (2014) and Zhang *et al.* (2010), reported increased MCs concentrations after boiling of clams (*Corbicula fluminea*) and bighead carp (*Aristichthys mobilis*), respectively. Both studies also detected MCs in the cooking water. Freitas *et al.*, (2014) reported the combined MCs concentration of clam tissue and water used for boiling. Hence, it is not clear if the concentration in muscle tissue itself decreased or increased. Zhang *et al.*, (2010) performed their experiment three and six hours after the carp had been injected with 500 µg kg^{-1} bw MC-LReq. The reported increase of MC-LReq detected after boiling could be explained by protein denaturation. Consequently, the increase might have been MCs released from denatured PPase. Similar to the observations made for the effect of boiling, the effect of microwaving on MC recovery caused increased levels in some studies, but decreased levels in others (Guzmán-Guillén *et al*., 2011; Morais *et al*., 2007; Freitas *et al*., 2014; section 1.5). Increases could again be associated with the denaturation of PPase and the release of bound toxin, while the reduction, on the other hand, is currently not understood. Potentially, molecular vibrations caused by the radiation lead to bond disruptions within the molecules (section 1.5). The temperature itself should not have affected the stability of MCs during either boiling or microwaving as they have been reported to be heat stable (Metcalf and Codd, 2000; Mekebri *et al.*, 2009; Dyble *et al.,* 2011; Guzmán-Guillén *et al*., 2011). These contradictory reports concerning the effect of boiling and microwaving on the MCs recovery in fish and shellfish, highlight the need for further research to fully understand the mechanism involved. This will be vital for future investigations of health risks associated with MC contaminated fish and shellfish.

Nonetheless, to ensure public safety, the total toxin burden of seafood should be considered to assess the risks associated with cyanotoxin contaminated seafood. Currently the detection of covalently bound MCs is performed via a Lemieux oxidation in combination with GC-FID (Sano *et al*., 1992), GC-MS (Williams *et al.,* 1997a), or LC-MS/MS (Neffling *et al.,* 2010; Cadel-Six *et al*., 2014; section 1.3). Challenges yet to be overcome are the effective recovery during the multistep procedure, especially when complex matrices are encountered (Table 5. 2). Analysis of river water showed excellent oxidation recoveries with minimal matrix effects (Roy-Lachapelle *et al*., 2014). However, when analysing more complex matrices such as snail and fish tissue, drastic

reductions in oxidation recovery occurred (Neffling *et al.,* 2010; Cadel-Six *et al*., 2014). Furthermore, additional concentration steps such as solid phase extraction (SPE) introduce another source of recovery loss. Due to the oxidation of the Adda moiety common to MCs and NOD, neither they nor their metabolites can be distinguished.

Table 5. 2 Reported recovery of total MCs during Lemieux oxidation analysis

Matrix	Oxidation recovery (%)	Matrix effect (%)[1]	SPE efficiency (%)	Reference
River water	97-109	87-97	NA	Roy-Lachapelle *et al.* (2014)
L. stagnalis	29-40	16-37	62-97	Neffling *et al*. (2010)
Juvenile trout Intestine*	6	122	27	Cadel-Six *et al*. (2014)
Liver	17	NA	28	
Gills	12	NA	18	
Muscle	5	142	27	

SPE - solid phase extraction, NA - not applicable ***** also includes stomach and spleen
1 – calculated as spike recovery

In order to gain a clear understanding of the uptake and depuration of NOD and six MC variants in a closed system, the common blue mussel *M. edulis* was exposed to both *N. spumigena* KAC66 and *M. aeruginosa* PCC 7813. This simulated the short term exposure of *M. edulis* to cyanobacteria native to brackish waters, but also the rising threat of the influx of freshwater strains into the marine environment. Due to the non-covalent interaction of NOD and PPases (section 1.2.1) it was intended to utilise NOD as internal standard within the system to assess covalent binding of MCs. In addition, in an effort to increase cyanotoxin recovery from mussel tissue the effect of autoclaving on the extraction of cyanotoxins was investigated, which also simulated a form of cooking.

5.2 Materials and Methods

5.2.1 Chemicals

As described in section 4.2.1. NOD and LNOD were purified from *N. spumigena* KAC66 (Liu *et al*., 2005).

5.2.2 Effect of autoclaving on toxin recovery in *Mytilus edulis*

To assess potential effects of heat on the extraction of MCs and NOD from *M. edulis*, similar to the process of cooking, 5.00 ± 0.01 g of MC or NOD contaminated mussel flesh were weighed into clean glass bijou bottles. Potentially, this step could release protein bound cyanotoxins and positively effect cyanotoxins recovery from mussel flesh, which has presented challenges during the last feeding trial (section 4.3.6). Contaminated mussel flesh from the two previous feeding studies, stored at -80°C, was thawed and thoroughly mixed to obtain a NOD contaminated homogenate and a second MC contaminated homogenate. The two homogenates were extracted as follows (n=3): the homogenate was autoclaved at 121°C for 15 min. Once cooled 5 mL of MeOH was added and samples were vortexed for 2 min. A 1 mL subsample was transferred to a 1.5 mL centrifuge tube and centrifuged at 10,433x g for 10 min. Subsequently, samples were syringe filtered (0.2 µm, nylon) into UPLC vials. The second set of samples was treated as described above except that 5 mL of water (purified to 18 MΩ; Purelab flex, Veolia Water Technologies, UK) was added to the sample, which was vortexed for approximately ten seconds prior to autoclaving. These samples were extracted as above using 10 mL MeOH. The last set of samples was extracted in 5 mL of MeOH without being autoclaved (Figure 5. 1).

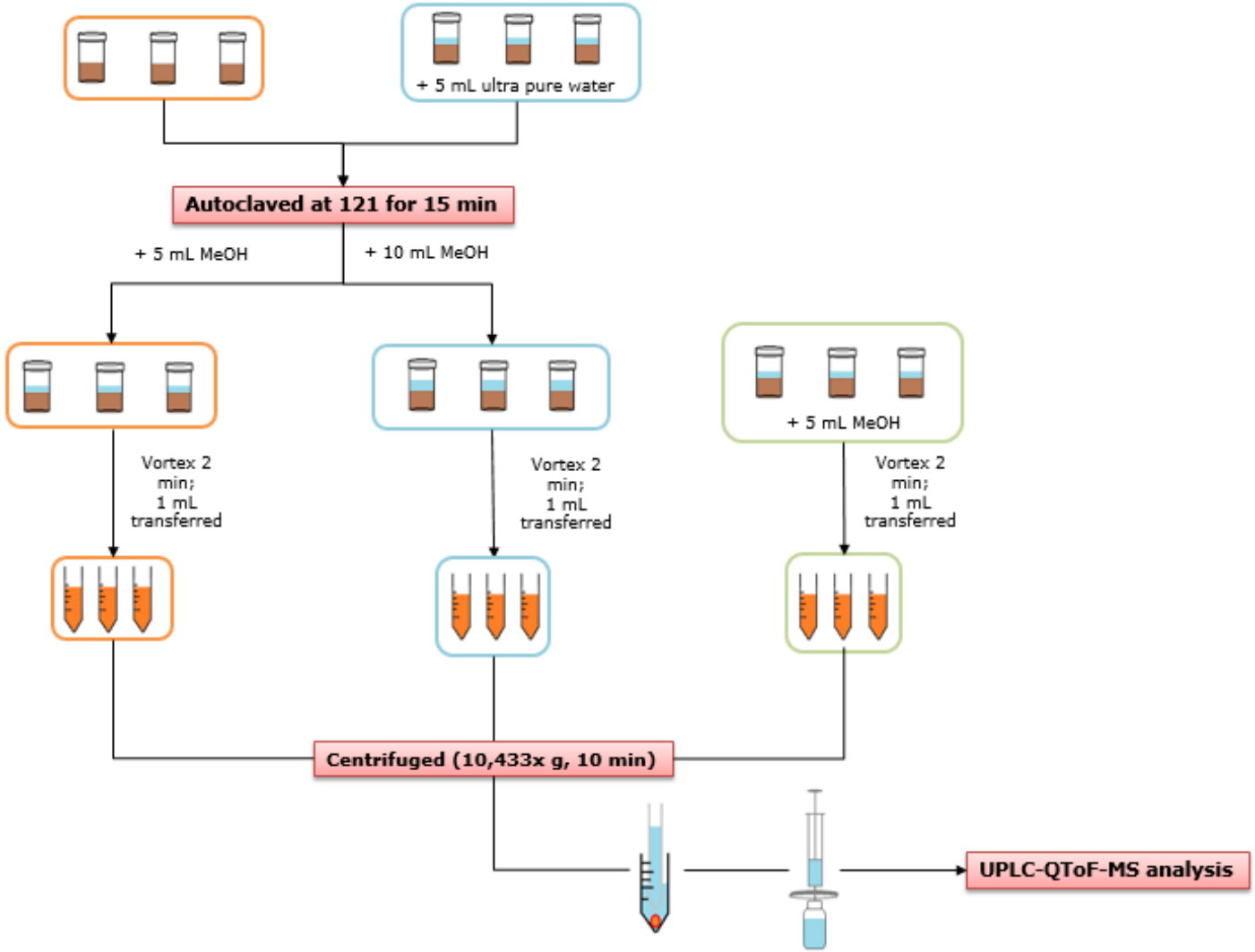

Figure 5. 1 Different extraction procedures utilised for the detection of NOD and MCs in mussel homogenate by UPLC-QToF-MS analysis.

5.2.3 Culturing of cyanobacteria cultures

N. spumigena KAC66 (Kalmar Algae Collection, Kalmar, Sweden) and *M. aeruginosa* PCC 7813 (Pasteur Culture collection of Cyanobacteria, Paris, France) cultures were grown in BG-11 medium and scaled up from laboratory cultures as previously described (section 3.2.3; section 4.2.2). They were maintained at 21 ± 1°C with continuous illumination (10-15 µmol m^{-2} s^{-1}). To obtain a homogenous culture stock for each cyanobacterial strain cell properties were exploited as follows. Following the cessation of sparging four days prior to transport *N. spumigena* KAC66 cells concentrated at the surface due to the presence of intracellular gas vesicles and concentrated cells were transferred into two 10 L carboys. *M. aeruginosa* PCC 7813 on the other hand lacks gas vesicles and settled following discontinued aeration. Concentrated cells were transferred into two additional 10 L carboys (Figure 5. 2). After transport the two 10 L carboys of each strain were combined to give a uniform stock culture of each strain, which was maintained in a 25 L carboy with mild aeration provided via PVC tubing. During the experiment both cultures were maintained at 17 ± 1°C. The light cycle was set to 17 hours of light (24 µmol m^{-2} s^{-1}) and 7 hours of darkness.

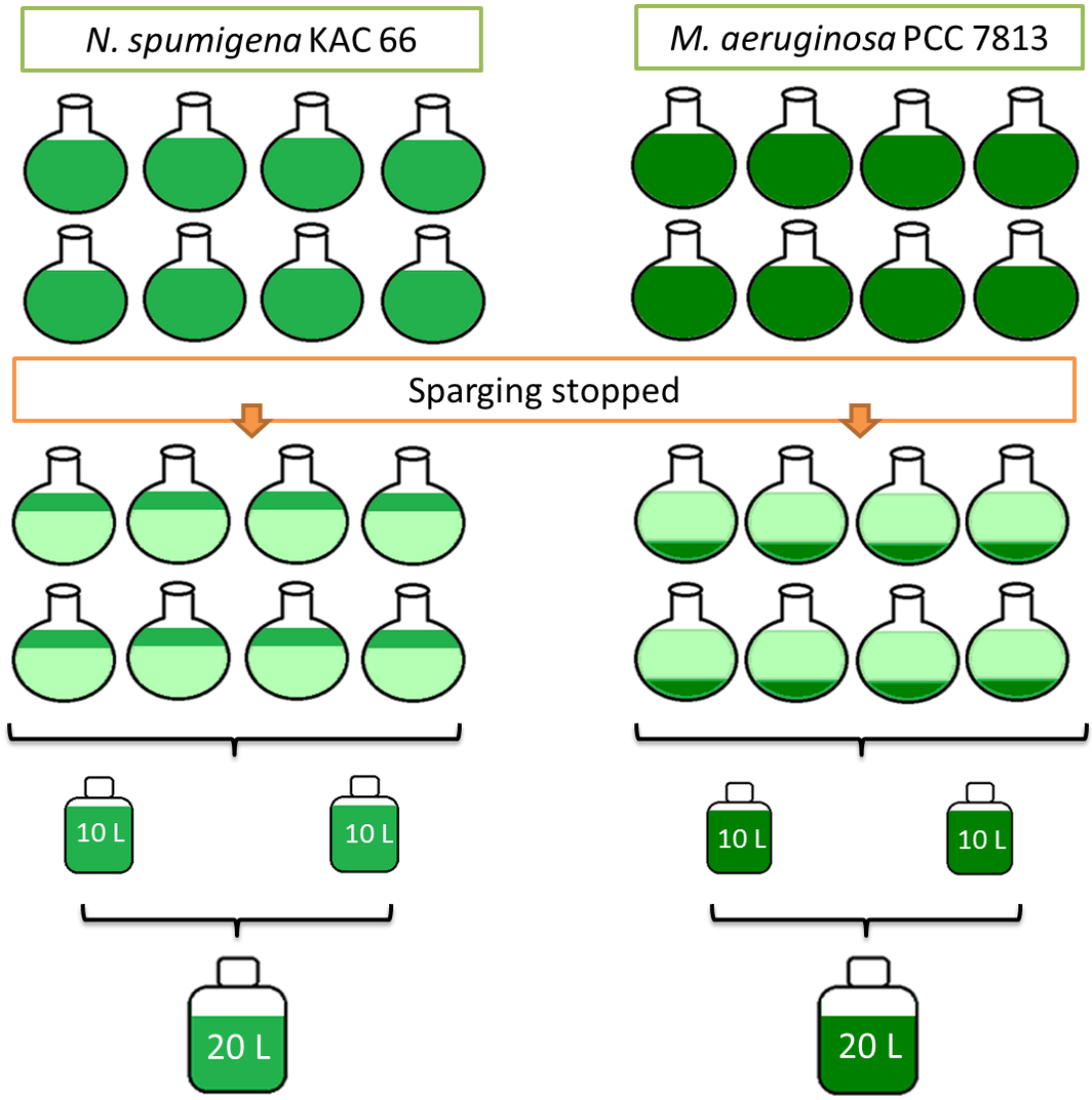

Figure 5. 2 Preparation of homogenous culture stocks of *N. spumigena* KAC66 and *M. aeruginosa* PCC 7813 utilised for the exposure of *M. edulis.*

5.2.4 Accumulation and depuration of cyanotoxins in *M. edulis*

All mussels (shell length of 50-65 mm), originating from the UK, were acclimatised to the laboratory tank conditions for one week and cleaned of barnacles and other debris prior to the experiment. Containers (12) were filled with 10 L of UV sterilised, filtered seawater (~35 psu), housing 70 mussels supported by mesh baskets. Gentle aeration was provided via PVC tubing (4.00 mm bore size, 1.00 mm wall thickness). Six of the twelve experimental tanks were utilised for the exposure of *M. edulis* to *M. aeruginosa* PCC 7813 and *N. spumigena* KAC66, whereas the remaining six tanks acted as control tanks (Figure 5. 3). Exposure and negative control samples were separately maintained in 300 L tanks filled with ~ 150 L seawater, which were fitted with heating devices to maintain water temperatures of 16 ± 1°C. In addition, outer tanks were equipped with ultraviolet (UV) sterilisers (class 1 IP64, twin UV 24 W, 240 V, 50 Hz, Tropical Marine Centre, UK).

Each day for three days the six cyanotoxin exposure tanks received a combined dose of 47 µg L^{-1} NOD with the addition of 300 mL *N. spumigena* KAC66 and 85 µg L^{-1} total MCs from 400 mL of *M. aeruginosa* PCC 7813. These values include both the intracellular and extracellular amount of the respective cyanotoxin. To ensure culture homogeneity, carboys were rigorously shaken each day prior to use for feeding. To encourage feeding by the shellfish 0.14 mL of shellfish diet (approximately 7.4 x 10^{11} cells per mL, Shellfish diet 1800, ReedMariculture Inc., US) was diluted into 500 mL of water and added to the exposure tanks. All negative control tanks were fed 0.33 mL of shellfish diet diluted into 1.2 L of water. To account for the salt content in the *N. spumigena* KAC66 culture, the shellfish diet was diluted into a water-seawater mixture (10:0.86). From day four onwards exposure tanks were treated identically to negative controls. A full water exchange was performed daily for each of the individual tanks housing the mussels prior to feeding.

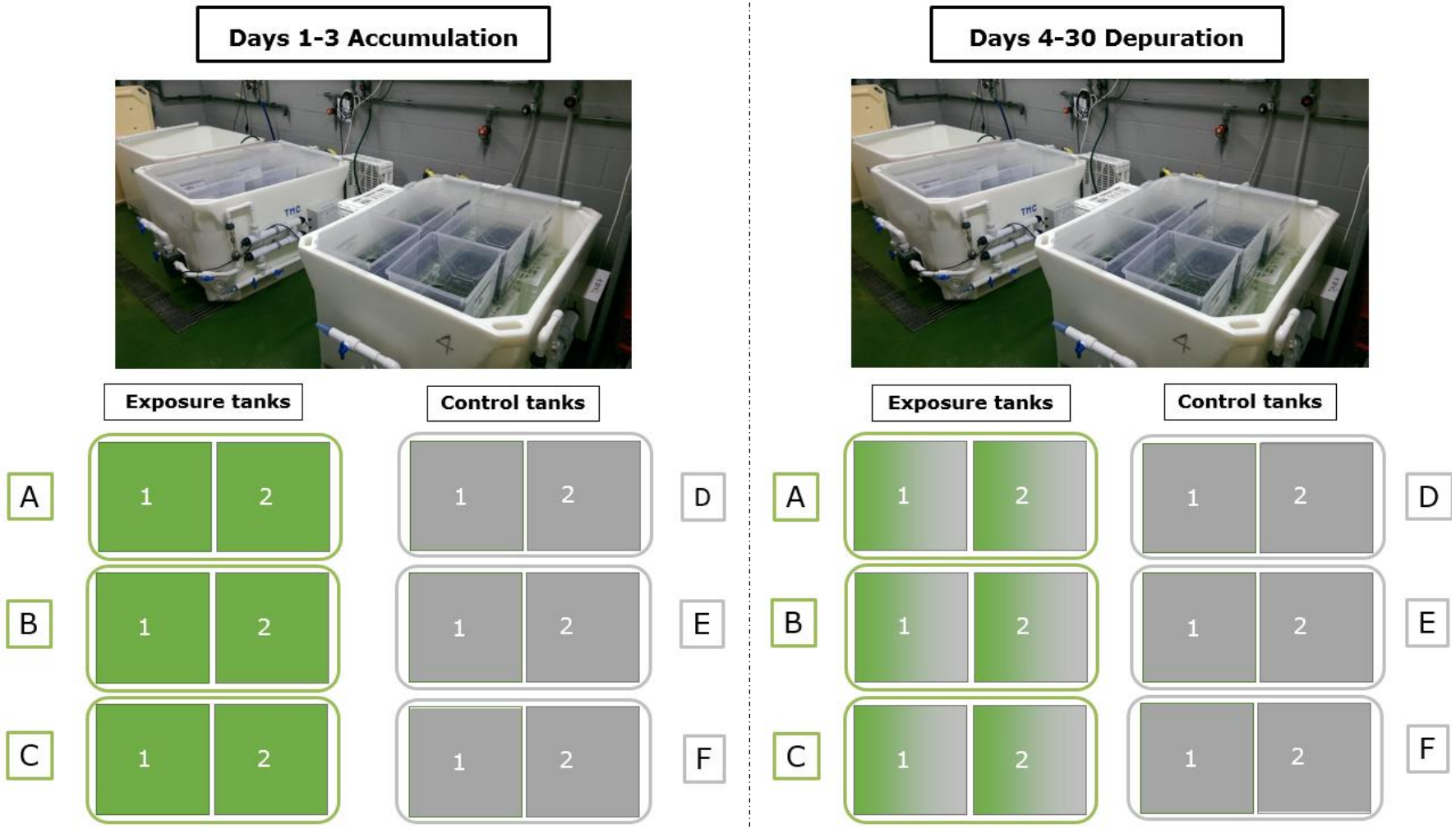

Figure 5. 3 Combined exposure of *M. edulis* to 300 mL *N. spumigena* KAC66 (~1.67 µg mL^{-1} NOD) and 400 mL of *M. aeruginosa* PCC 7813 (~1.2 µg mL^{-1} MC-LR; green). Negative controls (grey) were fed with shellfish diet. All twelve tanks were filled with 10 L of filtered seawater housing 70 mussels supported on mesh baskets. From the start of the depuration period (day 4) exposure tanks were treated like negative controls (gradient green to grey).

Cyanobacteria feed stock cultures were sampled (1 mL) prior to feeding to assess intracellular and extracellular cyanotoxin levels. Based on the slow depuration observed during previous feeding trials (chapter 3 and 4), the depuration period was extended to 26 days during the present study. To accommodate this lengthy depuration period, two experimental tanks (sub-tanks; 1 and 2; Figure 5. 3) were treated as one overall sample of 140 mussels from which 10 mussels were sampled (five from each sub-tank; Figure 5. 3). Mussels were sampled, on the following days: 1, 2, 3, 5, 7, 9, 12, 15, 18, 21, 24, 27, and 30. For each mussel sampled a 'stunt double' (SD) mussel, which had not been exposed to the cyanobacteria, was placed into a separate mesh basket kept within the containers to maintain a constant ratio of mussels exposed to the cyanobacteria (Amorim and Vasconcelos 1999, Bricelj *et al*. 1990; Figure 5. 4). Similarly to the mussel samples, the two water sub-samples (100 mL) of sub-tanks 1 and 2 were combined and thoroughly mixed (i.e. A1 and 2; Figure 5. 3). Water samples were taken twice daily: prior to the daily water exchange (approximately 8:30 am) and post-feeding (approximately 1:30 pm). To ensure identical t_0 times for all samples, mussels had been removed from tanks prior to sampling. Once the morning water sample had been taken, the water of both sub-tanks (1 and 2) was filtered (80 µm steel mesh) and the collected faecal material was washed into pre-weighed 50 mL centrifuge tubes using deionised water to collect the faecal material.

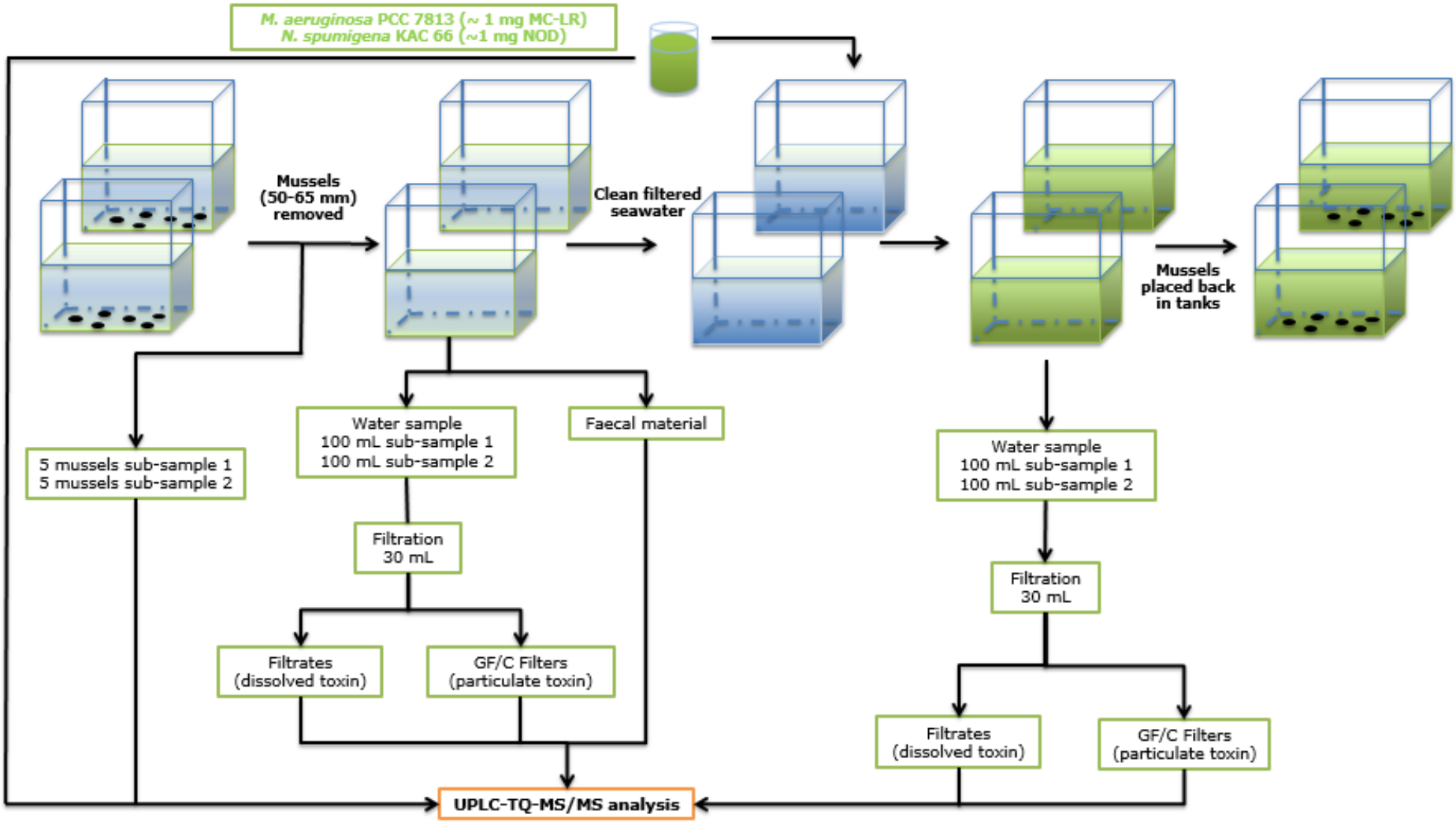

Figure 5. 4 Schematic illustration of sampling procedure of exposure tank. Control containers were treated identically except for shellfish diet being added to each tank rather than cyanobacteria.

5.2.5 Sample processing

5.2.5.1 Extraction of cyanotoxins from *M. aeruginosa* PCC 7813 feed stock cultures

Culture samples were centrifuged (12,470 g; 10 min; Sigma 1-14 K, Osterode, Germany) and supernatants transferred into LC-MS vials (extracellular). The pellet samples (intracellular) were extracted with 1 mL of 80% aqueous methanol for an hour. Samples were vortexed for 10 s every 15 min. Following extraction, samples were centrifuge (12,470 g; 10 min) and the supernatant was transferred into LC-MS vials. Both pellet extracts and supernatants were stored at -80°C until UPLC-MS/MS analysis.

5.2.5.2 Extraction of cyanotoxins from tank water

Water samples (30 mL) were filtered through 55 mm glass microfiber filters (GF/C, GE Health Care, Little Chalfont, Buckinghamshire, UK). Filtrate samples (1 mL) were transferred directly into UPLC vials and stored at -80°C until analysis. Filters were transferred to 15 mL centrifuge tubes and extracted with 5 mL aqueous methanol (20:80). Samples were vortexed for 2 min at 2500 rpm (DVX-2500 Multi-Tube Vortexer, VWR International, Pennsylvania, USA). Extracts were then filtered through nylon syringe filters (0.2 µm) into amber LC-MS vials and stored at -80°C.

5.2.5.3 Extraction of cyanotoxins from *M. edulis*

Mussels (n=10) were shucked and drained to allow separation from the excess liquid, the extrapallial fluid. Following, the drained and shucked tissue of 10 mussels was weight. This fluid was collected in 50 mL centrifuge tubes of which a 1 mL sub-sample was centrifuged (12,470 g, 10 min). The supernatant was transferred to LC-MS vials and stored at -80°C until analysis. The drained tissue was blended into a smooth homogenous paste, by pulse blending four times for 15 s (Waring Commercial, USA). In between pulses samples were rested for 5 s. Triplicates of the homogenate were weighed (2 g ± 0.05 g) into 50 mL centrifuge tubes and extracted with 8 mL of aqueous methanol (20:80). Samples were vortexed for 2 min at 2,300 rpm (Advanced Multi-Tube Vortexer,

VWR International, USA) and centrifuged (Sorvall ST 40R, Thermo Scientific, UK) at 2,279 g for 10 minutes. Samples were then filtered into LC-MS vials and stored at -80°C until analysis (Figure 5. 5).

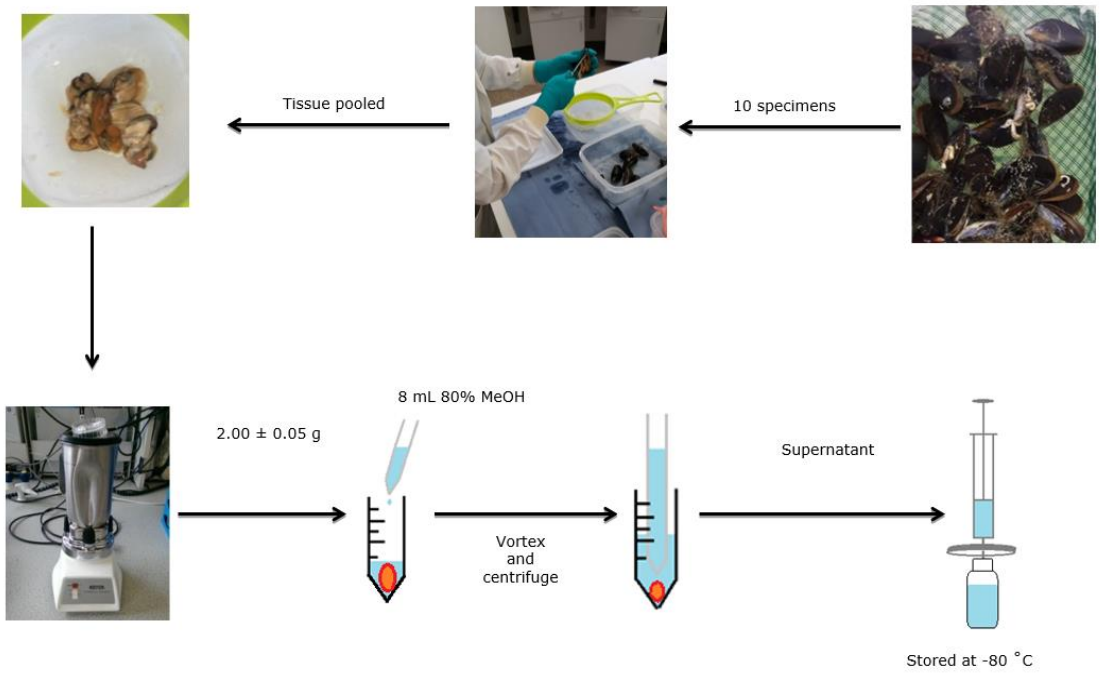

Figure 5. 5 Extraction procedure for the analysis of *M. edulis* exposed to *N. spumigena* KAC66 and *M. aeruginosa* PCC 7813. Ten specimen were pooled, pulse blended and extracted in 80% MeOH. Thereafter samples were vortexed (2 min; 2,300 rpm), centrifuged (10 min; 2,279 xg) and supernatants transferred into UPLC vials. Extracts were stored at -80 until analysis.

5.2.5.4 Extraction of cyanotoxins from faecal material

The collected material was centrifuged at 2,279 g for 40 min (Sorvall, ST 40R, ThermoScientific, USA) to obtain the faecal pellet. The supernatant was discarded and toxins were extracted in 5 mL 80% aqueous MeOH. After vortexing the sample for 2 min at 2,500, rpm approximately 1 mL of extract was transferred into 1.5 mL centrifuge tubes. Samples were centrifuged (12,470 g, 20 min) and the supernatant transferred into LC-MS vials. Samples were stored at -80°C until further analysis.

5.2.6 UPLC analysis of samples obtained during the simultaneous exposure of *M. edulis* to *N. spumigena* KAC66 and *M. aeruginosa* PCC 7813

Sample analysis was performed as described in section 4.2.5. In addition to the six MCs NOD and LNOD were included in the analysis. SRMs were used to optimise the method and LOD and LOQ determined in various matrices (Table 5. 3, Table 5. 4). Due to the lack of a commercially available LNOD standard it could not be included in the calibration standards. Quantitation of LNOD was achieved based on a Relative Response Factor (RRF) calculated from the NOD:LNOD SRM peak area ratio (primary transition) determined following the injection of NOD and an in-house purified LNOD standards (n=5, 1 µg mL^{-1} both; Table 5. 5): 0.808. This was utilised to quantify LNOD based on the NOD external calibration.

Table 5. 3 SRM transitions used for the detection and quantitation of NOD and LNOD

Cyanotoxin	Mode	SRM transitions	Cone, V	CE, eV
NOD	+ve	825.5 > <u>135.1</u>; 103.1	55	60; 100
LNOD	+ve	692 > 175, <u>135</u>, 107	55	37; 54; 58

CE = Collision energy; underlined are the primary transitions

Table 5. 4 LODs and LOQs of NOD in various matrices following UPLC-TQ-MS/MS analysis

Matrix	LOD	LOQ
Algal cells (µg L^{-1})	0.0004 ± 0.0002	0.0012 ± 0.0008
Water (µg L^{-1})	0.04 ± 0.02	0.13 ± 0.06
Mussels (µg kg^{-1})	0.05 ± 0.01	0.16 ± 0.03

Table 5. 5 Peak areas detected following the injection (n=5) of NOD and LNOD standards (1 µg mL^{-1}) utilising UPLC-TQ-MS/MS analysis

Cyanotoxin	1	2	3	4	5	mean	SD	%RSD
LNOD	1029	698	1288	909	693	923	249	26.98
NOD	744	739	776	761	712	746	24	3.24

5.2.7 Statistical evaluation

A statistically significant value of p=0.05 was set. The Excel software (Microsoft Office Professional Plus 2013) was used to perform a single factor ANOVA analysis to determine potential presence of significant differences between cyanotoxin recoveries following the utilisation of three extraction procedures (section 5.2.2). Similarly, single factor ANOVA was utilised to determine the presence of significant difference between extracellular cyanotoxin at dosing and approximately 19 h later.

5.3 Results and Discussion

5.3.1 The effect of autoclave extraction on toxin recovery in *Mytilus edulis*

Generally, low precision was obtained following determination of NOD concentration in mussel flesh utilising all three extraction procedures (n=3; Figure 5. 6). No significant difference was seen between NOD concentrations obtained utilising the three extraction methods (p=0.43). For LNOD a significant higher concentration was detected utilising the MeOH extraction method without autoclaving (p=0.003; Figure 5. 6). Similarly, highest MC-LR concentration was recovered following the MeOH extraction with no pre-treatment by autoclave (Figure 5. 6). The observed differences were statistically significant (p=0.001). It was shown that autoclaving did not increase recovery, hence, it was not incorporated into the sample extraction procedure.

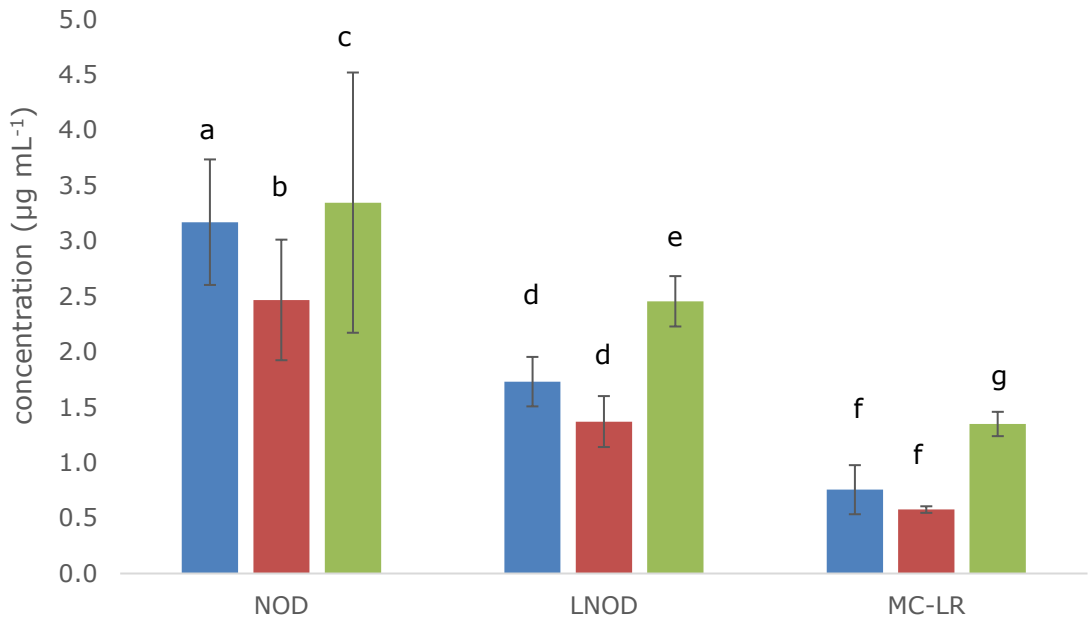

Figure 5. 6 Concentration of nodularin, linear nodularin, and microcystin-LR following different extraction procedures. Blue: samples (5 ± 0.05 g) were autoclaved with 5 mL Elga water and extracted with 10 mL methanol. Red: samples (5 ± 0.05 g) were autoclaved and extracted with 5 mL methanol. Green extraction with 5 mL of methanol. Same letters show results that are not significantly different. Denotation with different letters marks statistically significant differences between the three extraction procedures for the respective cyanotoxin only.

5.3.2 Quantification of cyanotoxins in feed stock cultures

Both cultures showed excellent stability of toxin content during the feeding trial. Variability between the three day treatments was below 6% for *N. spumigena* KAC66. The majority of NOD was contained within cells. The extracellular toxin fraction ranged from 4-9 µg L^{-1} (0.2-0.5%; Table 5. 6). This corresponded with the extracellular toxin levels reported in the literature. Møgelhøj *et al*., (2006) reported extracellular NOD only being detectable in the stationary growth phase (after 12 days of inoculation) where it amounted to up to 12% of overall NOD. The culture used during the present feeding study was older than this, but still only small concentrations of extracellular NOD were detected (maximum 0.5%). NOD in its linear form, LNOD, was also present during the current study where it was detected at levels three orders of magnitude lower than NOD (intracellular and extracellular ~1 µg L^{-1}; Table 5. 6).

Table 5. 6 NODs detected in *N. spumigena* KAC66 culture utilised for the exposure of *M. edulis*. Samples were analysed utilising UPLC-TQ-MS/MS

Cyanotoxin (µg L^{-1})	Day	Time (days)			mean	SD	%RSD
		1	2	3			
NOD	Intracellular	1807	1774	1623	1735	98	6
	Extracellular	4	9	5	6	3	50
LNOD	Intracellular	1	1	1	1	0	0
	Extracellular	1	1	1	1	0	0

The *M. aeruginosa* PCC 7813 samples contained six MC variants: MC-LR, Asp^3-MC-LR/[Dha^7]-MC-LR, MC-HilR, MC-LY, MC-LF, and MC-LW. MC-LR was the dominant variant detected at intracellular concentrations ranging from 1120-1339 µg L^{-1} (Table 5. 7). The remaining variants in decreasing abundance were: MC-LF, MC-LW, MC-LY, Asp^3-MC-LR/[Dha^7]-MC-LR, and MC-HilR. Extracellular MC content was low, generally <15% of total toxin. Only Asp^3-MC-LR/[Dha^7]-MC-LR and MC-LY showed slightly higher values of 18-22%. Yokoyama and Park (2002) reported extracellular toxin levels of 2% of the total toxin content during monitoring of a *Microcystis sp*. bloom in a hypereutrophic lake in Japan. Values obtained in this study were slightly higher which could be due to extracellular MCs being more susceptible to both biotic and abiotic degradation in the environment. In addition, increases in extracellular MCs could be attributed to the stress cells experienced during the 13 hour transport to the experimental site. However, results still illustrate that healthy cultures were utilised for the experiment and that cyanotoxin content was consistent over the three exposure dates.

Table 5. 7 MC variants detected in *M. aeruginosa* PCC 7813 utilised for the exposure of *M. edulis*. Samples were analysed utilising UPLC-TQ-MS/MS

Cyanotoxin (µg L^{-1})	Toxin Fraction	Time (days)			mean	SD	%RSD
		1	2	3			
MC-LR	Intracellular	1120	1146	1339	1202	120	10
Asp^3-MC-LR/[Dha^7]-MC-LR		76	77	93	82	10	12
MC-HilR		19	19	23	20	2	9
MC-LY		122	121	144	129	13	10
MC-LF		388	378	464	410	47	11
MC-LW		240	227	281	249	28	11
MC-LR	Extracellular	141	141	142	141	1	1
Asp^3-MC-LR/[Dha^7]-MC-LR		21	20	22	21	1	4
MC-HilR		3	3	3	3	0	3
MC-LY		27	29	33	29	3	11
MC-LF		61	47	67	59	10	18
MC-LW		28	23	31	27	4	15

The amount of each cyanobacteria strain had been adjusted to expose *M. edulis* to approximately equal amounts of the two major cyanotoxins of the respective strain, NOD and MC-LR. Analysis showed that this was achieved as no significant difference was detected between the total amount of NOD and MC-LR available in each container (intracellular and extracellular) over the three day feeding period (p= 0.67; Table 5. 8).

Table 5. 8 Total (intracellular and extracellular) NOD and MC-LR administered to 140 *M. edulis*

Cyanotoxins (µg)	Time (days)			mean	SD	%RSD
	1	2	3			
Total NOD	1086	1070	976	1044	59	6
Total MC-LR	1009	1030	1185	1074	96	9

5.3.3 Quantification of cyanotoxins in tank water

As previously described (section 5.2.5.2) the water samples obtained were filtered to assess intracellular and dissolved cyanotoxin concentration within the system.

5.3.3.1 Intracellular cyanotoxins

Analysis of extracted filters showed that on all three days exposure to MCs and NOD was consistent within exposure tanks (Table 5. 9). No intracellular cyanotoxins were detected in water of negative control tanks. LNOD was consistently below LOQ. It was noticed that despite the end of cyanotoxin exposure, small quantities of all six MC variants were detected in filter samples after feeding on day four (total of 5.6 µg L^{-1} MCs). On this day all mussels had been fed exclusively on shellfish diet. Potentially, MCs could have been present in faecal material, which was suggested by results obtained during the single exposure of *M. edulis* to *M. aeruginosa* PCC 7813 (chapter 4). A total of ~7 µg MCs were detected in faecal material after 24 h exposure (section 4.3.5). In the present study, each morning after the water sample had been taken a full water exchange was performed on all experimental tanks. Due to space limitations mussels were placed back into their tanks after the water exchange and remained in them until feeding commenced approximately 3h later. Consequently, during this time mussels were able to excrete MCs in their faeces which can be associated with the levels detected on day four (Table 5. 9).

Table 5. 9 Intracellular cyanotoxins (µg L^{-1}) detected in water samples taken after addition of *N. spumigena* KAC66 and *M. aeruginosa* PCC 7813. Results are shown as mean ± SD (n=3).

Toxin (µg L^{-1})	Time (days)			
	1	2	3	4*
NOD	41.0 ± 3.07	35.4 ± 0.24	37.1 ± 1.08	Not detected
MC-LR	32.9 ± 1.85	25.9 ± 3.26	34.7 ± 1.59	2.2 ± 0.55
Asp^3-MC-LR/[Dha^7]MC-LR	2.73 ± 0.29	2.20 ± 0.18	3.07 ± 0.16	0.27 ± 0.06
MC-HilR	0.81 ± 0.04	0.64 ± 0.07	0.82 ± 0.01	0.13 ± 0.00
MC-LY	5.33 ± 0.29	3.92 ± 0.46	5.46 ± 0.16	0.67 ± 0.06
MC-LF	13.1 ± 0.93	8.91 ± 1.21	12.9 ± 0.49	1.27 ± 0.18
MC-LW	10.2 ± 0.69	6.96 ± 1.03	10.2 ± 0.62	1.08 ± 0.18
Total MCs	65.1 ± 2.22	48.5 ± 3.66	67.2 ± 1.79	5.61 ± 0.61

***** Start of depuration period, MCs potentially excreted in faecal material

Maximum cyanotoxin concentrations encountered in water bodies can vary drastically (section 1.1). Generally, values reported for MC concentrations ranged from 0.0198 to 658 µg L^{-1} (Table 1.1). The form of cyanotoxins available is also important for their accumulation as intracellular levels result in higher accumulation (section 4.3.4; Gibble *et al*., 2016). The majority of MC values reported were below the exposure concentration utilised in this study (~ 25-35 µg L^{-1} MC-LR). However, MC concentrations as high as 658 µg L^{-1} (Tango and Butler), 298 µg L^{-1} (De Pace *et al*., 2014), and 184 µg L^{-1} (Park *et al*., 1998) have been reported, which illustrates that environmentally relevant concentrations were utilised for the exposure of *M. edulis* during the present study.

NOD concentrations reported in the literature ranged from 2-25,852 µg L^{-1} (section 3.3.4.3). Compared to the exposure concentration utilised in the present study (35-41 µg L^{-1}) the maximum NOD concentration reported was approximately 630 times higher (Gulf of Gdansk; Mazur-Marzec *et al*., 2006a). This indicates that NOD could potentially reach considerably higher concentrations in seafood compared to values obtained during the present study. Hence, potential NOD contamination in seafood represents a real threat to wildlife and the general public.

Based on the cyanotoxin concentrations detected in feeding cultures, the theoretically expected concentrations of toxins in the filter samples were

calculated (Table 5. 10). Generally, 80-120% of the exposure concentrations were detected in the particulate fraction of the water sample. This was an improvement compared to MC recoveries (43-105%, Table 4.6) observed during the previous feeding study in which *M. edulis* had been exposed to *M. aeruginosa* PCC 7813 (chapter 4). As shown by Lawton *et al*. (1994) sacrifices in recovery need to be accepted to enable the simultaneous detection of a range of MCs and NOD with varying polarity. Coyle and Lawton (1996) showed that after a single MeOH extraction of *M. aeruginosa* PCC 7820 cells 86% MC-LR were recovered. Cells had been filtered onto GF/C filter disks which had been utilised in the present study and more than 90% recovery was observed for MC-LY, MC-LW, and MC-LF. For these variants a second extraction was sufficient to extract all cyanotoxins according to Coyle and Lawton (1996). In order to fully recover MC-LR, three extractions with MeOH were found to be necessary (Coyle and Lawton 1996). This suggests that potentially, due to the single dispersive 80% aqueous MeOH extraction utilised during the present study, discrepancies in recovery could be explained as this method has not been reported in the literature. In addition, the seawater matrix could have interfered with toxin recovery (Ott and Carmichael 2006).

Table 5. 10 Recoveries of intracellular MCs and NOD in water samples (filter) detected immediately after feeding compared to values detected in feed stock cultures.

Cyanotoxins	Day	Detected concentration (µg L^{-1})	Theoretical concentration (µg L^{-1})*	% Recovery
NOD	1	40.98	48.40	85
	2	35.38	47.52	74
	3	37.09	43.46	85
MC-LR	1	32.91	40.00	82
	2	25.91	40.92	63
	3	34.71	47.83	72
Asp^3-MC-LR/ $[Dha^7]$-MC-LR	1	2.73	2.71	101
	2	2.20	2.76	80
	3	3.07	3.33	92
MC-HilR	1	0.81	0.69	116
	2	0.64	0.68	93
	3	0.82	0.81	102
MC-LY	1	5.33	4.37	122
	2	3.92	4.31	91
	3	5.46	5.16	106
MC-LF	1	13.08	13.87	94
	2	8.91	13.52	66
	3	12.87	16.56	78
MC-LW	1	10.24	8.56	120
	2	6.96	8.12	86
	3	10.22	10.03	102

***-** Based on intracellular cyanotoxins detected in feed stock cultures and their expected theoretical concentration in tank water according to the dilution applied

5.3.3.2 Dissolved cyanotoxin

Extracellular concentrations of NOD, MC-LR, and MC-LY were considerably lower than intracellular toxin concentrations as expected based on toxin levels in feed stock cultures which is representative of healthy cyanobacteria cultures. No extracellular cyanotoxins were detected in water of negative controls. The remaining cyanotoxins were not detected or were present only at concentrations below LOQ (section 0). Extracellular cyanotoxin concentrations remained consistent over the three exposure days (Table 5. 11). For the MC congeners MC-LF and MC-LW the lowest calibration standard (0.66 µg L^{-1}) had to be disregarded due to extremely high variabilities between calibration standard injection replicates (>300%). Hence, the second lowest standard (3.28 µg L^{-1}) was set as a limit of quantification (LOQ) in this assessment. Unfortunately, no results were found to be above the LOQ for dissolved toxins. MC-LF and MC-LW are the two variants with the lowest polarity which could account for the higher variabilities at low levels.

Table 5. 11 Extracellular cyanotoxins detected in water samples taken after addition of *N. spumigena* KAC66 and *M. aeruginosa* PCC 7813. Results are shown as mean ± SD (n=3).

Cyanotoxin (µg L^{-1})	Time (days)		
	1	2	3
NOD	1.28 ± 0.26	1.36 ± 0.04	1.30 ± 0.22
MC-LR	3.40 ± 0.79	3.70 ± 0.24	3.82 ± 0.69
MC-LY	1.22 ± 0.28	1.20 ± 0.10	1.20 ± 0.06

In comparison to extracellular cyanotoxin levels reported during blooms, dissolved NOD was lower in the present study: approximately 1.3 µg L^{-1} compared to 2.6-90 µg L^{-1} (Table 5. 12). Total dissolved MC concentration in this study was comparable to most concentrations reported in the literature: 1.3-9.5 µg L^{-1} compared to approximately 5 µg L^{-1}. During blooms, the majority of the cyanotoxins are contained within the cells. However, due to cell senescence and consequently cell death, cyanotoxins are released into the water following cell lysis. Therefore, extracellular cyanotoxins levels have been reported on occasion to reach very high levels in water: 13,000-19,000 µg L^{-1} (Graham *et al*., 2010). As healthy cultures were utilised during the present study only small concentrations of extracellular toxin were observed. Recoveries of dissolved MC-LR, MC-LY, and NOD were calculated based on extracellular

concentrations detected in feed stock culture samples compared to those expected in tank water according to the dilution applied (Table 5. 13). MC-LR showed recoveries of 68-76%. A slight increase in extracellular MC-LY was observed (102-128% recovery). Free, unconjugated MC-LR can be eliminated via urine and faeces in mice and bivalves (Schmidt *et al*., 2014). This confirms the results of the present feeding study in which dissolved MC-LR was detected at levels above LOD but below LOQ on day five. This effect was even more pronounced for NOD which was detected in water (>LOD, <LOQ) until day 17. MC-LY was only detected on day four, the first day of depuration. These results were in agreement with observations made during the single exposure of *M. edulis* to *M. aeruginosa* PCC 7813, in which dissolved MC-LR, MC-LY, MC-LF, and MC-LW were also detected during the depuration period (section 4.3.3.2).

Dissolved NOD increased by factors between 5 and 12. This could indicate that *N. spumigena* KAC 66 did not tolerate the change in salinity as well as *M. aeruginosa* PCC 7813, suggesting cells lysed and released cell bound NOD. However, compared to overall NOD the extracellular fraction accounted for only approximately 3% indicating that the majority of *N. spumigena* KAC 66 was unaffected by the change in salinity. Alternatively, dissolved NOD could have also been excreted by mussels.

Table 5. 12 Reported extracellular cyanotoxin levels encountered during blooms

Water body	Country	Toxin	c_{max} µg L^{-1}	Reference
Lake Marathonas	Greece	MCs	1.342	Kaloudis *et al*. (2013)
Gulf of Finland	Finland	NOD	2.6	Kankaanpää *et al.* (2001)
Fish pond Novoveský	Czech Republic	MCs	9.51	Kopp *et al*. (2013)
Gulf of Gdańsk	Poland	NOD	95	Mazur-Marzec *et al.* (2006a)
Rock Creek	United States of America	MCs	13,000* 19,000†	Graham *et al.* (2010)
Vancouver Lake	Canada	MCs	0.5	Lee *et al*. (2015)

c_{max} **-** maximum concentration ***** ELISA analysis **†** Sum of MCs detected by LC-MS/MS

Table 5. 13 Difference (%) of dissolved MCs and NOD in water samples detected immediately after feeding compared to values detected in feed stock cultures

Cyanotoxin	Day	Detected concentration (µg L^{-1})	Theoretical concentration (µg L^{-1})*	% Difference
NOD	1	1.28	0.10	1269
	2	1.36	0.25	553
	3	1.30	0.12	1062
MC-LR	1	3.40	5.02	68
	2	3.70	5.02	74
	3	3.82	5.02	76
MC-LY	1	1.22	0.95	128
	2	1.20	1.03	117
	3	1.20	1.18	102

* - Based on extracellular cyanotoxins detected in feed stock cultures and their expected theoretical concentration in tank water according to the dilution applied.

5.3.3.3 Clearance of cyanotoxins from tank water

Comparing the intracellular toxin content detected in samples taken immediately prior to feeding (at dosing) with those obtained approximately 19 h later showed decreased cyanotoxin concentrations, which was an indicator of cyanobacteria clearance by mussels (Figure 5. 7). The largest clearance was observed for NOD which was nearly removed altogether (3-5% remaining after 19 h). MCs on the other hand all showed considerably lower clearance. Approximately 50% of MCs available were not cleared except on day two. On that day the highest concentrations were observed ranging from 75% for MC-LW to 60% for MC-LR. It was noticed that variants of decreasing polarity showed lower reduction (Table 5. 14). The results obtained during the previous exposure of *M. edulis* to *M. aeruginosa* PCC 7813 (chapter 4) did not show the same trends. In addition, less MCs were cleared by mussels over the 19 h time period during the present study. Following the single cyanobacteria exposure, MCs detected after feeding were at levels ranging from only 8% to a maximum of 48% of the concentrations detected at dosing, except for the minor variant MC-HilR which had been cleared completely (4.3.3.3).

Table 5. 14 Average intracellular NOD and MCs (%) remaining in tank water following the exposure of *M. edulis* to *N. spumigena* KAC66 and *M. aeruginosa* PCC 7813 over a period of ~19 hours (n=3).

Day	NOD	MC-LR	Asp^3-MC-LR/[Dha^7]-MC-LR	MC-HilR	MC-LY	MC-LF	MC-LW
1	3	44	47	48	47	47	48
2	3	60	62	63	72	73	75
3	5	46	51	47	50	49	49

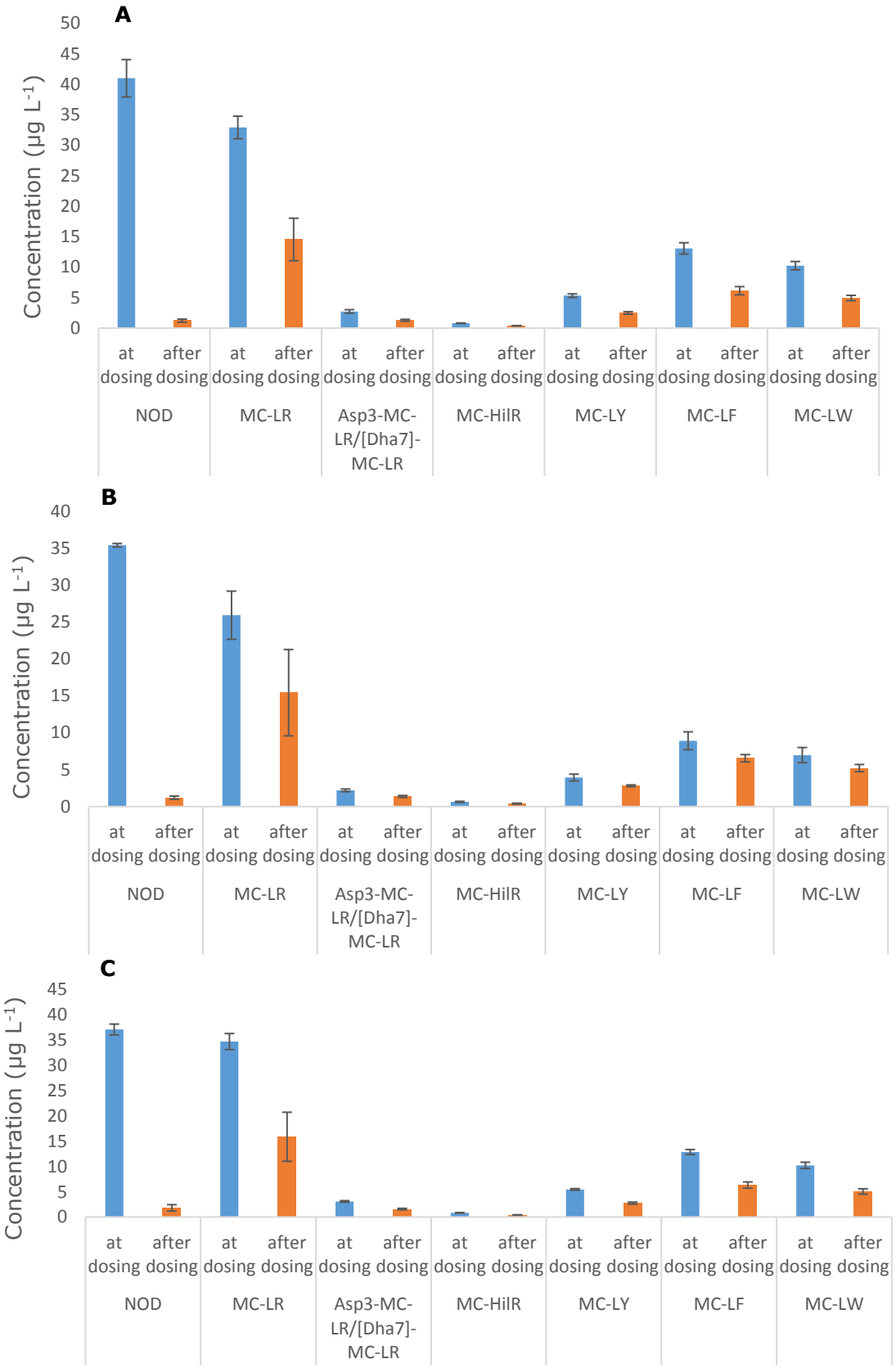

Figure 5. 7 Intracellular NOD and MCs on day one (A), day two (B), and day three (C). Concentrations detected before exposure to *M. aeruginosa* PCC 7813 and *N. spumigena* KAC 66 (at dosing, blue) and in the morning of the following day prior to the daily water exchange (after dosing, orange)

The observed higher clearance of NODs from tank water compared to MCs suggested that potentially *M. edulis* filtered the filamentous *N. spumigena* KAC66 more easily compared to the unicellular *M. aeruginosa* PCC 7813. Opposed to the single cell appearance of axenic *M. aeruginosa* in laboratory cultures (Damerval *et al*., 1989), environmental samples show aggregation of cells which are embedded in mucilage which might potentially be more easily ingested by bivalves (Reynolds *et al*., 1981). The previous two feeding studies have shown that when exposed to *M. aeruginosa* PCC 7813 and *N. spumigena* KAC66 individually, *M. edulis* was able to clear MCs and NOD, respectively (sections 3.3.3.1 and 4.3.3.3). Results of the present study suggest that the filamentous *N. spumigena* KAC66 was more easily ingested compared to *M. aeruginosa* PCC 7813 when present simultaneously. Pseudo-faeces production of *M. edulis* in response to the exposure to *N. spumigena* KAC66 (section 3.3.3.1) was not seen during the present study. In addition, no aggregation of *N. spumigena* KAC 66 cells on the water surface, which had been observed during a previous feeding study (section 3.3.3.1), were observed (Figure 5. 8). Previous investigations have shown that *M. aeruginosa* PCC 7813 was not affected by the seawater environment as no evidence of cell lysis and increased extracellular cyanotoxin concentration was observed for eight hours of salt exposure (section 4.3.1). These preliminary results were supported by lack of extracellular MC increase in water samples taken 19h post exposure.

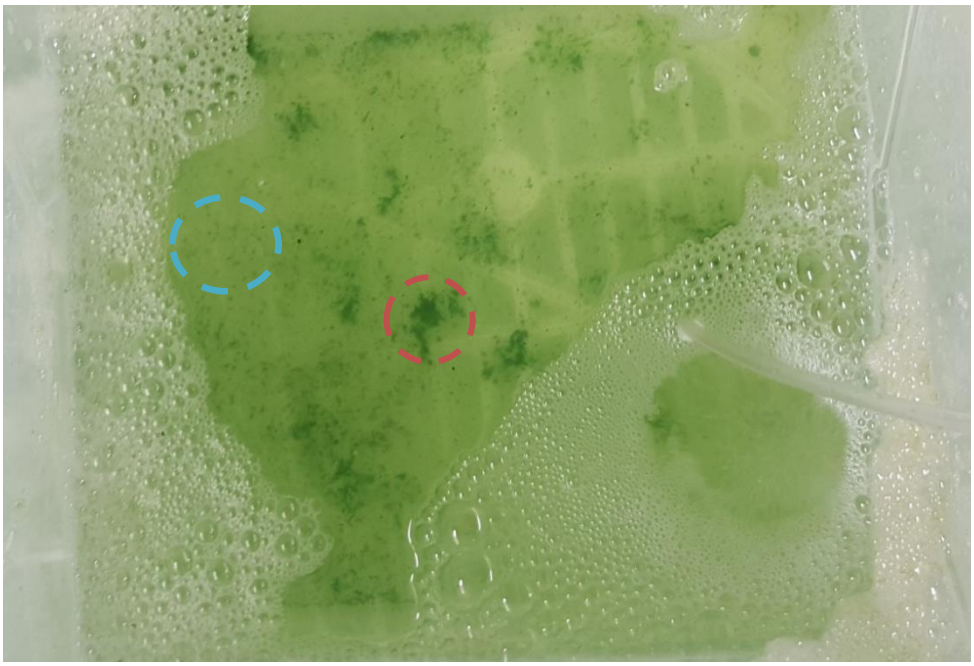

Figure 5. 8 Experimental tank on day three (7:24 am) illustrating the observed lower clearance of *M. aeruginosa* PCC 7813 associated with the combined effect of aggregation and settling of *M. aeruginosa* PCC 7813 cells.

Faecal pellets

Aggregated *M. aeruginosa* PCC 7813 cells

Concentrations of dissolved MC-LR and MC-LY showed no statistically significant differences between concentrations obtained after feeding and those detected on the following morning (Figure 5. 9). This suggested that little to no dissolved MCs were taken up by *M. edulis*. These results correlate with results of Novaczek *et al*. (1991) who showed that compared to dissolved domoic acid *M. edulis* accumulated more toxin when food-borne. In addition, Prepas *et al*. (1997) showed that *A. grandis simpsoniana* did not accumulate MC-LR when exposed to dissolved MC-LR at levels ranging from 51-55 μg L^{-1}. An alternative reason for the level remaining relatively constant could be that, mussels may have excreted MCs at concentrations similar to those of the dissolved exposure dose.

Extracellular NOD on the other hand increased on all three exposure days (Figure 5. 9). Statistically significant increases were observed on day two ($p= 0.015$) and on day three ($p=0.001$). Potentially this could be a result of NOD excretion by *M. edulis*. Alternatively, *N. spumigena* KAC66 cells could have lysed following the change in salinity. As previously mentioned, *N. spumigena* KAC66 was grown in BG-11 medium adjusted to a salinity of 6.6 psu. The water utilised

during this study contained salt at a concentration of approximately 30-32 psu. This change in salinity could have caused the cells to lyse. However, overall the extracellular NOD concentration in water remained low. Similar to reports of MC accumulation, dissolved NOD in *M. balthica* was accumulated to a considerably lower degree compared to NOD accumulated after exposure to *N. spumigena* cultures (Lehtonen *et al*., 2003). Conversely to these reports, Kankaanpää *et al*. (2007) showed that exposing *M. edulis* to NOD extracts (70-110 μg L^{-1} in seawater) resulted in the accumulation of 1.1 μg g^{-1} detected in the hepatopancreas 24 h post exposure. Generally cell bound MCs and NOD seem to represent a greater potential for cyanotoxins accumulation in seafood.

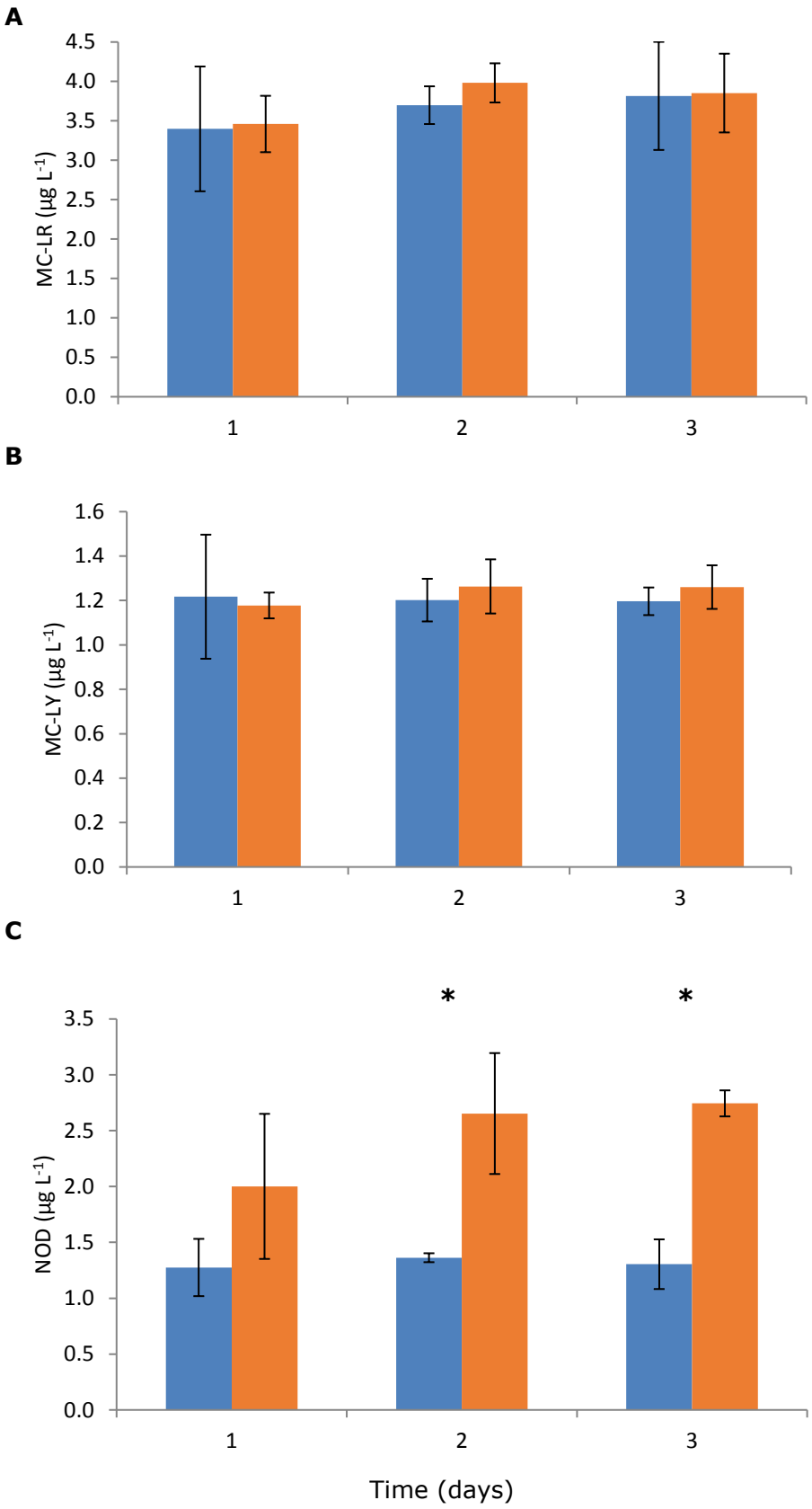

Figure 5. 9 Extracellular MC-LR (A), MC-LY (B) and NOD (C) concentration at exposure to *M. aeruginosa* PCC 7813 (blue) and approximately 19 h later (orange) * significant difference $p < 0.05$

5.3.4 Quantification of cyanotoxins in *M. edulis* flesh

The determination of the weight of ten shucked mussels supported the previous assumption of 1 mussel of 4 g (section 3.3.4 and 4.3.4) was reasonable based on 39.9 ± 4.3 (n=78) being the average weight of 10 shucked mussels (Table 5. 15).

Table 5. 15 Weight (g) of ten shucked mussels (*M. edulis*) determined throughout the feeding study

Time (days)	A	B	C	D	E	F
1	37.2	43.5	41.9	37.4	43.9	45.6
2	41.0	36.4	43.4	37.6	39.9	40.0
3	48.4	49.4	56.9	40.0	38.8	52.6
5	37.1	46.5	35.0	41.5	37.6	42.7
7	38.0	36.1	36.6	41.8	41.3	41.1
9	37.5	33.6	38.4	41.7	42.8	38.2
12	43.8	37.9	35.5	41.0	35.5	37.5
15	31.1	36.0	42.6	38.0	39.4	45.6
18	35.1	36.3	41.9	42.0	41.6	40.8
21	43.3	35.8	38.9	37.3	40.6	37.9
24	34.0	40.8	44.3	41.6	37.1	38.0
27	38.7	36.3	40.0	38.0	39.5	37.9
30	34.5	34.2	41.5	43.5	35.4	42.6
Average	39.9 ± 4.3					

All MCs and NOD present in feed stock cultures were detected in mussel tissue after one day of exposure to cyanobacteria cells except for MC-HilR, which was detected but below the LOQ of 0.45 ± 0.26 (Figure 5. 10). In addition to NOD, the linearised form (LNOD) was also detected in tissue samples. The total amount of cyanotoxins quantified was 0.77 ± 0.65 µg g^{-1}. A steep increase from day one to two was observed in which the total cyanotoxin concentration increased by approximately a factor of three to 2.18 ± 0.25 µg g^{-1}. Remarkably, even after the end of the exposure period (day three) the toxin content within the tissue increased during the next four days and reached maximum values of 3.40 ± 0.64 µg g^{-1} on day seven. Over the following eight days a reduction to 1.19 ± 0.09 µg g^{-1} was observed followed by a slight increase to 1.73 ± 0.30 µg g^{-1} on day 18. The demethylated MC variants (Asp^3-MC-LR/[Dha^7]-MC-LR) were not detected after this day and the combined amount of remaining MCs and NOD decreased steadily until day 30 to a concentration of 0.49 ± 0.27 µg g^{-1} (black line, Figure 5. 10). Generally, the same trend of accumulation and depuration was observed for all cyanotoxins present in the feed stock cultures

(Figure 5. 10; Figure 5. 11 A and B). Concentrations of Asp^{3}MC-LR/[Dha^{7}]-MC-LR, MC-HilR and MC-LY were below LOQ by days 21, 24, and 27, respectively.

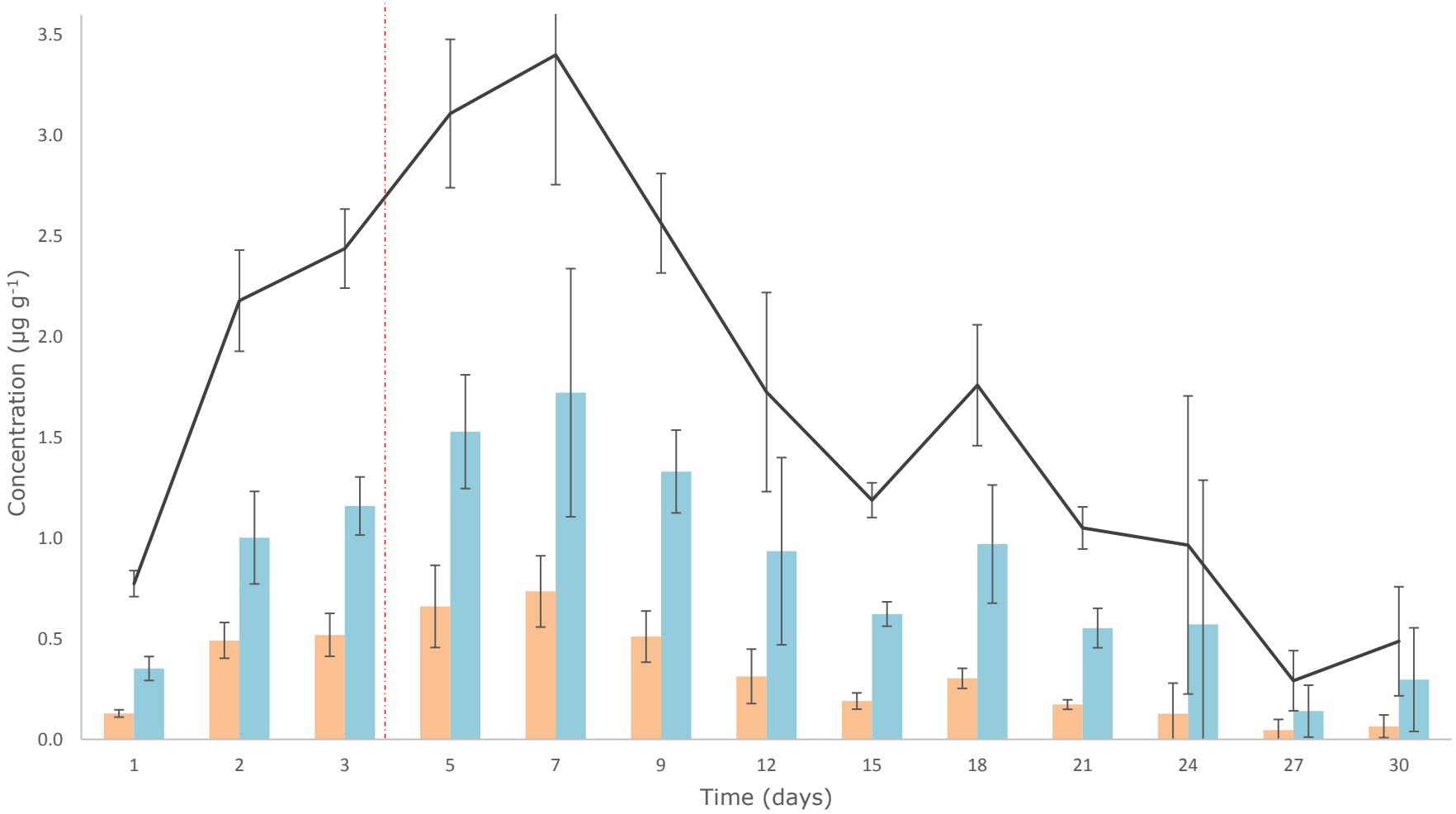

Figure 5. 10 Total cyanotoxins (black), MC-LR (orange), and NOD (light blue) detected in *M. edulis* after a three day exposure to a mixture of *N. spumigena* KAC 66 and *M. aeruginosa* PCC 7813 followed by a 27 day depuration period. Values are shown as mean ± standard deviation (n=3). The red line shows the end of the exposure period.

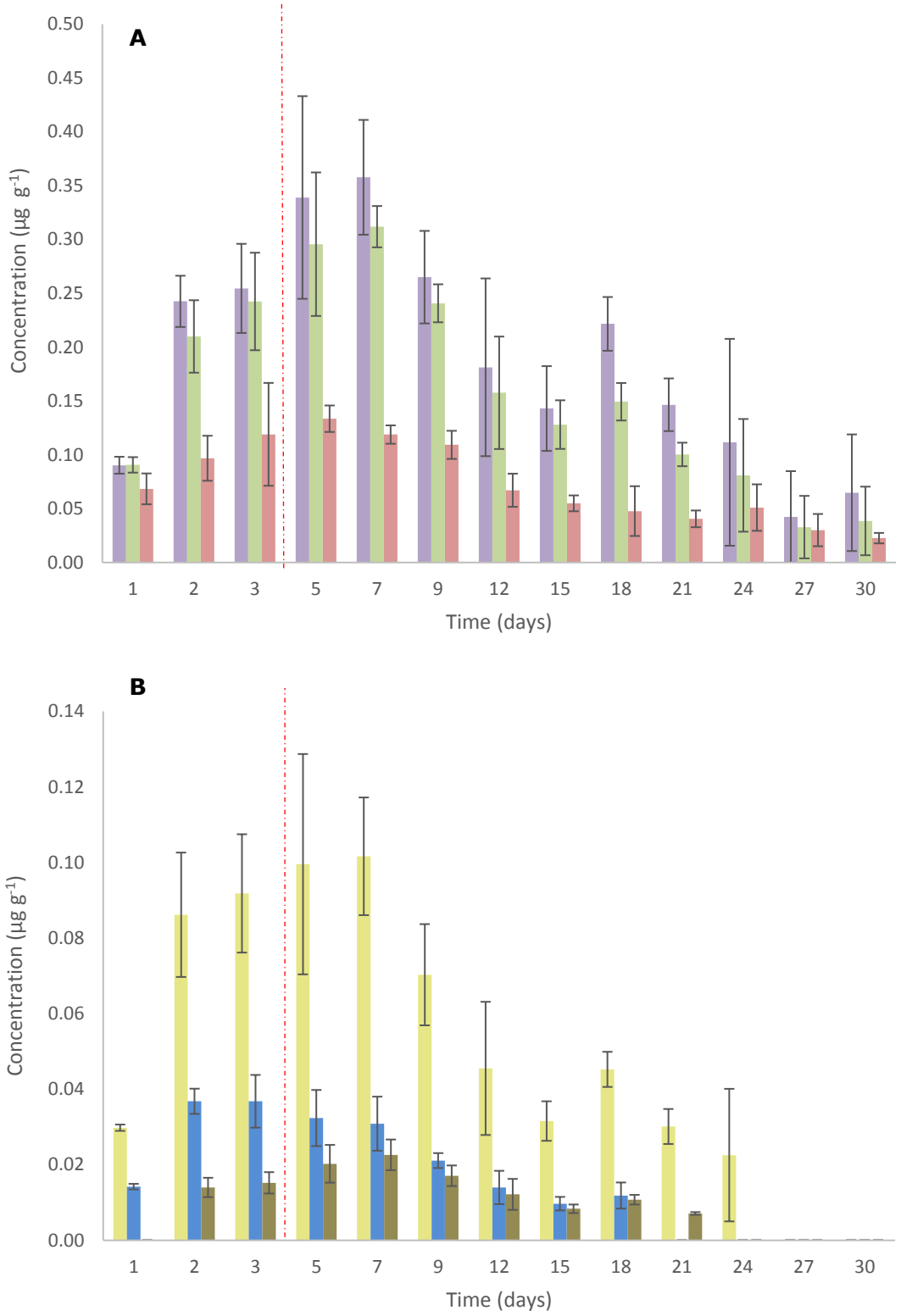

Figure 5. 11 Remaining cyanotoxins detected in *M. edulis* after a three day exposure to a mixture of *N. spumigena* KAC 66 and *M. aeruginosa* PCC 7813. The red line shows the end of the exposure period. Values are shown as mean ± standard deviation (n=3). A - Microcystin-LF (purple), MC-LW (green), and LNOD (red). B - MC-LY (yellow), Asp^{3}MC-LR/[Dha^7]-MC-LR (blue), and MC-HilR (brown)

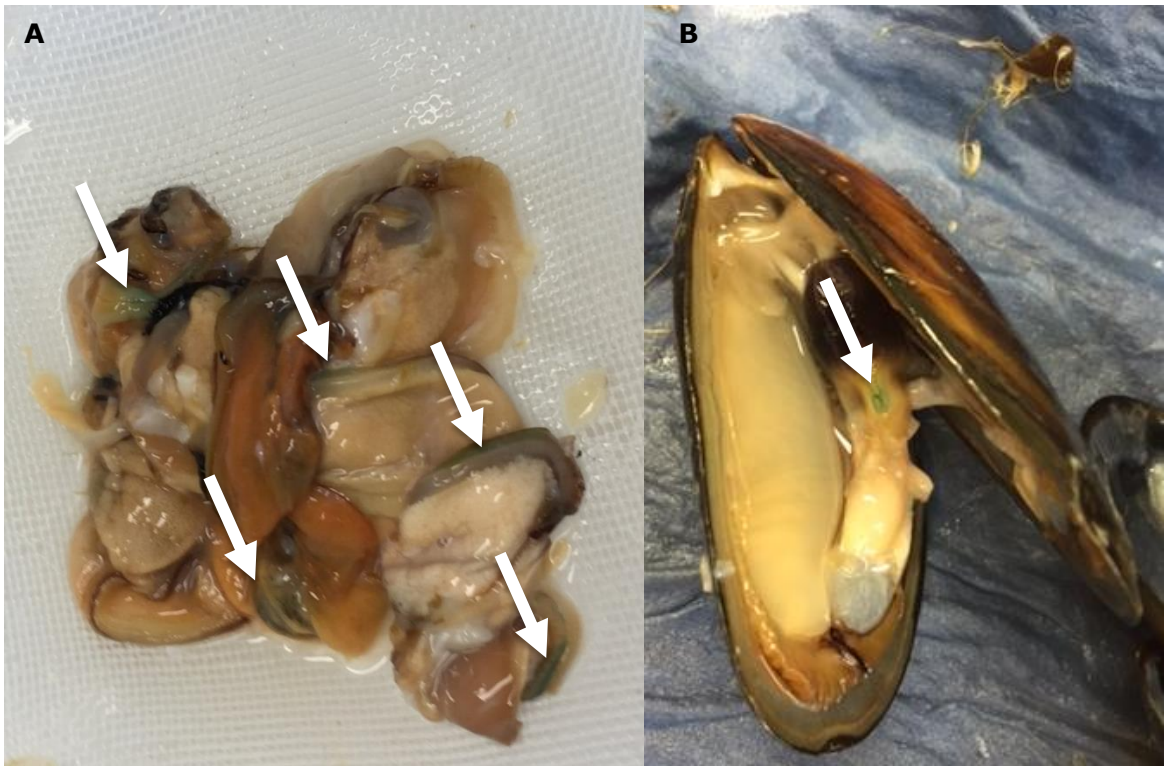

Figure 5. 12 Example of observed green discolouration (white arrow) in *M. edulis* after the combined exposure to *N. spumigena* KAC66 and *M. aeruginosa* PCC 7813. A - mantle skirt (day 21) and B - base of mussel foot (day 3).

Accumulation of cyanobacteria by *M. edulis* was evident even by visual inspection. A green discolouration of mainly the mantle skirt and the base of the mussel foot were observed starting from day three. This discolouration was observed throughout the remainder of the experiment (Figure 5. 12)

The rapid accumulation observed for this study is consistent with numerous reports (Table 5. 16). Kankaanpää *et al*. (2007) exposed *M. edulis* to extracts of freeze-dried Baltic Sea phytoplankton containing *N. spumigena* (480 µg NOD mL^{-1}). They reported high concentrations of NOD after only 6 h as indicated by NOD detected in the hepatopancreas (1.10 µg g^{-1} dw) and the remaining tissue (approximately 0.2 µg g^{-1}). This was further supported by findings of Vasconcelos (1995), who exposed *M. galloprovincialis* to *M. aeruginosa* and observed detectable MC-LR concentrations after one day of exposure. Our results showed an increase in detectable MCs and NOD concentration in mussel tissue even when exposure to the two cyanobacteria had ceased. In addition, the total cyanotoxin concentration showed a small increase during the depuration period from day 15 to day 18 (1.19 ± 0.09 µg g^{-1} versus 1.73 ± 0.30

μg g^{-1}). Similar results have been reported for MC-LR in *M. galloprovincialis* (Amorim and Vasconcelos 1999, Vasconcelos 1995). Following an initial 50% decrease of MC-LR in tissue after three days of depuration, an increase was observed over the following four days (Vasconcelos 1995). Similarly, MC-LR concentration in *M. edulis* after five days exposure to *Microcystis aeruginosa* IZANCY A2 showed an increase during the depuration phase (Amorim and Vasconcelos 1999). ELISA analysis of *Tilapia rendalli* also showed an increase in MC-LR in muscle, liver and faeces during depuration (Soares *et al.* 2004). The authors suggested that potentially a fraction of the covalently bound MC-LR had been released. However, in the present study the same trend was observed for NOD (Figure 5. 10). As previously mentioned, NOD does not covalently bind to PPase, but could be closely associated with proteins and therefore be unavailable.

Table 5. 16 Accumulation and depuration of cyanotoxins in mussels reported in the literature.

Cyanobacteria	Dose µg mL^{-1}*	Mussel	Uptake (d)	Depuration (d)	Maximum toxin concentration µg g^{-1}†	Levels after depuration µg g^{-1}†	Mortality	Reference
N. spumigena (NOD)	480	*M. edulis*	1	6	1.10 dw (hepatopancreas) 0.2 dw (Soft tissue)	0.6 dw (hepato-pancreas) 0.1 dw (Soft tissue)	0%	Kankaanpää *et al.* (2007)
M. aeruginosa (MC)	0.7 ± 0.08 0.4 ± 0.04	*Mesodesma mactroides*	12	None	5.27±0.23 dw (hepatopancreas) ~ 3.7 (based on graph)	Not applicable	13%	Leão *et al*. (2010)
M. aeruginosa IZANCYA2 (MC-LR)	153 ± 73.79 µg per feeding	*M. gallopro-vincialis*	1	15	10.52 mg g dw (day 10)	not detected on day 13	6%	Vasconcelos (1995)
M. aeruginosa (MCs)	16.2 mg MCs	*M. edulis*	3	53	336.9 ± 45.8 ww 0.204 ww	not detected on day 11	Not reported	Williams *et al.* (1997a)

* Unless specified values are shown as µg mL^{-1}

† If not specified concentrations (mean ± standard deviation were available) are given for the whole organisms

Table 5. 16 continued

Cyanobacteria	Dose µg L^{-1}*	Mussel	Uptake (d)	Depuration (d)	Maximum toxin concentration µg g^{-1}†	Levels after depuration µg g^{-1}†	Mortality	Reference
N. spumigena (NOD)	3.2 µg 31.6 µg not clear	*M. edulis*	0.5 1	none 3	1.18 ± 1.09 dw 13.8 ± 5.26 dw Not applicable	Not applicable 75% reduction	0%	Strogyloudi *et al*. (2006)
M. aeruginosa (MC-LR)	10^5 cells mL^{-1}	*M. gallo-provincialis*	4	14	10.7 dw	2.5	0%	Amorim and Vasconcelos (1998)
C. raciborskii (CYN)	14-90 µg L^{-1}	*Anodonta cygnea*	16	14	2.52 dw	~50%	0%	Saker *et a*l. (2004)
N. spumigena KAC66 (NOD)	100-480	*M. edulis*	7	7	17.13 ± 3.61	7.46 ± 2.47	<1%	Present study chapter 3
M. aeruginosa PCC 7813 (MCs)	240-390	*M. edulis*	7	22	8.53 ± 0.94	1.15 ± 0.54	7%	Present study chapter 4
N. spumigena KAC66 (NOD) *M. aeruginosa* PCC 7813 (MCs)	44-48 80-94	*M. edulis*	3	27	3.4 ± 0.64	0.49 ± 0.27	4.5%	Present study chapter 5

* Unless specified values are shown as µg mL^{-1}

† If not specified concentrations (mean ± standard deviation were available) are given for whole organisms

In agreement with other studies, depuration of both MCs and NOD was slow in the present study. Persson *et al*. (2009) provided evidence for the slow depuration of NOD in seafood. It was suggested that NOD detected in flounder livers was a remnant of previous blooms as the sample sites as well as *M. edulis* collected at these did not contain NOD. The previous year however, a bloom of *N. spumigena* had occurred and mats of cyanobacterial material had been found on a beach. These mats contained 16 mg NOD g^{-1} dw. The authors concluded that it was likely that mussels, being a primary food source for flounders, had been the route of NOD exposure during earlier months. The results indicate different rates of depuration of NOD in fish and mussels although no statement can be made regarding NOD concentrations in flounders and mussels of the previous year. In addition, approximately 50% of cylindrospermopsin (CYN) remained detectable in tissue of swan mussel even after 14 days of depuration (Saker *et al*., 2004). Mussels had been exposed to *C. raciborskii* cells (14-90 µg L^{-1}) for 16 days. The results obtained in the present study show very similar depuration patterns: after 15 days of depuration cyanotoxin concentration in mussel tissue was 1.73 ± 0.30 µg kg^{-1} (~52% of the maximum concentration).

In contrast to results presented here, faster depuration of NOD in *M. edulis* after exposure to extracts of *N. spumigena* has been reported (Kankaanpää *et al*., 2007). At one day post exposure NOD concentration in tissue decreased by 30%, however, by day six only approximately half of the NOD had been eliminated from *M. edulis* tissue (Kankaanpää *et al*., 2007). In comparison to the present study a similar decrease of 25% of the maximum toxin concentration in tissue was reached only on day nine, six days post exposure. Variations in depuration kinetics could potentially be attributed to the differences in toxin exposure. In the present study *M. edulis* were exposed to cell cultures containing both intracellular and extracellular toxins. In addition, mussels will not only be exposed to MCs and NOD but also to other metabolites produced by cyanobacteria. Hence this interaction of various bioactive compounds could interfere with cyanotoxin accumulation and depuration. Kankaanpää *et al*. (2007) on the other hand exposed mussels to cell extracts (aqueous MeOH; 25:75) and therefore dissolved cyanotoxins only. This could account for the difference in toxin depuration observed.

Vasconcelos (1995) reported complete depuration of MC-LR from *M. galloprovincialis* (maximum concentration 10.52 µg g^{-1}) following 13 days. In addition, rapid clearance of MCs was reported *in M. edulis*: after four days of depuration only 3.6% of MCs were detected in mussels utilising the Lemieux oxidation method in combination with GC-MS (Williams *et al*., 1997a). However, when applying PPase inhibition assays approximately 7% of the MC-LR concentration was still detected. This discrepancy between the two methods applied can be explained by their different mode of actions. PPase inhibition assays are generally less specific as any substance interacting with PPases will contribute to the results. These could be, for example, potential degradation or metabolism products of cyanotoxins or other hepatotoxins. Similarly, the Lemieux oxidation GC-MS method, based on the oxidation of the Adda group specific for MCs and NODs will result in potential metabolites and microbial degradation products being detected. Furthermore, low toxin recoveries due to multi-step approach have been reported (Roy-Lachapelle *et al*., 2014; Neffling *et al.,* 2010; Cadel-Six *et al*., 2014). Finally, both the PPase inhibition assay and the Lemieux oxidation method cannot differentiate between different MC variants and/or the presence of NOD. There is a need for the validation of accurate, precise, and robust methods to determine the total MCs and NOD burden in samples of varying matrices. Nonetheless, both methods are an excellent way to obtain an overview of potential total cyanotoxin presence.

Rapid depuration of NOD from *M. edulis* was reported by Strogyloudi *et al*. (2006) who utilised LC-ESI-MS. *M. edulis* which had been exposed to *N. spumigena* KAC13 showed a 75% decrease in NOD tissue concentration after three days of depuration. In contrast to the present study mussels were not fed during the depuration period. The present study was set out to mimic a natural influx of brackish and freshwater cyanobacteria into the marine environment, where constant feeding is likely to be maintained. It was therefore decided to feed the mussels during the depuration period as the phytoplankton community would also be present under natural circumstances.

The total detectable concentration of all MCs, NOD, and LNOD was used to calculate the amount of toxin the average adult would be exposed to, based on a typical portion of mussels (section 3.3.4.). Values were calculated for day

seven when highest toxin concentration was observed and for day 30 at the end of the depuration period (Equation 5.1).

Equation 5.1 Cyanotoxin load expected in a serving of 20 mussels

On day seven

$$m_{MCs\ and\ NOD} = m_{mussel} \times n_{mussel} \times c_{MCs\ and\ NOD}$$

$$m_{MCs\ and\ NOD} = 4\ g \times 20 \times 3.40\ \mu g\ g^{-1}$$

$$m_{MCs\ and\ NOD} = \mathbf{272.08\ \mu g}$$

On day 30

$$m_{MCs\ and\ NOD} = m_{mussel} \times n_{mussel} \times c_{MCs\ and\ NOD}$$

$$m_{MCs\ and\ NOD} = 4\ g \times 20 \times 0.49\ \mu g\ g^{-1}$$

$$m_{MCs\ and\ NOD} = \mathbf{38.97\ \mu g}$$

m = amount in µg n= number of specimen c= concentration

Based on the maximum cyanotoxin content observed in this study (day seven) the derived acute and seasonal tolerable intake limits would be exceeded by a factor of approximately 1.4 and 9, respectively (Ibelings and Chorus 2007). In addition eating a portion of the mussels even after a 27 day depuration period would still result in the seasonal tolerable intake being exceeded. This therefore demonstrated that even short term bloom events in close proximity to shellfish beds can cause significant cyanotoxin accumulation in *M. edulis* and hence represent a major risk of cyanotoxin exposure to higher predators including humans.

5.3.5 Quantification of cyanotoxins in faecal material

Immediately after one day of exposure NOD and MCs were detected in faecal material of *M. edulis* exposed to the two cyanobacteria. No cyanotoxins were detected in faeces of negative control mussels. Values were stable over the next day but showed a steep increase from 2.01 ± 0.53 on day two to 5.64 ± 1.18 µg g^{-1} detected on day three. Following the end of the exposure period (day three), cyanotoxin concentration decreased to 2.82 ± 0.63 µg g^{-1} (~ 50%). Already on day six cyanotoxin levels had decreased to 0.29 ± 0.03 µg g^{-1} (~5%). Linear NOD was not detected at concentrations above LOQ. The less dominant MC variants Asp^3-MC-LR/[Dha^7]-MC-LR, MC-HilR, MC-LY, MC-LF and MC-LW reached levels below LOQ on day seven, six, 24, 27 and 24, respectively. For the remainder of the study the cyanotoxin concentration in faecal material decreased with only MC-LR and NOD detectable on day 30 (total 0.016 ± 0.004 µg g^{-1}; Figure 5. 13).

In contrast to the present study, Amorim and Vasconcelos (1999), observed a steady increase in MC concentration in faecal material during the first three days of depuration. This was followed by a steep decline of toxin levels which remained low in faecal material for the last ten days of depuration. Previously, mussels (*M. galloprovincialis*) had been exposed to *M. aeruginosa* IZANCY A2 cells for five days. Similarly, steadily increasing levels of MCs in faeces were observed after exposing *T. rendalli* to *M. aeruginosa* cells for 15 days (Soares *et al.,* 2004). Concentration of MCs had not been monitored during the exposure period. Nonetheless, concentration of MCs increased from approximately 0.01 µg g^{-1} $fish^{-1}$ to approximately 0.07 µg g^{-1} $fish^{-1}$ on day 30 (Soares *et al.,* 2004). Due to the use of ELISA the presence of potential conjugates could not be evaluated.

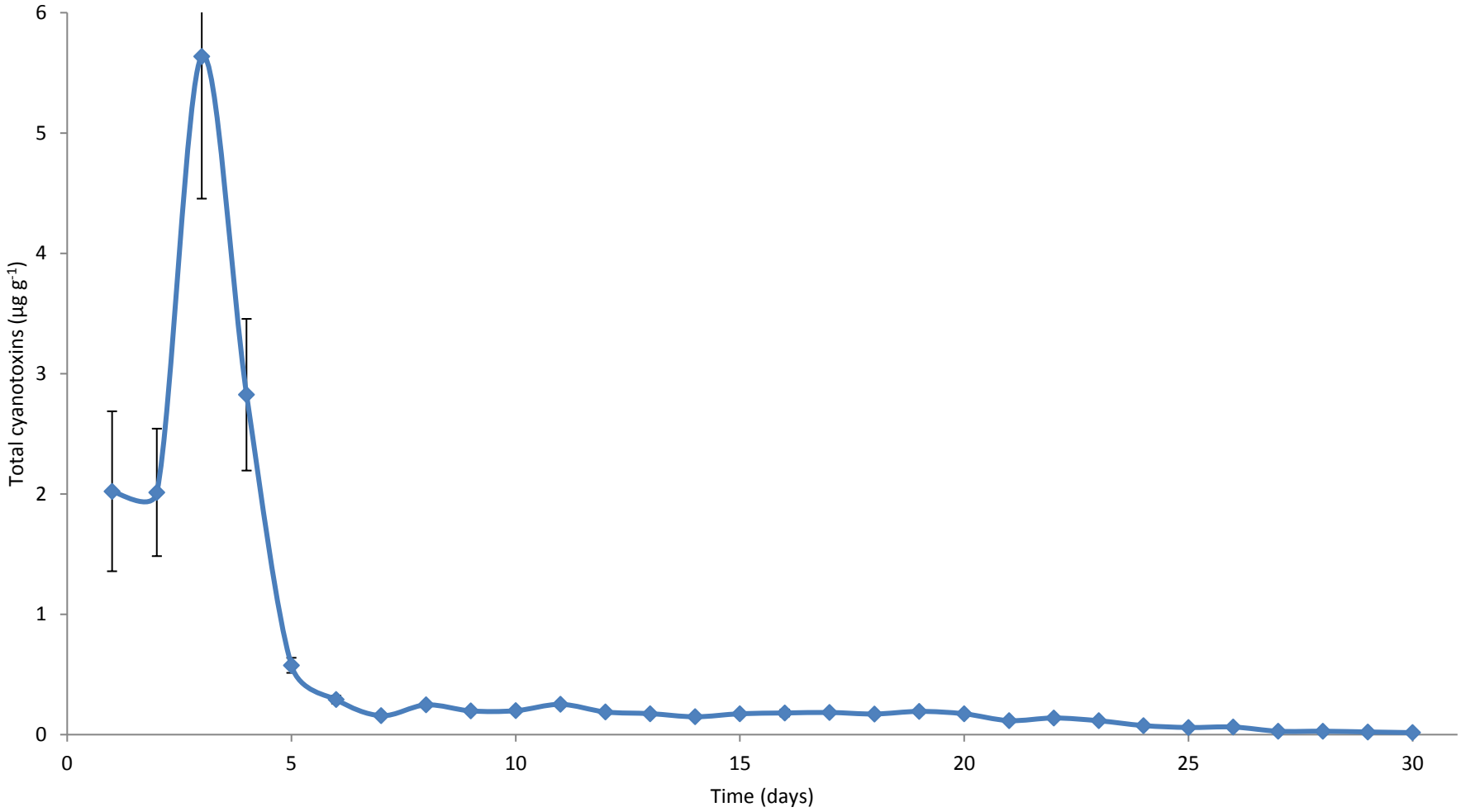

Figure 5. 13 Total cyanotoxins in faecal material of *M. edulis* after a three day exposure to a mixture of *N. spumigena* KAC66 and *M. aeruginosa* PCC 7813. Values are shown as mean ± standard deviation (n=3)

5.3.6 Quantification of cyanotoxins in extrapallial fluid of *M. edulis*

Low concentrations of NOD, LNOD and MCs were determined from day one onwards. The only MC variant not detected in extrapallial fluid was MC-HilR. The remaining MC variants were only detected during the exposure period except for day seven on which MC-LW contributed to the overall toxin load. From day one to two cyanotoxin concentration increased (~2x) to a maximum concentration of 281.42 ± 17.98 µg L^{-1}. Following this, a rapid decrease in concentration occurred two days after cyanobacteria exposure had ceased on day seven: 22.75 ± 9.44 µg L^{-1} (~8% of maximum concentration). The concentration of NOD and MCs continued to decrease until day 18 and could not be detected for the remainder of the experiment (Figure 5. 14). No cyanotoxins were detected in extrapallial fluid of negative control mussels.

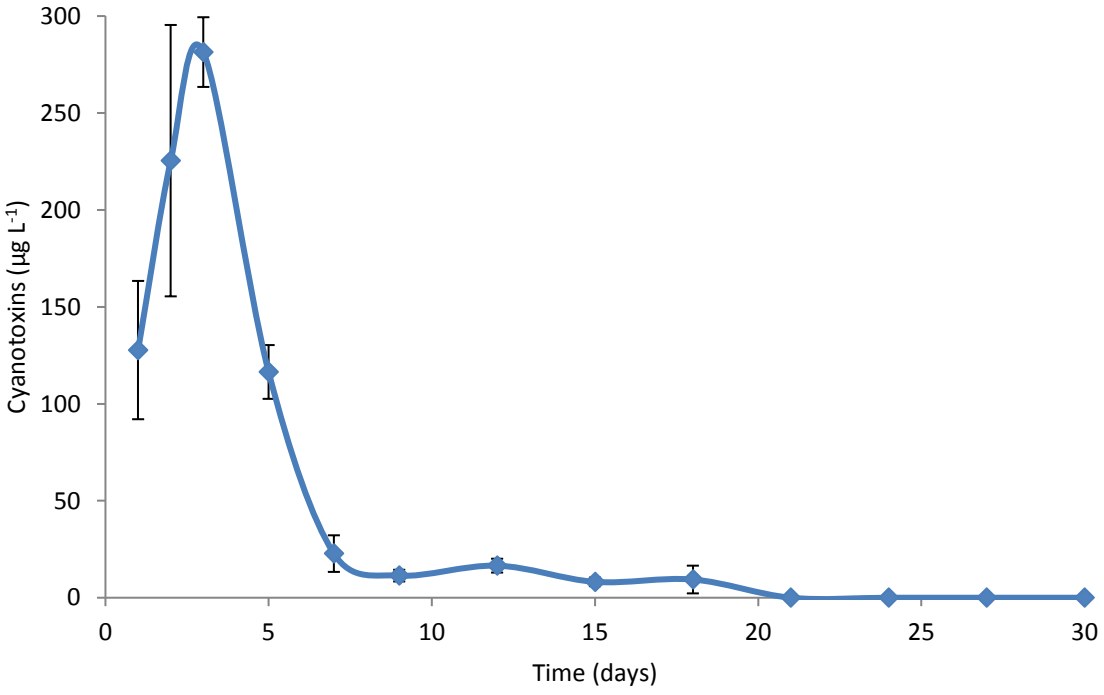

Figure 5. 14 Total cyanotoxins in extrapallial fluid of *M. edulis* after a three day exposure to a mixture of *N. spumigena* KAC66 and *M. aeruginosa* PCC 7813. Values are shown as mean ± standard deviation (n=3).

5.3.7 Determination of the cyanotoxin budget within the experimental system

A significant proportion of the MCs (42-47%) were unaccounted for on all three exposure days. MCs were dominant in the intracellular fraction representing approximately one third of the total MC amount (37-38%). In decreasing order the remaining MCs were detected in mussel tissue (8-11%), dissolved toxin (~6%) and in extrapallial fluid (up to 1.7% MCs). The faecal material contained the lowest amount of MCs (0.1-0.2%, Figure 5. 15 A). Based on the percentage of MCs and NODs accumulated in mussel tissue and extrapallial fluid after one day of exposure, the amount of MCs and NODs within stunt doubles was extrapolated and combined with the values obtained in mussels. Evaluating the NODs distribution showed that an even greater proportion of NODs was unaccounted for (61-69%; Figure 5. 15 B). In contrast to MCs, the second largest fraction of NODs was found in mussel flesh (19-27%). Dissolved toxin accounted for 4-5% similar to the toxin contained in extrapallial fluid (around 4%). Intracellular NODs accounted for only minute fractions of the total NODs (2-3%). Similar to MC results, the faecal material contained the least amounts of NODs (<0.1%). The distribution of individual cyanotoxins can be found in the appendix (section 5.5).

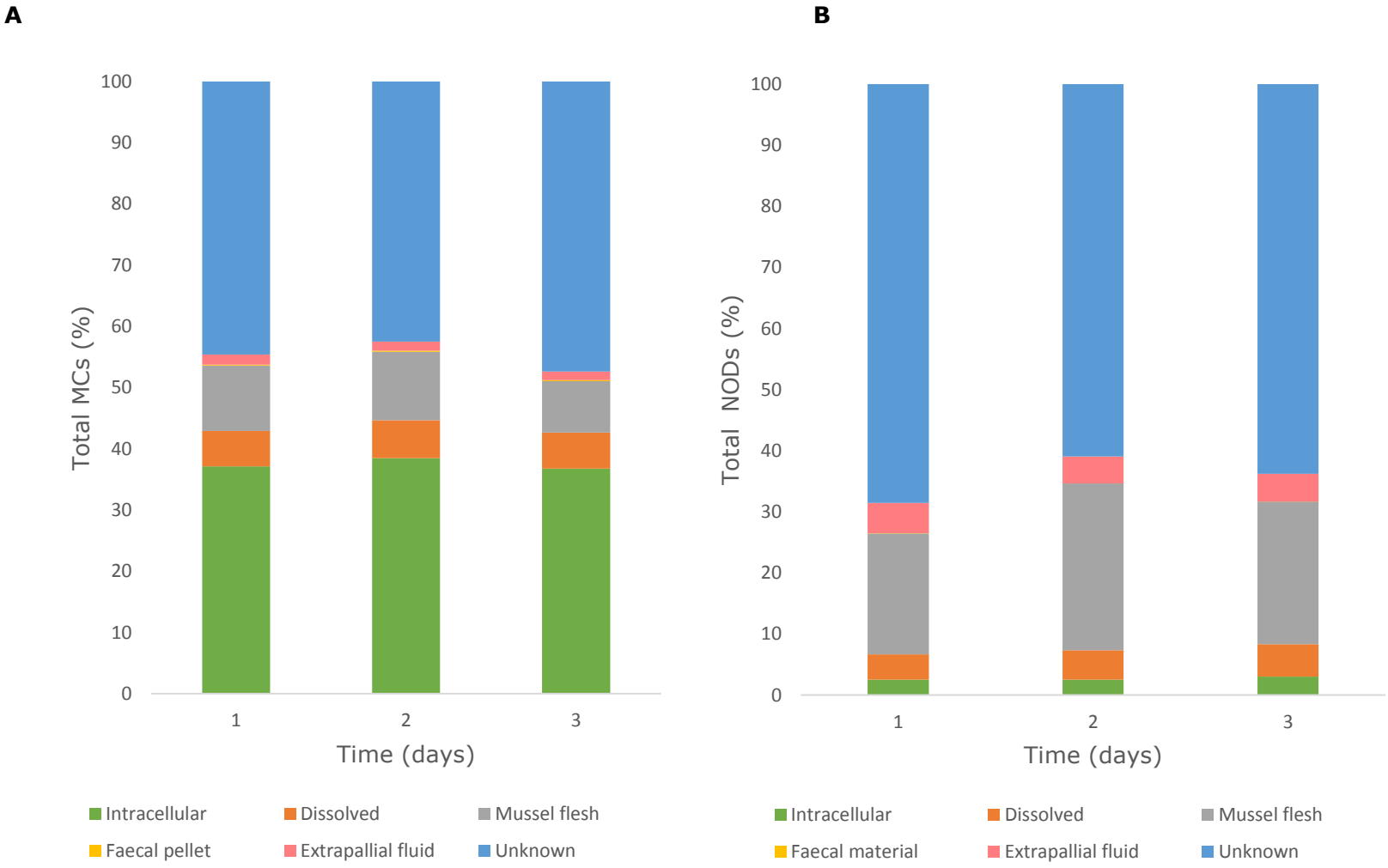

Figure 5. 15 Distribution of MCs (A) and NODs (B) within the experimental system following the simultaneous exposure of *M. edulis* to *N. spumigena* KAC66 and *M. aeruginosa* PCC 7813.

Table 5. 17 Comparison of total MC distribution (%) in the experimental system following the exposure of *M. edulis* to single or multiple cyanobacteria

Exposure strains	Day	Intracellular	Dissolved	Tissue	Faecal pellet	Unknown
N. spumigena KAC66, *M. aeruginosa* PCC 7813	1	37	6	11	0.2	45
	2	38	6	11	0.1	42
	3	37	6	8	0.2	47
M. aeruginosa PCC 7813 (chapter 4)	1	5	21	19	0.3	55
	2	8	16	26	0.2	49
	3	7	14	30	0.2	48
	4	9	15	34	0.2	42
	5	11	16	27	0.1	45
	6	12	17	33	0.1	38
	7	12	18	32	0.1	38

Compared to the single exposure of *M. edulis* to *M. aeruginosa* PCC 7813 (chapter 4) large changes in toxin distribution were observed (Figure 5. 15), which could potentially be associated with the filamentous *N. spumigena* KAC66 being more easily ingested compared to the unicellular *M. aeruginosa* PCC 7813 (5.3.3.1). In addition, the relatively large proportion of extracellular MCs detected in *M. aeruginosa* PCC 7813 utilised during the earlier feeding study (up to 21%; section 4.3.3.2; Table 5. 17) has likely contributed to the observed differences as generally, dissolved MCs were accumulated to a lesser extent compared to intracellular MCs (Gibble *et al*., 2016; Lance *et al*., 2010b). The overall amount of unaccounted MC was comparable: 42-47% during the present study compared to 38-55% during the single cyanobacterium exposure (chapter 4). Similarly, the amount of MCs detected in faeces were similar.

Compared to the single *N. spumigena* KAC66 exposure (section 3.3.4), where 43-83% of the NOD dose (NOD + LNOD) were detected in *M. edulis*, only 20-26% of the available NODs dose were detected in mussel flesh during the present study. Dose recovery in faecal pellet, ~0.03% in the present study, was also considerably lower compared to the *N. spumigena* KAC66 feeding trial (0.1-0.4%). Strogyloudi *et al.* (2006) reported 10-12% of the NOD exposure dose within tissue and faecal samples of *M. edulis*. In comparison to the present study values obtained for NODs in the three mussel associated samples (tissue, extrapallial fluid, faecal material) ranged from 21% on day one to 38% on day three. Higher recoveries of 24-55% were reported for MC-LR in *M. galloprovincialis* tissue following exposure to a toxic *Microcystis* strain (Vasconcelos 1995). The recovery of mussel associated MCs in this study was considerably less 13-18%. Compared to MCs the mussels accumulated approximately twice as much NOD. Furthermore, only minute amounts of intracellular NODs were found within the filter sample collected on the morning after feeding. A large proportion of MCs on the other hand were found in the intracellular sample. As previously discussed (section 5.3.3.3) these differences were most likely a consequence of the different structure of cyanobacteria available (unicellular versus filamentous).

Overall, for both MCs and NODs the majority of the theoretically available amount was unaccounted for. Suggested reasons for this were: 1. metabolism of MCs and NOD into glutathione or cysteine conjugates; 2. Biotic and/or abiotic

degradation of toxins; 3. covalent binding of MCs to PPases and other proteins and finally losses of NOD and MCs during sample analysis.

Closer observation revealed the presence of linear NOD (LNOD) in the mussel tissue and extrapallial fluid samples. It was found at concentrations ranging from 5-35% of that of NOD in mussel tissue (Table 5. 18) and from 11-30% in extrapallial fluid (Table 5. 19), respectively. LNOD in extrapallial fluid was only detectable at levels above LOQ until day six and again on day 13. In feed stock cultures LNOD represented approximately 0.05% of the detected NOD. This change in relative LNOD concentration could indicate that LNOD was produced due to activities and processes within the experimental system. As LNOD was only observed in samples taken from *M. edulis*, it could be suggested that LNOD was produced by metabolism processes changing NOD to LNOD within the mussel. No LNOD was detected in water or faecal samples at levels >LOQ.

Table 5. 18 Average percentage of LNOD compared to NOD detected in tissue of *M. edulis* after three day exposure to *N. spumigena* KAC66 and *M. aeruginosa* PCC 7813 (n=3).

Day	%LNOD	SD	%RSD
1	19.60	1.20	6
2	9.70	0.83	8
3	10.09	2.90	29
5	8.95	2.03	23
7	7.37	2.08	28
9	8.26	0.83	10
12	7.79	2.05	26
15	8.86	1.26	14
18	4.76	1.52	312
21	7.45	1.88	25
24	18.71	12.81	68
27	35.11	33.49	95
30	23.05	30.26	131

Table 5. 19 Average percentage of LNOD compared to NOD detected in extrapallial fluid of *M. edulis* after three day exposure to *N. spumigena* KAC66 and *M. aeruginosa* PCC 7813 (n=3)

Day	%LNOD	SD	%RSD
1	19.40	2.12	11
2	12.37	2.19	18
3	9.36	1.02	11
5	10.54	3.08	29

As previously described LNOD could have been produced either by the cyanobacteria themselves, by abiotic and/or biotic degradation, or by metabolism processes within *M. edulis* (section 3.3.4). Generally LNOD concentration mirrored that of NOD (Figure 5. 16). This could indicate that potentially both degradation processes and/or metabolism of NOD were rapid. No lag phase was observed for the appearance of LNOD. Samples of mussel tissues had only been taken daily. Potentially, sampling points might not have been detailed enough to observe patterns of LNOD formation.

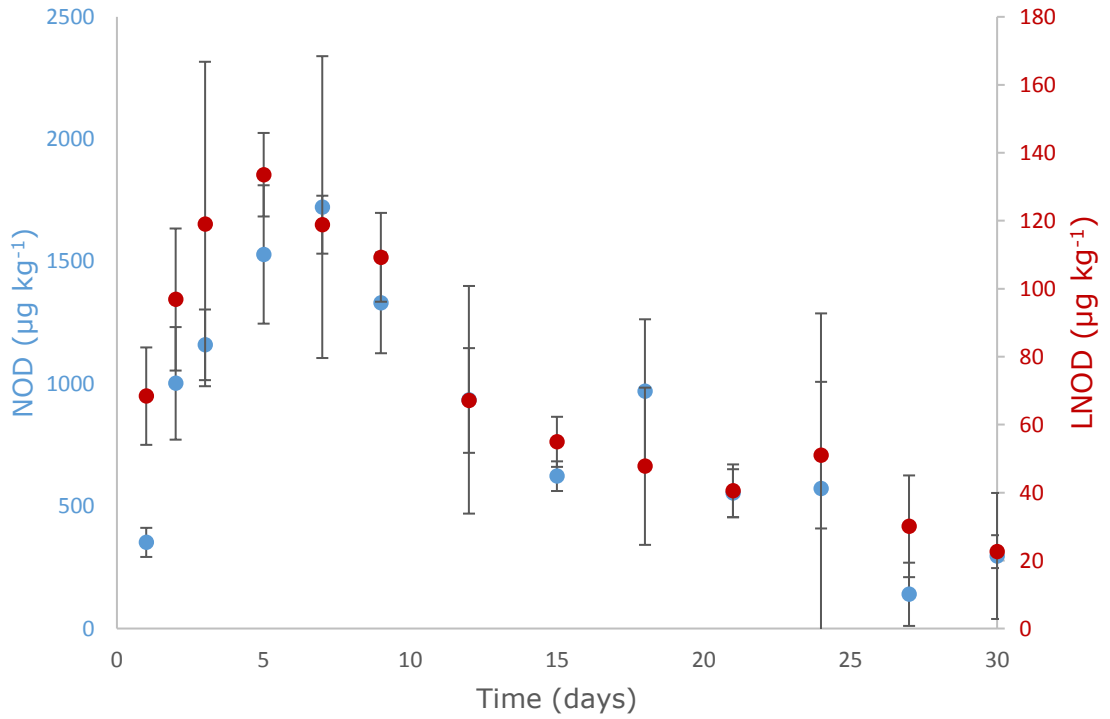

Figure 5. 16 NOD (blue) and LNOD (red) in *M. edulis* after a three day exposure to a mixture of *N. spumigena* KAC66 and *M. aeruginosa* PCC 7813. The dashed red line shows the end of the exposure period. Values are shown as mean ± SD (n=3).

Microbial degradation has also been reported for MC-LR. Bourne *et al.* (1996), showed that a *Sphingomonas* species was able to cleave the Adda-Arg peptide bond. Comparable to the degradation of NOD, this enzymatic hydrolysis resulted in linearised MC-LR and a tetrapeptide. Similar results were reported by Imanishi *et al*. (2005) utilising a bacterial strain isolated from Lake Tsukui in Japan. This bacterium was also found to degrade NOD, MC-LF, MC-RR, and geometrical isomers of MC-LR and MC-RR (Imanishi *et al*., 2005). The authors also suggested a sequential enzymatic hydrolysis initially via the Arg-Adda bond. The mussels utilised during the present study were a potential source of bacterial contamination which could have contributed to a loss of MCs and/or NOD. The tank water in which mussels were maintained was not tested for the presence of MCs and/or NOD degrading bacteria. In addition, due to the lack of appropriate analytical standards for the degradation products of NOD and MC products it was not possible to investigate their presence, except for that of LNOD. Nonetheless, samples have been stored at -80°C post analysis and could be re-analysed and scanned for possible degradation products. This could help to assess if bacterial degradation could have contributed to loss of MCs and NOD observed in this study.

Despite microbial or abiotic degradation, metabolism processes within *M. edulis* could have contributed to the unaccounted fraction of MCs and NOD. A large body of evidence regarding the formation of MC GSH and Cys conjugates has recently been compiled (section 4.3.6). The formation of NOD-conjugates on the other hand has only rarely been reported (Table 5. 20). Only the GSH conjugates were detected in mussel samples obtained from the Baltic Sea. Nonetheless, increased activity of mGST and cGST in brine shrimp (*A. salina*) suggested that these enzymes were also involved in the NOD detoxification (Beattie *et al*., 2003). However, the presence of NOD-GSH could only be proven in mussel tissue as reported by Sipiä *et al.* (2002). Numerous other studies could not confirm NOD-GSH presence in flounders and mussels (Mazur-Marzec *et al*., 2007; Karlsson *et al*., 2003; Vuorinen *et al*., 2009; Kankaanpää *et al*., 2007; Mazur-Marzec *et al*., 2007; Karlsson *et al*., 2003; Sipiä *et al*., 2002).

Table 5. 20 Formation of NOD conjugates reported in seafood

Experimental animal	Form of administration	Time post exposure in h	Organ	Conjugate	Reference
Blue mussel *M. edulis*	Extract of *N. spumigena*	24	whole organism	none	Kankaanpää *et al.* (2007)
Blue mussel *M. edulis*	Unknown (field samples)	unknown	whole organism	NOD-GSH	Sipiä *et al.* (2002)
Blue mussel Flounder	Unknown (field samples)*	unknown	whole organism liver	none	Mazur-Marzec *et al*. (2007)
Flounder *Platichthys flesus* Blue mussel *M. edulis*	Unknown (field samples)	unknown	liver whole organism	no GSH-NOD†; dmNOD	Karlsson *et al.* (2003)
Brine shrimp *Artemia salina*	*In vitro* assay	24	whole organism	NOD-GSH	Beattie *et al.* (2003)
Mallards *Anas platyrhynchos*	Oral gavage (200-600 µg NOD kg^{-1} bw)	24, 96, 168	liver muscle	none	Sipia *et al*. (2008)

i.p. - intraperitoneal, *** -** area known to be affected by *N. spumigena* blooms in summer, bw - body weight
† Authors investigated the presence of glutathione conjugates, but not that of cysteine conjugates

Evidence suggests that MCs and NOD could have been metabolised during this study. However, the analysis of samples obtained on day eight and ten utilising a full scan UPLC-QToF-MS system (section 2.2.5.2) did not show any evidence of conjugate formation. In addition, no potential products formed as a consequence of biotic or abiotic degradation, were detected. At this point the unaccounted toxin fraction of both MCs and NOD cannot be explained. As only a fraction of the total samples were re-run, currently no conclusion regarding metabolite presence and/or the potential formation of degradation products can be drawn at this point. A full re-analysis of all obtained samples would be necessary in order to achieve this. Recent studies have suggested that the conjugation of MCs could be reversible. Miles *et al*. (2016) showed that free MCs were released from thiol conjugated MCs under basic conditions when reacted with nucelophiles. Examples of these were mercaptoethanol, methanethiol, *L*-cysteine, and glutathione. The reaction rate increased with increasing pH. Potentially, this could suggest that during transition through the duodenum (pH 7.0-8.5) bound MCs could become bioavailable. This should therefore be considered when deriving safety guidelines for MCs in seafood. Li *et al*. (2014) also reported reversible conjugation of MCs *in vivo*. Despite i.p. injection of only MC-RR-Cys and MC-RR-GSH in bighead carps, analysis of liver, kidney, intestine, and blood samples all showed the presence of MC-RR in addition to the two conjugates. This release of GSH and or Cys bound MCs could potentially account for the continued increase seen in MCs concentration detected in mussel tissue (day four until day eight), despite the end of the exposure period. It would be worth investigating if similar effects can be observed for NOD.

As discussed in section 4.3.6 covalent bond formation of MCs could have attributed to the fraction of unaccounted MCs in the system. In contrast to MCs NOD do not covalently bind to PPases due to their different structure (section 1.2.1) The present study therefore intended to utilise NOD as a non-binding 'internal standard' to estimate the degree of potential covalent binding of MCs. Volumes of cultures fed to *M. edulis* had been adjusted based on concentrations of MC-LR and NOD present. This aimed to expose mussels to approximately the same amounts of both toxins. However, compared to MC-LR significantly more NOD was accumulated by *M. edulis*. This discrepancy was linked to the differential ingestion of the filamentous *N. spumigena* KAC66 which was

ingested more readily compared to *M. aeruginosa* PCC 7813 (unicellular) as analysis of the water sample suggested (section 5.3.3.1).

Comparing the recoveries of both MCs and NOD in tissue samples based on the total amount of cyanotoxin added to the system showed only small differences. NOD recovery ranged from 19-27%, whereas 11-22% of MC-LR was recovered. Remaining MC variants showed slightly better recovery. Values ranged from 13-21% (Asp3-MC-LR/[Dha^{7}]-MC-LR), 23-41% (MC-HilR), 41-68% (MC-LY), 20-31% (MC-LF), and 40-60% (MC-LW). Based on the overall lowest recovery for each toxin variant, correction factors should be considered for the determination of safety guidelines for cyanotoxins in seafood (Table 5. 21). In comparison to all MC variants NOD showed recoveries either within the same order or slightly lower values. This could suggest that covalent binding of MCs to PPase and other proteins did not show significant effects in this study. This hypothesis was supported by spike recoveries of more than 80% for MCs in mussel organs (Ame *et al.*, 2010).

Table 5. 21 Dose recovery (%) of NODs and MCs in mussel tissue based on cyanotoxins available in feed stock cultures along with proposed correction factors for the analysis of cyanotoxins in *M. edulis*.

Cyanotoxin	Day	Added µg	Total $_{\text{no tissue}}$ µg	Difference to added µg	c in Mussel µg	% Recovery	Correction factor
NODs*	1	1086.39	117.87	968.52	180.17	19	5
	2	2156.32	238.87	1917.45	513.22	27	
	3	3132.66	378.90	2753.75	595.18	22	
MC-LR	1	1008.53	420.41	588.12	65.67	11	10
	2	2038.26	870.71	1167.55	251.88	22	
	3	3223.43	1329.95	1893.47	267.82	14	
Asp^3-MC-LR/[Dha^7]-MC-LR	1	77.43	32.23	45.20	7.30	16	8
	2	155.35	65.11	90.23	18.86	21	
	3	247.59	102.23	145.35	18.95	13	
MC-HilR	1	17.89	8.78	9.11	2.08	23	4
	2	35.42	17.75	17.67	7.16	40	
	3	55.77	26.43	29.35	7.84	27	
MC-LY	1	119.29	84.71	34.58	15.30	44	2.5
	2	238.92	173.85	65.07	44.09	68	
	3	380.78	266.06	114.72	47.27	41	
MC-LF	1	359.81	143.59	216.21	46.39	21	5
	2	700.19	293.65	406.54	124.34	30	
	3	1124.85	436.89	687.96	131.11	19	
MC-LW	1	214.08	113.66	100.42	46.59	46	2.5
	2	414.16	232.77	181.39	107.38	59	
	3	663.60	347.72	315.88	124.82	40	

c – concentration, * combined LNOD and NOD

5.4 Conclusion

Simulating the influx of a freshwater toxic cyanobacterium into the coastal environment showed that both NOD and MCs were rapidly accumulated in *M. edulis*. Maximum concentrations (NOD and MCs combined 3283 µg kg^{-1}) were observed on day eight, four days after cyanobacteria exposure had ceased. Potentially this could have been caused by the release of bound and/or conjugated NOD and/or MCs. These results suggest that contamination of the marine mussel *M. edulis* by freshwater cyanotoxins does represent a risk to public health, hence, their presence in seafood should be considered as a risk to be managed in future monitoring programs. Depuration of NOD and MCs was incomplete even 27 days after toxin exposure had ceased. This highlights the importance of cyanotoxin monitoring even in the month following bloom events. Assessing the toxin budget within the experimental system has proven to be a highly complex process. Influences of metabolism, abiotic and/or biotic degradation in combination with analytical recoveries have been observed and discussed. In addition, due to the large fraction of unaccounted toxins, inclusion of uncertainty factors should be considered when determining safety guidelines for NOD and MCs in seafood. This is particularly important since recent reports suggest that conjugation of MCs to GSH and Cys can be reversible (Miles *et al*., 2016; Li *et al*., 2014) and therefore made bioavailable to seafood consumers. To accurately assess the overall risk cyanotoxin contaminated seafood represent to the general public, further research elucidating the metabolism of these toxin in various seafood is required.

5.5 Appendix

Table 5. 22 Distribution of individual cyanotoxins (µg) within the experimental system following the simultaneous exposure of *M. edulis* to *M. aeruginosa* PCC 7813 and *N. spumigena* KAC66.

	Time (days)	MC-LR	Asp3-MC-LR/[Dha7]-MC-LR	MC-HilR	MC-LY	MC-LW	MC-LF	NOD	LNOD
Feed culture stock (cumulative)	1	1008.53	77.43	17.89	119.29	214.08	359.81	1086.39	1.16
	2	2038.26	155.35	35.42	238.92	414.16	700.19	2156.32	2.57
	3	3223.43	247.59	55.77	380.78	663.60	1124.85	3132.66	3.84
Intracellular	1	325.67	28.79	8.74	55.89	110.54	137.36	27.50	0.00
	2	671.13	59.38	17.68	118.78	227.17	283.91	54.26	0.00
	3	1026.92	94.46	26.30	180.41	340.26	425.68	94.83	0.00
Extracellular	1	77.50	0.00	0.00	26.38	0.00	0.00	44.83	0.00
	2	166.69	0.00	0.00	54.68	0.00	0.00	104.26	0.00
	3	252.99	0.00	0.00	82.91	0.00	0.00	165.74	0.00
Mussel flesh	1	65.67	7.30	2.08	15.30	46.59	46.39	180.17	34.97
	2	251.88	18.86	7.16	44.09	107.38	124.34	513.22	49.51
	3	267.82	18.95	7.84	47.27	124.82	131.11	595.18	60.80
Extrapallial fluid	1	15.72	3.31	0.00	2.19	2.51	5.86	45.21	8.46
	2	30.36	5.53	0.00	0.00	4.63	8.99	79.89	9.18
	3	44.46	7.33	0.00	1.96	5.69	9.70	117.33	10.90
Faecal material	1	1.52	0.12	0.04	0.25	0.61	0.38	0.33	0.00
	2	2.53	0.21	0.06	0.39	0.97	0.76	0.46	0.00
	3	5.59	0.45	0.12	0.78	1.77	1.51	1.01	0.00

CHAPTER 6

Discussion

This investigation has clearly demonstrated the rapid accumulation of cyanotoxins in the filter feeding bivalve, *M. edulis* followed by very slow depuration. These findings are of significant concern in areas where shellfish may be exposed to toxic cyanobacteria either where it naturally occurs in a brackish water system or due to the flushing of freshwater blooms into estuaries.

Exposure to the brackish water strain *N. spumigena* KAC66 resulted in a maximum combined concentration of NOD and LNOD of 17 μg g^{-1} following seven days exposure (single daily dosing). The depuration was incomplete after an additional seven days and complete elimination was estimated to occur after ~40 days of depuration based on the logarithmic trend line. In addition to mussel flesh, NODs were also excreted in faeces of *M. edulis*.

Similar trends were observed during the second feeding study in which *M. edulis* was exposed to the freshwater cyanobacteria *M. aeruginosa* PCC 7813 once a day for seven days. The saltwater tolerance experiment showed that this strain was unaffected by the increased salinity for ~ 8 h, during which the majority of cells would be filtered by *M. edulis*. The maximum concentration of MCs detected was 8.5 μg g^{-1}. The depuration period of 22 days was not sufficient to completely eliminate MCs from mussel tissue. Based on the estimated logarithmic decrease of MCs in *M. edulis* complete depuration would be expected after 38 days, which was in good agreement with the estimate of 40 days suggested by results of the first feeding trial. In addition, differential excretion of MC variants in faeces produced by *M. edulis* was reported for the first time. Results showed that MC-LW and MC-LF were selectively eliminated to a greater extent than other variants. Results also suggested that *M. edulis* eliminated MCs not only in faeces, but also in dissolved form, which has been suggested in the literature.

The simultaneous exposure of *M. edulis* to *N. spumigena* KAC66 and *M. aeruginosa* PCC 7813 over a three days exposure period, aimed to simulate the reported influx of freshwater cyanobacteria into the marine environment, resulted in significant accumulation of cyanotoxins. The maximum concentration of cyanotoxins accumulated by *M. edulis* was detected on day seven, three days after the end of the exposure period (3.4 μg g^{-1}). This

continued increase of cyanotoxin concentration following the cessation of cyanobacteria exposure had not been observed during the previous two feeding trials. Potentially, the release of covalently bound and/or protein associated cyanotoxins could have caused the observed increase.

Furthermore, similar to the two previous feeding trials, depuration of cyanotoxins was very slow and incomplete after 27 days, the longest depuration period investigated during the feeding trials. Results suggested that during the depuration period bound cyanotoxins were released, which had not been observed during the two previous feeding trials. Estimating the complete depuration of cyanotoxins from *M. edulis* was therefore challenging, as the release of bound cyanotoxins could further prolong depuration in a ways currently not completely understood.

All three of the feeding trials resulted in rapid cyanotoxin accumulation of *M. edulis*. The consumption of a portion of 20 mussels would result in cyanotoxin ingestion well above the recommended acute and seasonal tolerable intake for a 75 kg adult of 190 and 30 µg MC-LR and/or NOD, respectively (Table 6. 1; Ibelings and Chorus 2007). Remarkably, even after more than 20 days of depuration the seasonal recommended limit was still exceeded in all three studies. This study has therefore demonstrated the dangers cyanotoxin contaminated *M. edulis* can pose to the general public and highlighted the need of cyanotoxin evaluation in bivalves obtained from areas potentially exposed to cyanobacteria.

Table 6. 1 Comparison of accumulation and depuration of cyanotoxins in *M. edulis* observed during the three feeding trials.

	NOD and LNOD (Chapter 3)	**MCs (Chapter 4)**	**NODs and MCs (Chapter 5)**
Exposure strain/strains	*N. spumigena* KAC66	*M. aeruginosa* PCC 7813	*N. spumigena* KAC66, *M. aeruginosa* PCC 7813
Maximum exposure concentration (µg L^{-1})	480	390	140
Exposure duration (days)	7	7	3
Maximum concentration in mussel tissue (µg g^{-1})	17	8.5	3.4
In portion of 20 mussels (µg cyanotoxins)	**1370***	**680***	**270***
Duration depuration (days)	7	22	28
Concentration in mussel tissue after depuration (µg g^{-1})	7	1.2	0.5
Estimated complete depuration (days)	40	38	>27
In portion of 20 mussels (µg cyanotoxins)	**600***	**96***	**39***

***** The acute and seasonal tolerable intakes derived for MCs and/or NOD are 190 and 30 µg for a 75 kg adult, respectively.

During the warmer months, when cyanobacteria are more likely to bloom in nutrient enriched (eutrophic) water bodies in the UK, toxins could be transported to the marine environment. A likely scenario would be an increased freshwater influx in coastal areas after prolonged sunshine followed by heavy rainfall events (Figure 6. 1). In addition, the increasing temperatures recorded during recent years could prolong cyanobacteria bloom periods and the potential exposure of mussels to cyanotoxins. In contrast to the observed rapid accumulation, depuration of cyanotoxins was incomplete even after nearly a month. It is therefore vital to identify potential risk areas in which mussels could be exposed to cyanotoxins via freshwater influx. Monitoring of mussels from these risk areas should also be considered not only during, but also in the month following bloom events. Moreover, the potential co-occurrence of brackish water cyanobacteria needs to be considered during future risk assessments as these can add to the overall cyanotoxin burden.

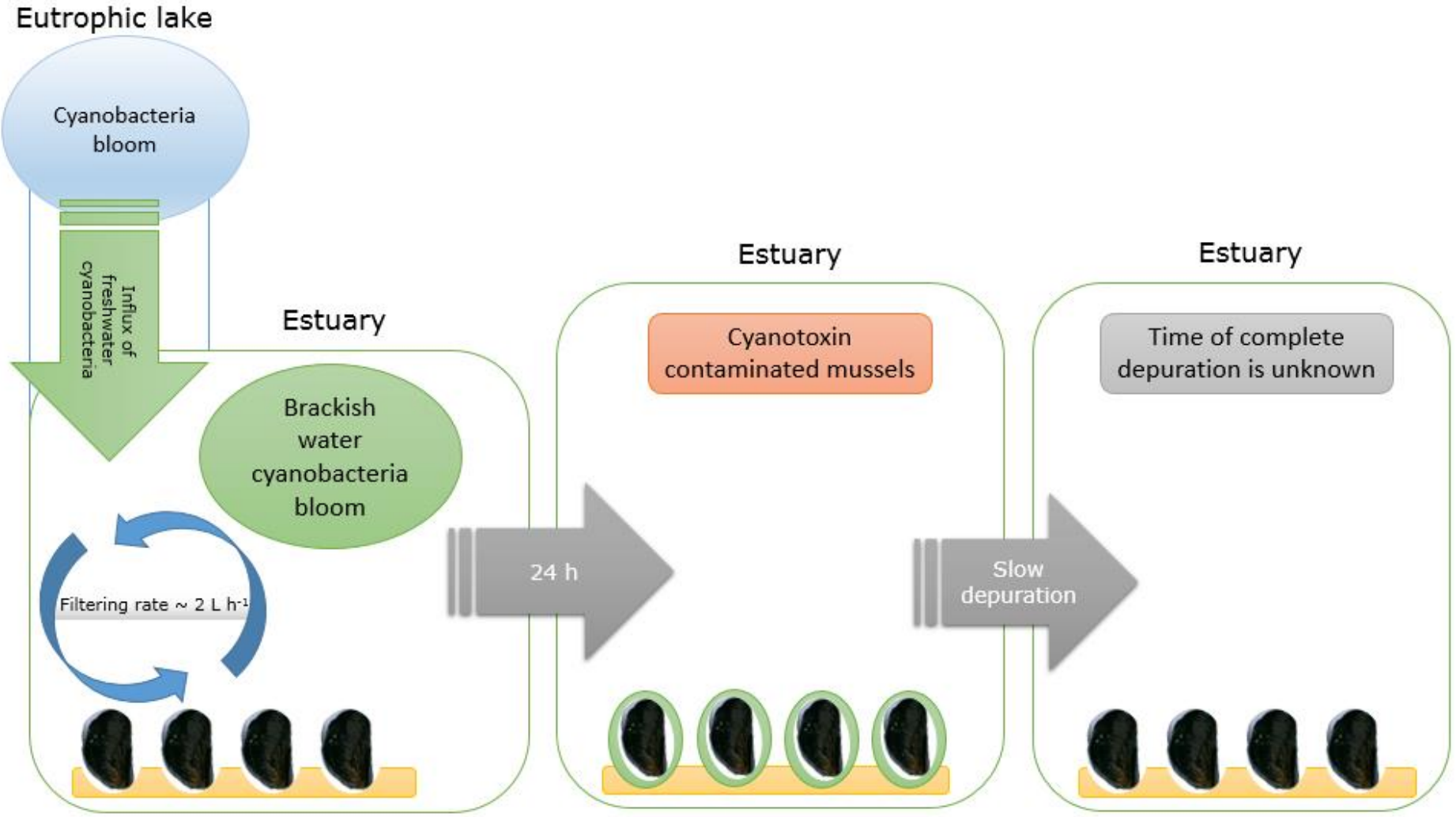

Figure 6. 1 Scenario for the exposure of marine bivalves to freshwater and brackish water cyanobacteria highlighting the rapid clearance of cyanobacteria and accumulation of cyanotoxins. Depuration of cyanotoxins from *M. edulis,* even after short exposure periods associated with influx of freshwater potentially containing cyanobacteria, has shown to be very slow.

Assessing the cyanotoxin budget during the feeding studies revealed a large proportion of unaccounted cyanotoxins within the experimental system. The combined effect of cyanotoxin metabolism within mussels, biotic and abiotic degradation, covalent binding and losses during sample extraction were likely to have contributed to the observed differences. The metabolism of cyanotoxins within *M. edulis,* in particular, is currently not well understood with conflicting reports within the literature. It is therefore vital, for the accurate assessment of risks associated with cyanotoxin contaminated mussels, to further investigate their metabolism in *M. edulis*.

In addition, due to low dose recovery of cyanotoxins in mussel flesh, the application of correction factors for the determination of potential cyanotoxin load in mussels is suggested. Comparing the correction factors determined in each of the three feeding studies showed good agreement between those utilising single cyanobacteria exposure of *M. edulis* (section 3.3.4; section 4.3.6; Table 6. 2). However, when simultaneously exposing mussels to *N. spumigena* KAC66 and *M. aeruginosa* PCC 7813 consistent values were only obtained for MC-LY and MC-LW. Suggested correction factors of the remaining cyanotoxins increased (Table 6. 2). This showed that the exposure of *M. edulis* to a mixture of two cyanobacteria*,* which may represent some environmental conditions more closely, potentially effected the cyanotoxin recovery in mussel flesh. Moreover, the bioavailability of bound MCs, especially following simulated human GI passage, requires further investigation as current findings are inconclusive. A review of the literature also highlighted species dependent accumulation and depuration of cyanotoxins consequently illustrating the need for more detailed research specific to individual consumer relevant seafood species.

Table 6. 2 Suggested correction factors determined during the three feeding studies based on cyanotoxin recovery in mussel flesh

Cyanotoxin	*N. spumigena* KAC66 (chapter 3)	*M. aeruginosa* PCC 7813 (chapter 4)	*N. spumigena* KAC66, *M. aeruginos*a PCC 7813 (chapter 5)
NOD	2.6*	NA	5.0*
MC-LR	NA	2.8	10
Asp3-MC-LR/[Dha7]-MC-LR	NA	2.1	8.0
MC-HilR	NA	2.3	4.0
MC-LY	NA	2.6	2.5
MC-LF	NA	2.4	5.0
MC-LW	NA	2.3	2.5

NA – not applicable * - combined LNOD and NOD

This investigation has demonstrated the emerging threat MCs produced by freshwater cyanobacteria can pose to the marine environment. Consequently, it is vital to identify risk areas in which marine organisms could be exposed to these toxins via the influx of freshwater. Moreover, cyanotoxins such as NOD and LNOD produced by brackish water cyanobacteria were also rapidly accumulated by *M. edulis*. Therefore, the incorporation of cyanotoxins into existing bivalve monitoring programs should be considered in order to protect the public from exposure to cyanotoxin contaminated seafood.

CHAPTER 7

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