# Occurrence of microcystin congeners in surface waters and food in Belgium as a source of exposure to humans

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# Foreword

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# Abbreviations

| | |
|---|---|
| Adda | (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid |
| ACN | Acetonitrile |
| AHI | Aggregated hazard index |
| ATX | Anatoxin-a |
| BFCS | Belgium Food Consumption Survey |
| BLAST | Basic Local Alignment Search Tool |
| BMAA | Beta-methylamino-L-alanine |
| bw | Body weight |
| CCFS | *Chlorella*- and cyano-based food supplements |
| CYN | Cylindrospermopsin |
| CAGR | Compound annual growth rate |
| CV | Coefficient of variation |
| DART | Direct Analysis in Real Time |
| DON | Dissolved Organic Nitrogen |
| EDI | Estimated daily intake |
| ELISA | Enzyme-Linked-Immunosorbent-Assays |
| EPA | Environmental Protection Agency |
| $Exp_R$ | Daily exposure values for recreational surface waters |
| FDA | Food and Drug Administration |
| FTICR | Fourier transformation ion cyclotron resonance |
| GAC | Granulated active carbon |
| GNT | Guanitoxin |
| HP | Hydrogen Peroxide |
| ICBN | International Code of Botanical Nomenclature |

| | |
|---|---|
| ICNP | International Code of Nomenclature of Prokaryotes |
| HPLC-DAD | High-performance liquid chromatography-photodiode array detection |
| HRMS | High-Resolution Mass Spectrometry |
| LB | Lower bound |
| $LD_{50}$ | Lethal dose for half of the test population |
| LOD | Limit of detection |
| LOQ | Limit of quantification |
| LPS | Lipopolysaccharides |
| MALDI | Matrix-assisted laser desorption ionization |
| MCs | Microcystin congeners |
| Mdhb | Methyldehydrobutyrine |
| MEOH | Methanol |
| MMPB | 2-methyl-3-methoxy-4-phenyl butyric acid |
| MRL | Maximum Residue Limit |
| MRP2 | Multidrug-resistant protein 2 |
| MS | Mass Spectrometry |
| MS/MS | Tandem Mass Spectrometry |
| MU | Measurement Uncertainty |
| NGS | Next-generation sequencing |
| NRPS | Nonribosomal peptide synthetase |
| NOAEL | No Observed Adverse Effect Level |
| NOD | Nodularin |
| NOM | Natural organic matter |
| OATPs | Organic anion-transporting polypeptides |
| ORF | Open Reading Frame |
| PAC | Poly-aluminum chloride |

| | |
|---|---|
| PDA | Photodiode-Array Detection |
| PFS | Plant food supplements |
| PKS | Polyketide synthetase |
| PP | Protein phosphatase |
| Q | Research question |
| RfD | Reference dose |
| RQ | Risk daily exposure index |
| RSD | Relative standard deviation |
| sGSTs | Soluble glutathione S-transferases |
| SPE | Solid phase extraction |
| STX | Saxitoxin |
| S/N | Signal-to-noise |
| TDI | Tolerable daily intake |
| TOF | Time-of-flight |
| UB | Upper bound |
| UHPLC | Ultra high-performance liquid chromatography |
| WHO | World Health Organization |

# Summary

Increased awareness in the media and strict control by competent authorities resulted in an overall risk perception of toxic cyanobacteria blooms among the global population in Belgium. There is a general concern about the negative impact of the blooms and their toxins on the environment and public health. Yet, limited data about cyanotoxins in food, drinking and recreational water are available in Belgium. Among the main reasons for this data shortage is a lack of publicly sourced, reliable methods to analyze cyanotoxins in food and environmental samples. This has a negative downstream effect on possible risk assessment studies that would establish the exposure and influence of the different cyanotoxins on public health, and on the final risk management through concrete policy measures.

This doctoral dissertation discusses the development and validation of fit-for-purpose analytical methodologies to quantify the most prevalent cyanotoxin group worldwide, the microcystin congeners (MCs). After method validation, the quantitative UHPLC-MS/MS approaches were applied to Belgian samples to characterize potential human exposure sources in conjunction with and compared to other research tools. The MCs prevalence data was then used to make a preliminary assessment of human exposure and associated public health risk.

Initially, methodologies were successfully validated and applied to quantify MCs in drinking water and environmental samples. Drinking water has the highest potential to be an exposure source of MCs, as assumed by the World Health Organization. Yet, no toxins were found in Belgium's drinking water. However, the situation is very different for the Belgian fresh waterbodies (lakes, ponds, canals). There, MCs ranging from 0.11 µg $L^{-1}$ to 2798.81 µg $L^{-1}$ total microcystin, were quantified in 68 out of 79 samples during the monitoring of five lakes in Wallonia and occasional blooms in Flanders and Brussels, including a canal. MCs prevalence in Belgian waterbodies indicated the potential for exposure through recreational activities and also potential contamination of drinking water from reservoirs. The risk of the latter is aggravated by water shortages and droughts that will require the increased use of surface water for drinking water production in Belgium. Dominant cyanobacterial strains and the presence of the *mcy* gene cluster were also identified in the waterbody samples to better characterize the cyanobacteria blooms.

Algal-based food supplements were also identified as a potential exposure source. The presence of toxin-producing cyanobacteria during the production or harvesting of these products may cause health risks. However, data concerning sales and consumption of algal-based food supplements on the Belgian market, as well as possible contaminations with cyanotoxin, are

scarce. Again, a quantitative UHPLC-MS/MS approach was optimized, validated and applied. Overall, 35 algal-based food supplement samples were collected and analyzed, whereof nine samples contained MCs. Moreover, three samples exceeded the concentration of 1 µg $g^{-1}$ total microcystins, previously proposed as a guideline value. *Microcystis* sp. were determined as the producers of these toxins by amplification and sequencing of the *mcyE* gene. Yet, data on the MCs prevalence is not sufficient to accurately assess human exposure and risk. Additional consumption data were needed. Therefore, a consumption survey for algal-based food supplements was launched, showing that 13.00 % of the 554 respondents used these products. The majority of the users consumed these food supplements daily and all year round. Additionally, most users confirmed that they adhered to the recommended dose for consumption as advertised on the label of the product. This result supports our suggestion that the recommended dose values can be used as a measure for the consumption of algal-based food supplements since quantitative consumption data is not available for these products. The collected data were cumulated in a new exposure assessment for adults and children and a reaffirmation of the proposed guideline values.

Vegetables and fruits might also become a potential exposure source for MCs. These products can potentially accumulate cyanotoxins when contaminated water is used for irrigation, pesticide spraying or other pre- and post-harvest operations. Therefore, their potential role in foodborne exposure was studied. A new quantitative UHPLC-MS/MS approach was developed and validated to quantify MCs in strawberries, carrots and lettuce. These three matrices represented three groups of crops, fruits/berries, leafy and root vegetables, sequentially. Afterwards, a sampling of nine different kinds of fruits and vegetables from the Belgian market was accomplished. In total, 103 samples were analyzed with the method. Yet, MCs were not detected. However, the quality controls during the analysis reported acceptable recoveries for the different products, confirming the appropriateness of the method.

Finally, the results from the 79 samples from algal blooms, 75 drinking water samples, 35 algal-based food supplements, and 103 fruits and vegetable samples were combined with consumption data from the different sources to make a preliminary aggregated exposure and risk assessment for different exposure scenarios. Currently, our data suggest that algal-based food supplements and contaminated recreational water are the major exposure sources, with the food supplements posing a potential risk to public health, especially in children.

Of course, this preliminary assessment should be complemented with additional data on the MCs prevalence in the different products. Moreover, other exposure sources, like freshwater foods (fish), and other cyanotoxins

should be included in the future to further develop their risk assessment. The approaches described in this thesis can hopefully be used as a toolbox to guide and support continuing research.

# Resume

Une sensibilisation accrue dans les médias et un contrôle strict par les autorités compétentes ont donnés lieu à une perception globale du risque de prolifération de cyanobactéries toxiques parmi la population mondiale en Belgique. Il y a une prise de conscience générale quant à l'impact négatif des efflorescences et leurs toxines sur l'environnement et la santé publique. L'une des raisons principales du manque de données est le manque de méthodes officielles et fiables pour analyser les cyanotoxines dans les échantillons alimentaires et environnementaux. Cela a des conséquences négatif sur d'éventuelles études d'évaluation des risques qui établiraient l'exposition et l'influence des différentes cyanotoxines sur la santé publique, et sur la gestion finale des risques par le biais de mesures politiques concrètes.

Cette thèse de doctorat traite de nos efforts pour développer et valider des méthodologies analytiques, adaptée à l'objectif, afin de quantifier le groupe de cyanotoxines le plus répandu dans le monde, les congénères de microcystines (MCs). Après validation, une approche d'UHPLC-MS/MS quantitative a été appliquée à des échantillons belges pour caractériser les sources potentielles d'exposition humaine et cela, en parallèle avec des autres outils de recherche avec lesquels elles ont été comparées. Les données sur la prévalence des toxines ont ensuite été utilisées pour faire une évaluation préliminaire de l'exposition humaine et les risques associés pour la santé publique.

Initialement, les méthodologies ont été validées et appliquées avec succès pour quantifier les MCs dans l'eau potable et les échantillons environnementaux. L'eau potable représente une source majeure d'exposition aux MCs, comme déclaré par l'Organisation Mondiale de la Santé. Pourtant, aucune toxine n'a été détectée dans l'eau potable en Belgique lors de cette étude. Néanmoins, des concentrations, allant de 0.11 µg $L^{-1}$ à 2798.81 µg $L^{-1}$ de microcystine totale, ont été quantifiées dans 68 des 79 échantillons issus du suivi de cinq lacs en Wallonie et de blooms occasionnels en Flandre et à Bruxelles, y compris un canal. La prévalence des MCs dans les masses d'eau belges a montré une exposition potentielle par le biais d'activités récréatives et également une contamination potentielle de l'eau potable des réservoirs. Le risque de ce dernier est aggravé par les pénuries d'eau et des sécheresses qui nécessiteront l'utilisation accrue des eaux de surface pour la production d'eau potable en Belgique. L'identification des souches dominantes de cyanobactéries et de la présence de gènes *mcy* a

également été réalisée dans les échantillons de plans d'eau afin de mieux caractériser les efflorescences de cyanobactéries.

Des compléments alimentaires à base d'algues ont également été identifiés comme une source d'exposition potentielle. La présence de cyanobactéries qui produisent des toxines au cours de la production ou de la récolte de ces produits peut impliquer des risques pour la santé. Cependant, les données concernant la vente et la consommation de compléments alimentaires à base d'algues sur le marché belge, ainsi que les éventuelles contaminations par des cyanotoxines, sont rares. Une fois de plus, une approche quantitative UHPLC-MS/MS a été optimisée, validée et appliquée. Au total, 35 échantillons de compléments alimentaires à base d'algues ont été collectés et analysés, parmi lesquels neuf échantillons se sont révélés contenir des MCs. De plus, trois échantillons dépassaient la concentration de 1 µg $g^{-1}$ de microcystines totales, précédemment proposée comme valeur guide. Des espèces de *Microcystis sp.* ont été déterminées comme productrice de ces toxines grâce à l'amplification et le séquençage du gène *mcyE*. Cependant, les données belges sur la prévalence des MCs ne sont pas suffisantes pour évaluer avec précision les risques d'exposition humaine. Des données supplémentaires sur la consommation sont nécessaires. C'est pourquoi une enquête concernant la consommation de compléments alimentaires à base d'algues a été réalisée, montrant que 13.00% des 554 répondants utilisaient ces produits. La majorité des utilisateurs consomment ces compléments alimentaires quotidiennement et tout au long de l'année. De plus, la plupart des utilisateurs ont confirmé qu'ils respectaient la dose de consommation recommandée telle qu'annoncée sur l'étiquette du produit. Ces résultats renforçent notre suggestion d'utiliser ces données de posologie comme mesure de la consommation des compléments alimentaires à base d'algues puisque des données de consommation explicites et quantitatives ne sont pas disponibles pour ces produits. Les données recueillies ont été cumulées dans une nouvelle évaluation de l'exposition chez les adultes et les enfants et une réaffirmation des valeurs guides proposées.

Les légumes et les fruits sont la dernière source d'exposition potentielle étudiée. Ces produits peuvent potentiellement accumuler des cyanotoxines lorsque de l'eau contaminée est utilisée pour l'irrigation, la pulvérisation de pesticides ou d'autres opérations avant et après récolte. Par conséquent, leur rôle potentiel dans l'exposition alimentaire a été étudié. Une nouvelle approche quantitative UHPLC-MS/MS a été développée et validée pour quantifier les MCs dans les fraises, les carottes et les salades. Ces trois matrices représentent trois types de récolte différents, fruits/baies, légumes-feuilles et légumes racines. Ensuite, un échantillonnage de neuf sortes de fruits et légumes différents disponibles sur le marché belge a été réalisé. Au total, 103 échantillons ont été analysés avec la méthode mise au point. Aucune MC n'a été détectée. Les contrôles qualité effectués lors de l'analyse

ont rapporté des taux d'extraction affichant des rendements acceptables pour les différents produits, confirmant la pertinence de notre méthode.

Finalement, tous les résultats (79 échantillons environnementaux, 75 échantillons d'eau potable, 35 de compléments alimentaires à base d'algues et les 103 échantillons de fruits et légumes) ont été combinés avec des données de consommation provenant de différentes sources en vue de réaliser une évaluation préliminaire globale de l'exposition et des risques pour différents scénarios d'exposition. Actuellement, nos données suggèrent que les compléments alimentaires à base d'algues et les eaux récréatives contaminées sont les principales sources d'exposition de la population. Les compléments alimentaires représentent un risque potentiel pour la santé publique, surtout chez les enfants.

Bien entendu, cette évaluation préliminaire devrait être complétée avec des données supplémentaires sur la prévalence des MCs dans les différents produits. En plus, d'autres sources d'exposition, comme les aliments provenant des eaux douches, comme poissons, et d'autres cyanotoxines devraient être inclus à l'avenir pour développer davantage l' évaluation des risques qu'ils posent. Nous espérons que les approches, décrites dans cette thèse, pourront être utilisées comme 'boîte à outils' pour guider et soutenir la recherche future.

# Samenvatting

Een algemene risicoperceptie omtrend de bloei van toxische cyanobacteriën is gegroeid binnen de algemene bevolking in België door de recente media aandacht en een strikte controle van deze bloeien door de bevoegde autoriteiten. Er bestaat een algemene bezorgdheid over de gerelateerde negatieve impact van de bloeien en hun toxines op het milieu en de volksgezondheid. Toch zijn er maar een beperkte hoeveelheid gegevens beschikbaar in België over cyanotoxines in voeding en water voor consumptie en recreatief gebruik. Eén van de belangrijkste redenen voor het tekort aan gegevens is een gebrek aan openbare, betrouwbare methoden om de cyanotoxines te analyseren in voeding en omgevingsstalen. Dit heeft vervolgens een negatief effect op eventuele risicobeoordelingsstudies die de blootstelling en invloed van de verschillende cyanotoxinen op de volksgezondheid zouden vaststellen, en op het uiteindelijke risicobeleid door middel van concrete beleidsmaatregelen.
Dit proefschrift bespreekt onze inspanningen om analytische methodologieën te ontwikkelen en te valideren voor de kwantificatie van de meest voorkomende cyanotoxinegroep in de wereld, de microcystine congeneren (MCs). Na validatie, werden onze kwantitatieve UHPLC-MS/MS-methode toegepast op Belgische stalen om potentiële menselijke blootstellingsbronnen te karakteriseren in combinatie, maar ook als

vergelijking met andere onderzoeksmethode. Deze toxineprevalentie gegevens werden vervolgens gebruikt om een preliminaire menselijke blootstelling en de bijbehorende risico beoordeling voor de volksgezondheid te maken.

Aanvankelijk werden methodologieën succesvol gevalideerd en toegepast om MCs in drinkwater en omgevingsstalen te kwantificeren. Drinkwater heeft het grootste potentieel als blootstellingsbron van MCs, zoals aangenomen door de World Health Organization (WHO). Toch werden er geen MCs aangetroffen in het Belgische drinkwater. Desalniettemin werden MCs, variërend van 0.11 µg $L^{-1}$ tot 2798.81 µg $L^{-1}$ totaal microcystine, gekwantificeerd in 68 van de 79 stalen tijden de herhaaldelijke controle van vijf meren in Wallonië en incidentele bloeïen in Vlaanderen en Brussel, waaronder een kanaal. De prevalentie van MCs in Belgische waterlichamen toont dat potentiële blootstellingen door recreatieve activiteiten en ook potentiële verontreiniging van drinkwater uit reservoiren mogelijk zijn. Het risico van dit laatste zal zwaarder doorwegen wanneer drinkwaterproductie meer oppervlaktewater zal vereisen door watertekorten en droogte. Dominante cyanobacteriënstammen en de aanwezigheid van het *mcy*-gen cluster werden ook geïdentificeerd in de monsters van het waterlichaam om de bloei van cyanobacteriën beter te karakteriseren.

Voedingssupplementen op basis van algen werden ook geïdentificeerd als een potentiële bron van blootstelling. De aanwezigheid van toxine-producerende cyanobacteriën tijdens de productie of oogst van deze producten kan gezondheidsrisico's met zich meebrengen. Gegevens over de verkoop en consumptie van voedingssupplementen op basis van algen op de Belgische markt, evenals mogelijke aanvwezigheid van cyanotoxines, zijn echter schaars. Opnieuw werd een kwantitatieve UHPLC-MS/MS-methode geoptimaliseerd, gevalideerd en toegepast. In totaal werden 35 stalen van voedingssupplementen op basis van algen verzameld en geanalyseerd. Hiervan werden MCs in negen stalen teruggevonden. Bovendien overschreden drie stalen de concentratie van 1 µg $g^{-1}$ totale microcystines. Deze waarde werdt eerder voorgesteld als een richtwaarde voor voedselveiligheid. *Microcystis sp.* werden bepaald als de producenten van deze toxinen door amplificatie en sequentie bepaling van het *mcyE*-gen. Toch zijn gegevens over de prevalentie van MCs niet voldoende om de menselijke blootstelling en risico's nauwkeurig te beoordelen. Er waren aanvullende consumptiegegevens nodig. Daarom werd een consumptie-enquête voor voedingssupplementen op basis van algen gelanceerd. Hieruit bleek dat 13,00% van de 554 respondenten deze producten gebruikten. Het merendeel van de gebruikers consumeerde deze voedingssupplementen dagelijks en het hele jaar door. Bovendien bevestigden de meeste gebruikers dat ze zich hielden aan de aanbevolen dosis voor consumptie zoals geadverteerd op het etiket van het product. Deze resultaten ondersteunen onze suggestie dat de doseringsgegevens op de verpakking kunnen worden gebruikt als maatstaf

voor de consumptie van voedingssupplementen op basis van algen. Dit is nodig aangezien er voor deze producten geen expliciete, kwantitatieve consumptiegegevens beschikbaar zijn. Alle verzamelde gegevens werden gecombineerd in een nieuwe blootstellingsbeoordeling voor volwassenen en kinderen, waarbij de voorgestelde richtwaarden voor deze producenten kon worden herbevestigd.

Groenten en fruit waren de laatste potentiële blootstellingsbron die werd bestudeerd. Deze producten kunnen mogelijk cyanotoxinen opstapelen wanneer verontreinigd water wordt gebruikt voor irrigatie, tijdens het gebruik van pesticide en andere behandeling voor en na het oogsten. Daarom werd hun potentiele rol als voedingscontaminant bestudeerd. Een nieuwe kwantitatieve UHPLC-MS/MS-methode werd ontwikkeld en gevalideerd om MCs in aardbeien, wortelen en sla te bepalen. Deze drie matrices vertegenwoordigden achtereenvolgens drie gewasgroepen, fruit/bessen, bladgroenten en wortels. Vervolgens werd er stalen van negen verschillende soorten groenten en fruit van de Belgische markt genomen. Met de methode werden in totaal 103 monsters geanalyseerd. Toch werden MCs niet gedetecteerd. De kwaliteitscontroles toonde echter aanvaardbare terugvindings waarden voor de MCs in de verschillende producten, wat de geschiktheid van onze methode bevestigde.

Uiteindelijk werden de resultaten van de 79 stalen van algenbloeïen, 75 drinkwaterstalen, 35 stalen van op algen gebaseerde voedingssupplementen en 103 fruit- en groentestalen gecombineerd met consumptiegegevens uit verschillende bronnen om een voorlopige geaggregeerde blootstelling en een risicobeoordeling voor verschillende blootstellingen scenario's te maken. Momenteel suggereren onze gegevens dat voedingssupplementen op basis van algen en recreatiewater verontreinigd met MCs de belangrijkste blootstellingsbronnen zijn, waarbij de voedingssupplementen een potentieel risico vormen voor de volksgezondheid, vooral bij kinderen.

Deze voorlopige beoordeling moet wel worden vervolledigd met aanvullende gegevens over de prevalentie van MCs in de verschillende producten. Bovendien moeten in de toekomst andere blootstellingsbronnen, zoals voedsel afkomstig uit zoetwater, zoals vis, en andere cyanotoxines worden opgenomen om de risicobeoordeling verder te ontwikkelen. De methodes die beschreven zijn in mijn proefschrift kunnen hopelijk in de toekomst gebruikt worden als ‘toolbox’ om verder onderzoek te begeleiden en te ondersteunen.

# 1. General Introduction

## 1.1. Cyanobacteria

### 1.1.1. General characteristics

Cyanobacteria are photosynthetic bacteria that can be found all over the world in a large variety of lit environments. Their oxygenic photosynthetic capacity is assumed to have led to the Great Oxygenation Event some 2.4 billion years ago and changed the composition of our atmosphere [1–3]. They played an important role in the evolution of eukaryotic algae and plants thanks to the endosymbiosis of a free-living cyanobacterium by a primitive eukaryote [4]. Currently, they are well known for the formation of blooms (proliferations) by planktonic taxa in eutrophic waterbodies where a basic pH and access to phosphorous and nitrogen stimulate rapid growth. However, cyanobacteria also occur as symbiotic species (e.g. lichens) or benthic species growing in marine, freshwater and terrestrial biotopes, including in more extreme environments (hot springs, salines….). Several cellular mechanisms, like photosynthesis, nitrogen fixation, formation of gas vesicles, akinetes, nitrogen and phosphorous reservoirs and $CO_2$ concentration mechanisms provide these organisms with a competitive edge compared to other microorganisms (Figure 1.1).

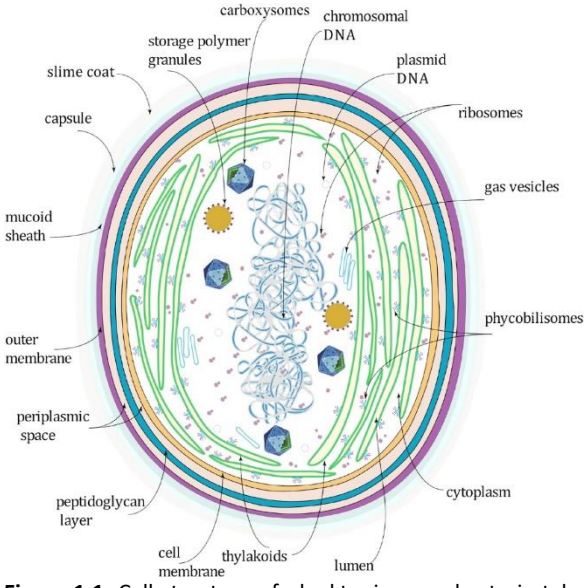



**Figure 1.1**. Cell structure of planktonic cyanobacteria taken from Noreña-Caro and Benton (2018) [5]

#### *1.1.1.1 Photosynthesis*

The oxygenic photosynthetic system in cyanobacteria is the oldest on our planet. Cyanobacteria helped provide the planet with a stable, oxygenated atmosphere by using water as an electron donor. Light energy captured by chlorophyll-a is used in photosystems I and II to produce organic compounds. For most cyanobacteria, these reactions take place in the thylakoids that are present in a regular or irregular position in the cells (Figure 1.1) [6–9]. However, an ancient lineage of cyanobacteria (*Gloeobacteraceae*) does not possess thylakoids. Instead, the photosynthetic systems are present in the cytoplasmic membrane [10]. Interestingly, early plastids that also contain thylakoids, originate from a cyanobacteria ancestor that diverged later in the cyanobacterial radiation (after the *Gloeobacteraceae*), as has been shown based on 16S rRNA data [11]. This data also provides evidence for the cyanobacterial origin of chloroplasts in eukaryotes [11–13].

#### *1.1.1.2 Gas vesicles*

Light availability is necessary for photosynthesis. Therefore, cyanobacteria have developed a way to position themselves in the water column to acquire sufficient light [14–20]. Gas vesicles are hollow, protein-based structures (Figure 1.1) that are produced at low light intensity and cause the cells to migrate upwards in the water column to increase photosynthesis efficiency. While differences exist between genera, after a period at the surface, the increase in turgor pressure through the production of photosynthetic sugar compounds will start the collapse of the vesicles, causing the cells to descend in the water column. The increase in dry weight of the cells has little effect on this process [14,15,17]. Regulation, molecular components, positioning, and size of gas vesicles are variable between different genera. For instance, the size of the gas vesicles can be species and habitat-dependent [18]. Less wide gas vesicles will be able to resist larger turgor pressure experienced at higher depths (deep lakes and the Baltic sea) while wider gas vesicles, with thinner protein membranes, are more common in species found in shallow lakes. The upside of wider gas vesicles is that fewer vesicles have to be produced and fewer resources are required. The cyclic vertical migration between the water surface and the sediment also provides the cyanobacteria with the opportunity to scavenge additional nutrients located lower in the water column, e.g. released by the sediments [14–18]. Primarily planktonic cyanobacteria use gas vesicles. However, benthic cyanobacteria might also develop gas vesicles during a short planktonic phase [21].

*1.1.1.3 Nitrogen fixation*

Some cyanobacteria also have the remarkable capacity to fix atmospheric nitrogen ($N_2$) by the use of a nitrogenase. The well-preserved *nif* gene cluster is present in some cyanobacteria. This is unusual as nitrogenase is irreversibly inhibited by oxygen, which is produced during photosynthesis [7,22]. Different families have developed different methods to prevent the inhibition of nitrogenase [7,22,23] based on spatial or temporal separation from oxygen. For instance, $N_2$ fixation in *Trichodesmium* is controlled by a circadian clock influenced by the presence of oxygen in the cell, while other genera only fix $N_2$ when light is absent. On the other hand, some filamentous cyanobacteria may produce specialized cells, called heterocysts (Figure 1.2), when nitrogen sources are limited [7,22,24]. Heterocysts have additional surface layers containing glycolipids and polysaccharides external to the cell wall that slow the flow of oxygen diffusion [24]. Photosystem II is also inactive, so oxygen is not produced. The heterocysts, therefore, depend upon carbon sources provided by the neighbouring vegetative cells [7,22]. The location of the heterocysts in the filaments is also genus-dependent [25]. Some heterocystous cyanobacteria can also use a secondary nitrogenase (Nif2) under anaerobic conditions. This enzyme is also found in some non-heterocyst-forming genera [7].

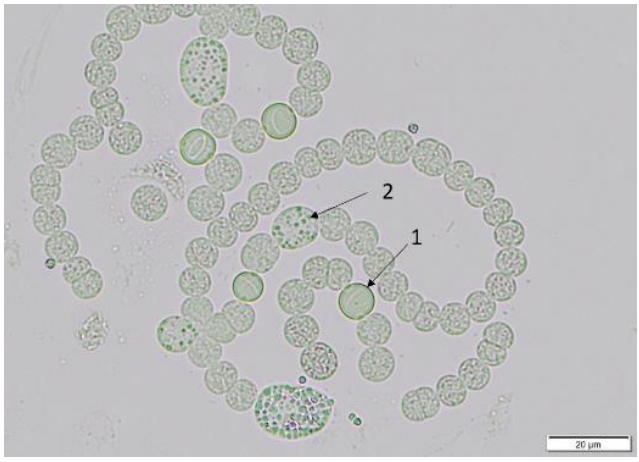



**Figure 1.2**. Filamentous cyanobacteria with heterocysts (1) and akinetes (2).

*1.1.1.4 Akinetes*

Another specialized cell type present in some filamentous, heterocystous cyanobacteria is the akinetes (Figure 2). These cells have a similar function as spores in Fungi or endospores in Bacteria [24,26–28]. Under unfavorable growth conditions (e.g.: low light, desiccation and phosphorous limitation),

akinetes were shown to develop [29–33]. When nitrogen was available, akinetes were positioned randomly in the filaments. However, if heterocysts were present under nitrogen restriction, the akinetes preserved a position relative to these cells [31]. The specific position was dependent on the cyanobacterial species. For instance, akinetes developed midway between heterocysts in *Anabaena sp.* strain CA, whereas they could be found directly adjacent to the heterocysts in *A. cylindrica.* Moreover, akinetes were shown to be separated from heterocysts by a few cells in *Anabaena circinalis* (now *Dolichospermum sigmoideum*) [24,34,35]. Akinetes were reported to be enclosed by a cell wall, a multilayer cellular envelope, not present in vegetative cells but similar to heterocysts. The cellular envelope contained layers of glycolipids, a mucilaginous and polysaccharidic layer [24,26,28,36]. During the maturation of the akinetes, cellular metabolism decreased. Thylakoids became disorganized in the cells and respiratory activities decreased [36]. Glycogen, lipid droplets and cyanophycin were stored in the cells until germination [29–31,36]. Interestingly, cyanophycin was long believed to be the main nitrogen source during germination [24,27,28]. However, the inability to produce cyanophycin in a deletion mutant did not affect the cyanobacteria's ability to form or germinate akinetes compared to the wild type in nitrogen-restricted conditions [26]. Many researchers suggested that the cell wall could also be used as a nitrogen source during germination [26,33]. Moreover, heterocysts were developed early after germination under nitrogen-restricted conditions [24]. Germination was induced when the growth conditions were favorable [24,26,37] and photosynthesis resumed after the reorganization of the thylakoids [37].

## 1.1.2. Taxonomy of cyanobacteria and phylogenetic relations

### *1.1.2.1 Early taxonomy and species concept*

Early taxonomy of cyanobacteria was exclusively based on morphological characteristics of which some were described in the previous subchapter. The current taxonomy of cyanobacteria is based on a polyphasic approach, where morphological characteristics and molecular data (generally 16S rRNA sequencing) were combined [25]. However, the taxonomical system of cyanobacteria is in constant evolution. Originally, cyanobacteria were named using the rules of the botanical taxonomical code [25,38–41]. However, after the recognition that cyanobacteria belonged to the Prokaryotes, the use of the bacteriological nomenclature was proposed. Merging the taxonomic practices for both phyla was difficult due to different definitions of the species concept. While the botanical taxonomic approach primarily focuses on morphological and physiological characteristics to define (new) species, current practices under the rules of the International Code of Nomenclature of Prokaryotes (ICNP) rely on a multitude of characteristics (e.g. genotypic,

chemotaxinomic and other phenotypic markers) for the same purpose [40]. Moreover, the concept of ecotypes has also often been used for bacteria and was therefore applicable for cyanobacteria as well. Strains in different environments could exhibit different morphological characteristics even though they were closely related at a molecular level. Recently, the status of cyanobacteria in the prokaryotic nomenclature was resolved by accepting taxa originally described in the International Code of Botanical Nomenclature (ICNB) to the ICNP and vice-versa [41].

*1.1.2.2 Families of cyanobacteria and key characteristics*

The structural variation of cyanobacteria is well illustrated in Figures 2 and 3 in Komárek et al. [25].

The *Gloeobacteriophycidae* contains the family *Gloeobacteraceae*. This family is distinguishable by their lack of thylakoids and is monophyletic. The *Gloeobacter* genus was already well-described. It is also considered the most ancient lineage [25].

*Synechococcales* and *Spirulinales* are two families belonging to the *Synechococcophycidae* subclass. Both families have been identified by parietal thylakoids. *Spirulinales* have typical regularly screw-like coiled trichomes, while *Synechococcales* are still morphologically (both filamentous and unicellular morphotypes) and ecologically poorly defined [43,44].

The family of *Chrooccoccales* have an irregular thylakoid arrangement and contains the well-studied genus of *Microcystis* [21]. This genus is monophyletic. However, the classification at the species level was long under discussion. Five *Microcystis* sp. were shown to be closely related based on DNA/DNA hybridization data and hypothesized to be different morphotypes of *Microcystis flos-aquae* [45]. Two other species, *M. viridis* and *M. wesenbergii*, are still considered independent [21].

The *Oscillatoriales* family consists of filamentous genera with irregular thylakoids. The *Microcoleaceae* includes the genera *Arthrospira (*previously *Spirulina* and recently revised as *Limnospira)* and *Planktothrix* which are monophyletic [46,47], while the *Oscillatoriaceae* contain polyphyletic genera like *Lyngbya, Oscillatoria* and *Phormidium* [25].

*Chroococcidiopsidales* are primarily extremophiles that were shown to form thin, yet firm, colorless sheaths of spherical or irregular groups or cells [25]. However, solitary cells have also been observed in nature. Members of this family were described to have irregularly distributed thylakoids in the cytoplasm of the cell [25].

The order *Nostocales* contains a large variety of filamentous genera with special cells ( e.g., heterocysts and akinetes). The *Aphanizomenonaceae* family was characterized as planktonic, isopolar organisms with unbranched heterocystous types. *Cylindrospermopsis, Nodularia* and *Raphidiopsis* are monophyletic members of this family. *Aphanizomenon* and *Dolichospermum*

(formerly known as planktonic *Anabaena*) [48] are polyphyletic and are even so similar that they could form one genus [25,49–54]. The *Nostocaceae* family contains the genera *Nostoc,* (benthic) *Anabaena* and *Cylindrospermum.* The genera are polyphyletic, could produce akinetes, were shown to be heteropolar and have isopolar unbranched heterocysts. Interestingly, the difference between *Dolichospermum* and *Anabaena* was based on the presence of gas vesicles, as not all members were conform to the molecular separation of these genera [49,55–57].
Important to note is that the allocation of species and genera to different families is still evolving with ongoing research.

*1.1.2.3 Phylogenic markers*

Different phylogenetic markers have been used since the discovery of molecular biology. Early methods used %GC-content and DNA-DNA hybridization, which was the official technique to determine a bacterial species [38,58]. After the invention of PCR, multiple sequencing and non-sequencing methods were used to determine phylogenetic relationships and identify unknown cyanobacterial isolates [11,25,58–63]. Multiple genes (16S rRNA, 23S rRNA, ITS, *nif* genes) were shown to be reliable phylogenetic markers for cyanobacteria [46,50,51,58,64]. Especially, 16S rRNA was commonly used due to its universal and conserved character and relatively stable mutation rate providing a suitable resolution at the genus level [58]. Moreover, the dominant use of 16S rRNA amplification over other genes provided large databases needed to construct phylogenetic trees. However, multiple genes should be compared when trying to elucidate phylogeny at a species level [46]. Moreover, third-generation sequencing could identify cyanobacteria and cyanobacteria diversity within environmental samples and help to study their geographical distribution [62,65,66]. These methods also have the potential to link phenotypical and genomic data that could further improve phylogenetic and taxonomic characterization of cyanobacterial strains.

## 1.1.3. Cyanobacteria as food and nutraceuticals

*Arthrospira platensis (*previously *Spirulina platensis,* and recently proposed to be renamed *Limnospira)* were being harvested in soda lakes and consumed in certain regions of Central America and Africa for centuries [47,67]. In addition, *Aphanizomenon flos-aquae* has been harvested in the Klamath lakes in the USA for a few decades for commercial exploitation [68]. More recently, *Arthrospira platensis* has also been cultivated on a more industrial scale but also used for development projects to help mitigate hunger in developing countries [69,70]. The high protein and polyunsaturated fatty acid content of 'spirulina' made it an ideal food to balance dietary needs [69,71]. Moreover, 'spirulina' was shown to contain

high amounts of essential molecules, like vitamin B12, carotene, thiamin, and riboflavin [70]. In contrast, the nutritional value of *Aphanizomenon flos-aquae* has not been thoroughly described, even though it has been consumed for the same reasons as 'spirulina' [70]. Due to the presence of phycobiliproteins, both *Aphanizomenon flos-aquae* and 'spirulina' have also been used as natural food colorants [70]. Besides the nutritional value and other uses in food, neutraceutical effects, like ergogenic, antioxidant, hypolipidemic and anti-inflammatory effects, have been ascribed to cyanobacteria–based products [72–75]. 'Spirulina' was recognized as GRAS (Generally Recognized As Safe) in 2002 by the US Food and Drug administration (FDA). The analysis of genomes of *Arthrospira* sp. strains did not show the presence of cyanotoxin genes clusters [76]. On the other hand, the production of different cyto- and neurotoxins have been reported in *Aphanizomenon flos-aquae* strains [68,77,78]. However, the added benefits and certain health safety aspects of cyanobacterial consumption should be evaluated together, which will be done in Chapters 6, 8 and 9.

## 1.2. Cyanobacterial metabolites

### 1.2.1. Major freshwater cyanotoxins

Some of the major cyanobacterial toxins and metabolites are described in this section. Cyanotoxins are classified either by structure or by their activity. Classification by activity is most commonly used in literature. Toxins are usually classified as hepatotoxins, neurotoxins, or dermatotoxins.

#### *1.2.1.1 Microcystin congeners*

Microcystin congeners (MCs) are the most studied and prevalent hepatotoxins produced by cyanobacteria. They were initially identified in a *Microcystis aeruginosa* strain, although were later also identified in *Planktotrix, Anabaena* (some are now *Dolichospermum)*¸*Lyngbya*, *Nostoc* and other taxa [79–84].

MCs were shown to be made up of seven amino acids, with the unique (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda) on position 5 [81,83,85] (Figure 1.3). Positions 1, 3 and 6 have primarily been identified as an Ala, D-Glu and derivative of Asp, sequentially [81–96]. These positions were constant in the structure for most of the MCs. Position 2 and 4, on the other hand, do differ between different congeners as amino acids vary [81,83,85,89,92,93]. Commonly, leucine was found at position 2 or arginine at position 4, combined with another amino acid at either position. Position 7 could be occupied by a dehydro alanine (Dha), a methyl-dehydro alanine (Mdha) or 2-amino-2-butonic acid (Dhb) [83,86–88,90,92,93,95,97–103]. The discovery of potential methylations at

positions 3 and 7, as well as demethylations or acetylations on the Adda further increased the possible variations in the congeners [82,92,97,104,105]. As of now, more than 300 different MCs have been reported [85,106].

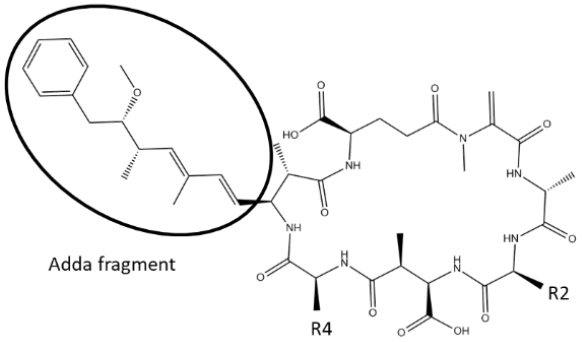



**Figure 1.3.** Microcystin core structure with annotated Adda-group. Different amino acids can be located at positions R2 and R4.

MCs were found to be produced by a gene cluster (*mcyA-J*) coding for an enzyme complex combining polyketide synthetase (PKS) and nonribosomal peptide synthetase (NRPS) (Figure 1.4) [107–111]. The structure of the gene cluster differed between different genera and some open reading frames can be missing [109–111]. The genetic cluster in *Microcystis* was first identified and contained two operons. *McyA-C* codes for the PKS genes, while the *mcyD-J* operon was situated in the opposite sense and codes for a PKS-NRPS module [109]. The *mcyA-J* cluster was orientated in the same sense in *Planktotrix* and thus formed one operon. An additional ORF (*mcyT)* was available, while two ORF (*mcyF and I*), available in *Microcystis*, were missing [110]. *Anabaena* strain 90 contained identical ORFs to *Microcystis*. However, *mcyD-J* ORFs were differently placed in the operons (Figure 1.4) [111]. The order of the ORFs in the operons was co-linear with the hypothetical synthesis of the MCs, which further suggested that the general structure of the *Anabaena* operon better represents the general structure of the common ancestor of the *mcy* genes in the three species. Therefore, shifts in the general structure of the ORFs in *Microcystis* and *Planktothrix* were hypothesized [112]. Variations and duplications in the adenylation domains of the *mcyA-C* genes would also influence the variation of different congeners by the addition of different amino acids on positions 2 and 4 [110]. However, MCs diversity was also dependent on differences between species, strains and different environmental factors, like nutrient availability [113–117].

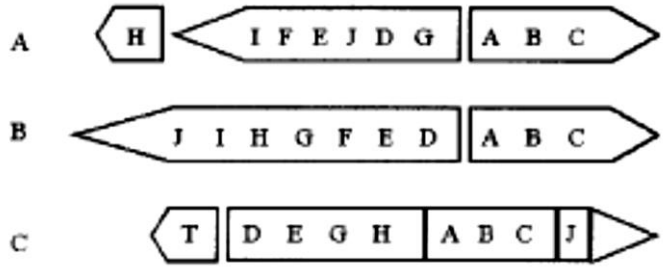



**Figure 1.4.** Order of *mcy* genes in *Anabaena* (A), *Mircocystis* (B) and *Planktothrix* (C). The figure is taken from Rouhiainen et al. 2004 [111].

Microcystin congeners were shown to inhibit protein phosphatase (PP) by non-covalent interactions between the Adda-tail and Glu residue on position 6 and the hydrophobic grove in the catalytic subunit of the enzyme. Additional hydrogen bonds between the two residues and metal-bound water molecules in the catalytic subunit fortified the interaction between MCs and PP. Modifications to the toxin side chains greatly reduced their toxicity. MCs also formed a thiol-bond to PP via Michael addition between position 7 and a serine residue in the catalytical site. However, the thiol bond was shown to be not essential for toxicity and the reaction speed depended on the variable side chain at position 7 [87,88]. Amino acid variability at positions 2 and 4 only caused minor differences in the inhibition potential of the MCs on the PPs. Additionally, MCs have different half-maximal inhibitory concentrations for different PPs [118,119]. Overall, small differences were reported for MC-LR, MC-RR, MC-LW and MC-LF for inhibition of PP1 and PP2A (Table 1.1) [118–120].

**Table 1.1.** The ($IC_{50}$) of MC-LR, MC-RR, MC-LW and MC-LF for protein phosphatase 1(PP1) and 2A (PP2A) based on Altaner et al. 2020 *[118]*.

| **Congener** | **PP1 (nM, $IC_{50}$)** | **PP2A (nM, $IC_{50}$)** |
|---|---|---|
| MC-LR | 0.3 | 1.6 |
| MC-RR | 1.5 | 0.5 |
| MC-LW | 1.2 | 0.7 |
| MC-LF | 2.0 | 1.4 |

Differences in toxicity for different MCs were potentially caused by differences in interactions between the MCs and membrane transporters. Organic anion-transporting polypeptides (OATPs) have been shown to transport MCs in the cells. OAPT1B1 and OATP1B3 were present in hepatocytes and were primary targets for MCs. Fischer et al. described higher uptake of MC-LF and MC-LW compared to MC-LR. MC-RR uptake was even lower compared to MC-LR. However, the higher uptake of MC-LF would not result in higher toxicity due to an increased efflux transport by multidrug-resistant protein 2 (MRP2) in hepatocytes compared to MC-LR and MC-RR [121,122]. Yet, it has also been shown that most hydrophobic MCs (e.g. MC-LF) were poorly detoxified in vitro compared to the more hydrophilic congeners (like MC-RR) [123]. Therefore, hydrophobic MCs were believed to be more toxic [123,124].

The localization of the MCs in the body was also dependent on OATPs present in different organs. OATPs located in the liver, kidneys and blood-brain barrier have been shown to facilitate transport [119,125–127]. Multiple studies have shown the accumulation of MCs in the gastrointestinal tract (especially a large portion of the small intestine), liver, blood urine, kidney, lungs and brains to different extents [128–132]. Similarly, MCs could be transported in central and peripheral human neural cells and rat astrocytes in vitro and cause neuro- and cytotoxicity, respectively [133,134]. MC-LF showed higher neurotoxicity compared to MC-LR.

Bound forms of MCs were also potentially toxic to humans. These bound MCs could be formed during bioaccumulation at different trophic levels (e.g. plants, fish) through the covalent serine linkage with PPs or linkage with glutathione (GHS) as a detoxification strategy [135–137]. When plants or fish were consumed by humans, bound MCs are also ingested. PP-linked MCs were partially released by proteolytic enzymes with the Adda fragment intact [138]. Therefore, they were still able to inhibit the catalytic site of PPs at half the efficiency of MC-LR. Similar results were found for GHS-linked MCs. A more extensive reduction of liver toxicity for GHS-linked MCs was found in mice compared to MC-LR, either due to blockage of transport to the liver or effective excretion of the bound toxins by ATP-dependent glutathione S-conjugate pumps [139]. Currently, legislation and regulatory limits do not take the bound forms of MCs into account.

Due to the protein phosphatase inhibition by MCs, they were considered as a Group 2B carcinogenic (e.g. possibly carcinogenic to humans) due to possible tumour-promoting effects in humans [122,140]. However, no evidence was found of DNA damage directly caused by exposure to pure MCs during genotoxicity studies [140,141].

The occurrence of MCs in the environment will be further discussed in detail in Chapters 5 and 10. The regulatory limits for the MCs will be discussed in Chapters 3, 5, 6 and 7 organized based on exposure source.

*1.2.1.2 Nodularin*

Nodularin (NOD) is structurally similar to the MCs (Figure 1.5). This toxin was initially observed in *Nodularia spumigena* [142–144]. Later on, NOD was also found in terrestrial *Nostoc* sp. [145]. A pentacyclic peptide structure has been identified for NOD, containing most of the structural elements of the MCs. While NOD lacked the Ala peptide and variable peptide on position 2, it did contain the typical Adda, D-Glu, D-MeAsp and Arg groups. The Dha/Dhb or Mdha group of the different MCs was also substituted by N-Methyldehydrobutyrine (Mdhb) [142]. Six other congeners have been identified [(6Z)Adda3]NOD-R, [DMAdda3]NOD, [DAsp1]NOD, [Glu4(OMe)]NOD, [DHb5]NOD, [MeAdda3]NOD and [L-Har$^2$]NOD [142,146–149]. NOD and [L-Har$^2$]NOD have been regularly found in the environment [19,147,150–154].

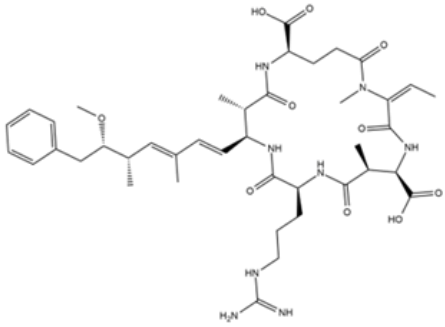


**Figure 1.5.** Structure of nodularin

The hepatotoxic effect of NOD was shown to be similar to the effect of MCs due to their similar structure [155]. The lethal dose for half of the test population ($LD_{50}$) for NOD was similar to microcystins [144]. Moreover, efficient inhibition for PP1 and PP2A and transport by OATPs (in zebra fish) were also shown [144,156,157].

The genetic cluster (*nda*) responsible for the production of NOD contained similar elements to the *mcy* cluster [158–160]. The *ndaA* and *B* ORFs upstream of the promoter code for the PKS enzymes were similar to the *mcyA* and *mcyB* ORFs in *Microcystis aeruginosa*. Missing modules in *ndaA*/*B* compared to the *mcyA/B* were responsible for the two missing peptides (Figure 1.5). Deletion events were believed to be responsible for these missing modules. The *ndaCDF* ORFs were situated downstream of the promoter codon and responsible for the NRPS-based synthesis of the Adda fragment, similar to the *mcyDEFG* ORFs (Figure 1.6). *NdaEGHI* encodes for additional tailoring enzymes, which also had counterparts in the *mcy* cluster. Due to the high similarity, it has been widely accepted that the *nda* and *mcy* gene clusters are evolutionary related [159]. Although, additional

phylogenetic data should be acquired to establish whether the *nda* cluster evolved from the *mcy* cluster or both clusters were derived from a common ancestor [158–160].

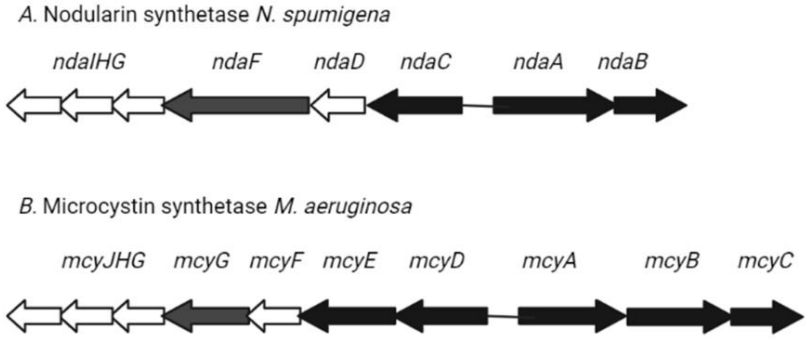



**Figure 1.6.** Representation of *nda* cluster in *N. spumigena* (A) compared to *mcy* cluster in *M. aeruginosa* (B). The figure is adjusted from Moffitt and Neilan 2004 [159] and created in BioRender.com

*1.2.1.3 Cylindrospermopsin*

Cylindrospermopsin (CYN) is yet another hepatotoxin with a different structure from the MCs [161–163]. The alkaloid was found to consist of a tricyclic guanidine moiety and a hydorxymethyluracil (Figure 1.7) [161,162]. CYN was initially discovered in *Cylindrospermopsis raciborskii (*previously*, Anabaena raciborskii)* [162,164]. However, the toxin was also later found in *Umezakia natans*, several *Aphanizomenon sp.,* other *Nostocales* and *Oscillatoriales* genera [78,161,165–179]. Four structural variants have also been observed. Deoxycylindrospermopsin was also found in *Cylindrospermopsis raciborkii* but was shown to be non-toxic in an intraperitoneal mouse assay [169,180,181]. 7-Epicylindrospermopsin was another variant isolated from *Aphanizomenon ovalisporum* (now *Chrysosporum ovalisporum*) [170]. Two additional analogues (7-deoxy-desulfo-cylindrospermopsin and 7-deoxy-desulfo-12-acetylcylindrospermopsin) have been reported by Wimmer et al. [182]. Toxic effects of CYN have been shown in liver, kidney, intestines, thymus, spleen and lungs [164,183–186]. CYN was initially shown to inhibit protein synthesis. The main targets for inhibition are not yet elucidated but shown to be a soluble protein associated with the eukaryotic translational system but not the ribosome [187]. Inhibition of glutathione synthetase was suggested to be the major cause of cytotoxicity in the hepatic cells [164,184,185,188,189]. Cytochrome P450 was shown to be involved in CYN metabolism, generating the acute hepatotoxic effect [184,188,189]. The uracil moiety in CYN was suggested to accomplish an important role in its toxicity [190]. However, exact mechanisms still need to be elucidated. Moreover, CYN has been

shown to exhibit genotoxicity in mammalian cell models, including human lymphoblast cell-lines and mouse liver (*in vivo*) [163,191–194]. The WHO suggested a no-observed adverse effect level (NOAEL) of 30 μg $kg_{bw}^{-1}$ $day^{-1}$ based on the existing toxicological data and advises reporting the sum of the concentration for all available CYN analogues [163,186].

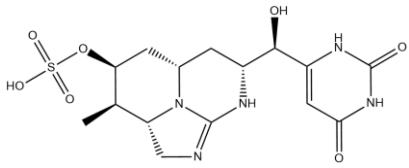



**Figure 1.7.** Cylindrospermopsin structure

The biosynthesis mechanisms for CYN have been identified in several species, which contained the same core enzymes, an amidinotransferease, NRPS and PKS [195–199]. The genetic sequences coding for the core enzymes have been named *aoaA*, *aoaB* and *aoaC* in *A. ovalisporum*. The genetic structure cluster in *C. raciborskii* has been further elucidated with *cyrA*, *cyrB* and *cyrC* being homologs of *aoaA*, *aoaB* and *aoaC*, in consecutive order [196]. Additional tailoring, transport and transposase genes were also identified [196]. The *cyr* cluster was also identified in *Oscillatoria* PCC 6506 showing genes with high sequence identity but different gene arrangements compared to *C. raciborskii* (Figure1.8) [199].The *cyrA* gene coded for the amidinotransferase. The *cyrB* and *cyrC* genes coded for a mixed NRPS-PKS and PKS module, respectively [196]. Interestingly, the *cyrJ* gene was responsible for the sulfotransferase that catalyzes the transfer of a phosphate group on 7-deoxy-desulfo-cylindrospermopsin. Moreover, the produced 7-deoxycylindrospermopsin was transformed to CYN by a hydroxylase (*CyrI*), showing that these compounds are precursors of CYN [200].

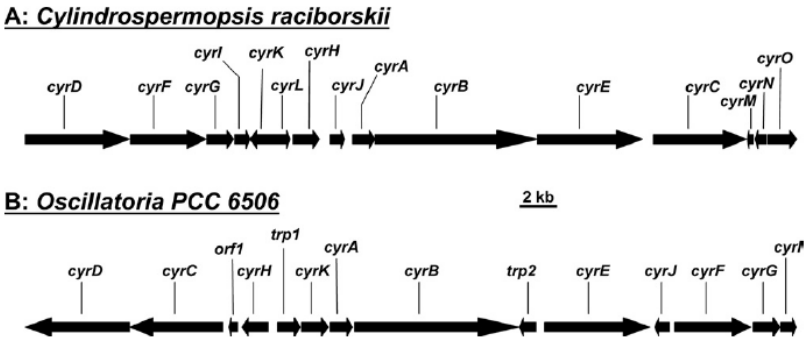



**Figure 1.8.** Organization of the *cyr* gene cluster in *C. raciborskii* (A) and *Oscillatoria* PCC 6506 (B) as published by Mazmouz et al 2010 [199].

Cylindrospermopsin has been identified in various regions across the world, including Europe, Asia, Australia, North and South America [162,164,167,172,173,175,176,178,179,201–205].

*1.2.1.4 Anatoxin-a*

Another group of cyanotoxins was defined by their neurotoxic activity. One of these neurotoxins, anatoxin-a (ATX), is an amine alkaloid produced by different cyanobacteria. ATX has already been observed in *Anabaena flos-aquae* (now *Dolichospermum flos-aquae*)*, Planktothrix rubescens* (previously *Oscillatoria rubescens*)*, Arthrospira fusiformis* (previously *Spirulina fusiformis), Phormidium* sp.*, Tychonema* sp. (previously *Oscillatoria*)*, Cylindrospermum* sp., *Cuspidothrix issatschenkoi* (previously, *Aphanizomenon issatschenkoi*) and many *Oscillatoria* sp. [206–213]. ATX was shown to have a cyclic structure (Figure 1.9) [206]. Three other homologues were also identified; dihydro-anatoxin-a (dhATX), homoanatoxin-a (HTX) and dihydro-homoanatoxin-a (dhHTX). ATX and HTX have been regularly found together, although at different ratios [206,214,215]. HTX was reported to be a homologue of ATX with the same toxic potential [215]. Both dhATX and dhHTX were produced by certain cyanobacteria but also occurred as derivatives resulting from interactions with the extracellular environment [212,216–218].

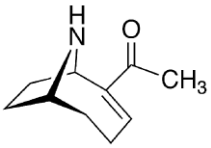



**Figure 1.9.** Anatoxin-a [210]

ATX was initially known as the Very Fast Death Factor due to its acute potential to block acetylcholine ion receptors causing hyperactivity of the muscles. Eventually, exhaustion of the muscles caused complete paralysis and breathing stopped [219–226]. ATX remained permanently bound with the receptor. Therefore, treatment after intoxication required removing ATX from the body and not only temporary respiration assistance [222,224,227]. ATX produced by benthic blooms has also been well-described as a cause of dog deaths during the summer [209,214,228–232].

The *ana* gene cluster, responsible for the production of ATX, was first elucidated in *Oscillatoria* sp. strain PCC 6506 (Figure 1.10) [210,211,233,234]. The PKS enzymes were coded by *AnaE*-G, while *AnaA-D* codes for the other enzymes necessary for the production [210,233,234]. *AnaG* was responsible for the eventual production of HTX due to methylation of ATX. Furthermore, *anaJ* and *anaI* coded for a cyclase and transporter, respectively [212]. The

*ana* clusters were also observed in *Anabaena flos-aquae* and *Cylindrospermum stagenale* PCC7417. Additional genes, unimportant for ATX synthesis, were detected between *anaG* and *anaI* in *Anabaena flos-aquae*, as well as a relocation of the *anaA*, *anaI* and *anaJ* genes downstream from *anaG* [211,235]. The *A. flos-aquae ana* cluster strongly resembled the other clusters with a shorter *anaG* gene and a new gene situated between *anaD* and *anaE* [211,236]. *AnaE* and *AnaF* were also shown to be the most variable in 15 *Cuspidothrix issatschenkoi* strains [237].

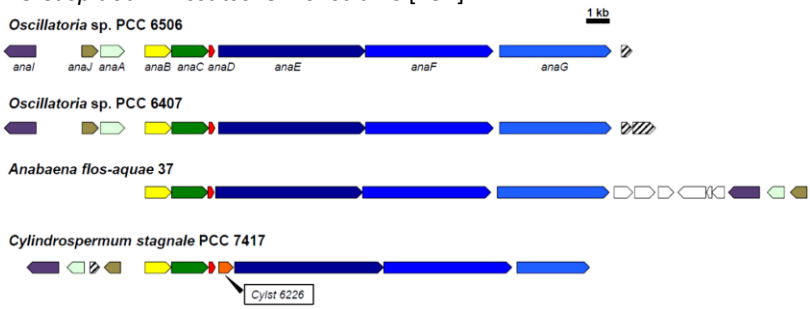



**Figure 1.10.** Differences in *ana* gene cluster structures between different cyanobacterial strains. The figure was published by Méjean et al. 2014 [211]. Genes unrelated to the ATX production are colored in white.

### *1.2.1.5 Anatoxin-a (S)/ Guanitoxin*

Another neurotoxic compound that caused similar symptoms to ATX is anatoxin-A(S). Anatoxin-A(S) lended its name, like ATX, from its initial identification in *Anabaena flos-aquae* NRC 525-17 (now *Dolichospermum flos-aquae)* and was later observed in *Sphaerospermopsis torques-reginae* (previously known as *Anabaena torques-reginae or Dolichospermum torques-reginae*) [238,239]. However, this organophosphate was shown to be structurally different from ATX (Figure 1.11) [77]. Additionally, the mechanism of neural disruption was completely different compared to ATX. Anatoxin-A(S) has been proposed to form a covalent bond with an acetylcholine esterase, irreversibly inhibiting the removal of acetylcholine, while ATX functions as a receptor antagonist, as discussed earlier [240–243]. Due to these differences, anatoxin-A(S) was recently renamed guanitoxin (GNT) [240]. Up to recently, very little additional information was available for GNT, due to its unstable structure and lack of commercially available standards. An acetylcholine esterase assay could be used to indicate GNT presence. However, this method was not specific to GNT [244,245]. HPLC-MS/MS methods have also been developed for GNT detection [239,246,247]. Yet no independent quantification could be performed without a standard. Therefore, only a few cases of GNT-producing cyanobacteria have been recorded worldwide [241,244–246,248–250]. Additionally, a case of bird

deaths and cases of dog deaths were suggested to be caused by GNT [245,251,252].

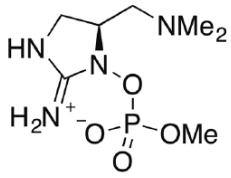



**Figure 1.11.** Guanitoxin [240]

Early studies of the GNT biosynthesis discovered the L-arginine precursor and some of its intermediates via labelling experiments [253,254]. However, the full biosynthesis process and gene cluster were not elucidated until recently. Using full genome sequencing, putative genes for GNT biosynthesis were postulated in *Sphaerospermopsis torques-reginae*. Ten metabolic genes (*gntA-J)* and a transporter gene *(gntT)* were identified [255]. L-arginine is converted to GNT in nine metabolic steps that each have been shown *in vitro* using expression of the responsible gene(s). The *gnt* genes were found in freshwater molecular datasets for an *Aphanizomenon* sp., *Cuspidothrix* sp. and multiple unidentified species [255]. The identification of the *gnt* cluster provided the opportunity to enhance screening for GNT worldwide.

*1.2.1.6 Beta-methylamino-L-alanine*

Beta-methylamino-L-alanine (BMAA) was believed to be a neurotoxin due to its possible role in the development of amyotrophic lateral sclerosis-parkinsonism-dementia complex (ALS-PDC), which is a neurodegenerative disease (Figure 1.12) [256,257].

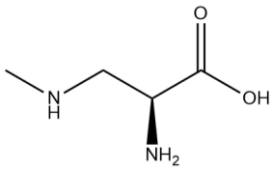



**Figure 1.12.** structure of BMAA

The initial link between BMAA and ALS-PDC was observed in indigenous people in Guam [258–260]. In this particular case, BMAA was initially produced by *Nostoc* sp., which lives in symbiosis with *Cycas micronesica* [258]. BMAA bioaccumulation progressed through the seeds of this plant further to flying foxes, which were consumed by the local population [259]. An additional cluster of an unusual amount of ALS-PDC cases was observed

around Lake Mascoma in Enfield, US [261]. BMAA presence has already been determined in multiple environments, cyanobacterial species and seafood with concentrations reaching up to µg/g levels [262–269]. BMAA has been shown to cause cell death of motor neurons and injured cortical neurons in vitro [270,271]. Exposure of *in vivo* models (e.g. rat and vervet monkeys) to BMAA also showed symptoms of neurodegeneration [272–275]. The BMAA concentrations used during these experiments were representative of the assumed exposure to the toxin in Guam based on the quantification of BMAA in different flying fox samples. However, more research towards the toxicity of BMAA and human exposure to its free, soluble and insoluble bound forms in the environment and foodstuffs should further elucidate the human health risk [276].

*1.2.1.7 Saxitoxin*

Saxitoxin (STX) and its analogues are the last neurotoxins presented here. This carbamate alkaloid (Figure 1.13) was initially identified in clams after bioaccumulation through microalgae consumption [277–279].

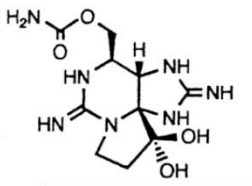



**Figure 1.13.** Saxitoxin [281]

Saxitoxin production has been shown for marine microalgae, like the well-known red tides formers ( *Alexandrium sp.*), as well as different cyanobacterial genera (e.g.: *Anabaena, Dolichospermum*, *Raphidiopsis*, *Planktotrix*, *Lyngbya, Aphanizomenon, Oxynema* and *Cylindrospermum*) [277–287]. The blockage of voltage-gated sodium channels in neural cells by this group of toxins was demonstrated, causing numbness and a tingling sensation in sensory cells [277,288]. However, when the toxins reached the neural motor cells, weakness and paralysis could follow, resulting eventually in death [280,288]. Other studies also found interactions of STX with calcium and potassium channels. Yet, the clinical relevance of these interactions has to be determined [280,289,290]. Toxicity equivalence factors for 19 of the 57 STX analogues were established compared to STX [291,292]. These values were used to accurately assess health risks when STX analogues could be observed in samples [280]. The different analogues are derived from STX, which can be modified at 4 different sidechains in its structure [293]. The unique biosynthesis of STX contains an uncommon Claisen condensation reaction and starts from an arginine [293]. The 26 proteins involved in the

synthesis are part of the 35Kb *stx* gene cluster [294,295]. Moreover, the common ancestry of the *stx* gene cluster in several cyanobacterial genera and even other prokaryotes has been shown as their *stx*I genes, coding for an O-carbamoyltransferase, were homologues. This homology strongly suggested multiple horizontal gene transfer events to different cyanobacterial genera [294].

*1.2.1.8 Other established cyanotoxins*

Some marine cyanobacteria (primarily *Lyngbya sp.*) were shown to also produce dermatotoxins, which could cause erythema and burning of skin covered by bathing suits. Lyngbyatoxin (A-C), debromoaplysiatoxin and aplysiatoxin have been identified [3,296–299]. These toxins were demonstrated to also affect microvilli in the gut if the cyanobacteria were ingested. However, due to their marine nature, we will not discuss them further in this thesis.
Furthermore, like other gram-negative bacteria, lipopolysaccharides (LPS) were identified in the cell wall of cyanobacteria. However, there has been no definitive proof presented that cyanobacterial LPS are a public health risk, like some LPS of heterotrophic bacteria [3].

## 1.2.2. Other important metabolites

Besides the well-studied toxins, other secondary peptides are being produced by the PKS and PKS/NRPS systems. Some of these peptides have already been identified and classified in related compound classes. The biological activity has also been elucidated for some of the compounds.

*1.2.2.1 Linear peptides*

Two related classes of linear peptides have been determined, aeruginosins and microginins (Figure 1.14). Aeruginosins typically contain a hydroxyl-phenyl lactic acid (Hpla) segment at the N-terminus and a 2-carboxy-6-hydroxycoctahydroindole (Choi) fragment at the c-terminus. Additionally, the c-terminus also contained a variable arginine derivative [89,151,300–311]. Different structural variants have been identified [89,97,300,306,308,311]. Some of the variants were even glycosylated, which is uncommon for cyanopetides [309]. Aeruginosins were already detected in cultures from *Microcystis, Planktothrix*, *Nodularia, Nostoc* and *Brasilonema* [97,151,302,303,305,306,308]. Potent thrombin inhibition was demonstrated for these compounds [300,304,307,308]. They also have been observed in environmental samples in Europe and Israel [302,303,307,312–315]. The *aer* gene cluster was shown to be responsible for the synthesis of aeruginosins and seemed to have a common ancestry in *Planktotrix* and *Microcystis*. The presence or absence of genes in the genetic cluster of

different cyanobacterial strains resulted in the production of different variants [309,310].
The linear microginins on the other hand were synthetized from 6 amino acids of which one was their distinctive 3-amino-2-hydroxy decanoic acid (Ahda) segment [316–319]. This peptide class has been identified in *Microcystis, Planktothrix, Anabaena* and *Nostoc* [302,320–322]. Most microginin variants have been established as angiotensin-converting enzyme inhibitors, while another peptide in this group was able to inhibit zink metalloprotease [316,318,319]. Multiple variants were already detected in environmental samples in Europe, Japan and the US [302,307,315,321,322].

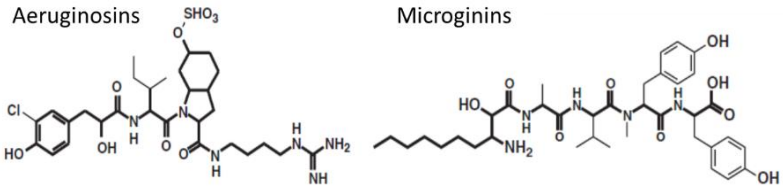



**Figure 1.14.** General structures of aeruginosins and microginins adapted from Welker et al. 2006 [89].

*1.2.2.2 Cyclic peptides*

The other peptide classes consist of cyclic peptides.
Cyanopeptolins were established as cyclic depsipeptides that contained a typical N-formylation and a 3-amino-6-hydroxy-2-piperidone (ApH) fragment (Figure 1.15) [89,306,317,323–329]. These peptides have already been identified in *Anabaena*, *Lyngbya, Microcystis* and *Planktothrix* [89,323,325,326,328,329]. Multiple variants showed protease inhibition activity [325,326]. Some variants inhibited (chymo-)trypsine [328,329]. Cyanopeptolin 1020 in particular was shown to be toxic to crustaceans because of their suggested serine protease inhibition activity [323]. Neurotoxicity in zebrafish was also demonstrated for this cyanopeptolin [324]. Cyanopeptolins have also been identified in multiple environmental samples [306,307,313,317,321,322,327].
The cyclic hexapeptides, anabaenopeptins, have been classified into their own group [89]. These secondary metabolites were identified by their lysine in position 2 while the other peptides varied. This lysine was shown to form the N-peptide ring using an ureido bond (Figure 1.15) [306,312,330–335]. Anabaenopeptins were found in *Anabaena, Planktothrix, Aphanizomenon, Microcystis* and other cyanobacterial taxa [153,302,303,306,312,314,330,330,331,331,332,332–334,334–337]. The gene cluster (*apt)* responsible for anabaenopeptin-production was reported to contain four genes producing multiple NRPS subunits [338]. There were differences within the gene cluster between species. *Anabaena* sp. strain 90 had two *aptA* (1 and 2) genes responsible for the synthesis of different

anabaenopeptins, while this was not the case for other cyanobacterial genera [112,339]. Anabaenopeptins showed the capacity to inhibit proteases, caboxypeptidase A and protein phosphatase 1 [334,340,341]. Anabaenopeptins were also observed and quantified in environmental samples [151,153,314,315,321,322,336,337,340,342–345].

Microviridins were identified by their multi-cyclic structure and were found in *Microcystis*, *Planktothrix* and *Nostoc.* An ester bond between a 4-carboxy group of aspartate and a hydroxyl group of threonine and a peptide bond between lysine and glutamate resulted in this structure (Figure 1.15) [346–353]. The KYPSD core structure was demonstrated to be distinctive for these compounds. Besides the cyclic nature, the sidechains vary from compound to compound [89,336,351,354,355]. The activity of several members of these groups was elucidated. Microviridin J was shown to cause a lethal moulting disruption in *Daphnia*, eventually causing death. This compound was hypnotized to be part of the anti-predation strategy of the cyanobacteria [350,354,356]. Microviridin A and B demonstrated tyrosinase and thrombin inhibition, respectively [352,353]. Generally, the *mdn* gene cluster responsible for the synthesis contained a pre-peptide gene (*mdnA),* two ligase genes, an ABC transport gene and an N-acetyl transferase [354,355,357].

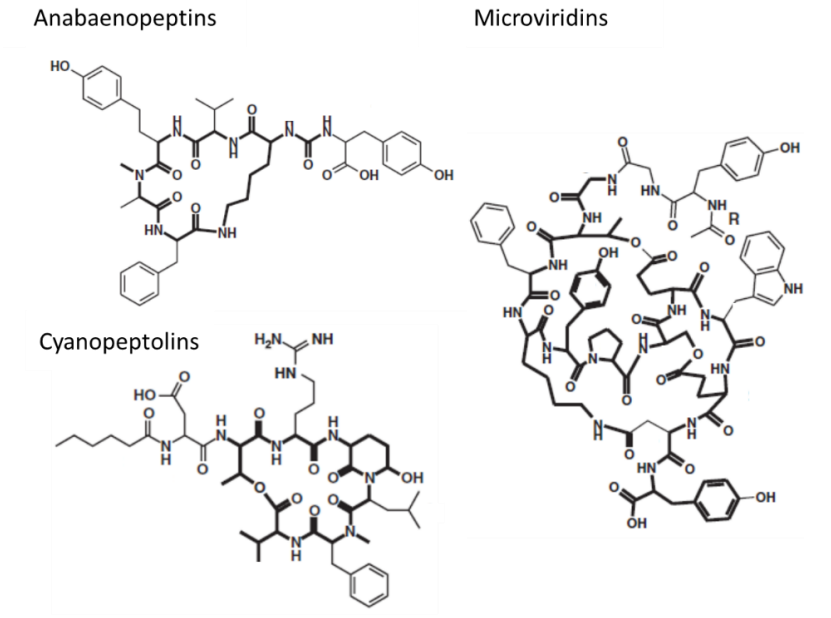



**Figure 1.15.** General structures of anabaenopeptins, microviridins and cyanopeptolins, represented by anabaenopeptin A, microviridin A and cyanopeptolin A consecutively, adapted from Welker et al. 2006 [89]

## 1.3. Methods for cyanotoxin detection

Different analytical methods can be used to detect and quantify cyanotoxin presence in samples from various origins.

### 1.3.1 Molecular methodologies

Molecular methods, like PCR, quantitative (q)PCR and DNA micro-array have already been developed to detect the toxin-producing potential of cyanobacteria. These methods were designed to identify one or more genes from the biosynthetic gene clusters, described in Chapter 1.2, for one or multiple toxins [358–367]. For instance, methods targeting the *mcy*/*nda*, *stx* and *cyr* gene clusters simultaneously have been developed for qPCR and DNA micro-array [358–360]. They could be useful as part of an 'early warning' strategy, due to the high sensitivity of the PCR amplification. Thus, the presence of potential producers could be detected, before the bloom and the cyanotoxin production [368]. Another interesting feature is that the sequences of genes responsible for their production are known for a number of toxins and differ between different taxa. Therefore, a PCR assay could be designed to sequence the amplicon and obtain the identity of the producing taxa. However, these techniques are only able to assess the toxin-production potential and can not directly link the presence of a toxin gene with the available quantity of toxins [369]. Molecular methods should therefore be used in combination with other quantification methods. Moreover, some potential samples, like vegetables and fish, might contain only toxins and (barely) no cyanobacterial DNA to amplify. As a research tool, sequencing approaches could be used to detect unknown, or mutated toxin-producing genes, as illustrated in Chapter 1.2. Moreover, they could help elucidate links between environmental drivers, active cyanotoxin production and the geographical spread of toxic cyanobacterial blooms.

### 1.3.2 Analytical methodologies

#### *1.3.2.1 Enzyme-based assays*

Enzymatic assays were developed for GNT and MCs/NOD, acetylcholine esterase inhibition and phosphatase inhibition assays, respectively [157,241,251]. Inhibition of PP1 was initially used to detect MCs and NOD by measuring the reduced activity of PP1 on *p*-nitrophenol phosphate. Reduced conversion of *p*-nitrophenol phosphate to *p*-nitrophenol resulted in a reduced colometric change [157]. This method was merited as a less expensive quantitative technique that could handle a large number of samples and required limited expertise. Yet, these assays were not specific for different congeners of a toxin and could display false positives or signal enhancing due to the presence of other compounds (e.g. okadaic acid,

calyculin A and tautomycin) in unknown samples that have the same inhibitory activity towards the target enzyme [370,371,374]. Clean-up steps using antibodies or C-18 solid phase extraction (SPE) have been developed to increase the specificity of the method [370,372,409]. Yet, confirmation with other methodologies was usually necessary for the identification of MCs composition and their quantification. Proteins inhibition assays have primarily been applied to water, bloom and urine samples [370–373].

Enzymatic assays were largely replaced with Enzyme-Linked-Immunosorbent-Assays (ELISA). A specific antibody interaction with a specific cyanotoxin was used for identification [157,410]. This mechanism reduced the occurrence of false positives, although they could not be completely excluded, especially interference by degradation products. Specific applications of this technique for MCs will be discussed in Chapters 3, 5, 6 and 7. ELISA's have been developed for all the major freshwater cyanotoxins and are commercially available [157,410–416]. Overall, several ELISAs were developed to detect MCs by targeting the Adda fragment or the variable amino acids in the MCs structure [157,417,418]. Targeting a variable amino acid was demonstrated to strongly influence the specific cross-reactivity of the antibody with different MCs. This approach could cause preferred interaction with certain congeners. For instance, most MCs antibodies have been raised to detect MC-LR, which could result in poor interactions with other MCs [419]. ELISAs targeting the Adda fragment were shown to detect most available MCs in a mixture but could overestimate their concentration due to the presence of MCs degradation products, like the free Adda. A newer approach used a selective mixture of conjugated MCs to raise antibodies that provided a broad specificity. This approach already resulted in a sensitive indirect competitive ELISA assay with well-described cross-reactivity for certain MCs [419].

ELISAs were established as valuable and easy-to-use screening approaches due to their sensitivity and rapid detection of MCs without a clean-up step. They have been implemented as a first screening method on water, serum, animal tissues and seafood [92,368,373–382]. Moreover, the detection of conjugated and protein-bound forms of MCs was also demonstrated. Again, the downside of ELISAs was reported as their inability to distinguish different MCs. Therefore, accurate quantification of MCs mixture could be strongly dependent on the cross-reactivity of the specific antibody to the different MCs, as well as the calibration standard used for quantification. Moreover, sufficient quality controls in complex matrices were shown to be necessary as ELISAs are sensitive towards matrix effects, resulting in signal enhancement [374].

**Table 1.2**. General overview of the major analytical methods for microcystin congeners (MCs) detection and quantification. The matrices for which the methods were validated are mentioned. Some characteristics of the methods are ranked low (+) to high (+++)*.

| Analytical methods | Validated matrices | Detection of separate MCs | Quantification of MCs | Quantification of bound MCs | Cost | Required Expertise | References |
|---|---|---|---|---|---|---|---|
| Phosphatase inhibition assays | Water, blooms, urine samples, algal supplements | + | ++ | +++ | + | + | [370–375] |
| ELISA | water, blooms, serum, algal supplements, animal tissues, plant material, seafood | + | ++ | +++ | + | + | [92,368,373–382] |
| LC-PDA | water, blooms, algal supplements, seafood | ++ | ++ | ++ | + | + | [368,374,375,375,383–386] |
| LC-MS(/MS) | water, blooms, algal supplements, plant material, seafood | +++ | +++ | + | ++ | ++ | [205,373,387–400] |
| LC-MS/MS (MMPB) | algal supplements, animal tissues | + | +++ | +++ | ++ | ++ | [92,128,378,401] |
| MALDI | water, blooms | +++ | + | + | ++ | ++ | [332,402–405] |
| HRMS | Water,blooms, algal supplements, plant material, seafood | +++ | ++ | + | +++ | +++ | [92,94,342,378,406–408] |

*Based on information obtained from the included references

*1.3.2.2 Ultra-High Performance Liquid chromatography based approaches*

Ultra-High Performance Liquid chromatography (UHPLC) (Figures 1.16 and 1.17) or other LC techniques have been used to analyze cyanotoxins. The LC techniques could be used to separate the cyanotoxins before detection. The separation is accomplished by temporarily retaining the toxins on an absorbent matrix contained in a column. Later, a selected solvent releases the compounds from the column based on their physio-chemical properties. Different detection methods, (e.g. Photodiode-Array Detection (PDA), Mass spectrometry (MS), Tandem-Mass spectrometry (MS/MS) and High-Resolution mass spectrometry (HRMS)) could then be used to detect the target toxins.

When using PDA for MCs detection, the absorption between wavelengths of 190 nm and 300 nm, with a maximum of 238 nm, was determined to assess their concentration and purity in samples [161,383,420]. For this setup, MCs were usually separated using a reverse phase C-18 column. Therefore, individual MCs could be identified and quantified based on their elution time and UV absorption spectra if the corresponding analytical standards were available. However, obtaining good resolution between peaks of different MCs during separation could be quite difficult due to small differences between congeners. Moreover, identification and quantification were constrained by the availability of commercial standards. Furthermore, successfully identifying MCs using HPLC required technical expertise, extensive sample cleanup and was time-consuming. This approach is usually more expensive than enzyme-based approaches. LC-PDA detection has been used in water, algal extracts, seafood and plants [327,368,376,383,384,421–423].

Alternatively, MCs could be ionized with MS to identify their mass-to-charge ratio as an indication of their true molecular weight. However, this approach is being replaced by MS/MS approaches. During the latter approach, the ionized toxin was initially selected based on its mass-to-charge ratio (also called precursor ion), degraded by collision with an inert gas in a collision cell and identified based on the produced ions. At least two production ions, also known as daughter ions, should be selected for the analysis. The ion with the highest response should be used as the quantifier ion. The second ion should be used to confirm the identity of the analyte and is also known as the qualifier ion. During most established methods, MCs were first separated using a reverse phase C-18 column. MS/MS could also be used to quantify specific MCs with high selectivity and sensitivity with the inclusion of the appropriate calibration standards [205,216,246,387,392,393,424–426]. Matrix effects could also be more easily taken into account, resulting in the use of this method for MCs detection in diverse, complex matrices (e.g. water, cyanobacterial extracts, plants and seafood) [388,394,398–400,427]. However, implementing these methods could be quite costly and require

experienced personnel. Moreover, complex sample preparation could be necessary before toxin identification and quantification is possible. Reference standards are also needed to identify optimal production ions [373].

**Figure 1.16**. UHPLC-MS/MS setup used for the present research, Waters, Xevo TQ-S

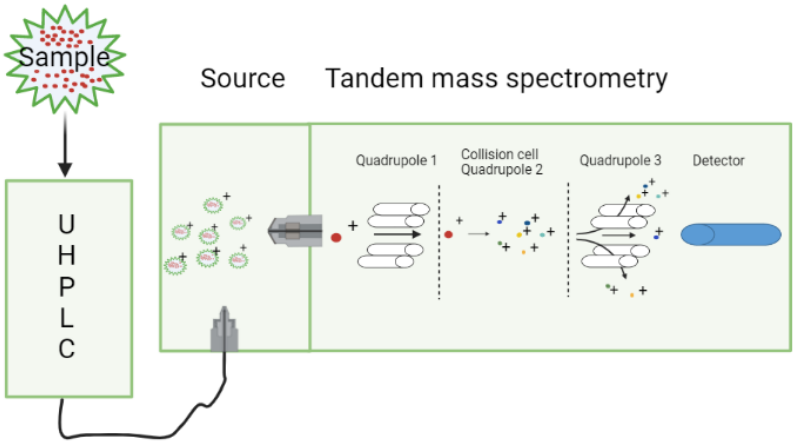



**Figure 1.17.** Identification of a compound using Electrospray ionization Tandem Mass spectrometry. Samples are injected into the UHPLC and ionized in the source. Target compounds are selected in quadrupole 1 based on their mass-to-charge ratio. In the collision cell, the target compound will be fragmented. A fragment will again be selected based on the mass-to-charge ratio for detection. Figure created with BioRender.com

A more sensitive technique to analyze MCs was also developed, namely HRMS. Two techniques have been described, either based on Time-Of-Flight (TOF) or Fourier transformation ion cyclotron resonance (FTICR). Both techniques could identify unknown compounds based on their ion fragmentation pattern, which includes all produced ions after the collision of the precursor ion in a collision cell [92,342,343,407,408,428,429]. These patterns should be compound-specific and could be compared to compound databases. This method has been used to detect unknown and bound MCs in a sample, without the need for reference standards. Moreover, this method was developed to reach high sensitivities with minimal sample preparation. However, HRMS is currently still a quite expensive technique requiring specialized skills to perform data-heavy analysis. Individual quantification of the selection of MCs was already shown in water using HRMS based on a matrix-matched calibration curve [342].

Matrix-assisted laser desorption ionization (MALDI) has been developed as an alternative to the UHPLC. MALDI can be coupled with MS approaches to identify and quantify compounds [403,429]. Samples were immobilized on a cartridge with a matrix. This laser-absorbing matrix was developed to assist the ionization of the compounds without fragmentation by ablation with a laser. Different matrix types were demonstrated for different targeted compounds. For instance, α-cyano-hydroxy cinnamic acid, 2,5-dihydroxy-benzoic acid or pure glycerol were already used as matrix for the detection of MC-LR [402–404].

As discussed earlier, analytical methodologies require commercially available analytical standards to quantify the targeted cyanotoxins. However, in some cases (e.g. MCs) the various identified congeners are not available as analytical standards. If standards are not available, Lemieux oxidation could be used to dissociate the 2-methyl-3-methoxy-4-phenyl butyric acid (MMPB) moiety of the MCs from the complete toxins as a part of the sample preparation [92,401,430]. MMPB corresponds to the Adda tail, shown in Figure 1.3. LC-MS/MS and HRMS were developed to detect MMPB and so quantify the concentration of all the MCs and their metabolites in a sample, similar to ELISA, without the need for a specific congener[92,406]. Moreover, this method was shown to detect bound MCs.

Applications and comparison of the different analysis techniques for MCs in different matrices will be further discussed in Chapters 3, 5, 6, 7 and 10.

### 1.3.3. Method validation

The development of an analytical method for the quantification of a certain toxin, or any compound of interest, is not sufficient to apply this method to unknown samples. Validation of the method has to be performed to prove that it is fit-for-purpose. The goal is to show that the method can measure concentrations of the selected analytes close enough to the true value of

these analytes in an unknown sample. This validation should follow a certain approach and show that defined parameters meet specific acceptation criteria. Depending on the purpose of the validation (scientific and/or regulative), different guidelines (e.g. 2002/657/EC or FDA guidelines) are available that utilize different validation approaches and criteria [431–435]. During this subchapter, the approach and parameters needed to validate an UHPLC-MS/MS methods, which were selected to perform the validations in this manuscript, will be discussed. The validations described in Chapters 3, 4, 6 and 7 are based on the 2002/657/EC [431].

*1.3.3.1. Validation approaches*

During the validation of a method, there is a need for a control and verification of the process. To realise this, a set of controls for either the presence or absence of a compound needs to be defined and used.
Blank matrix represents the analyzed matrix without the presence of the selected analytes. This can either be a blank or a solvent samples. These samples are necessary to estimate the interference of the sample matrix on the measurements. Therefore, it is always better to use exactly the same matrix as the real samples. If the identical matrix without a contamination with the analytes of interested is unavailable, a similar matrix should be used.
On the other hand, fortified samples, also referred to as spiked samples in the rest of the manuscript, are blank matrix samples where a known concentration of the selected analytes is added. These samples should be analyzed during the validation to assess the ability to accurately quantify the analytes. Alternatively, certified reference materials (CRMs) could be used for the same purpose. CRMs are samples of the target matrix that already contain the selected analytes and are accompanied by a certificate specifying the quantity of these analytes [431,432,435]. As an example, a wide range of CRMs can be obtained from the National Research Council Canada.
As discussed in the previous chapter, a (matrix-matched) calibration curve is necessary to quantify analytes with an analytical method. Therefore, these samples should also be included during the validation.
Overall, at least 3 blank samples, 27 spiked samples and two or three calibration curves are used during a validation [434]. Spiked samples should be fortified at three different concentrations ( n=9 for each concentration level). Determining the concentration levels depends on the guidelines that were followed and the information available about the analytes. If a maximum allowed residue level (MRL) was available in legislation, 0.5 MRL, 1 MRL and 1.5 MRL could be used as the different concentration levels. However, if no such value was available, the minimum required performance limit (MRLP) should be established and used as the lowest concentration level with the addition of two higher concentration levels [431,433].

The validation should be performed over two or three days, preferably by different (trained) analysts, to assess the applicability of the method during routine analysis over time. During each day, a calibration curve, one blank sample and three spiked samples at each of the three concentration levels should be analyzed. Based on the obtained data, the validity of the method could be checked based on certain parameters that will be discussed in the next subchapters [431,432,435].

*1.3.3.2. Validation parameters*

The parameters that should be checked during validation are specificity, selectivity, accuracy, trueness, precision, repeatability, reproducibility, measurement uncertainty, linearity, the limit of detection, the limit of quantification and apparent recovery.

1.3.3.2.1. Specificity and selectivity

The specificity of a method is its ability to distinguish the selected analytes from other substances that might be present in the matrix. The selectivity of the method should be demonstrated by showing that the matrix does not interfere with the measurement of the analytes. Ideally, only the targeted analytes were measured, without interference of any substances from the matrix (or despite the presence of interfering substances in the matrix). Blank samples should be analyzed to show that the obtained responses in these samples are 20% less than the response obtained in spikes samples at the lowest concentration level. Additionally, the retention time should be stable throughout the analysis and not shift more than +/- 2.5% for individual samples, compared to a quality control (QC), used as a reference. Finally, the ion ratio of the analyzed samples during validation should be assessed. The variation of the peak intensity for the qualifier ion (Chapter 1.3.2.2.) should stay within a certain percentage determined by the relative intensity for the qualifier peak compared to the quantifier peak. For routine analysis, the ion ratio should only differ up to +/- 30% of the QC [431].

1.3.3.2.2. Accuracy, trueness, precision, recovery, repeatability and reproducibility

The accuracy of the method is determined by its trueness and precision. However, the trueness of a method can only be evaluated when analysing CRMs. The trueness represents the bias of the measured concentration compared to the true value or accepted reference value of the CRM. The bias is represented as a percentage. If there was no CRM available to perform the validation, spiked samples should be used. In this case, the apparent recovery is calculated instead. The recovery is also represented by a percentage and calculated by dividing the measured concentration by the theoretically spiked concentration. Ideally, this parameter should range between 70 to

120%. However, this range is dependent on the order of magnitude of the mass fraction and used guidelines.
Precision is defined as the standard deviation or coefficient of variation between independent test results. It should be determined by quantifying 3 spiked samples for each of the three different concentration levels during validation [431,432,435].
Besides precision, repeatability and reproducibility also have to be determined to assess variation within the measured results obtained over short and long time intervals. The repeatability should be assessed by calculating the mean, the standard deviation and the coefficient of variance for each test day on each concentration level. Reproducibility also assesses different conditions (such as a different operator, equipment, measuring system, location or laboratory), besides the length of time. Therefore, the mean, the standard deviation and the coefficient of variance are determined for all days and concentration levels combined. The Horwitz equation should be used to determine if the repeatability and reproducibility are sufficient. However, the Horwitz equation provides unacceptably high CV values at mass fractions lower than 100 µg $kg^{-1}$. Therefore, a fitness-for-purpose approach could define alternative performance criteria in advance [431,432,435].

1.3.3.2.3. Measurement uncertainty

Furthermore, the measurement uncertainty (MU) provides a value that shows the range wherein a measured concentration can reasonably differ compared to the actual concentration. This value should be calculated by estimating the errors that can occur in different analysis steps, like sample preparation, extraction, calibration curves, etc.
However, the calculation of the MU could be accomplished by other approaches. A common approach uses precision and trueness to assess the total MU. Moreover, when spiked blank material was used, reproducibility can be used as a good estimate for the MU. Usually, acceptation criteria are defined with the specific analytes in mind [431,432,435].

1.3.3.2.4. Linearity

The linearity of an analytical method shows the direct correlation between the signal obtained from the analytes and their concentration within a certain range, usually the range of the calibration curve. For this assessment, the different points of the calibration curve could be injected repeatedly on, preferably, multiple days. The most common approach is to then define the regression parameters (slope and intercept) of the linear regression and use the $R^2$ to evaluate the goodness-of-fit. $R^2$ should be higher than 0.99 [431,432,435]. Yet, other tests should be done to check if a linear regression was truly the best fit. Approaches like Mandel's fitting test could be applied, where a linear fit is compared to a quadratic fit [436].

1.3.3.2.5. Limit of detection and limit of quantification

The limit of detection (LOD) and limit of quantification (LOQ) define the lowest concentration for which an analyte can be detected and quantified, respectively. These values could be theoretically estimated by calculating the standard deviation of 10 measurements for analytes in blank samples or spiked samples with low concentrations of the analytes. The standard deviation should then be multiplied by 3 and 10 for the LOD and LOQ, respectively. However, calculated LOD/LOQ could vary over time due to differences in matrix interference and cleanliness of the instruments. An alternative approach is to consider the lowest validated concentration level as LOQ. Moreover, the decision limit (CCα) and detection capability (CCβ) could also be used as alternative parameters for the LOQ and LOD. CCα provides the concentration level at which the response of a blank sample is the same or higher as a contaminated sample with a probability α and thus provides a false positive. CCβ establishes a concentration level for which a false negative result will be reported with a probability β, thus the concentration level where there is a probability β that the response will be lower than CCα. Probability α and β are regularly defined as 5% [431,432,435].

Overall, the discussed parameters are important to show the applicability of a developed method. In our case, our methods were validated using the 2002/657/EC guidelines [431]. A new version of these guidelines was published in 2021 after our validations were already completed [433]. The new document described concentration levels at which the method can be validated (e.g. reference point of action or MRL) more in detail, as well as new, concentration-dependent, acceptable coefficients of variation for reproducibility and a method to calculate relative matrix effect.

# 1.4. Origin, monitoring and remediation of cyanobacteria blooms and cyanotoxins

## 1.4.1. Eutrophication and environmental conditions affecting cyanobacterial growth and cyanotoxin production

### *1.4.1.1 Inorganic environmental drivers*

Environmental drivers, like temperature, nitrogen (N) and phosphorus (P) abundance, and inorganic carbon availability could determine when a cyanobacteria bloom occurred. Moreover, these drivers also helped determine, which potentially toxic species dominated a bloom.

N and P could be introduced into the environment from point sources or more general human activities like agriculture, fossil fuel combustion, animal feeding operations and sewage treatment [437]. A major source of N was shown to be the increased use of urea fertilizers that can run off in

watersheds of groundwater. Another source, Dissolved Organic Nitrogen (DON), was demonstrated to originate up to 40% from rain and could also increase bloom productivity. Rain was also shown as a potential source of iron (Fe), which was established as an important micronutrient for cyanobacteria. P enrichment has also been shown to increase chlorophyll concentration in waterbodies. Moreover, nutrient-enriched groundwater could slowly leak into surface water potentially stimulating blooms [438–440]. Phosphorous enrichment was also suggested to provide cyanobacteria with an advantage compared to other organisms in the phytoplankton community [441]. In general, P has been seen as the limiting factor for cyanobacteria growth as a number of cyanobacterial species are capable of N fixation, as explained in subchapter 1.1 [442,443]. However, when P was in sufficient abundance, nitrogen became the determining factor for the increased growth of the cyanobacteria [444,445]. Increasing dissolved organic carbon also had a positive effect on cyanobacteria blooms.

*1.4.1.2 Climate change*

Climate change, including global warming and an increase in extreme weather events, also had a potential impact on the prevalence of cyanobacterial blooms. Global warming resulting in increasing water temperatures was suggested to decrease the growth rate of eukaryotic phytoplankton and increase the growth of cyanobacteria, providing the latter with an advantage. Moreover, the viscosity of the water would also decrease, allowing more nutrients to reach the cell wall. Additionally, faster sedimentation of larger cells was demonstrated at higher temperatures [442]. Therefore, cyanobacteria were hypnotized to gain an additional advantage through their buoyancy. Longer periods of stratification of the water in the waterbodies were also suggested to occur, prohibiting nutrients to reach the water's surface. As discussed earlier, some cyanobacteria can be motile through the use of gas vesicles and were shown to gain an advantage in stratified waterbodies. Moreover, sedimentation of cyanobacteria to collect nutrients has been reported, whereafter the cells rose back to the surface for photosynthesis [14–18]. Increasing salinity could also contribute to cyanobacteria dominance in brackish and fresh waterbodies as cyanobacteria (like *Microcystis* and *Nodularia*) were shown to be more resistant than eukaryotic phytoplankton [441]. An increase in cyanobacterial blooms due to climate change was suggested to create a perpetual loop as greenhouse gasses were produced ($CO_2$ and $CH_4$) during bloom decomposition [446]. At last, increasing $CO_2$ concentrations in the air were proposed to be reflected in the increase of dissolved organic and inorganic carbon in waterbodies and a subsequent drop in pH. However, climate change could have a positive effect on cyanotoxin exposure as a shift from cyanotoxin-producers to non-cyanotoxin producers of the same species was

observed when inorganic carbon increases in a waterbody [447]. Yet, the dominance between producer and non-producer was difficult to predict as it was also shown that non-producers preferred low N and P concentrations, while toxin producers preferred higher nutrient concentrations [445,448]. It was further observed that under P-limiting conditions, more N-containing toxins were produced in most cyanobacteria species and strains (e.g. *Microcystis*), while under N-limiting conditions microcystins were produced in three *Microcystis* strains and a *Raphidiopsis raciborskii*. Effects of N limitation on saxitoxin production were also strain-dependent for the dinoflagellates, *Alexandrium* sp. and *Gymnodinium catenatum,* while there was no effect on cylindrospermopsin production in *Raphidiopsis raciborskii*. However, the latter seemed logical as cylindrospermopsin producers could usually also fix nitrogen [449].
Sequential occurrences of extreme weather events, like extreme rainfall and flooding followed by draughts, were also suggested to become more regular. These events have been shown to result in nutrient enrichment in waterbodies that could promote bloom formation [450].

*1.4.1.3 Environmental drivers in Belgium*

Environmental conditions that seemed to stimulate the growth of specific species were observed through the monitoring and modelling of five lakes in Belgium during the B-BLOOMS2 project [368]. When taking into account data for all lakes, total P, depth of the euphotic zone, epilimnion temperature, low dissolved organic N contributed most to the development of blooms. Interestingly, pH was excluded from the models, as an increase was observed when blooms developed. For Lake Falemprise, a more in-depth analysis was made. In this lake, *Aphanizomenon* was abundant when DIN < 1.5 mg $L^{-1}$ and total P > 0.030 mg $L^{-1}$. The abundance of P and the photoperiod contributed the most to a bloom event. The P abundance (>0.07 mg $L^{-1}$) also was the most important factor for *Planktothrix* dominance. A switch between these two species could be caused by the mixing of the sediment through the wind. For *Microcystis* blooms, beneficial environmental factors were low transparency, high total P concentration (>0.5 mg $L^{-1}$) and some rainfall in the period before the bloom [451]. Similar results were found for *Microcystis, Aphanizomenon* and *Planktorix* sp. when combing data for the four remaining lakes, which were more eutrophic and shallow. Moreover, *Anabaena (*now *Dolichospermum)* was predicted to thrive under relatively low total P conditions compared to other blooms, with low dissolved inorganic nitrogen, long photoperiods and a high epilimnion temperature [368].

## 1.4.2. Prevalence of cyanobacteria worldwide and their characteristics

Different environments worldwide were shown to provide opportunities for cyanobacterial growth, as they only need light, water, air and a few minerals to grow. More extreme environments, like cold and hot deserts, generally supported microbial mats or crusts containing cyanobacteria [62,94,251,452–456]. Generally, species like *Nostoc* sp., *Synechococcus* sp., *Leptolyngbya* sp., *Phormidium* sp. and *Microcoleus* sp., were reported, depending on the environment [62,94,453–456]. A limited amount of studies were already able to determine cyanotoxin presence in these environments [94,251,452].

Benthic cyanobacterial mats have also been described worldwide [178,209,214,218,228,229,232,457–462]. *Phormidium* sp., *Anabaena* sp., *Lyngbya* sp. and *Tychonema* sp. were identified in these mats. Toxins commonly found in the mats were ATX and CYN.

However, most reports deal with cyanobacterial blooms, due to their impacts [463]. The species diversity in these blooms covers many known cyanobacteria genera, which were earlier described in subchapter 1.1.2. Versions of all the cyanotoxin-types already described in subchapter 1.1.3 could be observed worldwide. Svirčev et al. published a comprehensive review of toxic cyanobacteria blooms in 2019 [463].

## 1.4.3. Prevalence of cyanotoxins in foodstuffs

Cyanotoxins, and especially MCs, have been found in multiple foodstuffs. The prevalence of cyanotoxins in *Chlorella*- and cyano-based food supplements have already been observed in the literature and will be further discussed in Chapter 6. The accumulation of MCs and CYN in crops have also been described by other authors and will be presented in detail in Chapter 7.

Accumulation of different cyanotoxins has already been shown in different freshwater organisms, used as food. Earlier research targeted MCs accumulation in fish [381,384,385,397,464–468] and shellfish [88,377,395,469]. MCs were found to accumulate in fish intestines, liver, muscles, kidneys and gills [381,384,385,396,397]. For noble crayfish, most MCs were found in the liver, intestines and stomach, with lower concentrations in the muscles [377]. In noble crayfish, MCs concentrations did not reach values above the tolerable daily intake (TDI) for MCs (0.04 µg $kg^{-1}_{bodyweight}$) taking into account the consumption of a normal boiled portion. Removing the intestines could be a preventive measure to reduce exposure to MCs [122,377]. Concentrations of MC-LR and MC-RR above the TDI were also reported in fish [396]. To evaluate the risk for humans, guideline values should be established. They indicate safe consumption of substances regarding current safety data, uncertainties in these data, and the likely

duration of consumption. A universal way to calculate guideline values for MCs has not been not yet harmonized. However, a health guideline value of 39 µg $kg^{-1}_{whole\ organism}$ was already proposed for fish and prawns [470]. The National Nutrition survey of Australia was used to assess 95$^{th}$ percentile consumption of fish and prawns [470,471]. Currently, detection and quantification in fish and shellfish tissues were achieved using UHPLC-MS(/MS), HRMS or ELISA approaches [88,377,381,384,385,395,397,464–469].

### 1.4.4. Monitoring techniques for cyanobacterial blooms and guidelines

Monitoring of cyanobacteria blooms usually occurs in several stages. Different parameters have been used to detect (toxic) cyanobacteria blooms in a waterbody [3].

For initial regular monitoring, water discoloration and transparency have been used. For most blooms, a color green or red was observed when a bloom was present [3]. The transparency of the waterbody below one meter could also indicate a bloom. Transparency was commonly measured with a Secchi disc and has been expressed as Secchi depth [3,472].

Once a bloom was observed, its severity should be determined. Morphological identification of the cyanobacterial species was used to determine its potential for toxin production. This method could provide results fast but requires well-trained analysts to correctly identify cyanobacterial species [3,472].

Cell counting was equally used as a measure of potential public health risk. The WHO suggested informing the water users of a potential risk from a threshold of 20000 cells $mL^{-1}$. From 100000 cells $mL^{-1}$, the waterbody should be further investigated and the water usage should be restricted [3,472,473]. Similar values were used as response thresholds in multiple countries (for example, Australia, Canada, Cuba, Czech republic) [473]. Visible scums warranted further investigations and possible restrictions in most countries.

The chlorophyll-*a* concentrations could be used similarly as cell-counting with 20 µg $L^{-1}$ and 50 µg $L^{-1}$ corresponding to the 20000 and 100000 cells $mL^{-1}$ suggested by the WHO, respectively [3,473].

Species identification using molecular tools has been more commonly used. Several sequencing methods were developed. Although these methods are very accurate, they are costly, require highly skilled analysts and usually take more processing time to report the final results [11,25,58–63]. Closing recreational waterbodies for an unnecessary amount of time due to long reporting times could cause avoidable economic losses [3]. (q)PCR's could also be used to detect the toxin-producing genes, as described earlier, to identify a potentially toxic bloom. These techniques also require a skilled analyst but are less expensive [63,358–360,362–367].

Toxin analysis has been another valuable and diverse tool to assess the potential health risks of a bloom. The immediate identification and quantification of the toxins provided a good measure of the risk to public health. Multiple techniques could be implemented to determine toxins. These techniques will be further discussed in Chapters 3, 5, 6 and 7. The reported downside of direct toxin analysis was that accurate quantification usually requires different methods for different toxin types. Moreover, some techniques required skilled analysts and were expensive. The sensitivity of toxin detection was also demonstrated to be highly dependent on the technique that was used.
A reliable health risk assessment when a bloom occurs, requires a correct sampling of the bloom. The type of sample collected during monitoring largely determines the information derived from the monitoring techniques and should fit the sampling goal. Two main sample types were commonly used in the past. Aggregated samples combined samples from different water layers to reflect a complete overview of the waterbody [3]. These samples provided a total image of the cyanobacteria population and an average concentration of the toxins, nutrients and so on. These kinds of samples were most used during monitoring programs that aimed to study or remediate the waterbodies.
Point samples on the other hand should be taken from a single point in the waterbody (e.g.: a point for water intake, a single point in a bathing area, or a position where scum or benthic mats are accumulating) [3]. These samples were commonly used to monitor waterbodies for potential public health risks and primarily reflect the maximum possible exposure to toxins that could occur at that time in the sampled waterbody. The goal of the sampling and type of sample is therefore important to take into account when developing a monitoring program.

## 1.4.5. Bloom mediation in surface waters

Different techniques have been proposed to remove cyanobacteria biomass during blooms or prevent blooms from occurring.
As earlier discussed, the presence of phosphorus in the water was suggested as a main driver of the blooms. Intermediate solutions, using mechanical, (biological) or chemical methods, should always be combined with a long-term plan to reduce nutrient content in the waterbody for an enduring effect, as was suggested in the new European water directive (Dec, 2020) [474].

### *1.4.5.1 Mechanical bloom mediation*

Bubble screens, surface aerators, oil screens, fountains, dredging and excavation were proposed as different mechanical methods to mediate blooms in eutrophic waterbodies. However, these techniques were only

applicable to certain waterbodies and have been implemented with variable success [475].

*1.4.5.2 Chemical bloom mediation*

Chemical solutions were believed to be more reliable to control blooms. Herbicides, like endothall, copper and potassium chloride, have been used in the past. These products were reported to be cheap, effective and easy to use but have the major downside of also targeting other organisms in the waterbody. Moreover, the risk of further accumulation in the food chain made these products unfit to treat water sources used for irrigation or animal and human consumption [476].
Bloom inhibition by barley straw or the application of hydrogen peroxide has been discussed as more biological solutions. The algistatic effect by inhibitory compounds of decomposing straw was shown for *C. glomerata, Microcystis, Anabaena flos-aquae, Oscillatoria ridoken, Synechococcus sp.* and *Aphanizomenon flos-aquae* [477–481]. Two cyanophyta, *Anabaena cylindrica* and *Aphanizomenon flos-aquae* were resistant to the effects of barley straw [480]. However, the overall effects were suggested to be most pronounced in smaller lakes.
The application of hydrogen peroxide (HP) to disrupt cyanobacteria blooms has been well established although not widely applied. Reduction in cyanobacteria biomass by HP was seen by Barroin and Feuillade [482]. The photosynthesis system of cyanobacteria was shown to be more inhibited compared to green algae. Due to this specificity, HP treatments could be implemented with minimal toxicity for other organisms in the ecosystem, compared to other chemical treatments. Moreover, HP was shown to rapidly degrade in the waterbodies compared to copper, for example, precipitated and might cause risks for sediment-dwelling organisms over time [476]. Application of HP in lakes showed successful suppression of cyanobacterial growth (like, *Planktothrix agardhii* (previously *Oscillatoria agardhii*)) and allowed green algae and diatoms to become dominant [483–485]. The HP treatment was more effective on *Planktothrix* compared to *Microcystis*, while *Cylindrospermopsis* was even less susceptible. However, these study also suggested that high HP concentrations could be damaging to all aquatic life [486]. Lab experiments also demonstrated that HP application on *Microcystis* cultures was more effective at higher light intensity in the orange spectrum (compared to the blue) [487]. HP was shown to also assist in the faster removal of MC-LR from the water when combined with UV-radiation [488].
Removal of MCs is important if the water is designated for direct usage as disruption of the cyanobacteria cells will cause a fast release of these toxins in the water. In larger waterbodies, this can be mitigated by dilution of the toxins.

*1.4.5.3 Bloom precipitation as a mediation strategy*

Another investigated techniques to remove the bloom from the water column without damaging the cells were the Flock- Lock and Flock-Sink principles. Flocculants like poly-aluminum chloride (PAC) or $FeCl_3$ were shown to aggregate the cyanobacteria and phosphate (Flock) [489–493]. The flocculants could be combined with a ballast ( e.g. gravel, bauxite or local sediment) to settle the flocks on the bottom. Alternatively, Lanthanum-modified bentonite (Phoslock®) helped the flocks settle as well but also ed the phosphorous on the bottom of the lake. The combined application of PAC and Phoslock showed the sedimentation of *Aphanizomenon flos-aquae* and the reduction of total phosphorous, whereas the separate application was not effective [489,490]. However, the sediment gradually released phosphorous over time [490]. Both Flock-Sink and Flock-Lock treatments prevented damage to the cell membrane and thus prevented the release of cyanotoxins [491]. When using one of these systems, care had to be taken that the treatment was optimized for the specific lake. For instance, two *Planktothrix rubescens* species from different sources responded differently to a Flock-Lock treatment [493].

## 1.4.6. Cyanobacterial blooms and drinking water exploitation

Surface waters are a prominent source of drinking water. However, they have been shown to be vulnerable to cyanobacterial blooms and the toxins that may accompany them. During a bloom, multiple strategies were developed to ensure water availability. An alternate water source could be used if available, which is also the most straightforward solution. In some cases, water intake could be moved to a higher depth in the waterbody, excluding the cyanobacterial cells and some of the toxins if they were primarily intracellular. Most blooms were found to be situated at the surface or a couple of meters below the surface. Another option has been the treatment of the water after exploitation [494]. Multiple techniques could be used and combined to remove and inactivate cyanobacteria cells and their toxins, but they are costly.

Flocculation and sedimentation of the cyanobacteria cells were utilized as one of these techniques. As discussed in the previous chapter, the flocculation of cells could be accomplished by adding aluminium sulphate or ferric chloride to the water. Additional powdered or granulated active carbon (PAC and GAC, respectively) could be added. Then, a filtration step, like slow sand filtration, removed the cells [203,495]. PAC was already used for toxin removal through hydrophobic and electrostatic reactions of the cyanotoxins with the activated carbon. The efficiency of the toxin retention depended on the pore size of the PAC [496–498]. However, removal was also dependent on the different MCs, as MC-RR was more easily adsorbed compared to MC-

YR, MC-LR and MC-LA in descending order [499]. Water characteristics like the natural organic matter (NOM), turbidity and background electrolytes influenced the effectiveness of PAC as well [500–503]. The PAC removal of STX was also shown [504]. Reverse osmosis and nanofiltration were also effective in removing dissolved toxins, while ultra- and microfiltration were effective in removing cyanobacteria cells [501].

Instead of separating the toxins from the drinking water, they could also be destroyed and the cyanobacteria cells deactivated using disinfectants. However, most of these techniques were shown to also (partially) destroy the cells and increase the concentration of dissolved cyanotoxins. Chlorine has commonly been used to degrade unwanted chemicals and toxins in water and inactivated cyanobacteria cells. However, the use of chlorine warrants additional caution because the degradation products were suggested to form toxic disinfection products [505]. Cyanobacterial cell inactivation was also dependent on species. *Cylindrospermopsis rasciborskii* and *Aphanizomenon issatschenkoi* (now *Cuspidothrix issatchenkoi*) were shown to be more susceptible compared to *Anabaena circinalis* and *Microcystis aeruginosa* [495,506]. Permanganate was proposed as an alternative to chlorine that would inactivate the cyanobacterial cell without the release of toxins and could degrade MCs and ATX effectively. Ferrate on the other hand was demonstrated to inactivate the cyanobacteria and flocculate the cells while degrading MCs [495]. Moreover, advanced oxidation processes were investigated to degrade most cyanotoxins. Furthermore, ultrasound was shown to be able to degrade MC-RR, lysis and deactivate cyanobacteria cells [507–509]. UV exposure was also revealed to degrade MCs and inactivate cyanobacteria cells. However, this deactivation was not permanent [495]. Equally, HP also has high reactivity with cyanotoxins. The combination of HP with UV radiation was very effective at degrading MCs [488]. Ozone was also regularly used to inactivate cells and degrade cyanotoxins [510–512]. Several bacterial species from the phyla *Proteobacteria* and *Actinobacteria* were shown to degrade MCs [495]. Ren at al. combined the active carbon approach with the microcystin-degrading *Sphinopyxi*s to degrade MCs [513].

In the new drinking water guideline published by the WHO, the effectiveness of PAC and GAC was stressed for the removal of cell-bound cyanotoxins. Chlorination and ozonation were described as effective tools to degrade dissolved STX, MCs and CYN, while only ozone degradation was recommended for ATX [122,163,280,514].

# 2. Goals and research questions

## 2.1. Research questions

A large-scale study (BelSPO project B-BLOOMS2) from 2007 to 2010 [368,515] in Belgium revealed that 89% and 83% of the 162 samples tested showed the presence of *mcyE* and *mcyA* genes, respectively. MCs were quantified in 40% of the bloom samples using HPLC, while ATX and STX were never detected. Furthermore, *Microcystis*, *Anabaena* (now taxonomically identified as *Dolichospermum*), *Aphanizomenon*, *Planktothrix, and Woronichinia* were the primary bloom-forming cyanobacterial taxa. Based largely on the B-BLOOMS2 report, the public authorities started to take action by informing the citizens and including cyanobacterial blooms in monitoring studies. Presently, Flemish and Walloon environmental agencies perform limited monitoring of recreational ponds (where bathing or other activities involving water contact are allowed) but using different protocols.
Nevertheless, further research regarding cyanobacterial toxins and food safety has not happened after B-BLOOMS2 in Belgium. Our literature search even showed a knowledge gap about the prevalence of cyanobacterial toxins in Belgium.
Therefore, multiple research questions (Q) were developed based on the B-BLOOMS2 data. No analytical methods for cyanotoxins were available in Belgium at the start of the project. As MCs were shown to be most prevalent during the previous study, method development was focused on this toxin group.

- Q1: Is the Belgium population exposed to MCs via drinking water?
- Q2: Are toxic cyanobacterial blooms prevalent in Belgium waterbodies, in which human contact is expected, and can these blooms be characterized?
- Q3: Is the Belgium population exposed to MCs via algal-based food supplements and foodstuffs?
- Q4: Can the general exposure of the Belgian population to MCs, and its associated risk, be quantified?

## 2.2. Goals

Overall, the application of validated UHPLC-MS/MS methodologies should accomplish the first assessment of MCs prevalence in multiple exposure routes for a complete population (e.g. Belgium). Moreover, these data from different exposure sources should suffice to obtain an unprecedented, preliminary aggregated exposure and risk assessment for MCs. Furthermore, the method design should permit the use of the methods in later research,

as tools in a toolbox. Therefore, drinking water (Q1), cyanobacterial blooms (Q2) and algal-based food supplements (Q3) were selected as primary matrices to determine their MCs content by the end of the thesis. Moreover, state-of-the-art analytical methodologies to quantify MCs had to be developed, validated and applied for drinking water, (Chapter 3), cyanobacterial biomass (Chapter 4 and 5) and algal-based food supplements (Chapter 6). Moreover, molecular methods were to be evaluated as a complement to the analytical approaches for cyanobacteria biomass (Q3) and algal-based supplements. PCRs were applied to detect genes responsible for MCs production in both matrices. To better understand cyanobacterial blooms in Belgium, the extracted DNA was further used to characterize the cyanobacterial diversity present.

Toxic cyanobacterial blooms were thus again characterized in Belgium for the first time in more than a decade. Therefore, the accumulation of MCs in other foods via (irrigation) water became a feasible research option. To further investigate this possibility, the analytical toolbox was extended to quantify MCs in fruits and vegetables, as a foodstuff. For the first time in Belgium, this analytical method was applied to samples from the Belgian market (Chapter 7) to assess MCs prevalence, specifically including the first described assessment of MCs strawberries, chicory and potatoes. Other foodstuffs likely to contain cyanotoxins, like fish, shellfish and crustaceans, were not incorporated in the thesis. Factors like funding and timing played a role in this decision. However, exposure of the Belgium population to MCs through these kinds of foodstuffs was considered unlikely as reports confirmed that mostly marine fish, shellfish and crustaceans have been consumed in Belgium instead of the freshwater varieties [516]. Therefore, it would be unlikely that human exposure would occur through these products.

As a last part of the toolbox, a first-time exposure and risk assessment of MCs for the Belgian population (Q4, Chapter 9) was performed using the obtained data for the different matrices. However, a lack of consumption data for algal-based supplements in Belgium and Europe prevented a proper exposure and risk assessment. To obtain this original data, a consumption survey had to be composed, distributed and analyzed for these products (Chapter 8).

The exposure assessment of the individual exposure sources and consumption data could be combined, resulting in an aggregated exposure for MCs in Belgium for adults and children (Q4, Chapter 10).

Finally, the toolbox encompassed the combination of analytical and molecular methodologies with the risk assessment. As such it may be used to further investigate the public health risks associated with MCs and toxic cyanobacterial blooms in the future, or used as a template to extend research towards other cyanotoxins.

## 2.3. Thesis road map

In accordance with our goals, we developed a quantitative method to analyze microcystin congeners in drinking water. The validation and application of the method will be discussed in detail in Chapter 3. The chapter has already been published in Water-MDPI (DOI: https://doi.org/10.3390/w14081195) and is adapted for this thesis.
Not only drinking water can cause exposure to MCs. Recreational use of waterbodies can also result in exposure when a bloom is present. In Chapter 4, the validation for MCs quantification in cyanobacterial biomass will be presented. Moreover, the results from our own sampling and investigation of Belgian bloom samples will be presented in Chapter 5. Both analytical and molecular approaches were used for this chapter. This chapter was published in Toxins-MDPI (DOI: https://doi.org/10.3390/toxins14010061) and adapted for the thesis.
Chapter 6 will discuss the prevalence of MCs in algal-based food supplements using analytical and molecular methods. This chapter was already published in Toxins-MDPI (DIO:https://doi.org/10.3390/toxins14080513) and was adapted for the thesis. Moreover, an analytical method is used in Chapter 7 to determine MCs prevalence in fruits and vegetables. This chapter was already published in Separations-MDPI (DOI: 10.3390/separations9100319) and was adapted for the thesis.
Chapter 8 will discuss the additional consumption survey for algal-based food supplements that was needed to calculate exposure and risk assessment for MCs to the Belgian population. Chapter 9 will show an exposure and risk assessment for which all the data from the previous chapters were used.
The general structure of the thesis is illustrated in Figure 2.1.

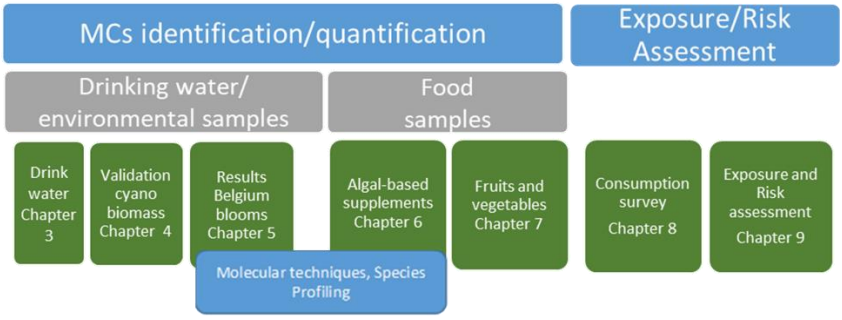



**Figure 2.1.** Thesis roadmap

# 3. Development, Validation and Application of a Targeted LC-MS Method for Quantification of Microcystins and Nodularin: Towards a Better Characterization of Drinking Water

**Contributing authors:** *Wannes Hugo R. Van Hassel* performed the optimization of analytical methodology, prepared the samples, performed the mass spectrometry, analyzed the data and wrote the chapter; *Bart Huybrechts*, *Julien Masquelier* and *Mirjana Andjelkovic*. supervised the study and were involved in critical analysis of the data, manuscript corrections and discussion; *Annick Wilmotte* supervised the study and was involved in critical analysis of the data, manuscript corrections and discussion.
This chapter has already been published in Water edited by MDPI (DOI: https://doi.org/10.3390/w14081195) and is adapted for this thesis.

**Abstract:** Cyanotoxins can be produced in surface waters by cyanobacterial blooms, mostly during summer and early autumn. Intoxications would result from consumption of water contaminated with the potent hepatotoxins, microcystins and nodularin. Therefore, the WHO has set a guideline value for drinking water quality concerning one congener of microcystin. Consequently, the design of a validated, public method to detect and quantify the hepatotoxins in drinking water is necessary. During this study, a method was developed to quantify cyanotoxins (eight microcystin congeners and nodularin) in water using liquid chromatography coupled with tandem mass spectrometry. Additionally, bottled and tap water samples were tested for the presence of cyanotoxins. No cyanotoxins were detected in any of the collected water samples. However, quality controls and the results of a proficiency test show the validity of the method.

## 3.1. Introduction

Water availability and quality are two crucial factors contributing to a healthy and well-functioning society. However, due to climate change and pollution, access to safe freshwater sources diminishes. Increasing salinization, increasing sea levels and the presence of organic and non-organic pollutants are some of the causes of the problem. Moreover, due to the increasing human population, water demand is also increasing, while water reserves in aquifers, groundwater and fossil water are decreasing. These resources can only slowly be replenished. An increase in water storage deficits in Europe was observed after the dry summers of 2018 and 2019 compared to the

water storage deficits after the droughts during the summers of 2003 and 2015, as shown by the GRACE and GRACE-FO data record [517]. A high water storage deficit was also observed in Belgium in 2018 and 2019 [517]. Freshwater is not only used for consumption but also for industrial processes, agriculture and other activities. In Flanders, 10% of the total consumed water is used for agriculture [518]. Currently, to produce tap water, ground and surface waters are used equally in Flanders, while in Wallonia, up to 80% of tap water originates from groundwater [519–521]. The remaining 20% is captured from the river Meuse, old mining sites and six dams [522,523]. Increasing the use of surface water could be necessary to meet the current and future water demand. However, the switch to surface water is accompanied by certain pitfalls, as mentioned by the Flanders Environment Agency [524].

One of these pitfalls is the development of cyanobacterial blooms in these waterbodies. These proliferations of certain cyanobacterial taxa are favored by environmental and meteorological factors and their prediction is still under study [437,441,451,525]. The presence of these blooms can have a detrimental effect on the water quality by producing compounds that lead to foul tastes and odors, or possibly worse, toxic compounds also known as cyanotoxins.

A major group of cyanotoxins are the hepatotoxins, categorized as such due to their main toxicological effect. Two other structurally related hepatoxins are the microcystin congeners (MCs) and nodularin (NOD) (Figure 3.1). Both contain in their structure an (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid (Adda) group connected to a peptide ring. However, NOD's ring contains five peptides, whereas the MCs have a heptacyclic peptide ring [152,526]. Both toxins inhibit protein phosphatase 1 (PPI) and 2A (PPIIA), disrupting cell growth and metabolism [527,528]. When ingested, these toxins are transported by the bile salts to the liver, potentially causing liver damage [119,157,529].

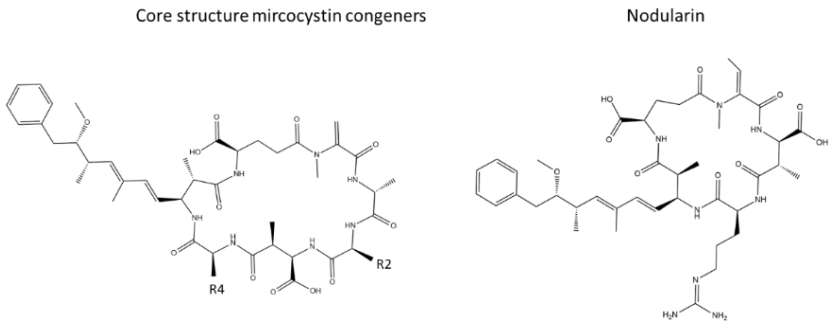



**Figure 3.1.** Microcystin core and nodularin structures. For microcystin, the two variable amino acids are annotated as R2 and R4.

Furthermore, MCs are the cyanotoxins most commonly observed worldwide, with MC-LR being the most prevalent in Western Europe [463,526]. The World Health Organization (WHO) has set a guideline value of 1 µg $L^{-1}$ for MC-LR in drinking water in 1994. Following the most recent assessment, the 1 µg $L^{-1}$ MC-LR value is to be considered as a provisional guideline value for the lifetime exposure via drinking water (WHO, 2020) [122]. Additionally, a provisional guideline value for short exposure of 12 µg $L^{-1}$ MC-LR was set based on the No Observed Adverse Effect Level (NOAEL) observed by Fawell et al. [530], the bodyweight of an adult (60 kg), assuming 100% exposure via drinking water and excluding the uncertainty factor of limited databases [531]. The short exposure is considered for a duration of maximum two weeks in one season until water treatment can be improved and the toxin removed. Due to the absence of the oral toxicity data for the other congeners, it is assumed that their values would be similar to MC-LR as the other congeners have a comparable activity. The sum of the congeners is currently calculated and presented as µg $L^{-1}$ MC-LR equivalent to assess the intoxication risk without any equivalency factors taken into account [531]. Nevertheless, to describe the risk accurately, toxicity equivalency factors for the other MCs and NOD need to be determined as in the case of other toxins potentially present in drinking water.

The European drinking water directive recently selected the 1 µg $L^{-1}$ MC-LR guideline value as a quality parameter for drinking water [474]. However, this directive does not include the other MCs, though a mixture of congeners is commonly found in nature.

Multiple methods have been developed to detect and quantify the MCs. HPLC-DAD was the first method used [383]. Later on, protein phosphates inhibition assays (PPIA) and ELISA tests were developed to quickly quantify the MCs and NOD. PPIAs were simple tests based on a colorimetric detection of the dephosphorylation of the p-nitrophenyl phosphate by PPI [410,529]. The presence of MCs prevents this process by inhibiting the PPI. A pitfall of this test is the lack of specificity for one particular MC or NOD. Therefore, identification is not possible. Interactions with unintended compounds can also result in false-positive results or concentrations in abnormal ranges. ELISA assays have similar shortcomings. However, there is now a tendency to use liquid chromatography–tandem mass spectrometry (LC-MS/MS) and liquid chromatography high-resolution mass spectrometry (LC-HRMS) techniques for the detection of MCs and NOD in water, as well as other matrices [94,388,532]. The main advantage of these methods is that the identification of each congener is based on its physicochemical properties and molecular mass, while the concentration can be determined simultaneously. However, LC methods require pure standards for each specific congener, with the exclusion of high resolution mass spectrometry.

Only a limited number of fully described, validated LC-MS/MS methods are available to evaluate drinking water contamination by hepatotoxins, where the matrix effect has been investigated or taken into account during quantification [205,388,391]. Considering that these toxins could jeopardize public health, the development and validation of a quantification method for MCs and NOD and the evaluation of the drinking water quality in Belgium appeared worthwhile. A method was developed to quantify eight MCs (MC-LR, MC-RR, MC-LA, MC-LY, MC-YR, MC-WR, MC-LF and MC-LW) and NOD using a matrix-matched calibration curve. Due to this approach, water samples from different sources could be quantified more reliably. Moreover, our validation used an alternate approach to calculate the limit of detection for the method, ensuring accurate quantification of the MCs at this level. Furthermore, the matrix effect was determined with a statistically relevant method. The method was fully validated to analyze bottled water from several countries in Europe and tap water from different sources in Belgium. The sampling and analysis of tap and bottled drinking water from Belgium will provide valuable information about MCs presence and human exposure to these toxins.

## 3.2. Materials and Methods

### 3.2.1. Reagents

UHPLC-MS grade solvents (Biosolve B.V., Valkenswaard, The Netherlands) were chosen to prepare the mobile phases and dilution solvents. Milli-Q water and acetonitrile (ACN) were used as mobile phase A and phase B, respectively. All the toxin standards, eight MCs (MC-RR, MC-YR, MC-WR, MC-LR, MC-LA, MC-LF, MC-LW, MC-LY) and NOD were obtained from Enzo Life Sciences® (Enzo Life Sciences, Antwerp, Belgium) as a solid powder. The toxin stock solutions were diluted in MeOH. Intermediate dilutions for the toxin standards were made with a MeOH: Milli-Q water mixture (50:50), supplemented with 1% acetic acid. The stock and the intermediate solutions were stored at −20 °C.

### 3.2.2. Water samples

Bottled water samples were obtained from major retail stores. A total of 51 water samples (various bottles and brands) were tested, with 23 samples of sparkling water and 28 samples of still water. The bottled water samples originate from all over Europe (Belgium, Luxembourg, Germany, France, Italy, The Netherlands). Furthermore, 24 tap water samples were included. These samples were collected in sterile amber glass bottles directly after opening the faucet. In the Flemish provinces, 18 samples were collected. Additionally, two samples were taken from faucets in the Brussels region, and four

samples were collected in Wallonia. More details on the samples are provided in Tables S1.1 and S1.2. The geographic distribution of the tap water samples is presented in Figure S1.1.
Additionally, we participated in the Eurofins Abraxis microcystins proficiency testing program 2021-01.

### 3.2.3. Sample preparation

Water samples (5 mL), independent of their source, were adjusted to pH 11. Using solid-phase extraction (SPE), toxins were extracted from the water as follows: conditioning 6 mL MeOH 100%, equilibration with 6 mL Milli-Q water (pH 11) and elution with 5 mL MeOH (80%). The elute was purified using a Phenomenex 0.2 µm RC-syringe filter (Phenomenex Inc., Utrecht, The Netherlands) and was transferred in amber glass vials with an insert. Each analysis of drinking water samples was accompanied by a quality control (QC) containing all nine toxins at a concentration of 1 µg $L^{-1}$. A calibration curve was made in a blank water matrix ranging from 0.1 ng $mL^{-1}$ to 20 ng $mL^{-1}$.

### 3.2.4. UHPLC-MS/MS conditions

Toxins were analyzed on a Xevo-TQ-S from Waters© (Waters, Eten-Leur, The Netherlands). The initial separation of the toxins was performed with a Waters Acquity UPLC H-class (Waters, Eten-Leur, The Netherlands) on a 1,7 µm, 2,1 mm × 100 mm Waters Acquity BEH C18 column (Waters, Eten-Leur, The Netherlands) proceeded with a Waters Acquity BEH C18 1,7 µM VANGUARD PRE-Col (Waters, Eten-Leur, The Netherlands).
The mobile phase was composed of phase A (Milli-Q water) and phase B (ACN). Formic acid was added to both mobile phases at a ratio of 0.025%. The flow rate used was 0.5 mL $min^{-1}$ and the applied gradient elution program was as follows for mobile phase B: 0 min, 2%; 1.00 min, 40%; 7.00 min, 55%; 7.20 min, 98%; 8.00 min, 98%; 9.00 min; 2%; 12 min, 2%. The column temperature was 60 °C, and the sample injection volume was 10 µL.
The mass spectrometer was operated in the ESI(+) mode. The MS parameters were set as follows: source and desolvation temperatures were 150 and 450 °C, respectively. The capillary voltage was 1.0 kV. Cone and desolvation gas flows were set at 150 and 1000 L $h^{-1}$, respectively. Collision gas flow was 0.15 mL $min^{-1}$. Source offset was 50 V.

### 3.2.5. Optimization of the MS/MS conditions

Initially, detection parameters for the toxins were optimized individually at concentrations of 1 µg $L^{-1}$. The precursor mass was determined and used as the selection parameter during the collision. After the precursor ion fragmentation, two product ions with the highest intensity were selected as a qualifier or quantifier ion. Collision energy and cone voltage were then

further optimized to maximize the signal intensity. An overview can be found in Table 3.1. Qualifier and Qauntifier ion fragments are represented in Figure 3.2.

**Table 3.1**. MS/MS parameters used for ion fragmentation.

| Toxins | Precursor Ion | Quantifier Ion (m/z) | Collison Energy (eV) | Cone Voltage (V) | Qualifier Ion (m/z) | Collison Energy (eV) | Cone Voltage (V) |
|---|---|---|---|---|---|---|---|
| MC-LR | 995.4 | 135.0 | 70.0 | 80.0 | 213.1 | 60.0 | 80.0 |
| MC-RR | 519.8 | 134.8 | 30.0 | 50.0 | 107.2 | 60.0 | 50.0 |
| MC-YR | 1045.5 | 135.3 | 80.0 | 60.0 | 212.9 | 60.0 | 60.0 |
| MC-WR | 1068.4 | 135.3 | 70.0 | 100.0 | 213.1 | 60.0 | 100.0 |
| MC-LY | 1002.4 | 135.4 | 60.0 | 50.0 | 213.0 | 50.0 | 50.0 |
| MC-LA | 910.3 | 135.1 | 60.0 | 50.0 | 107.1 | 80.0 | 50.0 |
| MC-LF | 986.3 | 135.0 | 60.0 | 70.0 | 213.1 | 60.0 | 70.0 |
| MC-LW | 1025.4 | 134.9 | 60.0 | 60.0 | 213.1 | 50.0 | 60.0 |
| NOD | 825.2 | 134.9 | 50.0 | 80.0 | 102.7 | 90.0 | 80.0 |

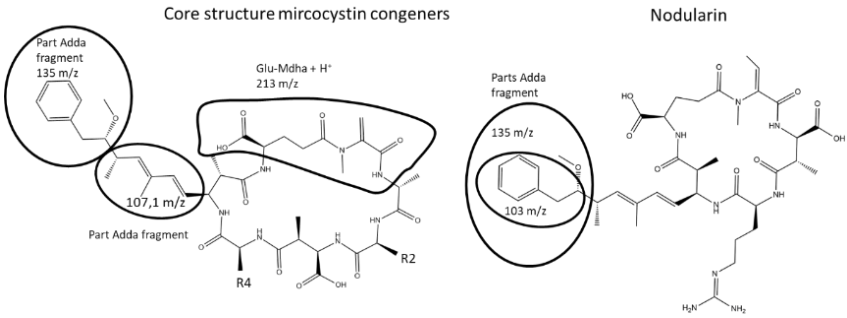



**Figure 3.2.** Qualifier and quantifier ions used during MS/MS analysis

The selectivity of the LC method was also optimized to minimize overlap between different toxin peaks. Different mixtures of mobile phases, such as methanol instead of ACN and neutral, acidified or alkalized versions of the mobile phases, were tested. Moreover, various total elution times and elution gradients were tested to provide the best elution pattern and peak shape, resulting in the use of the earlier described LC method. Peak selectivity is shown in Figure S1.2.

## 3.2.6. Method validation procedure

The validation study was performed using spiked bottled water. The following method parameters were evaluated: limit of detection (LOD), limit

of quantification (LOQ), specificity, linearity, recovery, repeatability, reproducibility, matrix effects, and measurement uncertainty (MU).

During the validation experiments, three toxin mixtures with different concentrations (0.5 µg $L^{-1}$, 2.5 µg $L^{-1}$ and 5 µg $L^{-1}$) for each toxin were selected. These concentrations were chosen around the 1 µg $L^{-1}$ WHO guideline value for chronic exposure for accurate quantification [531]. In food applications, concentrations for validation would be chosen as 0.5, 1.0 and 1.5 maximum residue limit (MRL), and in the case of MCs in water, 0.5 µg $L^{-1}$, 1.0 µg $L^{-1}$, 1.5 µg $L^{-1}$ [410]. Using this validation approach would result in a very narrow concentration range, which will not be able to accurately quantify higher concentrations of MCs present under validation in drinking or environmental water samples.

For each concentration level, 5 mL of bottled water (from a source) was spiked from a standards mix solution containing each toxin at 100 µg $L^{-1}$, in triplicate. Additionally, a blank (bottled water) sample was included as a negative control. The validation experiment was repeated on three different days.

A calibration curve was established using a serial dilution of the toxins at 20 µg $L^{-1}$, 10 µg $L^{-1}$, 5 µg $L^{-1}$, 1 µg $L^{-1}$, 0.5 µg $L^{-1}$, 0.25 µg $L^{-1}$, and 0.1 µg $L^{-1}$ for each toxin. The serial dilution was made in a blank water matrix to assess the matrix effects of the toxins in the water. To quantify the toxin content, the calibration curve was weighted at $1/X^2$.

The linearity of the curve was assessed based on the Mandle's fitting test [533]. This statistical test compares a linear model to a quadratic regression model based on the area under the peak for the different concentrations of a calibration curve.

The LOD and LOQ were accepted as the lowest point in the calibration curve (0.1 µg $L^{-1}$) and the lowest validated quantified concentration (0.5 µg $L^{-1}$), respectively, if the value of the signals was at least 3 times higher than the noise for LOD (S/N >3) and 10 times higher for the LOQ (S/N >10).

Furthermore, the specificity and selectivity of the signal were checked by monitoring the difference in elution time (<5%), the lack of signal in the negative control, the peak shape and the presence of both the quantifier and qualifier ion in spiked samples.

As part of the validation, the ion ratios were also taken into account, following the guidelines of the EU directive 2002/657/EC [431]. The tolerance of the ion ratio is determined based on the relative intensity of the qualifier compared to the quantifier. Inspired by the same directive, the Horwitz equation was used to calculate the reproducibility and repeatability, represented as the coefficient of variation (CV) and average variance, respectively. To determine the repeatability, spiking experiments were performed at three concentration levels in triplicate on the same day, while

for within-laboratory reproducibility evaluation, the same experiments were carried out on three separate days.
During the validation, matrix effects were assessed by comparing the slopes of calibration curves prepared in the matrix extract and neat solvent (MeOH: Milli-Q water (50:50) + 1% acetic acid). The t-test was used for the statistical evaluation of the matrix effect data. The matrix effect can also be observed visually when the curves intersect.
Eventually, the concentrations of the spiked samples were measured. The data analysis was performed in TargetLynx extension of the MassLynx software (Waters©).
Furthermore, the recovery was calculated as the mean of means divided by the theoretical spiked concentration of each of the MCs, separately. The lowest and upper threshold values for the recovery from the spiked samples were 60.0% and 120.0%, respectively. The MU was calculated by multiplying the CV value for the reproducibility by 2 and adding the difference of the recovery from 100.0%. Both the recovery and MU were calculated for each concentration level. The upper threshold value was 80.0%.
Additionally, the sum of the measured concentrations for all the eight MCs and NOD in one spiked sample were taken, resulting in concentration levels of 4.5 µg $L^{-1}$ total microcystin, 22.5 µg $L^{-1}$ total microcystin and 45.0 µg $L^{-1}$ total microcystin. Repeatability, reproducibility, recovery and MU were also calculated for these values and were evaluated using the same criteria as for the separate congeners.

## 3.3. Results

### 3.3.1. Method validation

Initially, the analysis of blank bottled water samples demonstrated the absence of MCs toxins. This means that no peak with an S/N higher than three was detected at the expected retention time of the hepatotoxins from this study, pointing out the good specificity of the method.
Afterwards, the validity of a linear fit was determined for all the nine toxins using the Mandle's fitting test on three separate days [436]. For five out of nine toxins, the tests showed a preference for a linear model. The results were slightly more ambiguous for four MCs congeners (MC-RR, MC-LA, MC-LW and MC-YR) due to the preference of a quadratic model during at least one of the days. This ambiguity resulted from variations between different days for the residual standard deviation for both models. The residual standard deviations for both models on the same day were very similar. Moreover, the $R^2$ value of the linear model was higher than 0.99 and thus suitable for quantification (Table 3.2). In similar methods for quantification of the hepatotoxins, the $R^2$ was also used as selection criteria for the linear

model [205,388,389,392]. The linear model was therefore selected for all nine toxins.

After spiking the water matrix at the same concentrations as the calibration curve, a matrix effect was observed for all toxins based on the difference between the slopes using a *t*-test (Figure 3.3 and Table 3.3). The presence of a matrix effect substantiates the need for a calibration curve in the matrix.

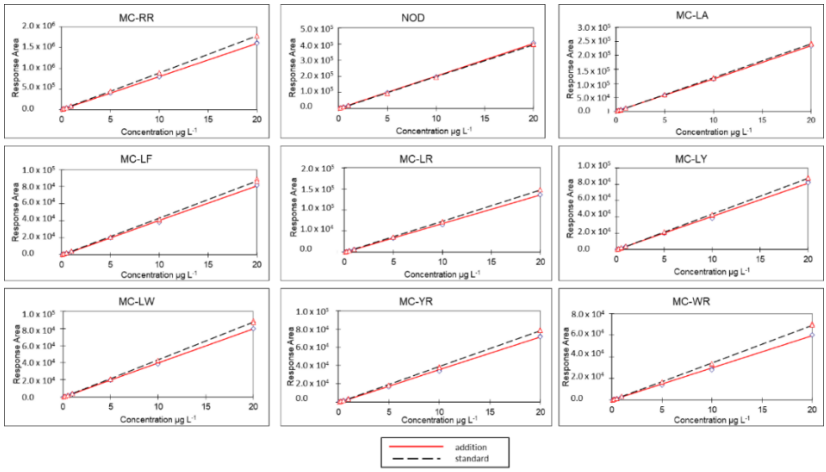



**Figure 3.3.** Matrix effect assessment for the different microcystin congeners and nodularin in drinking water. The presence of a matrix effect was established by comparing the difference in slope based on a student t-test. However, also a visual assessment can be made. If the curve runs in parallel, there is no matrix effect. If this is not the case, there is a matrix effect. All toxins displayed matrix effect in drinking water.

**Table 3.3.** Values for the calculated t(b) is compared with the tabulated t at the 95% confidence level. If t(b) is higher than t(95%), a matrix effect is present.

| | **MC-RR** | **NOD** | **MC-LA** | **MC-LF** | **MC-LR** | **MC-LY** | **MC-LW** | **MC-YR** | **MC-WR** |
|---|---|---|---|---|---|---|---|---|---|
| t(b) | 53.59 | 3.34 | 7.92 | 5.57 | 11.54 | 7.53 | 11.93 | 9.17 | 12.02 |
| t(95%) | 2.06 | 2.06 | 2.06 | 2.06 | 2.06 | 2.06 | 2.06 | 2.06 | 2.06 |

**Table 3.2.** Overview of validation results for eight microcystin congeners (MC), nodularin (NOD) and the sum of all toxins in water. Results for the recovery, repeatability, reproducibility, measurement uncertainty, $R^2$ and average signal to noise (S/N) for the limit of detection (LOD) and the limit of quantification (LOQ) are shown on average and at three concentration levels. * µg $L^{-1}$ total microcystin.

| Toxins | Spiked Concentration (µg $L^{-1}$) | Recovery (%) | Repeatability (%) | Reproducibility (%) | Measurement Uncertainty (%) | Average S/N for LOD (0.10 µg $L^{-1}$) | Average S/N for LOQ (0.50 µg $L^{-1}$) | $R^2$ |
|---|---|---|---|---|---|---|---|---|
| MC-RR | 0.50 | 96.00 | 5.03 | 9.12 | 18.25 | 361.85 | 1691.95 | 1.00 |
| | 2.50 | 97.00 | 2.04 | 4.99 | 9.98 | | | |
| | 5.00 | 103.00 | 6.39 | 7.98 | 15.96 | | | |
| | Average | 98.70 | 4.48 | 7.37 | 14.73 | | | |
| NOD | 0.50 | 95.00 | 5.23 | 7.14 | 14.29 | 193.38 | 7605.39 | 1.00 |
| | 2.50 | 98.00 | 2.80 | 5.08 | 10.15 | | | |
| | 5.00 | 103.00 | 7.26 | 8.10 | 16.20 | | | |
| | Average | 98.70 | 5.10 | 6.77 | 13.55 | | | |
| MC-LA | 0.50 | 90.00 | 4.66 | 6.43 | 12.85 | 60.53 | 222.93 | 1.00 |
| | 2.50 | 92.00 | 3.30 | 6.35 | 12.70 | | | |
| | 5.00 | 97.00 | 7.21 | 7.84 | 15.67 | | | |
| | Average | 93.00 | 5.06 | 6.87 | 13.74 | | | |
| MC-LF | 0.50 | 68.00 | 3.56 | 5.75 | 11.51 | 37.42 | 106.18 | 1.00 |
| | 2.50 | 66.00 | 4.87 | 14.05 | 28.09 | | | |
| | 5.00 | 72.00 | 7.04 | 7.04 | 14.08 | | | |
| | Average | 68.70 | 5.15 | 8.95 | 17.89 | | | |
| MC-LR | 0.50 | 88.00 | 2.27 | 7.19 | 14.37 | 95.72 | 432.98 | 1.00 |
| | 2.50 | 89.00 | 1.63 | 7.67 | 15.34 | | | |
| | 5.00 | 93.00 | 7.35 | 10.07 | 20.14 | | | |
| | Average | 90.00 | 3.75 | 8.31 | 16.62 | | | |

| Toxins | Spiked Concentration (μg $L^{-1}$) | Recovery (%) | Repeatability (%) | Reproducibility (%) | Measurement Uncertainty (%) | Average S/N for LOD (0.10 μg $L^{-1}$) | Average S/N for LOQ (0.50 μg $L^{-1}$) | $R^2$ |
|---|---|---|---|---|---|---|---|---|
| MC-LY | 0.50 | 88.00 | 5.29 | 10.39 | 20.78 | 49.53 | 174.58 | 1.00 |
| | 2.50 | 88.00 | 2.70 | 9.41 | 18.82 | | | |
| | 5.00 | 94.00 | 6.11 | 10.94 | 21.88 | | | |
| | Average | 90.00 | 4.70 | 10.25 | 20.49 | | | |
| MC-LW | 0.50 | 53.00 | 8.68 | 16.23 | 32.45 | 42.34 | 98.27 | 1.00 |
| | 2.50 | 53.00 | 8.10 | 19.64 | 39.28 | | | |
| | 5.00 | 59.00 | 6.01 | 11.16 | 22.32 | | | |
| | Average | 55.00 | 7.60 | 15.67 | 31.35 | | | |
| MC-YR | 0.50 | 83.00 | 6.32 | 10.69 | 21.38 | 54.48 | 192.34 | 1.00 |
| | 2.50 | 87.00 | 4.17 | 15.80 | 31.61 | | | |
| | 5.00 | 91.00 | 5.30 | 15.34 | 30.67 | | | |
| | Average | 87.00 | 5.26 | 13.94 | 27.89 | | | |
| MC-WR | 0.50 | 62.00 | 11.31 | 19.64 | 39.29 | 55.80 | 254.99 | 1.00 |
| | 2.50 | 67.00 | 6.43 | 21.05 | 42.10 | | | |
| | 5.00 | 72.00 | 3.59 | 16.14 | 32.28 | | | |
| | Average | 67.00 | 7.11 | 18.94 | 37.89 | | | |
| SUM | 4.50 * | 80.00 | 4.32 | 5.27 | 10.54 | / | / | / |
| | 22.50 * | 82.00 | 2.99 | 8.59 | 17.18 | | | |
| | 45.00 * | 87.00 | 5.92 | 7.08 | 14.15 | | | |
| | Average | 83.00 | 4.41 | 6.98 | 13.96 | | | |

Finally, our validation confirmed the accurate detection and quantification of the nine toxins. The retention time stability, peak shape, selectivity and ion ratios were all within the pre-set boundaries. Moreover, the LOD (0.10 μg $L^{-1}$) and LOQ (0.50 μg $L^{-1}$) for all nine toxins had a ratio of signal-to-noise higher than 3 and 10, respectively, as shown in Table 3.2. However, the recoveries for MC-LF, MC-LW and MC-WR were below the acceptable limit of 70.0% (Table 3.2 and Figure 3.4). These low recoveries are possibly due to retention of the more hydrophobic MCs on the plastic tubes used during pH adjustment. Rinsing these tubes with higher amounts of organic solvent would solve this [534] but cause early elution of MCs during SPE resulting in a decrease in recovery of the more hydrophilic MCs. Moreover, the lower recoveries for the hydrophobic MCs obtained with our method will suffice, as the results of samples' analyses will always be corrected with the value of the recovery of a quality control (QC) sample, and the results for reproducibility, repeatability and MU were acceptable. During sample analysis, acceptable recoveries for the QCs of MC-LF, MC-LW and MC-WR will need to be between 30.00% and 90.00% or they will be labelled as non-conform. The recoveries for the conform toxins ranged from 87.00% to 98.70% on average (Table 3.2 and Figure 3.4), with acceptable recoveries for the QC during sample analysis being between 60.00–120.00%. MUs for all the MCs and NOD were calculated to be between 10.00% and 42.10% (Table 3.2). The average variance (repeatability) and CV (reproducibility) for all the MCs and NOD were within the bounds set by the Horwitz ratio, 14.70% and 22.00%, respectively (Table 3.2 and Figure 3.4).

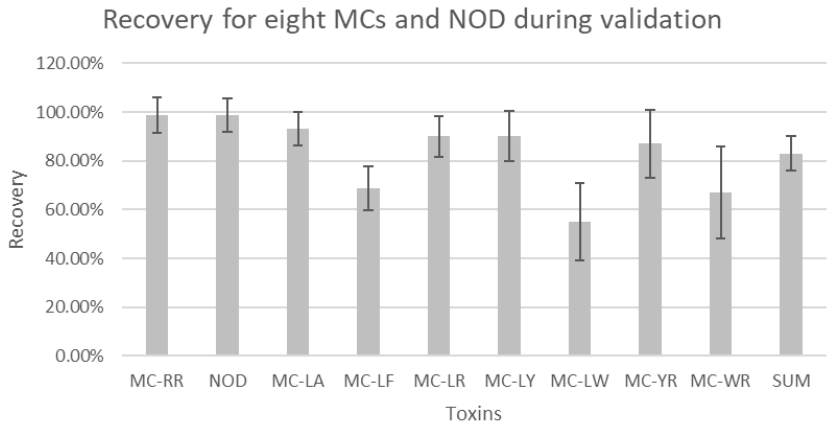



**Figure 3.4.** Recoveries for all eight microcystin congeners (MCs), nodularin (NOD) and their sum, are presented in the graph. The error bars represent the reproducibility.

### 3.3.2. Application of the method in drinking water

The validated UHPLC-MS/MS method was subsequently used to investigate the contamination of drinking water available on the Belgian market and Belgian tap water. In total, 51 samples of bottled water and 24 samples of tap water were collected (Tables S1.1 and S1.2) and analyzed during six different analysis days.

During the analysis, QCs were added to each analysis series to ensure the quality of the procedure. The recoveries of the QCs were calculated and were acceptable for each of the six analysis days (Table 3.4). The precision of the analysis was taken into account by assessing the standard deviation of the retention time, which should be below 0.05%. Additionally, the R2 values for the calibration curve were above 0.99, showing acceptability of the curve. The relative standard deviation (RSD) was calculated with the recovery and ranged between 1% to 10% for the different hepatotoxins. However, no MCs or NOD could be detected in the samples taken from stores or tap water.

**Table 3.4.** Average recoveries for quality controls (QC) of eight microcystin congeners and nodularin obtained from 6 different days of sample analysis.

| MC-RR | NOD | MC-LA | MC-LF | MC-LR | MC-LY | MC-LW | MC-YR | MC-WR |
|---|---|---|---|---|---|---|---|---|
| 97.00% | 90.00% | 81.00% | 61.00% | 72.00% | 74.00% | 43.00% | 77.00% | 60.00% |

Additionally, results were satisfactory ($|z| < 2$) for the Eurofins Abraxis microcystins proficiency testing program 2021-01.

## 3.4. Discussion

The screening results from bottled and tap water revealed that no MCs or NOD could be found, indicating that there is currently no safety risk for the population regarding contamination of drinking water with these hepatotoxins, based on this study of 75 samples. As expected, bottled water is not contaminated because it is generally exploited from sources or springs, where cyanobacterial blooms do not occur. On the other hand, tap water can be extracted from surface water and is thus at risk of contamination. However, in Belgium, groundwater sources are more frequently used than surface waters. In Flanders, only eight surface waterbodies are used in four of the nine distribution districts. Moreover, most of the surface water originates from the river Meuse and the Albert Canal. For Flanders, tap water was sampled from distribution districts that exploit groundwater, which might explain the lack of toxins. In Wallonia, 80% of the water is extracted from groundwater. Most of the surface water also originates from the river Meuse [521,535].

Furthermore, Belgian tap water is diligently treated before it is distributed, reducing the likelihood of toxins being present in the water. One instance of

a well-described treatment process for surface water uses grids and microsieves, flocculation, sand filtration, active carbon filters, consecutively, with additional chemical treatments if necessary to ensure the quality of the water [536]. Although the risk of MCs contaminated tap water seems small at present, our validated methods could be useful in case of any suspected contaminations. Due to extended dry periods, water reserves have dwindled every summer since a few years (e.g., in Flanders, four districts reported 'lower than normal' groundwater levels in 2020) [535]. This problem is expected to increase due to climate change, and alternate water sources, such as surface water, could be necessary to meet the water demand. Moreover, the flowing waterbodies such as the river Meuse and Albert Canal, are also susceptible to droughts, meaning that other waterbodies might need to be used in the future to supplement our water demand. Stagnant waterbodies, such as lakes and reservoirs, are more likely to harbor potentially toxic cyanobacteria blooms. Multiple studies have already shown that bloom occurrence in different waterbodies used as drinking water catchments is common [179,205,321,383,422,427,495,501,505,537–539]. Therefore, using the newly developed analytical methods to analyze contaminants, especially during summer and autumn, will ensure drinking water quality.

However, most studies found toxins in the raw water before but rarely after treatment. Although water treatment is still not yet universally available worldwide, certain techniques could remove cyanotoxins from raw water. The use of active carbon has been shown to remove cyanobacterial cells and, to a certain extent, free toxins from the raw water [203,495–500,502,503]. The use of chemical agents, such as chlorine, permanganate or ferrate, can inactivate cyanobacterial cells and, in some cases, degrade certain toxins [494,495,506,540]. Advance oxidation by ultrasound, ozone, UV, $H_2O_2$ or a combination of the latter two is also effective in degrading certain toxins and deactivating cyanobacterial cells [488,499,507–512]. Yet, the number of cyanobacterial cells and the concentration of the toxins influence the effectiveness of these methods. Therefore, accurate toxin quantification is vital for a successful treatment. Our validated UHPLC-MS/MS method could be applied for these control purposes.

Our validation shows that the method can quantify eight microcystin congeners (MC-LR, MC-RR, MC-YR, MC-LY, MC-LA, MC-WR, MC-LF and MC-LW) and nodularin. However, overall recoveries for MC-WR, MC-LF and MC-LW were lower than for the other toxins, and thus the acceptable limit for recovery had to be reduced for these hepatotoxins. Interestingly, this reduced recovery primarily affected the hydrophilic compounds, which was also observed in Zervou et al., where SPE was also used [391].

The LOQ is defined as the concentration level where a compound can be quantified with certain precision and accuracy during the method validation.

This definition would only be applicable for our lowest validation point, 0.50 µg $L^{-1}$. Pekar et al. accurately determined concentrations at 0.1 µg $L^{-1}$ for some MCs, while for NOD, MC-YR, MC-LW, MC-LY, MC-LF, this was only possible at a concentration of 0.50 µg $L^{-1}$ [205]. During the analysis, 0.5 µg $L^{-1}$ was chosen as the lowest level because it would be distinguishable from the 1 µg $L^{-1}$ guideline value proposed by the WHO. Lower concentrations would be of little concern during monitoring campaigns, even though the feasibility of quantifying concentrations below 0.50 µg $L^{-1}$ could be assessed. Instead, Turner et al. used an alternative term to approximate this definition of LOQ, namely limit of reporting (LOR). The LORs for the toxins ranged between 0.3 and 1.3 ng $mL^{-1}$ [388]. Zervou et al. used an alternate approach, first determining the LOD based on statistics and then defining the LOQ as 3 times the LOD [391]. They were able to obtain LODs in the ng $L^{-1}$ range. However, the lower LOD values can be explained by the higher sample volume used during SPE and the increased concentration of the extract after a nitrogen stream evaporation. Moreover, higher sample volumes and up concentration would increase the reporting time, which is an important factor when analyzing samples for contaminations. Our LOD is sufficient to accurately assess contaminations close to the WHO guideline values, with a fast reporting time (+/−1 day).

The LOD of the toxins analyzed in our method was set at 0.10 µg $L^{-1}$ as the lowest point of the calibration point and thus the lowest point for which we could assess the signal to noise ratio. Further investigation could result in lower values, but this would have little benefit to swiftly assess the public health threat.

The matrix effect in water seems to be variable depending on the source of the water used for validation. Pekar et al. reported a matrix effect for nearly all toxins in water, while Turner et al. found a matrix effect for only a few [205]. On the other hand, our study shows that a matrix effect could be measured for most MCs compared to the calibration solution MeOH:H2O 50:50 (*v*/*v*) solution with 1% acetic acid. However, the physicochemical properties (salts, metals and other micronutrients) of the water can probably influence the matrix effect. Therefore, we suggest that our approach could be a valuable asset to incorporate into a method design to analyze multiple water samples in one run by using a calibration curve in the water matrix. However, the water-based calibration curve could provide variability due to common adsorption by plastic lab equipment [534]. The SPE was used to collect the toxins in an organic solvent (MeOH 80%) before injection of the UHPLC-MS/MS, preventing this adsorption.

## 3.5. Conclusions

Our UHPLC-MS/MS method is the first publicly described and fully validated method for quantifying eight MCs and NOD for water in Belgium. This method shows reasonable specificity, linearity of the matrix-matched calibration curves, matrix effect, precision parameters, recovery, repeatability, reproducibility and measurement uncertainty for MC-RR, NOD, MC-LA, MC-LR, MC-LY, MC-YR, MC-WR, MC-LF and MC-LW in drinking water. However, the initial threshold recovery values for MC-WR, MC-LF and MC-LW were not reached. The obtained recoveries were sufficient for a valid method due to acceptable values for reproducibility and MU. All parameters for the other toxins were within the preset parameters.
Additionally, the implementation of our method on 51 bottled (from Europe) and 24 tap water (from Belgium) samples from different sources is exceptional. Dependent on the region in Belgium, hepatotoxins in drinking water are evaluated with undisclosed methods, which makes a comparison of the methods and results impossible. However, none of the nine hepatotoxins were detected during our study, showing that Belgian drinking water is most likely safe for the consumer. The QC measured during sample analysis does provide proof that our method is capable of quantifying the MCs and NOD, which was further supported by our participation in the Eurofins Abraxis proficiency test, resulting in satisfactory z-values.

**Supplementary data:** Following data is available in Chapter S1: Figure S1.1: Map showing the distribution of the tap water sampling in Belgium, Figure S1.2: The elution peaks and the intensity shown for the eight microcystin congeners and nodularin, Table S1.1: Overview of bottled water based on their country of origin, total volume of water and count of individual samples, Table S1.2: Overview of tap water samples based on region and count of individual samples.

# 4. Validation of an UHPLC-MS/MS method to quantify cyanobacterial toxins in environmental matrices

Contributing authors: *Wannes Hugo R. Van Hassel* performed the optimization of analytical methodology, prepared the samples, performed the mass spectrometry, analyzed analytical data and wrote the chapter; *Julien Masquelier, Bart Huybrechts* supervised the study and were involved in critical analysis of the analytical data, manuscript corrections and discussion.

Drinking water is not the only source of exposure to MCs. Recreational use of waterbodies can also result in exposure when a bloom is present. In Chapter 5, which was published in Toxins-MDPI (DOI: https://doi.org/10.3390/toxins14010061) and adapted for the thesis, sampling and analysis of cyanobacteria bloom samples are discussed. However, only an abridged version of the validation data was presented in the supplementary data of the published version of the chapter. The current chapter will therefore present the complete data of the validated method for the quantification of eight MCs and NOD in cyanobacterial biomass.

## 4.1. Validation approach

The analysis of bloom samples required a proper extraction of the cyanobacterial biomass due to the intracellular nature of MCs. Therefore, the cyanobacterial biomass was first separated from the water. Thereafter, cyanobacterial biomass and water were analyzed separately. The water was analyzed using the validated method described in Chapter 3. However, a new method had to be optimized and validated for the cyanobacteria biomass. The multiday validation approach and fortification scheme of the samples is described in Table 4.1. The biomass of strain *Arthrospira platensis* BCCM/ULC444 was filtered on GF/C Whatman filters to use as a blank matrix for the validation. The biomass was obtained from multiple cultures grown in 250 ml plastic flasks at 20°C under light. The spiked samples contained the same mixture of eight MCs and NOD, previously used during the drinking water validation.

**Table 4.1**. Cyanobacteria biomass samples were used for validation over three days. Samples were spiked with a mixture of eight MCs and NOD. Each toxin should be present at a concentration of either 12.50, 50.00 or 125.00 µg $kg^{-1}$.

| Concentration of Toxin spike µg $kg^{-1}$ | Blank | 12.50 | 50.00 | 125.00 |
|---|---|---|---|---|
| Day 1 | 1 X | 3 X | 3 X | 3x |
| Day 2 | 1 X | 3 X | 3 X | 3 x |
| Day 3 | 1 X | 3 X | 3 X | 3 X |

## 4.2. Validation parameters

The validation process includes the assessment and verification of certain parameters. Following the guidelines 2002/657/EC, the following criteria and their parameters had to be met:

- Recovery: 120% ≥ X ≥ 60% [431]
- Repeatability: Based on Horwitz ratio [541,542]
- Reproducibility: Based on Horwitz ratio [541,542]
- Ion ration: Based on guidelines 2002/657/EC [431]
- Limit of detection (*LOD*): 1/3 of the value for LOQ, if the signal-to-noise is higher than 3 [431].
- Limit of quantification (*LOQ*): lowest concentration for which the method is validated and signal-to-noise is higher than 10 [431].
- Linearity: Based on The Mandels Fitting test (22N16v3) [436].
- Specificity: Absences of peaks in solvent blanks and matrix blanks [431].
- Matrix effect: T-test comparing slopes of the standard curve in solvent to curve in the matrix [431].
- Measurement uncertainty: 80% ≥ 2* coefficient of variation [431].

## 4.3. Validation results

Overall, The results for the validation parameters were within the acceptable range for the different validation criteria.

### 4.3.1. Specificity and signal to noise

No residual peaks above 1 % of the mid-range concentration level (50.00 µg $kg^{-1}$) were observed in either the solvent or the matrix blanks. Both qualifier and quantifier ions were detected in spiked matrix samples. Therefore, the criteria for specificity were met. The same qualifier and quantifier ions were characterized as described in the previous chapter (Table 3.1.).
Signal-to-noise values for the LOD (0.25 µg $L^{-1}$ or 5.63 µg $kg^{-1}$) and LOQ (0.56 µg $L^{-1}$or 12.50 µg $kg^{-1}$) also met the acceptance criteria (Table 4.4). The LOQ was quite high due to the signal interference by the matrix but will suffice to quantify concentrations in environmental samples. When analysing these samples, the calculated concentration in µg $kg^{-1}$ should be corrected by the

weight of the tested biomass and the volume of water from which it was filtered. For instance, filtering 0.20 g of biomass from 150 mL of water results in a LOQ of 0.02 µg $L^{-1}$. This is far below any of the microcystin guidelines provided by the WHO [122].
The ion ratios (Table 4.2) were also within range of the maximum variance determined in the EU decision 2002/657/EC [431].

**Table 4.2**. Measured ion ratio and standard deviation of the ion ratio during validation.

| Toxins | Average ion ratio | Standard Deviation ion ratio |
|---|---|---|
| **MC-RR** | 13.37% | 0.39% |
| **NOD** | 46.65% | 2.08% |
| **MC-LA** | 51.91% | 7.59% |
| **MC-LF** | 41.51% | 2.43% |
| **MC-LR** | 31.66% | 1.26% |
| **MC-LY** | 45.82% | 3.68% |
| **MC-LW** | 49.20% | 6.15% |
| **MC-YR** | 33.21% | 3.19% |
| **MC-WR** | 40.21% | 8.11% |

## 4.3.2. Linearity of the calibration curve and matrix effect

The linearity was determined with the Mandels fitting test. The ideal fitting was a quadratic regression for most cases. However, the $R^2$ value of the linear regression was higher than 0.99 (Table 4.4). To simplify the calculations for the concentration, the linear regression was used.
The matrix effect was determined for each toxin by producing a calibration curve combining all the toxins in the dilution solvent (containing 50:50 MiliQ water: Methanol (+1% acidic acid)) and in the evaluated blank matrix after filtration. The difference in slope was analyzed using a student t-test. Results from the student t-test were illustrated in the graphs in Figure 4.1. T(b) values were higher for all toxins compared to t(95) (Table 4.3). Therefore, we could conclude that for all toxins, a matrix effect was observed

**Figure 4.1.** Assessment of the matrix effect for microcystin congeners and nodularin in cyanobacteria based food supplements.

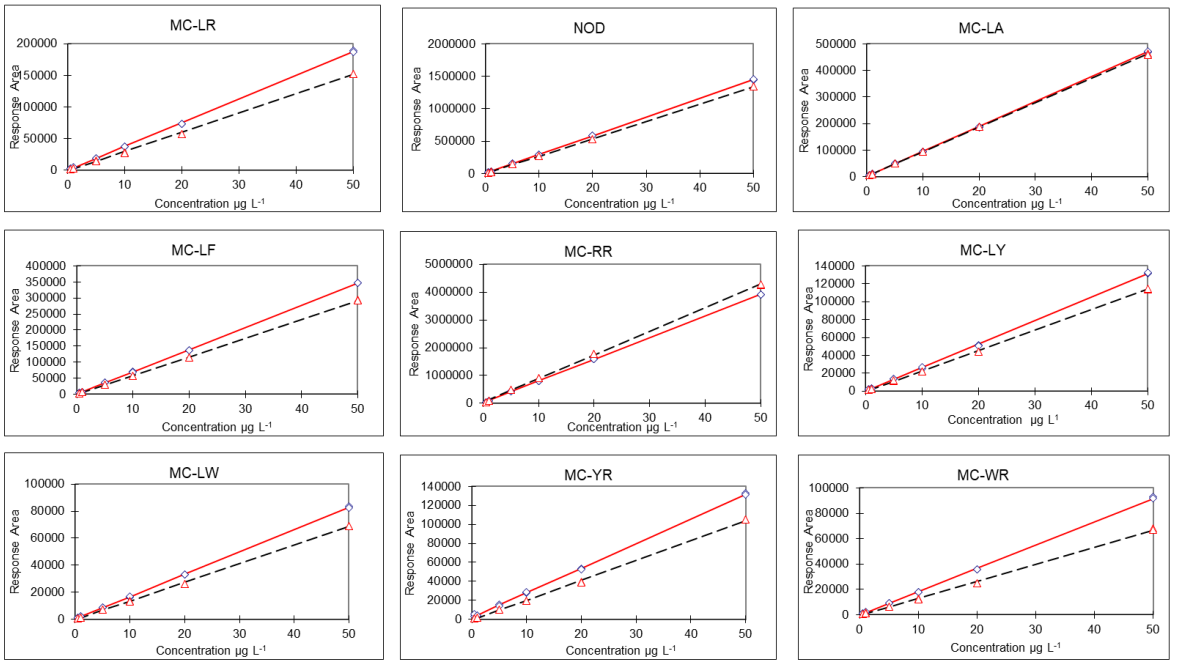


**Table 4.3.** The values for the calculated t(b) from the student t-test to assess matrix effect are compared with the tabulated t at the 95% confidence level. If t(b) is higher than t(95%), a matrix effect is present. The t(b) value compares a significant difference between the slope of standard and addition curve.

| Toxins | MC-RR | NOD | MC-LA | MC-LF | MC-LR | MC-LY | MC-LW | MC-YR | MC-WR |
|---|---|---|---|---|---|---|---|---|---|
| **t(b)** | 13.05 | 23.43 | 4.69 | 36.26 | 20.59 | 17.18 | 26.88 | 20.33 | 24.36 |
| **T(95%)** | 2.08 | | | | | | | | |

## 4.3.3. Recovery, repeatability, reproducibility and measurement uncertainty

The apparent recovery was calculated as shown in Chapter 3. Table 4.4 shows that the nine toxins separately, as well as, all the available toxins together were recovered at an efficiency between 60% and 120% during analysis.
For the repeatability of all nine toxins, the highest maximum allowed variance based on the Horwitz ratio was 14.70%. Max Horwitz variance was calculated for each toxin at each concentration range. Table 4.4 shows that for all different concentration levels and different toxins, the calculated CVs were below the calculated max Horwitz variance. The calculated CVs for the SUM of the toxins also were below the Max Horwitz ratio.
Continuing with the reproducibility, the Horwitz ratio provided different maximum average variances (<22%). The max Horwitz average variance for each toxin or the sum of the toxins on each concentration level was not exceeded as shown in Table 4.4.
The measurement uncertainty did not increase above 80%. The results for the individual MCs and their sum are shown in Table 4.4.
As all validation criteria were met, this method was successfully validated and used to analyze environmental samples as described in the following chapter.

**Table 4.4**: Validation results for the quantification of 8 microcystin congeners and nodularin in cyanobacteria biomass.

| Toxins | Spiked Concentration (µg/kg) | Recovery (%) | Repeatability (%) | Reproducibility (%) | MU (%) | Average S/N LOD (0.25 µg/L) | Average S/N LOQ (0.56 µg/L) | $R^2$ |
|---|---|---|---|---|---|---|---|---|
| MC-RR | 12.50 | 88.00 | 4.18 | 21.94 | 43.88 | 531.24 | 765.34 | 0.99 |
| | 50.00 | 89.00 | 10.54 | 10.54 | 21.07 | | | |
| | 125.00 | 92.00 | 5.82 | 15.65 | 31.29 | | | |
| | Average | 89.67 | 6.85 | 16.04 | 32.08 | | | |
| NOD | 12.50 | 95.00 | 4.18 | 11.50 | 23.00 | 386.91 | 721.74 | 0.99 |
| | 50.00 | 97.00 | 5.60 | 6.99 | 13.98 | | | |
| | 125.00 | 99.00 | 3.43 | 4.8 | 9.60 | | | |
| | Average | 97.00 | 4.40 | 7.76 | 15.53 | | | |
| MC-LA | 12.50 | 100.00 | 5.67 | 14.40 | 28.80 | 69.07 | 134.83 | 0.99 |
| | 50.00 | 89.00 | 5.46 | 7.71 | 15.41 | | | |
| | 125.00 | 101.00 | 2.45 | 6.81 | 13.62 | | | |
| | Average | 96.67 | 4.53 | 9.64 | 19.28 | | | |
| MC-LF | 12.50 | 95.00 | 3.12 | 18.51 | 37.02 | 64.03 | 139.69 | 0.99 |
| | 50.00 | 88.00 | 5.85 | 9.88 | 19.76 | | | |
| | 125.00 | 101.00 | 1.65 | 3.67 | 7.33 | | | |
| | Average | 94.67 | 3.54 | 10.68 | 21.37 | | | |
| MC-LR | 12.50 | 99.00 | 4.71 | 8.81 | 17.62 | 116.82 | 265.64 | 0.99 |
| | 50.00 | 92.00 | 6.53 | 7.04 | 14.09 | | | |
| | 125.00 | 101.00 | 1.45 | 3.21 | 6.43 | | | |
| | Average | 97.33 | 4.23 | 6.36 | 12.71 | | | |

| Toxins | Spiked Concentration (µg/kg) | Recovery (%) | Repeatability (%) | Reproducibility (%) | MU (%) | Average S/N LOD (0.25 µg/L) | Average S/N LOQ (0.56 µg/L) | $R^2$ |
|---|---|---|---|---|---|---|---|---|
| MC-LY | 12.50 | 96.00 | 4.45 | 13.11 | 26.21 | 51.57 | 78.84 | 0.99 |
| | 50.00 | 88.00 | 3.12 | 8.29 | 16.57 | | | |
| | 125.00 | 100.00 | 1.92 | 4.76 | 9.53 | | | |
| | Average | 94.67 | 3.16 | 8.72 | 17.44 | | | |
| MC-LW | 12.50 | 100.00 | 6.63 | 11.12 | 22.23 | 42.89 | 92.86 | 0.99 |
| | 50.00 | 90.00 | 3.39 | 11.93 | 23.87 | | | |
| | 125.00 | 102.00 | 2.21 | 6.26 | 12.51 | | | |
| | Average | 97.33 | 4.08 | 9.77 | 19.54 | | | |
| MC-YR | 12.50 | 100.00 | 6.38 | 9.52 | 19.05 | 75.26 | 139.15 | 0.99 |
| | 50.00 | 94.00 | 4.55 | 4.55 | 9.10 | | | |
| | 125.00 | 98.00 | 3.09 | 3.58 | 7.15 | | | |
| | Average | 97.33 | 4.67 | 5.88 | 11.77 | | | |
| MC-WR | 12.50 | 96.00 | 9.69 | 9.69 | 19.38 | 83.91 | 148.68 | 0.99 |
| | 50.00 | 91.00 | 9.57 | 12.86 | 25.73 | | | |
| | 125.00 | 100.00 | 3.73 | 4.90 | 9.81 | | | |
| | Average | 95.67 | 7.67 | 9.15 | 18.31 | | | |
| SUM | 12.50 | 96.00 | 3.24 | 11.27 | 22.55 | N.A. | N.A. | N.A. |
| | 50.00 | 91.00 | 2.84 | 5.46 | 10.91 | | | |
| | 125.00 | 99.00 | 2.09 | 3.76 | 7.52 | | | |
| | Average | 95.33 | 2.72 | 6.83 | 13.66 | | | |

# 5. A Summer of Cyanobacterial Blooms in Belgian Waterbodies: Microcystin Quantification and Molecular Characterizations

**Contributing authors:** *Wannes Hugo R. Van Hassel* performed the optimization of analytical methodology, prepared the samples, performed the mass spectrometry, PCRs and prepared DNA for sequencing, analyzed the molecular and analytical data and wrote the chapter; *Benoit Durie*u performed and analyzed the amplicon sequencing data and contributed to the writing of the chapter; *Viviana Almanza Marroquin* prepared the samples, extracted DNA for PCR, supervised the study and was involved in critical analysis of the data, manuscript corrections and discussion; *Mirjana Andjelkovic, Julien Masquelier, Bart Huybrechts* supervised the study and were involved in critical analysis of the analytical data, manuscript corrections and discussion; *Annick Wilmotte* supervised the study and was involved in critical analysis of the sequencing data, manuscript corrections and discussion.

This chapter was published in Toxins-MDPI (DOI: https://doi.org/10.3390/toxins14010061) and adapted for the thesis.

**Abstract:** In the context of increasing occurrences of toxic cyanobacterial blooms worldwide, their monitoring in Belgium is currently performed by regional environmental agencies (in two of three regions) using different protocols and is restricted to some selected recreational ponds and lakes. Therefore, a global assessment based on the comparison of existing datasets is not possible. For this study, 79 water samples from a monitoring of five lakes in Wallonia and occasional blooms in Flanders and Brussels, including a canal, were analyzed. A Liquid Chromatography with tandem mass spectrometry (LC-MS/MS) method allowed to detect and quantify eight microcystin congeners. The *mcyE* gene was detected using PCR, while dominant cyanobacterial species were identified using 16S RNA amplification and direct sequencing. The cyanobacterial diversity for two water samples was characterized with amplicon sequencing. Microcystins were detected above limit of quantification (LOQ) in 68 water samples, and the World Health Organization (WHO) recommended guideline value for microcystins in recreational water (24 µg $L^{-1}$) was surpassed in 18 samples. The microcystin concentrations ranged from 0.11 µg $L^{-1}$ to 2798.81 µg $L^{-1}$ total microcystin. For 45 samples, the dominance of the genera *Microcystis* sp.,

*Dolichospermum* sp., *Aphanizomenon* sp., *Cyanobium/Synechococcus* sp., *Planktothrix* sp., *Romeria* sp., *Cyanodictyon* sp., and *Phormidium* sp. was shown. Moreover, the *mcyE* gene was detected in 75.71% of all the water samples.

## 5.1. Introduction

A Belgian global picture of the diversity of cyanotoxins and the taxa producing them is currently lacking. This impairs a better understanding of their importance, their yearly variations and the need to prevent and mitigate blooms. Drinking water in Belgium is mostly provided by aquifers and barely depends on reservoirs [521,535]. Moreover, the water supply varies among the regions (Brussels regions, Flanders, and Wallonia). In Flanders, besides aquifers, the Meuse river and the Albert canal, only eight reservoirs supply water. If this is not sufficient, water is imported from Wallonia and neighbouring countries [535]. The drinking water in Brussels originates mainly (>90%) from Wallonia [543]. Water exploitation in Wallonia is dependent on ground water for 80% and is supplemented with water exploitation from the Meuse, some old mining sites and six dams [521–523]. Further, recreational waters are a sensitive issue, as there is an increasing societal demand for such areas in summer. External sources of eutrophication, such as untreated sewage discharge or agriculture run off, in these waterbodies may promote cyanobacterial bloom formation [437,441]. However, little information about eutrophication sources is available for fresh waterbodies in Belgium.

Ingestion of cyanotoxin contaminated water has been shown to be detrimental to human and animal health [128,228,231,544–547]. Yet in Belgium, no causative link has so far been found between toxic blooms and associated symptoms in humans and animals, such as gastroenteritis, vomiting, liver damage or convulsions [548,549]. However, suspicious bird deaths have been reported, which coincided with a toxic *Microcystis* bloom [550].

Between 1994 and 2008, a few studies identified the morphological and toxin diversity in toxic cyanobacterial blooms in Belgian lakes and ponds [21,368,515,550–554]. The concentrations of total microcystin (MC) measured by Ultra High Performance Liquid Chromatography (UHPLC) reached 18 to 2651 µg $g^{-1}$ dry weight (DW), and the bloom samples contained up to six variants [553,554]. *Microcystis* was found to be the most dominant genus, followed by *Planktothrix*. These results prompted a large-scale study (BelSPO project B-BLOOMS2) from 2007 to 2010 [368,515]. During this study, 89% and 83% of the 162 samples tested showed the presence of *mcyE* and *mcyA* genes, respectively. *Microcystis*, *Anabaena* (now taxonomically identified as *Dolichospermum*), *Aphanizomenon*, *Planktothrix, and Woronichinia* were the primary bloom-forming cyanobacterial taxa.

Furthermore, a quantitative toxin analysis of the samples showed that the total congeners concentration varied from 0.120 µg $L^{-1}$ to 37500 µg $L^{-1}$ total microcystin, analyzed with high performance liquid chromatography-photodiode array detection (HPLC-DAD) and ELISA, in parallel [368,451,515]. Based largely on the B-BLOOMS2 report, the public authorities started to take action by informing the citizens and including cyanobacterial blooms in monitoring studies. Presently, Flemish and Walloon environmental agencies perform limited monitoring of recreational ponds (where bathing or other activities involving water contact are allowed) but using different protocols. This approach precludes the possibility of obtaining a global overview of blooms in Belgium. Furthermore, the data is limited to a small number of waterbodies, as bathing in Belgian surface waters is very restricted. Our study will extend the data from the B-BLOOMS2 study by providing new toxin, molecular and cyanobacterial occurrence data for water samples after a 10-year hiatus, using a uniform protocol. Moreover, the MC quantification revealed the existence of new microcystin congeners.

MCs belong to the most common cyanotoxins group found worldwide and are produced by multiple taxa (e.g., *Microcystis aeruginosa*, *Planktothrix* sp., *Anabaena*/*Dolichospermum*, *Oscillatoria* and *Nostoc*) [463,526]. The MCs covalently bind the protein phosphatases 1 and 2A (PP1 and PP2A) in eukaryotes, inhibiting their functions and eventually causing cell death [527,528,555]. Upon ingestion by mammals, the congeners are primarily transported in the liver cells through specific organic anion transporting polypeptides (OATPS) [118,119,126,556], which results in a hepatotoxic effect, causing nausea, intestinal problems and liver damage [122,527,528,557–562]. These toxins can also effect other organs such as the lungs and kidneys [122,126,563]. Human exposure to the MCs through multiple routes have been described (e.g., drinking water, recreational exposure, cyanobacteria-based food supplements, contaminated crops,…) [3,122,376,548,549,559,564–568]]. The most prevalent congener found in Europe is MC-LR, though it is rarely detected in isolation [21,205,387,406,422,463,569,570]. Therefore, MC-LR is also commonly used in toxicological assays [530,558,559,561,571]. However, other congeners have single or multiple modifications in their structure, such as different amino acids in positions two and/or four, or methylations at different positions [85,89,572]. The structurally different congeners interact differently with the OATPS, PP1 and PP2A, resulting in different toxicities [85,119,120,573–575]. The half maximal inhibitory concentration ($IC_{50}$) for PP2a and lethal dose for half of the test population ($LD_{50}$) *in vivo* for MC-RR are shown to be lower than MC-LR [573–575]. More efficient uptake for MC-LF and MC-WR than MC-LR is suggested to correspond to higher *in vivo* toxicity, while the PP-inhibiting capabilities are comparable [119,126,573,574]. Although differences in toxicity between the congeners

are known, no uniform toxicity equivalency factors are available to adjust for the variation in their activity, as is the case for marine toxins [291,292]. An accurate risk assessment, when several congeners are present, is, therefore, difficult. Thus, the World Health Organization (WHO), Environmental Protection Agency (EPA) and other regulatory agencies use the sum of the concentrations for all MCs, described as MC-LR equivalent (MC-LR Equiv). The WHO published, in 1994, an initial tolerable daily intake (TDI) guideline of 0.04 µg $kg_{bodyweight}^{-1}$ $day^{-1}$, which translated to a concentration of 20 µg $L^{-1}$ in surface waters used for recreational activities [531]. In 2020, the WHO updated its provisional guideline value to 24 µg $L^{-1}$ [122]. The changed value results from a difference in calculations. The original value (20 µg $L^{-1}$) was based on the proposed TDI, the body weight of an adult and the involuntary ingestion of 100 mL of water during swimming activities [3]. The new guideline value is calculated from the no observed adverse effect level (NOAEL) (40 µg $kg^{-1}$ bodyweight) [530], a ten times reduced uncertainty factor compared to the proposed TDI due to the short-term nature of recreational exposure, a volume of 250 mL of involuntarily ingested water, and taking into account the bodyweight of a child instead of an adult. The WHO guideline values are calculated to provide an adequate margin of safety [122]. The US E.P.A. also provided a new guideline for recreational waters in 2019. A reference dose (RfD) of 0.05 µg $kg_{bodyweight}^{-1}$ $day^{-1}$, the mean body weight of children between 6 and 10 years and an incidental ingestion factor were used to calculate 8 µg $L^{-1}$ as the recommended value [576].

Since the B-BLOOMS2 study was finalized a decade ago, no studies with standardized protocols have been performed to monitor cyanobacterial diversity and toxins in water samples from all over Belgium. For the first time in Belgium, we utilized Ultra High Performance Liquid Chromatography with tandem mass spectrometry (UHPLC-MS/MS) to identify and quantify the most frequent microcystin congeners in the water samples. By also detecting the genetic potential for synthesizing MCs and the dominant species in the samples, we tried to obtain more insights concerning the bloom characteristics in Belgium. Our cooperation with the three regional environmental agencies in Belgium achieved a sampling on a wide spatial scale of 79 water samples, covering 23 aquatic ecosystems. The species and toxin profiling by a standard set of analyses reveal the importance of monitoring in space and time. Furthermore, by targeting various waterbodies, we aim to identify the locations where monitoring would be needed because there is a health risk. Additionally, the data could help to design more effective prevention and mitigation measures.

## 5.2. Materials and Methods

UPLC/MS grade solvents (Biosolve B.V., Valkenswaard, Netherlands) were used for extraction or basis for the mobile phase. The MCs standards were ordered as a solid powder from Enzo Life Sciences (Antwerp, Belgium)®, except for D-asp-Dhb-MC-RR from Cyano Biotech GmbH (Berlin, Germany) and Dm-MC-RR from Novakits (Nantes, France). They were initially dissolved in 100% methanol and used to prepare mixed stock solutions in 50% methanol with 1% acetic acid. The dissolved cyanotoxin standards were kept at -20 °C. Whatman GF/C grade filters were obtained from Sigma Aldrich (Overijse, Belgium).

### 5.2.1. Sampling

The sampling was performed from July until mid-September in 2019 at 23 different locations in the three Belgian regions: Wallonia (5 locations), Flanders (7 locations) and Brussels (11 locations). The sampling frequency was dependent on the region, type of waterbodies and access to the lakes (directly or via the environmental agency). Recreational waterbodies are defined as ponds and lakes where bathing is permitted. The water samples were either collected every week or only after a bloom was observed. Each sample was annotated by combining a three digit annotation of the sample site followed by a number giving the week of the year (e.g., XYZ.12). Names for the waterbodies can be found in Table S2.2 in Supplementary data.

In Wallonia, water samples were collected weekly in 5 recreational lakes (I01, I04, E04, B04 and H02), independently of the presence of a bloom following a standardized protocol. The environmental agency Institut Scientifique de Service Public (ISSEP) sampled the surface water with a 500 mL glass bottle at a fixed point. Samples were stored at 4 °C and later transported to Sciensano within three days of the collection for further processing.

In Flanders, water samples were taken from 3 recreational waterbodies (AN1, AN2 and AN3) by the environmental agency Vlaamse Milieu Maatschappij (VMM) only when a bloom was present. The samples were stored in plastic containers at 4 °C before further processing and analysis by Sciensano. Four ponds (GH1, VL1, VL2 and VL3) that were not used for recreation were sampled by Sciensano. GH1 is a sedimentation pond for wastewater, while VL1, VL2 and VL3 are shallow ponds in parks where fishing is allowed. The surface water was sampled with 500 mL sterilized glass bottles. They were processed the same day. In this case, public media had indicated the presence of the blooms. These waterbodies were only sampled a second time by Sciensano if a bloom was still present.

In Brussels, samples were taken from ponds where bathing is not allowed and thus are not considered as recreational waterbodies. Each waterbody was initially sampled after a bloom notification by the regional

environmental agency Environment.Brussels or Port.Brussels. The latter manages the port estate in the Brussels capital region. In total, 8 ponds (BL1-8) and 3 locations in the Brussels canal (BV1-3) were sampled. Each spot was sampled at least a second time independent of bloom presence, except for two spots in the canal. Samples were taken from the surface water with 500 mL sterilized glass bottles. They were processed the same day. An overview of all the sampling sites is shown in Figure 5.1. An overview of the sample sites with waterbody type can be found in the supplementary data (Table S2.2).

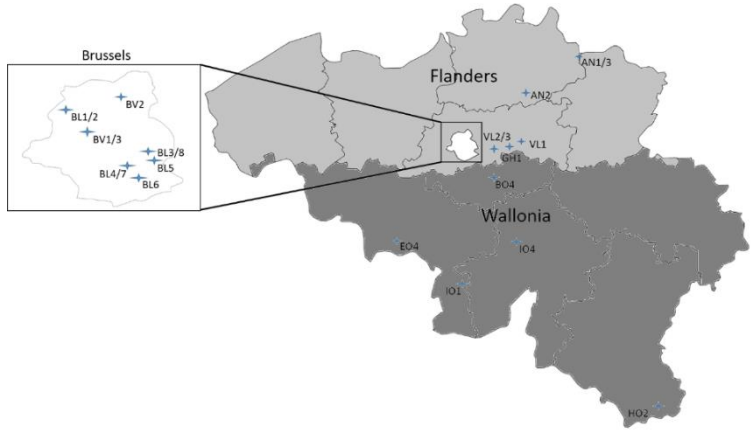



**Figure 5.1.** Map of Belgium showing the sample sites. The first three letters of the sample names are used as abbreviations. In Flanders, 7 sites were sampled (AN1-3, VL1-3 and GH1). In Wallonia, 5 recreational lakes were sampled (I01, I04, E04, B04 and H02). For clarity, the Brussels region is enlarged. Here 8 ponds were sampled (BL1-8), as well as the Brussels canal at 3 different sites (BV1-3). Place names for the waterbodies and their type can be found in Table S2 in Supplementary data.

In general, 150 mL of the sample was filtered on a GF/C Whatman® filter under vacuum to collect the biomass. Lower volumes were filtered due to clogging when dealing with high bloom density. The sample filters were weighed before and after filtration to determine the weight of the wet biomass. The sample filters were stored at −20 °C before analysis. Filtration was performed in duplicate. One filter was used for the quantification of the MCs, while the other was used for the molecular work. The filtrates were collected and stored at −20 °C to determine extracellular toxin concentration.

## 5.2.2. Quantitative analysis of microcystin congeners

### *5.2.2.1. Intracellular and extracellular microcystin extraction*

Only the most common cyanotoxins in Europe (MCs) were selected for our quantification method. Earlier studies in Belgium suggested that these toxins

are the most prevalent ones [368,553]. To properly validate the method, only commercially available MCs were selected.
The method used for analysis was validated in-house. Results of the validation are summarized in Chapter 4 Table 4.3 and Table S2.5 in the supplementary data. In short, the filters, containing biomass, used for toxin extraction underwent a freeze-thaw step and liquid extraction. When the filters were initially stored at −20 °C, they only need to be defrosted. The filters were cut in half and weighted. For the liquid extraction, 4.5 mL 80% methanol was added together with the filter in 50 mL plastic tubes. Solvent and biomass contact was increased by regularly mixing during 1 h. The samples were centrifuged for 10 min at 3900 rpm.
The extract was filtered through a Phenomenex 0.2 µm RC syringe filter (Utrecht, The Netherlands) to remove debris. Samples were stored in a 15 mL plastic tube at −20 °C. Samples with high concentrations of the MCs were diluted after the initial analysis to fit within the range of the calibration curve. The calibration curve was made in a blank matrix.
The sample filtrates (extracellular fraction) were also purified using a Phenomenex 0.2 µm RC syringe filter and analyzed separately through direct injection of 10 µL the Xevo TQ-S, similar to Turner et al., 2018 [388].

*5.2.2.2. Detection and quantification of cyanotoxins*

The detection and quantification parameters were identical for intra- and extracellular toxins analysis. A Waters Acquity UPLC H-class (Eten-Leur, The Netherlands) connected to a Waters XEVO TQ-S was used for the detection of the cyanotoxins. A 1.7 µm, 2.1 mm × 100 mm Waters Acquity BEH C18 column fitted with a Waters Acquity BEH C18 1.7 µM VANGUARD PRE-Col separated the toxins under the influence of a gradient elution program. The fraction of acetonitrile (B) in the eluent changed as followed: 0 min, 2% B; 1.00 min, 40% B; 7.00 min, 55% B; 7.20 min, 98% B; 8.00 min, 98% B; 9.00 min; 2% B; 12.00 min, 2% B. Both Organic and water phases were supplemented with 0.025% formic acid. The flow rate was 0.5 mL $min^{-1}$. The column was heated to 60 °C, and 10 µL of sample was injected.
Multiple reaction monitoring (MRM) was then used to detect the toxins by selectively quantifying compounds within complex samples. The triple quadrupole MS initially targeted the ions corresponding to the toxins of interest, referred to as the “precursor ion”. Two product ions from the collision-induced fragmentation were selected. One was used for quantification of the cyanotoxin, the other as a qualifier. The MS parameters were set according to the literature data and optimized to the instrument setting (Table 5.1). Qualifier and Qauntifier ion fragments are represented in Figure 3.2.

**Table 5.1.** MS/MS parameters for eight microcystin congeners (MCs).

| Toxins | Precursor Ion m/z | Quantifier Ion m/z | Collision Energy (eV) | Cone Voltage (V) | Qualifier Ion m/z | Collison Energy (eV) | Cone Voltage (V) |
|---|---|---|---|---|---|---|---|
| MC-LR | 995.4 | 135.0 | 70 | 80 | 213.1 | 60 | 80 |
| MC-RR | 519.8 | 134.8 | 30 | 50 | 107.2 | 60 | 50 |
| MC-YR | 1045.5 | 135.3 | 80 | 60 | 212.9 | 60 | 60 |
| MC-WR | 1068.4 | 135.3 | 70 | 100 | 213.1 | 60 | 100 |
| MC-LY | 1002.4 | 135.4 | 60 | 50 | 213.0 | 50 | 50 |
| MC-LA | 910.3 | 135.1 | 60 | 50 | 107.1 | 80 | 50 |
| MC-LF | 986.3 | 135.0 | 60 | 70 | 213.1 | 60 | 70 |
| MC-LW | 1025.4 | 134.9 | 60 | 60 | 213.1 | 50 | 60 |

After quantification, the concentration for each cyanotoxin was recalculated to µg $L^{-1}$, corrected with the mass of the original mass of the filter and the filtered volume of the sample. The concentration of each MC in the filtrate (extracellular fraction) was added to the final concentration of the MC extracted from the biomass. Thereafter, the sum of all the congeners was calculated to provide a µg $L^{-1}$ total microcystin value.
Congener proportions to the total MCs concentration were calculated in each sample. The differences in proportions for the separate congener were then compared for Belgium using the Wilcoxon test at $\alpha < 0.05$. Additionally, the same statistical test was used to compare the difference in congener proportions for samples containing MCs concentration higher and lower than 24 µg $L^{-1}$ total microcystin, separately. The samples without MCs were excluded. Proportions of MC-LR, MC-RR and MC-YR were also compared between the two concentration ranges.

## 5.2.3. Molecular analysis of the 16S rRNA and the mcyE gene

*5.2.3.1. DNA extraction*

First, 0.8 mL lysis buffer (40 mM EDTA 5, 50nM Tris-HCl, 0.75 M sucrose) was added to each sample filter (containing biomass), and a bead-beating step (at 30 m $s^{-1}$ for 30 s) was performed. Then, a lysozyme (Sigma-Aldrich, St. Louis, MI, USA) (20 mg $mL^{-1}$) digestion for 30 min at 37 °C was followed by a treatment with 22.22 mg $mL^{-1}$ proteinase K (Macherey-Nagel, Düren, Germany), supplemented with 80 µL SDS (100%), for 2 h at 55 °C. The lysate was transferred to a new Eppendorf tube. Subsequently, the filters were rinsed with 1 mL lysis buffer during a 10 min incubation at 55 °C. The second lysate was stored in another Eppendorf tube.
A Phenol-chloroform-isoamyl alcohol solution (25:24:1, pH 8) (VWR, Leuven, Belgium) was added in an equal volume to the extract volume (V:V) to both

lysates. Next, the samples were centrifuged at 14,000× *g* for 15 min. The upper phase of each tube was transferred to a new Eppendorf tube, and chloroform-isoamyl alcohol (24:1, pH 8) was added V:V. The tubes were centrifuged again at 14,000× *g* for 15 min to collect the upper phase. For each sample, the two lysates were combined.
Finally, the DNA was precipitated with 0.1 V:V of sodium acetate (3 M, pH 5.2) and 0.6 V:V of cold isopropanol. After centrifugation, the DNA was rinsed once with 300 µL ice-cold ethanol (Merck, Branchburg, NJ, USA) (100%) and once with 300 µL ice-cold ethanol (70%). The supernatant was removed, and the pellet was air dried. Finally, the DNA pellet was dissolved in 100 µL TE buffer (10mM Tris-HCl and 1mM EDTA, pH 8) and stored at −20 °C.

*5.2.3.2. Gene amplification of partial rRNA and mcyE gene sequences*

For the rRNA gene sequences, two protocols were tested. A long rRNA fragment was amplified with the cyano-specific primers 359F/23S30R [110] using the SuperTaq Plus© enzyme (HT Biotechnology, Cambridge, UK), buffer and dNTPs obtained from SpharoQ (NL). The amplification program was 95 °C—5 min, 35 times; 95 °C—30 s, 57 °C—45 s, 68 °C—1 min; followed by 69 °C—5 min, 16 °C—infinite. As a shorter PCR product could give a higher amplification efficiency, the primer pair 359F/781R [111] was tested later. However, the SuperTaq Plus© enzyme was no longer commercialized and was replaced by the Q5 High Fidelity polymerase (New England Biolabs, Ipswich, MA, USA) for the majority of the PCR reactions. The amplification program was: 98 °C—5 min, 35 times; 98 °C—30 s, 65 °C—45 s, 72 °C—1 min; 72 °C—5 min, 16 °C—infinite. The *mcyE* gene involved in the production of MCs was amplified with the primer pair mcyEF2/mcyER4 [64] using the SuperTaq Plus© enzyme. The amplification program was 94 °C—3 min, 30 times; 94 °C—30 s, 57 °C—45 s, 68 °C—1 min, followed by 68 °C—10 min and 16 °C infinite, as described in the final report of B-BLOOMS2 [368]. Amplifications were performed in a Thermal cycler T100 (Bio-Rad, Hercules, California, USA). The presence of PCR products of the right size was visualized by electrophoresis on a 1.5% agarose gel during a 95 min run at 90 V.

*5.2.3.3. Sanger sequencing and sequence analysis*

After PCR, the 16S rRNA amplicons were sent for Sanger sequencing with primers 359F, 781R or 23S30R at Giga Genomics (ULiege) [60,456]. Some sequences were of bad quality, which prohibited further analysis. These sequences probably resulted from a mixture of organisms without clear dominance by one taxon. The forward and reverse sequences were not obtained in all cases for each PCR product, and therefore, the individual sequences of a single strand were used for further analysis, admitting that some sequencing errors might be present but that the quality would be sufficient to determine the dominant genus. In three cases, the sequences

obtained on different PCR products (short or longer ones) for the same sample were affiliated to different genera. The sequences used during the further analysis can be found in the supplementary data (Table S2.7).
The NCBI nucleotide BLAST (basic local alignment search tool) was used to identify the most closely related strain sequences for the 16S rRNA sequences, using individual sequences obtained by the different primers tested and the identification was based on this data, as shown in Table S2.3.

*5.2.3.4. Amplicon Sequencing with the Illumina Technology*

For samples BL5.29 and VL1.36, partial 16S rRNA gene sequences were obtained by PCR using the primer set CYA359F and CYA781Ra/CYA781Rb, which amplifies the V3-V4 region of the cyanobacterial 16S rRNA gene [60]. Primers were modified to include a 10-bp sample-specific barcode tag at the 5' end to allow samples to be multiplexed for sequencing. PCR reactions were performed in triplicates in order to minimize the influence of amplification biases. These were pooled to equivalent concentrations and purified using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany). Purified samples were sent to Genewiz (South Plainfield, NJ, USA), where sequencing adapters were ligated to the amplicons and sequencing was performed using Illumina MiSeq (Illumina, San Diego, CA, USA) using 2 × 300 bp paired-end libraries. The bioinformatic analysis is adapted from a validated method by Pessi et al. and consists of processing raw reads to remove chimeric sequences, followed by the clustering into an operational taxonomic unit (OTU) [62]. Briefly, paired-end reads were merged, filtered and only reads containing both barcodes in the 3' and 5' ends were kept. Two and zero mismatches were allowed to the primer and barcode sequences, respectively, and reads with a maximum expected error of more than 0,5 and a length of less than 370 bp were removed. Singletons were removed, and remaining quality-filtered sequences were denoised to remove chimaeras and sequencing errors using unoise3 [65]. The denoised operational taxonomic units (ZOTUs) obtained were then clustered into OTUs at a 99% similarity threshold [66]. The representative sequence of an OTU is the most abundant unique sequence of each OTU cluster. Taxonomic classification was performed by extracting from Genbank the most closely related sequences of each OTU using BLAST.

## 5.3. Results

### 5.3.1. Toxin quantification

In 86.08% (68/79) of the water samples, at least one of the quantified MCs (MC-RR, MC-LA, MC-LF, MC-LR, MC-LY, MC-LW, MC-YR, MC-WR) could be found above the limit of quantification (LOQ = 12.50 µg $kg^{-1}$ before correction for the total sample weight and volume). Moreover, 22.78% (18/79) of the samples contained toxin concentrations higher than 24 µg $L^{-1}$ total microcystin [122]. A complete overview of the results can be found in Table S2.1 in the supplementary data. The concentration range was between 0.11 µg $L^{-1}$ and 2798.81 µg $L^{-1}$ total microcystin.

Furthermore, 82.61% (19/23) of the waterbodies contained, at least once during the summer, a quantifiable concentration of toxin (>LOQ), whereas concentrations higher than 24 µg $L^{-1}$ total microcystin were detected in 34.78% (8/23) of the waterbodies.

Interestingly, the canal samples (BV1, BV2, BV3) from Brussels contained congener concentrations higher than LOQ. One of the samples even reached 1831.32 µg $L^{-1}$ total microcystin. These results are the first reports on blooms in Belgium waterways.

Validation results for the UHPLC-MS/MS method used for analysis of the water samples can be found in Chapter 4 Table 4.3 and Table S2.5 in the supplementary data.

### 5.3.2. Toxin congener diversity

The detection frequency of MC-RR was the highest (84.81%), followed by the detection frequencies of MC-LR (81.01%) and MC-YR (50.63%).

MC-LR, MC-RR and MC-YR also contributed the most to the total MCs concentration in individual samples, compared to the other congeners. When comparing the proportions of the highest contributors in Belgium, the proportion of MC-RR was significantly higher than MC-LR, based on the Wilcoxon signed-ranked test ($\alpha < 0.05$). Proportionally, MC-LR is the second-highest contributor, followed by MC-YR. The Wilcoxon test shows a significant difference ($\alpha < 0.05$) between the proportional contributions of MC-YR compared to the other congeners (Figure 5.2a). Separate statistical analysis of samples containing a total MC concentration above or below the WHO guideline value for recreational water showed that the proportions of MC-YR are also significantly lower in relation to the proportions of MC-LR and MC-RR both above or below the 24 µg $L^{-1}$ total microcystin (Figure 5.2b,c).

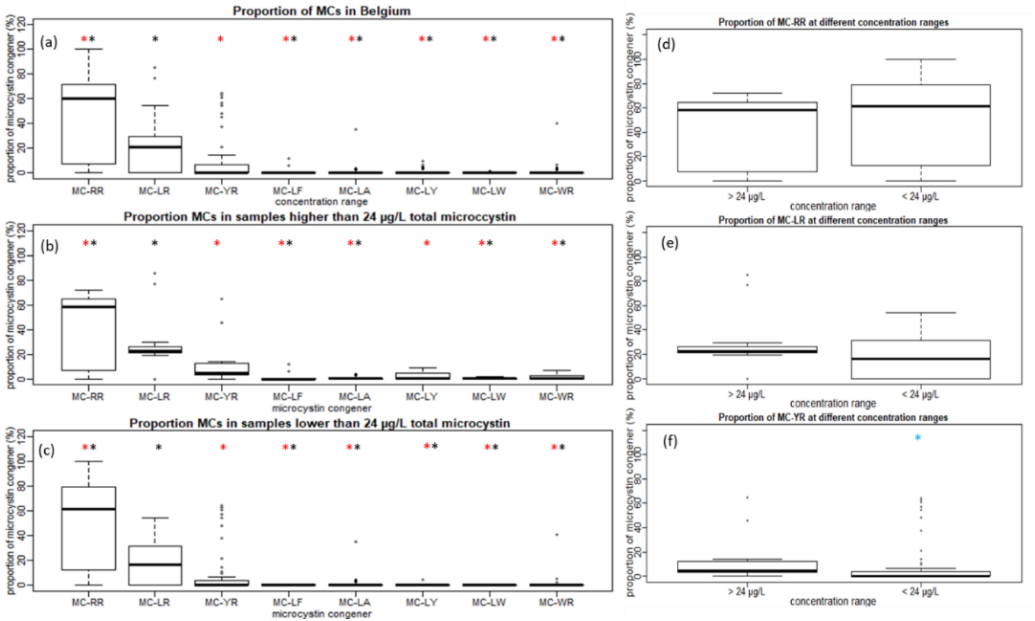



**Figure 5.2.** (**a**) The distribution of the proportion of microcystin congeners (MCs) calculated at an individual sample level for all Belgian samples. (**b**) Samples with concentrations higher than 24 µg $L^{-1}$ total microcystin. (**c**) Samples with concentrations lower than 24 µg $L^{-1}$ total microcystin. (**d**) Proportions of MC-RR are compared in samples below and above the World Health Organization (WHO) guideline value. (**e**) Proportions of MC-LR are compared in samples below and above the WHO guideline value. (**f**) Proportions of MC-YR are compared in samples below and above the WHO guideline value. * Proportion of MC is significantly different from MC-LR at $\alpha < 0.05$ using the Wilcoxon test. * Proportion of MC is significantly different from MC-YR at $\alpha < 0.05$ using the Wilcoxon test. * Proportion of MC is significantly different from the proportion of MC at concentration range > 24 µg $L^{-1}$ total microcystin with $\alpha < 0.05$ using the Wilcoxon test.

When comparing samples with a total MC concentration above or below the guideline value, several observations can be made. There was a significant difference in MC-YR (Figure 5.2f) but no significant difference in the proportions of MC-LR and MC-RR (Figure 5.2d,e). Comparing the two concentration ranges for the proportions of MC-LA, MC-LY, MC-LF and MC-LW, a significant difference was shown using the Wilcoxon signed-rank test ($\alpha < 0.05$) (data not shown). A higher diversity of congeners contributed to the total MCs concentration when the concentration was above the WHO guideline value for recreational water (24 µg $L^{-1}$ total microcystin) (Figure 5.2b,c).

Additionally, the water samples were screened for six other MCs (MC-HtyR, dm-MC-LR, D-asp- MC-LR, dm-MC-RR, D-asp-Dhb-MC-RR and MC-HilR), which are also commonly detected in other studies [391,427,553]. These toxins were not included in the initially designed validation process. However, due to their prevalence and possible toxicity, they were screened. The congeners are identified based on molecular mass, production ions and elution time with the UHPLC-MS/MS method. However, dm-MC-LR and D-asp-MC-LR as well as dm-MC-RR and D-asp-Dhb-MC-RR could not be separated based on these parameters and are reported together (Table 5.2). The limits of detection for the congeners were further established, shown in Table 5.2. Overall, dm-MC-RR/D-asp-Dhb-MC-RR were the most abundant congeners in the water samples, followed by dm-MC-LR/D-asp- MC-LR, MC-HilR and MC-HytR, sequentially. An overview of their detection frequency can also be seen in Table 5.2, as well as their frequency related to the total quantified microcystin concentration in the samples. A complete overview of the results per sample can be found in Table S2.6 in the supplementary data.

**Table 5.2.** Overview of precursor ion, product ions and limit of detection for not validated microcystin congeners. Additionally, the table also includes the detection frequency of the congeners in the analyzed samples at different total microcystin concentrations.

| **Toxins** | **MC-HtyR** | **dm MC-LR / D-asp MC-LR** | **D-asp-Dhb MC-RR / dm MC-RR** | **MC-HilR** |
|---|---|---|---|---|
| Precursor ion | 1059.5 | 981.1 | 512.7 | 505.3 |
| Product ions | 106.9; 135.3 | 106.8; 135.1 | 103.2; 135.1 | 127.0; 134.9 |
| Limit of Detection (μg $L^{-1}$) | 0.10 | 0.10 | 0.10 | 0.10 |
| All samples | 13.92% | 53.16% | 77.22% | 34.18% |
| Samples < 1 μg $L^{-1}$ total microcystin | 0.00% | 9.38% | 50.00% | 0.00% |
| Samples > 1 μg $L^{-1}$ total microcystin | 23.40% | 82.98% | 95.74% | 57.45% |
| Samples < 24 μg $L^{-1}$ total microcystin | 25.00% | 100.00% | 100.00% | 100.00% |

## 5.3.3. Molecular analysis of water samples

PCR amplification of the 16S rRNA fragment was attempted for 76 water samples. The fragment was successfully amplified for 45 samples. Some water samples were amplified twice (e.g., BL1.29, BV1.34, B04.29, I04.32), as can be seen in Table S2.3. Direct Sanger sequencing of the 16S rRNA fragment from the water samples resulted in 49 sequences of sufficient quality that could be analyzed with BLAST. They were of cyanobacterial origin, except for one plastid sequence in sample E04.32. The majority (42/49) of the analyzed 16S rRNA fragments showed 97% or higher similarities to sequences found in Genbank, as shown in Table S2.3. However, not all samples had a single dominant species, and 31 failed sequencings corresponded to mixtures of sequences that could not be analyzed. In four cases, the PCR with different primer pairs gave different dominant genera, and both are shown in Table S2.3. For all the samples analyzed with direct Sanger sequencing, *Microcystis* was the most dominant genus (12/76 or 15.79%), closely followed by *Dolichospermum* (11/76 or 14.47%). The third most abundant genus was *Aphanizomenon* (8/76 or 10.53%). Furthermore, the *Synechococcus* and *Planktothrix* genera were dominant in five and three samples, respectively. The *Cyanobium* genus was observed twice, while the *Cyanodictyon, Romeria* and, *Phormidium* genera were found once.

The amplicon sequencings by Illumina indicated a dominance of sequences from five OTUs belonging to the *Dolichospermum* genus for sample BL5.29 (71% of the reads), followed by *Microcystis* (20.5% of the reads) and a minor

fraction of *Aphanizomenon* and *Cyanobium*/*Synechococcus*. This corresponds to the dominance of *Dolichospermum* inferred from the direct Sanger sequencing. In contrast, sample VL1.36 was completely dominated by sequences of *Microcystis* (99.1% of the reads) followed by 0.60% of the reads affiliated to *Dolichospermum*. However, the direct Sanger sequencing did not give any readable sequence to compare (Table S2.4).
The dominance of *Microcystis* in VL1.36 coincides with a high diversity (seven microcystin congeners) and a high concentration of total microcystin (128.93 µg $L^{-1}$). In contrast, only two congeners and a lower total microcystin concentration (1.22 µg $L^{-1}$) were found in the *Dolichospermum* dominated bloom BL5.29 (Table S2.1).
The *mcyE* gene amplification was tested in 70 water samples. The *mcyE* gene was detected together with MCs in 71.43% of the samples. Moreover, 4.29% of the samples contained the *mcyE* gene though the presence of MCs could not be detected by UHPLC-MS/MS method. However, the presence of the *mcyE* gene only implies the potential to produce MCs. Microcystin will not be produced when *mcyE* or other genes of the *mcy* gene cluster are lacking, are silenced or contain mutations.

### 5.3.4. Cyanobacteria dominance at different MCs concentrations

In the water samples, there was a dominance of the genera *Microcystis*, *Dolichospermum*, *Cyanobium/Synechococcus*, *Aphanizomenon* and *Planktothrix*. Sequences affiliated with *Cyanodictyon*, *Romeria*, and *Phormidium* were each observed in one of the samples. In samples with quantified MCs below the WHO guideline value, *Aphanizomenon*, *Dolichospermum*, *Microcystis*, *Synechococcus*/*Cyanobium*, *Planktothrix* and a plastid were identified as dominant (Figure 5.3a). For water samples that contained MCs concentrations above the WHO guideline value for recreational waters, primarily *Microcystis* or *Dolichospermum* could be identified as dominant species, based on the direct Sanger sequencing (Figure 5.3b). However, in both cases, it was not always possible to determine the dominant taxon, as the direct sequencing was not successful. When MCs were present in samples where *Dolichospermum* was dominant, concentrations ranged between 0.67 to 2420.91 µg $L^{-1}$ total microcystin, while for samples with *Microcystis* as dominant species, concentrations ranged from 1.07 to 2798.81 µg $L^{-1}$ total microcystin. Samples, dominated by *Aphanizomemon*, contained concentrations between 0.11 and 4.35 µg $L^{-1}$ total microcystin. In two water samples from lake H02, where *Planktotrix* was dominant, total microcystin concentrations were 4.05 and 2.80 µg $L^{-1}$. In contrast, a concentration of 250.35 µg $L^{-1}$ total microcystin was quantified in a water sample from lake I04 containing *Planktothrix*. However, the origin of the MCs can be debated as a week earlier, *Microcystis* was abundant in lake

I04, and the bloom could have been in decline, as further shown in Figure 5.4a. Our results cannot support definitive conclusions that would link high toxin concentrations to a specific cyanobacterial taxon. However, higher total microcystin concentrations are observed when *Microcystis* or *Dolichospermum* are the dominant taxa.

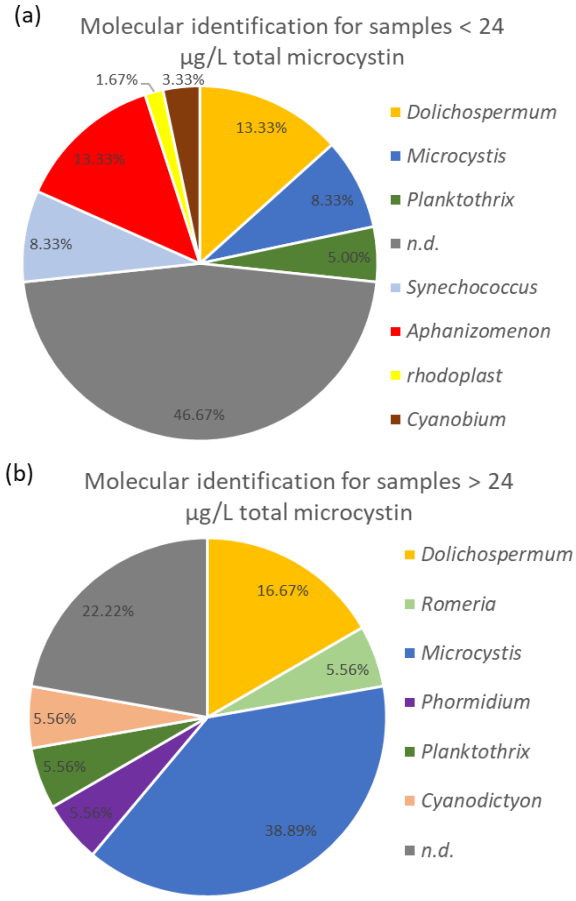



**Figure 5.3.** Identification of species using direct Sanger sequencing and BLAST analysis. Samples were divided based on the WHO guideline value for recreational ponds (24 µg $L^{-1}$ total microcystin (MC)). The "n.d." abbreviation refers to not exploitable 16S rRNA sequences. (**a**) Species distribution for samples containing total MCs concentration below the WHO guideline value. (**b**) Species distribution for samples containing total MCs concentration above the WHO guideline value.

## 5.3.5. Monitoring of walloon recreational Lakes

The weekly sampling of the Walloon lakes provided an opportunity to look at the evolution of the toxin concentration and dominant species with time. The samples from lake I01 (Falemprise) showed a total microcystin concentration higher than 1 µg $L^{-1}$ (I01.31) only once, and when the direct Sanger sequencing was possible, the dominant cyanobacteria belonged to the unicellular *Synechococcus*/*Cyanobium* (Table S2.1). This lake was designated as a reference recreational lake during the B-BLOOMS2 study and has been regularly monitored since then. In lake B04 (Renipont plage), the concentration of MCs only rose slightly above 1 µg $L^{-1}$ in three instances when the potentially toxic cyanobacteria genera, *Aphanizomenon* and *Planktothrix* were found (Table S2.1). MC concentrations in the samples from H02 (Saint Léger sport complex) never reached the WHO guideline for recreational use but were slightly increasing over the summer and peaked at 4.35 µg $L^{-1}$ at the end of August, coinciding with the presence of the potentially toxic *Aphanizomemon* genus. However, the values decreased in the following weeks (Table S2.1). The two lakes where the WHO guideline value was exceeded were E04 (Grand Large, Mons) and I04 (Lac de Bambois). There was an increase in MCs over the summer, ending with a decrease in September in these samples. However, in the latter lake, the MC values were much higher, reaching 250.35 µg $L^{-1}$, and the decrease was more gradual. For both lakes, *Microcystis* sp. was prevalent in the samples just before the MC increases (Figure 5.3a,b). However, in lake I04, a higher diversity of potentially toxic genera was detected by direct Sanger sequencing, *Aphanizomenon*, *Dolichospermum* and *Planktothrix* (Figure 5.3a).

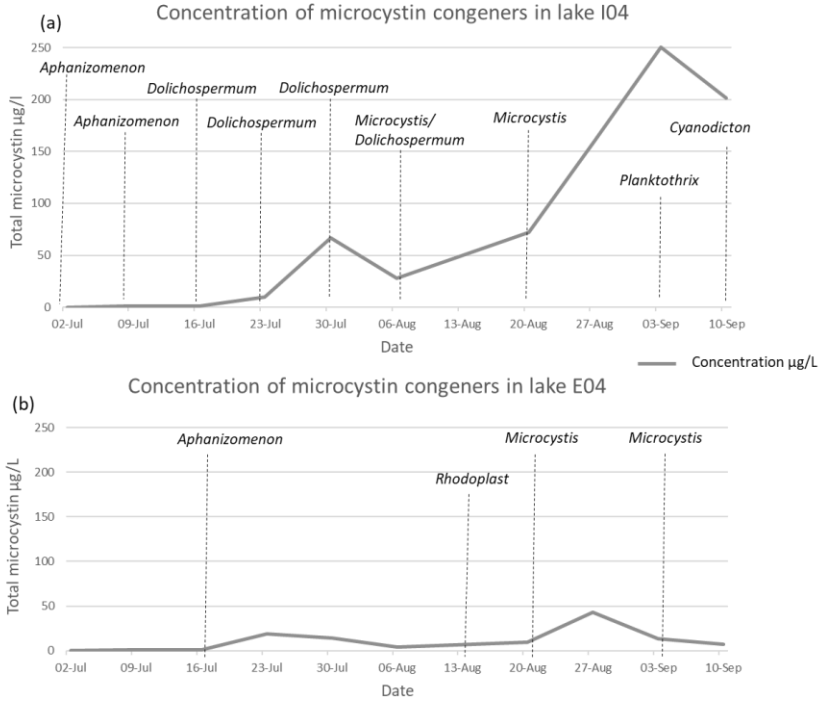



**Figure 5.4.** (**a**) Evolution of total microcystin concentrations in Lake I04 (lac de Bambois, Fosses-La-Ville) during the summer of 2019. Dominant genera detected in the samples are also indicated. (**b**) Evolution of total microcystin concentrations in Lake E04 (Grand large, Mons) during the summer of 2019. Dominant genera detected in the samples are also indicated.

## 5.3. Discussion

For the first time since the B-BLOOOMS2 study, microcystin congeners in Belgian surface waters have been reliably quantified by a standardized, state-of-the-art analytical method in all three Belgian regions. Moreover, unmonitored waterbodies in the Brussels region were included in the study and provided additional proof of toxic blooms.

True monitoring data was achieved in Wallonia due to the weekly sampling of five recreational lakes during the summer of 2019. Three recreational waters (H02, I01, B04) showed MC values lower than the WHO guideline. Falemprise (I01) and Bambois (I04) had already served as a reference recreational lake with regular monitoring and a sporadically analyzed lake, respectively, during the B-BLOOMS2 study. Falemprise had shown quite a

variable planktonic diversity, with a regular presence of potentially toxic taxa, the *mcy* genes and total microcystin concentrations ranging from 0.12 to 6.11 µg $L^{-1}$. In Bambois, *Anabaen*a (now *Dolichospermum*) formed blooms with MCs concentrations lower than 2.6 µg $L^{-1}$ [368]. In the present study, both lakes contained MCs, showing a persistent problem. Lake Bambois presented the highest values for the five Walloon lakes and exceeded the WHO guideline value for recreational use between the end of July to the middle of September (weeks 31 to 37). These results seem to indicate an increase in toxin concentration during the bloom events. *Aphanizomenon* and *Dolichospermum* were observed before the start of the MCs peaks.

Globally, 34.78% of the studied waterbodies contained total microcystin concentrations higher than the suggested guideline for recreational waters. The highest values were observed in waterbodies in Flanders. These samples also contained the highest fresh weights of filtered biomass (20.40 $10^{-6}$ g $L^{-1}$ average) compared to Brussels (4.60 $10^{-6}$ g $L^{-1}$ average) and Wallonia (2.30 $10^{-6}$ g $L^{-1}$ average). However, high concentrations of total microcystin could not always be linked to a high fresh weight of the biomass, as shown in Table S2.1. The total microcystin range (0.11 µg $L^{-1}$ to 2798.81 µg $L^{-1}$) found in 68 of the 78 analyzed water samples is similar to the one found a decade ago during the B-BLOOMS2 study (0.120 µg $L^{-1}$ to 37500 µg $L^{-1}$ total microcystin) by HPLC or ELISA [368]. The specific screening can explain the difference in certain MCs, resulting in underestimating the actual concentration, as the screening method only includes a selection of congeners and might miss certain ones. The ELISA assays, performed during the B-BLOOMS2 study, have the advantage of detecting all the possible hydrophobic Adda groups specific for MCs (Figure 1.4) in the samples. In contrast, our triple quadrupole MS/MS method is very specific and will detect only the targeted toxins selected a priori in the detection method. This type of method is not well suited for a full scan analysis of samples. Worldwide, total microcystin concentrations can reach a maximum of 42.7 mg $L^{-1}$, although concentrations are usually lower [205,364,387,391,402,427,539,570,577–582]. For instance, during a sampling campaign in the United Kingdom, only 18% of the samples contained a concentration above 20 µg $L^{-1}$ total microcystin [387].

In terms of congener diversity, MC-RR (84.81%), MC-LR (81.01%), and MC-YR (50.63%) were most prevalent in our study. Earlier, MC-LR was found to be the dominant congener (in 64% of analyzed water samples) in Wallonia and Luxemburg by Willame et al. [553]. MC-YR was the second most common congener, while MC-RR was not found [20]. Other reports confirm the presence of these most abundant congeners (MC-LR, MC-RR and MC-YR) in western Europe [202,205,387,391,427,526,570,583,584].

Moreover, five MCs (MC-LA, MC-LF, MC-WR, MC-LW and MC-LY) were also quantified in lower concentrations, while six other MCs (MC-HytR, dm-MC-LR, D-asp- MC-LR, dm-MC-RR, D-asp-Dhb-MC-RR and MC-HilR) were only

screened and not quantified during our study. For the five quantified MCs, only a minor contribution to the total microcystins concentration at different concentration levels was shown. The MCs that were not quantified were primarily present in samples with total microcystin concentrations above the WHO guideline limit (24 µg $L^{-1}$). The lower contribution or presence of these eleven congeners at higher total microcystin concentrations are consistent in the samples from independent waterbodies and suggest that the biosynthesis dynamics of these toxins is different compared to the more abundant congeners (MC-LR, MC-RR, MC-YR). However, shifts in environmental factors, cyanobacterial strain diversity, growth phase and toxin production dynamics could influence the overall toxin diversity and quota in the blooms [585].

Overall, the minor contribution or presence of these eleven MCs (quantified or not) to the total MC concentration might be of minor importance for the degree of health risk compared to the more abundant MCs (MC-LR, MC-RR and MC-YR). In most cases, the sum of the MC-LR, MC-RR and MC-YR concentrations already exceeded the new WHO guidelines for microcystin, without the contributions of the other congeners (Table S2.1) [122]. However, different geographical origins, species and environmental factors might influence which congeners are more abundant. Analysis of the most common congeners during monitoring is therefore still advisable [205,391,422,538,553,570,579,580,586].

Incorporation of the toxic demethylated congeners in the quantification methodology would be similarly advisable in the future [549].

Besides toxins, *mcyE* was detected in almost 76% of the water samples. Comparing these results is difficult due to differences in the monitoring frequency and amplification method. For instance, the B-BLOOMS2 study found *mcyE* in 89% of the analyzed samples, but the number of samples was larger due to more regular monitoring in surface waters prone to bloom events and the addition of occasional bloom samples [368]. Similarly, Moreira et al. (2020) also found variations in *mcyE* presence in various lakes in Portugal. During their sampling from April to September, the *mcyE* detection rate varied from 33.3% to 83.3% for different lakes, but they used a different amplification method than our study [364]. A good correlation between toxin content and the m*cyE* compared to other genes has been shown in the multiple studies reviewed in Pacheco et al. [369]. The authors also pointed out that correlations between *mcyE* copy number and toxin concentration are still controversial. Nonetheless, this data supports the need for our approach of assessing the potential of toxin production by the detection of the *mcyE*.

With the direct Sanger sequencing approach, only the most abundant species would be detected if there was a strong dominance in the sample. Indeed, if the cyanobacteria populations were too heterogeneous, the direct

sequencing would fail because the resulting chromatograms would not be interpretable. Therefore, only 45 out of 79 samples produced usable data due to species heterogeneity. Nevertheless, direct Sanger sequencing showed that *Microcystis, Dolichospermum/Anabaena, Synechococcus/Cyanobium, Planktothrix* and *Aphanizomenon* were generally observed in the samples, as earlier described in the B-BLOOMS2 study. However, *Woronichinia*, also present during B-BLOOMS2, was not detected. Our sampling season, which focused on the warmer months of the year, might have missed the occurrence of *Woronichinia*, which is more prevalent at temperatures below 15 °C [587]. Yet, they might also be present in the more heterogeneous samples but could not be detected with our current methods. Furthermore, the *mcyE* gene was found in samples containing *Cyanobium*, which raises the question of the possible toxicity of this genus, as discussed during B-BLOOMS [368]. Little information is available about the toxicity of picocyanobacteria in eutrophic waters. *Cyanobium* have previously been identified in freshwater ecosystems [588,589], although their toxicity has been put in question. Multiple studies showed low amounts of MCs being produced [221,548,589,590], while the sequenced genomes of *Cyanobium* strains seem to lack a complete NRPS/PKS gene clusters [236].

Amplicon sequencing by a high-throughput sequencing technique is an alternative to direct Sanger sequencing that allows the identification of species in complex populations, allowing the study of heterogeneous samples. However, amplicon sequencing protocol is more expensive and time-consuming. Thus, it was tested in two water samples, of which one (BL5.29) was used for both sequencing types. This comparison shows that the dominant taxon was quasi-identical with both methods. Indeed, the Sanger sequence was 99.8% similar to the most similar hit for the dominant OTU72 (*A. ellipsoides* Ana HB).

The samples from waterbodies were either collected through one of the regional monitoring programs or following a sporadic bloom notice by the regional environmental agencies in an unmonitored waterbody. The samples from Wallonia and some of the samples from Flanders were obtained from already established monitoring programs for recreational waterbodies, whereas the samples from ponds in Brussels were obtained specifically for the study when a bloom notice was received. In the cases with monitoring programs, the situation is followed by the public authorities and the recreational waterbodies in both regions get regularly closed if (toxic) blooms are observed. In the not monitored ponds in the Brussels region, we found that 66.67% and 16.67% of the samples were above LOQ and 24 µg $L^{-1}$ total microcystin, respectively [3,531]. By comparison, the total MC concentration rose above LOQ and 24 µg $L^{-1}$ total microcystin in 86.08% and 22.78% of the Belgian waterbodies, respectively, at one point in time. Even though the numbers seem to be lower in Brussels compared to the rest of the country,

this is probably an artifact and experimental bias. Namely, in Brussels, only a small number of waterbodies was sampled. Furthermore, these results were obtained outside the standardized protocol and from only three or fewer time points per site. Therefore, bloom peaks could have been missed during the summer. As we reported, the canal in Brussels also contained a total of 1831.32 µg $L^{-1}$ total microcystin at one sampling spot. Currently, there is a public dynamic in the region to make more waterbodies available for recreational use during the summer. Moreover, the unauthorized use of waterbodies as bathing areas was also common practice during hot summers. Without proper monitoring, this could create a public health risk for humans and domestic animals [128]. In addition, other usages of this water need to be considered. For instance, pond water could be used to irrigate urban vegetable gardens, and water from canals could be used in agriculture. Several research groups have already shown that plants could accumulate MCs when irrigated with contaminated water [392,398,565,566,591]. The connection between water usage and toxin accumulation in plants needs to be further investigated in the future.

Based on our results, it appears that monitoring of any potential bathing sites where unauthorized bathing occurs could be recommended. In the context of Belgium, this would be in the Brussels region. Monitoring could reveal links between public health issues and any potential hazard related to cyanobacterial blooms. The other two Belgian regions monitor their official bathing sites, using different sampling protocols and analysis techniques. Harmonizing the monitoring methods could provide insight into the species and toxin diversity in the blooms in Belgium. Moreover, it might help to uncover environmental drivers that promote the blooms. Techniques used during the monitoring could vary depending on the expertise and resources that are available. Cell counting and species identification are relatively low tech monitoring tools to determine the intensity of the blooms and quantify the potential toxin-producing species, but they are time-consuming and need taxonomic expertise. ELISA and *mcyE* amplification are fast, relatively cheap tests appropriate for screening MCs or MC-production potential, respectively, when a bloom is observed. However, these four techniques need to be supplemented with UHPLC-MS/MS to accurately determine the concentrations of each microcystin congener separately during and after the bloom to ensure public safety. When toxin equivalency factors become available for the different congeners, UHPLC-MS/MS approaches will be even more crucial to accurately determine the risk.

More detailed information about the bloom incidences in a country can also benefit possible mitigation strategies in the future. Preventive strategies could be designed to reduce the influx of nutrients where this is feasible. This strategy requires information about the sources of eutrophication, which is still lacking for most fresh waterbodies in Belgium. During this study; the

regional environmental agency listed agriculture, the discharge of purified sewage water, water influx by a canal and the feeding of fish during recreational fishing as potential sources of eutrophication in the Walloon lakes. For smaller ponds in Brussels, an overpopulation of waterfowl can cause nutrient loading due to an abundance of excrement. Another mitigation strategy is hydrogen peroxide treatments. This treatment can eliminate the bloom and MCs but needs to be optimized based on bloom density and the quantity of toxins. For toxin quantification and assistance to prevention, analytical methods, such as the one presented in this paper, would be suitable [482,483,486–488,592–595]. Approaches such as flock lock or flock sink techniques could be a viable solution to prevent bloom incidence by capturing phosphorous on the bottom of larger waterbodies with a greater depth. However, the sediments of shallow recreational lakes might be too frequently disturbed for this approach to be effective [489–492]. External phosphorous loading in the waterbodies after treatment (e.g., sewage disposal, floods,…) will also undermine the effectiveness of these treatments. To ensure public safety, monitoring of waterbodies will always be necessary and toxin quantification should be included as a part of the monitoring techniques.

## 5.4. Conclusions

This study described the validation and application of an UHPLC-MS/MS method for the first time to analyze Belgian water samples from the three different regions with an identical method. The microcystin concentrations found clearly illustrate a persistent problem of toxic blooms throughout Belgium with a potential health impact. The three most abundant MCs (MC-RR, MC-LR, MC-YR) contributed the most to the total microcystin concentrations. Our fast and efficient method can be applied to monitoring programs in Belgium and other parts of the world. PCR amplification of the *mcyE* gene linked its occurrence to the toxin presence for 71.43% of the water samples. Moreover, the dominant blooming taxa were also determined in a number of samples. Interestingly, this study also characterized a cyanobacterial bloom in a Belgian canal for the first time. The abundance of water samples that contained MCs shows the need to enlarge the sampling of waterbodies where there could be a risk of human exposure and include them in existing or new monitoring programs.

**Supplementary Data:** Data is available in Chapter S2. Table S2.1. Overview of experimental data for the samples, concentrations of MC congeners and total microcystin (µg $L^{-1}$), presence of genes coding for 16S rRNA and *mcyE*, molecular identification, coverage and identity % of the most similar hit by BLAST and primer used for Sanger sequencing. Table S2.2. Overview of

sampling sites in the different regions, sample annotation, waterbody type and specific monitoring for cyanobacteria. Table S2.3. Detailed information for taxonomic identification based on BLAST analysis. * Samples for which PCR with different primers gave different dominant taxa. Table S2.4. Number of reads and BLAST analysis of the OTUs obtained with the Illumina amplicon sequencing. Table S2.5: Results of the ion ratio for the validation of the UHPLC-MS/MS method quantification method of 8 MCs and Nodularin in filtered cyanobacterial biomass. Acceptation criteria are based on European Decision 2002/EC/657. Table S2.6. Detection results for six additional microcystin congeners with a limit of detection (LOD) at 0.10 µg $L^{-1}$. Not detected is abbreviated by "n.d.". Detected MCs are annotated as > LOD. Table S2.7: Overview of the single sequences amplified by the Sanger method used for taxonomic identification based on BLAST analysis.

# 6. LC-MS/MS Validation and Quantification of Cyanotoxins in Algal Food Supplements from the Belgium Market and Their Molecular Origins.

**Contributing authors:** *Wannes Hugo R. Van Hassel* performed the optimization of analytical methodology, prepared the samples, performed the mass spectrometry, PCRs and prepared DNA for sequencing, analyzed the molecular and analytical data and wrote the chapter; *Anne-Catherine Ahn* performed PCRs and prepared DNA for sequencing, contributed to the redaction of the chapter, and was involved in critical analysis of the data, manuscript corrections and discussion; *Mirjana Andjelkovic, Julien Masquelier, Bart Huybrechts* supervised the study and were involved in critical analysis of the analytical data, manuscript corrections and discussion; *Annick Wilmotte* supervised the study and was involved in critical analysis of the sequencing data, manuscript corrections and discussion.

This chapter is already published in Toxins-MDPI (DIO:https://doi.org/10.3390/toxins14080513) and was adapted for the thesis.

**Abstract:** Food supplements are gaining popularity worldwide. However, harmful natural compounds can contaminate these products. In the case of algae-based products, the presence of toxin-producing cyanobacteria may cause health risks. However, data about the prevalence of algal food supplements on the Belgian market and possible contaminations with cyanotoxins are scarce. Therefore, we optimized and validated a method based on Ultra High Performance Liquid Chromatography-Tandem Mass Spectrometry to quantify eight microcystin congeners and nodularin in algal food supplements. Our analytical method was successfully validated and applied on 35 food supplement samples. Nine out of these samples contained microcystin congeners, of which three exceeded 1 µg $g^{-1}$, a previously proposed guideline value. Additionally, the *mcyE* gene was amplified and sequenced in ten products to identify the taxon responsible for the toxin production. For seven out of these ten samples, the *mcyE* gene could be amplified and associated to *Microcystis* sp.. EFSA and posology consumption data for algal-based food supplements were both combined with our toxin prevalence data to establish different toxin exposure scenarios to assess health risks and propose new guideline values.

## 6.1. Introduction

The use of cyanobacteria- and *Chlorella*-based food supplements are becoming more and more common worldwide. Overall consumption of food supplements in the United States of America increased by 12% compared to a decade earlier as shown by the Council for Responsible Nutrition (CRN) Consumer Survey in 2017 [596]. The survey further revealed that the supplements are primarily consumed to improve overall health and energy [596]. However, similar consumption data are not yet available for Europe nor Belgium, which prevents a more realistic assessment of a health risk. Following the last National Consumption Survey in Belgium [597], food supplements on the basis of 'spirulina' and *Chlorella* were the third most consumed supplements, not belonging to the group of vitamins and minerals. They were ranked below yeast supplements and bee products. However, these results were obtained with food frequency surveys, which are only descriptive. A risk assessment for *Chlorella*- and cyano-based food supplements is necessary as a more frequent ingestion of these products could also lead to an increased exposure of the public to the ingredients used for their production, which might unintentionally contain harmful compounds. For instance, natural toxins such as phyto-, myco-, or phycotoxins can sometimes be found in these types of food supplements [69,598].

The use of algae biomass in food applications is still a growing market in Europe [599]. Several algae are recognized and authorised for human consumption (food/food supplement) and can be found on the European market. Two types of cyano-based food supplements are commonly found on the market. The first product is 'spirulina', which is mainly produced from cultivated *Arthrospira platensis* and occasionally *Arthrospira maxima* (formerly, *Spirulina maxima)*. The denomination 'spirulina' is a commercial one. It originates from the time before molecular taxonomy methods proved that the true *Spirulina* sp. were very different from the cyanobacteria of the *Arthrospira* genus, used as food supplement [600]. In this paper, any *Arthrospira* sp. based product will be referred under the general product name 'spirulina'. The cultivation of these cyanobacteria is generally performed in open air ponds and occasionally in closed incubators [76,601–603]. The culture medium required for growth of *Arthrospira* sp. has a high pH and high salinity, which protects against contamination by other cyanobacteria [76]. The second cyano-based food supplements product found on the market is based on the cyanobacterial species *Aphanizomenon flos-aquae*. The product names most associated to this food supplement are Klamath and AFA (*Aphanizomenon flos-aquae)*. *Aphanizomenon flos-aquae* occurs naturally in the Klamath Lake in Oregon, USA, and is being harvested there for already more than 30 years [68]. However, the harvest of naturally

occurring blooms is not unique to Oregon. In South America and Africa, local populations have been harvesting *Arthrospira* sp. from soda lakes for centuries [67].

Natural lakes usually contain mixed populations of cyanobacteria. By harvesting natural cyanobacteria, potentially toxic organisms could be included in the cyano-based food supplements. Moreover, food supplements can also be contaminated during the after-harvest processing. The presence of toxins produced by cyanobacteria was already shown in commercially available *Chlorella*- and cyano-based food supplements by several studies [376,390,406,598,604–606].

Different types of cyanotoxins exist based on their effect on the human body, and include neurotoxins and hepatotoxins. Neurotoxins consist of three major members: saxitoxin (STX), anatoxin-a (ATX), and β-methylamino-L-alanine (BMAA). STX is regularly monitored and might be found in shellfish (filter feeding organisms) but until now, it was not reported in *Chlorella*- and cyano-based food supplements [549]. In contrast, ATX and BMAA were already detected in *Chlorella*- and cyano-based food supplements either by HPLC-LFD or HPLC-MS/MS [390,406,598,604].

Another major group of cyanotoxins are hepatotoxins, including cylindrospermopsin, nodularin and microcystin. The first two were never detected in *Chlorella*- and cyano-based food supplements [374,390,393,406,605,607]. This is in strong contrast with the results obtained for the microcystin congeners (MCs). These heptacyclic peptides with their specific Adda ((2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid)-moiety are produced by different species of cyanobacteria (e.g: *Microcystis, Anabaena, Oscillatoria, Nostoc)* [526], and are commonly found in the environment as well as in *Chlorella*- and cyano-based food supplements [85,374,376,387,390,393,406,463,606–609].

The toxicity of microcystin and nodularin results from the interaction of their Adda tail with protein phosphatase 1 (PPI) or 2a (PPIIa), enabling the covalently binding of the hepatotoxin to the enzyme and inhibiting the protein activity. In particular, the toxicology of MCs is well studied. They are primarily transported to the liver by bile salts, where specific organic anion transporting polypeptides (OATPS) transport the MCs into the cells [118,119,125,556]. The function of the liver cells is disrupted, causing liver cirrhosis after prolonged exposure [560,561,610,611]. Based on the available toxicological research, the World Health Organization (WHO) suggested a tolerable daily intake (TDI) for MC-LR of 0.04 μg $kg_{bw}^{-1}$ $day^{-1}$. However, this does not take into account that multiple MCs can be present [122]. In addition, the structural variation between different congeners causes differences in chemical characteristics that can alter the toxicity of a congener [85]. Amino acids composition of the MCs at position 2 and 4 can be variable, while other positions can be methylated, demethylated and/or

acetylated [81,82,85,88–90,92,93,95,96,101,102]. Overall, 279 different congeners have been reported [85].

Several analytical methods have already been used to detect MCs in *Chlorella*- and cyano-based food supplements. The ELISA method was first used to detect MCs in *Chlorella*- and cyano-based food supplements originating from the Upper Klamath lakes. Concentrations of MCs reaching up to 16.4 μg $g^{-1}$ were found in 85 of the 87 tested products. MC-LR was determined by HPLC-separation and ELISA quantification as the most dominant congener in the products[376]. Other approaches, LC-MS, LC-MS/MS and Phospho-Inhibition Assay approach have been compared and used to determine MCs concentrations between 0.1 and 35.7 μg $g^{-1}$ [374].

During the past years, the LC-MS(/MS) has evolved to become a commonly used technique to detect MCs in *Chlorella*- and cyano-based food supplements. Multiple groups have developed and validated methods capable of accurately detecting MCs. The amount of different congeners that they were able to detect, was primarily dependent on the availability of standards [388,390,393,406,606–608].

Furthermore, molecular techniques allow to screen for cyanotoxin synthetase genes and thus to determine the capacity to produce cyanotoxins in a given sample. The gene clusters are identified based on the *mcyE* and the *ndaF* genes encoding a microcystin and nodularin synthetase, respectively [109,160]. For example, Saker et al. [366] amplified the *mcyA* and *mcyE* genes in *Chlorella*- and cyano-based food supplements and corroborated the result with an ELISA test.

Currently, only a limited number of studies have evaluated the contamination of *Chlorella*- and cyano-based food supplements by cyanotoxins and even fewer have tried to quantify the health risk for the consumers based on consumption data. In this work, we explored the presence of eight MCs and NOD in *Chlorella*- and cyano-based food supplements sold on the Belgium market using a validated UHPLC-MS/MS method. In addition, the *mcyE* gene was amplified and sequenced from the positive samples to determine the primary producer of the toxin. Elucidating the presence and the source of the contamination allows a more comprehensive assessment of the public health risks. A new assessment of the daily exposure to MCs was made and new guideline values were proposed based on the available posology and consumption data for *Chlorella*- and cyano-based food supplements.

## 6.2. Materials and Methods

### 6.2.1. *Chlorella*- and cyano-based food supplements sampling on the Belgium market

Samples were selected after an offline and online market inquiry. In total, 36 *Chlorella*- and cyano-based food supplements were selected with a balance between *Chlorella*, 'spirulina' and Klamath-based products and purchased within few weeks after the inquiry. Mixed products, with two or more of these ingredients were also included. More details on these samples are available in Table 6.4.

### 6.2.2. Reagents and materials

The solvents for the extraction and for the basis of the mobile phase were UHPLC-MS grade. All toxin standards for MCs (MC-LR, MC-RR, MC-LA, MC-LY, MC-LF, MC-LW, MC-YR, MC-WR) and NOD came from Enzo Life Sciences® (Antwerp, Belgium) and were received under the form of a solid powder. After dilution in 100% methanol (MeOH), mixed stock solutions were prepared in 50% methanol (MeOH) (50% Milli-Q water with 1% acetic acid (*v/v*)). The stock and the intermediate solutions were stored at −20 °C.

### 6.2.3. Sample preparation and extraction of cyanotoxins

Before extraction, the tablets were crushed to a powder using a mortar. If the *Chlorella*- and cyano-based food supplements was enclosed in a capsule, it was opened and the powder was removed. The complete package content was processed to ensure homogeneity within the sample. Then, 0.5 g *Chlorella*- and cyano-based food supplements powder was weighed, and dissolved in 80% MeOH (*v/v*). This mixture was blended for 1 h in an overhead shaker. Thereafter, the sample was centrifuged for 10 min at 2500 × *g*. The supernatant was removed and filtered through a Phenomenex 0.2 µm RC-syringe filter (Utrecht, The Netherlands).

### 6.2.4. Detection and quantification of cyanotoxins

To continue the analysis, each *Chlorella*- and cyano-based food supplements extract was diluted ten times. Samples were injected into the UPLC-MS/MS system (Xevo TQ-S triple quadrupole mass spectrometer —Waters, Milford, MA, USA) at a volume of 5 µL. UHPLC and MS/MS conditions were already described in detail in earlier published works [609,612]. In short, during injection, the congeners were separated on a 1.7 µm, 2.1 mm × 100 mm BEH-C18 column from Waters © (Eten-Leur, The Netherlands) preceded by a 1.7 µm BEH C-18 VANGUARD pre-column from Waters©. A gradient elution with

an initial high percentage (98%) water phase was used, while gradually increasing the proportion of acetonitrile over time.
The precursor, quantifier and qualifier ion used for detection can be found in Table 6.1, accompanied by corresponding collision energies and cone voltages. Qualifier and Qauntifier ion fragments are represented in Figure 3.2.

**Table 6.1.** MS/MS parameters used for the ion fragmentation for the detection of MCs and NOD in algal based supplements.

| toxins | precursor ion (*m/z*) | quantifier ion (*m/z*) | collision energy (eV) | cone voltage (V) | qualifier ion (*m/z*) | collision energy (eV) | cone voltage (V) |
|---|---|---|---|---|---|---|---|
| MC-LR | 995.4 | 135.0 | 70 | 80 | 213.1 | 60 | 80 |
| MC-RR | 519.8 | 134.8 | 30 | 50 | 107.2 | 60 | 50 |
| MC-YR | 1045.5 | 135.3 | 80 | 60 | 212.9 | 60 | 60 |
| MC-WR | 1068.4 | 135.3 | 70 | 100 | 213.1 | 60 | 100 |
| MC-LY | 1002.4 | 135.4 | 60 | 50 | 213.0 | 50 | 50 |
| MC-LA | 910.3 | 135.1 | 60 | 50 | 107.1 | 80 | 50 |
| MC-LF | 986.3 | 135.0 | 60 | 70 | 213.1 | 60 | 70 |
| MC-LW | 1025.4 | 134.9 | 60 | 60 | 213.1 | 50 | 60 |
| NOD | 825.25 | 134.9 | 50 | 80 | 102.7 | 90 | 80 |

Toxin concentrations were calculated by the TargetLynx extension included in the MassLynx V4.2 (Waters©) software based on dilution factors and a six-pointed calibration curve, which was made in blank matrix (sample extract containing no MCs or NOD) with concentrations between 0.50 µg $L^{-1}$ (45 µg $kg^{-1}$) and 50.00 µg $L^{-1}$ (4500 µg $kg^{-1}$). The calibration curve in the blank matrix corrects for the matrix effect. During each run, a quality control (QC) sample was also added to calculate the recovery of the toxins during the analysis run. A blank matrix sample was spiked with a mixture of all the toxin standards resulting in a final concentration of 800 µg $kg^{-1}$ for each of the MCs and NOD. The QC was further extracted and analyzed in accordance with our method. Eventually, the observed concentration in the unknown (BGAS or *Chlorella*) samples should be corrected with the recovery.

## 6.2.5. Validation of the MS/MS method

The validation study was performed using a blank (containing no MCs or NOD) 'spirulina' sample and completed before analysis of the *Chlorella*- and cyano-based food supplements samples. Aliquots of blank 'spirulina' powder were spiked with a toxin mix of eight MCs and NOD to validate the method. Each toxin was added at concentrations of 50.00 µg $kg^{-1}$, 400.00 µg $kg^{-1}$, 800.00 µg $kg^{-1}$ and 1200.00 µg $kg^{-1}$ in triplicate. The procedure was repeated on three different days. The following multiple parameters that are described below were evaluated to assess the validity of the method: specificity, limit

of detection (LOD) and the limit of quantification (LOQ), apparent recovery, reproducibility, repeatability, measurement uncertainty, linearity and matrix effect.
The specificity of the method was stated as sufficient if the quantifier ion and the qualifier ion were both present during detection, and the ion ratio adhered to the EU commission decision's 2002/657/EC limits [431]. Furthermore, no residual signal should be detected in the blank samples above 1% of the signal intensity found in the 800.00 µg $kg^{-1}$ spike.
The LOQ was determined as the lowest concentration, for which a toxin was fully validated and the signal-to-noise (S/N) ratio was 10. The LOD was defined as 1/3 of the value of the LOQ if the S/N signal is higher than 3. Moreover, the boundaries set for the apparent recovery were laid between 60.00% and 120.00% and the limits for reproducibility and repeatability were obtained using the Horwitz ratio. Reproducibility is calculated as the average variance of the validation results, representing the variability of the method over multiple days of analysis. Repeatability is calculated as the CV of the validation results representing variability of the method during one day of analysis. The measurement uncertainty was calculated as the double of the CV. Linearity of the standard curve was determined with a Mandel's fitting test between concentrations of 0.10 and 50.00 µg $L^{-1}$ for all toxins. However, if the $R^2$ of the linear fit was equal or higher than 0.99, a linear fit was used for quantification.
Furthermore, the matrix effect in the *Chlorella*- and cyano-based food supplements was determined with an additional method during the validation. For each toxin, a standard curve between 0.10 and 50.00 µg $L^{-1}$ was measured in the solvent solution (MeOH:$H_2O$ Milli-Q 50:50 + 1% acetic acid) and in the blank matrix (annotated as addition). The slopes of the resulting curves were compared using a student t-test, and a matrix effect was deduced when the slopes were significantly different (t(b) > t (95%)).

### 6.2.6. DNA extraction, PCR with HEP primers, and sequencing of PCR products

DNA was extracted from nine *Chlorella*- and cyano-based food supplements samples (Apha-430, Mx-582, Apha-584, Apha-585, Apha-587, Apha-650, Apha-696, Apha-862, Apha-1230), for which contamination with MCs was found by the LC-MS/MS method in this study. DNA extracted from the *Microcystis aeruginosa* strain PCC7806 and *Aphanizomenon gracile* Niva-Cya626 were used as positive controls in the PCR, while DNA from the 'spirulina' sample Sp-475, in which no MCs were found in this study, was used as a negative control. Approximately 50 mg of powder or tablets (previously grinded for toxin analysis) were used for DNA extraction with the NucleoSpin Tissue kit (Macherey-Nagel, Düren Germany), following the supplier's recommendations, with the addition of an initial crushing of the samples with

a pestle and glass beads. Thereafter, DNA samples were cleaned with the Genomic DNA Clean & Concentrator kit (Zymo, Tustin, CA, USA). The DNA samples were stored at −20°C until further processing. PCR was performed on these DNA samples with the HEP primers HEPF (5'-TTTGGGGTTAACTTTTTTGGGCATAGTC-3') and HEPR (5'-AATTCTTGAGGCTGTAAATCGGGTTT-3') amplifying the aminotransferase (AMT) domain situated in the *mcy*E and *nda*F genes encoding the microcystin and nodularin synthetase enzyme complexes [160]. The cycling protocol included an initial denaturation step at 98°C (1 min), followed by 40 cycles of a denaturation at 98°C (30 s), an annealing at 57°C (45 s) and an extension at 72°C (40 s), and a final extension at 72°C (7 min). The reaction mix contained in a total volume of 50 µL, 1U Q5 High Fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA), 1× Q5 Reaction Buffer, 200 µM dNTP, 1 mg $mL^{-1}$ BSA, 0.5 µM of both primers, and 1 µL gDNA. Negative controls without DNA were processed in parallel. The presence or absence of bands of the expected size was visualised after electrophoresis of 4 µL of PCR product on 1.0 % agarose gel. PCR products were purified with the NucleoSpin Gel and PCR Clean-up Purification kit (Macherey-Nagel, Düren Germany) according to the furnisher's protocol, and sent for sequencing with the HEPF primer to the GIGA-GenoTranscriptomics Platform (ULiege, Liège Belgium). Amplicon sequences were deposited under the GenBank accessions MW924666 to MW924672.

## 6.2.7. Exposure and risk assessment

The exposure to toxins via intake of food supplements should be estimated by using the consumption data for the food supplements. However, these data are scarce. For a more realistic approach, the posology data for each food supplement analyzed in the study were collected. These data were used to estimate the exposure by multiplying the daily dose suggested by the producer with the determined toxin concentration (average and maximum concentrations) to estimate the exposure. The worst-case exposure scenario was estimated only for the *Chlorella*- and cyano-based food supplements where MCs were detected (n = 8). Additionally, a comparison was performed where the consumption data from the EFSA Comprehensive Food Consumption data was used for the same calculations [613].

To estimate the exposure, the intake was calculated based on all analyzed samples (real case) and based on only the concentrations found in the contaminated samples (worst-case exposure scenario). For the latter, the values for non-detected were imputed according to the bounding approach. All non-detected values were replaced by LOD values and all not quantified values by LOQ value (upper bound approach). The calculation was performed using the following equation for estimated daily intake (EDI):

**Equation 6.1:** formula to calculate estimated daily intake (EDI) (μg $kg^{-1}_{bw}$ $day^{-1}$). With $C_{MCs}$ representing the total MCs concentration (μg $g^{-1}$) in a sample. DD (g) represents the suggested daily dose. Body weight is abbreviated as bw (kg).

$$EDI = \frac{C_{MCs} \times DD_{(BGAS)}}{\text{bw}}$$

# 6.3. Results

## 6.3.1. Validation of UPLC-MS/MS method

The validation of our method was successful for all nine toxins. The criteria for specificity were met. Namely, blank samples did not present toxin signals. All spiked samples showed signals for the quantifier and qualifier ion, and the ion ratio between both was within the limits set by the EU commission decision 2002/657/EC [431].

The LOQ for each toxin was determined as the lowest validated concentration (50.00 μg $kg^{-1}$). Signal to Noise (S/N) values were above 10 for each toxin, shown in Table 6.2. Usually, LOD is calculated as 1/3 of the LOQ, which in this case would be ± 16.66 μg $kg^{-1}$. However, the LOD was validated as 22.50 μg $kg^{-1}$ because this was also the lowest point, for which S/N values were obtained. Table 6.2 shows that the S/N values for the LOD are above 3, as dictated by the guidelines EU commission decision 2002/657/EC [431].

Furthermore, the Mandel's fitting test showed a preference for an exponential fit. However, the linear fits for all the curves showed $R^2$ values higher or equal to 0.99, resulting in this fit being chosen to determine concentrations in the samples, as it is more straightforward to calculate (shown in Table 6.2) [35].

Additionally, a matrix effect was observed for seven out of nine toxins in the *Chlorella*- and cyano-based food supplements matrix during the validation, as shown by the non-parallel relationship between the two calibration curves (Figure 6.1) and further evaluated with a student t-test (Table 6.3). All congeners except MC-LA and NOD were affected by a matrix effect. The calibration curve was therefore made in blank matrix, which reduces the effect of the matrix on the quantification.

The apparent recoveries were calculated for the four different spiking levels (50 μg $kg^{-1}$, 400 μg $kg^{-1}$, 800 μg $kg^{-1}$ and 1200.00 μg $kg^{-1}$) and additionally as average of the four levels. Recoveries were situated between 69.00% and 104.00% (Table 6.2).

For the repeatability and reproducibility, the Horwitz ratio determined maximum values from 10.30 to 14.70% average variance and 15.50 to 22.00% coefficient of variation (CV), respectively. These thresholds were calculated for each toxin at each concentration level. The actual average variance and CV obtained from the measured concentrations were both below their

respective threshold values for each toxin at each spiked concentration (Table 6.2).
At last, the measurement's uncertainty for all toxins for all spike levels was comprised between 6.26% and 37.48% as seen in Table 6.2.

**Table 6.3.** This table shows the values for the calculated t(b) compared with the tabulated t at the 95% confidence level (2.09) to determine the presence of a matrix effect. If t(b) is higher than t(95%), a matrix effect is present. The t(b) value compares a significant difference between the slope of standard and addition curve.

| **Toxins** | **MC-RR** | **NOD** | **MC-LA** | **MC-LF** | **MC-LR** | **MC-LY** | **MC-LW** | **MC-YR** | **MC-WR** |
|---|---|---|---|---|---|---|---|---|---|
| **t(b)** | 19.46 | 1.76 | 1.24 | 7.70 | 17.53 | 2.42 | 3.14 | 10.45 | 15.60 |

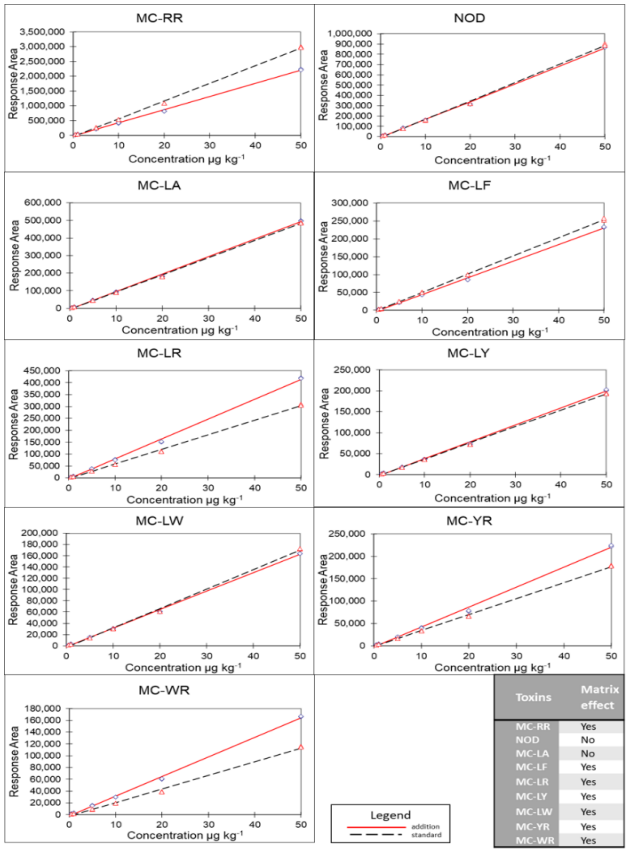



**Figure 6.1.** Matrix effect in a blank 'spirulina' matrix is assessed during the validation by comparing the response area of a calibration curve in blank matrix (e.g., addition) to a calibration curve in solvent (e.g. standard). Matrix effect is not observed when the curves are parallel to each other. However, when the curves intersect, there is a matrix effect.

**Table 6.2.** Overview of results for the validation parameters: recovery, repeatability, reproducibility and measurement uncertainty are calculated for MCs and NOD and the sum at each independent concentration level and on average.

| Toxins | Spiked Concentration (µg kg$^{-1}$) | Recovery (%) | Repeatability (%) | Reproducibility (%) | MU (%) | Average S/N* LOD (22.50 µg kg$^{-1}$) | Average S/N LOQ (50.00 µg kg$^{-1}$) | $R^2$** |
|---|---|---|---|---|---|---|---|---|
| **MC-RR** | 50.00 | 80.00 | 4.20 | 13.26 | 26.53 | 224.16 | 445.87 | 1.00 |
| | 400.00 | 76.00 | 8.87 | 9.46 | 18.91 | | | |
| | 800.00 | 84.00 | 1.64 | 14.02 | 28.05 | | | |
| | 1200.00 | 83.00 | 1.10 | 11.68 | 23.36 | | | |
| | Average | 80.75 | 3.95 | 12.11 | 24.21 | | | |
| **NOD** | 50.00 | 95.00 | 3.30 | 7.73 | 15.46 | 220.22 | 328.44 | 1.00 |
| | 400.00 | 86.00 | 8.94 | 15.46 | 30.92 | | | |
| | 800.00 | 89.00 | 2.50 | 15.55 | 31.11 | | | |
| | 1200.00 | 89.00 | 0.91 | 13.19 | 26.39 | | | |
| | Average | 89.75 | 3.91 | 12.99 | 25.97 | | | |
| **MC-LA** | 50.00 | 102.00 | 2.79 | 10.52 | 21.03 | 35.10 | 74.09 | 1.00 |
| | 400.00 | 95.00 | 8.47 | 13.60 | 27.20 | | | |
| | 800.00 | 99.00 | 2.06 | 6.95 | 13.90 | | | |
| | 1200.00 | 99.00 | 1.17 | 4.33 | 8.65 | | | |
| | Average | 98.75 | 3.62 | 8.85 | 17.69 | | | |
| **MC-LF** | 50.00 | 102.00 | 3.72 | 8.06 | 16.11 | 34.01 | 78.61 | 1.00 |
| | 400.00 | 99.00 | 9.37 | 10.96 | 21.91 | | | |
| | 800.00 | 103.00 | 1.40 | 14.05 | 28.10 | | | |
| | 1200.00 | 103.00 | 1.48 | 14.33 | 28.67 | | | |
| | Average | 101.75 | 3.99 | 11.85 | 23.70 | | | |

| Toxins | Spiked Concentration (μg $kg^{-1}$) | Recovery (%) | Repeatability (%) | Reproducibility (%) | MU (%) | Average S/N* LOD (22.50 μg $kg^{-1}$) | Average S/N LOQ (50.00 μg $kg^{-1}$) | $R^2$** |
|---|---|---|---|---|---|---|---|---|
| **MC-LR** | 50.00 | 92.00 | 5.61 | 7.06 | 14.12 | 73.46 | 142.57 | 1.00 |
| | 400.00 | 79.00 | 8.86 | 18.74 | 37.48 | | | |
| | 800.00 | 82.00 | 2.54 | 13.72 | 27.45 | | | |
| | 1200.00 | 82.00 | 1.88 | 7.04 | 14.08 | | | |
| | Average | 83.75 | 4.72 | 11.64 | 23.28 | | | |
| **MC-LY** | 50.00 | 102.00 | 3.50 | 8.01 | 16.02 | 25.53 | 49.62 | 1.00 |
| | 400.00 | 94.00 | 9.07 | 15.53 | 31.06 | | | |
| | 800.00 | 96.00 | 1.21 | 8.80 | 17.61 | | | |
| | 1200.00 | 96.00 | 0.75 | 3.13 | 6.26 | | | |
| | Average | 97.00 | 3.63 | 8.87 | 17.74 | | | |
| **MC-LW** | 50.00 | 103.00 | 3.77 | 3.85 | 7.70 | 23.62 | 50.09 | 1.00 |
| | 400.00 | 100.00 | 8.50 | 11.25 | 22.50 | | | |
| | 800.00 | 104.00 | 2.24 | 15.13 | 30.26 | | | |
| | 1200.00 | 103.00 | 1.64 | 14.54 | 29.07 | | | |
| | Average | 102.50 | 4.04 | 11.19 | 22.38 | | | |
| **MC-YR** | 50.00 | 86.00 | 3.31 | 9.85 | 19.70 | 33.86 | 68.87 | 1.00 |
| | 400.00 | 73.00 | 10.21 | 16.81 | 33.61 | | | |
| | 800.00 | 75.00 | 1.85 | 16.21 | 32.42 | | | |
| | 1200.00 | 75.00 | 1.93 | 12.24 | 24.47 | | | |
| | Average | 77.25 | 4.33 | 13.78 | 27.55 | | | |

| **Toxins** | Spiked Concentration (µg $kg^{-1}$) | Recovery (%) | Repeatability (%) | Reproducibility (%) | MU (%) | Average S/N* LOD (22.50 µg $kg^{-1}$) | Average S/N LOQ (50.00 µg $kg^{-1}$) | $R^2$** |
|---|---|---|---|---|---|---|---|---|
| **MC-WR** | 50.00 | 90.00 | 3.24 | 16.00 | 32.00 | 29.17 | 79.90 | 1.00 |
| | 400.00 | 69.00 | 9.59 | 15.89 | 31.78 | | | |
| | 800.00 | 69.00 | 2.04 | 13.88 | 27.66 | | | |
| | 1200.00 | 69.00 | 2.01 | 9.65 | 19.30 | | | |
| | Average | 74.25 | 4.22 | 13.86 | 27.69 | | | |
| **Sum** | 450.00 | 95.00 | 1.90 | 4.98 | 9.95 | N.A. | N.A. | N.A. |
| | 3600.00 | 86.00 | 8.98 | 13.17 | 26.34 | | | |
| | 7200.00 | 89.00 | 1.62 | 11.98 | 34.96 | | | |
| | 10800.00 | 89.00 | 0.83 | 9.48 | 30.22 | | | |
| | Average | 89.75 | 3.33 | 9.90 | 25.37 | | | |

* Signal to Noise (S/N) is assessed as parameter for the limit of quantification (LOQ). LOQ is assessed at the lowest spiked concentration with an acceptable threshold of 10. S/N is assessed for the limit of detection (LOD) at the lowest concentration in the calibration curve with an acceptable threshold of 3. **Linearity of the calibration curve for each toxin is determined by $R^2$.The LOD and LOQ are assessed for each toxin separately. LOD and LOQ is not calculated for the sum of the concentrations for all toxins and is therefore not applicable (N.A.)

## 6.3.2. Toxin occurrence in food supplements

Cyanotoxins, under the form of six MCs congeners, were detected in nine out of the 35 analyzed samples. Nodularin, MC-LW, and MC-LF were not detected in any of the samples. All contaminated products contained 100% *Aphanizomenon flos-aquae* with the exception of Mx-852, which was a mix of the 'spirulina', *Chlorella* sp. and *A. flos-aquae*. However, only MC-RR below LOQ was found in Mx-852 below LOQ. The other eight contaminated products contained total MCs concentration between 238.45 and 5645.33 µg $kg^{-1}$, as shown in Table 6.4.

The congener compositions varied between the different products. Between two and six different congeners were detected in the eight MCs contaminated samples by our validated method (Figure 6.2). It should be noted that the samples with the highest congener diversity (Apha-430 and Apha-1230) contained MC-WR below LOQ, whereas the five other congeners could be quantified. MC-LA and MC-LR were the most prevalent toxins found in the products, as shown in Figure 6.2.

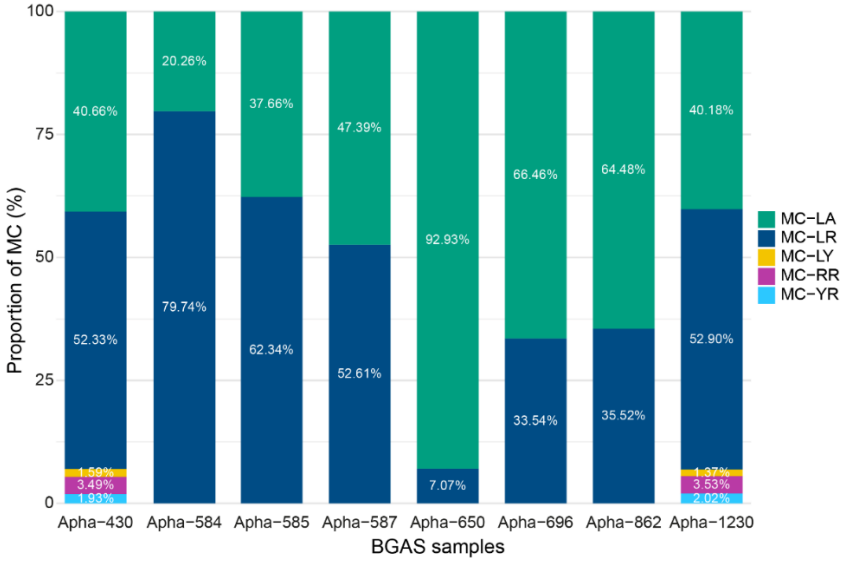



**Figure 6.2.** The proportionate contribution (%) of each microcystin congener to the total concentration of microcystin congeners (MCs) per sample of *Chlorella*- and cyano-based food supplements.

**Table 6.4.** Overview of samples ID with the type of *Chlorella*- and cyano-based food supplements (pill, powder or tablet), the species indicated on the package, the type of retailer, the total microcystin concentration found, the proposed dose per day and estimated daily consumption.

| sample ID | type | species indicated on the package | type of retailer | total microcystin (µg $kg^{-1}$) | Proposed dose $day^{-1}$ * | Estimated daily consumption (g) |
|---|---|---|---|---|---|---|
| **Apha-430** | powder | *Aphanizomenon flos-aquae* | Health food store | 5645.33 | 2 teaspoons or sprinkle | 4 |
| **Apha-584** | powder | *Aphanizomenon flos-aquae* | webshop | 431.26 | 1 teaspoon (2 g) | 2 |
| **Apha-585** | powder | *Aphanizomenon flos-aquae* | webshop | 308.11 | 1 teaspoon (1.5 g) | 1.5 |
| **Apha-587** | capsules | *Aphanizomenon flos-aquae* | webshop | 238.45 | 2 capsules (800 mg/capsule) | 1.6 |
| **Apha-650** | powder | *Aphanizomenon flos-aquae* | webshop | 1106.06 | 1 or 2 teaspoons (2 = 3 g) | 3 |
| **Apha-696** | tablets | *Aphanizomenon flos-aquae* | webshop | 499.52 | first 15 days: 2 capsules, after 4 caplsules (400 mg/tablet) | 1.6 |
| **Apha-862** | capsules | *Aphanizomenon flos-aquae* | webshop | 623.39 | 2 capsules (800 mg/capsule) | 1.6 |
| **Apha-1230** | powder | *Aphanizomenon flos-aquae* | Health food store | 5436.31 | 2 teaspoons or sprinkle | 4 |
| **Sp-589** | tablets | *Arthrospira maxima* | webshop | < LOD | 3 capsules (600 mg/tablet) | 1.8 |
| **Sp-698** | capsules | *Arthrospira maxima* | webshop | < LOD | 3 capsules (600 mg/tablet) | 1.8 |
| **Sp-433** | tablets | *Arthrospira pacifica* | Health food store | < LOD | 6 tablets (500 mg/tablet) | 3 |
| **Sp-431** | powder | *Arthrospira platensis* | Health food store | < LOD | Not mentioned on package | 2 |

| sample ID | type | species indicated on the package | type of retailer | total microcystin (µg kg$^{-1}$) | Proposed dose day$^{-1}$ * | Estimated daily consumption (g) |
|---|---|---|---|---|---|---|
| **Sp-432** | tablets | *Arthrospira platensis* | webshop | < LOD | up to 6 tablets (500 mg/tablet) | 3 |
| **Sp-475** | powder | *Arthrospira platensis* | Health food store | < LOD | 2 teaspoons (6 g) or sprinkle | 6 |
| **Sp-543** | powder | *Arthrospira platensis* | Health food store | < LOD | 1 teaspoon (1.5 g) | 1.5 |
| **Sp-544** | powder | *Arthrospira platensis* | Health food store | < LOD | 1 tablespoon (7 g) | 7 |
| **Sp-545** | tablets | *Arthrospira platensis* | Health food store | < LOD | 6–10 tablets (300 mg/tablet) | 3 |
| **Sp-579** | powder | *Arthrospira platensis* | webshop | < LOD | adults 3 g, children 1.5 g | 3 |
| **Sp-581** | powder | *Arthrospira platensis* | webshop | < LOD | Not on package | 2 |
| **Sp-583** | powder | *Arthrospira platensis* | webshop | < LOD | 2 teaspoons (6 g) or sprinkle | 6 |
| **sample ID** | type | species indicated on the package | type of retailer | total microcystin (µg kg$^{-1}$) | Proposed dose day$^{-1}$ * | Estimated daily consumption (g) |
| **Sp-586** | powder | *Arthrospira platensis* | webshop | < LOD | 1 teaspoon (1 g) | 1 |
| **Sp-588** | tablets | *Arthrospira platensis* | webshop | < LOD | 2–6 tablets (500 mg/tablet) | 3 |
| **Sp-651** | powder | *Arthrospira platensis* | webshop | < LOD | 1 teaspoon | 2 |
| **Sp-863** | tablets | *Arthrospira platensis* | webshop | < LOD | 2–3 tablets (500 mg/tablet) | 1.5 |
| **Sp-864** | powder | *Arthrospira platensis* | webshop | < LOD | 2 × 1 measuring spoon (± 3 g ) | 6 |
| **Sp-865** | powder | *Arthrospira platensis* | webshop | < LOD | 1 teaspoon | 2 |

| sample ID | type | species indicated on the package | type of retailer | total microcystin (µg kg$^{-1}$) | Proposed dose day$^{-1}$ * | Estimated daily consumption (g) |
|---|---|---|---|---|---|---|
| **Mx-582** | powder | *Arthrospira platensis , Chlorella vulgaris, Aphanizomenon flos-aquae* | webshop | < LOQ | 2 teaspoons or sprinkel | 4 |
| **Mx-821** | powder | *Arthrospira platensis, Chlorella vulgaris* | webshop | < LOD | 1 teaspoon first two weeks than 1 eatspoon (7.5 g) | 7.5 |
| **Mx-822** | powder | *Arthrospira platensis, Chlorella vulgaris* | webshop | < LOD | 2 × 1 teaspoon | 4 |
| **Ch-546** | powder | *Chlorella vulgaris* | Health food store | < LOD | 3 teaspoons | 6 |
| **Ch-547** | tablets | *Chlorella vulgaris* | Health food store | < LOD | 1–12 tablets (500 mg/tablet) | 6 |
| **Ch-580** | powder | *Chlorella vulgaris* | webshop | < LOD | Not on package | 2 |
| **Ch-649** | powder | *Chlorella vulgaris* | webshop | < LOD | 1 spoon (unspecified) | 7.5 |
| **Ch-652** | tablets | *Chlorella vulgaris* | webshop | < LOD | 6 tablet (500 mg/tablet) 12 in special cases | 3 |
| **Ch-697** | tablets | *Chlorella vulgaris* | webshop | < LOD | 4 to 6 tablets (400 mg/tablet) | 2.4 |
| **Sp-823** | powder | *Spirulina platensis/maxima* | webshop | < LOD | Not mentioned on package | 2 |

*The weight of *Chlorella*- and cyano-based food supplements measured as a teaspoon suggested by EFSA (2 g), was used when the proposed daily dose is not mentioned on the package or the weight of a teaspoon is not mentioned.

## 6.3.3. Microcystin synthetase encoding genes

The HEP amplicons (385 bp) of seven food supplements (Apha-430, Apha-584, Apha-585, Apha-587, Apha-650, Apha862 and Apha-1230) corresponded to the sequences of the *mcy*E gene of different *Microcystis* sp.. No HEP amplicons could be obtained from the samples Sp-475, Mx-582 and Apha-696. Moreover, the samples Apha-430, Apha-650, Apha-862 and Apha-1230 contained more than one *Microcystis* sp. strain as there were single nucleotide polymorphisms in their HEP amplicon sequence. Samples of Apha-584, Apha-585 and Apha-587 contained only a single HEP amplicon sequence, which was identical for the three samples and which could be associated with *Microcystis* sp. clone Bel-Nar12/07-1 (100% identity, accession: KF219536.1).

## 6.3.4. Exposure and risk assessment estimate

Based on the EFSA Comprehensive Food Consumption database, the average and 95$^{th}$ percentile consumption from the acute consumption data (e.g. taking into account consumption of each participant on individual reporting data) of algae-based formulations (e.g. 'spirulina', *Chlorella*) for all population groups (Adolescents, Adults, Elderly, Other children, Pregnant women, Vegetarians and Very elderly) is 2.60 g and 3.50 g, respectively. However, the data can be considered statistically robust only for the adults, with a number of observations higher than 60. Recalculating the consumption for adults, based on the data found in the EFSA database, provides values of 1.62 g and 2.58 g on average per day and at the 95$^{th}$ percentile per day, respectively. These values were considered during the toxin exposure estimations (estimated daily intake (EDI)) in Table 6.4.
Additionally, the posology data (suggested daily dose) found on the package of the tested products was also used as a measure of the consumption. The average suggested daily dose for each food supplement was 3.14 g day$^{-1}$, while for the 95$^{th}$ percentile, this was 6.25 g day$^{-1}$. Therefore, , using the Equation 6.1, it was estimated that an average EDI was 0.02 µg $kg_{bw}^{-1}$ day$^{-1}$ for an adult person of 70 kg (Table 8.5). In a worst- case exposure scenario (only upper bound approach), where only the contaminated samples were included, the average EDI was 0.08 µg $kg_{bw}^{-1}$ day$^{-1}$ and the 95$^{th}$ percentile exposure at the recommended doses was 0.32 µg $kg_{bw}^{-1}$ day$^{-1}$. For the comparison, the exposure estimation was also done using the mean consumption calculated for the adults using the EFSA Food Consumption Comprehensive Database (Table 6.5).

**Table 6.5.** Mean, median and 95th percentile estimated daily intake (EDI) (µg $kg_{bw}^{-1}$ $day^{-1}$) for an adult population to microcystins detected in *Chlorella*- and cyano-based food supplements.

| **exposure scenario** | **EDI µg $kg_{bw}^{-1}$ $day^{-1}$** | | |
|---|---|---|---|
| *adults (consumption following the posology recommendation)* | *mean* | *median* | *P95* |
| real (UB)* | 0.02 | 0.00 | 0.14 |
| worst (UB)** | 0.08 | 0.01 | 0.32 |
| *adults (mean consumption as calculated from the EFSA database***)* | *mean* | *median* | *P95* |
| real (UB)* | 0.01 | 0.00 | 0.06 |
| worst (UB)** | 0.04 | 0.01 | 0.13 |

*Real (UB), the exposure scenario where all analyzed *Chlorella*- and cyano-based food supplements were taken into account (upper bound approach); **worst (UB), only results of the positive samples (at least one MC detected) were taken into account (upper bound approach), ***EFSA, the mean chronic consumption was calculated from the EFSA Food Consumption Comprehensive Database.

In the worst-case exposure scenario, the tolerable daily intake (TDI) [122] of 0.04 µg $kg_{bw}^{-1}$ for an adult was exceeded for both the average (0.08 µg $kg_{bw}^{-1}$ $day^{-1}$) and 95th (0.32 µg $kg_{bw}^{-1}$ $day^{-1}$) percentile EDI. For children, the TDI values would be severely exceeded by the EDI in both exposure scenarios. This suggests that there might be a potential health concern risk for some consumers and in particular for children when contaminated products are consumed.

Furthermore, we suggest a maximum allowed MCs concentration in *Chlorella*- and cyano-based food supplements for adults, ranging from 0.70 to 1.08 µg MC-LR equivalent $g^{-1}$ $day^{-1}$. The lower limit was calculated based on the 95th percentile of consumption based on posology data (representing high consumers in worst-case exposure scenario. The upper limit represents the average consumer use (average consumption based on posology data). If children (15 kg average weight) might consume these *Chlorella*- and cyano-based food supplements, the maximal allowed level should be even lower (0.15 µg MC-LR equivalent $g^{-1}$ $day^{-1}$ *Chlorella*- and cyano-based food supplements). The average and 95th percentile of MCs concentration found in *Chlorella*- and cyano-based food supplements during our study for the worst-case scenario were 1.84 µg MC-LR equivalent $g^{-1}$ and 5.72 µg MC-LR equivalent $g^{-1}$, respectively, and both surpass the suggested maximum allowed MCs concentration for adults and children.

## 6.4. Discussion

Based on the data from the literature cited in this study, it is clear that a ± 80% MeOH extraction is the most appropriate way to extract the MCs and NOD from food supplements, with the addition of a mechanical extraction

and/or sonication [388,390,393,606,607]. Moreover, no additional purification seems to be needed for *Chlorella*- and cyano-based food supplements samples for MS/MS analysis, which significantly reduces material cost and analysis duration.

Several quantification methods for MCs in food supplements have already been validated. Turner et al. (2018), validated a method using the same extraction method but a different detection and quantification method compared to our method, which uses a calibration curve in the matrix. A matrix-matched calibration curve should result in a more accurate determination of the recovery, as the matrix effect is taken into account. Our recoveries seem to be similar to the results from Turner et al. (2018), except for the lower recoveries of MC-LF. The values of the other parameters (LOD, LOQ, repeatability,...) are difficult to compare as different calculations were used [388]. Other validated methods are even more difficult to compare as the methods used different techniques during extraction and/or quantification of the methods. Parker et al. (2015) included a SPE step in their validated method. Up concentration of the samples during SPE lowers the LOD and LOQ, while optimizing recovery for all MCs and NOD and minimizing the matrix effect. However, this methodology does increase the needed time and the associated costs to perform the method [393]. Ortelli et al (2008) validated a method for quantification of MC-RR, MC-YR, MC-LR, MC-LA and NOD using LC-MS. They used a time of flight MS instead of the triple quadrupole used in our method [607].

A more complex method for MCs quantification was also validated by Roy-Lachappelle et al. using LC-HRMS. The recovery and LOD/LOQ were comparable to our results, while the repeatability and reproducibility were lower [406].

During our screening of *Chlorella*- and cyano-based food supplements (*Chlorella* sp., 'spirulina' and Klamath derived products) on the Belgium food market, we found nine products that were contaminated with MCs with concentrations between 0.24 and 5.6 µg $g^{-1}$ total microcystin, and with MC-LR and MC-LA as most abundant congeners. They were exclusively observed in products containing *Aphanizomenon flos-aquae*. Our concentrations fit perfectly with the earlier reported toxin concentrations ranging between 0 and 60 µg $g^{-1}$ in *Chlorella*- and cyano-based food supplements using different methods (e.g. MS/MS, MS, ELISA, PPIA) [374,376,378,390,606,607,614]. Roy-Lachappelle et al. (2017) also found toxin concentrations between 0.25 and 8.2 µg $g^{-1}$ total microcystin, present in 'spirulina' products, which is uncommon [406].

Besides UPLC-MS/MS methods, PCR amplification of the *mcyE* gene could be a valuable simple tool during screening of *Chlorella*- and cyano-based food supplements and detection of possible contaminations during the production process. The sequencing of the *mcyE* amplicon allowed to identify

the producer of the microcystin. In seven MCs positive *Chlorella*- and cyano-based food supplements, the *mcyE* gene of *Microcystis* sp. could be detected by PCR [109]. In three other samples, PCR amplification failed, but this could be explained. The sample Sp-475 was a negative control made on a sample where no MCs were detected and the lack of amplification of the *mcyE* gene was expected. For sample Mx-582, MC-LR was detected but in too low concentration to be quantified. For sample Apha-696, a quantifiable level (499.52 total microcystin µg $kg^{-1}$) of MCs was measured but it was the only Apha sample in tablet form. Therefore, we hypothesize that the treatment used for tablet production sheared the DNA too much for proper PCR amplification. The presence of *Microcystis* sp. in food supplements has previously be reported in several studies [366,606,615,616]. Therefore, we advise the producers to take additional precautions to avoid the presence of such known toxin-producing cyanobacteria and control their absence by regular screenings using microscopy or PCR.

However, knowing the presence of toxin-producing cyanobacteria or even the concentration of toxins is not sufficient to assess the risk to public health. However, a risk assessment for cyanotoxins in *Chlorella*- and cyano-based food supplements is difficult to perform, due to a lack of occurrence and toxicity data for some microcystin congeners, as well as a lack of regulation on food supplements, in particular regarding these toxins. For instance, the average daily consumption of *Chlorella*- and cyano-based food supplements should be known to calculate the risk. Yet, recommended doses are not uniform between different products. Generally, the amount of a teaspoon (2 g $day^{-1}$) is recommended on a daily basis, although some products recommend a higher consumption or do not provide a recommended dose on the product packaging. Therefore, an average dose of 3.14 g $day^{-1}$ was calculated on the basis of the posology data of the tested products. Additionally, there is a chance that users will not comply with the recommended dose. For instance, Dietrich and Hoeger [564] mentioned that parents might increase the dose for their children above the recommendation in an attempt to further increase the beneficial results of the *Chlorella*- and cyano-based food supplements but obtain the inverse effect. Marsan et al. found products with a recommended dose of up to 15 g $day^{-1}$ [614]. Moreover, Gilroy et al. report recommended doses of *Chlorella*- and cyano-based food supplements up to 20 g $day^{-1}$ [376]. If we use the data from the EFSA food consumption database, we calculated an average daily dose of 1.6 g of *Chlorella*- and cyano-based food supplements and 2.58 g $day^{-1}$ in the highest $95^{th}$ percentile of consumption for adults in the food category “Algae formula” (e.g. ‘spirulina’, *Chlorella*) [549]. This dose was two times lower than the average calculated in our study. Unfortunately, the consumption of these products in Belgium has not yet been quantified. The

difference between recommended and real consumption complicates the evaluation and the recommendation of a guideline value.

The WHO considers a value of 0.04 µg $kg_{bw}^{-1}$ $day^{-1}$ as a tolerable daily intake (TDI) for MCs, which is assumed to be a safe dose for a lifetime ingestion. Using the suggested doses, we have estimated that in a worst-case exposure scenario (when MCs are present at the level found in the study), EDI might be on average as high as 0.08 µg $kg_{bw}^{-1}$ $day^{-1}$ for an adult person. However, for some consumers, this might be as high as 0.32 µg $kg_{bw}^{-1}$ $day^{-1}$. These estimated values exceed the TDI value. Nevertheless, only a small proportion of the samples showed a contamination, and it was linked to the presence of *Aphanizomenon flos-aquae*. Gilroy et al. already proposed a regulatory value of 1 µg $g^{-1}$ of *Chlorella*- and cyano-based food supplements consumption based on the TDI of MCs suggested by the WHO, which considers an average consumption of 2 g $day^{-1}$ for an average human of 60 kg. Based on the results of our study, where the average consumption is above 2 g and the average body weight was estimated as 70 kg following the EFSA guidance, a similar recommendation can be given [617]. The MCs concentration should not exceed 1.08 µg $g^{-1}$ *Chlorella*- and cyano-based food supplements for most consumers. Dietrich and Hoeger also mentioned that the supplements are also consumed by children, who, due to a lower body mass, are more susceptible to intoxication [564,618]. Recalculating a guideline value for infants and children would be advised, especially as *Chlorella*- and cyano-based food supplements consumption generally represents a long-term exposure. If the same values for the consumption of *Chlorella*- and cyano-based food supplements would be applied to adults and children, the regulatory threshold would decrease more than four times to ensure a safe consumption for all the population below the TDI level. Based on the concentrations found in our study, the recommended regulatory level could be as low as 0.15 µg $g^{-1}$. Similarly, the new WHO provisional guidelines for MCs in drinking water recommended a 3 µg $L^{-1}$ total microcystin for children during short exposure. They also used the average body weight of a child (15 kg) while calculating the guideline value for recreational waters, instead of the average body weight of an adult (60 kg) [122].

For the Belgium market, three products exceeded the value proposed by Gilroy et al. Eight products contained a MC-LR equivalent concentration higher than 0.50 µg $g^{-1}$. If the original consumption estimate from EFSA (3.5 g) was used, the guideline value for adults would become 0.80 µg $g^{-1}$ MC-LR equivalent, meaning that sample Apha-862 would also be considered as unsafe.

Further research should be done to obtain more data concerning the daily consumption of *Chlorella*- and cyano-based food supplements as well as the toxin presence in the *Chlorella*- and cyano-based food supplements to refine and improve the risk assessment for the consumers. A regular monitoring is

also advisable, as Gilroy et al showed that there could be variations in toxin concentration between different batches [376].

## 6.5. Conclusions

In this study, the validation of a quantitative UHPLC-MS/MS method to quantify eight MCs and NOD in *Chlorella*- and cyano-based food supplements is performed for the first time with a matrix-matched calibration curve. Validation results adhered to the pre-set parameters. Using this method, the presence of five MCs was determined in nine of the *Chlorella*- and cyano-based food supplements samples at concentrations that could pose a risk to the public health. Additionally, we were able to determine that the *mcyE* gene, involved in MCs production, originated from *Microcystis* sp.. Although the recommended maximum MCs level in *Chlorella*- and cyano-based food supplements were calculated, the actual risk to the public health is difficult to determine. A lack of *Chlorella*- and cyano-based food supplements consumption data, MCs occurrence and toxicological data for most MCs needs to be resolved to obtain a proper risk assessment for the Belgian population. However, using the currently available data, multiple exposure scenarios were discussed and new guidelines were suggested. Establishing proper regulation and monitoring programs for MCs in *Chlorella*- and cyano-based food supplements would be advisable, especially for products harvested from the environment. These products are a protein rich and eco-friendly food source that should not be dismissed out of hand due to the possibility for toxin contamination, which is also possible in more 'traditional' food sources (e.g. phyto-, myco- and bacterial toxins).

# 7. Towards a Better Quantification of Cyanotoxins in Fruits and Vegetables: Validation and Application of an UHPLC-MS/MS-Based Method on Belgian Products

**Contributing authors:** *Wannes Hugo R. Van Hassel* performed the optimization of analytical methodology, prepared the samples, performed the mass spectrometry, analyzed analytical data and wrote the chapter; *Mirjana Andjelkovic, Julien Masquelier, Andreja Rajkovic* supervised the study and were involved in critical analysis of the analytical data, manuscript corrections and discussion;
This chapter was already published in Separations-MDPI (DOI: 10.3390/separations9100319) and was adapted for the thesis.

**Abstract:** Vegetables and fruits can potentially accumulate cyanotoxins after water contaminated with cyanobacteria is used for irrigation. We developed and validated an analytical method to quantify eight microcystin congeners (MCs) and nodularin (NOD) using ultra-high-performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS) in three different matrices. Strawberries, carrots and lettuce were selected as model matrices to represent the fruits/berries, leafy and root vegetables, sequentially. The validation of an UHPLC-MS/MS method in the strawberry is novel. Matrix effects were observed in all three matrices. Our methodology used matrix-matched calibration curves to compensate for the matrix effect. The implementation of the method on 103 samples, containing nine different sorts of commercially obtained fruits and vegetables from the Belgian market, showed no presence of MCs or NOD. However, the recoveries of quality controls showed the effectiveness of our method, illustrating the appropriateness of this method in future research or monitoring as well as in official food controls in fruits and vegetables.

## 7.1. Introduction

Cyanotoxins can be produced in eutrophic waterbodies during blooms of photosynthetic, eukaryotic organisms called cyanobacteria [89]. Multiple studies and reviews have documented the occurrence of cyanotoxins in different foods [549,619]. The stationary nature of agriculture allows for the accumulation of these harmful compounds in food crops when vital irrigation water is contaminated with these toxins.

The most common cyanotoxin group, worldwide, is microcystin congeners (MCs). These are hepatotoxins produced by different cyanobacteria species (e.g., *Microcystis aeruginosa*, *Anabaena/Dolichospermum*, *Planktothrix* sp., *Oscillatoria* and *Nostoc*) [526]. Nodularin (NOD) has a similar structure to MCs and was initially found in *Nodularia* sp. [152,529]. A unique structural part of both toxins is the Adda fragment ((2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid), which contributes to the interaction with the target proteins [336]. Both toxins inhibit protein phosphatases 1 and 2A (PP1 and PP2A) in eukaryotes after transport in cells, causing cell death. The transport is facilitated by specific organic anion-transporting polypeptides (OATPs) [119,126,527,528]. Cylindrospermopsin (CYN) is another hepatotoxin that inhibits the protein synthesis of the affected cell and, thus, cell growth [164]. This toxin was first found in Australia. CYN is produced by *Cylindrospermopsis raciborskii*, although other producing cyanobacterial species have been identified [164]. Even though CYN is more commonly reported in warmer climates in the Southern Hemisphere, cases have been found in Europe since its discovery [204].

The effects of the other classes of cyanotoxins, such as neurotoxins, are not well studied in plants. Neurotoxins, such as saxitoxin, are also primarily found in warmer regions [203,620,621], while anatoxin-a is also regularly found in colder regions [526,622,623].

For studying the physiological effects and accumulation of cyanotoxins in plants, two methods are commonly used for the detection and quantification of these toxins. Initially, optimized immunoassays were used to (semi)quantify MCs in plant tissue [382,624–626]. Later, more robust and quantitative liquid chromatography (LC) approaches coupled to (tandem) mass spectrometry (MS(/MS)) were used to measure the concentrations of different MCs, CYN and their transformation products in plants [392,394,398,425,627].

The applicability of both the immune assays and the LC-MS(/MS) approaches depends on the goal of the analysis. To determine bound and unbound MCs in plant material, immunoassays might be the best option, as all the available Adda fragments will be detected [628]. However, immunoassays are known to produce false positive readouts and are not able to identify separate congeners or transformation products [627]. Moreover, insufficient toxicological data are available from a public health point of view to determine the effect and bioavailability of the bound/transformed MCs. Using immunoassays might thus cause an overestimation of the concentration of the bioavailable MCs, resulting in an overestimation of the risk to human health.

Various LC-MS(/MS) approaches have been developed to identify multiple MCs and CYN, separately or concurrently, in plant material [392,394,398]. Usually, multiple reaction monitoring (MRM) is used, which is capable of

identifying and quantifying multiple toxins. Yet, due to the selective nature of this method, uncommon congeners or transformation products will not be detected when they are not included in the method a priori. The inclusion of a sufficient number of MCs in an UHPLC-MS/MS method is crucial for accurately assessing the total microcystin concentration and, thus, possible public health risks.

Up till now, different MS methods have been validated. Generally, the methods use 75%–80% methanol (MeOH) as an extraction solvent, which in some cases is acidified [392,394,398,627]. Recoveries for MC-LR (56–65%), MC-RR (30–32%), MC-YR (79–81%) and NOD (97–118%) were reported in broccoli roots and stems, separately, using LC-MS selected ion recording (SIR) [627]. Signal enhancement was observed for nearly all toxins [627]. Only MC-LR and MC-YR were affected by signal suppression in the root samples at the highest spiked concentration. Later, an UHPLC-MS/MS method was validated for MC-LR, MC-RR and MC-YR in multiple crop plants (lettuce, turnip, water spinach, potato, cabbage, pumpkin, chio sum, cucumber, carrots and eggplants) [398]. Solid-phase extraction (SPE) was used during the method to purify the extract. A C18 cartridge was used for all crops except for eggplant and cucumber. For the latter, an HLB cartridge had to be used to optimize their recovery. A suppressive matrix effect was observed for all plant materials [398]. Another UHPLC-MS/MS method was validated for MC-LR and MC-RR in lettuce using 50% MeOH in conjunction with an HLB cartridge for the SPE [394]. The UHPLC-MS/MS method was also validated successfully for CYN in lettuce [425]. Furthermore, the quantification of MC-LR, MC-RR, MC-YR and CYN in lettuce was successfully optimized using 80% MeOH extraction, dual (C18 and PGC cartridge) SPE purification and UHPLC-MS/MS. The obtained recoveries were slightly lower compared to the earlier studies, probably due to the inclusion of CYN, which is hydrophilic compared to the more hydrophobic MCs [392]. Clearly, important developments in methods have already occurred. However, most methods still lack multiple chemically diverse microcystin congeners. These MCs could be present in the sample and interact differently with the matrix during analysis. Especially for the more hydrophobic congeners, for example, MC-LF and MC-LW, they are still missing. Other matrices, such as fruits and berries, should be validated separately as different matrix effects could occur.

The plant accumulation of different MCs and CYN through irrigation water has been studied in multiple (crop)plants. Lettuce is probably the best-studied vegetable, often selected based on its relevance in the human diet, massive production and its large leaf surface that may harbour variety of contaminants. The presence of MCs was initially detected with an immunoassay in lettuce after spray irrigation with *Microcystis aeruginosa* containing water [382]. The central leaves significantly retained higher concentrations compared to leaves from the basal or distal zones. Lettuce

shots were later also shown to accumulate MCs [565]. The accumulation of MCs in the roots and leaves of lettuce was also shown after irrigation with naturally contaminated water [423,629,630]. The bioaccumulation of different MCs has also been linked to the initial exposure concentrations in lettuce, showing higher accumulation with higher MC concentrations in the water [631,632], while depuration of 75% of the initially accumulated MC-LR was reported for lettuce after seven days [632]. Similar experiments were conducted for CYN, showing accumulation in lettuce [425,633]. Decreasing depuration was observed when the CYN exposure concentration was increased [633]. The combined accumulation of CYN and MCs in lettuce and spinach was shown when these plants were exposed to irrigation water containing both toxins. Only CYN was detected in the leaves, while both MCs and CYN were found in the roots [567]. The accumulation of MCs in root vegetables such as rape, carrots and radishes was also shown and was dependent on the exposure concentration [565,624,634,635]. For rice, the MCs' accumulation was found in the laboratory as well as in field studies [420,624,636]. MCs have also been observed in tomatoes and chilli plants [637,638]. While a lab study with radio-labeled MC-LR only found accumulation in the roots and stems of the tomato plants [637], field studies have shown the accumulation of only MC-RR in the tomatoes when water contaminated with MC-LR and MC-RR was used [638]. In the same field study, MC-LR and MC-RR were found in the seeds of *Capsicum annuum* (sweet and chill pepper) and only MC-LR in the fruit tissue [638]. The accumulation of MCs in legumes, clover, ryegrass, broccoli and mustard has also been evaluated in the past [565,627,639].

A large field study showed the prevalence of MCs in different vegetables, with the highest concentrations of total MCs found in leafy vegetables, fruits and root vegetables. MC-RR contributed the most to the total MC concentration, while MC-LR and MC-YR combined only contributed up to 30% of the MCs' concentration in the samples [640].

Moreover, the presence of MCs in irrigation water does not only endanger public health, but it can also influence the growth of crops. Initially, the conjugation of MC-LR with glutathione by soluble glutathione S-transferases (sGSTs) was shown in the rhizome and stem parts of *Phragmites australis* [625]. Additionally, a reduction in sGST activity was observed with increasing MC concentrations for lettuce [631]. During development, MCs can influence the growth of different crop plants, resulting in shorter shoots, necrosis or a lack of primary roots and yellowing of the leaves [565,591,624,627,630,634,639,641,642]. However, the effects of MCs are shown to differ between different crop species [565,591]. Furthermore, oxidative stress responses occur when irrigation water is contaminated with MCs but differ depending on the plant [624,631,641,642].

The gas exchange parameters in the lettuce were shown to be elevated when treated with MCs [631]. When lettuce plants were irrigated with MC-contaminated water at different development stages, an overall increase in the photosynthetic rates and a reduction in the root biomass for plants irrigated from the seed stage were shown [630]. In strawberries, carotenoids and chlorophyll-a and -b concentrations were reduced after treatment for 60 days with irrigation water containing 20 µg/L of MCs (primarily MC-LR and MC-RR) [642].
Adult *Lycopersicon esculentum* was irrigated with pure MC-LR and crude toxic cyanobacteria extract. The treatment with pure MC-LR decreased the plant's capacity to synthesize ATP and the performance of photosynthesis, while the crude extract caused increases in carbon fixation and decreased carbohydrate metabolism [566].
To provide a tool to accurately quantify the possible accumulation of different MCs in plant materials, we successfully developed and validated an UHPLC-MS/MS method capable of doing this for eight common MCs (i.e., MC-LR, MC-RR, MC-LF, MC-LA, MC-LY, MC-LW, MC-WR and MC-YR) and NOD in lettuce, carrots and strawberries. These crops represent major groups of leaf and root vegetables as well as fruits and berries. Moreover, a validated method has not yet been reported for strawberries up to now. Our method is also validated for the quantification of the highest amount of microcystin congeners in plant materials. To further evaluate the method and show that it is applicable to other vegetable matrices, a screening of nine different vegetables and fruits from The Belgian market was performed in more than 100 independent samples. The recovered concentration for the MCs from a spiked sample in each matrix was used to assess the quality of the method for each of the MCs.

## 7.2. Materials and Methods

The solvents used for the mobile phase and extraction were all UPLC/MS grade solvents (Biosolve B.V., Valkenswaard, the Netherlands). The NOD and MC standards were ordered as a solid powder from Enzo Life Sciences (Antwerp, Belgium)®. The initial dilution with 100% methanol was followed by dilutions with 50% methanol with 1% acetic acid to prepare a mixed stock solution. The dissolved cyanotoxin standards were kept at −20 °C.

### 7.2.1. Quantification of cyanotoxins

#### *7.2.1.1. Sample preparation and extraction*

Fruit and vegetable samples were cut into small pieces (+/−1 $cm^3$) and mixed with a 1000 W mixer (Moulinex). Consequently, 0.5 g of the samples were stored in a 50 mL plastic tube for analysis. During the analysis, the samples of each sort of vegetable or fruit, which were previously shown to be

uncontaminated, were used as blank and quality control (QC) samples. For the QC, 25 µL of a toxin standard solution (100.00 ng $mL^{-1}$ per toxin), containing the eight MCs and NOD, was added.
The samples were subjected to extraction with 4.5 mL MeOH (80%) and incubated for 15 min in a sonic disrupter (BRANSON 2510). The samples were then mixed for 30 min in an overhead shaker (Heidolph Reax 2 Mixer) and centrifuged (Sorvall Legend XT centrifuge Thermo Scientific) at 15,303 g for 15 min. The supernatant was evaporated (Evaporator organomation N-EVAP 112) under a nitrogen flow for 90 min at 45 °C, after which some liquid remained.
Solid-phase extraction (SPE) was used to purify the remaining liquid. An Agilent C18 cartridge (6 mL, 500 mg) was conditioned with 6 mL MeOH (100%) and equilibrated with Milli-Q water at pH 11. The remaining liquid was then loaded, and the cartridge was dried under vacuum for 5 min. The toxins were then eluted with 3 mL MeOH (80%). The filtrate was further purified with a 5 mL syringe attached to a 0.2 µm Phenomenex RC-filter.
Purified samples were added to amber glass vials for injection at 10 µL. A matrix-matched standard calibration curve (MCC) was made in a blank matrix in a range between 0.10 and 50.00 µg $L^{-1}$.

*7.2.1.2. UHPLC-MS/MS parameters*

The UHPLC-MS/MS method was used earlier in similar methods developed by our lab [609,612,643]. In short, a Waters Acquity UPLC H-class was used in tandem with a Waters XEVO TQ-S. The UHPLC was fitted with a Waters Acquity BEH C18 1.7 µM VANGUARD PRE-Col and Waters Acquity BEH C18 column, 1.7 µm, 2.1 x 100 mm for the separation of the toxins. A gradient elution was used where the fraction of acetonitrile (B) in the eluent changed as followed: 0 min, 2% B; 1.00 min, 40% B; 7.00 min, 55% B; 7.20 min, 98% B; 8.00 min, 98% B; 9.00 min; 2% B; 12 min, 2% B. The elution solvent A was made of Milli-Q Water. Both elution solvents were supplemented with 0.025% formic acid, the flow rate was 0.5 mL $min^{-1}$ and the column was heated to 60 °C.
The parameters for the detection of the different toxins can be found In Table 7.1. The electrospray was used in the positive mode at a capillary voltage of 1 kV, nebulizer gas pressure of 7.0 bar and a source temperature of 150 °C. The desolvation temperature and desolvation gas flow were 450 °C and 1000 L $h^{-1}$, respectively. Qualifier and Qauntifier ion fragments are represented in Figure 3.2.
The cone gas flow and collision gas flow were 150 L $h^{-1}$ and 0.15 mL $min^{-1}$, respectively. The elution peaks of the eight MCs and NOD in the three different matrices are shown in Figures S3.1–S3.3 in the Supplementary data.

**Table 7.1.** Mass-to-charge ratio (m/z) of the precursor, quantifier and qualifier ions for the eight microcystin congeners and nodularin. The collision energy (eV) and cone voltage (V) are shown for the quantifier and qualifier ions.

| Toxin | Precursor Ion (m/z) | Quantifier Ion (m/z) | Collision Energy (eV) | Cone Voltage (V) | Qualifier Ion (m/z) | Collision Energy (eV) | Cone Voltage (V) |
|---|---|---|---|---|---|---|---|
| MC-LR | 995.4 | 135.0 | 70 | 80 | 213.1 | 60 | 80 |
| MC-RR | 519.8 | 134.8 | 30 | 50 | 107.2 | 60 | 50 |
| MC-YR | 1045.5 | 135.3 | 80 | 60 | 212.9 | 60 | 60 |
| MC-WR | 1068.4 | 135.3 | 70 | 100 | 213.1 | 60 | 100 |
| MC-LY | 1002.4 | 135.3 | 60 | 50 | 213.0 | 50 | 50 |
| MC-LA | 910.3 | 135.1 | 60 | 50 | 107.1 | 80 | 50 |
| MC-LF | 986.3 | 135.0 | 60 | 70 | 213.1 | 60 | 70 |
| MC-LW | 1025.4 | 134.9 | 60 | 60 | 213.1 | 50 | 60 |
| NOD | 825.25 | 134.9 | 50 | 80 | 102.7 | 90 | 80 |

*7.2.1.3. Calculations of toxin concentration*

The quantification of the MCs was performed using the MassLynx TargetLynx software based on a matrix-matched calibration curve for each toxin from 0.10 to 50.00 ng $mL^{-1}$, except for MC-RR in strawberries, where 0.10 ng $mL^{-1}$ was excluded and the curve started at 0.50 ng $mL^{-1}$. The results were recalculated to µg $kg^{-1}$ and corrected with the recovery based on the QC.

## 7.2.2. Validation parameters

Three different matrices (i.e., lettuce, strawberries and carrots) were validated for our method at 3 concentration levels (i.e., 1.00, 5.00 and 25.00 µg $kg^{-1}$). However, the lowest validation point for MC-RR in strawberries did not meet the requirements for the limit of quantification (LOQ). Therefore, the lowest validation point (1.00 µg $kg^{-1}$) was replaced by 5.00 µg $kg^{-1}$ for MC-RR in strawberries and an additional point was validated at 10.00 µg $kg^{-1}$.

*7.2.2.1. Specificity*

The specificity was successfully validated if no identifiable signal was observed in the blank samples or lower than 1.00% of the obtained signal for the analysis of 5.00 µg $kg^{-1}$. Further, the signal for both the quantifier and qualifier ions must be present in a spiked or analytic sample to confirm that the signal coincides with the detection of a toxin.

*7.2.2.2. Ion Ratio*

The maximum variance of the ion ratios is determined in EU Decision 2002/657/EC [431]. Depending on the contribution of the individual ion to the total peak, a different variation is allowed.

*7.2.2.3. Limit of Detection (LOD) and Limit of Quantification (LOQ)*

The LOQ was defined as the lowest concentration where the method was fully validated and the signal-to-noise was higher than 10. The LOD was defined as the lowest concentration and the signal-to-noise was higher than 3.

*7.2.2.4. Linearity and matrix effect*

The linearity was evaluated based on a seven-point calibration curve ranging from 0.10 to 50.00 ng $mL^{-1}$, except for MC-RR in strawberries, where the calibration curve ranged from 0.50 to 50.00 ng $mL^{-1}$. Each level was injected twice. The ideal fitting of the curve was determined with Mandel's fitting test. If a quadratic regression was proposed, the $R^2$ of the linear regression was first evaluated. When this value was equal to or greater than 0.98, a linear regression was chosen over a quadratic one to simplify the subsequent calculations.
The matrix effect was determined for each toxin by producing a calibration curve combining all the toxins in Milli-Q water and methanol (+1% acidic acid) mix (50:50, v/v) and in a blank matrix (either carrots, strawberries or lettuce). When the fits of the calibration curves for the dilution solvent and blank matrix intersect, there is a matrix effect. A visual inspection of the linear fit was performed to determine the matrix effect. Additionally, the difference in the slope between the dilution solvent and the blank matrix was analyzed using the Student's *t*-test. The calculated t-value was compared with the tabulated at the 95% confidence level. If the difference between slopes is significant, a matrix effect is present.

*7.2.2.5. Recovery*

Recoveries were calculated based on the different spiked concentrations and should be between 70% and 120%. The recovery was calculated as follows:

$$\text{Apparent Recovery} = \frac{C_{mm}}{C_p}$$

where $C_{mm}$ = the mean of the means of each concentration level ($n$ = 9); $C_p$ = the spiked concentration.

*7.2.2.6. Repeatability, reproducibility and measurement uncertainty*

The acceptance criteria for reproducibility and repeatability were established based on EU Decision 2002/657/EC [431]. In this decision, the upper boundaries for both parameters are determined by the Horwitz ratio based on the coefficient of variation (CV) and average variance, respectively. In addition, the boundaries for the measurement uncertainty were set below or equal to 80%. This high amount of variation can be justified by the lack of internal standards. The measurement uncertainty was calculated as twice

the coefficient of variation. The statistical analysis was performed according to ISO5725-2 from 1994.

### 7.2.3. Sampling from the Belgium market

A sampling of fruits and vegetables at the point of sale was conducted for two reasons. The first was to further test the developed method. The second was to perform a screening of potential cyanotoxin presence in actual foods bought by the consumers. The selection of fruits and vegetables from the Belgium market was based on multiple criteria. EFSA food consumption data were used to identify the most consumed foods in Europe. Fruits and vegetables were ranked based on the available consumption data (Table 7.2). Only fruits and vegetables for which consumption data were available were taken into account for sampling. Other selection criteria were raw consumption, contact of edible part with irrigation water, harvest during or after cyanobacteria's bloom season and scientific and cultural relevance. The last two criteria were based on a lack of data present in the literature and vegetables or fruits typically consumed in Belgium, respectively. Moreover, the included products were preferably harvested in Belgium.

**Table 7.2.** Criteria for the sample matrix selection: food consumption rank, raw consumption, (direct) contact with irrigation water, harvested during cyanobacteria's bloom period and scientific relevance and cultural relevance.

| Sample Matrix | Consumption Rank * | Eaten Raw | Contact with Water | Harvested during Bloom Period | Scientific Relevance | Cultural Relevance |
|---|---|---|---|---|---|---|
| Carrots | 4 | X | | X | | |
| Onions | 5 | X | | X | | |
| Potato | 5 | X | | X | X | |
| Chicory | 3 | X | X | X | X | X |
| Radish | 2 | X | | X | X | X |
| Strawberries | 4 | X | X | X | X | |
| Tomato | 4 | X | X | X | | |
| Cherry Tomato | 4 | X | X | X | | |
| Lettuce | 4 | X | X | X | | |

* Rank 1 = no consumer data; rank 2 > 1 consumer; rank 3 > 100 consumers; rank 4 > 1000 consumers; rank 5 > 2000 consumers.

In total, nine different fruits and vegetables were selected based on these criteria. Carrots, chicory, radish, onions and potato were selected and grouped as root vegetables. Strawberries, tomatoes and cherry tomatoes were grouped as fruits. Only lettuce was selected as a leafy vegetable under our selection criteria. Table 7.2 provides a summary of the criteria applicable

to each selected group. A complete overview of all independent samples can be found in Table S3.2 of the Supplementary data. These data also include sampling data and the origin of the samples (as read on the label). The sample analysis for a particular matrix was conform if the recoveries calculated from the QC samples for this specific matrix adhered to the validation guidelines.

## 7.3. Results

### 7.3.1. Validation results for the different matrices

The validation for the eight MCs and NOD was successful in lettuce, strawberries and carrots. All toxins showed specific signals for qualifier and quantifier ions in all matrices adhering to the validation criteria. Ion ratios were also found within the boundaries set by EU Decision 2002/657/EC [431], as shown in Table 7.3.

For the most part, the validation parameters were met for the LOD and LOQ. The LOQ was set at 1.00 µg $kg^{-1}$, as this was the lowest validated concentration. The average signal-to-noise values for the LOQ were above 10, as shown in Table S3.1 of the Supplementary data. The LOD was set at the lowest concentration in the calibration curve (0.1 equal to 0.6 µg $kg^{-1}$) if the signal-to-noise was higher than three, as can also be seen in Table S3.1 of the Supplementary data. All toxins in all matrices adhered to this parameter for the LOD and LOQ, except MC-RR in strawberries, where the average signal-to-noise was not high enough. Therefore, the LOQ and LOD were instead validated at 5.00 and 3.00 µg $kg^{-1}$ (or LOD equal to 0.50 µg $L^{-1}$), respectively, for MC-RR in strawberries. An additional concentration level (10.00 µg $kg^{-1}$) was added specifically for this toxin to assess recovery, repeatability, reproducibility and measurement uncertainty later on during the validation.

**Table 7.3.** Results for the average ion ratio (%) and standard deviation (%) in carrots, lettuce and strawberries for the eight microcystin congeners and nodularin.

| | **Carrots** | | **Lettuce** | | **Strawberries** | |
|---|---|---|---|---|---|---|
| | Average Ion Ratio | Standard Deviation Ion Ratio | Average Ion Ratio | Standard Deviation Ion Ratio | Average Ion Ratio | Standard Deviation Ion Ratio |
| MC-RR | 15.28 | 5.43 | 16.15 | 5.82 | 13.13 | 2.63 |
| NOD | 42.66 | 4.69 | 41.24 | 1.65 | 46.58 | 2.29 |
| MC-LA | 47.36 | 7.07 | 51.46 | 6.07 | 40.28 | 1.78 |
| MC-LF | 40.45 | 1.77 | 41.14 | 2.14 | 39.31 | 0.89 |
| MC-LR | 31.98 | 1.20 | 32.57 | 2.81 | 31.75 | 1.68 |
| MC-LY | 50.01 | 3.87 | 49.58 | 6.26 | 51.80 | 5.08 |
| MC-LW | 45.36 | 2.05 | 44.85 | 2.32 | 45.19 | 2.00 |
| MC-YR | 36.45 | 3.98 | 35.84 | 4.49 | 38.88 | 4.65 |
| MC-WR | 43.05 | 10.74 | 41.22 | 4.03 | 38.93 | 2.21 |

The values for the recovery stayed within the preset parameters of 70.00–120.00% for all toxins in all matrices, as can be seen in Figure 7.1 and in Table S3.1 of the Supplementary data. The recovery for the sum of all toxins was also calculated for each concentration level and adhered to the preset parameters.

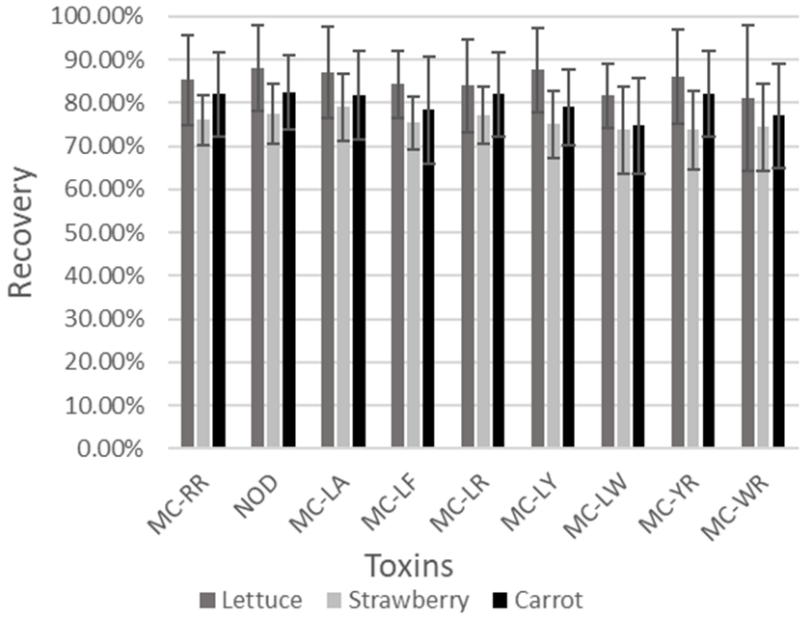
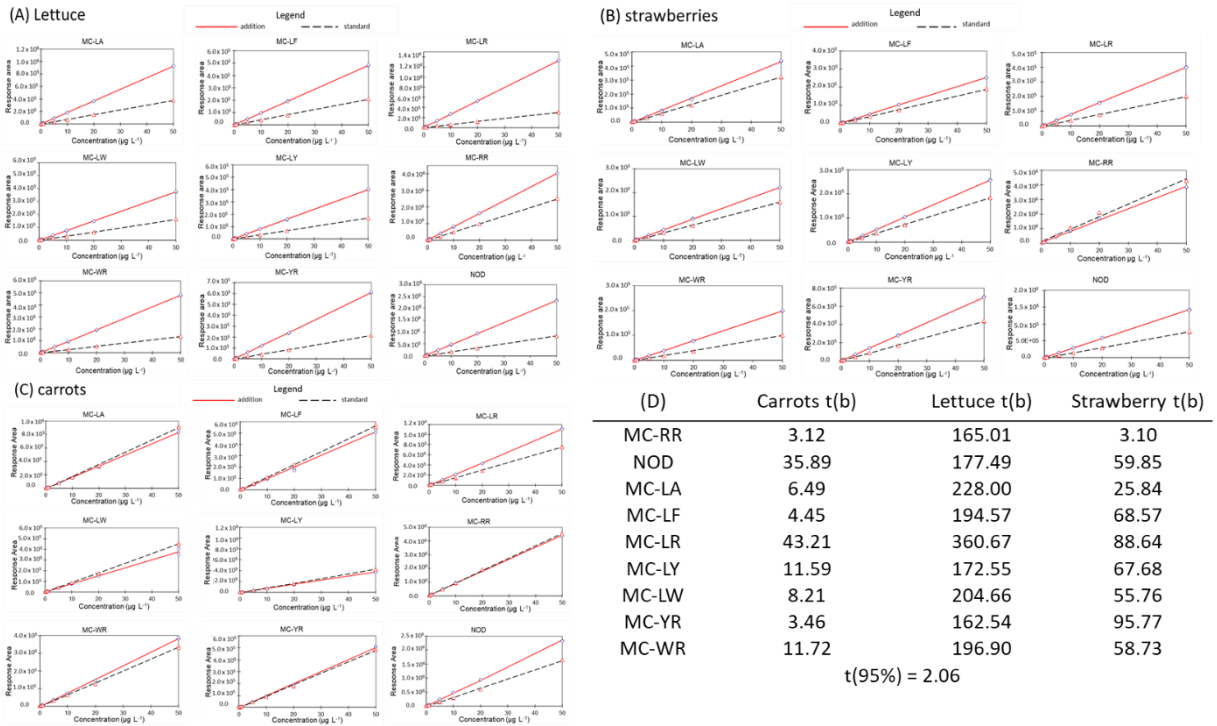



**Figure 7.1.** Average recoveries (%) obtained during the validation of the eight microcystin congeners (MCs) and nodularin (NOD) in the validated matrices (i.e., lettuce, carrots and salads). The average recovery for each toxin in each matrix was calculated from the recoveries at the different concentration levels.




| (D) | Carrots t(b) | Lettuce t(b) | Strawberry t(b) |
|---|---|---|---|
| MC-RR | 3.12 | 165.01 | 3.10 |
| NOD | 35.89 | 177.49 | 59.85 |
| MC-LA | 6.49 | 228.00 | 25.84 |
| MC-LF | 4.45 | 194.57 | 68.57 |
| MC-LR | 43.21 | 360.67 | 88.64 |
| MC-LY | 11.59 | 172.55 | 67.68 |
| MC-LW | 8.21 | 204.66 | 55.76 |
| MC-YR | 3.46 | 162.54 | 95.77 |
| MC-WR | 11.72 | 196.90 | 58.73 |

t(95%) = 2.06

**Figure 7.2.** Visual assessment of the matrix effect for the eight microcystins and nodularin in lettuce (**A**), strawberries (**B**) and carrots (**C**). Table (**D**) presents the calculated t(b) values of the Student's *t*-tests compared to t(95%), with t(b) < t(95%) if no significant difference was present between the slopes of the curve and, thus, no matrix effect.

The linearity of the calibration curves of the different toxins in the different matrices was evaluated with Mandle's fitting test. The ideal fit (quadratic or linear) always varied for the same toxin in the same matrix due to the R2 values that were similar for both fits. We eventually chose to calculate the concentrations with a linear fit, as the calculations are easier and linearity of the calibration curve is expected when using MS/MS. The R2 values are presented in Table S3.1 of the Supplementary data to illustrate the validity of the linear fit.

The matrix effect was observed for all of the toxins in all of the matrices based on a t-test on the slopes of the calibration curves in the matrix and dilution solvent. Plotting both curves, the presence of the matrix effect could be evaluated based on the intersection. When the lines intersect, a matrix effect is present, as illustrated in Figure 7.2.

Furthermore, Horwitz ratios determined the maximum repeatability and reproducibility for each toxin and the sum of all toxins in each matrix. The repeatability and reproducibility calculated with the results from the validation were below the maximum values calculated with the Horwitz ratio (Table S3.1 in the Supplementary data).

The measurement uncertainties were also below the boundary of 80.00% for all the toxins in all the matrices, as can be seen in Table S3.1 of the Supplementary data.

### 7.3.2. Method application on different vegetables and fruits

Multiple vegetables and fruits were collected from the Belgian supermarket from the end of July to the middle of November. Some radishes and potatoes originated from other countries, as during the sampling period, insufficient samples were available from Belgian origins (Table S2 in the Supplementary data).

Overall, no MCs or NOD were found in any of the vegetable or fruit samples. An additional quality control (QC) was added to each batch of analysis for each different matrix. The recoveries of the QCs for the matrices were within the boundaries (70–120%) used during validation (Table 7.4). The successful use of our validated method on vegetables and fruits other than carrots, salad and strawberries shows that our method can be used to analyze diverse matrices in these groups of products.

**Table 7.4.** Average recoveries (%) of quality controls in the market samples for the different matrices.

| Sample Type | MC-RR | NOD | MC-LA | MC-LF | MC-LR | MC-LY | MC-LW | MC-YR | MC-WR |
|---|---|---|---|---|---|---|---|---|---|
| Chicory | 98.53 | 97.07 | 95.87 | 96.93 | 99.93 | 105.20 | 110.27 | 89.47 | 94.80 |
| Onion | 84.05 | 87.50 | 89.85 | 83.70 | 87.40 | 83.60 | 75.45 | 87.65 | 80.60 |
| Cherry Tomato | 87.38 | 85.78 | 90.38 | 79.33 | 86.70 | 88.58 | 70.15 | 83.68 | 80.38 |
| Raspberry | 80.10 | 79.50 | 95.10 | 81.20 | 82.10 | 90.50 | 72.80 | 86.50 | 86.30 |
| Tomato | 91.40 | 99.20 | 106.80 | 100.30 | 89.50 | 98.30 | 104.40 | 108.20 | 109.50 |
| Carrot | 81.58 | 86.02 | 91.05 | 103.15 | 96.28 | 84.70 | 101.65 | 81.90 | 80.15 |
| Potato | 79.38 | 88.05 | 89.58 | 97.60 | 95.48 | 85.53 | 96.75 | 87.87 | 80.57 |
| Straw -berries | 79.93 | 91.13 | 84.47 | 75.07 | 87.73 | 82.13 | 84.47 | 80.00 | 93.07 |
| Lettuce | 70.0 | 82.67 | 89.07 | 98.47 | 105.67 | 77.07 | 83.13 | 76.00 | 70.87 |

## 7.4. Discussion

We successfully validated an UHPLC-MS/MS method for the highest number of MCs (i.e., eight) described in the literature and NOD in three plant matrices (i.e., carrots, lettuce and strawberries). Moreover, this method describes the first validation for strawberries, where, due to the fact of their different chemical composition compared to the other matrices, it clearly showed a slightly lower recovery for MCs. The matrix effects caused by the strawberries significantly masked the signal of MC-RR, resulting in an increased LOD and LOQ. Chemical diversity among different fruits and vegetables is not uncommon, as Li et al. (2014) already reported the use of an HLB SPE cartridge for cucumbers and eggplants to increase MC recovery instead of the C-18 cartridges used for the validation of other vegetables [398]. The influence of the matrix effect on different methods of analysis has been shown [392,394,627]. In the three matrices we tested, the matrix effect was shown to affect the quantification if the calibration curve was prepared in a solvent. To account for the matrix effect, we opted for a matrix-matched calibration curve.

Separate parameters (LOD, LOQ, recovery and MU) are difficult to compare between the existing validated methods due to the use of different matrices, state of the matrix (dry or fresh weight), methodology or calculation of the parameters. One method used MS [627], while another was developed to extract MCs from different matrices (i.e., fish, soil and vegetables), resulting in a completely different methodology [394]. Similarly, in 2018, Díez-Quijada

et al. developed an UHPLC-MS/MS method for CYN and three MCs, altering the methodology using a duplicate SPE, which caused a generally reduced recovery for all compounds [392]. Moreover, they reported a calculated LOD and LOQ, while our LOD and LOQ were based on the signal-to-noise values for the lowest point in the calibration curve and the lowest validated concentration, respectively. Our more experimental approach assured the measurability of the concentrations at the LOD and LOQ, while the calculated LOD and LOQ should be confirmed by the measurements after the validation. However, this confirmation is often not presented in the literature. Li et al. (2014) validated an UHPLC-MS/MS method similar to ours with only three MCs but in multiple matrices [398]. Our recoveries were similar to theirs. Furthermore, their similar recoveries for vegetables were different from our matrices, suggesting that the validation of our three matrices as model matrices for different fruit and vegetable types is valid. Using a QC as a control, we can use our validated method to quantify MCs and NOD in other leaf and root vegetables as well as in fruits and berries without additional validation for each matrix. However, if the recovery of the QC is not sufficient, an additional validation or adjustment to the method could be warranted, as shown for cucumbers and eggplants [398].

Although our study did not find any MCs or NOD in the fruit and vegetables taken from Belgian markets, multiple studies suggest that the accumulation of MCs in crops under real agricultural production is possible [398,420,423]. Up to 3 µg $g_{freshweight}^{-1}$ of the total microcystin concentration was found in the edible parts of different plants after irrigation with contaminated water from a groundwater well [423]. MC-RR, MC-LR and MC-YR were also found in lettuce, water spinach, cabbage and chio sum irrigated with contaminated lake water, with the total microcystin concentrations ranging up to 108.2 µg $kg_{freshweight}^{-1}$ [398]. MC-LR was also detected in the edible tissues of two rice variants (20.97 and 18.19 µg $kg^{-1}$) and Ipomoea aquatica (132.86 µg $kg^{-1}$) [420]. These results suggest that when sampling vegetables for monitoring of MCs, the number of samples per species and variety of samples should be increased. Additionally, for more targeted analysis, one could collect crops produced near locations where (toxic) blooms occur (i.e., “hot spots”). This approach requires the availability of reliable and rich data on bloom monitoring in various types of waterbodies, including those that could be used for the irrigation of plants and crops. However, only recreational waterbodies are monitored for toxic blooms in Belgium [609], but they cannot be exploited for irrigation purposes. Therefore, reliable monitoring of

cyanotoxin accumulation in crops has to coincide with an increased scope of monitoring of blooms in waterbodies. Alternatively, the establishment of citizen science initiatives to report blooms might increase awareness of this issue and allow for a more accurate assessment of the presence of MCs in crops.

## 7.5. Conclusions

Our UHPLC-MS/MS method quantified eight MCs and NOD in three crops (i.e., carrots, lettuce and strawberries). Moreover, the quantification of MCs in strawberries as a model matrix for fruits is novel. Together with the validation of our uniform method in leafy and root vegetables, these matrices can be used as models for the quantification of the eight MCs and NOD in other edible plants. This approach does require adequate quality controls.
Following the initial screening of nine different vegetables and fruits from Belgian markets, it appears that there is currently no accumulation of MCs. Despite a lack of detection of MCs or NOD in the market samples, the quality control of the developed method was acceptable and reliable. Therefore, the methodology is adequate for future support of food market monitoring and MCs research in general.

**Supplementary data:** Data available in Chapter S3, Table S3.1: Validation results for eight microcystin congeners (MCs) and nodularin (NOD) in carrots, lettuce and strawberries at three concentration levels and on average. The included parameters are recovery, repeatability, reproducibility, measurement uncertainty (MU), average signal-to-noise for LOD, average signal-to-noise for LOQ and $R^2$; Table S3.2: Overview of the samples taken from Belgian markets showing separate results, origin, sample type and sample annotation. Figure S3.1: The elution peaks for eight microcystin congeners and NOD in carrot matrix at validation level 5 ng $g^{-1}$. The peaks are presented together at representable ratios based on the peak intensities by overlaying the chromatograms of the different toxins. However, during analysis, the chromatograms for each toxin are analyzed separately. Figure S3.2: elution peaks for eight microcystin congeners and NOD in lettuce matrix at validation level 5 ng $g^{-1}$. The peaks are presented together at representable ratios based on the peak intensities by overlaying the chromatograms of the different toxins. However, during analysis, the chromatograms for each toxin are analyzed separately. Figure S3.3: elution peaks for eight microcystin congeners and NOD in lettuce matrix at validation level 5 ng $g^{-1}$. The peaks are presented together at representable ratios based on the peak intensities by overlaying the chromatograms of the different toxins. However, during analysis, the chromatograms for each toxin are analyzed separately.

# 8. Belgian consumption survey for *Chlorella*- and cyano-based food supplements

**Contributing authors:** *Wannes Hugo R. Van Hassel* prepared the survey, analyzed data and wrote the chapter; *Mirjana Andjelkovic,* supervised the study and was involved in the critical analysis of the data; *Annick Wilmotte* and *Andreja Rajkovic* supervised the study, helped with the dissemination of the survey, and were involved in manuscript corrections and discussion.

## 8.1. Introduction

In Chapter 6, the prevalence of MCs was quantified in *Chlorella*- and cyano-based food supplements and a preliminary exposure assessment was described. However, consumption data for *Chlorella*- and cyano-based food supplements were not included in the exposure assessment. This data was not included because detailed consumption data for *Chlorella*- and cyano-based food supplements, and food supplements in general, have been lacking in Belgium. EFSA Comprehensive Consumption Database, which is a source of information on food consumption across the European Union (EU), also has only limited information on this consumption type. The data regarding the consumption of food supplements like vitamins and some algal-based supplements were present, but there was an insufficient number of survey respondents for relevant conclusions. Therefore, to evaluate the consumption, the posology data, i.e. recommendations on how many and how often to use a food supplement, was used as a basis during the *Chlorella*- and cyano-based food supplements-specific exposure assessment in Chapters 6 and 9. Yet, assumptions had to be made about consumer profile, adherence to the proposed dose, and consumption frequency (on a weekly and yearly basis).

Many studies have already described the general use of food supplements (also referred to as dietary supplements) in different populations all over the world and at different times [597,644–657]. Nearly all these studies have usual limitations, specifically regarding the targeted populations which were either not specific or not representing the whole population. However, some conclusions seem to be plausible. Generally, women were more likely to use food supplements [597,644,647,650–652,654,656], while supplement use was more reported in higher-educated cohorts of the investigated

populations. Overall, vitamin and micronutrient supplements were more often used than plant-based products [597,644,647,650–652,654,656]. However, similar to *Chlorella*- and cyano-based food supplements, it was reported that the consumption of plant food supplements (PFS) was also rising, as shown in a PFS-specific survey in six European countries [658]. Moreover, differences in the marketing of products, like PFS, depended on national legislation, which differs widely across European Member States and could contribute to differences in consumption [658].

The main reported motivation for supplement consumption was to improve the health of the user [597,644,647,650–652,654,656]. Yet despite these surveys, information about consumption frequency and the dose is variable or missing. This lack of information is understandable as different types of food supplements require different posologies and consumption frequencies. Moreover, specific population cohorts (supplement users, students, elderly, et cetera) were targeted during specific studies, potentially skewing the results for generalization of the outcomes [648,652,655]. The most relevant results for Belgium were obtained from the Belgium food consumption survey conducted in 2014 and a Dutch study from 2017 [597,654].

Specific consumption data for *Chlorella*- and cyano-based food supplements was obtained during the Belgium food consumption survey. The data was collected from the population aged 3 to 64 years. *Chlorella*- and cyano-based food supplements, which were defined as ‘‘spirulina’’ and *Chlorella* in the survey, were reported as part of the consumption of “other food supplements”, next to general food supplements like vitamins and multivitamins. Overall, *Chlorella*- and cyano-based food supplements contributed to 11% of the category “other food supplements”, which was consumed by 15% of all participants [597]. A study from Taiwan showed that 3.9% of adults aged 19 to 44 consumed “algal food supplements” [657]. However, this category of supplements could also contain green algae supplements. The only study specifically focused on *Chlorella*- and cyano-based food supplements (e.g. *Chlorella*, ‘spirulina’ and *Aphanizomenon* products) surveyed Polish consumers (n = 150) [655]. ‘Spirulina’, *Chlorella* and *Aphanizomenon* products were used in descending order of frequency. The products were consumed most frequently as tablets (annotated as pills in our study).

To better estimate the consumption of *Chlorella*- and cyano-based food supplements in Belgium, a new survey on the consumption of food supplements and *Chlorella*- and cyano-based food supplements was created

and distributed at the University of Liège and Ghent. Amongst others, the type of product, frequency and adherence to the indicated posology were surveyed as important criteria for future exposure assessments for these products. The consumption was represented in function of the demographical information of the participants and their general use of various classes of food supplements. Moreover, the consumption data was used to reassess the exposure of the Belgian population to MCs from *Chlorella*- and cyano-based food supplements in Chapter 9.

## 8.2. Methodology

The anonymous online survey, using the open-source software LimeSurvey (version 3.22.24+200630) [659] was conducted. After performing the literature search for any validated survey study and consulting the Food Frequency Questionary used in the Belgian National Food Consumption Survey [597], a survey was designed to evaluate consumer habits regarding food supplements and in particular *Chlorella*- and cyano-based food supplements. The survey with two main parts (general and specific) was distributed primarily in the universities of Liège and Ghent between 28 November 2021 and 1 April 2022. However, the internet (e.g. Facebook, LinkedIn, Instagram and the Sciensano websites) was also used as a distribution platform. The survey was constructed in two forks using LimeSurvey in two languages, French and Dutch [659]. Eight general questions were asked to obtain information about age, diet, level of education, general health and fitness from the participant. Then, five questions about all food supplements were posed to the participants in the first fork. The food supplement users were asked for additional information about the type and the number of products and their reasons for consumption, while non-users were asked why they did not consume these products. In the second fork, all participants were asked if they used certain algal-based food supplements (*Chlorella*- and cyano-based food supplements). If the answer was positive, the users were required to complete seven additional questions to complete the survey. Non-users skipped to the end of the survey. The additional questions concerned the frequency of consumption, the formulation of the product, adherence to indicated dose, the reasons for consumption and prior research sources before consumption. An overview of the questions and responses can be found in Chapter S4 in the supplementary data. Most results have been presented as descriptive data. For some data, the statistical analysis was performed using $\chi$-statistics for $p < 0.05$ in Rstudio ®.

## 8.3. Results

### 8.3.1. Survey on food supplement

In total, 558 participants participated in the survey between 28 October 2021 and 1 April 2022. An initial selection of participants between ages 18 and 74 left 554 completed responses. The average and median ages were 37.64 and 32 years, respectively. More women (64.26%) than men (34.66%) participated in the survey. An additional 1.08% of the participants identified as another gender or preferred not to divulge this information. The surveyed population primarily constituted higher educated participants (University diploma or PhD (72.56%) ) and Bachelor's degree (19.86%). The largest part of the surveyed population did not follow any specific diets (66.42%), while flexitarians and vegetarians represented 14.80% and 8.66% of the population respectively. Participants were asked to rate their physical health on a scale of "bad", "reasonable", "good" or "excellent", resulting in a 57.94% "good" rating and a 21.30% "excellent" rating. Yet, physical activities excluding and including sports, were "less than 1 hour" and "between 1 to 3 hours" respectively for the largest part of the population.

Most participants used one (21.48%) or multiple (42.78%) food supplements in general. In winter and autumn, users consumed significantly ($p < 0.05$) more food supplements (80.23% and 78.65%, respectively) compared to spring (60.67%) and summer (54.47%) (Figure 8.1).

The food supplement users consumed micronutrients, single vitamins and multivitamins in descending order as described in Table 8.1. *Chlorella*- and cyano-based food supplements were consumed by 13.00% and 17.98% of the 554 respondents and food supplement users, respectively (Table 8.1). The most cited reasons for food supplement use by the users were: "To stay healthy", "To support/improve my immune system", "For medical reasons", "As an addition to my regular diet". The majority of the non-users did not consume the products because they believe they receive sufficient nutrients from their regular diet. The non-users of food supplements also did not believe in their promised effect (21.21%).

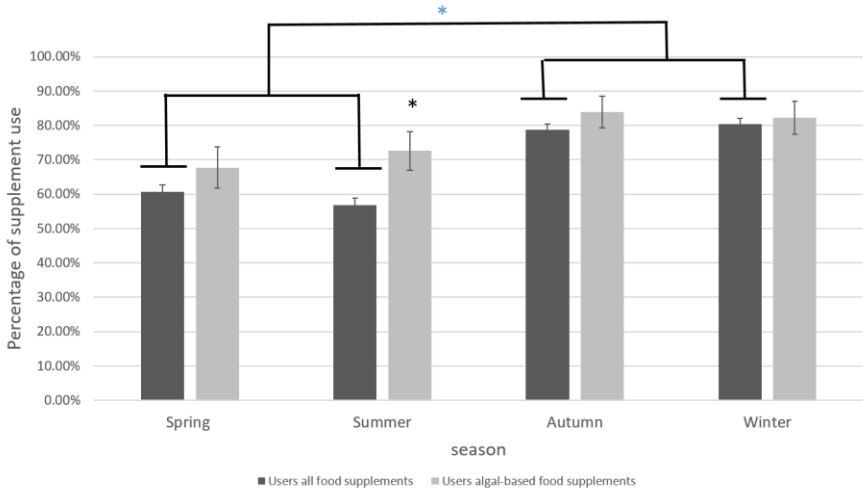



**Figure 8.1**. Consumption frequency during the different seasons for all food supplements and *Chlorella*- and cyano-based food supplements users. * significant difference ( $p < 0.05$) for the general food supplement consumption between different seasons using χ-statistics. * significant difference ( $p < 0.05$) between the general food supplement consumption and the *Chlorella*- and cyano-based food supplements consumption during the same seasons using χ-statistics. The error bars show the standard deviation for the different conditions

**Table 8.1.** Independent consumption of each food supplement group divided by the number of participants that use at least one food supplement product. The participants can consume multiple products. However, each participant is counted only once as food supplement user.

| ***Supplement categories*** | ***Consumption by food supplement users*** |
|---|---|
| *Multivitamins* | 41.57% |
| *Combination of antioxidants* | 16.29% |
| *Single vitamin* | 59.83% |
| *Micronutrients* | 65.45% |
| *plant-based food supplements* | 29.21% |
| *Algae-based food supplements* | 17.98% |
| *Collagen* | 5.06% |
| *Fishoil* | 17.42% |
| *Other* | 23.60% |

On the other hand, of the *Chlorella*- and cyano-based food supplements users (n= 62), 75.81% used 'spirulina' and 16.13% used *Chlorella* (Figure 8.2). One participant (1.61%) used Apha or Klamath products. Multiple algal-based products were used by 30.65% of product-specific users. In general, only 2.25% of the food-supplement users consumed exclusively algal-based products.

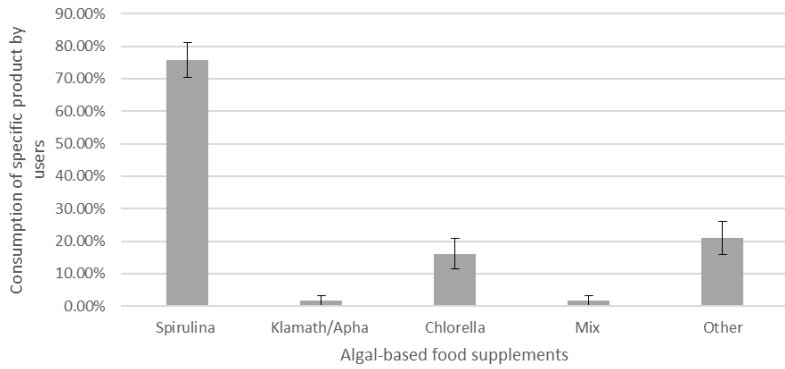



**Figure 8.2.** Consumption of different products by algal-based food supplement users. The error bars show the standard deviation for the different conditions.

Daily consumption (45.31%) of the *Chlorella*- and cyano-based food supplements was most common among the *Chlorella*- and cyano-based food supplements users (Figure 8.3). The users also seemed to prefer pills (46.77%) compared to powder or capsule products, in descending order. Some users also mixed different algal-based products (3.13%). Most users (43.55%) also followed the indicated daily dose on the package. However, 17.74% of the users reported consuming more than the indicated dose and 9.38% were not aware of the existence of the indicated dose. Interestingly, the seasonal usage of algal-based food products was different from the general consumption of food supplements. Algal-based food supplements were consumed significantly more throughout the summer compared to other supplements as shown in Figure 8.1. Moreover, consumption of the *Chlorella*- and cyano-based food supplements was not significantly different between the different seasons. Significantly more *Chlorella*- and cyano-based food supplements users (59.32%, n=59) consumed the product during the whole year compared to the overall use of food supplements (44.10%).

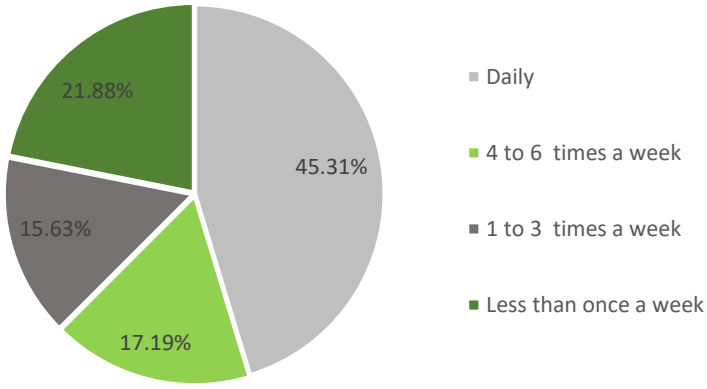



**Figure 8.3**. Consumption frequency of algal-based food supplements by users during one week.

The reasons for the use of *Chlorella*- and cyano-based food supplements were similar to those for food supplements in general. *Chlorella*- and cyano-based food supplements were also primarily consumed to boost the user's general health. Most consumers of these products quoted the internet (59.38%) and their medical practitioner or pharmacist (25.00%) as information sources about these products. Most of the users trusted the activity, quality and safety of the *Chlorella*- and cyano-based food supplements. However, 31.25% of the users indicated that they did not know.

## 8.3.2. Limitations of the survey

The survey was designed to verify the use of recommended dose and to get insight into the consumption. By its design it has limitations. The study was not organised as a national survey taking into account the representativeness of the whole Belgian population. In the study, the highest proportion (92.42%) of the participants had obtained a higher degree of education. If this is compared to the proportion of higher educated people in the general population (36.1%) in Belgium, this might suggest an imbalance in the surveyed population. This imbalance might be caused by the primary distribution of the survey in universities [660]. However, many studies have shown that people with higher education were more likely to use food

supplements [597,644,647,650]. In the Belgium food consumption survey, 45% of the participants with a higher education aged 3 to 64 consumed food supplements, compared to 34 to 37% of participants with a lower grade of education [597].

Another limitation was the lack of data for the population aged 0 to 16. The online format of our survey was not ideal to reach children. Moreover, children would have needed assistance from a parent to complete the survey. However, the consumption of food supplements by children would probably be decided by their parents. Although the survey was also distributed online, teenagers were not incentivized to complete the survey, probably resulting in a lack of response.

## 8.4. Discussion

In total, 64.28% of participants reported consuming at least one food supplement. These values were similar to the surveys of different populations in The United States, Denmark, Italy and The Netherlands [651–654]. However, our value was higher than the value (38%) in the Belgian Food Consumption Survey (BFCS) of 2014 [597]. This latter survey summarized consumption data for the Belgian population between ages 3 and 64, while our survey took into account ages 18 to 74. Moreover, the BFCS was concluded in 2014, and as discussed in this chapter, the consumption of food supplements has increased [596].

The increased consumption of food supplements is part of a changing dietary habits and emerging trends. The eating habits, mostly in the Western World with diet that results in intake of (i) an excess of sugar, salt, fats, and chemical additives in processed foods and (ii) a shortage of fibres, amino acids, vitamins and minerals, brought about new emerging diet and lifestyle trends, such as attention to physical appearance and physical and mental well-being, slowing down of the ageing process, a need to cure metabolic disorders, specific health problems preventions and overall fitness [661]. Therefore, the Vitamins and dietary supplements global market has grown at a rate of 6,3% (compound annual growth rate (CAGR) 2014-2018), mainly driven by the segment of food supplements. In Western Europe, the healthy living trend supported growth among key players such as the UK and Italy, where the latter was already culturally used to taking supplements (eight out of 10 Italians consumed vitamins and dietary supplements). Meanwhile, in Germany, the second biggest market, demand was supported by the growing popularity of veganism and vegetarianism among consumers. Vitamins and dietary supplements sales in Western Europe increased at the growth rate in

2014-2018 with a 3,5% CAGR with a market value of almost 10 €B in 2018). The growth of demand for vitamins and dietary supplements in Eastern Europe was reported to be somewhat slower (CAGR 1,1%), but has been spurred by three main trends, health and wellness, convenience and increasingly hectic lifestyles. North America had the highest market value (around 26 €B in 2018) with respect to the European market, with a growth trend of 6,7% (CAGR) in the period 2014-2018 [661].

During our survey, women participated more often (64.25%) and their usage of supplements (68.8%) was higher than other genders, similar to all earlier publications [597,644–657]. During our study, the primary motive for the consumption of food supplements was to enhance health, like in all other surveys. The dominant intake of micronutrients and (multi-) vitamins by the population were equally similar to previous surveys. However, in our survey, increased use of other types of food supplements was seen compared to the consumption of micronutrient and (multi-) vitamin supplements in the general Belgian population. For instance, plant-based food supplement consumption was below 10% during the Belgium National Food Consumption Survey, while it was reported as 29.21% in this study [597]. Moreover, 17.98% of the participants reported using algal-based food supplements, which is an increase compared to the (+/-) 1.40% in the national food consumption survey [597]. Differences in the selected population might also partially explain this difference.

The most relevant point of comparison for *Chlorella*- and cyano-based food supplements use is a Polish survey of supplement-specific users [655]. Also in that study, 'spirulina' products were more often used, followed by *Chlorella* and Klamath, sequentially. However, the use of 'spirulina' was more often reported in our study, while the consumption of *Chlorella* and Klamath products was lower compared to the Polish study. Pill/tablet formulation was most commonly used by the participants in both studies. The internet was also reported as a primary information source in the Polish survey. In our study, more *Chlorella*- and cyano-based food supplement users gathered information from medical professionals and apothecaries compared to the Polish study. There were also fewer *Chlorella*- and cyano-based food supplement users in our study that did not know what the recommended dose on the package was, compared to the other survey. Furthermore, our study indicated a year-round consumption of the supplements compared to the Polish study where only 12% of the users were consuming the *Chlorella*- and cyano-based food supplements for 1 year or longer. The demographic characteristics of the Polish study were similar to the current study, with a

mean age of 35 years, mostly female, having a moderate income and a higher education [655].

## 8.5. Conclusion

Overall, the main target of the survey was to quantify the consumption of *Chlorella*- and cyano-based food supplements in the Belgium population for later use in exposure and health risk assessment. With 554 usable responses, the study is representative of the Belgian cohort between age 18 and 74, taking into account that the group of higher-educated people in Belgium are probably over-represented. In total 17.98% of the participants used Chlorella- and cyano-based food supplements. 'Spirulina' was used by most users. Moreover, most users used pill products (46.77%) daily (45.16%), throughout the year (59.32%) following the recommended dose on the package (43.55%). This data can now be used for exposure and health risk assessment. However, more comprehensive and quantitative data concerning individual consumption frequency and dose should be obtained in the future. This could be accomplished by including more questions about *Chlorella*- and cyano-based food supplements and Food supplements in general in the next Belgian Food Consumption Survey.

**Supplementary data:** Overview of survey questions and data is available in Chapter S4

# 9. Associated and aggregated health risks of MCs in foodstuffs and environment

**Contributing authors:** *Wannes Hugo R. Van Hassel* critical analysis of the data and wrote the chapter; *Mirjana Andjelkovic,* supervised the study and was involved in the critical analysis of the data and was involved in manuscript corrections and discussion; *Andreja Rajkovic* supervised the study;

## 9.1. Introduction

In the previous Chapters, MCs prevalence data was gathered for different exposure routes, e.g. drinking water, waterbodies, *Chlorella*- and cyano-based food supplements and fruit and vegetables. Moreover, additional Belgian consumption data for *Chlorella*- and cyano-based food supplements was obtained. These data can now be used in preliminary exposure and risk assessments to evaluate the impact of MCs on public health in Belgium. Risk assessments were initially focused on the relationships between exposure to a single chemical from a single source and a resulting adverse health outcome expressed in TDI. However, there has also been a need to simultaneously evaluate the impact of a chemical from various sources, or multiple chemicals from one or more sources on disease outcomes. As a result, there was an increasing need to better understand the complex mechanisms that influence risk of chemical and non-chemical stressors, beginning at their source and ending at a biological endpoint relevant to human or ecosystem health risk assessment.

First of all, the exposure sources of MCs should be assessed. For algal bloom material, exposure assessments were dependent on multiple assumptions during different exposure scenarios and were only scarcely explored in previous research. In most studies, only exposure to bloom through ingestion (e.g. involuntary ingestion of water during recreation activities) was counted as a risk [122,662,663]. Yet, no data concerning the frequency of recreational activities during bloom season is currently available. Therefore, a scenario of daily recreational activities was commonly used so that lifetime exposure values can be used [662]. However, this approach was changed during the newest WHO guidelines for recreational water, which used a value for acute exposure instead [122]. Dermal contact could be another exposure route but not enough indications have been found suggesting harmful effects could occur [662]. Moreover, the inhalation of bloom material via aerosols or

particles of dried bloom material has been considered as a possible exposure route. Yet, after evaluating potential inhalation exposure cases, no traces of cyanotoxins were found in human blood [662,664]. Nonetheless, a guideline concentration has been proposed of 4.58 ng MC-LR $m^{-3}$ [665], although such high levels have not yet been reported [662]. Furthermore, the frequency data for exposure of MCs through inhalation is also not available. Intravenous exposure of MCs was also observed in dialysis patients due to the use of contaminated water during treatment in Brazil [544].

Exposure of MCs through foods, like fruit and vegetables and freshwater organisms was already suggested. The prevalence of MCs was already discussed in subchapters 1.4.3 and 7.1. Moreover, the EFSA consumption database could also be used to estimate the consumption of these products [613]. Yet, the available prevalence toxin data is currently too limited to perform an extensive exposure assessment.

An initial exposure assessment for *Chlorella*- and cyano-based food supplements was already done in Chapter 6. The approach was verified using the *Chlorella*- and cyano-based food supplements consumption data presented in Chapter 8. This data was included in further calculations for the risk assessment. Comprehensive risk assessments have been overall lacking for cyanotoxins. However, these kinds of assessments were important to evaluate the effects of cyanotoxins as a whole. In most publications, risk assessments were approximated by using a limited data set of cyanotoxin prevalence, usually only for MCs, in one possible exposure source. The cyanotoxins concentration of individual samples was then compared with the TDI. Samples, for which the TDI was exceeded, were described as a potential risk. This approach was applied for risk characterization in vegetables [640,666], *Chlorella*- and cyano-based food supplements [390], crayfish [377], and fish [381,385,397]. However, this approach was limited since it only shows the exposure from one external source, namely food, and may significantly underestimate the risk.

To evaluate full exposure to one chemical via all exposure routes (dermal, oral and inhalation) and from different sources (drink water and accidentally ingested water), an aggregated exposure should be calculated. For example, parabens were found in personal care products in the last decade [667], leading to the development of the PACEM tool used to assess the exposure of consumers. Moreover, advancements in measurement technologies continued to result in an increasing abundance of occurrence data that could be combined with improving modelling capabilities to produce additional exposure information. The combination of exposure from different sources

(cosmetic products, diet and food supplements), as illustrated for example vitamins [668], provided a more realistic estimation of human health risk. In this way, the main contributing exposure route could be better understood, resulting in more effective risk mitigation in case of safety guideline exceedance [669].

Historically, aggregate risk calculations combining different exposure sources have not been performed, due to a lack of data or approaches. The only assumption that was made so far regarding MCs is that drinking water would be responsible for 80% of the exposure to MCs, followed by the consumption of foods and recreational activities, respectively [122,132,662,663,670,671]. However, aggregate exposure should be the sum of exposures of an individual or a defined population to a specific agent from all sources and pathways. Therefore, the relevance of exposure to MCs through different foods should also be assessed. Moreover, this assessment should be performed on a regional level if dietary habits were variable [671]. However, the aggregate exposure assessment for MCs in foods has not yet been possible on any of the currently established platforms in the scope of the European Projects (Merlin Expo, Integra, or similar) [672,673]. These platforms consolidated both, biomonitoring and physiologically based pharmacokinetic dose models, combining estimated exposures from multiple sources, pathways and routes to provide a projected single dose or biologically effective dose metric. In their vision by 2030, EFSA will work together with its partners on the development of harmonized cross-cutting methodology and regulatory guidance for aggregate exposure assessment for food contaminants that will take into account all relevant sources and routes of exposure to chemicals [674]. A valuable review of existing methodologies, models and tools for assessing external exposure from different sources and for aggregate exposure assessment will be performed. The hazard index system was suggested as one of the approaches for combined exposure assessment. In general, it was calculated as the sum of all ratios of exposure estimates over the health-based guidance value.

In this study, we have used the hazard index system for the estimation of the aggregated exposure to MCs from three different sources (environmental, food and water). Occurrence data from Belgium were used in a simple deterministic approach, using lower and upper-bound approaches. Through this analysis, the first assessment of aggregated exposure for MCs was performed.

## 9.2. Materials and methods

### 9.2.1. Concentration data

MCs concentration data for the 35 *Chlorella*- and cyano-based food supplements (Chapter 6) and 79 algal bloom samples (Chapter 4) collected in Belgium were taken into account for the exposure assessment. However, data from 107 fruit or vegetable samples (Chapter 7), 75 drinking water samples (Chapter 3) were not included due to the absence of MCs. The concentration data for these exposure sources were already discussed in depth in the previous chapters. For the exposure assessments, the median, average, 95$^{th}$ percentile and maximum of the total MCs concentrations were calculated for all samples (per exposure group) using an upper and lower-bound approach (Table 9.2). For the lower bound (LB) approach, all samples containing individual MCs concentrations below LOD or LOQ were assumed to be 0 for each toxin. Then the upper bound (UB) approach was used, equalizing individual MCs concentrations below LOQ to the value of the LOQ for environmental and *Chlorella*- and cyano-based food supplements samples, 0.56 µg $L^{-1}$ and 50.00 µg $kg^{-1}$ respectively. Moreover, samples with concentrations below LOD were equalized to the value of the LOD for environmental and *Chlorella*- and cyano-based food supplements samples, 0.25 µg $L^{-1}$ and 22.5 µg $kg^{-1}$ respectively.

### 9.2.2. Consumption data

Following the online survey results (Chapter 8), 43.55% of the *Chlorella*- and cyano-based food supplements users followed the recommended dose for *Chlorella*- and cyano-based food supplements indicated on the package. Yet, recommended doses on the package could differ from product to product. Therefore, several assumptions related to consumption had to be made to establish exposure scenarios. Firstly, the mean daily intake corroborated by the EFSA Comprehensive Consumption Database was used as the mean assumed reference dose. For the moderate- and worst-case doses, the mean and 95$^{th}$ percentile values calculated from the posology data for the analyzed products (Chapter 6) were used, respectively (Table 9.1). Independently of consumption dose, *Chlorella*- and cyano-based food supplements were consumed daily by 45.31% of the users. Therefore, daily consumption of the products was assumed during the exposure and risk assessment for the reference-, moderate- and worst-case doses.

MCs intake via recreational use of waterbodies was not available. To estimate it, calculations used to establish the WHO guidelines for recreational water were used as a basis. The daily (incidental) water intake during recreation activities was assumed to be 250 mL (for a child) [122]. This value was also used for adults due to a lack of an alternative value. Furthermore, no general data was available for the frequency of recreation in waterbodies by the Belgium population, neither in normal conditions nor when algal blooms were present. Assumptions were made for both conditions. Therefore, two conservative assumptions for the frequency were proposed to avoid overestimation of the exposure (Table 9.1). The mildest assumption was one event of recreation activities during the total bloom period (mid-June to mid-October or 122 days). The second assumption took into account weekly recreation activities (22 days) during the bloom season, as daily recreational activities in surface water were unlikely for the majority of the population.

### 9.2.3. Exposure scenarios

The different assumptions for the dose or frequency of consumption of *Chlorella*- and cyano-based food supplements and water intake during recreation were made, respectively. This resulted in three exposure scenarios (reference, moderate and worst case) which are shown in Table 9.1. For both exposure sources, the reference exposure case scenario assumed that a person is exposed to an LB mean MCs concentration. Whereas for the *Chlorella*- and cyano-based food supplements consumption a mean daily intake was used, for the water intake during recreation it was assumed that a person would be exposed only once during the blooming period, which is estimated to last 122 days. For the moderate- and worst-case scenarios, values were calculated for the LB and UB median, mean, 95$^{th}$ percentile or maximum of the MCs concentration in the samples by either consuming the mean or 95$^{th}$ percentile recommended daily quantity (*Chlorella*- and cyano-based food supplements) or through ingestion of water during recreational swimming (once or weekly during the bloom period). The most severe assumptions for the consumption of *Chlorella*- and cyano-based food supplements and water during recreation were used for the worst-case exposure scenario.

**Table 9.1**. Overview of exposure scenarios for *Chlorella*- and cyano-based food supplements (CCFS) and recreational water use. For recreational water use, one exposure event is the accidental ingestion of 250 mL of recreation activities [122].

| **Exposure scenario** | **CCFS per day ($CN_{CCFS}$)** | | **Recreational water per day ($CN_R$)** | |
|---|---|---|---|---|
| | consumption | Concentration (µg $kg^{-1}$) | consumption/exposure rate | Concentration (µg $kg^{-1}$) |
| **reference case** | 1.62 g $day^{-1}$ (Average consumption in EFSA database) | Median<br>Mean<br>95$^{th}$ percentile<br>Max | One-time exposure ($ES_A$) during bloom period (122 days ($ES_B$)) | Median<br>Mean<br>95$^{th}$ percentile<br>Max |
| **Moderate case** | 3.14 g $day^{-1}$ (average recommended dose from sample packages) | Median<br>Mean<br>95$^{th}$ percentile<br>Max | One-time exposure ($ES_A$) during bloom period (June to October 122 days ($ES_B$)) | Median<br>Mean<br>95$^{th}$ percentile<br>Max |
| **Worst case** | 6.25 g $day^{-1}$ (95$^{th}$ percentile recommended dose from sample packages) | Median<br>Mean<br>95$^{th}$ percentile<br>Max | Weekly (22 days ($ES_A$)) exposure during bloom period (June to October 122 days ($ES_B$)) | Median<br>Mean<br>95$^{th}$ percentile<br>Max |

## 9.2.4. Exposure assessment and hazard index

In general, exposure expressed as estimated dietary intake ($EDI_{CCFS}$) was calculated by multiplying the MCs concentration( C, µg $kg^{-1}$product) with a daily dose (DD, g) and dividing by body weight (bw, kg). Body weight was taken into account as a measure to normalize the differences in the consumption rate between children and adults. This deterministic approach was a one-point estimation for the different exposure scenarios and was expressed by the following equation.

**Equation 9.1.** The formula for estimated dietary intake (EDI µg $kg^{-1}{}_{bw}$ $day^{-1}$) in *Chlorella*- and cyano-based food supplements (CCFS). MCs concentration( C, $\mu g_{MCs\ total}$ $kg^{-1}{}_{product}$) with a daily dose ($DD_{CCFS}$, g) and divided by body weight (bw, kg)

$$EDI_{CCFS} = \frac{C \times DD_{CCfs}}{\text{bw}}$$

Additionally, daily exposure values for recreational surface waters (ExpR) were modified to include assumed involuntary ingestion of water for the different exposure scenarios.

**Equation 9.2.** The formula for daily exposure values for recreational surface waters ($Exp_R$). $ES_A$ (days) and $ES_B$ (days) are described in Table 9.1. C represents the MCs concentration (μg $L^{-1}$) ) in the recreational water. DIW is the daily incidental water consumption (250 mL).

$$\mathrm{Exp_R} = \frac{\mathrm{C} * \mathrm{DIW} * (\frac{\mathrm{ES_A}}{\mathrm{ES_B}} * \frac{1}{365\ \mathrm{days}})}{\mathrm{bw}}$$

The ratio of $ES_A$ and $ES_B$ described the frequency of exposure in recreational water where $ES_A$ is how many days a person was effectively exposed and $ES_B$ estimates how many days the cyanobacterial bloom season lasts with an assumption that a person can be exposed to MCs only during a bloom (Table 9.1). DIW is the daily incidental water consumption (250 mL), which was used by WHO in their latest drinking water guideline for MC-LR as a default for children [122]. Body weight (bw) was assumed to be either 70 kg or 15 kg for adults and children, respectively.

### 9.2.5. Aggregated exposure assessment

In order to evaluate the aggregated exposure from all possible sources, the hazard index was proposed. The risk quotient for each exposure source was calculated by dividing the EDI for each source by the known TDI. The sum of all risk quotients for each exposure source resulted in the aggregated hazard index (AHI). If a risk index is higher than 1, there is a risk concern for exposure.

**Equation 9.3.** The formula for aggregated hazard index (AHI). The sum of the EDIs for different exposure sources, is divided by the TDI.

$$\mathrm{AHI_{MC}} = \frac{\mathrm{EDI_{CCFS}}}{\mathrm{TDI}} + \frac{\mathrm{EXPI_R}}{\mathrm{TDI}}$$

## 9.3. Results

### 9.3.1. Concentration data

For the evaluation of the aggregated exposure, a lower and upper bound approach was used to determine the MCs concentration values in all samples to further calculate the median, mean, 95$^{th}$ percentile and maximum

concentrations for the exposure sources. These MCs concentration data are given in Table 9.2. From the concentration data, it was already clear that the exposure will give a range for most values between the lower and upper bound approach.

**Table 9.2**. The median, mean and 95$^{th}$ percentile values of total MCs concentrations for the *Chlorella*- and cyano-based food supplements (CCFS) and surface water samples from Belgium waterbodies. The values were calculated based on all analyzed samples using a lower bound (LB) and upper bound (UB) approach.

| Exposure source | CCFS (µg kg$^{-1}$) ($C_{CCfs}$) | | Surface waters (µg L$^{-1}$) ($C_R$) | |
|---|---|---|---|---|
| | All analyzed samples_LB | All analyzed samples_UB | All analyzed samples_LB | All analyzed samples_UB |
| Median | 0.00 | 202.5 | 1.33 | 3.18 |
| Mean | 369.90 | 519.35 | 137.92 | 139.57 |
| 95$^{th}$ percentile | 2188.62 | 2353.00 | 619.93 | 620.41 |
| Maximum | 5645.33 | 5762.83 | 2798.81 | 2799.06 |

## 9.3.2. Exposure assessment for different scenarios

An overview of the calculated range between LB and UB exposure values for the different scenarios (Table 9.1.) is presented in Table 9.3. The daily exposure to MCs by *Chlorella*- and cyano-based food supplements consumption for adults and children was expanded upon compared to Chapter 6. In this chapter, EDI was calculated for a specific exposure scenario, whereas the EDI was first calculated for each contaminated sample based on their recommended dose before using this data to determine the median, average and 95$^{th}$ percentile values in Chapter 6. More exposure scenarios were proposed, supported by the consumption data gathered from the survey (Chapter 8).

Daily consumption of *Chlorella*- and cyano-based food supplements resulted in MCs exposure exceeding the TDI (0.04 µg $kg_{bw}^{-1}$) for the 95$^{th}$ percentile in moderate-case scenarios for adults, as well as for the mean, 95$^{th}$ percentile in moderate-case scenarios for children. TDI was equally exceeded for these concentration levels in the worst-case scenario, with the addition of the mean exposure for adults. The results for children in the reference-case were not reported because the consumption data is not robust for this population. The exposure scenarios for recreational waters showed a low exposure to MCs, below the TDI. MCs daily exposure through recreational activities did not exceed TDI in any exposure scenario.

**Table 9.3.** Calculated exposures for *Chlorella*- and cyano-based food supplements (CCFS) and recreational water during reference-, moderate- and worst-case scenarios, including concentration levels at the median, mean, 95$^{th}$ percentile and maximum. Values are shown as a range between lower bound (LB) and upper bound (UB) calculations for adults and children.

| **Consumption scenario** | **Concentration level** | **CCFS daily exposure ($EDI_{CCFS}$) (µg $kg_{bw}^{-1}$) LB - UB** | | **Recreational water daily exposure ($EDI_R$) (µg $kg_{bw}^{-1}$) LB - UB** | |
|---|---|---|---|---|---|
| | | Adult | Children | Adult | Children |
| Reference-case | Mean | 0.01 | /* | $1.11\ 10^{-5}$ | $5.16\ 10^{-5}$ |
| Moderate-case | Median | $0.00 - 9.08\ 10^{-3}$ | $0.00 - 4.24\ 10^{-2}$ | $1.07\ 10^{-7}$ – $2.55\ 10^{-7}$ | $4.98\ 10^{-7}$ - $1.19\ 10^{-6}$ |
| | Mean | 0.02 - 0.03 | 0.08 - 0.12 | $1.11\ 10^{-5}$ - $1.12\ 10^{-5}$ | $5.16\ 10^{-5}$ - $5.22\ 10^{-5}$ |
| | 95$^{th}$ percentile | 0.10 - 0.11 | 0.46 - 0.49 | $4.97\ 10^{-5}$ - $4.98\ 10^{-5}$ | $2.32\ 10^{-4}$ - $2.32\ 10^{-4}$ |
| | Maximum | 0.25 - 0.26 | 1.18 - 1.21 | $2.24\ 10^{-4}$ - $2.24\ 10^{-4}$ | $1.05\ 10^{-3}$ - $1.05\ 10^{-3}$ |
| Worst-case | Median | $0.00 - 1.81\ 10^{-2}$ | 0.00 - 0.08 | $2.35\ 10^{-6}$ - $5.61\ 10^{-6}$ | $1.10\ 10^{-5}$ - $2.62\ 10^{-5}$ |
| | Mean | 0.04 - 0.5 | 0.17 - 0.25 | $2.43\ 10^{-4}$ - $2.46\ 10^{-4}$ | $1.14\ 10^{-3}$ - $1.15\ 10^{-3}$ |
| | 95$^{th}$ percentile | 0.20 - 0.21 | 0.91 - 0.98 | $1.09\ 10^{-3}$ - $1.09\ 10^{-3}$ | $5.10\ 10^{-3}$ - $5.10\ 10^{-3}$ |
| | Maximum | 0.50 - 0.51 | 2.35 - 2.40 | $4.49\ 10^{-3}$ - $4.49\ 10^{-3}$ | $2.30\ 10^{-2}$ - $2.30\ 10^{-2}$ |

*EFSA consumption data for *Chlorella*- and cyano-based food supplements only robust for adults.

## 9.3.3. Aggregated risk assessment of MCs for the Belgium population

To further calculate the risk for each exposure source, the risk daily exposure quotient (RQ) was used for the individual exposure sources. The aggregated hazard index ($AHI_{MC}$) was also calculated based on the sum of the RQ's for *Chlorella*- and cyano-based food supplements ($RQ_{CCFS}$) and recreational water ($RQ_R$). An overview of the RQ's and $AHI_{MC}$ can be found in Table 9.4. When $AHI_{MC}$ >1, the health risk is high.

**Table 9.4.** Calculated hazard index for *Chlorella*- and cyano-based food supplements (CCFS) and reactional waterbodies for reference-, moderate and worst-case scenarios. Aggregated hazard index was calculated based on the sum of the individual hazard indexes. Values are shown as a range between lower bound (LB) and upper bound (UB) calculations.

| **Consumption scenario** | **Concentration level** | **Risk Quotient CCFS ($RQ_{CCFS}$) LB - UB** | | **Risk Quotient Recreational water ($RQ_R$) LB - UB** | | **Aggregated hazard index ($AHI_{MC}$) LB - UB** | |
|---|---|---|---|---|---|---|---|
| | | Adult | Children | Adult | Children | Adult | Children |
| reference case | Mean | 0.23 | /* | $2.77\ 10^{-4}$ | $1.29\ 10^{-3}$ | 0.23 | /* |
| Moderate case | Median | 0.00 - 0.23 | 0.00 - 1.06 | $2.67\ 10^{-6}$ - $6.38\ 10^{-6}$ | $1.24\ 10^{-5}$ - $2.98\ 10^{-5}$ | $2.67\ 10^{-6}$ - 0.23 | $1.24\ 10^{-5}$ - 1.06 |
| | Mean | 0.45 - 0.66 | 2.07 - 3.9 | $2.77\ 10^{-4}$ - $2.80\ 10^{-4}$ | $1.29\ 10^{-3}$ - $1.31\ 10^{-3}$ | 0.45 - 0.66 | 2.07 - 3.10 |
| | $95^{th}$ percentile | 2.45 - 2.64 | 11.45 - 12.31 | $1.24\ 10^{-3}$ - $1.24\ 10^{-3}$ | $5.80\ 10^{-3}$ - $5.81\ 10^{-3}$ | 2.46 - 2.64 | 11.46 - 12.32 |
| | Maximum | 6.33 - 6.46 | 29.54 - 30.16 | $5.61\ 10^{-3}$ - $5.61\ 10^{-3}$ | $2.62\ 10^{-2}$ - $2.62\ 10^{-2}$ | 6.34 - 6.47 | 29.57 - 30.18 |
| Worst case | Median | 0.00 - 0.45 | 0.00 - 2.11 | $5.87\ 10^{-5}$ - $1.40\ 10^{-4}$ | $2.74\ 10^{-4}$ - $6.55\ 10^{-4}$ | $5.87\ 10^{-5}$ - 0.45 | $2.74\ 10^{-4}$ - 2.11 |
| | Mean | 0.89 - 1.32 | 4.13 - 6.16 | $6.08\ 10^{-3}$ - $6.15\ 10^{-3}$ | $2.84\ 10^{-2}$ - $2.87\ 10^{-2}$ | 0.89 - 1.33 | 4.16 - 6.19 |
| | $95^{th}$ percentile | 4.89 - 5.25 | 22.80 - 24.51 | $2.73\ 10^{-2}$ - $2.74\ 10^{-2}$ | $1.28\ 10^{-1}$ - $1.28\ 10^{-1}$ | 4.91 - 5.27 | 22.93 - 24.64 |
| | Maximum | 12.60 - 12.86 | 58.81 - 60.03 | $1.23\ 10^{-1}$ - $1.23\ 10^{-1}$ | $5.76\ 10^{-1}$ - $5.76\ 10^{-1}$ | 12.72 - 12.99 | 59.38 - 60.60 |

The $AHI_{MC}$ showed already a potential health risk for children beginning at the moderate-case scenario for the median at the higher end (UB) of the calculated range. The UB limit was relevant as it was used more during initial risk assessments compared to the LB limit. This scenario showed that children could already be at risk when they consume the daily indicated dose of *Chlorella*- and cyano-based food supplements contaminated with rather low concentrations of MCs, while they are only once exposed to MCs through recreational activities in water that would be considered safe. Moreover, the health risk was high for the range of $AHI_{MC}$ starting at the moderate-case when the mean concentration level was taken into account for children.

It should also be noted that the independent RQ for recreational water barely contributed towards $AHI_{MC}$, in most cases. The $RQ_R$ only significantly contributed to the $AHI_{MC}$ for children at the $95^{th}$ percentile and maximum concentration level during the worst-case scenario. Therefore, recreational exposure to MCs seemed to contain low risks during our conservative scenarios.

For adults, the $RQ_{CCFS}$ had again the highest contribution to the $AHI_{MC}$. Adults were also at high risk ( $AHI_{MC}$ between 2.46 - 2.64) when exposed to $95^{th}$ percentile values of MCs under moderate consumption scenarios. This scenario showed that consuming the daily indicated dose of *Chlorella*- and cyano-based food supplements can be harmful if a product contains MCs in the higher observed concentrations. This could be representative of daily consumption of highly contaminated *Chlorella*- and cyano-based food supplements of the same product batch due to the long use of the same brand. In the worst-case scenarios, adults could already be at risk when they were exposed to concentrations close to the upper bound of the mean concentration level ($AHI_{MC}$ between 0.89 - 1.33). This situation could represent the use of a contaminated product and use at doses above the recommended consumption.

## 9.4. Discussion

Legislation and regulatory guidance documents now specifically call for estimates of total or aggregate human exposure in order to accurately cover multiple sources and routes of exposure to contaminants [669,675–677]. Deterministic and probabilistic approaches can be used. Deterministic approaches are simpler and use the available prevalence and consumption data for each exposure source to produce a point estimate [676]. This approach should provide a conservative assessment with a high level of uncertainty. By including an LB - UB approach in the deterministic approach, a range of exposure replaced the point estimates and provided a measure to approximate a part of the uncertainty associated with the risk assessment. In the case of this study, variation of MCs concentrations in *Chlorella*- and cyano-based food

supplements was taken into account. On the other hand, different probabilistic assessments would be more realistic as they take into account both the distribution of the contaminant (e.g. MCs) in the food product and the distribution of food consumption of individuals in a population [669]. To enable these kinds of assessments, high-quality consumption and prevalence data should be accessible to apply different probabilistic approaches [678–680]. These approaches have already been implemented for pesticides [676], mycotoxins [680], heavy metals [679] and other compounds [668,681–683], for which sufficient data of each type was available. However, the currently available occurrence data of MCs in any of the possible exposure sources in Belgium is still too limited on its own to assess the exposure with probabilistic approaches. Similarly, *Chlorella*- and cyano-based food supplements' specific consumption data for individual consumers was also limited to inexistent.

Therefore, different exposure scenarios were proposed during this study to deterministically assess the exposure of the Belgian population to each exposure source identified and evaluated in the previous chapters. Based on these scenarios, a deterministic aggregated risk index was used to assess aggregated risk in Belgium for MCs exposure. Moreover, the LB and UB approach was used to provide a range of possible risks for each scenario. This range was quite small for the 95$^{th}$ percentile and maximum values due to small differences in the calculated values for the LB and UB. For the median and mean values, the ranges were bigger.

The proposed risk assessment showed a risk of harmful MCs exposure in the moderate and worst-case scenarios for adults and children at different concentration levels. However, this risk primarily originated from the consumption of *Chlorella*- and cyano-based food supplements and not from recreational activities. Drinking water, fruit and vegetables were not included in the assessment, as no MCs prevalence have been detected in Belgium this far.

The moderate- and worst-case exposure scenarios for *Chlorella*- and cyano-based food supplements were developed based on the posology data of the analyzed samples as this is the only data that was available in Belgium (Chapter 6). The use of the posology data was supported by the results from the *Chlorella*- and cyano-based food supplements consumption survey (Chapter 8), as the majority of the *Chlorella*- and cyano-based food supplements users (43.55%) consumed the indicated daily dose. Chapter 6 also showed that all the contaminated products were from one particular origin (Klamath/Apha). Therefore, taking into account all consumption of all *Chlorella*- and cyano-based food supplements would cause an overestimation of the risk. Yet, both moderate- and worst-case scenarios could represent cases where the consumption of contaminated *Chlorella*- and cyano-based food supplements occur due to consumer loyalty to a specific product or brand, showing a clear health

risk. Our initial deterministic risk assessment substantiates the decades-old concern of the scientific community that there could be risks involved in the consumption of *Chlorella*- and cyano-based food supplements products, especially for children [376,562,564]. For future work, it would be important to also directly assess *Chlorella*- and cyano-based food supplements consumption by children. However, performing these kinds of surveys comes with additional difficulties (e.g. legislation, GDPR, outreach to participants) that could not be tackled during this PhD thesis.

Our current deterministic approach could further be refined by aggregating consumption and MCs concentration of different *Chlorella*- and cyano-based food supplements products ( e.g. *Chlorella*, Apha, 'Spirulina' and mixtures) in different product groups during the exposure assessment. By this aggregation, a more representative mean, median and P95 concentration could be calculated. This would be relevant as only one type of supplement was observed to be contaminated with MCs. Moreover, additional refinement of this data should be possible with the product-specific consumption information during risk assessment. However, this approach would need to be implemented in the future as the current thesis was more focused on the analytical aspects of the work. Similarly, extensive modelling could be used to prepare the posology and survey data for a probabilistic approach. However, this kind of modelling was also outside the scope of our current work. Moreover, acquiring additional MCs prevalence data and consumer data for *Chlorella*- and cyano-based food supplements would enable the use of more traditional probabilistic approaches to better assess the exposure risk.

From our risk assessment, chronic recreational exposure to MCs showed little risk to public health. However, there were some caveats in our risk assessment that might underestimate the overall risk. The most important factor was the complete lack of data concerning the frequency of recreation in waterbodies by the Belgian population. Therefore, quite arbitrary exposure scenarios were proposed to assess exposure rates (days of exposure during bloom season) of the population during recreation activities. More data should be gathered concerning the use of recreational waterbodies during bloom season to produce a more accurate risk assessment. Moreover, additional MCs prevalence data should be collected to represent all recreational waterbodies in Belgium. Our current maximum UB, 2799.06 µg $L^{-1}$ is low compared to maximum concentrations reported in other studies (up to 42.7 mg $L^{-1}$) [205,364,387,391,402,427,539,570,577–582]. This nearly 10-fold increase combined with our worst-case scenario would provide an assessment of where there is a risk to public health.

The eventual risk was calculated by comparing the exposure in different scenarios to the TDI. However, TDI was a measure of chronic exposure [122]. For *Chlorella*- and

cyano-based food supplements, this seemed appropriate as the consumption survey showed that most *Chlorella*- and cyano-based food supplements users used the product daily and during the whole year. However, recreation activities in surface waters and cyanobacteria blooms were expected to be largely seasonal. Therefore, exposure rates to MCs in the different scenarios were normalized to exposure throughout a whole year, as an approximation of chronic exposure.

Ideally, probabilistic approaches should be used in the future, but more high-tier data should be available. Moreover, additional data should be gathered for other exposure sources to enhance the risk assessment. Monitoring of Belgian drinking water for the MCs presence should be continued since the increased use of surface water as a drinking water source during hot summers could result in contamination due to inadequate water treatment (Chapter 3). Moreover, additional sampling of fruit and vegetable products should be performed to evaluate MCs prevalence more extensively. Multiple studies have shown MCs accumulation is possible in the lab for rice [420,624,636], leafy vegetables [420,565,632], beans [639] and fruits [637] and under agricultural conditions [638,640,666]. Other relevant exposure sources were the food products from freshwater ecosystems (e.g. fish, shellfish, molluscs), which were shown to accumulate MCs at potentially dangerous levels [132,377,381,384,465,467,671,684,685]. MCs prevalence data for these products should be obtained, as well as proper assessments of their consumption in Belgium. Indeed, the daily consumption of a single contaminated product would be limited due to the large choice of different food sources and fishing for sustenance is very limited and even prohibited in most Belgium fresh waterbodies.

## 9.5. Conclusion

A preliminary risk assessment for MCs exposure in Belgium was made based on a deterministic approach using lower- and upper-bound approaches for the available data incorporated in three different scenarios. In the reference-case scenario, no health risk was observed for adults. However, moderate- and worst-case scenarios showed the range of exposures that result in a health risk for adults and children that primarily originated from consumption of *Chlorella*- and cyano-based food supplements when they are contaminated by MCs. Higher-tier exposure assessment approaches will rely on the MCs prevalence data and consumption data availability. While refinement of the deterministic approach is still possible in future research. Additional data should be acquired to enable the use of probabilistic approaches and calculate the uncertainty for the risk assessment.

# 10. General discussion and future perspectives

**Contributing authors:** *Wannes Hugo R. Van Hassel* wrote the chapter; *Mirjana Andjelkovic, Annick Wilmotte* and *Andreja Rajkovic* supervised the study and were involved in manuscript corrections and discussion.

This last chapter will be used to summarize and discuss the results as a whole and suggest future research topics, methodological developments for monitoring of cyanotoxins in Belgium and management approaches to reduce the public health risk caused by cyanotoxins. At the end of this chapter, a perspective on the planned, but not executed, experiment to co-cultivate *Microcystis* and *Arthrospira* will also be provided. This experiment was not pursued due to time constraints. The reasoning behind abandoning these experiments and approaches for the experiments will be discussed in the last subchapter (10.4).

## 10.1. Evaluation of methodology and future perspectives for detection and quantification of cyanotoxins in Belgium

During my PhD study, methodologies for the detection and quantification of eight MCs and NOD were developed in four different matrices. While most matrices required a different extraction approach, the approach for detection and quantification of the toxins with UHPLC-MS/MS was universal. The ability to use our quantitative analytical method for different matrices can be ascribed to the use of matrix-matched calibration curves that mitigate matrix effects. Moreover, the run time during analysis was 12 minutes in total. Therefore, the method length situates itself in the middle of the already published quantification methods [21–28]. Proper separation of the different analytes was chosen above speed. Yet, a 12-minute run is certainly still fit to acquire a fast final reporting speed. A report could be produced in 3 to 5 days' time when sample transport, preparation and extraction and analysis speed were taken into account. These kinds of reporting speeds are necessary to quickly evaluate cyanobacterial bloom material in recreational lakes, toxin accumulation in food and human intoxication events. Speed is of the essence in these cases to assess possible hazards to public health, but also reduce economic damage when no intoxication risk can be observed. Indeed, when the production or sale of uncontaminated food is halted due to a possible health threat, products might needlessly perish when analytical results take too long to be obtained. These delays can then result in economic losses for the producer or seller. Similarly, prolonged

prohibitions of the use of recreational waterbodies can cause extensive economic losses for the owner of the accommodation but also the surrounding food, beverage and lodging establishments. Economic losses, caused by analysis time should be mitigated as much as possible. Therefore, extraction methodologies were developed fit for the purpose with an emphasis on analysis time. However, longer extractions, containing SPE and evaporation steps, were necessary to develop a reliable method and adhere to validation criteria.

The selection of validation criteria and adherence to them was also an important factor influencing the development of the method. Proper validation was required to ensure the quality of the results. The selected criteria were based on requirements for analytical analysis for food safety under (BELAC) accreditation. These requirements resulted in more stringent criteria than those used in some other published methods. Recovery should be at least above 60.00% and preferably above 70.00%. The LOQ was also calculated differently for most other methods [21–28]. We applied a newer approach where the LOQ is defined as the lowest point for which the accurate quantification of the compounds has been shown for all validation criteria, thus the lowest level of validation. Most other methods either define a concentration for the LOQ based on a signal-to-noise above a cutoff of 10 calculated by analytical software or measure dilutions of a compound until a concentration has been found for which the signal-to-noise is 10. Yet, other validation parameters for this concentration have not been evaluated, which would be necessary to define this quantifiable concentration as the limit for a method. Although our LOQ was usually higher compared to other methods, and thus reduced the resolution of the method, we believe it to be more accurate. The ion ratios for the qualifier ion were also evaluated during the validation of all the methods to ensure both the qualifier and quantifier ions are good identifiers for the targeted MCs and NOD. This was required for HPLC-MS/MS methods by the European directive for validation of analytical methods [431]. However, most validation studies did not present this data [388,392,394,398].

Repeatability and reproducibility were compared to and remained below their respective maximum variance calculated by the Horwitz equation, as suggested by most guidelines for validation [431,432,435]. A more statistical approach, thus far not yet implemented for MCs, was used to evaluate the linearity of the calibration curve. For the linearity, a Mandels fitting test was used instead of only determining the $R^2$, while in most publications, only the latter was determined. Our approach assured that the best fit is assessed to further inform decisions made during the validation. Similarly, matrix effects were established by comparing the slopes of a matrix-matched and solvent calibration curve, which showed matrix effects for nearly all MCs

and NOD in all matrices. This approach could be considered an improvement on the method used by other studies, where the overlay of the calibration curves was compared [388,394]. During analysis, matrix effects were mediated by using a matrix-matched curve. Therefore, the matrix effect did not need to be quantified. Other studies have quantified the matrix effect by dividing the slopes of the matrix-matched and in-solvent calibration curves, subtracting this number by 1 and taking the percentage of the result [392,398].

**Equation 10.1** Calculation matrix effect based on Li et al. 2014

$$Matrix\ effect = 100\ x\ (\frac{slope\ matrix}{slope\ solvent} - 1)$$

However, it was also suggested that values below |10|% and between |10|% and |20|% could be ignored or were of low relevance, respectively, due to variation during the detection caused by the measurement equipment [398]. Yet, we believe these values to be inconsequential as the use of a matrix-matched calibration curve to obtain the best possible quantification is preferred. Moreover, it was clear from the development of our methods that usually at least one of the MCs will experience major influences of the matrix during quantification and detection.

Furthermore, the targeted UHPLC-MS/MS approach was selected during our research as it was capable of identifying specific MCs separately and accurately quantifying the compounds in the matrix. Both specific detection and accurate quantification were crucial characteristics of the method for its future implementation in food safety programs and support in food legislation. Lacking either of these characteristics could result in a lack of clarity in future legislation, which could prevent its implementation. Moreover, vague legislation could cause contention of analytical results by producers resulting in potentially contaminated foods circulating on the market and invalidating time-consuming and expensive monitoring resources. Yet, no official regulation for MCs in food was in place in Belgium or on a European level. However, Belgium does follow the European regulatory value for MC-LR (1 µg $L^{-1}$) in drinking water [474]. Due to this regulation, methodologies for testing MC-LR concentrations should be in place from 2024 onward, showing the necessity for reliable analytical methods to quantify MCs. In the future, we can also expect an extension of this regulation for other MCs and cyanotoxins, as well as similar regulations for foodstuffs and recreational waterbodies.

The selective nature of UHPLC-MS/MS also has its downsides. Some of the many MCs will not be able to be accurately quantified due to a lack of analytical standards, resulting in an underestimation of the total MCs concentration [29–31]. cPPIA, ELISA and MMPB methods could be used instead as they detect the universal Adda fragment [128,157,410,529]. cPPIA and ELISA are overall cheaper and require less expertise to use. However, these methods were reported to be more susceptible to matrix effects and activities of other compounds (false positives). Utilizing these methods in matrices other than water also requires thorough validation as most available kits are only validated for use in water. Usually, analysis of MCs with cPPIA and ELISA would require a secondary confirmation of the results using UHPLC-MS/MS methods. MMPB methods could be a good alternative to cPPIA and ELISA as their quantification was supposed to be more reliable in more complex matrices. However, MMPB is as expensive and requires as much expertise as other UHPLC-MS/MS methods. Moreover, the additional oxidation step increases the analysis time. Furthermore, most competent authorities on food and water safety will also require quantification of independent toxin congeners, analogues or metabolites to determine the total sum of the toxin group available in a product to enact legislation. For instance, different STX congeners need to be analyzed separately to be able to assess the risk of their presence in seafood based on their different toxicity factors [291,292]. Similarly, only MC-LR is regulated in drinking water. If a non-selective method is used, like cPPIA, ELISA and MMPB, the results could be questioned as MC-LR was not specifically identified and thus should be verified with UHPLC-MS/MS. Similarly, UHPLC-MS/MS will need to be used to quantify a selection of MCs if they would be included in future legislation.

In the future, newer approaches using quantitative HRMS could also provide all the needed information by accurately assessing the concentration of all MCs, and other cyanotoxins in a sample [342,378,686]. However, at the start of the thesis, these methodologies were in their infancy and even now, they are not widely used. The cost and skills needed to procure and utilize these machines, respectively, are important factors for the slow start of implementation of these methods in the food safety field. Yet, fast quantification of ATX and hATX at 1 ng $mL^{-1}$ was accomplished using direct analysis in real-time (DART)-HRMS on a Q Exactive HF Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) [686]. Further developing and applying these technologies to obtain increased sensitivity for the quantification of different cyanotoxins in different matrices can help reduce analysis time. Moreover, orbitrap mass spectrometers are more and more available in scientific institutes, including Sciensano.

Future perspectives on the application and extension of ELISA and UHPLC-MS/MS methodologies in Belgium will be discussed in subchapter 10.3, as these applications are linked with the targeted toxins and matrices.

## 10.2. Evaluation and future perspectives of MCs prevalence and risk assessment in Belgium

Overall, this dissertation presented the first compilation of validated UHPLC-MS/MS methodologies in a toolbox to assess MCs prevalence in different exposure routes for a complete population. Moreover, the data, obtained by applying these methodologies for the different exposure sources, was used for a preliminary, yet unprecedented aggregated risk assessment.

Applying the toolbox, to quantify MCs provided an opportunity to assess MCs prevalence in the different matrices (fruits and vegetables, drinking water, environmental samples and *Chlorella*- and cyano-based food supplements). During our research, *Chlorella*- and cyano-based food supplements and drinking water were thoroughly sampled for the first time from Belgium's market and analyzed with a state-of-the-art methodology. Moreover, the assessment for fruits and vegetables with UHPLC-MS/MS was also the first data acquired for Belgium samples. However, this data was only preliminary as greater numbers of samples should be examined to thoroughly get a grasp on the time-dependent occurrence of cyanotoxins in these kinds of samples. The data obtained for *Chlorella*- and cyano-based food supplements, fruit and vegetables provided valuable insights on the global occurrence of MCs in these products, as currently, this data is still limited, especially for risk assessment purposes.

From our current sampling of different matrices, MCs were found in particular *Chlorella*- and cyano-based food supplements and environmental samples showing the potential exposure of the public to these toxins. The lack of MCs in the drinking water samples was a result of the frequent use of underground water sources in Belgium, especially in Flanders [519–521]. These kinds of sources should normally not be susceptible to MCs contamination and cyanobacteria blooms in general due to a lack of sunlight. Moreover, surface water that is used in Belgium as drinking water should be tested for MC-LR by the producer and intensively treated before introduction in the general waterworks. However, diligent testing remains necessary as more surface water sources will be needed in the future due to dwindling water supply in aquifers, lower groundwater replenishment rates and hotter summers propagated by climate change [517]. Moreover, toxic bloom monitoring of bathing

waters and other waterbodies used for recreation should be continued and expanded as hotter summers might also induce an increase in water-related recreation, even potentially in waterbodies where this is prohibited in Belgium.

Due to decreasing availability of water, surface water will probably be used more frequently to irrigate crop plants. The MCs contaminated water can potentially cause accumulation of these toxins in the plants [41–43]. Although MCs were not detected during our limited sampling of nine fruit and vegetable types from the Belgian market, accumulation in crops has been shown in multiple studies, as extensively discussed in Chapter 7. With increasing and prolonged occurrences of cyanobacterial blooms, research on the mechanisms of accumulation for all cyanotoxins should be continued in vegetables and fruits, as well as the monitoring of these toxins in these products. Similarly, the consumption of freshwater aquatic organisms could cause potential exposure to MCs. However, research towards the accumulation of MCs in fruit and vegetables was given priority as consumption of freshwater aquatic organisms is very low in Belgium. Fishing for sustenance in fresh waterbodies is very limited due to prohibitory legislation. Moreover, seafood from marine environments (North Sea) or import is preferred by Belgium customers [516]. MCs are not expected to be prevalent in these products.

For public risk assessment, the dietary and environmental exposure through the exposure sources (food, recreational activities) and the toxic potency of the compounds themselves are important. Yet, in these two areas, more information is needed to develop complete risk assessments. The consumption of different foods and *Chlorella*- and cyano-based food supplements as well as the frequency of recreational activities in surface waters should be further quantified for the Belgium population, especially kids. However, quantifying such activities is difficult as it can vary in time (for instance, no recreational activities due to COVID-19 lockdown), is region dependent and can be very specific for different population groups based on age, gender, physical fitness, et cetera. This thesis contributed to this work by trying to evaluate the consumption of *Chlorella*- and cyano-based food supplements. However, the scope of follow-up surveys could still be increased to enhance the resolution of the consumption data in different population groups. Similarly, more quantitative data could be obtained for the daily consumed dose of *Chlorella*- and cyano-based food supplements.

Also, the approach for the risk assessment itself could be refined in future work. As discussed in Chapter 9, the deterministic exposure assessment approach could be refined by separating the risk assessment for product groups within different foods. For instance, separating the *Chlorella*- and cyano-based food supplements based on the genus used for their production (e.g. *Arthrospira, Chlorella or Aphanizomenon*) as

our data showed that only the latter was contaminated in our sample. The fact that the food supplements were clearly labelled would facilitate their precise distinction. However, no data is available on whether consumers also recognize the distinctions between the different products, which also should be taken into account during risk assessment. Besides *Chlorella*- and cyano-based food supplements, similar groupings could be made for fruits or vegetables as more information becomes available. Ideally, exposure and risk assessments should be made for each individual fruit and vegetable. However, aggregation in larger groups would be possible for products wherefore insufficient data is available. The aggregation of certain fruit and vegetables could be decided based on the classification of foods used in the EFSA food consumption database (FoodEx2) [613]. In this case, MCs prevalence data for each group could be evaluated separately. Moreover, the risk assessment could then be even more refined if group-specific consumption data is available.

Using models to simulate the consumption of *Chlorella*- and cyano-based food supplements based on the data provided in this thesis could also permit the use of probabilistic risk assessment approaches. This modeling approach was not pursued as it would require additional development time which was not available within the framework of this thesis. Providing a risk assessment based on a probabilistic approach that includes prevalence data for MCs and individual consumption data would be the ideal situation. However, sufficient individual consumption data for *Chlorella*- and cyano-based food supplements was still lacking in Belgium and in Europe.

For the overall risk assessment, the toxicological insight for the different cyanotoxins should be improved as well. Taking MCs as an example, the effect of their variable structures on their mechanism of action and their general human health effects (dependent on bioavailability and transport) are not well known. Currently, all congeners are assumed to be as toxic as MC-LR even though multiple studies have shown that the inhibitory and transport capacities of different congeners are not the same in vitro [53–58]. Moreover, some congeners might interact differently during digestion compared with the well-studied MC-LR. These knowledge gaps exist for most other cyanotoxins, except saxitoxin. Due to the high prevalence of saxitoxin variants in mollusks and the economic implications, toxicity equivalence factors have already been determined [292]. Such toxicity factors would be crucial to further develop unambiguous legislation for food and water safety that can include other MCs than MC-LR. Additionally, it is well known that during MCs accumulation in foods (e.g. aquatic foods, crops and potentially meat or milk), transformed and conjugated compounds can arise [86,87,92]. Some of these compounds have been shown in vivo and in vitro to retain some of their toxicity when ingested [138,139]. However,

insufficient data are available on their interaction with the human digestive system to properly assess human health risks.

Interactions between MCs, other cyanotoxins or other environmental pollutants should also be further elucidated so that they can be taken into account during future aggregated risk assessments. Multiple types of cyanotoxins have been observed during a bloom [314,342,391,570], as well as linear and circular peptides. Yet, little research has been done on their combined effects. However, increased protein expression was observed in a human kidney cell line after combined exposure to MC-LR and CYN [687]. Different effects of co-exposure to MCs and ATX have also been observed in different organisms [688]. Microplastics have been established to interact and adsorb different MCs, with different specificities [689]. Microplastics could potentially serve as vectors to allow MCs to reach different parts of the human body and alter their toxic capacity [690]. Microplastics have been found in water and could potentially help MCs circumvent current drinking water treatments. Other biological contaminants in food like mycotoxins, specifically aflatoxins, could also enhance, add to or interfere with the toxicological effects of MCs, as they also target the liver [688]. The presence of heavy metals combined with cyanotoxins should also be further investigated, especially for aquatic foods and *Chlorella*- and cyano-based food supplements. Those products have already been shown to contain both contaminants [69,384,390,691].

## 10.3. Approaches for future management and monitoring of cyanotoxins in the Belgian environment, water and food

Thorough management and monitoring of cyanotoxins in Belgium was not a part of the thesis. However, the next step for cyanotoxin research and public health assessments in Belgium would be the increase in monitoring and management of cyanobacterial blooms and cyanotoxins in the environment as well as continued monitoring of cyanotoxins in drinking water and increased monitoring in foods.

Collaborative monitoring programs between the responsible regional organizations could greatly increase insights into the factors that stimulate toxic cyanobacterial bloom occurrence. The toolbox could provide these programs with a starting point to combine analytical and molecular to characterize blooms throughout Belgium.

Different molecular approaches are available and ready to implement in Belgium. The detection of *mcyE*, as shown in Chapters 5 and 6, could be utilized as an early warning system and expanded to other toxin-producing gene clusters. Moreover, next-generation sequencing (NGS) technologies could be implemented to assess toxin-producing capacity and identify cyanobacteria diversity in the blooms. The genomic data acquired with these techniques during monitoring could also be easily used

during research projects to find correlations between genomic diversity in blooms and their environmental drivers and stressors (for example: phosphorous, nitrogen, climate and predation). These correlations could then support the development of remediation strategies. Illumina sequencing was used in multiple studies outside of Belgium to investigate the relationship between cyanobacterial species diversity and environmental drivers [692–694], environmental stressors [693], toxin-producing capacity [692,695], remediation techniques [694] and used as early warning tool [696]. However, most of these approaches amplified a specific genomic regions like toxin-producing gene clusters or 16S rRNA, which limits the obtained amount of genomic data. The development of third-generation sequencing approaches might provide a solution to this problem, as they would be able to amplify longer DNA fragments. Moreover, these new technologies are being developed as benchtop or portable machines that can produce fast results on-site, ideal for monitoring purposes.

The current analytical methodologies used in Belgium are not sufficient to identify and quantify all the potential toxins in a bloom. The UHPLC-MS/MS methods included in the toolbox are a good start but should be extended and validated to include more microcystin congeners and transformation products, ATX, CYN, STX and GNT. To obtain proper quantification, adherence to validation parameters, especially recovery, should be respected. This might result in separate analytical methods for some of these toxins. To reduce the analysis time and cost caused by using multiple methods for different toxins, semi-quantitative methods like ELISA could also be used to screen samples for toxin presence. These commercially available tests are usually faster and cheaper than UHPLC-MS/MS approaches. Positive hits of certain toxins can then reduce the number of UHPLC-MS/MS analyses that have to be performed. Once new quantitative HRMS approaches are more accessible, they will hopefully be able to deliver a vast overview of the different compounds available in the samples. These approaches could then replace ELISAs and UHPLC-MS/MS altogether.

Preventing or mitigating blooms in waterbodies starts with characterizing blooms and the conditions in which they occur. The current paradigm for managing blooms was based on a waterbody-specific approach [3]. Data should be collected not only on the blooms themselves but also on the nutrient (N, P and carbon) availability and influx in the waterbody and watersheds. This data would cumulate in a management plan to remediate the waterbody with short and long-term perspectives. In the short term, the use of HP, chemical or biological solutions was suggested to inhibit bloom formation, while flocculants could capture cyanobacteria cells and phosphorous on the bottom of a waterbody [476,482,483,489,491,697]. Longer-term solutions should involve the removal of nutrients from the waterbody and the prevention of an

additional influx of new nutrients. Another important feature of this approach is the inclusion of the different stakeholders surrounding the waterbody. Also in Belgium, this management approach would be recommended but might have additional complexities as the interests of multiple stakeholders (private, NGO's, commercial, regional and federal) can be conflicting. For instance, the runoff of fertilizers from fields in nearby waterbodies was suggested to promote blooms. Ideally, all waterbodies in Belgium should be managed to prevent the public health risk caused by toxic cyanobacterial blooms and possible accumulation in foods. Yet, management plans are too expensive and time-consuming to develop for all the waterbodies. However, recreational waterbodies could be prioritized initially for the application of the management plan paradigm in Belgium. These waterbodies have public, biological and economic value that could motivate different stakeholders to collaborate on the management plan. Moreover, they were identified as a source of MCs exposure during this thesis. Other biological valuable waterbodies due to their high biodiversity should also be protected or remediated by implementing management plans.

Because not all waterbodies can be managed, some might still be used to irrigate plants, capture mollusks, crayfish or fish, or provide drinking water for cattle. Therefore, research and potential monitoring of cyanotoxins prevalence in these foods should be done. New UHPLC-MS/MS methods for the different cyanotoxins (MCs, ATX, CYN, STX and GNT) should be extended from the toolbox or newly developed in crops, freshwater fish, crayfish and molluscs, and even potentially milk and meat. However, the development of all these methods will take time as each matrice will have to be validated separately. Methodologies for different matrices will also vary as different matrices will provide unique challenges in sample preparation, extraction and toxin quantification. ELISA test could again be developed and validated for each matrix. However, it is important to note that the commercially available tests are not specifically designed to analyze cyanotoxins in food matrices. Therefore, significant validation efforts should be made as required for their use in a food safety context. The presence of conjugated and transformed MCs should be investigated, as well as their overall toxicity and bioavailability. For the analysis of these compounds, MMPB and ELISA approaches could be used. Again, quantitative HRMS could in time replace ELISA and UHPLC-MS/MS approaches, once they become more accessible to use.

Furthermore, The approaches developed for *Chlorella*- and cyano-based food supplements during this thesis should also be expanded to include other MCs and other cyanotoxins. From this thesis, it was already clear that MCs were prevalent in some *Chlorella*- and cyano-based food supplements, specifically *Aphanizomenon*

based-products. Therefore, it might be possible that other cyanotoxins are also present in these products. Yet only limited studies have attempted to identify them [390,406,698]. Moreover, it would be recommended to start monitoring these products for MCs, as high levels were observed in particular products from the Belgium market.

In our preliminary aggregated risk assessment a public health risk due to ingestion of MCs was shown. Yet, it was clear that more data about Mcs prevalence and food consumption and recreational activities is necessary to definitively assess this risk. Moreover, information about MCs prevalence in other foodstuffs should be established to come to a complete aggregated risk assessment. With this additional data, more advanced risk assessment approaches could be used to finalize the risk assessment. Yet any risk assessment should be continuously updated with new data as MCs prevalence and consumption data will change over time.

## 10.4. Co-cultivation of *Microcystis* and *Arthrospira* as potential risk during *Chlorella*- and cyano-based food supplements production*:* background and approaches

As a final research question of the thesis, a co-cultivation experiment for *Microcystis* and *Arthrospira* was planned. However, these experiments were not pursued. Yet, these experiments could be interesting to evaluate concerns about MCs contamination in *Chlorella*- and cyano-based food supplements.

Production and consumption of *Chlorella*- and cyano-based food supplements were mentioned in earlier chapters. Specifically, the consumption of naturally harvested *Chlorella*- and cyano-based food supplements was shown to be accompanied by a certain risk of MCs contamination. This thesis showed that the *mcyE* gene was present in particular *Chlorella*- and cyano-based food supplements that contained *Aphanizomenon* from Klamath lake, and the producer was *Microcystis sp.*. Therefore, *Aphanizomenon sp.* and *Microcystis sp.* were hypothesized to co-occur in the natural bloom during harvesting, as was already published in the past [699]. While no MCs were detected in other *Chlorella*- and cyano-based food supplements products during this thesis, it cannot be ruled out that such products could be contaminated with toxin-producing cyanobacteria during the cultivation of *Arthrospira platensis* or *Chlorella vulgaris.* The risk of different species co-occurring during cultivation was initially of low concern as *C. vulgaris* is commonly cultivated in well-sterilized, closed systems [700–702] and *A. platensis* is cultivated at high salinity and pH [454,601,602,703–710], which is detrimental for other freshwater, toxin-producing species (e.g. *Microcystis* strains) [711–715]. However, the adaptation of *Microcystis*

to high salinity and new alterations of production methods for *A. platensis* and *C. vulgaris* could provide opportunities for toxin contaminations to occur.
Therefore, Vieira Costa et al. executed co-cultivation experiments for *A. platensis* and *Microcystis* strain RST 9501 and showed no cell growth for the *Microcystis* strain [711]. However, other *Microcystis* strains, like the PCC7806, have shown an innate higher resistance to high salt concentrations (10 g $L^{-1}$ and 17.5 g $L^{-1}$ during addition to medium and salt shock, respectively) [712]. This innate resistance is logical as PCC7806 was originally isolated from brackish water. This trait might also be quite recently acquired through horizontal gene transfer, explaining the limited number of strains that have currently acquired it [714]. Moreover, *Microcystis aeruginosa* was shown to adapt to salinity over time through spontaneous mutations, using a selective evolution approach [715]. These results suggest that *Microcystis* might be able to adapt to high salinity in the future, supported by increasing salinization of freshwater bodies through droughts due to climate change. These strains would then also be able to colonize open or half-open cultivation ponds, potentially resulting in MCs contamination in the product.
Recent developments in the cultivation process for *Arthrospira* could also result in potential contamination of the culture with toxic *Microcystis* strains. Many studies have shown that *Arthrospira* biomass production is increased when up to 80% of the Zarrouk medium is replaced with water [601,705,711]. A dilution of Zarrouk medium would result in a substantial decrease in production costs. However, the reduced use of Zarrouk medium would also cause a reduced salt concentration, resulting in possible contamination with toxic *Microcystis* strains. Indeed, this possibility was tested already with one *Microcystis* strain by Vieira Costa et al. [711]. However, strains with a higher salt resistance already exist, as discussed earlier [712,714]. Furthermore, the use of naturally salinized water (brackish and sea) is shown to be a good replacement for the current medium [602,708]. Again, salt concentrations in certain water sources used for cultivation could potentially be insufficient to inhibit *Microcystis* cell growth. Moreover, other (marine) cyano/micro-algae toxins, like saxitoxin, could form a potential threat if water sources are not properly sterilized before use. An additional example of a potential contamination risk is the use of activated sludge from wastewater treatment as a nutrient source to grow *Chlorella* sp. for consumption in bioreactors [716]. If this sludge is contaminated with toxic cyanobacteria or their akinetes, the toxic cyanobacteria might also be able to grow.
Nonetheless, the production of *Chlorella*- and cyano-based food supplements should not be dismissed due to these risks. The potential of these nutrient-rich alternative foods to combat food insecurity is too great. Continuing research to optimize safe cultivation conditions and proper monitoring for cyanotoxins in food products would

be advisable. A different step can be taken to ensure the safety of *Chlorella*- and cyano-based food supplements. Cultures could be checked for contaminating cyanobacteria species by microscope or high-throughput sequencing. Alternatively, methodologies presented in the toolbox could be used. The presence of toxin-producing genes could be monitored using PCR during cultivation and in finished products as a screening procedure. However, when doubt arises about the quality of the *Chlorella*- and cyano-based food supplements, quantification of the toxins should still occur using analytical approaches (ELISA, UHPLC-MS/MS,…). Therefore, it is interesting to note that the producers in France are working towards a guide of good practices that will be reviewed by the federal food agency (ANSES) to ensure the quality of their product. They also work on risk management for the production of spirulina [717].

The initial idea during the thesis for a co-cultivation experiment with the *Microcystis* strain PC7806 and *Arthrospira platensis* was based on the combination of the studies of Vieira Costa et al. and Melero-Jiménez et al. [711,715]. Initially, we planned to test if it would be possible to increase the salt resistance of the *Microcystis* strain PC7806 using the selective evolution approach by growing the strain sequentially in media with increasing salt concentrations. MCs presence would be tested before proceeding to the next step, in the case that the production of MC would be impacted. Indeed, mutations responsible for salt resistance or tolerance have been shown for MCs-phenotypes [712,714,718]. Once a sufficient (or maximum) resistance is obtained, the strain would be co-cultivated with *A. platensis.* Acceptable salt concentrations would be 36.795 ppt and 7.36 ppt of NaCl for Zarrouk and 20% Zarrouk medium, respectively.

However, we were not able to perform these experiments due to time constraints within the thesis. First, the focus was to develop the quantification methods for the toxins. At the moment this was finished, less than two years were left in the project. We assessed that only obtaining the salt resistance through directed evolution would probably take more time, partially due to the longer growth cycle of cyanobacteria, compared to other bacteria. Thus the co-cultivation experiment could not have been performed within the project. Instead, we focused on developing other quantification methods to investigate other possible exposure sources through food (e.g. vegetables and fruits).

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# Supplementary data

## S1 Drinking water paper

**Table S1.1.** Overview of bottled water based on their country of origin, total volume of water and count of individual samples.

| Country of origin | Still/Sparkling | Volume (L) | Count of individual samples |
|---|---|---|---|
| **Belgium** | **sparkling** | 1.0 | 3 |
| | | 1.25 | 1 |
| | | 1.5 | 2 |
| | **still** | 1.0 | 2 |
| | | 2.0 | 1 |
| | | 5.0 | 1 |
| | | 0.33 | 1 |
| | | 1.5 | 2 |
| **France** | **sparkling** | 1.0 | 4 |
| | | 0.5 | 4 |
| | | 1.25 | 3 |
| | **still** | 1.0 | 5 |
| | | 20 | 2 |
| | | 0.5 | 3 |
| | | 1.5 | 5 |
| **Germany** | **still** | 1.0 | 1 |
| | | 0.75 | 1 |
| **Italy** | **sparkling** | 1.0 | 1 |
| | | 1.,5 | 1 |
| | **still** | 1.0 | 1 |
| | | 1.5 | 1 |
| **Luxemburg** | **sparkling** | 1.5 | 1 |
| **The Netherlands** | **sparkling** | 0.33 | 1 |
| | | 1.5 | 2 |
| | **still** | 0.5 | 1 |
| | | 1.5 | 1 |
| **Grand Total** | | | **51** |

Table S1.2. Overview of tap water samples from Belgium based on region and count of individual samples

| Region | Count of individual samples |
|---|---|
| Flanders | 18 |
| Wallonia | 4 |
| Brussels | 2 |

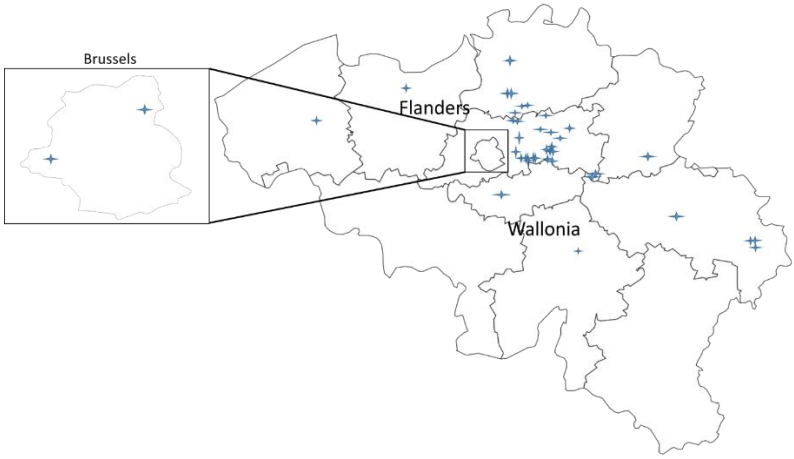


**Figure S1.3.** Map showing the distribution of the tap water sampling in Belgium

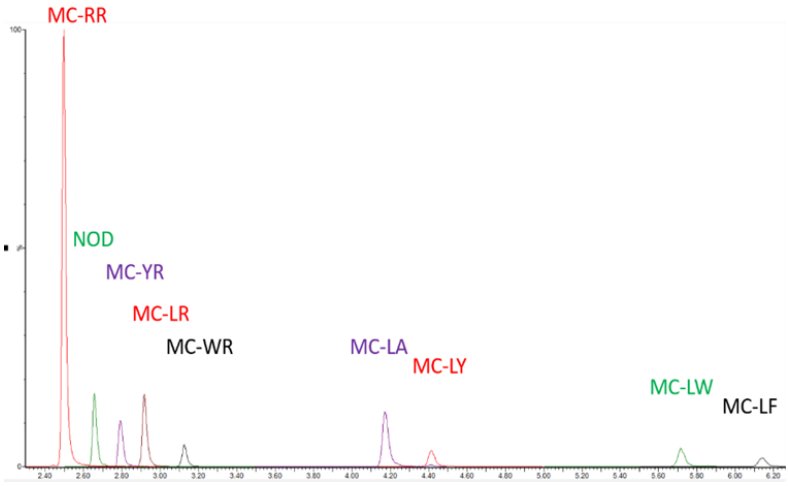


**Figure S1.2.** The elution peaks and the intensity are shown for the eight microcystin congeners and nodularin.

# S2. A summer of cyanobacterial blooms in Belgian waterbodies: analytical and molecular characterizations: Supplementary data

**Table S2.1.** Overview of experimental data for the water samples, concentrations of M congeners and total microcystin (µg $L^{-1}$), presence of genes coding for 16S rRNA and *mcyE*. molecular identification, coverage and identity % of the most similar hit by BLAST and primer used for Sanger sequencing. The first three characters of the samples annotation indicate the sample location, while the last two numbers indicate the week of the year when the sample was collected. Samples are grouped by origin, Flanders. Brussels and Wallonia. N.A. indicates samples for which no PCR was performed. "-" represent samples where no visible bands were obtained after gel electrophoresis of the PCR products or sequencing results were off to pore a quality to provide a reliable result. "?" represents inconclusive PCR results where the band was visible but was too faint or not at the correct height. The asterisk * indicates that the sequence was obtained on the basis of a PCR reaction produced with the primer pair 359F-781R, whereas the others sequences were obtained with 359F-32S30R. "i" denotes the samples that were analyzed using Illumina technology.

| Sample | MC-RR µg $L^{-1}$ | MC-LA µg $L^{-1}$ | MC-LF µg $L^{-1}$ | MC-LR µg $L^{-1}$ | MC-LY µg $L^{-1}$ | MC-LW µg $L^{-1}$ | MC-YR µg $L^{-1}$ | MC-WR µg $L^{-1}$ | Total MCs µg $L^{-1}$ | 16s rRNA | Fresh biomass ($10^{-6}$ g $L^{-1}$) | *mcyE* | Molecular identification (genus) | Cover-age (%) | Per. Identity (%) | primer |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **AN1.32** | 0.44 | 0.00 | 0.00 | 0.23 | 0.00 | 0.00 | 0.00 | 0.00 | 0.67 | + | 5.4 | + | - | - | - | |
| **AN2.32** | 0.00 | 0.00 | 8.74 | 121.77 | 9.75 | 2.22 | 0.00 | 0.00 | 142.48 | + | 15.4 | + | *Dolichospermum* | 100 | 100 | 359F |
| **AN2.37** | 12.77 | 0.00 | <LOQ | 6.16 | <LOQ | 0.00 | 1.41 | 0.44 | 20.78 | + | 5.6 | + | *Synechococcus* | 100 | 99.16 | 359F |
| **AN3.37** | 0.00 | 0.00 | 16.64 | 105.94 | 12.75 | 2.35 | 0.00 | 0.00 | 137.68 | + | 3.7 | + | *Romeria* | 99.71 | 98.65 | 359F |
| **GH1.30** | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | - | 4.9 | - | - | - | - | |
| **VL1.32** | 206.29 | 3.73 | 0.00 | 75.43 | 0.13 | <LOQ | 43.79 | 24.04 | 353.40 | + | 37.7 | + | *Microcystis* | 100 | 98.98 | 781R* |
| **VL1.36** | 357.34 | 30.31 | 0.90 | 196.15 | 2.66 | 3.44 | 103.32 | 52.56 | 746.69 | + | 30.4 | + | - | - | - | |
| **VL2.34** | 1565.98 | 4.28 | 0.52 | 538.41 | 1.16 | 0.83 | 241.32 | 68.41 | 2420.91 | + | 13.2 | + | *Dolichospermum* | 100 | 100 | 359F |
| **VL2.36i** | 75.24 | 0.39 | <LOQ | 30.00 | 0.11 | 0.00 | 18.45 | 4.74 | 128.93 | + | 2.2 | + | *Microcystis* | 100 | 97.81 | 781R* |
| **VL2.2.36** | 1726.08 | 7.40 | 3.02 | 594.51 | 3.49 | 1.60 | 277.00 | 185.71 | 2798.81 | + | 102.8 | + | *Microcystis* | 100 | 99.68 | 359F* |
| **VL3.34** | 0.16 | 0.24 | 0.00 | <LOQ | 0.00 | 0.00 | 0.00 | 0.27 | 0.67 | + | 3.6 | + | *Dolichospermum* | 100 | 99.70 | 359F |
| | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | |

| Sample | MC-RR µg $L^{-1}$ | MC-LA µg $L^{-1}$ | MC-LF µg $L^{-1}$ | MC-LR µg $L^{-1}$ | MC-LY µg $L^{-1}$ | MC-LW µg $L^{-1}$ | MC-YR µg $L^{-1}$ | MC-WR µg $L^{-1}$ | Total MCs µg $L^{-1}$ | 16s rRNA | Fresh biomass ($10^{-6}$ g $L^{-1}$) | *mcyE* | Molecular identification (genus) | Cover-age (%) | Per. Identity (%) | primer |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **BL1.29** | 66.89 | 7.79 | <LOQ | 75.62 | 0.00 | 0.32 | 97.30 | 1.25 | 249.18 | + | 4.3 | + | *Microcystis* | 100 | 100 | 781R* |
| | | | | | | | | | | | | | *Synechococcus/Merismopedia* | 100 | 98.78 | 359F |
| **BL1.30** | 143.54 | 13.45 | <LOQ | 132.23 | 0.00 | 0.59 | 179.54 | 3.67 | 473.01 | + | 2.3 | + | *Microcystis* | 100 | 99.13 | 781R* |
| **BL1.35** | 132.87 | 14.41 | 0.19 | 179.06 | <LOQ | 0.56 | 275.91 | 2.84 | 605.85 | + | 2.9 | + | - | - | - | |
| **BL2.29** | 0.00 | 0.00 | 0.00 | 0.68 | 0.00 | 0.00 | 0.00 | 0.00 | 0.68 | + | 3.8 | - | - | - | - | |
| **BL3.30** | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | + | 10.22 | - | - | - | - | |
| **BL4.30** | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | + | 3.5 | - | - | - | - | |
| **BL4.35** | 0.11 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.11 | + | 1.5 | - | *Aphanizomenon* | 100 | 100 | 359F |
| **BL5.29i** | 0.81 | 0.00 | 0.00 | 0.41 | 0.00 | 0.00 | 0.00 | 0.00 | 1.22 | + | 0.6 | + | *Dolichospermum* | 100 | 100 | 359F |
| **BL5.35** | 0.89 | 0.00 | 0.00 | 0.44 | 0.00 | 0.00 | <LOQ | 0.00 | 1.33 | + | 12.2 | + | *Dolichospermum* | 100 | 100 | 359F |
| **BL6.29** | 0.00 | 0.00 | 0.00 | 0 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | + | 17.8 | - | *Dolichospermum* | 100 | 100 | 359F |
| **BL6.35** | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | + | 3.7 | + | - | - | - | |
| **BL7.30** | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | + | 5.5 | + | *Dolichospermum* | 99.82 | 99.26 | 359F |
| **BL7.35** | 0.00 | 0.00 | 0.00 | <LOQ | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | + | 2.2 | + | - | - | - | |
| **BL8.35** | 0.51 | 0.00 | 0.00 | 0.39 | 0.00 | 0.00 | 0.16 | 0.00 | 1.07 | + | 2.5 | + | *Microcystis* | 100 | 99.65 | 359F* |
| **BV1.34** | 7.68 | 0.00 | 0.00 | 3.57 | 0.00 | 0.00 | 1.40 | <LOQ | 12.65 | + | 1.4 | + | *Microcystis* | 100 | 97.94 | 781R* |
| **BV2.34** | 13.65 | 0.00 | 0.00 | 3.92 | 0.00 | 0.00 | 3.04 | 1.03 | 21.63 | + | 2.3 | + | *Microcystis* | 100 | 99.82 | 23S30R |
| **BV2.35** | 4.53 | 0.00 | 0.00 | 1.70 | <LOQ | 0.00 | 0.80 | 0.31 | 7.34 | + | 1.9 | + | *Synechococcus* | 99.81 | 96.85 | 359F |
| **BV3.35** | 1213.11 | 0.71 | 0.56 | 364.91 | 1.42 | 0.95 | 188.58 | 61.09 | 1831.32 | + | 2.5 | - | *Phormidium* | 100 | 98.02 | 359F |
| **B04.27** | 0.65 | 0.00 | 0.00 | 0.41 | 0.00 | 0.00 | 0.00 | 0.00 | 1.06 | + | 2.3 | + | *Aphanizomenon* | 100 | 97.97 | 359F |
| **B04.28** | 0.57 | 0.00 | 0.00 | 0.45 | 0.00 | 0.00 | 0.00 | 0.00 | 1.02 | + | 2.7 | + | - | - | - | |
| **B04.29** | 0.25 | 0.00 | 0.00 | <LOQ | 0.00 | 0.00 | 0.00 | 0.00 | 0.25 | + | 3.0 | + | *Aphanizomenom* | 100 | 100 | 359F |
| | | | | | | | | | | | | | *Planktothrix* | 100 | 98.42 | 781R* |
| **B04.30** | <LOQ | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | + | 0.9 | N.A. | - | - | - | |
| **B04.31** | 0.98 | 0.00 | 0.00 | 0.45 | 0.00 | 0.00 | 0.00 | 0.00 | 1.43 | + | 1.2 | + | *Dolichospermum* | 100 | 94.52 | 359F |
| **B04.32** | 0.72 | 0.00 | 0.00 | 0.49 | 0.00 | 0.00 | 0.00 | 0.00 | 1.21 | + | 2.1 | + | - | - | - | |

| Sample | MC-RR µg $L^{-1}$ | MC-LA µg $L^{-1}$ | MC-LF µg $L^{-1}$ | MC-LR µg $L^{-1}$ | MC-LY µg $L^{-1}$ | MC-LW µg $L^{-1}$ | MC-YR µg $L^{-1}$ | MC-WR µg $L^{-1}$ | Total MCs µg $L^{-1}$ | 16s rRNA | Fresh biomass ($10^{-6}$ g $L^{-1}$) | *mcyE* | Molecular identification (genus) | Cover-age (%) | Per. Identity (%) | primer |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **B04.34** | 1.03 | 0.00 | 0.00 | 0.83 | 0.00 | 0.00 | 0.00 | 0.00 | 1.86 | + | 1.8 | - | *Aphanizomenon* | 99.83 | 98.09 | 359F |
| **B04.35** | 0.38 | 0.00 | 0.00 | 0.32 | 0.00 | 0.00 | 0.00 | 0.00 | 0.70 | + | 2.2 | ? | - | - | - | |
| **B04.36** | 0.19 | 0.00 | 0.00 | 0.22 | 0.00 | 0.00 | 0.00 | 0.00 | 0.41 | + | 2.0 | + | - | - | - | |
| **B04.37** | 0.27 | 0.00 | 0.00 | 0.29 | 0.00 | 0.00 | 0.00 | 0.00 | 0.56 | + | 2.1 | + | - | - | - | |
| **E04.27** | 0.12 | 0.00 | 0.00 | <LOQ | 0.00 | 0.00 | 0.00 | 0.00 | 0.12 | N.A. | 6.1 | ? | N.A. | N.A. | N.A. | |
| **E04.28** | 0.36 | <LOQ | 0.00 | 0.33 | 0.00 | 0.00 | 0.18 | 0.00 | 0.87 | + | 1.7 | + | - | - | - | |
| **E04.29** | 0.29 | 0.00 | 0.00 | 0.49 | 0.00 | 0.00 | 0.47 | 0.00 | 1.26 | + | 2.3 | + | *Aphanizomenon* | 99.85 | 99.85 | 359F |
| **E04.30** | 1.93 | 0.80 | 0.00 | 6.96 | 0.00 | 0.00 | 9.12 | 0.15 | 18.96 | + | 1.6 | N.A. | - | - | - | |
| **E04.31** | 1.57 | 0.40 | 0.00 | 3.25 | 0.00 | 0.00 | 8.85 | 0.00 | 14.07 | + | 1.4 | N.A. | - | - | - | |
| **E04.32** | 0.51 | <LOQ | 0.00 | 0.94 | 0.00 | 0.00 | 2.60 | 0.00 | 4.05 | + | 2.2 | + | *rhodoplast* | 98.9 | 95.48 | 781R |
| **E04.34** | 1.71 | 0.24 | 0.00 | 2.31 | 0.00 | 0.00 | 5.13 | 0.00 | 9.39 | + | 2.3 | + | *Microcystis* | 99 | 97.00 | 359F* |
| **E04.35** | 3.17 | 1.50 | 0.00 | 10.64 | 0.00 | 0.00 | 28.02 | <LOQ | 43.34 | + | 2.1 | ? | - | - | - | |
| **E04.36** | 1.70 | 0.46 | 0.00 | 3.21 | 0.00 | 0.00 | 8.41 | 0.00 | 13.78 | + | 2.1 | + | *Microcystis* | 100 | 94.00 | 359F |
| **E04.37** | 0.99 | 0.21 | 0.00 | 1.97 | 0.00 | 0.00 | 4.20 | 0.00 | 7.38 | N.A. | 2.4 | + | N.A. | N.A. | N.A. | |
| **H02.27** | <LOQ | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | + | 3.1 | ? | *Synechococcus* | 100 | 99.59 | 359F |
| **H02.28** | 0.60 | 0.00 | 0.00 | <LOQ | 0.00 | 0.00 | 0.00 | 0.00 | 0.60 | + | 2.2 | + | - | - | - | |
| **H02.29** | 0.53 | 0.00 | 0.00 | 0.17 | 0.00 | 0.00 | 0.00 | 0.00 | 0.69 | + | 1.1 | N.A. | - | - | - | |
| **H02.30** | 2.18 | 0.00 | 0.00 | 0.87 | 0.00 | 0.00 | <LOQ | 0.00 | 3.05 | + | 1.0 | N.A. | - | - | - | |
| **H02.31** | 3.16 | 0.00 | 0.00 | 0.71 | 0.00 | 0.00 | 0.18 | 0.00 | 4.05 | + | 0.8 | + | *Planktothrix* | 99.44 | 94.99 | 359F |
| **H02.32** | 3.66 | 0.00 | 0.00 | 0.69 | 0.00 | 0.00 | <LOQ | 0.00 | 4.35 | + | 2.1 | + | *Aphanizomenon* | 100 | 99.16 | 359F |
| **H02.34** | 3.32 | 0.00 | 0.00 | 0.69 | 0.00 | 0.00 | 0.18 | 0.00 | 4.18 | + | 1.9 | + | - | - | - | |
| **H02.35** | 2.37 | 0.00 | 0.00 | 0.43 | 0.00 | 0.00 | <LOQ | 0.00 | 2.80 | + | 1.9 | ? | *Planktothrix* | 100 | 100 | 359F |
| **H02.36** | 2.33 | 0.00 | 0.00 | 0.36 | 0.00 | 0.00 | <LOQ | 0.00 | 2.69 | + | 3.5 | ? | *Synechococcus* | 99.80 | 99.45 | 23S30R |
| **H02.37** | 0.64 | 0.00 | 0.00 | 0.12 | 0.00 | 0.00 | <LOQ | 0.00 | 0.76 | N.A. | 3.7 | - | - | - | - | |
| **I01.27** | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | + | 4.6 | + | - | - | - | |
| **I01.28** | 0.13 | 0.00 | 0.00 | <LOQ | 0.00 | 0.00 | 0.00 | 0.00 | 0.13 | + | 2.7 | ? | *Cyanobium* | 100 | 98.10 | 781R |
| **I01.29** | 0.18 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.18 | + | 1.3 | ? | - | - | - | |

| Sample | MC-RR µg $L^{-1}$ | MC-LA µg $L^{-1}$ | MC-LF µg $L^{-1}$ | MC-LR µg $L^{-1}$ | MC-LY µg $L^{-1}$ | MC-LW µg $L^{-1}$ | MC-YR µg $L^{-1}$ | MC-WR µg $L^{-1}$ | Total MCs µg $L^{-1}$ | 16s rRNA | Fresh biomass ($10^{-6}$ g $L^{-1}$) | *mcyE* | Molecular identification (genus) | Cover-age (%) | Per. Identity (%) | primer |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **I01.30** | 0.46 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.46 | + | 1.9 | N.A. | - | - | - | |
| **I01.31** | 1.26 | 0.00 | 0.00 | 0.24 | 0.00 | 0.00 | 0.15 | 0.00 | 1.66 | + | 0.8 | N.A. | - | - | - | |
| **I01.32** | 0.19 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.19 | + | 2.3 | N.A. | - | - | - | |
| **I01.34** | 0.44 | 0.00 | 0.00 | 0.12 | 0.00 | 0.00 | 0.00 | 0.00 | 0.56 | + | 1.7 | + | - | - | - | |
| **I01.35** | 0.15 | 0.00 | 0.00 | <LOQ | 0.00 | 0.00 | 0.00 | 0.00 | 0.15 | + | 1.8 | + | *Synechococcus* | 100 | 99.81 | 359F |
| **I01.36** | 0.12 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.12 | + | 2.1 | - | - | - | - | |
| **I01.37** | 0.44 | 0.00 | 0.00 | <LOQ | 0.00 | 0.00 | 0.00 | 0.00 | 0.44 | + | 2.7 | + | *Cyanobium* | 100 | 97.30 | 781R* |
| **I04.27** | <LOQ | 0.00 | 0.00 | 0.04 | 0.00 | 0.00 | 0.00 | 0.00 | 0.04 | + | 1.9 | + | *Aphanizomenon* | 100 | 99.64 | 359F |
| **I04.28** | 0.91 | 0.00 | 0.00 | 0.48 | 0.00 | 0.00 | <LOQ | 0.00 | 1.39 | + | 1.5 | + | *Aphanizomenon* | 99.84 | 98.85 | 359F |
| **I04.29** | 0.83 | 0.00 | 0.00 | 0.33 | <LOQ | 0.00 | <LOQ | 0.00 | 1.16 | + | 1.1 | + | *Dolichospermum* | 100 | 100 | 359F |
| **I04.30** | 6.11 | 0.00 | 0.00 | 2.90 | 0.42 | 0.00 | 0.36 | 0.00 | 9.79 | + | 3.3 | + | *Dolichospermum* | 99.83 | 99.16 | 359F |
| **I04.31** | 42.34 | 0.00 | 0.00 | 17.62 | 3.74 | 0.52 | 2.38 | <LOQ | 66.61 | + | 2.5 | + | *Dolichospermum* | 100 | 99.39 | 359F |
| **I04.32** | 17.70 | 0.00 | <LOQ | 6.92 | 1.34 | 0.27 | 1.73 | <LOQ | 27.96 | + | 3.3 | + | *Microcystis* | 100 | 99.66 | 359F* |
| | | | | | | | | | | | | | *Dolichospermum* | 99.75 | 98.25 | 359F |
| **I04.34** | 51.87 | <LOQ | 0.15 | 13.95 | 2.05 | 0.46 | 3.44 | 0.27 | 72.18 | + | 3.5 | + | *Microcystis* | 100 | 98.65 | 359F |
| **I04.35** | 106.98 | 0.86 | 0.45 | 36.09 | 7.50 | 1.16 | 7.19 | 0.32 | 160.54 | + | 2.2 | + | - | - | - | |
| **I04.36** | 167.40 | 1.15 | 0.77 | 56.88 | 11.22 | 1.76 | 10.59 | 0.58 | 250.35 | + | 2.9 | + | *Planktothrix* | 99.11 | 95.63 | 359F |
| **I04.37** | 140.98 | 0.73 | 0.58 | 43.02 | 7.40 | 1.12 | 7.44 | 0.28 | 201.54 | + | 3.9 | + | *Cyanodictyon* | 100 | 98.62. | 359F |

**Table S2.2.** Overview of sampling sites in the different regions, water sample annotation, waterbody type and specific monitoring for cyanobacteria.

| Annotation | Name | Waterbody type | Monitored | Annotation | Name | Waterbody type | Monitored |
|---|---|---|---|---|---|---|---|
| **Wallonia** | | | | **Brussels** | | | |
| **IO1** | Lac de Falemprise, Cerfontaine | Recreational lake | Yes | BL1 | Boudewijn park fase 1. Jette | Shallow pond | No |
| **EO4** | Grand large, Mons | Recreational lake | Yes | BL2 | Boudewijn park fase 2. Jette | Shallow pond | No |
| **BO4** | Renipont-Plage, Lasne | Recreational lake | Yes | BL3 | Big Mellaert pond. Sint-Pieters-Wolluwe | Shallow pond | No |
| **HO2** | Sport complex Saint-Leger, Saint-Lèger | Recreational lake | Yes | BL4 | Tercoigne laan. Watermaal-Bosvoorde | Shallow pond | No |
| **IO4** | Lac de Bambois, Fosses-La-Ville | Recreational lake | Yes | BL5 | Red cloister 3.Oudergem | Shallow pond | No |
| **Flanders** | | | | BL6 | Leybeekpond. Watermael-Bosvoorde | Shallow pond | No |
| **GH1** | Sedimentation pond Gasthuisberg, Leuven | Sedimentation pond | No | BL7 | Rue de pecheries.Watermaal-Bosvoorde | Shallow pond | No |
| **AN1** | Resort De kempen, Mol | Recreational shallow pond | Yes | BL8 | Little Mellaert pond. Sint-Pieters-Wolluwe | Shallow pond | No |
| **AN2** | Hof van Eden, Westerloo | Recreational shallow pond | Yes | BV1 | Quai des Péniches. Sint-Jan-Molenbeek | Canal | No |
| **VL1** | Kleine vijver provincial domain Kessel-Lo, Kessel-Lo | Shallow pond | No | BV2 | Qaui de Heembeek Ferry stop. Heembeek | Canal | No |
| **VL2** | Vossem pond Park, Vossem | Shallow pond | No | BV3 | Quai des Charbonnages. Sint-Jan-Molenbeek | Canal | No |
| **VL3** | Gordaal pond, Tervuren | Shallow pond | No | | | | |
| **AN3** | Zilverstrand, Mol | Recreational Lake | Yes | | | | |

**Table S2.3.** Detailed information for taxonomic identification based on BLAST analysis. * Samples for which PCR with different primers gave different dominant taxa

| Place | Sample | qseqid | Accession number | Title | % identity | length | Mis-match | Gaps | e-value | Bit-score |
|---|---|---|---|---|---|---|---|---|---|---|
| **Hof van Eden, Westerloo** | AN2.32 | GS20-C085-AWil-20-359F | MH669067 | *Dolichospermum circinale* NRERC-107 | 100.000 | 577 | 0 | 0 | 0 | 1066 |
| | AN2.37 | GS20-C088-AWil-26-359F | AM709627 | *Synechococcus* sp. PCC 7920 | 99.165 | 599 | 5 | 0 | 0 | 1079 |
| **Zilverstrand, Mol** | AN3.37 | GS20-C089-AWil-27-359F | JQ819251 | *Romeria* sp. KLL-H-201 | 98.651 | 667 | 5 | 4 | 0 | 1179 |
| **Kleine vijver provincial domain Kessel-Lo, Kessel-Lo** | VL1.32 | GS20-C114-AWil-19-781R | LC557463 | *Microcystis aeruginosa* NIES-90 | 98.985 | 197 | 2 | 0 | 1.71E-93 | 353 |
| **Vossem pond Park, Vossem** | VL2.34 | GS20-C086-AWil-21-359F | FN691909 | *Dolichospermum flos-aquae* 04-57 | 100.000 | 427 | 0 | 0 | 0 | 789 |
| | VL2.36 | GS20-C116-AWil-24-781R | LC557463 | *Microcystis aeruginosa* NIES-90 | 97.81 | 274 | 4 | 2 | 6.57E-129 | 472 |
| | VL2.36 bis | GS20-C064-AWil-25-359F | LC557463 | *Microcystis aeruginosa* NIES-90 | 99.681 | 313 | 1 | 0 | 2.04E-159 | 573 |

| Place | Sample | qseqid | Accession number | Title | % identity | length | Mis-match | Gaps | e-value | Bit-score |
|---|---|---|---|---|---|---|---|---|---|---|
| **Gordaal Vijver, Tervuren** | VL3.34 | GS20-C087-AWil-22-359F | FN691909 | *Dolichospermum flos-aquae* 04-57 | 99.703 | 673 | 2 | 0 | 0 | 1232 |
| **Boudewijn park fase 1, Jette** | BL1.29* | GS19-G066-AWil-1-781R | LC557461 | *Microcystis aeruginosa* NIES-104 | 100.000 | 341 | 0 | 0 | 1.31E-176 | 630 |
| | BL1.29* | GS20-C072-AWil-1-359F | MT376739 | *Synechococcus* sp. SR-R4S2 | 98.785 | 494 | 6 | 0 | 0 | 880 |
| | | GS20-C072-AWil-1-359F | EF088332 | *Merismopedia sp*. CENA106 | 98.785 | 494 | 6 | 0 | 0 | 880 |
| | BL1.30 | GS19-G068-AWil-5-781R | LC557461 | *Microcystis aeruginosa* NIES-104 | 99.13 | 345 | 0 | 3 | 1.03E-172 | 617 |
| **Tercoigne laan, Watermaal-Bosvoorde** | BL4.35 | GS20-C080-AWil-11-359F | MN104685 | *Aphanizomenon flos-aquae* NRERC-020 | 100.000 | 649 | 0 | 0 | 0 | 1199 |
| **Red cloister 3, Oudergem** | BL5.29 | GS20-C073-AWil-3-359F | KC955174 | *Dolichospermum flos-aquae* CHAB 1657 | 100.000 | 509 | 0 | 0 | 0 | 941 |
| | BL5.35 | GS20-C081-AWil-12-359F | MT558568 | *Dolichospermum planctonicum* ANCD0809 | 100.000 | 429 | 0 | 0 | 0 | 793 |

| Place | Sample | qseqid | Accession number | Title | % identity | length | Mis-match | Gaps | e-value | Bit-score |
|---|---|---|---|---|---|---|---|---|---|---|
| **Leybeek-pond, Watermael-Bosvoorde** | BL6.29 | GS20-C074-AWil-4-359F | MT558568 | *Dolichospermum planctonicum* ANCD0809 | 100.000 | 540 | 0 | 0 | 0 | 998 |
| **Rue de pecheries, Watermaal-Bosvoorde** | BL7.30 | GS20-C077-AWil-7-359F | LC096267 | *Dolichospermum flos-aquae* NIES-75 | 99.259 | 540 | 3 | 1 | 0 | 977 |
| **Little Mellaert pond, Sint-Pieters-Wolluwe** | BL8.35 | GS19-G073-AWil-15-359F | LC557461 | *Microcystis aeruginosa* NIES-104 | 99.655 | 290 | 0 | 1 | 4.1E-146 | 529 |
| **Quai des Péniches, Sint-Jan-Molenbeek** | BV1.34* | GS19-G072-AWil-8-781R | LC557463 | *Microcystis aeruginosa* NIES-90 | 97.938 | 194 | 4 | 0 | 1.67E-88 | 337 |
| | BV1.34* | GS20-C131-AWil-8-23S30R | CP039373 | *Synechococcus sp*. CB0101 | 95.161 | 62 | 1 | 1 | 2.74E-16 | 97.1 |
| **Qaui de Heembeek** | BV2.34 | GS20-C132-AWil-9-23S30R | AP019314 | *Microcystis viridis* NIES-102. | 99.818 | 550 | 1 | 0 | 0 | 1011 |
| **Ferry stop, Heembeek** | BV2.35 | GS20-C083-AWil-16-359F | MT376735 | *Synechococcus sp*. SR-C1 | 96.846 | 539 | 16 | 1 | 0 | 900 |

| Place | Sample | qseqid | Accession number | Title | % identity | length | Mis-match | Gaps | e-value | Bit-score |
|---|---|---|---|---|---|---|---|---|---|---|
| **Quai des Charbonnages, Sint-Jan Molenbeek** | BV3.35 | GS20-C084-AWil-17-359F | EU196622 | *Phormidium* cf. tergestinum Drak | 98.023 | 354 | 7 | 0 | 3E-173 | 619 |
| **Renipont-Plage, Lasne** | B04.27 | GS20-C107-AWil-59-359F | MT443970 | *Aphanizomenon gracile* FBCC-A274 | 97.967 | 541 | 11 | 0 | 0 | 939 |
| | B04.29* | GS19-G118-AWil-69-781R | LC455659 | *Planktothrix agardhii* NIES-905 | 98.423 | 317 | 5 | 0 | 5.78E-155 | 558 |
| | B04.29* | GS20-C111-AWil-69-359F | MT443970 | *Aphanizomenon gracile* FBCC-A274 | 100.000 | 442 | 0 | 0 | 0 | 817 |
| | B04.31 | GS20-C092-AWil-31-359F | MT443970 | *Aphanizomenon gracile* FBCC-A274 | 94.521 | 292 | 16 | 0 | 9.21E-123 | 451 |
| | | GS20-C092-AWil-31-359F | CP051206 | *Dolichospermum flos-aquae* CCAP 1403/13F | 94.521 | 292 | 16 | 0 | 9.21E-123 | 451 |
| | B04.34 | GS20-C104-AWil-53-359F | MT443970 | *Aphanizomenon gracile* FBCC-A274 | 98.087 | 575 | 10 | 1 | 0 | 1000 |

| Place | Sample | qseqid | Accession number | Title | % identity | length | Mis-match | Gaps | e-value | Bit-score |
|---|---|---|---|---|---|---|---|---|---|---|
| **Grand large, Mons** | E04.29 | GS20-C112-AWil-71-359F | MN104685 | *Aphanizomenon flos-aquae* NRERC-020 | 99.846 | 650 | 0 | 1 | 0 | 1194 |
| | E04.32 | GS20-C121-AWil-46-781R | LT622877 | Liagora brachyclada chloroplast isolate J.0126 | 95.48 | 177 | 8 | 0 | 2E-72 | 283 |
| | E04.34 | GS19-G105-AWil-51-359F | LC557461 | *Microcystis aeruginosa* NIES-104 | 97.000 | 309 | 1 | 0 | 4E-145 | 518 |
| | E04.36 | GS20-C067-AWil-43-359F | LC557461 | *Microcystis aeruginosa* NIES-104 | 95.082 | 297 | 1 | 9 | 4E-120 | 435 |
| **Sport complex Saint-Leger, Saint-Lèger** | H02.27 | GS20-C106-AWil-58-359F | MT376735 | *Synechococcus sp*. SR-C1 | 99.591 | 489 | 2 | 0 | 0 | 893 |
| | H02.31 | GS20-C093-AWil-32-359F | LC455659 | *Planktothrix agardhii* NIES-905 | 94.986 | 359 | 12 | 6 | 7.15E-155 | 558 |
| | H02.32 | GS20-C102-AWil-49-359F | LC037454 | *Aphanizomenon sp*. NIES-3732 | 99.161 | 477 | 2 | 2 | 0 | 857 |
| | H02.35 | GS20-C096-AWil-36-359F | LC455659 | *Planktothrix agardhii* NIES-905 | 100.000 | 498 | 0 | 0 | 0 | 920 |
| | H02.36 | GS19-G115-AWil-67-359F | MT376735 | *Synechococcus sp*. SR-C1 | 99.660 | 296 | 1 | 0 | 5.39E-150 | 542 |

| Place | Sample | qseqid | Accession number | Title | % identity | length | Mis-match | Gaps | e-value | Bit-score |
|---|---|---|---|---|---|---|---|---|---|---|
| **Lac de Falemprise, Cerfontaine** | I01.28 | GS20-C124-AWil-65-781R | KM218863 | *Cyanobium* sp. UMPCCC 1208 | 98.099 | 263 | 5 | 0 | 4.88E-125 | 459 |
| | I01.35 | GS20-C097-AWil-37-359F | MT376735 | *Synechococcus sp*. SR-C1 | 99.815 | 541 | 1 | 0 | 0 | 994 |
| | I01.37 | GS19-G098-AWil-42-781R | AM710381 | *Cyanobium sp*. JJR2A5 | 97.297 | 296 | 8 | 0 | 2.55E-138 | 503 |
| **Lac de Bambois, Fosses-La-Ville** | I04.27 | GS20-C105-AWil-57-359F | AJ293129 | *Aphanizomenon flos-aquae* PMC9706 | 99.638 | 552 | 2 | 0 | 0 | 1009 |
| | I04.28 | GS20-C109-AWil-62-359F | EU078530 | *Aphanizomenon gracile* LMECYA 9 | 98.851 | 609 | 6 | 1 | 0 | 1085 |
| | I04.29 | GS20-C113-AWil-72-359F | AP018316 | *Dolichospermum compactum* NIES-806 | 100.000 | 588 | 0 | 0 | 0 | 1086 |
| | I04.30 | GS20-C090-AWil-29-359F | AP018316 | *Dolichospermum compactum* NIES-806 | 99.157 | 593 | 4 | 1 | 0 | 1066 |
| | I04.31 | GS20-C091-AWil-30-359F | AP018316 | *Dolichospermum compactum* NIES-806 | 99.391 | 657 | 4 | 0 | 0 | 1192 |
| | I04.32* | GS19-G101-AWil-47-359F | LC557461 | *Microcystis aeruginosa* NIES-104 | 99.656 | 291 | 1 | 0 | 3.18E-147 | 532 |
| | I04.32* | GS20-C101-AWil-47-359F | GU197633 | *Dolichospermum circinale* CHAB233 | 98.250 | 400 | 6 | 1 | 0 | 699 |

| Place | Sample | qseqid | Accession number | Title | % identity | length | mismatch | gaps | e value | bitscore |
|---|---|---|---|---|---|---|---|---|---|---|
| **Lac de Bambois, Fosses-La-Ville** | w19.I04.34 | GS20-C069-AWil-52-359F | LC557461 | *Microcystis aeruginosa* NIES-104 | 98.653 | 297 | 1 | 3 | 1.96E-144 | 523 |
| | I04.36 | GS20-C100-AWil-45-359F | LC455659 | *Planktothrix agardhii* NIES-905 | 95.633 | 229 | 5 | 5 | 3.3E-96 | 363 |
| | I04.37 | GS20-C098-AWil-39-359F | AM710382 | *Cyanodictyon* sp. JJCD | 98.618 | 217 | 3 | 0 | 6.72E-103 | 385 |

**Table S2.4.** Number of reads and BLAST analysis of the representative sequence of the OTUs obtained with the Illumina amplicon sequencing

| #OTU ID | BL5.29. Cloister Oudergem | Red VL1.36. Kessel-Lo Provincial Domain | Accession number of the most similar cultured hit | Name of the most similar cultured hit | % identity | length | Mis-match | gaps | e value | bitscore |
|---|---|---|---|---|---|---|---|---|---|---|
| OTU_72 | 5027 | 12 | AY701560 | *Anabaena ellipsoides* Ana HB | 100.000 | 362 | 0 | 0 | 0 | 669 |
| OTU_28 | 1517 | 37200 | LC557463 | *Microcystis aeruginosa* NIES-90 | 100.000 | 362 | 0 | 0 | 0 | 669 |
| OTU_231 | 318 | 230 | HE975013 | *Aphanizomenon flos-aquae* CCAP 1401/7 | 100.000 | 362 | 0 | 0 | 0 | 669 |
| OTU_346 | 185 | 0 | AB610891 | *Synechococcus sp*. Suigetsu-CG2 | 97.790 | 362 | 8 | 0 | 6E-175 | 625 |
| OTU_559 | 127 | 1 | EU078524 | *Anabaena spiroides* LMECYA 161 (now *Dolichospermum spiroides*) | 99.448 | 362 | 2 | 0 | 0 | 658 |
| OTU_582 | 76 | 1 | LC319779 | *Dolichospermum sp*. NIES-1951 | 99.171 | 362 | 3 | 0 | 0 | 652 |
| OTU_678 | 61 | 0 | FN691909 | *Dolichospermum flos-aquae* 04-57 | 100.000 | 362 | 0 | 0 | 0 | 669 |
| OTU_537 | 50 | 41 | CP003495 | *Cyanobium gracile* PCC 6307 | 100.000 | 362 | 0 | 0 | 0 | 669 |
| OTU_649 | 25 | 0 | AM710380 | *Cyanobium sp*. JJM10D5 | 99.724 | 362 | 1 | 0 | 0 | 664 |
| OTU_627 | 20 | 0 | GU936926 | Chlorophyta symbiont of *Lubomirskia* sp. isolate R53 | 100.000 | 362 | 0 | 0 | 0 | 669 |
| OTU_4 | 0 | 7 | LT634149 | *Timaviella circinata* GR4 | 99.448 | 362 | 2 | 0 | 0 | 658 |
| OTU_5 | 0 | 7 | MH688842 | *Hormoscilla* cf. pringsheimii Us-s-6-2 | 98.619 | 362 | 5 | 0 | 6E-180 | 641 |
| OTU_6 | 0 | 7 | KM052844 | *Leptolyngbya sp*. LCR-CYANT35 | 99.724 | 362 | 1 | 0 | 0 | 664 |

**Table S2.5.** Results of the ion ratio for the validation of the UHPLC-MS/MS method quantification method of 8 MCs and Nodularin in filtered cyanobacterial biomass. Acceptation criteria are based on European Decision 2002/EC/657

| Toxin | MC-RR | NOD | MC-LA | MC-LF | MC-LR | MC-LY | MC-LW | MC-YR | MC-WR |
|---|---|---|---|---|---|---|---|---|---|
| Average ion ratio (%) | 13.37 | 46.65 | 51.91 | 41.51 | 31.66 | 45.82 | 49.20 | 33.21 | 40.21 |
| Standard Deviation ion ratio (%) | 0.39 | 2.08 | 7.59 | 2.43 | 1.26 | 3.68 | 6.15 | 3.19 | 8.11 |

**Table S2.6.** Detection results for 6 additional microcystin congeners with a limit of detection (LOD) at 0.10 µg $L^{-1}$. Not detected is abbreviated by "n.d.". Detected MCs are annotated as > LOD.

| Samples | MC-HtyR | dm MC-LR/D-asp MC-LR | D-asp-Dhb MC-RR/ dm MC-RR | MC-HilR |
|---|---|---|---|---|
| AN1.32 | n.d. | n.d. | > LOD | n.d. |
| AN2.32 | n.d. | > LOD | > LOD | > LOD |
| AN2.37 | > LOD | > LOD | > LOD | > LOD |
| AN3.37 | n.d. | > LOD | n.d. | > LOD |
| GH1.30 | n.d. | n.d. | n.d. | n.d. |
| VL1.32 | n.d. | > LOD | > LOD | > LOD |
| VL1.36 | n.d. | > LOD | > LOD | > LOD |
| VL2.34 | n.d. | > LOD | > LOD | > LOD |
| VL2.36i | n.d. | > LOD | > LOD | > LOD |
| VL2.2.36 | n.d. | > LOD | > LOD | > LOD |
| VL3.34 | n.d. | n.d. | n.d. | n.d. |
| BL1.29 | > LOD | > LOD | > LOD | > LOD |
| BL1.30 | > LOD | > LOD | > LOD | > LOD |
| BL1.35 | > LOD | > LOD | > LOD | > LOD |
| BL2.29 | n.d. | > LOD | n.d. | n.d. |
| BL3.30 | n.d. | n.d. | n.d. | n.d. |
| BL4.30 | n.d. | n.d. | n.d. | n.d. |
| BL4.35 | n.d. | n.d. | n.d. | n.d. |
| BL5.29i | n.d. | n.d. | n.d. | n.d. |
| BL5.35 | n.d. | n.d. | > LOD | n.d. |
| BL6.29 | n.d. | n.d. | n.d. | n.d. |
| BL6.35 | n.d. | n.d. | n.d. | n.d. |
| BL7.30 | n.d. | n.d. | n.d. | n.d. |
| BL7.35 | n.d. | n.d. | n.d. | n.d. |
| BL8.35 | n.d. | n.d. | > LOD | n.d. |
| BV1.34 | n.d. | > LOD | > LOD | > LOD |
| BV2.34 | > LOD | > LOD | > LOD | > LOD |
| BV2.35 | > LOD | > LOD | > LOD | > LOD |
| BV3.35 | n.d. | > LOD | > LOD | > LOD |

| Samples | MC-HtyR | dm MC-LR/D-asp MC-LR | D-asp-Dhb MC-RR/dm MC-RR | MC-HilR |
|---|---|---|---|---|
| B04.27 | n.d. | n.d. | > LOD | n.d. |
| B04.28 | n.d. | n.d. | > LOD | n.d. |
| B04.29 | n.d. | n.d. | > LOD | n.d. |
| B04.30 | n.d. | n.d. | > LOD | n.d. |
| B04.31 | n.d. | n.d. | > LOD | n.d. |
| B04.32 | n.d. | > LOD | > LOD | n.d. |
| B04.34 | n.d. | n.d. | > LOD | n.d. |
| B04.35 | n.d. | n.d. | > LOD | n.d. |
| B04.36 | n.d. | n.d. | > LOD | n.d. |
| B04.37 | n.d. | n.d. | > LOD | n.d. |
| E04.27 | n.d. | n.d. | > LOD | n.d. |
| E04.28 | n.d. | > LOD | > LOD | n.d. |
| E04.29 | n.d. | > LOD | > LOD | n.d. |
| E04.30 | > LOD | > LOD | > LOD | > LOD |
| E04.31 | n.d. | > LOD | > LOD | > LOD |
| E04.32 | n.d. | > LOD | > LOD | n.d. |
| E04.34 | n.d. | > LOD | > LOD | > LOD |
| E04.35 | > LOD | > LOD | > LOD | > LOD |
| E04.36 | > LOD | > LOD | > LOD | > LOD |
| E04.37 | n.d. | > LOD | > LOD | > LOD |
| H02.27 | n.d. | n.d. | > LOD | n.d. |
| H02.28 | n.d. | n.d. | > LOD | n.d. |
| H02.29 | n.d. | n.d. | > LOD | n.d. |
| H02.30 | n.d. | > LOD | > LOD | n.d. |
| H02.31 | > LOD | > LOD | > LOD | n.d. |
| H02.32 | n.d. | > LOD | > LOD | n.d. |
| H02.34 | n.d. | > LOD | > LOD | n.d. |
| H02.35 | n.d. | > LOD | > LOD | n.d. |
| H02.36 | n.d. | > LOD | > LOD | n.d. |
| H02.37 | n.d. | > LOD | > LOD | n.d. |
| I01.27 | n.d. | n.d. | n.d. | n.d. |
| I01.28 | n.d. | n.d. | n.d. | n.d. |
| I01.29 | n.d. | n.d. | n.d. | n.d. |
| I01.30 | n.d. | n.d. | n.d. | n.d. |
| I01.31 | n.d. | n.d. | > LOD | n.d. |
| I01.32 | n.d. | n.d. | n.d. | n.d. |
| I01.34 | n.d. | n.d. | > LOD | n.d. |
| I01.35 | n.d. | n.d. | > LOD | n.d. |
| I01.36 | n.d. | n.d. | > LOD | n.d. |
| I01.37 | n.d. | n.d. | > LOD | n.d. |
| I04.27 | n.d. | n.d. | n.d. | n.d. |
| I04.28 | > LOD | > LOD | > LOD | n.d. |
| I04.29 | n.d. | > LOD | > LOD | n.d. |
| I04.30 | n.d. | > LOD | > LOD | n.d. |
| I04.31 | n.d. | > LOD | > LOD | > LOD |
| I04.32 | n.d. | > LOD | > LOD | > LOD |

| Samples | MC-HtyR | dm MC-LR/D-asp MC-LR | D-asp-Dhb MC-RR/dm MC-RR | MC-HilR |
|---|---|---|---|---|
| I04.34 | n.d. | > LOD | > LOD | > LOD |
| I04.35 | n.d. | > LOD | > LOD | > LOD |
| I04.36 | n.d. | > LOD | > LOD | > LOD |
| I04.37 | n.d. | > LOD | > LOD | > LOD |

**Table S2.7.** Overview of the single sequences amplified by the Sanger method used for taxonomic identification based on BLAST analysis

| Sample number and primer number | Genus name | Sanger sequence |
|---|---|---|
| AN2.32_359F | *Dolichospermum* | AGGCTCTTGGGTTGTAAACCTCTTTTCTCAGGGAAGAAAAAAATGACGGTACCT GAGGAATAAGCATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGAT GCAAGCGTTATCCGGAATGATTGGGCGTAAAGGGTCCGCAGGTGGCATTGTAA GTCTGCTGTTAAAGAGTCTAGCTCAACTAGATAAAAGCAGTGGAAACTACAAAG CTAGAGTTTGGTCGGGGCAGAAGGAATTCCTGGTGTAGCGGTGAAATGCGTAG ATATCAGGAAGAACACCAGTGGCGAAGGCGTTCTGCTAGGCCGAGACTGACAC TGAGGGACGAAAGCTAGGGGAGCGAATGGGATTAGATACCCCAGTAGTCCTAG CCGTAAACGATGGATACTAGGCGTAGCTCGTATCGACCCGAGCTGTGCCGGAG CTAACGCGTTAAGTATCCCGCCTGGGGAGTACGCAGGCAACTGTGAAACTCAAA GGAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCA ACGCGAAGAACCTTACCAAGGCTTGACATGTCACGAATCCTGTG |
| AN2.37_359F | *Synechococcus* | TTCCGTGCCAGCAGCCGCGGTAATACGGGAGTGGCAAGCGTTATCCGGAATTA TTGGGCGTAAAGCGTCCGCAGGCGGCCTTGTAAGTCTGTCGTTAAAGCGTGGA GCTCAACTCCATTTAAGCGATGGAAACTACAAGGCTGGAGTGTGGTAGGGGCA GAGGGAATTCCCGGTGTAGCGGTGAAATGCGTAGATATCGGGAAGAACACCAG TGGCGAAGGCGCTCTGCTGGGCCATAACTGACGCTCATGGACGAAAGCCAGGG GAGCGAAAGGGATTAGATACCCCTGTAGTCCTGGCCGTAAACGATGAACACTA GGTGTCGGGGGAATCGACCCCCTCGGTGTCGTAGCCAACGCGTTAAGTGTTCC GCCTGGGGAGTACGCACGCAAGTGTGAAACTCAAAGGAATTGACGGGGGCCC GCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCA GGGCTTGACATGCTGCGAATCCCCTGGAAACGAGGGAGTGCCTTCGGGAGCGC AGAGACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTA AGTCCCGCAACGAGCG |
| AN3.37_359F | *Romeria* | AGGAGGCCTTAGGGTTGTAAACCTCTTTTCTCTGGGAAGAAGAACTGACGGTAC CAGAGGAATAAGCCTCGGCTAACTCCGTGCCAGCAGCCGCGGTAAGACGGAGG AGGCGAGCGTTATCCGGAATTATTGGGCGTAAAGCGTCCGCAGGCGGTTTATC AAGTCTGCTGTCAAAGACTACAGCTTAACTGTGGGCAGGCAGTGGAAACTGAT GAACTAGAGAGCGGTAGGGGTAGAGGGAATTCCCGGTGTAGCGGTGAAATGC GTAGATATCGGGAAGAACACCAGTGGCGAAGGCGCTCTACTGGGCCGTTACTG ACGCTGAGGGACGAAAGCTAGGGGAGCGAAAGGGATTAGATACCCCTGTAGT CCTAGCTGTAAACGATGGATACTAGGTGTTGGGCGTATCGACCCGTCCAGTACC GTAGCTAACGCGTTAAGTATCCCGCCTGGGGAGTACGCACGCAAGTGTGAAAC TCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGGATGTGGTTTAATTCG ATGCAACGCGAAGAACCTTACCAAGGCTTGACATCCTGCGAATCCTTCAGAGAT GAGGGAGTGCCTTCGGGAGCGCAGAGACAGGTGGTGCATGGCTGTCGTCAGC TCGTGTCGTGAGATGTTGGGTTAAGTC |

| Sample number and primer number | Genus name | Sanger sequence |
|---|---|---|
| VL1.32_781R | *Microcystis* | GCCACCGATGTTCTTCCCAATCTCTACGCATTTCACCGCTACACTGGGAATTCCTGCTACCCCTACTGCTCTCTAGTCTGCCAGTTTCCACCGCCTTTATGTCGTTAAGCAACCTGATTTGACAGCAGACTTGGCTGACCACCTGCGGACGCTTTACGCCCAATAATTCCGGATAACGCTTGCCTCCCCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGATGCTGATTCCTCAAGTACCGTCA |
| VL2.34_359F | *Dolichospermum* | TCTGAAGGAAGCATGACGAGGAAATCACTACGCTCTGGGGTTGTAGCCTCTTTTCTAATGGAAGAAGTGCTGTTGGTACCTGGGTGTTAATCATCGTCTAACTCCGGGGAAGCTGCCGCGGTCTTACGGAGGATGCAAGCGTTATCCGGAATGATTGGGCGTAAAGGGTCCGCAGGTGGCATTGAAAGTCTGCTGTTCAGAGGTTGGGTCAACCAAATAAGAGCAGTGGAAACTACAAAGCTAGAGTTTGGTCGGGGCAGAGGGAATTCCTGGTGTAGCGGTGAAATGCGTAGATATCAGGAAGAACACCAGTGGCGAAGGCGCTCTGCTAGGCCGAGACTGACACTGAGGGACGAAAGCTAGGGGAGCGAATGGGATTAGATACCCCAGTAGTCCTAGCCGTAAACGATGGATACTAGGCGTAGCTCGTATCGACCCGAGCTGTGCCGGAGCTAACGCGTTAAGTATCCCGCCTGGGGAGTACGCAGGCAACTGTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATGTCACGAATCCTGTAGAAATATAGGAGTGCCTTCGGGAGCGTGAACACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTC |
| VL2.36.bis_359F | *Microcystis* | TCTGACGGTACTTGAGGAATCAGCCTCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGGGGAGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGTCCGCAGGTGGTCAGCCAAGTCTGCTGTCAAATCAGGTTGCTTAACGACCTAAAGGCGGTGGAAACTGGCAGACTAGAGAGCAGTAGGGGTAGCAGGAATTCCCAGTGTAGCGGTGAAATGCGTAGAGATTGGGAAGAACATCGGTGGCGAAAGCGTGCTACTGGGCTGTATCTGACACTCATGGACGAAAGCTAGGGGAGCGAAAGGGATTAA |
| VL2.36_781R | *Microcystis* | TCTCTACGCATTTCACCGCTACACTGGGAATTTCCTGCTGCCCCTACTGCTCTCTAGTCTGCCAGTTTCCACCGCCTTTAGGTCGTTAAGCAACCTGATTTGACAGCAGACTTGGCTGACCACCTGCGGACGCTTTACGCCCCAATAATTCCGGATAACGCTTGCCTCCCCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGATGCTGATTCCTCAAGTACCGTC |
| VL3.34_359F | *Dolichospermum* | TCTTGGGTTGTAAACCTCTTTTCTCAGGGAAGAACAGAATGACGGTACCTGAGGAATAAGCATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATGCAAGCGTTATCCGGAATGATTGGGCGTAAAGGGTCCGCAGGTGGCATTGAAAGTCTGCTGTTAAAGAGTTTGGCTCAACCAAATAAGAGCAGTGGAAACTACAAAGCTAGAGTTTGGTCGGGGCAGAGGGAATTCCTGGTGTAGCGGTGAAATGCGTAGATATCAGGAAGAACACCAGTGGCGAAGGCGCTCTGCTAGGCCGAGACTGACACTGAGGGACGAAAGCTAGGGGAGCGAATGGGATTAGATACCCCAGTAGTCCTAGCCGTAAACGATGGATACTAGGCGTAGCTCGTATCGACCCGAGCTGTGCCGGAGCTAACGCGTTAAGTATCCCGCCTGGGGAGTACGCAGGCAACTGTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATGTCACGAATCCTGTAGAAATATAGGAGTGCCTTCGGGAGCGTGAACACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAAC |
| BL1.29_359F | *Synechococcus/ Merismopedia* | AGCCGCGGTAATACGGGGGAGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGTCCGCAGGTGGTCTGCCAAGTCTGCTGTCAAATCATGTTGCTTAACTACCTAAAGGCGGTGGAAACTGGCAGACTAGAGAGCATTAGGGGTAGCAGGAATTCCCAGTGTAGCGGTGAAATGCGTAGATATTGGGAAGAACATCGGTGGCGAAAGCGTGCTACTGGGCTGTATCTGACACTCAGGGACGAAAGCTAGGGGAGCGAAAGGGAT |
| BL1.29_781R | *Microcystis* | AATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGGGAGGAAGGTCTTTGGATTGTAAACCTCTTTTCTCAAGGAAGAAGTTCTGACGGTACTTGAGGAATCAGCCTCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGGGGAGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGTCCGCAGGTGGTCAGCCAAGTCTGCCGTCAAATCAGGTTGCTTAACGACCTAAAGGCGGTGGAAACTGGCAGACTAGAGAGCAGTAGGGGTAGCAGGAATTCCCAGTGTAGCGGTGAAATGCGTAGAGATTGGGAAGAACATCGGTGGCGAAAGCGTGC |

| Sample number and primer number | Genus name | Sanger sequence |
|---|---|---|
| BL1.30_781R | *Microcystis* | CAATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGGGAGGAAGGTCTTTG<br>GATTGTAAACCTCTTTTCTCAAGGAAGAAGTTCTGACGGTACTTGAGGAATCAG<br>CCTCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGGGGAGGCAAGCGTTA<br>TCCGGAATTATTGGGCGTAAAGCGTCCGCAGGTGGTCAGCCAAGTCTGCCGTCA<br>AATCAGGTTGCTTAACGACCTAAAGGCGGTGGAAACTGGCAGACTAGAGAGCA<br>GTAGGGGTAGCAGGAAATTCCCAGTGTAGCGGTGAAATGCGTAGAGATTGGG<br>AAGAAACATCGGTGGCGAAAAGCGTGC |
| BL4.35_359F | *Aphanizomenon* | GGCTCTTGGGTTGTAAACCTCTTTTCTCAGGGAAGAACAGAATGACGGTACCTG<br>AGGAATAAGCATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATG<br>CAAGCGTTATCCGGAATGATTGGGCGTAAAGGGTCCGCAGGTGGCATTGTAAG<br>TCTGCTGTTAAAGAGTTTGGCTCAACCAAATAAGAGCAGTGGAAACTACAAAGC<br>TAGAGTGTGGTCGGGGCAGAGGGAATTCCTGGTGTAGCGGTGAAATGCGTAG<br>ATATCAGGAAGAACACCAGTGGCGAAGGCGCTCTGCTAGGCCGAGACTGACAC<br>TGAGGGACGAAAGCTAGGGGAGCGAATGGGATTAGATACCCCAGTAGTCCTAG<br>CCGTAAACGATGGATACTAGGCGTAGCTCGTATCGACCCGAGCTGTGCCGGAG<br>CTAACGCGTTAAGTATCCCGCCTGGGGAGTACGCAGGCAACTGTGAAACTCAAA<br>GGAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCA<br>ACGCGAAGAACCTTACCAAGGCTTGACATGTCACGAATCCTATTGAAAGATGGG<br>AGTGCCTTCGGGAGCGTGAACACAGGTGGTGCATGGCTGTCGTCAGCTCGTGT<br>CGTGAGATGT |
| BL5.29_359F | *Dolichospermum* | TCCGATTATGTTCTACTTTCTCGGGAGCAGCTCTGTGGGATTGTAAACCTCTTTA<br>TCTCAGGGTAAGAAACAATGACGGTACGTGAGGAATAGGCATCGGCTAACTCC<br>GTGCCAGCAGCCGCGGTAATACGGAGGATGCAAGCGTTATCCGGAATGATTGG<br>GCGTAAAGAGTCCGTAGGTGGCATTGAAAGTCTGCTGTTAAAGAGTCTAGCTCA<br>ACTAGATAAGAGCAGTGGAAACTACAAAGCTAGAGTTTGGTCGGGGCAGAAG<br>GAATTCCTGGTGTAGCGGTGAAATGCGTAGATATCAGGAAGAACACCGGTGGC<br>GAAGGCGTTCTGCTAGGCCGAGACTGACACTGAGGGACGAAAGCTAGGGGAG<br>CGAATGGGATTAGATACCCCAGTAGTCCTAGCCGTAAACGATGGATACTAGGC<br>GTAGCTCGTATCGACCCGAGCTGTGCCGGAGCTAACGCGTTAAGTATCCCGCCT<br>GGGGAGTACGCAGGCAACTGTGAAACTCAAAGGAATTGACGGGGGCCCGCAC<br>AAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCAAGG<br>CTTGACATGTCACGAATCCTGTAGAAATATAGGAGTGCCTTCGGGAGCGTGAAC<br>ACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATG |
| BL5.35_359F | *Dolichospermum* | TCCGGAAGGGGCCGCACGGTCGCGTAGGACGCTCTGGCGTTCTCTCCTCTTTTC<br>TCACGGAAGAAAAGATGACGGTACCTGAGGAATAGGCATCGGGTAACTCCGTG<br>CCAGCAGCCGCGGTAATACGGAGGATGCAAGCGTTATCCGGAATGATTGGGCG<br>TAAAGGGTCCGCAGGTGGCATTGAAAGTCTGCTGTTAAAGAGTCTAGCTCAACT<br>AGATAAGAGCAGTGGAAACTACAAAGCTAGAGTTTGGTCGGGGCAGAAGGAA<br>TTCCTGGTGTAGCGGTGAAATGCGTAGATATCAGGAAGAACACCAGTGGCGAA<br>GGCGTTCTGCTAGGCCGAGACTGACACTGAGGGACGAAAGCTAGGGGAGCGA<br>ATGGGATTAGATACCCCAGTAGTCCTAGCCGTAAACGATGGATACTAGGCGTAG<br>CTCGTATCGACCCGAGCTGTGCCGGAGCTAACGCGTTAAGTATCCCGCCTGGGG<br>AGTACGCAGGCAACTGTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCG<br>GTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGAC<br>ATGTCACGAATCCTGTGGAAACATAGGAGTGCCTTCGGGAGCGTGAACACAGG<br>TGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGG |

| Sample number and primer number | Genus name | Sanger sequence |
|---|---|---|
| BL6.29_359F | *Dolichospermum* | AGGCTCTTGGGTTGTAAACCTCTTTTCTCAGGGAAGAAAAAAATGACGGTACCTGAGGAATAAGCATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATGCAAGCGTTATCCGGAATGATTGGGCGTAAAGGGTCCGCAGGTGGCATTGAAAGTCTGCTGTTAAAGAGTCTAGCTCAACTAGATAAGAGCAGTGGAAACTACAAAGCTAGAGTTTGGTCGGGGCAGAAGGAATTCCTGGTGTAGCGGTGAAATGCGTAGATATCAGGAAGAACACCAGTGGCGAAGGCGTTCTGCTAGGCCGAGACTGACACTGAGGGACGAAAGCTAGGGGAGCGAATGGGATTAGATACCCCAGTAGTCCTAGCCCGTAAACGATGGATACTAGGCGTAGCTCGTATCGACCCGAGCTGTGCCGGAGCCTAACGCGTTAAGTATCCCGCCTGGGGAGTACGCAGGCAACTGTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAA |
| BL7.30_359F | *Dolichospermum* | GGCTCTTGGGTTGTAACCTCTTTTCTCAGGGAAGAACAGAATGACGGTACCTGAGGAATAAGCATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATGCAAGCGTTATCCGGAATGATTGGGCGTAAAGGGTCCGCAGGTGGCATTGAAAGTCTGCTGTTAAAGAGTTTGGCTCAACCAAATAAGAGCAGTGGAAACTACAAAGCTAGAGTTTGGTCGGGGCAGAGGGAATTCCTGGTGTAGCGGTGAAATGCGTAGATATCAGGAAGAACACCGGTGGCGAAGGCGCTCTGCTAGGCCGAGACTGACACTGAGGGACGAAAGCTAGGGGAGCGAATGGGATTAGATACCCCAGTAGTCCTAGCCGTAAACGATGGATACTAGGCGTAGCTCGTATCGACCCGAGCTGTGCCGGAGCTAACGCGTTAAGTATCCCGCCTGGGGAGTACGCAGGCAACTGTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAG |
| BL8.35_359F | *Microcystis* | GCCTCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGGGGAGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGTCCGCAGGTGGTCAGCCAAGTCTGCCGTCAAATCAGGTTGCTTAACGACCTAAAGGCGGTGGAAACTGGCAGACTAGAGAGCAGTAGGGGTAGCAGGAATTTCCCAGTGTAGCGGTGAAATGCGTAGAGATTGGGAAGAACATCGGTGGCGAAAGCGTGCTACTGGGCTGTATCTGACACTCAGGGACGAAAGCTAGGGGAGCGAAAGGGAT |
| BV1.34_23S30R | *Synechococcus* | TATAGCATCGTTTCATTCCCAATTGTTTAATTTCAAACGTGGGTTGAAAAGACGCTGGGTTCACCAGATACTTGCATTTGAAGTGTAATTATTTGGAGATTCAGCAGAACCTTGACAACTGCATAGGTAAGTCTGGAAAGAAAGCATCTCATAGATGTCCAGAAGCGAAAGCGAC |
| BV1.34_781R | *Microcystis* | TCCGTGCCAGCAGCCGCGGTAATACGGGGGAGGCAAGCGTTATCCGGAATCATTGGGCGTAAAGCGTCCGCAGGTGGTCAGCCAAGTCAGCTGTCAAATCAGGTTGCTTAACGACATAAAGGCGGTGGAAACTGGCAGACTAGAGAGCAGTAGGGGTAGCGGGAATTCCCAGTGTAGCGGTGAAATGCGTAGAG |
| BV2.34_23S30R | *Microcystis* | AGCAAACCCGGCGTCAGTTCAGATTGCAGGCTGCAACTCGCCTGCATGAAGGAGGAATCGCTAGTAATCGCCGGTCAGCATACGGCGGTGAATTCGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGAAGCTGGTCACGCCCGAAGTCATTACCTCAACCGCAAGGAGGGGGATGCCTAAGGCAGGGCTAGTGACTGGGGTGAAGTCGTAACAAGGTAGCCGTACCGGAAGGTGTGGCTGGATCACCTCCTTAAAGGGAGACCTAATTCAGGTATAAGACGAAAAAAAAGTAGTCCCTACCAAGAATCAATCCCAAAAGGTCGGAACGAGGTATGAGGCTTTCAAACTAGGTTCTGGGTTCATAAAAGACCTGAATCAGGAACAAGGGCTATTAGCTCAGGTGGTTAGAGCGCACCCCTGATAAGGGTGAGGTCCCTGGTTCGAGTCCAGGATGGCCCACCTGCACAGGTGGCAAAAACAAGAGAAGCGAGGAATCAGCACCTTATCTTATATACATATATAAGAGAGAATGCTGGCTCTGAGT |

| Sample number and primer number | Genus name | Sanger sequence |
|---|---|---|
| BV2.35_359F | *Synechococcus* | TGTAACCTCTTTTCTCAAGGAAGAAGTTCTGACGGTACTTGAGGAATCAGCCAC GGCTAATTCCGTGCCAGCAGCCGCGGTAATACGGGGGTGGCAAGCGTTATCCG GAATCATTGGGCGTAAAGCGTCCGCAGGTGGCTTTGTAAGTCTGCTGTTAAAGC GTGGAGCTTAACTCCCTTTCAGCGGTGGAAACTGCAAAACTTGAGTGTGGTAGG GGCAGAGGGAATTCCCGGTGTAGCGGTGAAATGCGTAGATATCGGGAAGAAC ACCAGTGGCGAAGGCGCTCTGCTGGGCCATATCTGACACTCATGGACGAAAGC CAGGGGAGCGAAAGGGATTAGATACCCCTGTAGTCCTGGCCGTAAACGATGAA CACTAGGCGTCGGGGGAATCGACCCCCTCGGTGTCGTAGCCAACGCGTTAAGT GTTCCGCCTGGGGAGTACGCACGCAAGTGTGAAACTCAAAGGAATTGACGGGG GCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCT TACCAGACTTGACA |
| BV3.35_359F | *Phormidium* | TCCGCTTAACAACACAGCCGCTCAGGCTATGTACGTTCTGTGGATGTAACCTCTT TTCTCAGGGAAGACATCTGACGGTACCTGAGGAATAAACGTCGAAGTAACTCCG TGCCAGCAGCCGCGGTAATACGGAGGATGCAAGCGTTATCCGGAATGATTGGG CGTAAAGCGTCCGCAGGTGGTTTTTCAAGTCTGCTGTTAAAGACCGGGGCTTAA CTCCAGGCAAGCAGTGGAAACTGAAAGACTAGAGTATGGTAGGGGCAGAGGG AATTCCTGGTGTAGCGGTGAAATGCGTAGAGCTCAGGAAGAACATCGGTGGCG AAGGCGCTCTGCTAGGCCGAAACTGACACTCAGGGACGAAAGCTAGGGGAGC GAATGGGATTAGATACCCCAGTAGTCCTAGCTGTAAACGATGGATACTAGGTGT TGTCTGTATCGACCCGAACTGTGCCGTAGCTAACGCGTTAAGTATCCCGCCTGG GGAGTACGCACGCAAGTGTGAAACTCAAAGGAATTGACGGGGGCCCGCACAA GCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGA |
| B04.27_359F | *Aphanizomenon* | CTTTTCTCAGGGAAGAACACAATGACGGTACCTGAGGAATAAGCATCGGCTAAC TCCGTGCCAGCAGCCGCGGTAATACGGGGGATGCAAGCGTTATCCGGAATGAT TGGGCGTAAAGGGTCCGCAGGTGGCATTGCAAGTCTGCTGTTAAAGAGTTTGG CTCAACCTCATAAAAGCAGTGGAAACTGCAAAGCTAGAGTGTGGTCGGGGCAG AGGGAATTCCTGGTGTAGCGGTGAAATGCGTAGAGATCAGGAAGAACACCGGT GGCGAAGGCGCTCTGCTAGGCCATAACTGACACTGAGGGACGAAAGCTAGGG GAGCGAATGGGATTAGATACCCCAGTAGTCCTAGCCGTAAACGATGGATACTA GGCGTGGCTCGTATCGACCCGAGCTGTGCCGTAGCTAACGCGTTAAGTATCCCG CCTGGGGAGTACGCACGCAACTGTGAAACTCAAAGGAATTGACGGGGGCCCGC ACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCAA GGCTTGACATCTC |
| B04.29_359F | *Aphanizomenon* | TACGGAGGATGCAAGCGTTATCCGGAATGATTGGGCGTAAAGGGTCCGCAGGT GGCATTGTAAGTCTGCTGTTAAAGAGTTTGGCTCAACCAAATAAAAGCAGTGGA AACTACAAAGCTAGAGTGTGGTCGGGGCAGAGGGAATTCCTGGTGTAGCGGTG AAATGCGTAGATATCAGGAAGAACACCGGTGGCGAAGGCGCTCTGCTAGGCCA AGACTGACACTGAGGGACGAAAGCTAGGGGAGCGAATGGGATTAGATACCCC AGTAGTCCTAGCCGTAAACGATGGATACTAGGCGTAGCTCGTATCGACCCGAGC TGTGCCGTAGCTAACGCGTTAAGTATCCCGCCTGGGGAGTACGCAGGCAACTGT GAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTA ATTCGATGCAACGCGA |
| B04.29_781R | *Planktothrix* | CGGAGCAAGACCGCGTGGGGGAGGAAGGTTCTTGGATTGTCAACCCCTTTTTTC AGGGAAGAACACAATGACGGTACCTGAGGAATAAGCATCGGCTAACTCCGTGC CAGCAGCCGCGGTAATACGGGGGATGCAAGCGTTATCCGGAATGATTGGGCGT AAAGAGTCCGTAGGTGGTCATCCAAGTCTGCTGTTAAAGAGCGAGGCTTAACCT CGTAAAGGCAGTGGAAACTGGAAGACTAGAGTGTAGTAGGGGCAGAGGGAAT TCCCGGTGTAGCGGTGAAATGCGTAGATATCAGGAAGAACACCGGTGGCGA |

| Sample number and primer number | Genus name | Sanger sequence |
|---|---|---|
| B04.31_359F | *Aphanizomenon/Dolichospermum* | GTTGTAAACCTCTTTTCTCAGGAAGAACATCTTGCGGGTCTTGGAGGATTAACC ATCGGTTATTTCCGGGCCACCACCCGGGGAATTCCGGAGGGTGCAGCGTTTTTC GGGATTGTTGGGGGGTAAGGGGCCCGCAGGGGGCTTTGTAGTTCGGCTGTTAA ACATGTTGCTTCACTCCATTTCAGCGATGGAAACTACAAGGCTAGAGTGTGGTC GGGGCAGAGGGAATTCCTGGTGTAGCGGTGAAATGCGTAGATATCAGGAAGA ACACCGGTGGCGAAGGCGCTCTGCTGGGCCATAACTGACACTGATGGACGAAA GCTAGGGGAGCGAATGGGATTAGATACCCCTGTAGTCCTGGCCGTAAACGATG AATACTAGGCGTAGCTCGAATCGACCCCCTCTGTGTCGTAGCTAACGCGTTAAG TGTTCCGCCTGGGGAGTACGCACGCAACTGTGAAACTCAAAGGAATTGACGGG GGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGA |
| B04.34_359F | *Aphanizomenon* | CTTGGGTTGTAACCTCTTTTCTCAGGGAAGAACAAATTGACGGTACCTGAGGAA TAACCATCGGCTAATTCCGGGCCACCAGCCGCGGTAATACGGAGGATGCAAGC GTTATCCGGAATGATTGGGCGTAAAGGGTCCGCAGGGGGCATTGTAATTCTGC TGTTAAAGAGTTTGGCTCAACCAAATAAAAGCAATGGAAACTACAAAGCTAGA GTGTGGTCGGGGCAGAGGGAATTCCTGGTGTAGCGGTGAAATGCGTAGATATC AGGAAGAACACCGGTGGCGAAGGCGCTCTGCTAGGCCAAGACTGACACTGAG GGACGAAAGCTAGGGGAGCGAATGGGATTAGATACCCCAGTAGTCCTAGCCGT AAACGATGGATACTAGGCGTAGCTCGAATCGACCCGAGCTGTGCCGTAGCTAA CGCGTTAAGTATCCCGCCTGGGGAGTACGCACGCAACTGTGAAACTCAAAGGA ATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACG CGAAGAACCTTACCAAGGCTTGACATGTCACGAATTCCGTTGAA |
| E04.29_359F | *Aphanizomenon* | CTCTTGGGTTGTAACCTCTTTTCTCAGGGAAGAACAGAATGACGGTACCTGAGG AATAAGCATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATGCAA GCGTTATCCGGAATGATTGGGCGTAAAGGGTCCGCAGGTGGCATTGTAAGTCT GCTGTTAAAGAGTTTGGCTCAACCAAATAAGAGCAGTGGAAACTACAAAGCTA GAGTGTGGTCGGGGCAGAGGGAATTCCTGGTGTAGCGGTGAAATGCGTAGAT ATCAGGAAGAACACCAGTGGCGAAGGCGCTCTGCTAGGCCGAGACTGACACTG AGGGACGAAAGCTAGGGGAGCGAATGGGATTAGATACCCCAGTAGTCCTAGCC GTAAACGATGGATACTAGGCGTAGCTCGTATCGACCCGAGCTGTGCCGGAGCT AACGCGTTAAGTATCCCGCCTGGGGAGTACGCAGGCAACTGTGAAACTCAAAG GAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAA CGCGAAGAACCTTACCAAGGCTTGACATGTCACGAATCCTATTGAAAGATGGGA GTGCCTTCGGGAGCGTGAACACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTC GTGAGATGTTGG |
| E04.32_781R | rhodoplast | TCTTCCTATCTCTACGCATTTCACCGCTACACTGGGAATTCCCTCTACCTCTACTG CACTCTAGTCTGCTAGTTTCCACCGCCTTTATGACGTTAAGCCCCGAGATTTAAC AGCAGACTTGGTAGGCCACCTACAGACGCTTTACGCCCAGTGATTCCGGATAAC GCTTGCATCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGATGCTTATT CCTCAAGTACCGTCAGTACTTCTTCC |
| E04.34_359F | *Microcystis* | CTTGAGGTACTTGAGGAACAGCCTCGGCTCACTCGTGCCAGCAGCCGCGGTAAT ACGGGGGAGGCAAGCGTTTTCCGGAATTATTGGGCGTAAAGCGTCCGCAGGTG GTCTTCCAAGTCTGCCGTCAAATCACGTTGCTTAACCACCTAAAGGCGGTGGAA ACTGGCAGACTAGAGAGCAGTAGGGGTAGCAGGAATTCCCAGTGTAGCGGTG AAATGCGTAGAGATTGGGAAGAACATCGGTGGCGAAAGCGTGCTACTGGGCTG TATCTGACACTCAGGGACGAAAGCTAGGGGAGCGAAAGGGATA |
| E04.36_359F | *Microcystis* | GTACTGAGGAAAAGCCTGGCTAACTCTGTGCCGCAGCCGCGGTAAACGGGGGA GGCAAGCGTTTTTCCGGATTATTGGGCGTAAAGCGTCCGCAGGTGGTCTGCCAA GTCTGCCGTCAAATATCGTTGCTTAACCACCTAAAGGCGGTGGAAATGGCAGAC TAGAGTGCAGTAGGGGTAGCAGGAATTCCCAGTGTAGCGGTGAAATGCGTAAT ATTGGGAAGAACATCGGTGGCGAAAGCGTGCTACTGGGCTGTATCTGACACTC AGGGACGAAAGCTAGGGGAGCG |

| Sample number and primer number | Genus name | Sanger sequence |
|---|---|---|
| H02.27_359F | *Synechococcus* | GCCTCTGGGCTGTAAACCTCTTTTCTCAAGGAAGAAGATCTGACGGTACTTGAGGAATAAGCCACGGCTAATTCCGTGCCAGCAGCCGCGGTAATACGGGAGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGTCCGCAGGCGGCCTTGTAAGTCTGCCGTTAAAGCGTGGAGCTTAACTCCATTTCGGCGATGGAAACTACAAGGCTTGAGTGTGGTAGGGGCAGAGGGAATTCCCGGTGTAGCGGTGAAATGCGTAGATATCGGGAAGAACACCAGTGGCGAAGGCGCTCTGCTGGGCCATAACTGACGCTCATGGACGAAAGCCAGGGGAGCGAAAGGGATTAGATACCCCTGTAGTCCTGGCCGTAAACGATGAACACTAGGTGTCGGGGGAATCGACCCCCTCGGTGTCGTAGCCAACGCGTTAAGTGTTCCGCCTGGGGAGTACGCACGCAAGTGTGAAACTCAAAGGAATTGACGGG |
| H02.31_359F | *Planktothrix* | TGCGTTGGAAGGGTGGGCTGGAGGGAGAGCCTCTTGGGACTGTCAACCTCTTTTCTCAGGGAAGAACACCATGACGGTCCCTGAGGATAAAGCATCGGCTAACTCCGTGCCAGCACCCGCGGTAATACGGGGGATGCAAGCGTTATCCGGAATGATTGGGCGTAAAGAGTCCGTAGGTGGTCATCCAAGTCTGCTGTTAAAGATGAAGCTTAACCTCTTTCGGCAGTGGAAACTGGAAGATAGAGTGTAGTAGGGGCAGAGGGAATTCCTGGTGTAGCGGTGAAATGCGTAGAGTCAGGAAGAACACCGGTGGCGAAGGCGCTCTGCTGGGCTATAACTGACACTGAGGGCGAAAGCTAGGGGAGCGAATGGGATTAGATACCCC |
| H02.32_359F | *Aphanizomenon* | AACCTCTTTTCTCAGGGAAGAAAAACACGACGGTACCTTGAGGAATAAGCATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATGCAAGCGTTATCCGGAATGATTGGGCGTAAAGGGTCCGCAGGTGGTAGTGTAAGTCTGCTGTTAAAGAATCACGCTCAACGTGATCAAAGCAGTGGAAACTACACAACTAGAGTACGGTAGGGGCAGAAGGAATTCCTGGTGTAGCGGTGAAATGCGTAGATATCAGGAAGAACACCGGTGGCGAAAGCGTTCTGCTAGACCTGTACTGACACTGAGGGACGAAAGCTAGGGGAGCGAATGGGATTAGATACCCCAGTAGTCCTAGCCGTAAACGATGGATACTAGGTGTGGCTTGTATCGACCCGAGCCGTACCGTAGCTAACGCGTTAAGTATCCCGCCTGGGGAGTACGCACGCAAGTGTGAAACTCAAAGGATTGACGGG |
| H02.35_359F | *Planktothrix* | CCGTGCCAGCAGCCGCGGTAATACGGGGGATGCAAGCGTTATCCGGAATGATTGGGCGTAAAGAGTCCGTAGGTAGTCATCCAAGTCTGCTGTTAAAGAGCGAGGCTTAACCTCGTAAAGGCAGTGGAAACTGGAAGACTAGAGTGTAGTAGGGGCAGAGGGAATTCCTGGTGTAGCGGTGAAATGCGTAGAGATCAGGAAGAACACCGGTGGCGAAGGCGCTCTGCTGGGCTATAACTGACACTGAGGGACGAAAGCTAGGGGAGCGAATGGGATTAGATACCCCAGTAGTCCTAGCGGTAAACGATGGAAACTAGGTGTGGCCTGTATCGACCCGGGCCGTGCCGAAGCAAACGCGTTAAGTTTCCCGCCTGGGGAGTACGCACGCAAGTGTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCAGGACTTGACATCTCTGGAATCTC |
| H02.36_359F | *Synechococcus* | CTCTGGGCTGTAACCTCTTTTCTCAAGGAAGAAGATCTGACGGTACTTGATGAATAAGCCACGGCTAATTCCGTGCCAGCAGCCGCGGTAATACGGGAGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGTCCGCAGGCGGCCTTGTAAGTCTGTCGTTAAAGCGTGGAGCTTAACTCCATTTCAGCGATGGAAACTGCAAGGCTTGAGTGTGGTAGGGGCAGAGGGAATTCCCGGTGTAGCGGTGAAATGCGTAGATATCGGGAAGAACACCAGTGGCGAAGGCGCTCTGCTGGGCCATAACTGACGCTCATGGACGAAAGCCAGGGGAGCGAAAGGGATTAGATACCCCTGTAGTCCTGGCCGTAAACGATGAACACTAGGTGTCGGGGGAATCGACCCCCTCGGTGTCGTAGCCAACGCGTTAAGTGTTCCGCCTGGGGAGTACGCACGCAAGTGTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTAC |
| I01.28_781R | *Cyanobium* | ACCGCTACACCGGGAATTTCCTCTGCCCTACCACACTCTAGTTCTACAGTTTCCATCGCTGAAATGGAGTTAAGCTCCACGTTTTAACGACAGACTTGTAAAACCGCCTGCGGACGCTTTACGCCCAATAATTCCGGATAACGCTTGCCACTCCCGTATTACCGCGGCTGCTGGCACGGAATTAGCCGAGGCTTATTCATCAAGTACCGTCAGATCTTCTTCCTTGATAAAAGAGGTTTACAGCCCACAGGCCTTCATCCCTCACGCGGCGTTGCTCCGTCAGGCTTTC |

| Sample number and primer number | Genus name | Sanger sequence |
|---|---|---|
| I01.35_359F | *Synechococcus* | AGGCCTCTGGGCTGTAAACCTCTTTTCTCAAGGAAGAAGATCTGACGGTACTTG AGGAATAAGCCACGGCTAATTCCGTGCCAGCAGCCGCGGTAATACGGGAGTGG CAAGCGTTATCCGGAATTATTGGGCGTAAAGCGTCCGCAGGCGGCCTTGTAAGT CTGTCGTTAAAGCGTGGAGCTTAACTCCATTTCAGCGATGGAAACTACAAGGCT TGAGTGTGGTAGGGGCAGAGGGAATTCCCGGTGTAGCGGTGAAATGCGTAGA TATCGGGAAGAACACCAGTGGCGAAGGCGCTCTGCTGGGCCATAACTGACGCT CATGGACGAAAGCCAGGGGAGCGAAAGGGATTAGATACCCCTGTAGTCCTGGC CGTAAACGATGAACACTAGGCGTCGGGGGAATCGACCCCCTCGGTGTCGTAGC CAACGCGTTAAGTGTTCCGCCTGGGGAGTACGCACGCAAGTGTGAAACTCAAA GGAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCA ACGCGAAGA |
| I01.37_781R | *Cyanobium* | ATGGGCGAAAGCCTGACGGAGCAACGCCGCGTGAGGGATGAAGGCCTGTGGG CTGTAAACCTCTTTTCTCAAGGAAGAAGATCTGACGGTACTTGAGGAATAAGCC ACGGCTAATTCCGTGCCAGCAGCCGCGGTAATACGGGAGAGGCAAGCGTTATC CGGAATCATTGGGCGTAAAGCGTCCGCAGGCGGCCTTGCAAGTCTGTCGTTAA AGCGTGGAGCTTAACTCCATAAAAGCGGTGGAAACTACAAGGCTAGAGTGTGG TAGGGGCAGAGGGAATTCCCGGTGTAGCGGT |
| I04.27_359F | *Aphanizomenon* | GCTCTTGGGTTGTAAACCTCTTTTCTCAGGGAAGAACAAAATGACGGTACCTGA GGAATAAGCATCGGCTAATTCCGTGCCAGCAGCCGCGGTAATACGGAGGATGC AAGCGTTATCCGGAATGATTGGGCGTAAAGGGTCCGCAGGTGGCATTGTAAGT CTGCTGTTAAAGAGTTTGGCTCAACCAAATAAGAGCAGTGGAAACTACAAAGCT AGAGTGTGGTCGGGGCAGAGGGAATTCCTGGTGTAGCGGTGAAATGCGTAGA TATCAGGAAGAACACCGGTGGCGAAGGCGCTCTGCTAGGCCAAAACTGACACT GAGGGACGAAAGCTAGGGGAGCGAATGGGATTAGATACCCCAGTAGTCCTAG CCGTAAACGATGGATACTAGGCGTAGCTCGTATCGACCCGAGCTGTGCCGTAGC TAACGCGTTAAGTATCCCGCCTGGGGAGTACGCAGGCAACTGTGAAACTCAAA GGAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCA ACGCGAAGAACCTTACCAAGG |
| I04.28_359F | *Aphanizomenon* | GCTCTTGGGTTGTAACCTCTTTTCTCAGGGAAGAACAACATGACGGTACCTGAG GAATAAGCATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCA AGCGTTATCCGGAATGATTGGGCGTAAAGGGTCCGCAGGTGGCATTGTAAGTC TGCTGTTAAAGAGTTTGGCTCAACCAGATAAGAGCAGTGGAAACTACAAAGCTA GAGTGTGGTCGGGGCAGAGGGAATTCCTGGTGTAGCGGTGAAATGCGTAGAT ATCAGGAAGAACACCGGTGGCGAAGGCGCTCTGCTAGGCCAAGACTGACACTG AGGGACGAAAGCTAGGGGAGCGAATGGGATTAGATACCCCAGTAGTCCTAGCC GTAAACGATGGATACTAGGCGTAGCTCGTATCGACCCGAGCTGTGCCGTAGCTA ACGCGTTAAGTATCCCGCCTGGGGAGTACGCAGGCAACTGTGAAACTCAAAGG AATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAAC GCGAAGAACCTTACCAAGGCTTGACATGTCACGAATTCTGTTGAAATATGGGAG TGCCTTCGGGAGCGTGAACACA |
| I04.29_359F | *Dolichospermum* | CTTGGGTTGTAAACCTCTTTTCTCAGGGAAGAACAGAATGACGGTACCTGAGGA ATAAGCATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATGCAAG CGTTATCCGGAATGATTGGGCGTAAAGGGTCCGCAGGTGGCATAGTAAGTCTG CTGTTAAAGAGTCACGCTCAACGTGATAAGAGCAGTGGAAACTACAAAGCTAG AGTATGGTCGGGGCAGAGGGAATTCCTGGTGTAGCGGTGAAATGCGTAGATAT CAGGAAGAACACCGGTGGCGAAGGCGCTCTGCTAGGCCAAAACTGACACTGAG GGACGAAAGCTAGGGGAGCGAATGGGATTAGATACCCCAGTAGTCCTAGCCGT AAACGATGGATACTAGGCGTAGCTCGTATCGACCCGAGCTGTGCCGTAGCTAAC GCGTTAAGTATCCCGCCTGGGGAGTACGCAGGCAACTGTGAAACTCAAAGGAA TTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGC GAAGAACCTTACCAAGGCTTGACATGTCACGAATCCTGTAGAAATATAGGAGTG CC |

| Sample number and primer number | Genus name | Sanger sequence |
|---|---|---|
| I04.30_359F | *Dolichospermum* | GGCTCTTGGGTTGTAACCTCTTTTCTCAGGGAAGAACAGAATGACGGTACCTGAGGAATAAGCATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATGCAAGCGTTATCCGGAATGATTGGGCGTAAAGGGTCCGCAGGTGGCATAGTAAGTCTGCTGTTAAAGAGTCTGGCTCAACGAGATAAGAGCAGTGGAAACTACAAAGCTAGAGTTTGGTCGGGGCAGAGGGAATTCCTGGTGTAGCGGTGAAATGCGTAGATATCAGGAAGAACACCGGTGGCGAAGGCGCTCTGCTAGGCCAAAACTGACACTGAGGGACGAAAGCTAGGGGAGCGAATGGGATTAGATACCCCAGTAGTCCTAGCCGTAAACGATGGATACTAGGCGTAGCTCGTATCGACCCGAGCTGTGCCGTAGCTAACGCGTTAAGTATCCCGCCTGGGGAGTACGCAGGCAACTGTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATGTCACGAATCCTGTAGAAATATAGGAGTGCCT |
| I04.31_359F | *Dolichospermum* | CTTGGGTTGTAAACCTCTTTTCTCAGGGAAGAACAGAATGACGGTACCTGAGGAATAAGCATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGATGCAAGCGTTATCCGGAATGATTGGGCGTAAAGGGTCCGCAGGTGGCATAGAAAGTCTGCTGTTAAAGAGTCTGGCTCAACCTGATAAGAGCAGTGGAAACTACAAAGCTAGAGTATGGTCGGGGCAGAGGGAATTCCTGGTGTAGCGGTGAAATGCGTAGATATCAGGAAGAACACCGGTGGCGAAGGCGCTCTGCTAGGCCAAAACTGACACTGAGGGACGAAAGCTAGGGGAGCGAATGGGATTAGATACCCCAGTAGTCCTAGCCGTAAACGATGGATACTAGGCGTAGCTCGTATCGACCCGAGCTGTGCCGTAGCTAACGCGTTAAGTATCCCGCCTGGGGAGTACGCAGGCAACTGTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATGTCACGAATCCTGTAGAAATATAGGAGTGCCTTAGGGAGCGTGAACACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCC |
| I04.32_359F | *Microcystis* | ATCAGCCTCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGGGGAGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGTCCGCAGGTGGTCATCCAAGTCTGCCGTCAAATCAGGTTGCTTAACGACCTAAAGGCGGTGGAAACTGGCAGACTAGAGAGCAGTAGGGGTAGCAGGAATTCCCAGTGTAGCGGTGAAATGCGTAGAGATTGGGAAGAACATCGGTGGCGAAAGCGTGCTACTGGGCTGTATCTGACACTCAGGGACGAAAGCTAGGGGAGCGAAAGGG |
| I04.32_359F | *Dolichospermum* | CTGAGGAATAAGCATCGGCTAACTCCGGGCCACCAGCCGCGGTAATACGGAGGATGCAAGCGTTATCCGGAATGATTGGGCGTAAAGGGTCCGCAGGTGGCATTGAAAGTCTGCTGTTAAAGAGTCTGGCTCAACCTGATAAGAGCAGTGGAAACTACAAAGCTAGAGTTTGGTCGGGGCAGAGGGAATTCCTGGTGTAGCGGTGAAATGCGTAGATATCAGGAAGAACACCGGTGGCGAAGGCGCTCTGCTAGGCCAAACTGACACTGAGGACGAAAGCTAGGGGAGCGAATGGGATTAGATACCCCAGTAGTCCTAGCCGTAAACGATGGATACTAGGCGTAGCTCGTATCGACCCGAGCTGTGCCGGAGCTAACGCGTTAAGTATCCCGCCTGGG |
| I04.34_359F | *Microcystis* | GGAATTCAGCCTCGGCTAACTTCCGTGCCAGCAGCCGCGGTAATACGGGGGAGGCAAGCGTTTATCCGGAATTATTGGGCGTAAAGCGTCCGCAGGTGGTCAGCCAAGTCTGCCGTCAAATCAGGTTGCTTAACCACCTAAAGGCGGTGGAAACTGGCAGACTAGAGAGCAGTAGGGGTAGCAGGAATTCCCAGTGTAGCGGTGAAATGCGTAGAGATTGGGAAGAACATCGGTGGCGAAAGCGTGCTACTGGGCTGTATCTGACACTCAGGGACGAAAGCTAGGGGAGCGAAAGGG |
| I04.36_359F | *Planktothrix* | AGTCTGCTGTTAAAGAGGGAGGCTTAACCTCTTTCGGCAGTGGAAACTGGAAGATAGAGTGTGGTAGGGGCAGAGGGAATTCCTGGTGTAGCGGTGAAATGCGTAGAGTCAGGAAGAACACCGGTGGCGAAGGCGCTCTGCTGGGCTATAACTGACACTGAGGGACGAAAGCTAGGGGAGCGAATGGGTTAGATACCCCAGTAGTCCTAGCGGAAACGATGGAACT |
| I04.37_359F | *Cyanodictyon* | GGAGCTTAACTCCATTTCAGCGGTGGAAACTACAAAGCTTGAGTGTGGTAGGGGCAGAGGGAATTCCCGGTGTAGCGGTGAAATGCGTAGATATCGGGAAGAACACCAGTGGCGAAGGCGCTCTGCTGGGCCATAACTGACACTCATGGACGAAAGCCAGGGGAGCGAATGGGATTAGATACCCCTGTAGTCCTGGCCGTAAACGATGAACACCTAGG |

# S3. Towards a better quantification of cyanotoxins in fruits and vegetables: validation and application of an UHPLC-MS/MS-based method on Belgian products: Supplementary data

**Table S3.1.** Validation results for eight microcystin congeners (MCs) and nodularin (NOD) in carrots, lettuce and strawberries at three concentration levels and on average. Included parameters are recovery, repeatability, reproducibility, measurement uncertainty (MU), average signal to noise for LOD, average signal to noise for LOQ, $R^2$.

| **Validation data Carrots** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Toxins** | Spiked Concentration | Recovery (%) | Repeatability (%) | Reproducibility (%) | Measurement Uncertainty (%) | Average Signal to noise for LOD | Average Signal to noise for LOQ | $R^2$ |
| **MC-RR** | 1.00 µg/kg | 82.00 | 4.42 | 13.20 | 26.39 | 133.07 | 174.79 | 0.99 |
| | 5.00 µg/kg | 80.00 | 5.30 | 5.30 | 10.61 | | | |
| | 25.00 µg/kg | 84.00 | 8.30 | 10.97 | 21.94 | | | |
| | Average | 82.00 | 6.01 | 9.82 | 19.65 | | | |
| **NOD** | 1.00 µg/kg | 84.00 | 3.69 | 11.41 | 22.82 | 154.23 | 172.07 | 0.99 |
| | 5.00 µg/kg | 81.00 | 5.71 | 7.38 | 14.76 | | | |
| | 25.00 µg/kg | 82.00 | 5.88 | 7.02 | 14.04 | | | |
| | Average | 82.33 | 5.10 | 8.60 | 17.21 | | | |
| **MC-LA** | 1.00 µg/kg | 85.00 | 8.92 | 13.50 | 27.00 | 50.08 | 76.93 | 0.99 |
| | 5.00 µg/kg | 79.00 | 7.16 | 9.21 | 18.42 | | | |
| | 25.0 µg/kg | 81.00 | 8.11 | 8.11 | 16.23 | | | |
| | Average | 81.67 | 8.06 | 10.27 | 20.55 | | | |

| Toxins | Spiked Concentration | Recovery (%) | Repeatability (%) | Reproducibility (%) | Measurement Uncertainty (%) | Average Signal to noise for LOD | Average Signal to noise for LOQ | $R^2$ |
|---|---|---|---|---|---|---|---|---|
| **MC-LF** | 1.00 µg/kg | 83.00 | 3.51 | 11.30 | 22.59 | 75.82 | 78.92 | 0.99 |
| | 5.00 µg/kg | 74.00 | 5.45 | 14.37 | 28.74 | | | |
| | 25.00 µg/kg | 78.00 | 11.83 | 11.83 | 23.66 | | | |
| | Average | 78.33 | 6.93 | 12.50 | 25.00 | | | |
| **MC-LR** | 1.00 µg/kg | 86.00 | 4.89 | 12.26 | 24.53 | 63.12 | 68.34 | 0.99 |
| | 5.00 µg/kg | 79.00 | 5.48 | 9.17 | 18.35 | | | |
| | 25.00 µg/kg | 81.00 | 7.60 | 7.60 | 15.24 | | | |
| | Average | 82.00 | 5.99 | 9.68 | 19.37 | | | |
| **MC-LY** | 1.00 µg/kg | 79.00 | 10.87 | 10.87 | 21.74 | 25.58 | 37.39 | 0.99 |
| | 5.00 µg/kg | 77.00 | 3.92 | 7.20 | 14.40 | | | |
| | 25.00 µg/kg | 81.00 | 8.49 | 8.49 | 16.98 | | | |
| | Average | 79.00 | 7.76 | 8.85 | 17.71 | | | |
| **MC-LW** | 1.00 µg/kg | 76.00 | 5.24 | 6.50 | 12.99 | 62.44 | 68.68 | 0.99 |
| | 5.00 µg/kg | 71.00 | 5.96 | 14.15 | 28.31 | | | |
| | 25.00 µg/kg | 77.00 | 12.77 | 12.77 | 25.55 | | | |
| | Average | 74.67 | 7.99 | 11.14 | 22.28 | | | |

| Toxins | Spiked Concentration | Recovery (%) | Repeatability (%) | Reproducibility (%) | Measurement Uncertainty (%) | Average Signal to noise for LOD | Average Signal to noise for LOQ | $R^2$ |
|---|---|---|---|---|---|---|---|---|
| **MC-YR** | 1.00 µg/kg | 87.00 | 8.27 | 12.87 | 25.73 | 26.99 | 40.33 | 0.99 |
| | 5.00 µg/kg | 79.00 | 6.11 | 10.59 | 21.17 | | | |
| | 25.00 µg/kg | 80.00 | 6.18 | 6.18 | 12.37 | | | |
| | Average | 82.00 | 6.86 | 9.88 | 19.76 | | | |
| **Toxins** | Spiked Concentration | Recovery (%) | Repeatability (%) | Reproducibility (%) | Measurement Uncertainty (%) | Average Signal to noise for LOD | Average Signal to noise for LOQ | $R^2$ |
| **MC-WR** | 1.00 µg/kg | 79.00 | 7.86 | 9.59 | 19.18 | 29.18 | 33.62 | 0.99 |
| | 5.00 µg/kg | 75.00 | 8.87 | 17.35 | 34.71 | | | |
| | 25.00 µg/kg | 77.00 | 8.98 | 8.98 | 17.96 | | | |
| | Average | 77.00 | 8.57 | 11.97 | 23.95 | | | |
| **SUM** | 9.00 µg/kg | 82.00 | 3.72 | 7.97 | 15.93 | N.A. | N.A. | N.A. |
| | 45.00 µg/kg | 77.00 | 5.69 | 9.40 | 18.79 | | | |
| | 225.00 µg/kg | 80.00 | 8.46 | 8.46 | 16.93 | | | |
| | Average | 79.67 | 5.96% | 8.61 | 17.22 | | | |

| Validation data lettuce | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Toxins** | Spiked Concentration | Recovery (%) | Repeatability (%) | Reproducibility (%) | Measurement Uncertainty (%) | Average Signal to noise for LOD | Average Signal to noise for LOQ | $R^2$ |
| **MC-RR** | 1.00 µg/kg | 85.00 | 7.76 | 7.76 | 15.51 | 126.37 | 139.99 | 0.99 |
| | 5.00 µg/kg | 87.00 | 4.52 | 9.59 | 19.17 | | | |
| | 25.00 µg/kg | 84.00 | 6.49 | 13.73 | 27.46 | | | |
| | Average | 85.33 | 6.25 | 10.36 | 20.71 | | | |
| **NOD** | 1.00 µg/kg | 84.00 | 7.64 | 11.54 | 23.08 | 105.79 | 129.43 | 0.99 |
| | 5.00 µg/kg | 93.00 | 5.41 | 6.92 | 13.84 | | | |
| | 25.00 µg/kg | 87.00 | 6.29 | 11.20 | 22.41 | | | |
| | Average | 88.00 | 6.45 | 9.89 | 19.77 | | | |
| **MC-LA** | 1.00 µg/kg | 86.00 | 7.52 | 13.12 | 26.23 | 37.36 | 50.85 | 0.99 |
| | 5.00 µg/kg | 90.00 | 5.76 | 7.64 | 15.28 | | | |
| | 25.00 µg/kg | 85.00 | 5.71 | 10.68 | 21.35 | | | |
| | Average | 87.00 | 6.33 | 10.48 | 20.96 | | | |
| **MC-LF** | 1.00 µg/kg | 81.00 | 7.67 | 8.75 | 17.49 | 55.52 | 68.48 | 0.99 |
| | 5.00 µg/kg | 89.00 | 6.01 | 6.01 | 12.02 | | | |
| | 25.00 µg/kg | 83.00 | 7.40 | 8.67 | 17.35 | | | |
| | Average | 84.33 | 7.03 | 7.81 | 15.62 | | | |

| Toxins | Spiked Concentration | Recovery (%) | Repeatability (%) | Reproducibility (%) | Measurement Uncertainty (%) | Average Signal to noise for LOD | Average Signal to noise for LOQ | $R^2$ |
|---|---|---|---|---|---|---|---|---|
| **MC-LR** | 1.00 µg/kg | 85.00 | 5.48 | 8.69 | 17.39 | 53.46 | 72.95 | 0.99 |
| | 5.00 µg/kg | 86.00 | 5.93 | 8.38 | 16.75 | | | |
| | 25.00 µg/kg | 81.00 | 6.54 | 15.24 | 30.48 | | | |
| | Average | 84.00 | 5.99 | 10.77 | 21.54 | | | |
| **MC-LY** | 1.00 µg/kg | 87.00 | 6.48 | 11.64 | 23.29 | 27.24 | 35.25 | 0.99 |
| | 5.00 µg/kg | 91.00 | 7.38 | 7.40 | 14.80 | | | |
| | 25.00 µg/kg | 85.00 | 6.12 | 10.11 | 20.22 | | | |
| | Average | 87.67 | 6.66 | 9.72 | 19.43 | | | |
| **MC-LW** | 1.00 µg/kg | 79.00 | 6.36 | 7.25 | 14.50 | 41.82 | 58.34 | 0.99 |
| | 5.00 µg/kg | 85.00 | 6.60 | 6.57 | 13.31 | | | |
| | 25.00 µg/kg | 81.00 | 8.74 | 8.74 | 17.47 | | | |
| | Average | 81.67 | 7.23 | 7.52 | 15.10 | | | |
| **MC-YR** | 1.00 µg/kg | 83.00 | 13.03 | 14.25 | 28.51 | 26.14 | 32.56 | 0.99 |
| | 5.00 µg/kg | 90.00 | 4.45 | 6.36 | 12.72 | | | |
| | 25.00 µg/kg | 85.00 | 7.00 | 11.87 | 23.75 | | | |
| | Average | 86.00 | 8.16 | 10.83 | 21.66 | | | |

| Toxins | Spiked Concentration | Recovery (%) | Repeatability (%) | Reproducibility (%) | Measurement Uncertainty (%) | Average Signal to noise for LOD | Average Signal to noise for LOQ | $R^2$ |
|---|---|---|---|---|---|---|---|---|
| **MC-WR** | 1.00 µg/kg | 80.00 | 7.28 | 20.35 | 40.70 | 25.79 | 25.90 | 0.99 |
| | 5.00 µg/kg | 84.00 | 6.53 | 12.51 | 25.03 | | | |
| | 25.00 µg/kg | 79.00 | 8.81 | 17.60 | 35.20 | | | |
| | Average | 81.00 | 7.54 | 16.82 | 33.64 | | | |
| **SUM** | 9.00 µg/kg | 83.00 | 4.75 | 9.26 | 18.51 | N.A. | N.A. | N.A. |
| | 45.00 µg/kg | 88.00 | 5.53 | 6.96 | 13.92 | | | |
| | 225.00 µg/kg | 83.00 | 6.85 | 11.41 | 22.82 | | | |
| | Average | 84.67 | 5.71 | 9.21 | 18.42 | | | |
| **Validation data Strawberry** | | | | | | | | |
| **MC-RR** | 5.00 µg/kg | 77.00 | 2.32 | 5.80 | 11.60 | 710.17 | 927.28 | 0.99 |
| | 10.00 µg/kg | 82.00 | 5.53 | 12.73 | 25.46 | | | |
| | 25.00 µg/kg | 76.00 | 7.29 | 7.29 | 14.58 | | | |
| | Average | 76.00 | 4.61 | 5.91 | 11.82 | | | |
| **NOD** | 1.00 µg/kg | 77.00 | 6.39 | 8.03 | 16.07 | 212.13 | 259.61 | 0.99 |
| | 5.00 µg/kg | 78.00 | 4.52 | 6.52 | 13.03 | | | |
| | 25.00 µg/kg | 77.00 | 3.65 | 6.33 | 12.65 | | | |
| | Average | 77.33 | 4.85 | 6.96 | 13.92 | | | |

| Toxins | Spiked Concentration | Recovery (%) | Repeatability (%) | Reproducibility (%) | Measurement Uncertainty (%) | Average Signal to noise for LOD | Average Signal to noise for LOQ | $R^2$ |
|---|---|---|---|---|---|---|---|---|
| **MC-LA** | 1.00 µg/kg | 82.00 | 9.91 | 11.19 | 22.37 | 66.56 | 86.60 | 0.99 |
| | 5.00 µg/kg | 78.00 | 4.06 | 6.58 | 13.16 | | | |
| | 25.00 µg/kg | 77.00 | 3.87 | 5.37 | 10.74 | | | |
| | Average | 79.00 | 5.95 | 7.71 | 15.42 | | | |
| **MC-LF** | 1.00 µg/kg | 74.00 | 7.61 | 7.61 | 15.23 | 88.72 | 89.63 | 0.99 |
| | 5.00 µg/kg | 76.00 | 3.02 | 5.82 | 11.64 | | | |
| | 25.00 µg/kg | 76.00 | 4.04 | 5.06 | 10.12 | | | |
| | Average | 75.33 | 4.89 | 6.16 | 12.33 | | | |
| **MC-LR** | 1.00 µg/kg | 79.00 | 7.06 | 7.16 | 14.32 | 66.36 | 89.14 | 0.99 |
| | 5.00 µg/kg | 77.00 | 5.13 | 6.05 | 12.11 | | | |
| | 25.00 µg/kg | 75.00 | 4.84 | 6.73 | 13.46 | | | |
| | Average | 77.00 | 5.68 | 6.65 | 13.29 | | | |
| **MC-LY** | 1.00 µg/kg | 77.00 | 6.50 | 8.38 | 16.76 | 35.86 | 53.57 | 0.99 |
| | 5.00 µg/kg | 74.00 | 3.64 | 8.15 | 16.30 | | | |
| | 25.00 µg/kg | 74.00 | 4.78 | 6.89 | 13.79 | | | |
| | Average | 75.00 | 4.97 | 7.81 | 15.62 | | | |

| Toxins | Spiked Concentration | Recovery (%) | Repeatability (%) | Reproducibility (%) | Measurement Uncertainty (%) | Average Signal to noise for LOD | Average Signal to noise for LOQ | $R^2$ |
|---|---|---|---|---|---|---|---|---|
| **MC-LW** | 1.00 µg/kg | 75.00 | 7.95 | 13.47 | 26.94 | 76.89 | 81.55 | 0.99 |
| | 5.00 µg/kg | 73.00 | 3.79 | 8.66 | 17.32 | | | |
| | 25.00 µg/kg | 73.00 | 5.75 | 8.14 | 16.29 | | | |
| | Average | 73.67 | 5.83 | 10.09 | 20.18 | | | |
| **MC-YR** | 1.00 µg/kg | 73.00 | 8.09 | 12.77 | 25.54 | 33.53 | 40.28 | 0.99 |
| | 5.00 µg/kg | 74.00 | 4.10 | 7.88 | 15.75 | | | |
| | 25.00 µg/kg | 74.00 | 4.27 | 6.81 | 13.61 | | | |
| | Average | 73.67 | 5.49 | 9.15 | 18.30 | | | |
| **MC-WR** | 1.00 µg/kg | 77.00 | 11.5 | 11.54 | 23.07 | 33.72 | 32.12 | 0.99 |
| | 5.00 µg/kg | 73.00 | 6.01 | 9.19 | 18.38 | | | |
| | 25.00 µg/kg | 73.00 | 5.28 | 9.27 | 18.54 | | | |
| | Average | 74.33 | 7.61 | 10.00 | 20.00 | | | |
| **SUM** | 8.00 µg/kg | 77.00 | 6.89 | 6.89 | 13.78 | N.A. | N.A. | N.A. |
| | 45.00 µg/kg | 76.00 | 3.35 | 5.95 | 11.90 | | | |
| | 225.00 µg/kg | 75.00 | 3.86 | 5.31 | 10.63 | | | |
| | Average | 76.00 | 4.54 | 5.90 | 11.79 | | | |

**Table S3.2.** Overview of samples taken from the Belgium market showing separate results, origin, sample type and sample annotation.

| Sample annotation | Sample type | origin | Sample date | Total concentration MCs (μg $kg^{-1}$) |
|---|---|---|---|---|
| **S22FD00063** | Strawberries | Hoogstraten, Belgium | 29/06/2021 | <LOD |
| **S22FD00109** | Strawberries | Hoogstraten, Belgium | 30/06/2021 | <LOD |
| **S22FD00110** | Strawberries | Belgium | 30/06/2021 | <LOD |
| **S22FD00111** | Strawberries | Belgium | 30/06/2021 | <LOD |
| **S22FD00112** | Strawberries | Hoogstraten, Belgium | 30/06/2021 | <LOD |
| **S22FD00113** | Strawberries | Hoogstraten, Belgium | 30/06/2021 | <LOD |
| **S22FD00114** | Strawberries | Sint-Katelijne Waver, Belgium | 30/06/2021 | <LOD |
| **S22FD00115** | Radish | Nederland | 27/07/2021 | <LOD |
| **S22FD00116** | Radish | Belgium | 27/07/2021 | <LOD |
| **S22FD00117** | Radish | Belgium | 27/07/2021 | <LOD |
| **S22FD00118** | Radish | Belgium | 27/07/2021 | <LOD |
| **S22FD00119** | Radish | Nederland | 27/07/2021 | <LOD |
| **S22FD00120** | Lettuce | Belgium | 10/08/2021 | <LOD |
| **S22FD00121** | Lettuce | Belgium | 10/08/2021 | <LOD |
| **S22FD00122** | Lettuce | Belgium | 10/08/2021 | <LOD |
| **S22FD00123** | Lettuce | Sint-Katelijne Waver, Belgium | 10/08/2021 | <LOD |
| **S22FD00124** | Lettuce | Sint-Katelijne Waver, Belgium | 10/08/2021 | <LOD |
| **S22FD00125** | Lettuce | Belgium | 15/08/2021 | <LOD |
| **S22FD00126** | Lettuce | Belgium | 16/08/2021 | <LOD |
| **S22FD00127** | Lettuce | Belgium | 17/08/2021 | <LOD |
| **S22FD00128** | Lettuce | Belgium | 18/08/2021 | <LOD |
| **S22FD00129** | Lettuce | Belgium | 17/11/2021 | <LOD |
| **S22FD00130** | Lettuce | Belgium | 17/11/2021 | <LOD |

| Sample annotation | Sample type | origin | Sample date | Total concentration MCs (μg $kg^{-1}$) |
|---|---|---|---|---|
| **S22FD00131** | Lettuce | Belgium | 17/11/2021 | <LOD |
| **S22FD00132** | Lettuce | Belgium | 17/11/2021 | <LOD |
| **S22FD00133** | Potato | Belgium | 10/08/2021 | <LOD |
| **S22FD00134** | Potato | Germany | 10/08/2021 | <LOD |
| **S22FD00135** | Potato | Spain | 10/08/2021 | <LOD |
| **S22FD00136** | Potato | Belgium | 10/08/2021 | <LOD |
| **S22FD00137** | Potato | Belgium | 10/08/2021 | <LOD |
| **S22FD00138** | Potato | Belgium | 10/08/2021 | <LOD |
| **S22FD00139** | Potato | Belgium | 10/08/2021 | <LOD |
| **S22FD00140** | Potato | Belgium | 17/11/2021 | <LOD |
| **S22FD00141** | Potato | Belgium | 17/11/2021 | <LOD |
| **S22FD00142** | Potato | Spain | 17/11/2021 | <LOD |
| **S22FD00143** | Potato | Belgium | 17/11/2021 | <LOD |
| **S22FD00144** | Onion | Belgium | 10/08/2021 | <LOD |
| **S22FD00145** | Onion | Belgium | 10/08/2021 | <LOD |
| **S22FD00146** | Onion | Belgium | 10/08/2021 | <LOD |
| **S22FD00147** | Onion | Belgium | 10/08/2021 | <LOD |
| **S22FD00148** | Onion | Belgium | 10/08/2021 | <LOD |
| **S22FD00149** | Onion | Belgium | 15/08/2021 | <LOD |
| **S22FD00150** | Onion | Belgium | 16/08/2021 | <LOD |
| **S22FD00151** | Onion | Belgium | 17/08/2021 | <LOD |
| **S22FD00152** | Onion | Belgium | 17/08/2021 | <LOD |
| **S22FD00153** | Onion | Belgium | 17/08/2021 | <LOD |
| **S22FD00154** | Onion | Belgium | 17/08/2021 | <LOD |
| **S22FD00155** | Carrots | Belgium | 10/08/2021 | <LOD |
| **S22FD00156** | Carrots | Belgium | 10/08/2021 | <LOD |
| **S22FD00157** | Carrots | Belgium | 10/08/2021 | <LOD |
| **S22FD00158** | Carrots | Belgium | 10/08/2021 | <LOD |
| **S22FD00159** | Carrots | Belgium | 10/08/2021 | <LOD |
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| **S22FD00162** | Carrots | Belgium | 10/08/2021 | <LOD |
| **S22FD00163** | Carrots | Belgium | 10/08/2021 | <LOD |

| Sample annotation | Sample type | origin | Sample date | Total concentration MCs (μg $kg^{-1}$) |
|---|---|---|---|---|
| **S22FD00164** | Carrots | Belgium | 17/11/2021 | <LOD |
| **S22FD00165** | Carrots | Belgium | 17/11/2021 | <LOD |
| **S22FD00166** | Carrots | Belgium | 17/11/2021 | <LOD |
| **S22FD00167** | Carrots | Belgium | 17/11/2021 | <LOD |
| **S22FD00168** | Tomato | Belgium | 27/07/2021 | <LOD |
| **S22FD00169** | Tomato | Belgium | 27/07/2021 | <LOD |
| **S22FD00170** | Tomato | Belgium | 27/07/2021 | <LOD |
| **S22FD00171** | Tomato | Belgium | 27/07/2021 | <LOD |
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| **S22FD00175** | Tomato | Belgium | 27/07/2021 | <LOD |
| **S22FD00176** | Tomato | Belgium | 27/07/2021 | <LOD |
| **S22FD00177** | Tomato | Belgium | 27/07/2021 | <LOD |
| **S22FD00178** | Tomato | Belgium | 27/07/2021 | <LOD |
| **S22FD00179** | Tomato | Belgium | 27/07/2021 | <LOD |
| **S22FD00180** | Tomato | Belgium | 27/07/2021 | <LOD |
| **S22FD00181** | Cherry tomato | Belgium | 27/07/2021 | <LOD |
| **S22FD00182** | Cherry tomato | Belgium | 27/07/2021 | <LOD |
| **S22FD00183** | Cherry tomato | Hoogstraten, Belgium | 27/07/2021 | <LOD |
| **S22FD00184** | Cherry tomato | Belgium | 27/07/2021 | <LOD |
| **S22FD00185** | Cherry tomato | Belgium | 27/07/2021 | <LOD |
| **S22FD00186** | Cherry tomato | Belgium | 27/07/2021 | <LOD |
| **S22FD00187** | Cherry tomato | Belgium | 27/07/2021 | <LOD |
| **S22FD00188** | Cherry tomato | Belgium | 27/07/2021 | <LOD |
| **S22FD00189** | Cherry tomato | Belgium | 27/07/2021 | <LOD |
| **S22FD00190** | Cherry tomato | Belgium | 27/07/2021 | <LOD |
| **S22FD00191** | Cherry tomato | Belgium | 27/07/2021 | <LOD |
| **S22FD00192** | Cherry tomato | Belgium | 27/07/2021 | <LOD |
| **S22FD00193** | Cherry tomato | Belgium | 27/07/2021 | <LOD |
| **S22FD00194** | Cherry tomato | Belgium | 27/07/2021 | <LOD |
| **S22FD00195** | Chicory | Belgium | 29/06/2021 | <LOD |
| **S22FD00196** | Chicory | Belgium | 30/06/2021 | <LOD |

| Sample annotation | Sample type | origin | Sample date | Total concentration MCs (μg $kg^{-1}$) |
|---|---|---|---|---|
| **S22FD00197** | Chicory | Belgium | 30/06/2021 | <LOD |
| **S22FD00198** | Chicory | Belgium | 30/06/2021 | <LOD |
| **S22FD00199** | Chicory | Belgium | 30/06/2021 | <LOD |
| **S22FD00200** | Chicory | Belgium | 30/06/2021 | <LOD |
| **S22FD00201** | Chicory | Belgium | 30/06/2021 | <LOD |
| **S22FD00202** | Chicory | Belgium | 30/06/2021 | <LOD |
| **S22FD00204** | Chicory | Belgium | 30/06/2021 | <LOD |
| **S22FD00205** | Chicory | Belgium | 30/06/2021 | <LOD |
| **S22FD00206** | Chicory | Belgium | 30/06/2021 | <LOD |
| **S22FD00207** | Chicory | Belgium | 30/06/2021 | <LOD |
| **S22FD00208** | Chicory | Sint-Katelijne Waver, Belgium | 30/06/2021 | <LOD |
| **S22FD00211** | Chicory | Sint-Katelijne Waver, Belgium | 30/06/2021 | <LOD |
| **S22FD00212** | Chicory | Belgium | 30/06/2021 | <LOD |

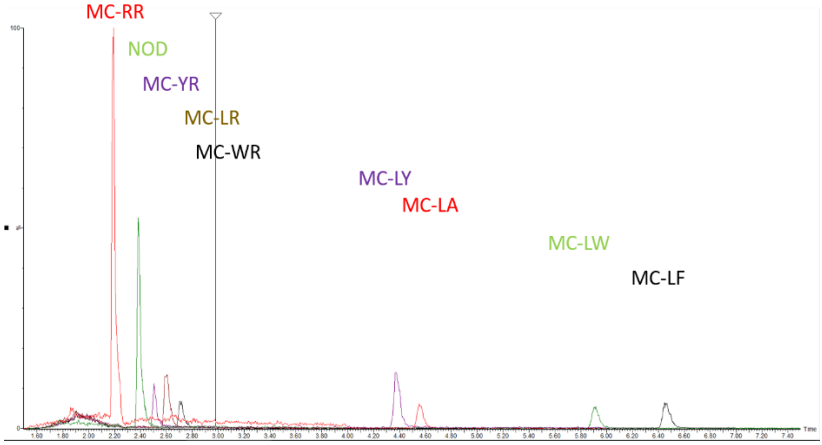


**Figure S3.1.** The elution peaks for 8 microcystin congeners and NOD in carrot matrix at validation level 5.00 ng $g^{-1}$. The peaks are presented together at representable ratios based on the peak intensities by overlaying the chromatograms of the different toxins. However, during analysis, the chromatograms for each toxin are analyzed separately.

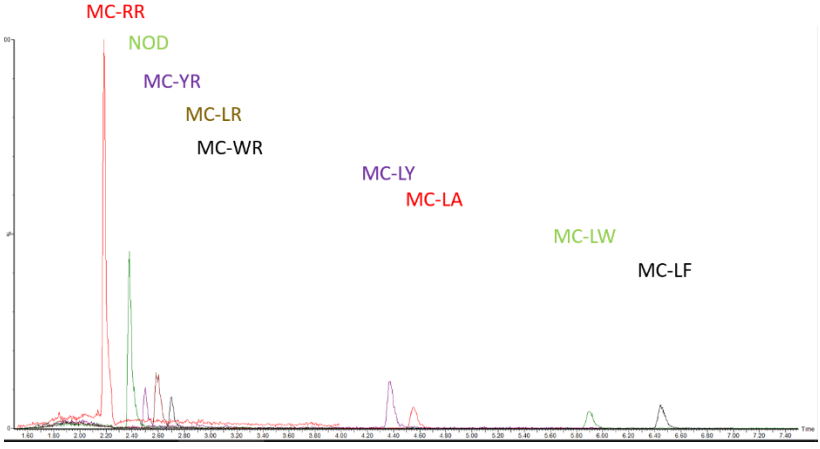


**Figure S3.2.** The elution peaks for 8 microcystin congeners and NOD in lettuce matrix at validation level 5.00 ng $g^{-1}$. The peaks are presented together at representable ratios based on the peak intensities by overlaying the chromatograms of the different toxins. However, during analysis, the chromatograms for each toxin are analyzed separately.

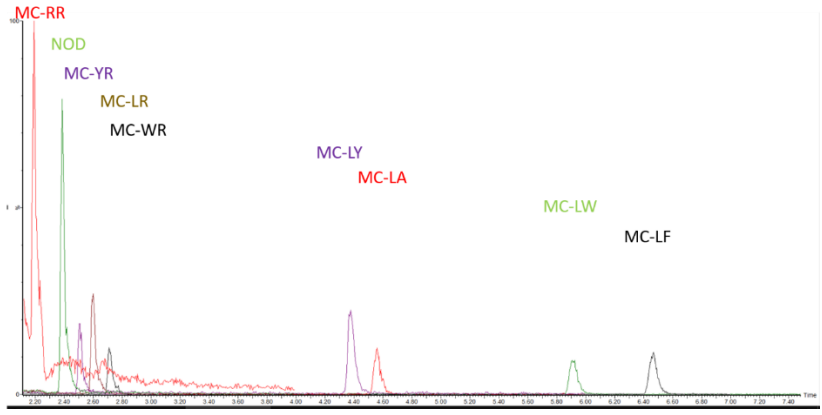


**Figure S3.3.** The elution peaks for 8 microcystin congeners and NOD in lettuce matrix at validation level 5.00 ng $g^{-1}$. The peaks are presented together at representable ratios based on the peak intensities by overlaying the chromatograms of the different toxins. However, during analysis, the chromatograms for each toxin are analyzed separately.

## S4. *Chlorella*- and cyano-based food supplements consumption survey for Belgian consumers: Overview of survey questions and data

| | |
|---|---|
| Number of records in this query: | 554 |
| Total records in the survey: | 558 |
| Percentage of total records | 99.28% |

| *What age were the participants?* | *Result* |
|---|---|
| *Count* | 554 |
| *Sum* | 20855 |
| *Standard deviation* | 13.62 |
| *Average* | 37.64 |
| *Minimum* | 18 |
| *1st quartile (Q1)* | 27 |
| *2nd quartile (Median)* | 32 |
| *3rd quartile (Q3)* | 48.25 |
| *Maximum* | 78 |

| *As which gender did the participant identify?* | *Count* | *Percentage* |
|---|---|---|
| *Man* | 192 | 34.66% |
| *Women* | 356 | 64.26% |
| *X* | 3 | 0.54% |
| *I rather don't provide this information* | 3 | 0.54% |
| *other* | 0 | 0.00% |

| *What is the level of education of the participants?* | *Count* | *Percentage* |
|---|---|---|
| *Primary school* | 3 | 0.54% |
| *Secondary school* | 39 | 7.04% |
| *Bachelor degree* | 110 | 19.86% |
| *University diploma or doctorate* | 402 | 72.56% |

| *Which diet did the participants follow?* | *Count* | *Percentage* |
|---|---|---|
| *No* | 368 | 66.43% |
| *Flexitarian* | 82 | 14.80% |
| *Pescotariar* | 17 | 3.07% |
| *Vegetarian* | 48 | 8.66% |
| *Veganist* | 8 | 1.44% |
| *Other* | 31 | 5.60% |

| *How did the participants assess their physical health?* | *Count* | *Percentage* |
|---|---|---|
| *Excellent* | 118 | 21.30% |
| *Good* | 321 | 57.94% |
| *Reasonable* | 104 | 18.77% |
| *Bad* | 11 | 1.99% |

| *How many hours did the participants spend performing moderate or heavy physical activities (excluding sports) on average?* | *Count* | *Percentage* |
|---|---|---|
| *Less than 1 hour* | 258 | 46.57% |
| *Between 1 to 3 hours* | 187 | 33.75% |
| *Between 3 to 6 hours* | 66 | 11.91% |
| *Between 6 to 9 hours* | 25 | 4.51% |
| *More than 9 hours* | 18 | 3.25% |

| *How many hours do the participants spend performing moderate or heavy sports activities on average?* | *Count* | *Percentage* |
|---|---|---|
| *Less than 1 hour* | 181 | 32.67% |
| *Between 1 to 3 hours* | 222 | 40.07% |
| *Between 3 to 6 hours* | 103 | 18.59% |
| *Between 6 to 9 hours* | 32 | 5.78% |
| *More than 9 hours* | 16 | 2.89% |

| *Did the participants use food supplements in the last 12 months* | *Count* | *Percentage* |
|---|---|---|
| *Yes, but only one product* | 119 | 21.48% |
| *Yes, but only multiple products* | 237 | 42.78% |
| *No* | 198 | 35.74% |

| *In which periods did the participants take food supplements during the last 12 months* | *Count* | *Percentage* |
|---|---|---|
| *Spring* | 216 | 60.67% |
| *Summer* | 202 | 56.74% |
| *Autumn* | 280 | 78.65% |
| *Winter* | 286 | 80.34% |

| *What food supplements are taken by users?* | *Percentage* |
|---|---|
| *Multivitamine* | 41.57% |
| *Combination of antioxidants* | 16.29% |
| *Single vitamine* | 59.83% |
| *Micronutrients* | 65.45% |
| *plant-based food supplements* | 29.21% |
| *Algae-based food supplements* | 17.98% |
| *Collagene* | 5.06% |
| *Fish oil* | 17.42% |
| *Other* | 23.60% |

| *What were the main reasons for using food supplements?* | *Count* | *Percentage* |
|---|---|---|
| *As an addition to my regular diet* | 164 | 46.07% |
| *To stay healthy* | 177 | 49.72% |
| *To support/improve my immune system* | 192 | 53.93% |
| *For medical reasons* | 125 | 35.11% |
| *To compensate for certain food allergies, sensitivities or intolerances* | 24 | 6.74% |
| *To maintain a stable body weight* | 16 | 4.49% |
| *To enhance my sport performance* | 28 | 7.87% |
| *To increase and maintain my power or muscle mass* | 21 | 5.90% |
| *To faster recovery from physical exercise* | 37 | 10.39% |
| *To increase my energy* | 104 | 29.21% |
| *To reduce hunger or thirst* | 5 | 1.40% |
| *The food supplements have an agreeable taste* | 4 | 1.12% |
| *The food supplements were recommended by acquaintances (coach, parents, friends, …)* | 40 | 11.24% |
| *Because others take these products as well.* | 3 | 0.84% |
| *Not displayed* | 200 | 36.10% |

| *Why were food supplements not used?* | *Count* | *Percentage* |
|---|---|---|
| *I did not know of their existence* | 6 | 3.03% |
| *I do not believe in their promised effects* | 42 | 21.21% |
| *I do not think they are safe to use* | 20 | 10.10% |
| *the food supplements are too expensive* | 23 | 11.62% |
| *I receive all the necessary nutrients from my regular diet* | 160 | 80.81% |
| *other* | 23 | 11.62% |

| *Which type of algal-based food supplements were used during the last 12 months by users?* | *Count* | *Percentage* |
|---|---|---|
| *'spirulina'* | 47 | 75.81% |
| *Klamath/Apha* | 1 | 1.61% |
| *Chlorella* | 10 | 16.13% |
| *Mix* | 1 | 1.61% |
| *Other* | 13 | 20.97% |

| *How many users used multiple types of algal-based food supplements?* | *Percentage* |
|---|---|
| | 30.65% |

| *How many users only used algal-based food supplements and no other classes of food supplements?* | *Percentage* |
|---|---|
| | 2.25% |

| *How many times were algal-based food supplements in one week by users?* | *Count* | *Percentage* |
|---|---|---|
| *Daily* | 28 | 45.16% |
| *4 to 6 times a week* | 11 | 17.74% |
| *1 to 3 times a week* | 10 | 16.13% |
| *Less than once a week* | 13 | 20.97% |

| *In which form were the algal-based food supplements taken by users?* | *Count* | *Percentage* |
|---|---|---|
| *Pil* | 29 | 46.77% |
| *Capsule* | 12 | 3.23% |
| *Powder* | 16 | 25.81% |
| *A combination of forms above* | 2 | 19.35% |
| *Other* | 3 | 4.84% |

| *How many spoons of algal-based food supplement powder (1.5g), capsules or pils were consumed on average during 24 hours by users?* | *Count* | *Percentage* |
|---|---|---|
| *As indicated on the package* | 27 | 43.55% |
| *Less than indicated on the package* | 18 | 29.03% |
| *Higher than indicated on the package* | 1 | 17.74% |
| *The indicated amount was not known by the participant* | 11 | 8.06% |
| *Not applicable* | 5 | 1.61% |

| *Why were algal-based food supplements consumed?* | *Count* | *Percentage* |
|---|---|---|
| *On the advice of an acquaintance* | 5 | 8.06% |
| *To enhance sport performance* | 8 | 12.90% |
| *To support general health* | 52 | 83.87% |
| *Other reasons* | 7 | 11.29% |

| *Did the participants research algal-based food supplements before buying?* | *Count* | *Percentage* |
|---|---|---|
| *Yes, from friends, family, and acquaintances* | 14 | 22.58% |
| *Yes, from the internet* | 36 | 58.06% |
| *Yes, from a doctor or apothecary* | 15 | 24.19% |
| *Yes, from a store* | 9 | 14.52% |
| *Yes, through social media* | 7 | 11.29% |
| *Yes, through advertisements* | 4 | 6.45% |
| *No* | 3 | 4.84% |
| *Other* | 12 | 12.00% |

| *Did the participants believe in the activity, quality and safety of the algal-based food supplements?* | *Count* | *Percentage* |
|---|---|---|
| *Yes* | 34 | 53.13% |
| *No* | 8 | 12.50% |
| *The participant does not know* | 20 | 31.25% |

# S5. Curriculum Vitae

Wannes Hugo Rosa Van Hassel
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1 May 1994
Belgium
Common law

**PhD-student/ Young scientist adapt in microbiology, analytical biochemistry and molecular biology. A primary focus on Cyanobacteria and their toxins in the environment and in food.**

## Work experience

| | |
|---|---|
| July 2022 - Present | Scientist at Sciensano in the Department of chemical contaminants and additives -Toxin unit |
| April 2022 - Present | PhD-candidate at Sciensano / uLiège |
| Mei 2021- Present | CYANTIR project Sciensano |
| April 2018- Present | PhD-candidate at Sciensano / uLiège<br>Promoter: Dr Annick Wilmotte<br>Co-Promoter: Dr Mirjana Andjelkovic<br>Project title: Development of methodologies for the quantitative determination of microcystin congeners and assessment of their associated and aggregated health risk in foodstuffs and Belgian surface waters |
| July 2017 – December 2017 | Applicant for FWOsb grant (not acquired)<br>Promoter Prof. Dr Patrick Van Dijck<br>Co-Promoter Dr Ann Packeu |

## Education

| | |
|---|---|
| Sept 2015- July 2017 | Kuleuven Master biochemistry en biotechnology<br>Cum Laude<br>acquired in July 2017<br>Title Master thesis: Investigation of the putative role of Dog1 in glycerol metabolism of *Candida albicans* |

| | |
|---|---|
| Feb 2016 – July 2017 | Internship at Scientific Institute of Public Health (WIV-ISP), Mycology department. |
| Sept 2013 – July 2015 | Kuleuven Bachelor biochemistry and biotechnology. acquired in June 2013<br>Title Bachelor thesis: Study of dendritic/synaptic remodelling in the zebrafish retina during optic nerve regeneration |

## List of publications

Van Hassel, W.H.R.; Huybrechts, B.; Masquelier, J.; Wilmotte, A.; Andjelkovic, M. Development, Validation and Application of a Targeted LC-MS Method for Quantification of Microcystins and Nodularin: Towards a Better Characterization of Drinking Water. Water 2022, 14, 1195, doi:10.3390/w14081195.

Van Hassel, W.H.R.; Masquelier, J.; Andjelkovic, M.; Rajkovic, A. Towards a Better Quantification of Cyanotoxins in Fruits and Vegetables: Validation and Application of an UHPLC-MS/MS-Based Method on Belgian Products. Separations 2022, 9, 319, doi:10.3390/separations9100319.

Van Hassel, W.H.R.; Huybrechts, B.; Masquelier, J.; Wilmotte, A.; Andjelkovic, M. Development, Validation and Application of a Targeted LC-MS Method for Quantification of Microcystins and Nodularin: Towards a Better Characterization of Drinking Water. *Water* **2022**, *14*, 1195. https://doi.org/10.3390/w14081195

Van Hassel, W.H.R.; Andjelkovic, M.; Durieu, B.; Marroquin, V.A.; Masquelier, J.; Huybrechts, B.; Wilmotte, A. A Summer of Cyanobacterial Blooms in Belgian Waterbodies: Microcystin Quantification and Molecular Characterizations. *Toxins* **2022**, *14*, 61. https://doi.org/10.3390/toxins14010061

Abdallah, M.F.; Van Hassel, W.H.R.; Andjelkovic, M.; Wilmotte, A.; Rajkovic, A. Cyanotoxins and Food Contamination in Developing Countries: Review of Their Types, Toxicity, Analysis, Occurrence and Mitigation Strategies. *Toxins* **2021**, *13*, 786. https://doi.org/10.3390/toxins13110786

## Posters during international conferences

Toxic Cyanobacterial Blooms in Brussel: a case study

Screen for cyanotoxins in cyanobacteria based food supplements on the Belgian market

The influence of pH on the recovery of microcystincongeners in water samples by HPLC-MS/MS detection

Molecular origin of microcystins in dietary supplements originating from the Klamath Lake, Oregon.

Belgian cyanobacterial blooms during the summer of 2019

Accumulation and depuration assessment of microcystin congeners in Basil grown on a hydroculture