Chapter from the book *Current Topics in Public Health*

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1. Introduction

Cyanobacteria (or “blue-green algae”) are among the oldest known groups of organism on Earth, with fossil records spanning approximately 3.5 billion years, and inhabit nearly every ecological niche on the planet. In addition to their conspicuous occurrence as aquatic “blooms” (e.g. visible scums on ponds and lakes; Fig. 1), as well as various colonial or macrophytic forms, in marine and freshwater habitats, these photosynthetic prokaryotes are widely found in terrestrial soils, and as part of numerous symbiotic relationships with a range of organisms including animals, plants and fungi. To illustrate the extent of their global abundance and importance, approximately 50% of all primary productivity, and related oxygen production, occurs in the ocean, and the majority of this is derived from two genera of cyanobacteria, \textit{Synechococcus} and the picophytoplankton, \textit{Prochlorococcus} (Ting et al., 2002). Likewise, nitrogen-fixing cyanobacteria, and particularly the genus, \textit{Trichodesmium}, are the largest source of nitrogen in ocean systems (Carpenter and Romans, 1991). Moreover, several lines of evidence (see, for example, Paerl and Huisman, 2009) point to an increase in the abundance, distribution and persistence of cyanobacterial blooms in marine and freshwater system, specifically driven by regional and global changes in climate (e.g. elevated water temperature, stratification of the water column, changes in interseasonal weather patterns). The latter, in particular, underscores the potential of cyanobacteria – and specifically toxin-producing cyanobacterial blooms (discussed below) – as a rapidly emerging concern for public health.

Alongside their global biological importance, the cyanobacteria are widely recognized as producers of a chemically diverse array of biologically active secondary metabolites (see, for example, reviews by Gerwick et al., 2001; Tan, 2007; Tan, 2010). Considerable work over the past four decades (see Tan, 2007) has, in particular, focused on exploring this chemical diversity as a source of bioactive compounds with possible relevance to biomedicine, and specifically
development of potential chemotherapeutics. Furthermore, a rather convincing body of evidence (Proksh et al., 2002; Simmons et al., 2008) also suggests that many of the pharmacologically active marine natural products, originally isolated from various marine animal sources – and including several currently either in clinical trials, or being commercially developed as drugs - may, in fact, originate from cyanobacterial (or other microbial) sources as a result of trophic transfer (e.g. herbivory, filter-feeding), and symbiotic or commensal relationships.

Figure 1. Bloom of toxin-producing cyanobacteria on Lake Patzcuaro (Michoacan, Mexico), and subsistence fishing occurring within the bloom. Photo courtesy of Alan Wilson (Auburn University).

In addition to their potential in biomedicine, however, a number of these bioactive metabolites have been identified as naturally occurring toxins, and have been associated – as so-called “cyanotoxins” - with various human and environmental health concerns. Perhaps most notably, in freshwater systems, cyanobacterial populations can proliferate to the extent that they form large “blooms,” typically manifesting as “films” or “scums” on lakes, ponds and other freshwater systems (see Fig. 1). When comprised of toxin-producing representatives, their occurrence is generally categorized as “harmful algal blooms” (HABs), or frequently as “cyanoHABs” (to distinguish from similar blooms of several, unrelated, but likewise toxigenic, marine microalgae). In particular, toxins from cyanoHABs - or even simply high abundance of cyanobacterial cells – can contaminate water, and exposure to toxins via drinking water, recreational exposure and related routes has been linked to various cases of human and animal intoxication, as well as possible sub-acute and/or chronic health effects (e.g. increased rates of certain cancers, effects on fetal development). As these direct routes of exposure are beyond the scope of this chapter, and have been thoroughly covered by many previous authors, the reader is direct to several good reviews on the topic (e.g. Rao et al., 2002; Stewart et al., 2006; Funari and Testai, 2008).
Although the vast majority of studies related to the health impacts of cyanobacteria have focused on direct exposure to cyanotoxins via drinking water and related routes, there is a growing body of evidence to suggest that toxic cyanobacterial metabolites can bioaccumulate in aquatic food-webs, and may consequently pose additional health concerns as food-borne contaminants. The relatively limited number of studies on food-borne cyanobacterial contaminants may be attributed, in part, to the perceived lack of a mechanism for their bioaccumulation. Unlike more lipophilic contaminants, many of the recognized, water-soluble cyanotoxins would, as such, not be expected to biomagnify by otherwise well-documented mechanisms (i.e. storage, and subsequent transfer, in fatty tissues of animals) to higher trophic levels most frequently consumed by humans. However, despite the lack of a clear means transfer of these hydrophilic toxins in food webs, numerous studies have, indeed, demonstrated presence and apparent bioaccumulation in a range of trophic levels. Also likely limiting the attention paid to cyanobacterial toxins in food-webs is the fact that best documented cases of intoxication have been generally limited to direct exposure to these toxins, and specifically acute human or animal poisonings with clear links to consumption of contaminated water, or various related route, whereas there are - at present - few, if any, clear cases of recognized human intoxication by food-borne cyanotoxin. That said, growing recognition that cyanobacterial toxins may contribute to a sub-acute and/or chronic health effects – ranging from increased rates of cancers, neurodegeneration and development toxicity - which are considerably more difficult to identify, would suggest that, despite the lack of currently documented toxicoses, health threats posed by diet-derived toxins remains a very real concern.

The following chapter will present the current state of knowledge regarding the bioaccumulation of cyanobacterial toxins in the food web, and the possible role of these food-borne toxins as it relates to human and environmental health. To begin, the chapter will present a brief summary of the recognized cyanobacterial toxins, and their known toxicology and health effects. Subsequently, the current evidence related to the bioaccumulation, trophic transfer and bioavailability of these cyanobacterial toxins in food webs will be reviewed, along with related methodologies (including methodological limitations and innovations) for investigating these aspects. In addition to the widely recognized water-soluble cyanotoxins, cyanobacteria produce a host of bioactive metabolites, including a number of lipophilic representatives. Accordingly, the discussion will include a consideration of the less characterized bioactive metabolites that, despite relatively unknown health effects, may represent – due to their potential for biomagnification –relevant food-web contaminants. Finally, the chapter will summarize the current state of knowledge regarding the impacts of cyanobacterial toxins as it relates to human and environmental (i.e. ecosystem, animal) health.

2. Recognized cyanobacterial toxins: Chemistry and toxicology

2.1. Hepatotoxins

Detoxification of a wide range of toxic metabolites occurs - via multiphasic enzymes, and associated processes (e.g cellular transporter and “pumps”) - in the liver or equivalent organ
systems in animals. Accordingly, many toxic metabolites are actively transported (for subsequent detoxification) to, and thus accumulate primarily in, hepatocytes. Not surprising, therefore, two of the most commonly recognized cyanobacterial toxins are, in fact, associated with hepatotoxicity. However, aside from active transport of these toxins to – and consequent toxicity in - hepatocytes, it is becoming increasingly clear that the same metabolites may accumulate in a range of tissues (even if not associated with acute toxicity in these cells), and may – along with their generally uncharacterized toxicology in these tissues - be thusly transferred to higher trophic levels.

2.1.1. Microcystins (MCs) and nodularin (NOD)

Perhaps the most widespread, and consequently well studied, of the cyanobacterial toxins, microcystins (MCs) and nodularin (NOD) are, respectively, hepatotoxic hepta- and pentapeptide toxins. Both share structural similarity (Fig. 2), specifically characterized by a peptide macrocycle incorporating common and unusual amino acids. However, the former (i.e. MCs) represents a chemically diverse group of toxins, comprised of more than ninety variants (Welker and Van Dohran, 2006). Although structural variation throughout the macrocycle of the MCs has been reported, the primary differences occur in “X” and “Y” positions (Fig. 2), as per the accepted nomenclature for the group. As an example, the most common, and generally considered the most toxic, of these variants is MC-LR in which the X and Y positions, respectively, are occupied by leucine (L) and arginine (R) residues. Although chemical variations exist, both NOD and most MC variants are characterized by a relatively well-conserved unusual β-amino acid, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda), which is involved (see below) in the toxicology of these metabolites (Gulledge et al., 2003). Finally, whereas NOD is generally limited to a single late summer blooming species, Nodularia spumigens, the MCs are produced by a taxonomically wide array of cyanobacterial species including, most notably, the widespread species, Microcystis aeruginosa, but also a growing list of other diverse taxa (e.g. Aphanizomenon, Oscillatoria, Planktothrix, Anabaena, Nostoc).

Toxiologically, both NOD and the MCs are inhibitors of Ser/Thr type 1 and 2A protein phosphatases (PPases). Data generally suggest that Adda of NOD and MCs are involved in the binding of the metabolite to the active site of PPases (Nishiwaki-Matsumishi et al., 1991; Gulledge et al., 2003). To demonstrate this, several analogues of the MCs, specifically comprised of only a single amino acid (i.e. Gly, or L- or D-Ala) coupled via peptidyl linkage to the carboxylic acid of the N-acetylated Adda, were synthesized (Gulledge et al. 2003) and evaluated for toxicity. Although orders of magnitude lower than intact MC-LR, these analogs retained substantial PPase inhibitory activity, suggesting that Adda significantly contributes to the toxicophore of the MCs and NOD. On the other hand, the unusual amino acid, N-methyldehydroalanine (Mdha), found in many MC variants has been shown to covalently bind via a Michael addition to Cys\(_{273}\) of type 1/2A Ser/Thr PPases (MacKintosh et al., 1995). Accordingly, it has been suggested that this distal (to Adda) amino acids is, therefore, also involved in irreversible binding of the toxin to the PPase targets, as well as considerable
underestimation of bioaccumulation (as a non-extractable “bound” form) in organisms exposed to the toxin (see 4.2 Methodologies for evaluating cyanobacterial toxins in food-webs, below).

Figure 2. Chemical structures of hepatotoxic microcystins (A) and nodularin (B). In the former, structural variability is primarily based on variation in the “X” and “Y” amino acid positions indicated; for reference, the common variant, MC-LR, in which X and Y positions are leucine (L) and arginine (R) is shown.

Given the importance of PPases in a wide range of cellular functions, inhibition of these enzymes, following exposure to NOD/MCs, can result in a range of acute toxicoses. Accumulating primarily in hepatocytes (i.e. liver) and associated organ systems, inhibition of PPase by MCs and NOD most typically manifests as acute failure and hemorrhaging in these systems. However, recent studies, specifically pointing to the presence of similar active transporters in mammalian (e.g. rat) brains, have proposed a possible connection between MC uptake and apparent inhibitory effects on short- and long-term memory (Maidana et al., 2006). Moreover, emerging evidence supports an additional role of MCs in various chronic health effects, and particularly, as recognized tumor promoters, increased rates of certain cancers. Most notably,
studies in China (Yu, 1995; Ueno et al., 1996; Yu et al., 2001) have linked chronic exposure to MC through ingestion of contaminated surface (i.e. ditch) waters to endemically high rates of primary liver cancers. These studies suggest, in particular, that health concerns associated with exposure to even quite low (e.g. sub-picogram per day) doses of cyanobacterial toxins, such as MC/NOD, may promote negative health effects that might not clearly manifest as acute intoxication. This may be particularly germane to discussion of the bioaccumulation of these toxins since there have been to-date no known cases of overt intoxication from food-borne MCs or NOD, whereas the possible long-term effects associated with chronic exposure to these toxins (and others, e.g. BMAA) in the diet continues to present a possible concern.

2.1.2. *Cylindrospermopsis* (CYN)

Cylindrospermopsis (CYN) is a zwitterionic tricyclic alkaloid, specifically containing a unique hydroxyuracil (Fig. 3). It was first identified following a relatively large intoxication event (the so-called “Palm Island Mystery”) in Queensland, Australia. In this original case, children from more than one hundred families on Palm Island, and nearby mainland community of Townsville, were stricken with severe gastroenteritis. Subsequent studies (Bourke et al., 1983; Hawkins et al., 1985) linked the illness to the Solomon Dam – the primary water reservoir for the community – and identified several bloom-forming species of cyanobacteria. Among these, a toxic (in mouse bioassay) strain of the species, *Cylindrospermopsis raciborskii*, was identified (Hawkins et al., 1985). More than ten years after the incident, CYN was identified as the toxic principle of the *C. raciborskii* blooms in the reservoir (Ohtani et al., 1992), and following subsequent stereoselective synthesis (Heintzelman et al., 2001), assigned the structure shown in Figure 3. Originally thought to be a strictly tropical species, *C. raciborskii*, has been subsequently shown to occur worldwide in both tropical and temperate freshwater systems, possibly the result of recent expansion in its geographic distribution (Gugger et al., 2005). Moreover, since its initial identification from *C. raciborskii*, CYN has been subsequently found to be produced by several other members of the Nostocales, including the closely related *Aphanizomenon* and *Anabaena* (Banker et al., 1997; Spoof et al., 2006; Preußel et al., 2006), as well as at least one member of the Stigonematales (i.e. *Umezakia*; Harada et al., 1994), suggesting relatively widespread production of the toxin.

![Figure 3. Chemical structure of the hepatotoxin, cylindrospermopsin (CYN).](image-url)
Toxicological studies primarily suggest that CYN is an inhibitor of protein synthesis (Terao et al., 1994; Froscio et al., 2001). Specifically, Terao et al. (1994) demonstrated ribosomal detachment (from endoplasmic reticula) in hepatocytes treated with CYN, and in vitro studies using the rabbit reticulocyte translation system showed that CYN inhibits protein synthesis at nanomolar concentrations (e.g. IC\textsubscript{50} = 120 nM; Froscio et al., 2001). However, a range of possible mechanisms of toxicity, and associated biomarkers of the toxin, have been additionally identified and/or proposed. In accordance with inhibition of protein synthesis, Runnegar et al. (1994, 1995) showed a reduction of the tripeptide, glutathione (GSH), in rat hepatocytes exposed to CYN, particularly via apparent inhibition of GSH synthesis, leading to a presumptive reduction in the detoxifying capacity of cells. Studies (e.g. Shaw et al., 2000; Humpage et al., 2005) have similarly pointed to possible interaction of CYN with cytochrome P450, and suggested a role of this detoxifying enzyme system. Based on structural similarity to nucleotides, specifically as guanidine alkaloid, and more specifically the hydroxyuracil moiety contained in the tricyclic structure (Fig. 3), it has been suggested that CYN may additionally exert toxicity by means of interaction with DNA and/or RNA. Indeed, CYN has been found to form covalent linkages with DNA, leading to consequent chromosomal strand breakage (Shaw et al., 2000; Shen et al., 2002), as well as other apparent genotoxic effects (Bazin et al., 2010). This is notable as the uracil moiety of CYN has been shown as required for toxicity (Banker et al., 2001). Finally, in vivo studies, particularly in the mouse model, point to a range of histopathological effects, particularly in cells of the liver, but additionally in several organ systems (e.g. kidney, adrenal glands, lungs, intestines), following exposure via intraperitoneal or oral exposure (Hawkins et al., 1985; Shaw et al., 2000; Humpage and Falconer, 2003). Moreover, recent studies (in mice) show that long-term oral exposure to low-doses of CYN leads to measurable effects (e.g. reduced hematocrit levels; Sukenik et al., 2006), and, likewise, exposure to CYN during gestation induces fetal toxicity (Rogers et al., 2007), indicating that (similar to MCs) sub-acute effects may occur with relatively low doses, but may be missed by simple assessment of intoxication.

2.2. Neurotoxins

Several of the prominent cyanobacterial toxins are known to presumably cross the blood-brain barrier, and have been consequently associated with neurotoxicity. Neurotoxic cyanotoxins have been particularly identified based on observation of acute toxicity following exposure to these toxins (see below). However, in at least one case (i.e. BMAA; see below), toxicity has been associated with possible chronic neurodegeneration.

2.2.1. Anatoxin-a (ATX-a) and anatoxin-a(s)

Although chemically unrelated, two of the most active neurotoxins produced by cyanobacteria are related both in name and mode of action. First identified from species of Anabaena, anatoxin-a (ATX-a) is a tropane alkaloid (Fig. 4) with structural, but not pharmacological, similarity to cocaine. In contrast, the relatively less common anatoxin-a(s), so-named due to hyper-salivation associated with its neurotoxicity, is a phosphate ester of N-hydroxyguanine (Fig. 4), likewise, isolated primarily from Anabaena spp. Although structurally very different,
the two metabolites (along with a few chemically related variants, e.g. homoanatoxin-a) share related toxicological mechanisms of action. Known as the “very fast death factor,” ATX-a and its analogues are potent inhibitors of nicotinic acetylcholine receptors (nAChRs), specifically mimicking the endogenous neurotransmitter, acetylcholine, whereas anatoxin-a(s) inhibits related acetylcholinesterases (Aráoz et al., 2010). Interestingly, ATX-a is not degraded by acetylcholinesterases, and thus irreversibly inhibits nAChRs (Aráoz et al., 2010). With regards to food-webs, it should be noted that – compared to other cyanobacterial toxins – the “ana‐toxins” are relatively unstable chemically, as well as being generally limited in their distribution and occurrence, and thus rather few studies have reported their bioaccumulation (e.g. Mejean et al., 2010; Osswald et al., 2011). That said at least one case of apparent bioaccumulation of ANTX-a and its analogue (i.e. homoANTX-a) is, in fact, among the very few cases of acute intoxications being possibly linked to food-borne cyanotoxins (see 3. Evidence for bioaccumulation of cyanobacterial toxins in aquatic food-webs, below).

2.2.2. Saxitoxin (STX) and “Paralytic Shellfish Toxins” (PSTs)

Potent inhibitors of voltage-gated sodium channels, saxitoxin (STX) and several chemically related metabolites have been frequently associated with contamination of shellfish, and consequent toxicity (i.e. “paralytic shellfish poisoning” [PSP]), as the so-called “paralytic shellfish toxins” (PSTs). Specifically, in marine systems, the origins of STX/PSTs have been identified as *Alexandrium* and several related species of dinoflagellates. However, in the late 1960s, apparent STX was identified (Jackim and Gentile, 1968) in the cyanobacterial species, *Aphanizomenon flos-aquae*. Over the subsequent four decades, STX and related PSTs have been identified from a wide range of cyanobacterial genera (e.g. Mahmood and Carmichael, 1986; Humpage et al., 1994; Negri et al., 1995; Carmichael et al., 1997; Lagos et al., 1999; Beltran and
Neilan, 2000; Pomati et al., 2000; Smith et al., 2011) including members of the Nostocales (e.g. \textit{Anabaena}, \textit{Scytonema}, \textit{Cylindrospermopsis}) and Oscillatoriales (e.g. \textit{Lyngbya}, \textit{Planktothrix}).

**Figure 5.** Chemical structure of saxitoxin (STX) and selected variants of the related “paralytic shellfish toxins” (PSTs).

STX/PSTs are potent inhibitors of voltage-gated sodium channels in neuronal cells, specifically acting on (via binding to, and consequent blockage of) sodium passage through channel pores (Aráoz et al., 2010). Inhibition of sodium channels, by blocking sodium influx involved in the propagation of action potentials in neurons, leads to the aforementioned PSP syndrome which manifests in a range of neurotoxic symptoms including numbness, tingling, weakness and difficulty breathing as a result of the neuromuscular paralysis (Etheridge, 2010). Interestingly, STX/PST binds to the nearly identical location as the equally potent neurotoxin, tetrodotoxin (TTX), associated with poisoning by consumption of several species of pufferfish (Stevens et al., 2011), and STX has, in fact, been identified alongside TTX in pufferfish (Nakamura et al., 1984; discussed below). Notably, despite the identification of STX/PSTs from numerous cyanobacteria species found in freshwater systems, as well as apparent bioaccumulation of presumptively cyanobacteria-derived toxin in fish and shellfish consumed by humans, reported poisoning by the PSTs has been generally limited to ingestion of shellfish contaminated by apparent marine dinoflagellate sources.

### 2.2.3. β-Methylamino-L-alanine (BMAA)

As perhaps the best studied case of apparent long-term toxicity resulting from a cyanobacterial toxin bioaccumulated within food webs, the non-essential, non-protein amino acid, β-methylamino-L-alanine (BMAA; Fig. 6) has been linked to high rates of the otherwise rare amyotrophic lateral sclerosis (ALS), and possibly other related neurodegenerative diseases (e.g. Parkinson’s Disease, Alzheimer’s Disease). Indeed, the first reports of BMAA as a neurotoxic cyanobacterial metabolite specifically stemmed from studies of extraordinarily high rates of ALS among the indigenous Chamorro populations on the island of Guam. BMAA
was originally identified as a plant-derived natural product, and specifically found in nonflowering plants of the genus, *Cycas* (Vega & Bell, 1967). However, the origin of the metabolite was ultimately traced to an endosymbiotic species of the cyanobacterial genus, *Nostoc*, found within roots these cycads. The occurrence of BMAA in this cycad species (Spencer et al., 1987), and its apparent biomagnification by fruit bats (or “Flying Foxes”) which consume the fruits of the cycad, which are, in-turn, consumed as a delicacy by the Chamorro of Guam (Cox et al., 2003; discussed below), has been suggested to provide a route of exposure to the neurotoxin, and was consequently linked to ALS among the Chamorro. Indeed, subsequent studies have accordingly identified both apparent biomagnification of the metabolite in this rather short “food chain” (i.e. an approximately 10^4-fold increase from cyanobacteria to cycad to fruit bat), as well as measurable levels of BMAA in the brains of Chamorro patients that died from ALS and related syndromes (Cox et al., 2003).

![Chemical structure of β-Methylamino-L-Alanine (BMAA).](image)

Toxicologically, BMAA is a recognized agonist of glutamate receptors. Staton and Bristow (1997) found that BMAA excited glutamate receptors, leading to apoptotic and necrotic cell death, in cerebellar granule cells. Subsequent studies (e.g. Rao et al., 2006; Lobner et al., 2007; Cucchiaroni et al., 2010) have confirmed a similar effect in a range of relevant neurons (e.g. spinal motor neurons, cortical neurons, dopaminergic substantia nigra pars compacta cells). More recently, however, it has been proposed that BMAA – as an amino acid – may become erroneously incorporated via translation into proteins. One of the hallmarks of several related neurodegenerative diseases (including ALS, Alzheimer’s Disease, Parkinson Disease/Dementia) is the formation of misfolded protein aggregates, and it has consequently been proposed that the possible mis-incorporation of BMAA may represent an alternative mechanism of action for this putative toxin.

Although first identified in the Chamorro/ALS case, potential health concerns associated with BMAA have continued to grow with recent reports of a widespread occurrence of the metabolite among cyanobacteria, and its bioaccumulation in a wide range of systems, as well as additional epidemiological findings that link the compound to a complex of related neurodegenerative diseases. In a study by Cox et al. (2005) chemical analysis of a wide range of cyanobacteria, including marine, freshwater and terrestrial representatives, indicated that as many as 95% of the genera produce BMAA, and pointed to a potentially widespread occurrence of the metabolite. Since this study, the analytical techniques used with respect to BMAA

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**Figure 6.** Chemical structure of β-Methylamino-L-Alanine (BMAA).

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Current Topics in Public Health
have rapidly evolved (see 5. Methodologies for analysis of cyanobacterial toxins in food-webs, below). Accordingly, several investigators (e.g. Li et al., 2012) have, in fact, argued - specifically based on re-evaluation of the analytical methods previously used - that these prior estimates regarding occurrence among cyanobacteria are perhaps exaggerated. However, despite this standing controversy, a growing number of studies have concurrently pointed to the apparent bioaccumulation of the metabolite in food webs and relevant human foods (discussed below) which, along with increasing experimental evidence to support toxicological effects related to neuronal and memory function (e.g. Karlsson et al., 2009 and 2009b; Liu et al., 2009; Purdie et al., 2009 and 2009b; Karlsson et al., 2011), and links to additional clusters of these disease, have continued to fuel emerging hypotheses regarding BMAA and neurodegeneration. With regards to the latter, post mortem studies have identified BMAA in the brain tissues of patients who had died from ALS and Alzheimer’s Disease (AD), but not from either strictly hereditary neurodegenerative disease (e.g. Huntington’s Disease) or unrelated causes (Pablo et al., 2009). Several studies have, likewise, suggested links between BMAA and so-called “sporadic” occurrence of these diseases ranging from Canadian AD patients (Murch et al., 2004) to clusters of ALS associated with exposure to water blooms in New England (Caller et al., 2009) to sporadic occurrence among Gulf War veterans purportedly exposed to BMAA through cyanobacteria in desert dust (Cox et al., 2009). As these questions regarding occurrence of BMAA among cyanobacteria, as well as its toxicological relevance, continue to be answered, it is becoming clear that understanding the potential role of food-web bioaccumulation, as a route of exposure to this metabolite, will be particularly critical.

3. Evidence for bioaccumulation of cyanobacterial toxins in aquatic food-webs

Toxins from a number of marine HAB species – particularly including diverse eukaryotic taxa within the dinoflagellates and diatoms (Bacillariophyta) – bioaccumulate and/or biomagnify in marine food-webs, and have been clearly linked to contamination of fish and seafood, and consequent intoxication of humans and wildlife (Van Dolah, 2000). Notably, marine algal toxins are (1) frequently associated with commercially important seafood species, including filter-feeding/grazing shellfish (e.g. clams, mussels) and several plantivorous fish species, and/or (2) alternatively characterized by relatively high lipophilicity enabling uptake and storage in fat tissues as a means of biomagnifications to higher trophic levels, including marine fish species eaten by humans (Van Dolah, 2000). Relevant examples of the former include dinophysotoxins and various other metabolites associated with “diarrhetic shellfish poisoning” (DSP), domoic acid associated with “amnesic shellfish poisoning” (ASP) and contamination of shellfish by so-called PSP toxins (i.e. STX and other PSTs, see above) derived from dinoflagellates (e.g. Alexandrium spp.). Examples of the latter, on the other hand, include biomagnification of ciguatoxin, and several chemically related lipophilic metabolites (e.g. maitotoxin), by various top-level predator species of fish in relation to the well documented “ciguatera poisoning.”
Compared to marine HAB toxins, bioaccumulation of cyanobacterial toxins in food webs, and its consequent relevance to human and environmental health, has been relatively much less studied. There are likely several reasons for this. The most obvious is that, in contrast to the well-documented contamination of fish and other seafood by marine algal toxins, there are very few recognized cases of acute human or animal intoxication via consumption of bioaccumulated cyanobacterial toxins. It is further proposed that this may be due, in part, to less commercial fishing in freshwater water habitats - and thus consumption of freshwater fish and shellfish - compared to marine fisheries. Indeed, a recent report by the United Nations’ Food and Agriculture Organization (FAO) estimated the 2008 global fisheries catch as approximately 90 million tonnes, but it was comprised of only a “record 10 million tonnes from inland waters,” compared to more than 80 million tonnes from marine sources (FAO, 2010).

Regardless of the relatively limited focus on cyanobacterial toxins in freshwater fish and shellfish, a growing body of knowledge – summarized in Table 1 – does, in fact, support the occurrence of cyanobacterial toxins in a range of trophic levels, including species with direct potential for human exposure, as well as possible implications for ecosystem health (see 7. Implications for Ecosystem Health, below). Indeed, a number of thorough reviews on the topic have recently appeared (e.g. Ibelings and Chorus, 2007; Ferrao-Filho et al., 2011; Kozlowsky-Suzuki et al., 2012). Owing to the relatively polar (i.e. water-soluble) nature of the recognized cyanobacterial toxins, it has been generally suggested that bioaccumulation in the food-web will be limited to relatively “low” trophic positions. Indeed, in freshwater food webs, this has included particularly high rates of bioaccumulation of toxins in planktivorous fish, and filter-feeding or other grazing invertebrates (Table 1). Reported concentrations of accumulated cyanobacterial toxins are, at first glance, typically quite low (Table 1). However, levels are obviously expected to vary - as shown in experimental studies (e.g. Osswald et al., 2011) - with concentrations of toxins and/or algal cell density to which animals are exposed. Moreover, it is suggested by various studies that levels may be sufficient for long-term (and consequently difficult to study) health effects, and that these reported data (as discussed further below) may underestimate - due to limitations of typical analytical methodologies, food preparation techniques used and other variables - the possible contribution of these food-borne toxins.

From inspection of the available data on the accumulation of cyanobacterial toxins in fish and shellfish (Table 1), it is clear that MCs are, by far, the most commonly reported. Indeed, MCs are generally considered the most widespread of the freshwater cyanobacterial toxins. Frequently, these data are reported in terms of “MC-LR equivalents,” as typical quantitative analyses (e.g. ELISA, LC-MS) use this common variant as a reference standard, despite the fact that as many as ninety variants have been reported (see Welker and von Döhren, 2006). In addition to being among the most commonly detected of the microcystins, MC-LR is also one of the most toxic variants (Zurawell et al., 2005). That said, studies suggest variability in the uptake and detoxification of the variants. Xie et al. (2004), for example, studied MC-LR and MC-RR distribution and depuration in phytoplanktivorous carp, and proposed, based on these findings, a possible preferential uptake of MC-RR, or inhibited uptake of and/or active mechanism to “degrade” MC-LR.
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<th>Toxin Conc. (\mu g , g^{-1})(^b)</th>
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<td>MC-LR (eq)</td>
<td>Whole(^c)</td>
<td>0.0185</td>
<td>Berry et al., 2011a</td>
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<tr>
<td>Redbreast Tilapia (Tilapia rendalli)</td>
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<td>Muscle</td>
<td>0.002-0.337</td>
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<td>Nile Tilapia (Oreochromis niloticus)</td>
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<td>Muscle</td>
<td>0.102</td>
<td>Mohammed et al., 2003</td>
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<tr>
<td>Blue Tilapia (Oreochromis aureus)</td>
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<td>0.00009</td>
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<td>STX/PSTs</td>
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<td></td>
</tr>
<tr>
<td>Topote (Dorosoma mexicana)</td>
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<td>0.0008</td>
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</tr>
<tr>
<td></td>
<td>STX/PSTs</td>
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<td>0.0003</td>
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</tr>
<tr>
<td>Flounder (Platichthys flesus)</td>
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<td>Muscle</td>
<td>0.0005-0.1</td>
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<td>Muscle</td>
<td>0.0004-0.2</td>
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<td>Trout (Oncorhynchus mykiss)</td>
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<td>Muscle</td>
<td>0.035</td>
<td>Wood et al., 2006</td>
</tr>
<tr>
<td></td>
<td>ATX-a</td>
<td>Whole (juveniles)</td>
<td>3.9-23.6</td>
<td>Osswald et al., 2011 (experimental studies)</td>
</tr>
<tr>
<td>Yellow Perch (Perca flavescens)</td>
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<td>Muscle</td>
<td>0.0008 (max)(^d)</td>
<td>Wilson et al., 2008</td>
</tr>
<tr>
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<td>MC-LR (eq)</td>
<td>Muscle</td>
<td>0.04</td>
<td>Magalhaes et al., 2003</td>
</tr>
<tr>
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<td>NOD</td>
<td>Muscle</td>
<td>0.0007-0.025</td>
<td>Van Buynder et al., 2001</td>
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**Shellfish**

**Bivalves: Mussels**

<table>
<thead>
<tr>
<th>Shellfish</th>
<th>Toxin(s)(^a)</th>
<th>Tissue(s)</th>
<th>Toxin Conc. (\mu g , g^{-1})(^b)</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anodonta woodiana</td>
<td>MC-LR (eq)</td>
<td>Muscle/foot (mean)</td>
<td>0.009 (mean)</td>
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<tr>
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<td>Whole(^c) (max)</td>
<td>0.026 (max)</td>
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<td>0.064</td>
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<tr>
<td>Anodonta cygnea</td>
<td>STX/PSTs</td>
<td>Whole(^c)</td>
<td>2.6</td>
<td>Pereira et al., 2004 (experimental study)</td>
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<td>Alathyria condola</td>
<td>STX/PSTs</td>
<td>Whole(^c)</td>
<td>57</td>
<td>Negri &amp; Jones, 1995 (experimental study)</td>
</tr>
<tr>
<td>H Bryopsis cumingii</td>
<td>MC-LR (eq)</td>
<td>Muscle/foot (mean)</td>
<td>0.022 (mean)</td>
<td>Chen &amp; Xie, 2005a</td>
</tr>
<tr>
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<td></td>
<td>Whole(^c) (max)</td>
<td>0.039 (max)</td>
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<td>Cristaria plicata</td>
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<td>Muscle/foot (mean)</td>
<td>0.023 (max)</td>
<td>Chen &amp; Xie, 2005a</td>
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<tr>
<td></td>
<td></td>
<td>Whole(^c) (max)</td>
<td>0.096</td>
<td></td>
</tr>
<tr>
<td>Fish</td>
<td>Toxin(s)</td>
<td>Tissue(s)</td>
<td>Toxin Conc. (µg g⁻¹)</td>
<td>Reference(s)</td>
</tr>
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<td>-------------------------------------------</td>
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<td>Lamprotula leai</td>
<td>MC-LR (eq)</td>
<td>Muscle/foot</td>
<td>0.021 (mean)</td>
<td>Chen &amp; Xie, 2005a</td>
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<tr>
<td></td>
<td></td>
<td>Whole</td>
<td>0.058 (max)</td>
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<td>Mytilus galloprovincialis</td>
<td>MC-LR</td>
<td>Whole</td>
<td>1.8 (max)e</td>
<td>Vasconcelos, 1995 (experimental study)</td>
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<td>ATX-a</td>
<td>Soft tissue</td>
<td>0.006 (max)</td>
<td>Osswald et al., 2008 (experimental study)</td>
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<td>Saker et al., 2004</td>
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<td>Whole</td>
<td>2.5</td>
<td>Van Buynder et al., 2001</td>
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<td><strong>Gastropods: Snails</strong></td>
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<td>Apple Snails (Pomacea patula catemacensis)</td>
<td>CYN</td>
<td>Whole</td>
<td>0.003</td>
<td>Berry and Lind, 2010</td>
</tr>
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<td>STX/PSTs</td>
<td>Whole</td>
<td>0.001</td>
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<tr>
<td>Crayfish (Procambarus clarkia)</td>
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<td>Muscle</td>
<td>0.005 (mean)</td>
<td>Chen &amp; Xie, 2005b</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.010 (max)</td>
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<tr>
<td>Red Claw Crayfish (Cherax quadrarcarinatus)</td>
<td>CYN</td>
<td>Muscle</td>
<td>0.18' (mean)</td>
<td>Saker &amp; Eaglesham, 1999</td>
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<td>Hepato-pancreas</td>
<td>0.86' (mean)</td>
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<tr>
<td>Freshwater Shrimp (Palaemon modestus)</td>
<td>MC-LR (eq)</td>
<td>Muscle</td>
<td>0.006 (mean)</td>
<td>Chen &amp; Xie, 2005b</td>
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<tr>
<td></td>
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<td>Freshwater Shrimp (Macrobrachium nipponensis)</td>
<td>MC-LR (eq)</td>
<td>Muscle</td>
<td>0.004 (mean)</td>
<td>Chen &amp; Xie, 2005b</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>0.012 (max)</td>
<td></td>
</tr>
<tr>
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<td></td>
<td></td>
<td>0.051</td>
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</tr>
<tr>
<td>Unidentified crab species</td>
<td>MC-LR (eq)</td>
<td>Muscle</td>
<td>0.103</td>
<td>Magalhaes et al., 2003</td>
</tr>
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<td>Unidentified prawn species</td>
<td>CYN</td>
<td>Muscle</td>
<td>0.205</td>
<td>Saker et al., 2004</td>
</tr>
<tr>
<td></td>
<td>NOD</td>
<td>Muscle</td>
<td>0.005-0.022</td>
<td>Van Buynder et al., 2001</td>
</tr>
</tbody>
</table>

* Total MC content frequently reported as MC-LR equivalents ("MC-LR(eq)" in the table).

b Toxin concentrations given as either range, or maximum ("max") or mean (if not otherwise indicated).

c Fish or shellfish eaten whole including muscle and viscera. In the case of shellfish, shell or exoskeleton/carapace is typically removed, and the inner flesh consumed.

d Converted from dry weight to wet weight using conversion factor of 5 as per U.S. EPA recommendation, assuming ~80% water content of fish (Holcomb et al., 1976).

e Conversion from dry weight to wet weight using conversion factor of 5.8 as per Ricciardi and Bourget (1998).

f Conversion from dry weight to wet weight using conversion factor of approximately 5 as per Headon and Hall (xxx)

Table 1. Measured concentrations of cyanobacterial toxins in freshwater fish and aquatic invertebrates eaten by humans. Adapted, in part, from Ibelings and Chorus (2007).
As shown in Table 1, concentrations of MC in these tissues are generally quite low, and might imply a consequently low concern with respect to human exposure. However, there is evidence – as discussed above - to suggest that chronic exposure to low levels of these toxins may pose concern for long-term health (e.g. increased rates of cancer). Moreover, not shown in this table is the generally higher accumulation of MCs by liver and associated organ systems due to active transport of these toxins to hepatocytes and related cells (as discussed above). Although, in the case of fish, in particular, muscle tissues (i.e. “flesh,” e.g. filets, etc.) are most typically eaten, there are exceptions. Berry et al. (2011a), for example, evaluated the MC content (see Table 1) of fish caught from a persistent cyanobacterial bloom in Lake Patzcuaro (Mexico), and specifically reported considerable levels for those fish (i.e. “charales” and Goodea spp.) that are locally eaten in their entirety, including muscle and associated viscera. Accordingly, these results suggest that preparation technique can have a key role in assessing the potential for human exposure.

Bioaccumulation, however, is not limited to the MCs, and a growing number of studies (see recent review by Kinnear, 2010) have, for example, also reported variable levels of the hepatotoxic CYN in relevant fish and shellfish species (Table 1). In fact, soon after the identification of CYN as the toxin responsible for the Palm Island Mystery (Ohtani et al., 1992), Saker and Eaglesham (1999) reported quite high levels of the toxin in both fish (“Rainbow Fish,” Melanotaenia eachemensis) and invertebrate (i.e. “Red Claw Crayfish,” Cherax quadricarinatus) species. Although, the former is not generally considered edible, the latter is, in fact, extensively aquacultured as freshwater “seafood” commercially. Since that time, the potential for bioaccumulation CYN has been reported in several field and laboratory studies (Norris et al., 2001; Nogueira et al., 2004; White et al., 2006; White et al., 2007), although most have focused on species not – or rarely (e.g. Swan Mussel, Anodonta cygnea; Saker et al., 2004) – eaten by humans, and therefore, not generally relevant to human diet and health. As a notable exception, Seifert et al. (2007) reported CYN from Eel-Tailed Catfish (Tandanus tandanus), an omnivorous species of game fish; the toxin, however, was not detected in several other less planktivorous species of fish (e.g. perch, bass). More recently, evaluation of CYN in the endorheic lake system of Lake Catemaco (Mexico) identified the toxin in species of both finfish (Berry et al., 2012) and relevant species of invertebrates (i.e. freshwater snails; Berry and Lind, 2010) consumed in this region.

Although not as well recognized (nor investigated), emerging evidence suggests that cyanobacterial neurotoxins may also accumulate in relevant freshwater species (Table 1). With regards to human health, STX and related “paralytic shellfish toxins” (PSTs) are – based on their well-described association to intoxication via seafood – perhaps of most obvious concern. STX/PSTs have widely documented as contaminants of marine shellfish, and particularly bivalves, representing a recognized concern for public health (Van Dolah, 2000). More recently, there have been increasing reports of STX/PSTs in fish, and particularly species of “pufferfish” (Family Tetraodontidae), alongside the toxicologically related (i.e. voltage-gated sodium channel blocking) tetrodotoxins that have been well described from these species. Similar to contamination of shellfish, however, it has been recently shown (Landsberg et al., 2006) that marine dinoflagellates (e.g. Pyrodinium spp.) are likely the source of STX/PSTs in the case of
these typically estuarine fish. That said, studies (Negri and Jones, 1995; Pereira et al., 2004) have shown that – like marine bivalves – freshwater mussels can also accumulate cyanobacterially derived STX/PSTs. Although the mussel species (*Alathyria condola*) examined in these studies (e.g. Negri and Jones, 1995) are not one typically eaten by humans (although frequently by other animal species), more recent studies (Pereira et al., 2004) have measured considerable levels of PSTs (fed via PST-producing *Aphanizomenon issatschenkoi*) in the Swan Mussel (*Anodonta cygnea*) that, as previously mentioned, is occasionally consumed within certain human populations in Europe and elsewhere. Even more recently, while evaluating the apparent bioaccumulation of CYN associated with a bloom of *C. raciborskii* in Lake Catemaco (Mexico), it was found that both edible “tegogolo” snails (Berry and Lind, 2010), and locally consumed species of freshwater finfish (Berry et al., 2012), were found to accumulate STX/PSTs. These studies, furthermore, point to a shared source of CYN and STX/PSTs, and specifically *C. raciborskii* that is abundant in this lake system (Berry et al., 2012).

In addition to STX/PSTs, cyanobacteria are known to produce several other neurotoxic metabolites, including (as discussed above) the toxicologically related ATX-a and anatoxin-a(s). Compared to other cyanotoxins, the neurotoxic ATX-a is generally considered chemically quite labile, and it is generally anticipated that the potential for bioaccumulation of this unstable toxin would be, accordingly, rather low. That said, in experimental studies, it has been shown that both fish – including trout (*Oncorhynchus mykiss*; Osswald et al., 2011) and juvenile carp (*Cyprinus carpio*; Osswald et al, 2007) - and shellfish (e.g. mussel, *Mytilus galloprovincialis*; Osswald et al., 2008) can, in fact, accumulate the toxin presented in either dissolved (added to tank water) or cell-bound form. Moreover, in a recent study (Mejean et al., 2010), bioaccumulation of this toxin in the giant clam (*Tridacna maxima*), frequently consumed in the South Pacific, was evaluated in relation to several “ciguatera-like” intoxication cases reported in the region, and found to contain the potently toxic analog, homoanatoxin-a, as well as possible traces of ATX-a. Though confirmation of these toxins, as the causative agent of these reported poisoning, remains to be made, this study represents of the very few examples of possible acute intoxication by a food-borne cyanobacterial toxin.

Similarly, despite the emerging picture of its biomagnification in terrestrial species (e.g. fruit bats feeding on cycads; see above) over the past several decades, as well as the particularly conspicuous abundance of cyanobacteria in aquatic systems, relatively limited attention has been paid to the possible bioaccumulation of neurotoxic BMAA in aquatic food-webs. Several recent studies (Jonasson et al., 2010; Brand et al., 2010; Mondo et al., 2012), however, have suggested both accumulation, and possible biomagnifications of BMAA in marine systems, including those species (i.e. fish, seafood) directly related to human health. In one very recent case, the fins of several species of sharks, as “apex” marine predators, were examined, and found to be laden with BMAA (Mondo et al, 2012), and consequently proposed to present – via widespread consumption in the form of “sharkfin soup” – a potentially important route of exposure to this toxin, and thus a public health concern, in Asian countries where shark fins are considered a delicacy. Likewise, one of these studies, specifically evaluating BMAA in South Florida waters, and more specifically including Caloosahatchee River, did, in fact, detect this putatively toxic amino acid in both invertebrate (i.e. mussel) and fish species, including
those consumed – at least occasionally - by humans (e.g. bass, bowfin, alligator gar) in this freshwater system. Most interestingly perhaps, it was found in this, as well as concurrent studies of marine food-webs, that measured BMAA levels were, in fact, higher in higher trophic levels suggesting the possibility of biomagnifications of this metabolite. As a highly water-soluble amino acid - with a low octanol/water-partitioning coefficient - it is not expected that BMAA would biomagnify by conventional means (i.e. via deposition in fat bodies, etc.); however, alternative mechanisms to this end are proposed (discussed below).

Finally, it bears mention that a growing number of studies have documented apparent uptake of cyanobacterial toxins by various plant species via toxin-contaminated irrigation water. Uptake of cyanobacterial toxins by plants was first suggested in a study by Pflugmacher et al. (2001) that reported both uptake - and associated metabolism - of MC-LR by the water reed (*Phragmites australis*). Subsequently, uptake and metabolism was similarly found to occur in several agriculturally important species including various legumes, maize, wheat and alfalfa (Peuthert et al., 2007). Similarly, it was recently reported that various cruciferous vegetables (e.g. *Brassica* spp., *Sinapis alba*) are capable of accumulating 10-21% of CYN provided to roots, reaching as high as 49 µg/g (fresh weight) in the leafy components (Kittler et al., 2012). Although not bioaccumulation *per se* (i.e. via trophic transfer), the rather high levels of these compounds found in exposed plants, and specifically several agriculturally important crop plant species, suggest that exposure to cyanotoxins through plant crops may pose a very real public health concern.

![Figure 7](http://dx.doi.org/10.5772/55111)  
*Figure 7.* Depiction of biomagnification (left) and biodilution (right) of toxins in food-webs.
4. Trophic transfer and bioavailability of cyanobacterial toxins

Despite emerging evidence to suggest the bioaccumulation of cyanobacterial toxins within food webs (as summarized above; Table 1), relatively little is known regarding the process of trophic transfer, and the subsequent bioavailability of “food-derived” cyanotoxins. For lipophilic contaminants, including recognized anthropogenic pollutants (e.g. PCBs, DDT) and even some HAB toxins (e.g. ciguatoxins), uptake and storage in fat tissues have been largely implicated as a means of trophic transfer. However, there is no clear mechanism for bioaccumulation and/or biomagnification of the most widely recognized and, moreover, typically water-soluble cyanobacterial toxins. Likewise, although growing evidence suggests that cyanobacterial toxins are, in fact, present in relevant components of freshwater food webs (see section 3. Evidence for bioaccumulation of cyanobacterial toxins in food-webs, above), a very limited number of studies have investigated whether toxins contained within ingested tissues are, in fact, released, available and/or taken up in the digestive process. The following sections will summarize the current state of knowledge regarding both of these aspects.

4.1. Trophic transfer

As discussed in the previous section, a growing number of studies do, indeed, suggest that cyanobacterial toxins are transferred via dietary/trophic transfer within aquatic food-webs. It has been shown, in particular, that a range of phytoplanktivorous species, including zooplankton, fish, benthic grazers and filter-feeders consume toxin-laden algal cells, and directly accumulate these toxins. Alternatively, it has been shown (Karjalainen et al., 2003) that certain species, specifically including zooplankton, can accumulate, i.e. bioconcentrate, dissolved toxins directly from water as might be found, in particular, during algal bloom senescence. However, given the generally water-soluble (i.e. non-lipophilic) nature of the best known cyanobacterial toxins (see 2. Recognized cyanobacterial toxins: chemistry and toxicology, above), as well as currently available data on the apparent accumulation of these toxins within food webs (see 3. Evidence for accumulation of cyanobacterial toxins aquatic food-webs, above), it has been largely argued that transfer of these toxins follows a trophic pattern of biodilution rather than biomagnification (e.g. Ibelings et al., 2005; Ibelings and Chorus, 2007; Kozlowsky-Suzuki et al., 2012; Fig. 7). In a very recent, and particularly thorough, meta-analysis of existing data by Kozlowsky-Suzuki et al. (2012), it was shown that biodilution generally prevails. However, the authors of this study do highlight several exceptions and related caveats with relevant implications for potential exposure to toxins via food webs. Likewise, although concerns regarding human exposure to aquatic toxins – particularly in freshwater systems – are most frequently focused on higher trophic levels (i.e. sport and commercially caught fish species) as sources of toxins, a growing number of studies (Ibelings and Chorus, 2007; Berry and Lind, 2009; Berry et al., 2011; Berry et al., 2012) have documented accumulation of cyanobacterial toxins by species from lower trophic levels (i.e. freshwater shellfish, phytoplanktivorous fish) that are, indeed, consumed by humans, such that lack of biomagnification would not preclude possible human dietary exposure.
The potential for trophic transfer of cyanobacterial toxins is, generally speaking, controlled by three interrelated factors: selection, chemical availability, toxin uptake and detoxification/elimination (Fig. 8). Chemical availability will be discussed in the next section (4.2. Bioavailability). With regard to the former (i.e. selection), this factor would be most likely expected to be limited to initial consumption of toxin-producing algal cells by planktivores (that can subsequently serve as vectors for the toxins). This would be expected since production of toxins by cyanobacteria cells has been suggested to be linked to possible chemical defenses (i.e. feeding deterrence) against potential grazer, and evidence (see below) does, in fact, suggest that toxins may deter potential phytoplanktivorous grazers. As a corollary of this, it is suggested by this avoidance that potential grazers are capable of “detecting” the presence of toxins in algal cells. On the other hand, little or no evidence exists to suggest that toxin subsequent present in animal tissues can be so detected, and thus it is generally assumed that selection further “up the chain” is not a factor in higher trophic transfer. That said, other aspects of feeding behavior, including both general feeding preferences/strategies (e.g. herbivory versus carnivory), and more specific behaviors, particularly including selection of certain tissues by predators (e.g. preference of human consumers toward fish muscle versus other organs/tissues), might be argued to contribute to selection, and consequently the potential for trophic transfer.

A preponderance of evidence, in fact, supports a possible avoidance of toxigenic cyanobacteria by phytoplanktivores. In particular, selectivity with regards to trophic transfer is perhaps best demonstrated by numerous studies that have investigated feeding by zooplankton, and particularly *Daphnia* spp., as a widespread cladoceran micrograzer, in relation to the MCs. Microcystins have been shown to be toxic to *Daphnia* and other micrograzers, and toxicity has been shown to specifically correlate with rate of ingestion of the toxin. However, subsequent studies – specifically using “knock-out”non-toxic strains of *Microcystis* – have also, in contrast, indicated that *Daphnia* may not have the ability to distinguish toxic versus non-toxic algal cells (Rohrlack et al., 2001). Moreover, it has been generally found that interaction between grazers and their cyanobacterial prey are dependent on class of grazer (e.g. micro- versus mesozooplankton), species and even inter-specific genetic differences (Kurmayer and Jüttner, 1999; Davis and Gobler, 2011). More recently, it has been proposed (e.g. Wilson et al., 2005 and 2006; Lemaire et al., 2012) that differences in the observed feeding deterrence, relative to toxin content, may be explained by so-called “genotype x genotype interactions” whereby effects on feeding behavior are determined by the combination of grazer genotype (e.g. tolerance or susceptibility to toxin) and algal genotype (i.e. toxic or non-toxic). Finally, studies have suggested (e.g. Kurmayer and Jüttner, 1999; Reinkainen et al., 2001) that avoidance of potentially toxic cyanobacterial cells may be related to currently unknown, and particularly lipophilic, metabolites rather than recognized toxins (e.g. MCs). Understanding of the role of selection in trophic transfer, therefore, will rely on our increasing knowledge of genetic and chemical variability of both cyanobacteria and their grazers.

Even if toxin-containing items are selected for ingestion, however, several lines of evidence suggest both rather limited uptake of diet-derived toxin, as well as active and passive mechanisms for detoxification and/or elimination of these toxins, which together would be expected to limit the potential for trophic transfer. Understanding the contribution of these factors to
trophic transfer, requires knowledge of – and/or means to investigate - the physiological, cellular and possible molecular processes involved in both uptake and potential detoxification/elimination. In the cases of MCs, for example, it has been suggested by multiple studies that the gastrointestinal tract, and particularly mid-gut wall, of fish may be an important site for toxin absorption (Chen et al., 2007; Dyble et al., 2011). Given the assumption that bioaccumulation is generally limited to lower trophic levels, it is not perhaps surprising, however, that most insight in this regard has been, likewise, largely limited to phytoplanktivore models. In order, for example, to evaluate the uptake (and subsequent elimination) of MC-LR by fish, in relation to possible human exposure, specifically using the juvenile yellow perch (*Perca flavescens*) model, Dyble et al. (2011) fed known doses of the toxin to fish orally via diet (i.e. “toxin-doped” pellets), and determined concentration and distribution within relevant tissues (i.e. muscle and liver) over time. Consistent with active transport of MCs to (for subsequent detoxification in) hepatocytes, higher levels were found in fish livers, and kinetically speaking, achieved a maximum level in liver cells (8-10 hours following dosing) prior to a subsequent peak in muscle tissue (12-16 hours post-dosing). Moreover, the concentrations measured represented orders of magnitude lower levels than those expected if the entire dose was assimilated. Moreover, roughly equivalent concentrations were observed for two doses used (5 and 20 µg), suggesting a possible maximum capacity for uptake. Furthermore, studies showed a rapid increase in the toxin concentration (dependent in magnitude on dose) following peak levels measured in the fish tissues (after approximately 10 hours), implying a rather rapid elimination of the toxin by excretion (i.e. urine, feces). These finding support both rather limited uptake, and rapid elimination of the toxin, however, it should be noted that these data also correspond to a single dosing of the fish, whereas in aquatic habitats (i.e. during blooms, or through persistent occurrence of toxic cyanobacteria) it would be expected that possible grazers (and even other higher trophic levels) would be exposed more continuously to toxins, such that the effective “window” of time for trophic transfer would be considerably longer.

As pointed-out, the initial step for trophic transfer (from cyanobacterial cell to grazer) might be expected to represent, in terms of selectivity, uptake and detoxification/elimination, a rather distinct process compared to subsequent uptake by higher trophic levels. To understand this higher-level transfer, therefore, it is necessary to evaluate the role of toxin derived from primary consumer with respect to secondary (and subsequent) consumers. In a particularly elegant example, Karjalainen et al. (2005) experimentally demonstrated uptake of NOD by planktivorous fish larvae (i.e. Northern Pike) and invertebrate (i.e. mysid shrimp, *Neomysis integer*) via pre-exposure of relevant zooplankton prey to the toxin both in pure form, and as cell-free extracts of *N. spumigens* (as would be representative of the toxin released by decaying blooms). Prior studies had shown that zooplankton accumulate NOD directly from water (Karjalainen et al., 2003). In these subsequent studies, equal amounts of NOD (approximately 0.20 ng produced by an individual per 24 h) were detected in fecal pellets of pike larvae - suggesting diet derived uptake and passage of the toxin - fed zooplankton exposed to both pure toxin and NOD-containing extracts. Moreover, the authors of the study utilized radiolabeled NOD (i.e. 3H-dihydronodularin) to quantify the transfer of the toxin from zooplankton to planktivores. Indeed, radiolabel was detected in both *N. integer* and pike larvae fed zoo-
plankton, previously exposed to $^3$H-NOD, with a maximum calculated accumulation of the toxin at 12 h (0.31 ng/individual) and 48 h (0.47 ng/individual), respectively, for the two species. Levels of NOD calculated based on these studies, however, were quite low (approximately 0.12% and 0.03%, respectively, for shrimp and fish larvae) compared to the amounts predicted based on measured ingestion rate and concentration of toxin in zooplankton. These results are, therefore, generally consistent with a proposed biodilution, rather than biomagnifications, of this toxin within this food-chain. Furthermore, although ingestion rates were quite different (i.e. more than 5-fold higher) for fish larvae compared to shrimp, both accumulated rather similar levels (i.e. concentration per weight or individual) of the toxin. Accordingly, these studies point to both a difference in the type of potential planktivore vector (i.e. fish versus invertebrate systems), as well as a likely role of uptake and/or detoxification (and subsequent elimination) of the toxin in relation to the observed biodilution.

In addition to understanding physiological, cellular and molecular aspects of potential grazers/predators, uptake and detoxification can also be closely tied to the chemistry of the toxin. Certainly, among the cyanobacterial toxins, the potential for uptake, and subsequent detoxification/elimination, might be expected – due the chemically diverse nature of these compounds – to vary considerably with this chemical variability. This is most obviously exemplified by the distinction of so-called “hepatotoxins” (e.g. CYN, MCs) that, as implied by this classification, and unlike other cyanotoxins (e.g. PSTs, ATX-a, BMAA), are actively transported via characterized organic anion transporter (OAT) proteins to hepatocytes for subsequent detoxification/elimination. However, even with toxin families, variability in uptake and elimination has been reported. For example, in studies on the uptake of MCs, Xie et al. (2004) compared relative distribution following dosing with two common variants, namely MC-LR and MC-RR, in phytoplanktivorous silver carp. Interestingly, dietary exposure to MC-LR and MC-RR (in algal cells) resulted in considerably higher levels of the latter distributed in various tissues, but rather limited tissue concentration/distribution of the former, and more toxic, variant (Xie et al., 2004). Moreover, detection of relative amounts of the two variants in gut and feces specifically supported an apparent barrier to uptake of MC-LR, compared to the less toxic MC-RR (Xie et al, 2004). It should be pointed-out, however, that results in this phytoplanktivorous model differ substantially from similar studies in a generally carnivorous fish model, namely rainbow trout, particularly with respect to apparently rapid uptake of MC-LR by the latter species, and consequently suggest a role of both consumer species, and differential species physiology, relative to the potential for uptake (and subsequent trophic transfer). Finally, uptake (and subsequent trophic transfer) may even be determined, in part, by chemical presentation of the toxin. For example, it has been shown that in a benthic grazer model, namely the snail, Lymnaea stagnalis, that MCs are more readily taken-up from ingested cyanobacterial cells compared to dissolved toxin (Lance et al., 2010a). Furthermore, concurrent studies comparing the fate of MC-LR presented in either dissolved or cell-bound form (Lance et al., 2010b) suggested that, whereas no toxin from ambient/dissolved dosings was found covalently bound in tissues, as much as 67% of cell-derived MCs were accumulated via covalent binding (to targeted PPase enzymes) representing a potentially considerable reservoir of the toxin for subsequent trophic transfer (as discussed further below).
The potential for trophic transfer is not likely limited to the most studied cyanobacterial hepatotoxins (i.e. MCs). Although relatively few studies have evaluated their bioaccumulation, the transfer of the neurotoxic STX/PSTs, for example, has been studied with respect to its accumulation in marine animals, and specifically in relation to non-cyanobacterially (i.e. marine dinoflagellate) derived toxin. As a particularly important vector for PSTs, several filter-feeding mollusks are recognized to accumulate toxin-containing algal cells, and represent a possible route for both direct exposure (i.e. “shellfish” consumption), as well as possible indirect exposure to these toxins (i.e. trophic transfer to, and consumption of, secondary consumers/vectors, e.g. fish). There is, in fact, emerging (albeit currently limited) evidence to suggest that these neurotoxins may be transferred, to some extent, from filter-feeding invertebrates to higher trophic levels. In particular, however, these studies suggest that biotransformation via metabolism of PSTs – represented, as a group, by as many as fifty variants (Wiese et al., 2010) - may be a critical consideration. In studies by Kwong et al. (2006), black sea bream were exposed to green-lipped mussels, previously exposed to the PST-producing dinoflagellate, Alexandrium fundyense. Through these studies, it was generally found that relatively little PST was transferred to the fish, and that the toxins were rapidly depurated after transferring to toxin-free mussels. Moreover, toxin profiles suggested considerable biotransformation, and particularly conversion of C2 to C1 variants (see 2.2.2. Saxitoxins and “Paralytic Shellfish Toxins,” above). Likewise, subsequent studies (Costa et al., 2010) using white seabream, similarly exposed to PST-contaminated cockles, confirmed the rather low uptake of the toxin, and additionally reported an apparently selective uptake/elimination and/or biotransformation such that only B1 and dcSTX were found in fish. This conversion and/or selective uptake/elimination during trophic transfer would have clear implications for subsequent bioavailability of this neurotoxin, and associated health concerns, as there is considerable variability in the toxicity of the PST congeners. Most generally, these studies point to the importance of the vectors (e.g. fish versus shellfish) for the toxin.

Before moving on, to consider bioavailability, perhaps the one exception to the observed pattern of biodilution, which consequently bears discussion, appears to be the trophic transfer of BMAA. In the limited studies that have investigated BMAA in marine and freshwater foodwebs, it was shown, in fact, that levels of the toxic amino acid were higher for top trophic levels (e.g. predatory fish) compared to lower trophic levels (e.g. predatory fish) compared to lower trophic levels (e.g. Brand et al., 2010; Jonasson et al., 2010). In a recent study, for example, Jonasson et al. (2010) examined BMAA within food webs of the Baltic Sea, and reported a discernible positive correlation between levels of the toxin and trophic level. It seems likely, though, that the pattern is not quite as simple as classic biomagnification. For example, the Jonasson et al. (2010) and other studies (e.g. Brand et al., 2010) also suggest particularly high levels for benthic versus pelagic species (of both vertebrates and invertebrates). These studies also suggest differences in tissue distribution of the toxin with highest levels of BMAA observed in brain compared to, for example, muscle, and, therefore, underscore the importance of feeding ecology within food-webs, as well as the likely important role of subsequent bioavailability (including uptake and metabolism) and tissue distribution of toxins, in regards to trophic transfer.
4.2. Bioavailability

Just as selection, chemical availability, uptake and detoxification/elimination would determine trophic transfer of cyanobacterial toxins through food-webs, these factors are, likewise, expected to primarily determine the bioavailability of these toxins to human as would be ostensibly considered – with respect to the current discussion of emerging public health concerns – the “top predator” in this regard. Although not perhaps, strictly speaking, a “bioavailability factor” selectivity with respect to human consumption can certainly contribute to the potential for exposure to food-borne cyanotoxins. In a general sense, several Tiers of selectivity can dictate the likelihood of exposure (in concert with other bioavailability factors) to cyanobacterial toxins in food. As mentioned previously, the generally limited consumption of freshwater fish and shellfish, relative to much more common consumption of fish and other seafood from marine sources, would be expected – given the recognized abundance of cyanobacterial toxins in freshwater systems – to, likewise, generally limit the possible exposure to these toxins. Even within freshwater systems, the relative consumption of fish and shellfish species from lower trophic (i.e. phytoplanktivorous) levels of food webs would similarly contribute to the possible exposure. However, as detailed above (3. Evidence for bioaccumulation of cyanobacterial toxins in aquatic food-webs), there are certainly numerous documented cases of toxin bioaccumulation by phytoplanktivorous species of freshwater fish and invertebrates (e.g. snails, bivalves) which are, indeed, consumed by humans. Finally, even with species, and particularly fish species, selectivity of certain tissues/organs can influence possible exposure scenarios. Most notably, with respect to this latter tier, the general preference for fish flesh (i.e. muscle) versus viscera (e.g. toxin-accumulating liver, etc.) has clear implications for the potential for exposure to these toxins. Selectivity aside, however, the real issue of bioavailability is clearly expected to be most closely linked to those biochemical and physiological processes of digestion (i.e. uptake) and possible detoxification/elimination.

Cyanobacterial toxins, as discussed previously (see 2. Recognized cyanobacterial toxins: chemistry and toxicology), have been traditionally classified based on their “target” organs. Specifically, the most commonly studied cyanotoxins have been grouped into those targeting either the liver/hepatocytes or brain/CNS, respectively, in the case of the so-called “hepatotoxins” (i.e. MCs, CYN) and “neurotoxins” (i.e. STX/PSTs, ATX-a, BMAA). Based on both evaluation of toxin distribution (e.g. high levels of MC and CYN in livers of exposed animals), and recognized manifestations of toxicity (e.g. neurotoxicity of STX/PSTs and ATX-a), following exposure, effective bioavailability to these organs is largely assumed. In the case of the MCs, however, active transport of the toxin to hepatocytes has actually been shown to be specifically
facilitated by a family of organic anion transporter polypeptides (OATPs) that are particularly abundant in these cells (Fischer et al., 2005; Lu et al., 2008; Fischer et al., 2010), and even suggested to play a role in the selective uptake of certain MC congeners by hepatocytes (Fischer et al., 2010). That said, OATPs are, in fact, found in other cell types, and it has been also been suggested, for example, that OATPs in the brain may allow passage of MCs across the blood-brain barrier (Fischer et al., 2005), and they have, accordingly, been linked to oxidative stress in neurons, and subsequent effects on short and long term memory, caused by the toxin in a rat model (Maidana et al., 2006). Although, likewise, considered a hepatotoxin, and found to accumulate primarily in hepatocytes, the mechanism for CYN is not currently known. On the other hand, studies of neurotoxic cyanotoxins have demonstrated the apparent ability of STX/PSTs, ATX-a and BMAA to cross the blood-brain barrier (BBB) as a fundamentally limiting step for all toxins that affect the CNS. For example, STX - as a representative PST - was detected through brain tissues (from sacrificed animals), and consequently suggested to cross the BBB, following both intravenous (Andrinolo et al., 1999) and intraperitoneal (Cervantes Cianca et al., 2007) in mammalian (i.e. cat, rat) models. In support of the implied passage to - as suggested by its purported toxicity, and measured presence in the brain - studies of BMAA, dating back more than twenty years, and well prior to the recent resurgence of interest in this putative neurotoxin, not only have shown that this unusual amino acid is capable of crossing the BBB, but that transport might be specifically facilitated by large neutral amino acid carriers at the blood-brain interface (Smith et al., 1992).

Although the potential for bioavailability of cyanobacterial toxins to target organs is implied by their patterns of bioaccumulation, and observed toxic effects on certain organ systems, as well as limited number of in vivo studies, most studies have focused on either exposure to toxins via water ingestion, or in the case of laboratory studies, have examined fate of the toxin, following intraperitoneal injection, or related forms of administering the toxin. The actual bioavailability, with respect to foodborne toxins, is obviously limited by the prior chemical availability (discussed further below), as determined by release (from food), uptake and detoxification/elimination, prior to transport to target organs. To-date, however, studies on uptake and detoxification/elimination of cyanobacterial toxins derived from foods are essentially non-existent.

Rather, as with other aspects of health concerns regarding cyanobacterial toxins, the very few studies that have considered bioavailability of these toxins – and implicitly uptake and detoxification/elimination as key factors - have generally relied on data, and subsequent inferences, extrapolated from water-borne cyanotoxins, including dissolved or algal cell-derived toxins. Most notably, several authors have considered World Health Organization (WHO) guidelines regarding acceptable concentrations of MCs - as the clearly most widespread cyanobacterial toxin family - in water, and subsequently derived guideline values for total daily intake (TDI) of this toxin. Values of TDI are generally based on observed no or lowest observable adverse effect levels (NOAELs and LOAELs, respectively) from very a very limited number of oral exposure studies in mouse and pig models (Falconer et al., 1994; Fawell et al., 1999). Accordingly, acceptable values for lifetime, one-time and occasional TDI have been estimated, respectively, as 0.04, 25 and 0.4 µg per kg body weight.
(Fromme et al., 1999; Ibelings and Chorus, 2007). In a particular thorough treatment, Ibelings and Chorus (2007) extrapolated this to proposed guideline values that incorporate exposure via both water and food (and particularly “seafood”). Acknowledging a high variability in the amounts of food consumed, as well as other relevant factors (e.g. body weight), the guidelines estimated in this way ranged greatly from 6 µg/kg for daily lifetime exposures to 1900 µg/kg for acute (i.e. “one-time”) exposures for adults, with corresponding lower values for children (i.e. 0.08-250 µg/kg body weight). That said, all such values regarding intake (and, implicitly, the necessary consideration of subsequent uptake and detoxification/elimination) are, as mentioned, solely based on (very limited) estimates derived from oral exposure to a single toxin in water, and out of necessity, ignore bioavailability from a more complex “matrix” of animal tissues. Of course, as more information is obtained with respect to the uptake, and subsequent detoxification/elimination, of toxins from animal-based diet, it is hoped that a more realistic understanding of the potential for food-borne exposure will emerge. In particular, a clearer understanding of these toxins in relation to the dietary matrix, and consequently those factors (e.g. digestion, cellular uptake) that determine fate is still needed.

Aside from considerations of uptake and subsequent detoxification/elimination, as it relates to bioavailability, it has become clear that, in certain cases, the potential (or lack thereof) for human bioavailability may be considerably affected by chemical availability. This has been specifically studied, to-date, in two cases: (1) irreversible, covalent binding of MCs to PPases targets; and (2) erroneous translation and consequent incorporation of BMAA into proteins. In the latter case, for example, it has been suggested that BMAA, as a non-essential amino acid, can be potential incorporated (via faulty translation mechanisms) into growing protein chains. Specifically, investigating this possibility, Murch et al. (2004) analyzed BMAA in cyanobacteria, and brains of patients who died of ALS/Parkinsonism dementia complex (along with cycads and flying foxes from Guam as suggested vectors for the toxin), both with and without prior acid hydrolysis. Measured levels of BMAA were on the order of 10- to 240-fold higher following acid hydrolysis, suggesting an apparent release of this amino acid from proteins in these samples. This finding, therefore, not only supported incorporation of BMAA into proteins as a mechanism of toxicity (see 1. Recognized cyanobacterial toxins: chemistry and toxicology, above), as well as possible limitations in the analytical methodologies applied to this toxin (discussed further below; see Methodologies for evaluating cyanobacterial toxins in food-webs), but furthermore, pointed to an “endogenous reservoir” of the toxin. Such a reservoir would specifically provide a means of “slow release,” of the toxin as possible mechanism for BMAA bioavailability, and would correlate with the generally late onset of these diseases. It is, of course, implied from these studies that BMAA bound in proteins in this way would, in fact, be readily available following peptidolytic digestion, however, this remains to be confirmed.

As discussed earlier in the chapter (see 1. Recognized cyanobacterial toxins: chemistry and toxicology), MCs are known to bind to PPases found ubiquitously in cells of all known organisms. In addition, however, to reversible binding to the active site of PPases, it has been shown (MacKintosh et al., 1995; Pereira et al., 2012) that the toxin, once in the active
site, will form covalent bonds (via Michael addition) between the Mdha (present in many MCs) and a cysteine (Cys273) found in the active site of Ser/Thr Type 1/2A PPases. Accordingly, it has been suggested that a portion of all Mdha-containing MCs might become bound in this way (Williams et al., 1997a and 1997b; Yuan et al., 2006; Suchy and Berry, 2012), and indeed, estimates - based on specific analysis of the bound toxin (see 5. Methodologies for analysis of cyanobacterial toxins in the food-web) - suggest a considerable pool of so bound MCs. In classic studies by Williams et al. (1997a and 1997b), for example, analysis of bound MC demonstrated that as little as 24% of MC administered (via i.p. injection) to salmon could be recovered by conventional solvent extraction and analyses, and likewise that as much as 10,000-fold more of the toxin, measured in Dungeness crab larvae, could be detected in the presumptively “bound form” compared to the “free form.” More recently, Hilborn et al. (2007) measured both free and bound MCs in dialysis patients exposed (through improperly treated water) to the toxin, and similarly measured significantly higher levels when total (i.e. free and bound) levels were compared to those of the unbound toxin (i.e. measured by solvent extraction and conventional detection, e.g. ELISA), and specifically that only approximately 8-51% of MCs were measured by the latter method, compared to the former.

A preponderance of evidence continues to suggest that bound MCs do, indeed, represent a considerable pool of the toxin, however, very few studies have investigated whether these bound MCs are, in fact, biologically available. To address this question, Smith et al. (2010) recently investigated the potential for digestive enzymes to release covalently bound MC from PPases. Whereas digestive proteases (e.g. trypsin, chymotrypsin, pepsin) were found, as expected, to effectively hydrolyze a control protein (i.e. angiotensin), they had no effect on the cyclic peptides (i.e. MC-LR and MC-LY). Furthermore, based on the assumption that protein-bound MCs could be partially released by these peptidolytic enzymes, the investigators synthesized four Cys-containing MC-oligopeptide adducts, specifically predicted for hydrolytic digestion of the PPase active site by these enzymes, and subsequently evaluated them for toxicity (i.e. inhibition of protein phosphatase). Although inhibition was reduced (compared to MC-LR alone) to approximately 58% for MC-peptide adducts - composed of the cyclic MC-LR covalently bound, via cysteine, to predicted tetra- and nonomeric peptide fragments - this residual biological activity supports the possible bioavailability of potentially toxic bound MCs following protein hydrolysis in the digestive system. Interestingly, concurrent studies (Zhang et al., 2010) evaluated the effects of cooking as an alternative mechanism for release of covalently bound MCs with respect to potential availability of the toxin. In these studies, it was specifically found that levels of MC-LR in carp (injected intraperitoneally with the toxin) were significantly higher (approximately 4-fold) in both muscle tissue and water following boiling, compared to lyophilization and subsequent solvent extraction only, and it was suggested that elevated levels were due to release of covalently bound toxin from these tissues. Although such studies do point to the possible chemical availability of covalent bound toxins, clearly further studies are needed to fully elucidate the possible bioavailability of these in relation to human exposure.
5. Methodologies for evaluating cyanobacterial toxins in the food-web

Techniques for chemical detection and quantitative analysis of cyanobacterial toxins have evolved alongside recognition of their potential health impacts. The majority of the previously established analytical methods (e.g. HPLC-UV, LC-MS, ELISA) have, therefore, primarily focused on the identification of toxin in algal cells and/or dissolved in water, with water (i.e. contamination of drinking water, recreational exposure) being an established direct route of exposure. As for these applications, analytical techniques applied to measurement of toxins in food webs have, likewise, generally focused on two approaches (Sivonen, 2008; Humpage et al., 2010). The first, and arguably most common - given the complex nature of these biological matrices (discussed further below) - has included a number of so-called “hyphenated methods” in which analytical separation, including liquid chromatography (LC) and capillary electrophoresis (CE), in particular, are coupled to one or more suitable detection/measurement technique, including UV absorbance, fluorescent derivatization/detection (FL), mass spectrometry (MS) and electrochemical detection. Alternatively, with the relatively recent commercial availability of enzyme-linked immunosorbent assay (ELISA) kits for several cyanobacterial toxins, as well as growing understanding of the toxicology of these metabolites - and thus development of several biochemical techniques (e.g. protein phosphatase inhibition assays for MCs) - these bioanalytical techniques have been also applied to the evaluation of cyanobacterial toxin in relation to food-webs and bioaccumulation (e.g. Lance et al., 2006; Berry and Lind, 2010; Berry et al., 2011; Berry et al., 2012). However, unlike detection of several non-cyanobacterial, marine algal toxins as contaminants of fish and seafood for which there are validated analytical techniques, there are presently no validated methods for evaluating cyanobacterial toxins in biological matrices.

Both of the aforementioned approaches present potential limitations, but generally speaking, it would be argued that the two consequently complement one another. In particular, ELISA-based methods have been somewhat criticized (Metcalf et al., 2000; Mountfort et al., 2005) as being potentially susceptible to non-targeted molecules (i.e. matrix components) in samples that may immunologically cross-react with antibodies, or alternatively not being able to distinguish more toxic variants among co-occurring congeners within toxin groups. As antibodies used in ELISAs are typically generated in relation to a particular representative variant of these toxins, relying on chemical similarity and cross-reactivity to detect other variants, they do not enable co-occurring congeners to be distinguished from total toxin concentrations. This latter limitation is perhaps best exemplified by the ELISA-based analysis of MCs. Although commercially available ELISAs for MCs exploit the generally conserved Adda moiety found in most variants (Fig. 2), MC variants lacking (or containing modified versions of) Adda have been reported (Namikoshi et al., 1990 and 1992; Sivonen et al., 1992; Oksanen et al., 2004), and would be missed in these analyses leading to some degree of “false negatives,” or at least possible underestimation of MC content. Alternatively, studies have shown that improper use of ELISA kits – as well as factors such as organic solvents, salinity and pH - can contribute to the potential for false positives (Metcalf et al., 2005). Additionally, it is recognized that toxicity of MCs varies with congener, and therefore the inability to distinguish particular variants, with respect to this relative toxicity, does not enable what is
essentially a proxy of “total MC” to be evaluated in terms of actual relevance to toxicity (Mountfort et al., 2005). As an alternative, enzyme assays - and particularly the various PPase inhibition assays developed for MCs (Mountfort et al., 2005) – are, in fact, capable of assessing cyanobacterial toxins based on relevant biological activity. However, such assays are typically not as sensitive as, for example, ELISA, and likewise may be susceptible to matrix components, as well as being unable to chemically distinguish particular toxin variants. That said, sensitivity of methods such as ELISA are generally higher than for most other methods, and moreover, although coupling analytical separation to detection (e.g. LC-MS) may enable identification of particular chemical variants, this is only applicable to those variant which are specifically targeted. In other words, even though LC coupled to tandem mass spectrometry (MS/MS) can, for example, selectively detect several common MC variants based on characteristic molecular ions, and subsequent “daughter ions,” without prior knowledge of the optimal parameters (i.e. parent/daughter ions, ionization energy, etc.) - and/or availability of suitable analytical standards - to use for other for other less common (or perhaps yet uncharacterized) variants, and their metabolic products, these would be generally missed by such an approach (Mountfort et al., 2005). Accordingly, a strategy which incorporates both approaches and their relative benefits (i.e. highly sensitive detection of “total” MC by ELISA, target-based bioassay and selective analytical separation and detection of individual MC variants) might be expect to provide the most comprehensive toxin profile.

In general, the obvious challenge posed in adapting analytical techniques, originally developed for water (and, to a lesser extent, algal) samples, to bioaccumulation in food webs is the relatively more complex matrix of biological specimens (i.e. animal tissues). Other components of these biological matrices can interfere with analyses by specifically requiring a higher degree of selectivity (to discern the analyte from other components of the matrix), and as well as leading to suppression of the detection response (e.g. suppression of ionization in MS). To some extent, these challenges are inherently addressed when coupling detection to optimized analytical separation (e.g. LC-MS) that essentially isolates components (e.g. as chromatographic peaks based on retention time, etc.), but has also been generally supplemented by sample preparation steps prior to analysis. In particular, sample preparation steps have included selective extraction (e.g. Metcalf et al., 2002; Msagati et al., 2006), solid-phase extraction (SPE; e.g. James et al., 1998; Metcalf et al., 2002; McElhiney and Lawton, 2005; Scott et al., 2009) and other so-called “clean-up steps” to remove these potential interfering chemical species. Of course, in the case of less stable toxins (e.g. ATX-a; discussed below), extensive sample work-up can chemically jeopardize the analyte leading to underestimation or even complete non-detection of these compounds; generally speaking though, most approaches require some degree of sample preparation prior to analysis, particularly when dealing with complex bio-matrices.

One of the particular challenges of a biological matrix is due to the lack of specificity of certain detection methods. For example, although none of the recognized cyanobacterial toxins discussed have a particularly specific chromophore, as to enable unambiguous detection, HPLC coupled to UV spectrophotometric detection has been successfully used – specifically based on shortwave UV detection and established chromatographic retention time, and in
conjunction with analytical standards – to detect and measure several cyanobacterial toxins in water samples (e.g. Harada et al., 1994; Gugger et al., 2005; McElhiney and Lawton, 2005; Berry and Lind, 2010). However, in more complex bio-matrices, this lack of a distinguishing UV chromophore, and potential for co-eluting non-targeted components of the matrix generally limit this approach. Similarly, although fluorescence derivatization – and subsequent fluorescence detection coupled to chromatography or other analytical separation techniques (e.g. CE) - has been used as a highly sensitive means of detection/measurement of cyanobacterial toxins (e.g. Harada et al., 1997; James et al., 1998), the derivatization chemistry frequently employed in these approaches exploit common functional groups (e.g. amines, carboxylic acids, dienes). As such, non-toxin analytes (e.g. peptides) present in biological matrices can be coincidentally derivatized and, by co-elution and/or simply poor resolution, interfere with identification of the analyte of interest. Possible overlap in analytical response is not limited to these analytical separation/detection techniques, and indeed, it has been suggested that for ELISA, antibody cross-reactivity with chemically related components of the matrix might, likewise, lead to non-selective detection, and erroneous results in analyses.

Even in the case of highly selective detection techniques, interference due to the bio-matrix can arise. This is particularly seen with quantitative analyses based on mass spectrometry, including LC-MS, and particularly the most commonly used (at present) method of electrospray ionization (LC-ESI-MS). Components of the sample matrix, including inorganic (e.g. pH, salts/ions) and organic (e.g. proteins and other biomolecules) components, can both interfere with ionization; the former can directly interfere with ionization, whereas the latter can indirectly effect ionization of the analyte, particularly through competitive ionization. In the case of MCs, for example, it has been shown that dissolved organic carbon, pH and ionic strength can suppress signal in LC-MS with the latter being the most significant (Li et al., 2010). More notably in relation to the present discussion, Karlsson et al. (2005) investigated the effect of a biological matrix with respect to the LC-MS detection of MCs and NOD in biological tissues, including aquatic invertebrates (i.e. Blue Mussels, *Mytilus edulis*), fish (i.e. Rainbow Trout, *Oncorhyncus mykiss*) and waterfowl (i.e. Common Eider, *Somateria mollissima*). In these studies, it was found that ion signal varied from 16-134% of the expected signal (from spiked toxin), suggesting a good deal of both ionization suppression and enhancement, which varied with toxin variant (i.e. six MC variants and NOD) and biological matrix. In related studies, investigating the use of an MC oxidation product (discussed below) for LC-MS analysis, Ott and Carmichael (2006) reported losses in signal strength of more than 41% for this analyte. More recently, Li et al. (2012) examined the effects of ionization suppression with respect to quantitative analysis of BMAA, as well as its non-toxic isomer, 2,4-diaminobutyric acid (DAB), by LC-MS/MS. Although considerable matrix effects were observed for DAB, there appeared to be no contribution to the BMAA signal. Notably, however, this optimized method was used to suggest that BMAA was not seemingly present in several cyanobacterial samples evaluated, and to support the growing indication that this putative toxin is not as widespread as previously suggested. Indeed, although matrix effects represent an analytical challenge, the effects of signal suppression (e.g. ion suppression) can generally be addressed by various strategies, including the use of appropriate internal standards and techniques of standard addition to assess the extent of this effect on quantitation.
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<th>Biochemical Methods</th>
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<td>ELISA</td>
<td>Cost (~$400-500/plate)</td>
<td>Highly sensitive (&lt; ppb)</td>
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<td>No chemical information, e.g. identification of chemical variants</td>
<td>Rapid/analyze multiple samples at once</td>
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<td>No toxicity information</td>
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<td>Relatively inexpensive instrumentation (i.e plate readers)</td>
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<td>“Target-Based” e.g. PPase inhibition</td>
<td>No chemical information, e.g. identification of chemical variants</td>
<td>Provides toxicity information</td>
</tr>
<tr>
<td></td>
<td>Somewhat lower sensitivity</td>
<td>Relatively inexpensive (reagents)</td>
</tr>
<tr>
<td></td>
<td>Low selectivity, e.g. false positives</td>
<td>Rapid/analyze multiple samples at once</td>
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<tr>
<td></td>
<td></td>
<td>Easy/little training required</td>
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<tr>
<td></td>
<td></td>
<td>Relatively inexpensive instrumentation (i.e plate readers)</td>
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<tr>
<td>Instrumental Analysis (i.e. analytical separation/detection)</td>
<td></td>
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<tr>
<td>HPLC/CE-UV</td>
<td>Low sensitivity</td>
<td>Provides some chemical information, e.g. can identify chemical variants (if standards available)</td>
</tr>
<tr>
<td></td>
<td>Low selectivity, e.g. false positives</td>
<td>Analysis can be automated</td>
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<td></td>
<td>Requires training</td>
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<td></td>
<td>Limited chemical information, e.g. can’t identify unknown variants</td>
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<td></td>
<td>Moderately expensive instrumentation</td>
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<td></td>
<td>Relatively slow/can’t analyze multiple samples at once</td>
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<td></td>
<td>Requires sample clean-up</td>
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<tr>
<td>HPLC/CE-FL</td>
<td>Requires derivatization</td>
<td>Highly sensitive</td>
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<tr>
<td></td>
<td>Somewhat low selectivity, e.g. false positives</td>
<td>Provides some chemical information, e.g. can identify chemical variants (if standards available)</td>
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<td>Requires training</td>
<td>Analysis can be automated</td>
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<td>Limited chemical information, e.g. can’t identify unknowns</td>
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<td>Moderately expensive instrumentation</td>
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<td>Requires sample clean-up</td>
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<tr>
<td>HPLC/CE-MS</td>
<td>Requires training</td>
<td>Provides chemical information, e.g. can identify chemical variants (if standards available), possible information regarding unknowns</td>
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<tr>
<td></td>
<td>Expensive instrumentation</td>
<td>Analysis can be automated</td>
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<td></td>
<td>Relatively slow/can’t analyze multiple samples at once</td>
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<tr>
<td></td>
<td>Requires (some) sample clean-up</td>
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*Table 2.* Limitations and advantages of different analytical techniques used for detection/measurement of cyanobacterial toxins.
Aside from issues specifically related to the complex biological matrices encountered in food webs, some of the same general challenges associated with quantitative analysis of cyanobacterial toxins in water are, likewise, associated with analysis of bioaccumulation. A common consideration, in this regard, includes a requirement of selectively to detect, and discern, isomers and chemically related congeners. Indeed, this is exemplified by all of the recognized cyanobacterial toxins. For example, both PSTs and the MCs belong to rather large families of chemically related, but structurally distinct, variants – specifically represented, at present, by as many as fifty, and more than ninety, reported variants, respectively - with equally variable toxicity for each. In both cases, especially toxic and common variants (i.e. STX and MC-LR) are most frequently considered as a proxies for these toxin families, however, it is known that several other congeners from these groups can, in fact, potentially contribute to toxicity, and moreover, due to differential uptake and bioavailability hold, likewise, variable potential with respect to bioaccumulation. This presents, as discussed above, a clear limitation to ELISA-based analyses of the MCs that is unable to distinguish specific contributions of individual congeners. Likewise, although LC-MS and related methods, which employ analytical separation prior to detection, are able (if sufficiently optimized) to chromatographically resolve/separate congeners, the requirement for established molecular ionization and fragmentation parameters to detect these variants (by mass spectrometry) limits which variants will, and will not, be detected. Although considerably less complex, on the other hand, both CYN and ATX-a, likewise, have structurally related congeners and/or structural isomers, including for homoATX-a and 7-epiCYN, respectively, that are found alongside the “primary” toxins. In both of these cases, the congeners are also associated with toxicity, and evaluation of their contribution with respect to food-borne toxins needs to be included. On the other hand, although only BMAA (among several structural isomers) has been reported as potentially toxic, it has been suggested that natural occurrence of numerous possible isomeric congeners, all sharing the same molecular mass (Fig. 9), may greatly confound mass spectrometric analysis of this toxic amino acid (Banack et al., 2010), and specifically it has been suggested that, due to this, the once considered widespread occurrence of this neurotoxin may, in fact, be considerably over-estimated (Jiang et al., 2012; Li et al., 2012).

Although many of the cyanotoxins are generally considered chemically quite stable, and thus persistent in the environment, stability has, likewise, been suggested to limit quantitative analysis in some cases - and particularly ATX-a as perhaps the most chemically labile of the known toxins from cyanobacteria. Indeed, ATX-a has an estimated (Stevens and Krieger, 1991) half-life of only about 1-2 days – or even as low as 4-10 hours - in solutions emulating relevant biological conditions, including sunlight (i.e. photolysis) and pH (i.e. acidification). Degradation products, moreover, are generally not considered toxic (Stevens and Krieger, 1991). This instability poses obvious challenges, and particularly the potential for this toxin being underestimated or even missed in chemical analyses, and may, in fact, contribute to the absence of any reports on its bioaccumulation in food webs. Accordingly, it is generally advised that appropriate precautions regarding sample collection, transport and storage be taken to minimize recognized factors (i.e. light exposure, pH), and methods have been developed to increase speed of analysis (e.g. Smith and Lewis, 1987), to minimize this factor in assessing the possible role of ATX-a in relation to food-borne health concerns.
As we learn more about the fate of cyanobacterial toxins in food webs, it is becoming increasingly recognized that at least two of the recognized cyanotoxins, namely MCs and BMAA, may present specific analytical challenges due to their specific chemical toxicology. As previously discussed (see 4.2 Bioavailability), in addition to reversible binding of the MCs to “targeted” PPases, covalent and thus effectively irreversible binding of these toxins, specifically via Mdha (Fig. 10), may lead to considerable underestimation of the total MC content as quantitative chemical analyses based on solvent extraction, and subsequent detection of the non-bound molecule, would generally miss these bound-forms of the toxin. Indeed, it has been estimated that more than 75% of MC-LR (as representative Mdha-containing variant) in exposed fish, and as much as 99.9% in exposed invertebrates (e.g. mussels), is effectively “tied up” by covalent, irreversible binding (Williams et al., 1997a and 1997b). Similarly, as also discussed previously, one of the proposed mechanisms of toxicity for BMAA is erroneous incorporation into proteins, leading to aberrant protein aggregates which are hallmarks of neurodegenerative disease (e.g. ALS, Alzheimers’ Disease) linked to this toxic amino acid. Accordingly, this bound pool of the amino acid would not be immediately available to subsequent analyses.

Figure 9. Isomers of the toxic amino acid, BMAA, suggested to potentially confound MS analysis.
relying solely on solvent extraction from the biological matrix. Considering this, several recent developments have focused on analytical strategies for including these “bound-forms” of the toxins in the overall assessment of their contribution to food-web bioaccumulation.

Perhaps the most elegant of these has been the development of the so-called “MMPB method,” based on detection of the Adda oxidative cleavage product, 3-methyl-4-methoxy-phenylbutanonic acid (MMPB), as a proxy for total (i.e. bound and unbound) microcystins. As stated previously, Adda is both a key component of the MCs with respect to their toxicity, and is, indeed, conserved in the vast majority of variants (Fig. 2). Taking advantage of this conserved moiety, Sano et al. (1992) developed an analytical technique, specifically based on Lemieux oxidation that utilizes permanganate/periodate oxidation to cleave MMPB from the pendant Adda of microcystins (Fig. 10). This analyte can be subsequently quantified by several techniques, including GC-MS (Sano et al., 1992; Williams et al., 1997a and Williams et al., 1997b; Suchy and Berry, 2012) and LC-MS (e.g. Ott and Carmichael, 2006; Yuan et al., 2006). As Adda is distal to the Mdha, and site of covalent linkage to the PPase active site, the liberated MMPB can be used as a proxy – with no otherwise known naturally occurring counterpart – for both bound and free MCs. In fact, when subsequently applied to a range of fish and invertebrates, it became clear both that a vast majority of MCs in exposed animals are not detected by analytical methods based on solvent extraction of the free toxin (suggesting a considerable loss of the exposure dose), and that significantly more of the toxin were detectable.
by the MMPB method (suggesting covalent binding as a likely “sink” for this lost toxin). Williams et al. (1997a and 1997b) most famously applied this method to the analysis of experimentally (i.e. intraperitoneally) exposed salmon, as well as samples of Dungeness crab larvae and mussels (*Mytilus edulis*). In these studies it was found, for example, that while nearly 100% of exposure dose (from *Microcystis* cells) could be recovered by the MMPB method, only approximately 0.1% of MC of the expected levels were detected by MeOH extraction coupled to a PPase inhibition assay (Williams et al., 1997a). Likewise, for i.p.-exposed salmon, approximately 34% recovery of MC by Lemieux oxidation, specifically coupled to GC-MS, was reported, compared to approximately 8% recovery for MeOH extraction and PPase inhibition assay, and similarly more than 10,000-fold higher MC was detected by the MMPB method compared to extraction/PPase inhibition in populations of crab larvae (Williams et al., 1997b).

More recently, the modified MMPB method (i.e. SPME-GC-MS detection of MMPB) was applied to evaluation of MCs in several fish species, including those consumed by humans, from various freshwater lake systems, and it was similarly found that this approach measured significantly higher levels of the toxin (5- to 40-fold higher) than those detected by ELISA (Suchy and Berry, 2012). Since initial development of the technique, the MMPB has been further refined to include improvements in the sample preparation steps, including SPE (Ott and Carmichael, 2006) and solid-phase microextraction (SPME; Suchy and Berry, 2012), as well as subsequent detection methods (e.g. LC-MS, and LC-MS/MS, versus GC-MS; Ott and Carmichael, 2006; Yuan et al., 2006; Neffling et al., 2010), and it has been applied to a range of matrices including assessment of human exposure (e.g. Yuan et al., 2006). Accordingly, the MMPB method represents a tremendous advancement to both our understanding of trophic transfer/bioavailability of this toxin, as well as our ability to assess its role in food webs and bioaccumulation with respect to public health.

Similar to the covalent binding of MCs to PPase targets, the possible incorporation of BMAA into proteins, and consequent potential for underestimation, has been addressed in recent – albeit much fewer - studies. Incorporation of BMAA into proteins, with respect to both analytical challenges, and its possible role in bioavailability and trophic transfer of the toxin, was first reported by Murch et al. (2004). In this study, acid hydrolysis (i.e. 24 h boiling in 6 M HCl) – to digest proteins, and release BMAA - coupled to subsequent HPLC-FL analysis measured levels of the toxin in cycad flours exceeding those previously measured, following simple solvent extraction (i.e. presumptively unbound BMAA), by as much as 90-fold. In the same study, BMAA in brain tissues from patients who had died of Alzheimer’s Disease were, likewise, analyzed following acid digestion, and similarly showed levels of the toxin that were 60- to 130-fold greater than those measured following solvent extraction (i.e. “free BMAA” only). The presence of this bound pool of BMAA in proteins was accordingly suggested to represent a previously unrecognized reservoir of the toxin available for both trophic transfer (i.e. following proteolytic enzyme digestion), and as a slowly released form of the toxin with respect to the recurrent damage and “latency period” observed in the onset of these neurodegenerative diseases (Murch et al., 2004). Moreover, these results point to a limitation in the prior analytical approach, and specifically a likely underestimation of the toxin in food webs. Accordingly, subsequent studies (Rosen and Hellenäs, 2008; Baptista et al., 2011; Cervantes Cianca et al., 2012) have undertaken development and validation – as well as application - of various
analytical techniques (e.g. HPLC-FL, LC-MS/MS and CE-UV) that incorporate acid hydrolysis as a means of characterizing “total BMAA” content. As these methods are validated, they will no doubt provide a key tool for evaluating both the ecology (e.g. trophic transfer within food-webs) and toxicology of this cyanobacterial metabolite, and clarify its possible role in human health.

6. Other cyanobacterial toxins and their bioaccumulation

A rather limited number of secondary metabolites produced by cyanobacteria – as discussed throughout this chapter (i.e. MCs, CYN, STX/PSTs, ATX-a and BMAA) – are generally considered, specifically based on association with documented intoxication events, as “toxins.” However, the blue-green algae are, in fact, widely recognized to produce a myriad of biologically active metabolites (Gerwick et al., 2001; Tan, 2007; Tan, 2010). Rather than toxins though, the majority of these chemically diverse, bioactive compounds have been identified as part of “drug discovery” efforts (Tan, 2007). That said, many of the biological systems used to prospect for potential pharmaceuticals – most notably perhaps including cytotoxicity as a means of identifying anticancer drugs – could clearly be extended to include those compounds (i.e. “cytotoxins”) which may have negative impacts on human health, as toxins, rather than, or in addition to, their intended therapeutic targets. Indeed, it has been argued (e.g. Berry et al., 2008) that many (or perhaps even most) of the bioactive secondary metabolites from cyanobacteria – including several investigated as drug leads - are likely produced as allelochemicals, serving a role as chemical agents for deterrence, specifically through their “toxicity,” toward other organisms.

Although limited to no literature exists, due to the nature of their discovery (as drug candidates), on either the potential for either toxicity or bioaccumulation of these many previously identified bioactive metabolites, indirect evidence suggests that as-of-yet unidentified metabolites do, indeed, contribute to toxicity of the cyanobacteria. Specifically, several studies (e.g. Pietsch et al., 2001; Kurmayer and Jüttner, 2009) have compared relative toxicity of pure cyanobacterial toxins to crude extracts, and consistently found higher degrees of biological activity for the latter suggesting an additive or even synergistic role of congeners in these mixtures, including both chemically related (i.e. variants within a toxin family) and/or potentially unrelated, and likely uncharacterized, metabolites. These finding, therefore, generally point to the higher toxicity of metabolic mixtures, and specifically suggest the possibility of unknown toxins which might be relevant to bioaccumulation – and, thus, consequent health concerns - within food-webs. Identification of these additional toxic metabolites will, therefore, be essential to a holistic understanding of the health effects of cyanobacterial toxins, including their potential contribution to food-borne toxicity.

Due, perhaps in part, to the nature of the biological assays used (e.g. cytotoxicity assays, enzyme inhibition assays) that rely on water-solubility of test compounds, the majority of the bioactive metabolites identified from blue-green algae as potential drug candidates have typically focused on rather water-soluble or polar compounds, and particularly the diverse
non-ribosomal peptides (NRPs) characteristic of the cyanobacteria (Welker and Von Döhren, 2006). Indeed, a few such NRPs have, in fact, been identified alongside recognized toxins, specifically based on potential relevance to chemical ecology. A particularly notable example is the identification of several chemically related, and apparently quite widespread, peptides – isolated, alongside MCs, from Microcystis - as protease inhibitors (e.g. microviridin J, aeruginosin) with demonstrated ability to serve as feeding deterrents to the zooplankton, Daphnia (Harada et al., 1993; Martin et al., 1993; Okino et al., 1993; Tsukamoto et al., 1993; Rohrlack et al., 2004; von Elert et al., 2005). As for MCs, very recent studies suggest a genotype x genotype interaction between toxin and grazer, and consequent development of tolerance to these protease inhibitors (Schwarzenberger et al., 2012; von Elert et al., 2012). Accordingly, these latter findings would open up the door to possible bioaccumulation, and possible human exposure, although to-date there have not been any studies to investigate this aspect within freshwater food-webs.

In marine environments, a particularly salient example is lyngbyatoxin, and severally chemically related cyclic peptides (e.g. aplysiatoxin, debromoaplysiatoxin), isolated from marine species of the genus, Lyngbya, a well known producer of several known cyanobacterial toxins (i.e. MCs, CYN, STX/PSTs). Although these former peptide toxins, specifically acting as activators of protein kinase C, have been associated with direct human exposure (i.e. dermatoxins) – as well as being investigated with respect to possible pharmacological potential – the toxins, found in filamentous cyanobacteria in marine shallows, have also been reported to be incidentally grazed by marine animals, including invertebrates – and particularly various species of gastropods that accumulate the toxin (Capper et al., 2005) - and vertebrates, most notably including sea turtles (Yasumoto et al., 1998; Arthur et al., 2008). Given the recognized toxicity to mammals (e.g. Ito et al., 2002), it is suggested that possible bioaccumulation, and subsequent consumption, could represent a route of human exposure and toxicity. In fact, as one of the few examples of acute toxicosis possibly associated with foodborne cyanotoxins, the presence of these toxins in turtle meat has been linked (Yasumoto et al., 1998) to several cases of human intoxication in the South Pacific and Madagascar (Hashimoto et al., 1967; Champetier et al., 1998) specifically characterized by ulceration of mucosal membranes, as well as apparent neurotoxic effects. It is also worth noting that these toxins have been hypothesized (Arthur et al., 2008) to explain chronic health effects – and specifically promotion of tumors associated with fibropapillomatosis – in exposed turtles, and these results, therefore, underscore the largely unstudied, possible contribution of these toxins to similar human health concerns associated with long-term, food-borne exposure.

Although the vast majority of bioactive secondary metabolites identified have, for reasons stated above, particularly included a number of peptides and other hydrophilic compounds, the cyanobacteria are known to produce a variety of lipophilic metabolites as well. In support of a possible role of these metabolites in relation to food webs, when evaluating the effects of toxic metabolites from the recognized toxigenic cyanobacterial species, Planktothrix rubescens, Kurmayer and Jüttner (1999) observed that lipophilic extraction reduced feeding deterrence of the cyanobacterial cells with respect to zooplankton grazers, and pointed to an uncharacterized lipophilic metabolite in this species. In more recent
studies, Berry et al. (2009) evaluated non-polar extracts of the CYN-producing species, *Aphanizomenon ovalisporum* and *Cylindrospermopsis raciborskii*, specifically using the zebrafish embryo model, and identified apparent lipophilic toxins, chemically unrelated to CYN or other water-soluble toxins produced by these species. It is not, therefore, surprising that considerable research in this area has identified numerous lipophilic metabolites ranging from alkaloids to lipopeptides (Orjala et al., 1995; Wu et al., 2000; Li et al., 2001; Edwards et al., 2004) to fatty acids (e.g. polyunsaturated fatty acid [PUFAs], Reinikainen et al., 2001) and seemingly simple hydrocarbons (Jaja-Chimedza et al., 2012).

Although much of the chemistry behind these observations remains to be characterized, one particularly well-studied group of lipophilic metabolites is a diverse family of indole alkaloids produced by members of the relatively widespread, but otherwise understudied, Stigonemataceae (e.g. Raveh and Carmeli, 2007; Mo et al., 2010; Kim et al., 2012; and many more). Although this chemically diverse, and taxonomically restricted, group of metabolites have been generally identified based on antimicrobial activity (e.g. Raveh and Carmeli, 2007; Mo et al., 2012), and in fact, have been linked to possible allelopathy (i.e. inhibition of photosynthetic microbial competitors) in their natural environment, several biological activities supporting animal toxicity have also been reported (e.g. inhibition of RNA polymerase; Doan et al., 2001). This finding, and their seemingly widespread occurrence, raises the question as to whether these quite lipophilic metabolites may contribute to toxicity within food webs, including perhaps human health concerns.

Similarly, considerable work over the past 15 years or more has identified a diversity of lipopeptides, particularly isolated from the widespread marine cyanobacterial species, *Lyngbya majuscula* (Orjala et al., 1995; Orjala et al., 1996; Li et al., 2001; Nogle et al., 2001; Edwards et al., 2004; Choi et al., 2010) and others (e.g. *Anabaena*; Kaya et al., 2002). Although bioactivity associated with these metabolites runs the gamut from inhibition of specific enzymes to cytotoxicity in mammalian cells to toxicity in a range of organisms (e.g. antifungal, molluscidal, ichthyotoxicity), potent neurotoxicity has, in particular, been associated with a number of these (e.g. Li et al., 2001; Nogle et al., 2001; Edwards et al., 2004; Choi et al., 2010). However, although a wealth of information regarding toxicity has emerged alongside their chemical discovery, essentially no insight as to possible bioaccumulation – despite the global abundance of these taxa, e.g. *Lyngbya*, in marine and freshwater habitats – currently exists.

More recently – following-up on prior studies (Berry et al., 2009; as discussed briefly above) – an apparently widespread group of toxic, lipophilic metabolites have been identified from several species of otherwise toxigenic cyanobacteria. Utilizing the zebrafish (*Danio rerio*) embryo as a vertebrate model of so-called developmental toxicity, and specifically as a means of bioassay-guided fractionation, Jaja-Chimedza et al. (2012), in very recent studies, identified a family of isotactic polymethoxy-1-alkenes (PMAs) from *Aphanizomenon ovalisporum*, specifically as lipophilic inhibitors of developmental pathways in this model system. Both this species, and the genus (i.e. *Aphanizomenon*), more generally, are recognized producers of CYN (genus/species; e.g. Preussel et al., 2006), as well as the neurotoxic STX/PSTs and ATX-a (species; e.g. Mahmood and Carmichael, 1986 and Ballot et al, 2010, respectively). The identification of PMAs as lipophilic metabolites in this species, including CYN-producing
strains (Berry et al., 2009), suggests that they may contribute to the overall toxicity of this widely distributed species, as well as - given the highly lipid-soluble nature of these compounds - the potential for their bioaccumulation, and even biomagnification, within food-webs. Moreover, prior studies have identified the same or similar PMAs from a wide range of cyanobacteria (e.g. Mynderse and Moore, 1979; Mori et al., 1991), although previous studies did not originally link their presence to potential toxicity (but rather identified them based on chemical characterization), and subsequent studies (Jaja-Chimedza et al., forthcoming), using the same toxicity, have identified them in otherwise recognized toxigenic species (e.g. C. raciborskii, M. aeruginosä). These emerging findings suggest that this group of metabolites may be widespread, and further underscore the potential role in food-derived toxicity. In fact, identification of closely related metabolites in marine sponges (Rama and Faulkner, 2002) has been attributed to a cyanobacterial biosynthetic origin/source (likely via filter-feeding of algal cells), and further support the potential for their uptake and accumulation in higher trophic levels. To-date, however, studies on their bioaccumulation (beyond this example) in food-webs remains to be addressed.

7. Implications for ecosystem health

Finally, although beyond the scope of this chapter (and volume), it is worthwhile to consider – before concluding our discussion with respect to public health implications of cyanobacterial toxins in food webs – the apparent contribution of cyanobacterial toxins in food webs with respect to animal and ecosystem health. Obviously, understanding the impacts of these toxins on animal health with respect to ecosystems is essential to understanding the potential for trophic transfer of these toxins. Moreover, insights regarding the potential for human health concerns of food-borne toxins – especially as contaminants of shared food-webs - can be often studied indirectly, and to some extent extrapolated, by understanding the role of these toxins in non-human animals. Indeed, with regards to the toxicity of cyanobacterial metabolites, the first reported case of intoxication by a cyanobacterial bloom was made based on animal health, and specifically reported livestock poisonings associated with pond scums (later identified as cyanobacteria) in a so-called “poisonous lake,” famously detailed in the pioneering works by George Francis in the late 19th century (Francis, 1878).

Given that current evidence generally suggests limited trophic transfer of most of the known cyanobacterial toxins, and consequent restriction of foodborne cyanobacterial toxins to lower trophic levels in food-webs, it is not surprising that toxicity to animals has particularly focused on those vertebrate (i.e. fish) and invertebrates which are exposed either directly (through phytoplanktvory), or through “single vector” (e.g. zooplanktvory), transfer. For example, the previously discussed studies of Karjalainen et al. (2005), not only demonstrated that pre-exposure of zooplankton to cell-free, NOD-containing extracts of N. spumigens – representative of the toxin released during blooms – resulted in subsequent bioaccumulation of this toxin in zooplanktivorous, larval fish predators, but also decreased (compared to unexposed controls) ingestion, growth rate and fecal production of the experimentally fed zooplanktivorous larvae of Northern Pike (Esox lucius), suggesting a possible toxic effect to these predators without
direct exposure to the toxin-containing water or algal cells. Interestingly enough, no such statistically significant effects was observed for larvae fed zooplankton which were pre-exposed to pure NOD, suggesting a possible contribution of other, currently unknown, toxins (Karjalainen et al., 2005).

Numerous studies, encompassing essentially all of the other known cyanotoxins, as well as yet uncharacterized, but apparently toxic, metabolites, likewise, have demonstrated the potential toxic effects to various aquatic species exposed at this lower end of the trophic scale. At the level of zooplanktivorous grazers, numerous studies have documented the apparent toxic effects of, not only MCs (as discussed above), but also other cyanobacterial toxins, including CYN (Nogueira et al., 2004), STX/PSTs (Filho et al., 2008) and ATX-a (Sieroslawska et al., 2010) - as well as perhaps other unidentified metabolites (present in extracts) - on Daphnia, and various other species. Likewise, biochemical and histopathologic analyses of both benthic grazers, particularly including snail species (Lance et al., 2010), and filter-feeding bivalves (Puerto et al., 2011; Sabatini et al., 2011), exposed to cell-bound toxins, including MCs and CYN, as well as unidentified toxic metabolites, demonstrate toxicity – along with apparent bioaccumulation – corresponding to uptake and transport pathways from the digestive system. Similarly, toxicity of several cyanobacterial metabolites to fish – and particularly several, representative phytoplanktivorous species (e.g. carp, tilapia) - exposed to dietary sources of these toxins has been well documented (e.g. Jos et al., 2005; Osswald et al., 2007; El Ghazali et al., 2010; Qiao et al., 2012).

On the other hand, relatively fewer studies to-date have clearly documented toxic effects of subsequent predation on toxin-laden invertebrates (e.g. zooplankton, benthic invertebrates), or phytoplanktivorous fish. However, the limited studies that have do generally point to the potential for toxicity – along with toxin transfer – to higher trophic levels. In one particularly notable study, Qiu et al. (2007) examined – using biochemical and histopathological approaches - four trophic levels, comprised of a phytoplanktivorous, omnivorous and carnivorous fish species in a Chinese lake in relation to a MC-producing bloom. Surprisingly in this case, the most pronounced histopathological signs of toxicity were observed for carnivorous fish, whereas the largest biochemical response (i.e. particularly the production of several antioxidant enzymes/pathway, e.g. superoxide dismutase, catalase, glutathione, glutathione peroxidase), were measured for phytoplanktivorous species. These results suggest not only that carnivores can be exposed to cyanobacterial metabolites, and toxic effects via food webs, but that grazers of cyanobacteria (i.e. phytoplanktivores) may, as such, be specifically adapted to the direct exposure to these toxins.

The toxic effects of cyanobacterial metabolites on higher trophic levels, aside from fish, in aquatic food webs remain quite scarce, despite the fact that numerous taxa, including bird and mammalian species, are recognized as frequent “top consumers” in these systems. However, examples are beginning to emerge in the literature. In an especially insightful example, Miller et al. (2010) recently reported on the apparent toxic effects - including several animal deaths among - among southern sea otters, along the Pacific coast of the U.S., exposed to MCs bioaccumulated by bivalves (i.e. clams, mussels and oysters) consumed by these carnivorous predators. Following an unusually high number of sea otters
deaths in the Monterey Bay, and surrounding coastal areas, particularly during the period of 2005-2008, necropsy on these stranded animals was performed. Based on the detection of relatively high levels (up to 348 ppb) of several variants of microcystins, including MC-RR, -LR and -desmethyl LR, in livers of sea otters, along with gross and microscopic pathological indications - particularly in livers of the animals - consistent with MC intoxication, it was concluded that animals had, indeed, died from exposure to this cyanobacterial toxin. The source of the toxin was ultimately traced to outflow from the nearby Pinto Lake into the marine waters of Monterey Bay, and subsequent bioaccumulation of the toxin by mollusks, consumed by sea otters, in the Bay. Characterized as a “super-bloom” of cyanobacteria, levels of MC in Pinto Lake, during this time, were measured as high as 2,100 ppm (more than six orders of magnitude higher than the WHO limit of 1 ppb), and use of Solid Phase Adsorption Toxin Tracking (SPATT) samples enabled tracking of the toxin from the lake toward the Bay. Subsequent laboratory studies confirmed the bioaccumulation of MC - at levels as high 1,324 ppb - by various bivalve (i.e. clam, mussel, oyster) species that make up a primary component of the sea otters’ diet. Indeed, this case is particularly revealing as it not only supports hypothesis that higher trophic levels – including mammalian carnivores - can, in fact, be exposed to toxic (and even lethal) levels of cyanobacterial toxins through food-webs, but also that toxins can transfer not only within ecosystems, but between (in this case, freshwater and marine) systems. Moreover, although levels of MCs, in this case, were exceptionally high, this study further provides – through quantification of the toxin in (livers of) exposed animals – a first estimation of relevant (i.e. lethal) exposure doses for mammalian consumers in relation to environmental concentration both in water, and vectors (i.e. bioaccumulation in bivalves) of the toxin.

8. Conclusions

Cyanobacteria are prolific producers of toxic, and otherwise biologically (i.e. pharmacologically) active, metabolites. Although ubiquitous in the environment, the cyanobacteria are arguably most conspicuous, and generally more abundant, in aquatic systems, particularly in association with so-called “harmful algal blooms.” As such, exposure to several cyanobacterial toxins through contamination of drinking water, and related routes, has been clearly linked to both acute toxicoses, including human and animal mortalities, as well as being increasingly tied to several long-term health effects (e.g. cancer, neurodegenerative disease). Consequently, waterborne cyanotoxins are widely acknowledged as a global health concern.

Given this particularly widespread occurrence of the “blue-green algae” in marine and freshwater ecosystems, it is not, therefore, surprising, that bioaccumulation of nearly all of the “recognized” cyanobacterial toxins within relevant species of aquatic food webs has been reported. In light of the enormous human reliance on the world’s oceans and freshwater systems, particularly as a source of food (i.e. fish, seafood), as well as culminating evidence to suggest that global climate change is fueling an apparently rapid increase in cyanobacterial abundance, bloom frequency and perhaps even toxigenicity in aquatic systems, cyanobacterial toxins in food webs, likewise, represent a clearly important human health issue.
As an emerging concern, however, clearly more questions than answers remain, at present, regarding the potential role of cyanobacterial toxins in food webs, particularly in terms of possible human and environmental health concerns. Although a preponderance of evidence indicates that diverse taxa of aquatic animal species do, in fact, accumulate cyanotoxins, the biochemical, physiological and ecological processes that control trophic transfer within food-webs remains to be clarified. Likewise, although trophic transfer of the largely water-soluble “known” cyanobacterial toxins appears to follow a pattern of biodilution, rather than biomagnification to top consumers, a growing number cases indicate that species, known to be consumed as part of human diets, do bioaccumulate significant quantities of these toxins, yet the potential implications for human health remains to be elucidated. This, it is argued is due, in part, to the lack of information regarding bioavailability, as well as limitations and challenges in current analytical methodologies used to assess this contribution.

Although the scientific evidence that does exist strongly suggests a potential (and, in fact, high probability) for human exposure to, and consequent health concerns associated with, food-borne cyanobacterial toxins, the implications with respect to public health policy remains almost entirely to be addressed. Indeed, while our scientific understanding of the bioaccumulation of cyanobacterial toxins in relation to human health currently remains rather limited - particularly relative to many other environmental health concerns – the public health implications, including relevance to policy makers and stakeholders, lags even more so. And, in fact, it is asserted that it is many of the same gaps in knowledge that limit our scientific understanding which, likewise, limit public health policy with regards to food-borne cyanotoxins, and thus addressing these gaps will be critical in this regard. First, and perhaps foremost, is the preeminent need to understand the toxicology of food-borne cyanotoxins, including both laboratory and (currently non-existent) epidemiological studies to elucidate what (if any) health effects exist, and which groups are most likely affected. Similarly, clarifying the actual health effects, including relevant doses, mechanisms of action, bioavailability, etc., will be fundamental to developing effective regulatory guidelines. Although, as described above, attempts have been made (e.g. Ibelings and Chorus, 2007) to extrapolate current (albeit limited) toxicological knowledge in this regard to possible acceptable levels for food-borne cyanotoxins, these are based entirely on data from water-borne toxins, and are not likely to be accurate in terms of exposure through food. Furthermore, even the provisional guidelines that exist for cyanobacterial toxins in water are only recommendations, and policy will not only need to clarify acceptable levels, but also address monitoring and enforcement of these guidelines. As such, improvements, validation and standardization of methods for chemical analysis of cyanobacterial toxins – toward effective monitoring and enforcement - in food will be key. Continued investigations in these areas will, therefore be of the crucial toward developing a comprehensive picture of this emerging public health concern.
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References


tion of microcystins derivatized with a highly fluorescent dienophile. *Natural Toxins*, 5, 201-207.


toxin cylindrospermopsin, and its recovery from environmental eutrophic waters by solid-phase extraction. *FEMS Microbiology Letters*, 216, 159-164.


Rosen, J., & Hellenäs, K. E. Determination of the neurotoxin BMAA (beta-N-methylamino-L-alanine) in cycad seed and cyanobacteria by LC-MS/MS (liquid chromatography tandem mass spectrometry). Analyst, 133, 1785-1789.


