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Current Techniques for Detecting and Monitoring Algal Toxins and Causative Harmful Algal Blooms

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Abstract

The detection and monitoring techniques for algal toxins and the causative harmful algal blooms (HABs) are essential for the protection of aquatic lives, shellfish safety, drinking water quality, and public health. Toward the development of fast, easy, and reliable techniques, much progress has been made during the last decade for the qualitative and quantitative analysis of algal toxins. This review highlights the recent progress and new trends of these analytical and monitoring tools, ranging from in-situ quick screening protocols for the monitoring of algal blooms to mass spectrometric analysis of trace levels of various algal toxins and structural elucidation. Solid-phase adsorption toxin tracking (SPATT) deployed in the field for the passive sampling of algal toxins has been recently validated, and improved ELISA-based methods with lower detection limits for more toxins have become commercially available for both screening and routine monitoring purposes. Liquid chromatography-mass spectrometry with several recent mass spectrometric innovations has expanded our understanding of traditional toxins, their metabolites along with newly discovered toxins of ecological importance. Several established *in vivo* and *in vitro* bioassays will continue to be used as benchmark toxicological testing of algal toxins; however, newly emerged molecular probing techniques such as real-time quantitative polymerase chain reaction (qPCR) have extended our ability to trace algal toxins from causative organisms at the molecular level. New chemical and biological sensors, lab-on-chip and remote sensing of blooms being developed will hold promise for early warning and routine monitoring to better manage and protect our freshwater, coastal and marine resources from adverse impact by harmful algal blooms.

Keywords: Emerging contaminant; Cyanobacteria; Phycotoxin; Microcystin; Biotoxin; HABs

Introduction

The detection of algal toxins and monitoring of causative harmful algal blooms (HABs) in marine and freshwater have become an increasingly important task globally in the recent decades [1,2]. Increased frequency of occurrence and expanded spatial extent of HAB incidents have been reported particularly in countries with frequent eutrophic fresh and coastal waters [2]. HABs refer to toxin producing algae as well as non-toxic blooms of microalgae (seaweeds), which can adversely impact habitat alteration and oxygen depletion [1]. It is widely acknowledged that HABs can result in fish kills, food poisoning, and public health issues from contaminated drinking water. However, an accurate account of its growing economic loss due to HABs is still not available. The total annual economic loss related to eutrophication and freshwater HABs was estimated to be US$2.2–4.6 billion in the U.S., and US$105–160 million in England and Wales [3,4]. A massive bloom in 2007 in Lake Tai cost over US$16.25 billion [5], and the cost to manage the impact of the green microalgae *Ulva prolifera* bloom during the summer 2008 exceeded US$100 million [6]. In the U.S alone, the monitoring and management due to HABs from 1987 to 1992 averaged $50 million per year [7].

Harmful algal blooms belong to five phyla of algae that produce toxins [8]. At least 60 marine species of microalgae and 20 freshwater and brackish water species of cyanobacteria are known to attribute toxins. These toxins belong to a variety of categories; each has its own structural analogues or congeners. For example, the most frequently reported microcystins (MCs) alone have more than 80 congeners isolated and characterized to date [9]. Similarly, more than 20 saxitoxin (STX) congeners, and 9 congeners of brevetoxin (PbTx) have been reported [10].

Algal toxins are emerging contaminants of public health significance [11]. The U.S. congressional legislation mandated the establishment of a National Research Plan for Coastal HABs, but no similar plan exists for freshwater HABs [3]. Surface water drinking supplies are particularly vulnerable to the growth of these toxin-generating organisms; however, current U.S. drinking water treatment practices do not actively treat for blue green algal toxins including the microcystins [12]. No regulatory methods have been established in the U.S. for the mandatory analysis of algal toxins in drinking water. Nevertheless, algal toxins, or specifically cyanotoxins, are in the Contaminant Candidate List 3 (CCL 3) among 116 chemical and microbiological contaminants based on a contaminant's potential to occur in public water systems and the potential for public health concern. Three cyanotoxins naturally produced and released by cyanobacteria (blue-green algae) were suggested: anatoxin-a, microcystin-LR, and cylindrospermopsin (CYL) (http://water.epa.gov/scitech/drinkingwater/dws/ccl/ccl3.cfm). The WHO recommended limit of 1 µg/L for MC-LR in drinking water.

From the analytical perspective, environmentally relevant concentrations of algal toxins are important considerations prior to

the adoption of any developed method. Table 1 lists the environmentally relevant concentrations reported recently for several algal toxins in selected water bodies of different regions worldwide. With the vast number of algal toxins in numerous HAB incidents, it is prudent not to give generalization but the spatial and temporal variations in the concentrations of algal toxins are clearly delineated. It is also evident that various algal toxins might predominate in different water bodies. Seasonal variations of MCs in the tidal freshwater segment of the James River Estuary, for example, occurred with first peak in May, maximal concentration in July and August, and undetectable after November, concurrent with the abundance of *Microcystis* and the *mcy*D genes responsible for MC generation [13]. The concentrations observed in various freshwater and marine water bodies ranged from undetectable at the ng/L to lower ng/L and could reach up to several hundreds of µg/L. Caution should be exercised for direct comparisons of the algal toxins among various waters because of the monitoring time relative to the algal bloom event. Even within the same water body, measured concentrations could be method dependent. Results from Bláhová et al. [14-24] indicated that although concentrations determined by enzyme-linked immunosorbent assay (ELISA) and liquid chromatography-mass spectrometry (LC-MS) showed good quantitative agreement, concentrations determined by ELISA were systematically higher than concentrations determined by LC-MS, which was attributed to matrix effects (both in ELISA and LC-MS) and ELISA cross-reactivity with other unidentified derivatives of toxin CYL (Table 1).

Water	Toxins	Causative Genera	Methods	Concentration Range	Ref
James River Estuary, Chesapeake Bay, Virginia, USA	MCs	Microcystis	ADDA- ELISA	< 0.10 µg/L (May), 0.92 µg/L (July), < 0.05 µg/L (Nov)	[13]
Monterey Bay, California, USA	MCs	Microcystis	LC-MS/MS	0.02–0.17 µg/L	[15]
Gulf of Alaska, USA	DA	Pseudo-nittzschia	cELISA	0.0002–1.4 ng/L	[16]
Hamilton Harbor, Lake Ontario, Canada	MCs	Microcystis	HPLC	60–400 µg/L	[17]
Wendt Beach, Lake Erie, Canada	MCs	Microcystis	HPLC	< 1 µg/L	[17]
Moreton Bay, Australia	PTX-2/ GD/OA	Dinophysis, Pseudo-nittzschia	LC-MS/MS	0.1–1.1/0.06–0.3/0.04–0.2 µg/L	[18]
Lakes in Czech Republic, Central Europe	CYL	Cylindrospermopsis	ELISA & LC-MS	0.4–4 µg/L (ELISA) vs. 0.01–0.3 µg/L (LC/MS)	[14]
Guanting Reservoir, Beijing, China	MCs	Microcystis	HPLC	< 1–1.15 µg/L	[19]
Coasta water of Qingdao City, China	OA /PTX-2	Marine algae	HPLC-MS /MS	1.41–89.52 ng/L/<1.70 ng/L	[20]
Wuli Lake and Meiliang Bay, Tai Lake, China	MC-LR/MC-RR/MC-YR	Microcystis	HPLC-ESI-MS	4.33–12.27 µg/L/8.36–16.91 µg/L/1.41–5.57 µg/L	[21]
Reservoirs in Brazil	MCs/ CYL	Planktothrix, Microcystis, Geitlerinema	ELISA	10.3–836280.0/0.5–2718.0 ng/g	[22]
Lambert's Bay, South Africa	STX/OA/DA	Alexandrium/Dinophysis /Pseudo-nitzschia	LC-FD	48/0.012/0.46 µg/L	[23]
Gotlandsea, Baltic Sea	ND	Nodularia	LC-MS	149–804 µg/L	[24]

Table 1: Algal toxins and their environmentally relevant concentrations in selected waters of various regions; CYL = cylindrospermopsin; DA = domoic acid; GD = gymnodimine; MCs = microcystins; ND = Nodularin; OA = okadaic acid; PTX-2 = pectenotoxin-2-seco acid; STX = saxitoxin; ADDA-ELISA = ADDA ELISA Kit; LC-MS/MS = liquid chromatography-tandem mass spectrometry; cELISA = competitive enzyme-linked immunosorbent assay; HPLC = high-performance liquid chromatography; HPLC-MS/MS = high-performance liquid chromatography-tandem mass spectrometry; HPLC-ESI-MS = high performance liquid chromatography - electrospray ionization-mass spectrometry; LC-FD = liquid chromatography with fluorescence detection.

The scope of this review is to highlight recent development and trends in detecting algal toxins and causative algae that pose both economic and environmental threats. Owing to the extensive number of reported work on the monitoring and analysis of algal toxins, we limited our review to representative papers mostly published after 2000 that were not included in two prior reviews [10] in 2001. Unlike two recent excellent reviews [25,26], this review is not intended to give an exhaustive list of all reported work, rather we attempted to offer a strategic sampling and analysis and trends for the detection of algal toxins as well as the field monitoring of HABs. Due to the unpredictable association between algal toxins and HABs [27], the monitoring of both toxins and causative species are needed. The

published papers selected are more relevant to surface and drinking water; however, most chemical and biological methods discussed in this review should be relevant to other sample matrices including phytoplankton and shellfish of significant economic values.

Monitoring and Analytical Challenges for Measuring Various Trace Algal Toxins

The detections of algal toxins as emerging contaminants are hampered by their low concentrations from µg/L to the ng/L (Table 1). The required detection limit lower than WHO's standard of 1 µg/L precludes the use of many UV-based HPLC methods without much effort in sample concentration and clean-up of other UV-absorbing interfering chemicals in the sample matrices. A large number of known and unknown congeners of algal toxins in surface and drinking water also make it hard for chromatographic separation. In some cases, the mixture of multiple congeners with different properties requires very different chromatographic conditions for needed separation, including reverse phase to ion-pair or ion-exchange chromatography.

Hummert et al. (2002) were able to develop a LC-MS method to simultaneously analyze several classes of algal toxins in a phytoplankton extract using a single solvent (50:50 (v/v) methanol-water [28]. The method developed by Dahlmann et al. [29] enabled simultaneous quantification of 7 toxins with a single chromatographic run within 30 minutes using a single quadrupole MS.

Table 2 illustrates a range of physicochemical and toxicological properties for several selected algal toxins, including the most commonly occurring algal toxins such as microcystins (MCs), nodularins (NOD), anatoxins (ANTX), cylindrospermopsin (CYL), and saxitoxins (STX). The high molecular weight hepatotoxic MCs and NOD are cyclic peptides, and ANTX, CYN, and STX are heterocyclic alkaloids. While ANTX and STX are neurotoxic, CYL is hepatotoxic [30]. In addition, the "red tide" toxins include neurotoxic brevetoxins (PbTx), which has heterocyclic polyether structures. Most of these toxins have relatively high-molecular weights and are highly hydrophilic and even ionic (polar). Certain algal toxins are lipophilic, including pectenotoxins (PTXs), yessotoxins (YTXs), azaspiracids (AZAs), and ciguatoxin (CTX), with low but highly variable log Kow values dependent of pH. As a result, most toxins with low log Kow values will elute fairly quickly in reverse phase HPLC and better resolution of chromatographic separations can be achieved with well-adjusted pH in the mobile phase.

Algal Toxin	Abbr.	Formula	MW	Soluble in Water/Lipid / log Kow Value	Toxic Syndrome	LD50 (µg/kg)
Anatoxin-a	ANTX	$C_{10}H_{15}NO$	165	Water / 1.12	NSP	25,000
Brevetoxin B	PbTx	$C_{50}H_{70}O_{14}$	895	Lipid	NSP	200
Ciguatoxin	CTX	$C_{60}H_{86}O_{19}$	1111	Lipid	CFP	0.25–0.9
Cylindrospermopsin	CYL	$C_{15}H_{21}N_5O_7S$	415	Water	hepatotoxin	52,000
Domoic acid	DA	$C_{15}H_{21}NO_6$	311	Water / -2.43 at pH5.3	ASP	120
Microcystin-LR	MCs	$C_{49}H_{74}N_{10}O_{12}$	995	Water / 1.49 / -1.1 / -1.76 (pH 2, 7, 10)	hepatotoxin	32.5
Nodularins	NOD	$C_{41}H_{60}N_8O_{10}$	825	N/A	hepatotoxin	50–500
Okadaic acid	OA	$C_{44}H_{68}O_{13}$	805 (ave)	Lipid	DSP	200
Saxitoxin	STX	$C_{10}H_{17}N_7O_4$	299	Water	PSP	10

Table 2: Physicochemical and toxicological properties of selected algal toxin; anatoxins = ANTX; CTX = ciguatoxin; CYL = cylindrospermopsin; DA = domoic acids; MCs = microcystins; NOD = nodularins; PbTx = brevetoxin; STX = saxitoxins; ASP = amnestic shellfish poisoning; CFP = ciguatera shellfish poisoning; DSP = diarrhetic shellfish poisoning; NOD = nodularins; NSP = neurotoxic shellfish poisoning; PSP = paralytic shellfish poisoning; MW = molecular weight; Kow = octanol - water partition coefficient; LD_{50} = half lethal dose. Source: (a) Kow values from De Maagd PGJ, Sijm DTHM (2005); Falk M, Seto PF, Walter JA (2011), Canadian J Chem 69(11):1740-1744; http://www.chemspider.com/. (b) LD_{50} values based on i.p. mouse from Yan T, Zhou M-J (2004), Biomed Environ Sci 17: 165-76; http://en.wikipedia.org/wiki.

The structural diversity and complexity further preclude the accurate quantitation and structural confirmation for new algal toxins of ecological and toxicological relevance. Many existing methods employed LC-MS with triple quadrupole mass spectrometers (MS/MS) to enhance the sensitivity and selectivity of toxins, which has become an essential tool for the search of new algal toxins. Apart from the need of such expensive mass spectrometers which are currently not available in most laboratories for routine monitoring, many algal toxins do not have commercially available standards, and in other cases, toxin standards were prepared from a minute amount from natural sources or unidentified source of unknown purity, making it impossible for accurate quantitation [31]. Burton et al. [32] examined the use of external algal toxin standard for quantitative measurement by ^{1}H-NMR of solutions contained in separate but identical sealed precision glass NMR tubes. This approach is particularly suitable for algal toxin calibration standards for subsequent use with LC-MS and other techniques where deuterated solvents should be avoided and safe handling is required.

Apart from the challenges in chemical analysis, the challenges for monitoring harmful algal blooms also arise from the difficulty in determining causative algae species in the event of algal blooms. The numbers of cells alone do not necessarily indicate the presence of

toxins [18], and the toxigenic algae may represent a small portion of the algae bloom population. Additionally, effective implementation of bloom mitigation strategies demands real-time monitoring tools in the time-scale of minutes to hours [33]. These point to the need of innovative techniques with fast response and field deployable sensors that can monitor algae species and toxin on site.

New Sampling and Sample Preparation Techniques for Algal Toxins

For most work involved in algal toxins, conventional grab sample collection followed by laboratory clean-up and analysis still remain to be the common approach by researchers and monitoring crews. Nevertheless, considerable progress has been made toward the use of passive sampling for time-integrated concentrations of algal toxins. MacKenzie et al. [34] introduced the first application of passive sampling in algal toxins using a device termed solid-phase adsorption toxin tracking (SPATT), which is conceptually similar to semi-permeable membrane device (SPMD) or polar organic chemical integrative samplers (POICS) that have already been used for other trace contaminants in water. The SPATT consists of bags sewn from polyester mesh containing activated polystyrenedivinylbenzene resin, which can adsorb lipophilic toxins dissolved in water. Like any other passive samplers, SPATT provides time-averaged algal toxin concentration prior to, or during algal blooms. This device was later improved by designing the frame in which the HP-20 resin is retained using disks between two layers of nylon mesh, and clamped tightly in the embroidery frame so as to form a thin layer of resin between the layers of mesh [35]. The SPATT collects relatively clean sample matrix which simplifies subsequent extraction and analysis using ELISA or LC-MS. The results of SPATT in several field studies have been described [36,37], implying its potential for use as an early warning for the onset of algal blooms.

Less work has been done on the adsorption of polar algal toxins such as the more polar STX and domoic acids (DA). The commercially available POCIS devices (www.est-lab.com) have not been tested for polar algal toxins to date [36]. Rodríguez et al. [38] compared a computationally designed polymer (CDP, based on the functional monomer ethylene glycol methacrylate phosphate) with a synthetic resin adsorbent (SEPABEADS SP700) for use in SPATT system. Results showed that CDP appears to be more appropriate for higher PSP (paralytic shellfish poisoning) adsorption, whereas SP700 adsorb both PSP and DSP (diarrheic shellfish poisoning). Both types of toxins can be employed for early warning for the monitoring of HABs. New sorbents for optimal sampling of toxins will continue to be developed. The suitability of a range of polymeric and lipophilic sorbents was examined for passive sampling of marine toxins. Their systematic evaluations also indicate the usefulness of the commercially available polymeric Oasis HLB and Strata-X sorbents in laboratory and field studies for various microalgal toxins. It was concluded that Strata-X and Oasis HLB are fast accumulators and better for daily or on-board evaluation of toxin presence, whereas HP-20 should be more appropriate for long exposure period (>5 days) [39].

The adsorbent-based solid phase extraction (SPE) and SPATT have become the preferred device for the concentration of analytes at the trace level. SPE, commonly used in sample clean-up, was also reported as a concentration method to enrich a large amount of high purity toxins and their metabolic products from the marine and freshwater environments. Pre-concentration and clean-up using SPE improves the detection limit from ppm to the sub-ppm level for HPLC-UV [40] and from sub-ppb to sub-ppt level for LC-MS/MS [41]. A large-scale pump of seawater was employed for concentration purpose; however, this sampling approach is prone to problems including cartridge clogging, long procedure steps and use of larger volumes of extraction solvents particularly for samples rich in chlorophyll pigment and other organic matrices [42]. To circumvent these issues, a supported liquid membrane based technique was explored as an alternative sample clean-up method for subsequent LC-ESI-MS of four MC variants (MC-RR, -YR, -LR, and -WR) from lyophilize algal cells [43].

Established and Newly Developed Bioassays for Algal Toxins

In vivo and *in vitro* bioassays for algal toxins provide toxicologically relevant information and reveal to some extent the causative agents responsible for the toxic action. They are suitable when a measure of total toxic potency is desired. Unlike direct chemical analysis of toxins, bioassays do not require extensive sample preparation. Although most bioassays are less expensive and do not need proficient personnel and equipment, the lower sensitivity than the instrumental methods is the major technical drawback. In a broad sense, the *in vitro* bioassays can be grouped into antibody-based immunological assays and receptor-based functional assays, with the latter further categorized into enzyme assays using purified enzyme, receptor assays using tissue homogenates, cytotoxicity assays using live mammalian cells, and molecular probing techniques [10,44]. The classical *in vivo* mouse bioassay is the established benchmark technique used as a regulatory method for toxin analysis. Along with other modifications using brine shrimp and fishes, the *in vivo* methods are less sensitive, selective, and quantitative to toxins. The intraperitoneal administration does not reflect natural exposures while requiring a large number of animals [45], which is commonly considered as socially undesirable. The enzyme assays using protein phosphatase PP1 or PP2A can be based on radiological, colorimetric or fluorometric, with the latter showing higher sensitivity and correlation with HPLC method (r=0.74) [46], although its application to natural samples have not been fully tested [10]. At present, the *in vitro* assays are still used mostly in research-based investigations for the purposes of identification of algal culture, shellfish, marine mammals, and human exposure to algal toxins. Three areas of technical advancement are noteworthy, i.e., the ELISA, cytotoxicity tests, and the molecular probing techniques, which are elaborated below.

The immunoassays are quick, inexpensive, and easy for screening program. Another preferred feature of ELISA compared to LC-MS is that ELISA could detect covalently bound algal toxin such as MCs, whereas LC-MS relies on the availability of congener standards and the proportion of non-covalently bound MC in the sample [47]. Its technical limitations are self-evident, such as the lack of selectivity and the occurrence of cross-reactivity due to the high variability of compounds that might be found in water samples. These limitations have also been the subject of extensive research and recent progress. One particular constrain is its lower sensitivity limited relatively to a narrow range of toxins. Consequently, it has the potential to give false negative for insensitive toxic congeners [45], false positive at low concentration [48] and overestimate some specific toxin congener concentration [47]. ELISA tends to give false positive detection at the low concentration range (0–0.15 μg/L), even though some good correlations were demonstrated between ELISA and HPLC for toxins in surface and drinking water [48,49].

Many recently developed ELISA and related bioassays achieved detection limit at the sub-ppb level which is well below the 1 µg/L limit proposed by the WHO (Table 3, and [25,26] for the detection limits of several commercial ELISA kits). As can be seen in Table 3, as low as 6 ng/L detection limit for MC-LR was achievable using a monoclonal antibody. The antibody was produced using an immunogen synthesized by a novel coupling chemistry to bind MC-LR via dehydroalanine to the carrier protein [50]. A rapid and sensitive (linear range: 0.1–3 µg/L) ELISA method was validated for the detection of domoic acid in environmental samples, which allows for the analysis of as few as 3 or as many as 36 duplicate samples within 1.5 hr in a standard 96-well format [51]. An ELISA-like time-resolved fluorescence immunoassay (TRFIA) was developed based on anti-MC-LR monoclonal antibodies (MAbs) and europium-labeled antimouse Ig conjugate. The MAbs showed a good cross reactivity with MC-LR, MC-RR and MC-YR, and a wide quantitative range between 0.01 and 10 µ/L MC-LR was achieved with the TRFIA performed at an indirect competitive mode [52] (Table 3).

There are some commercially available kits such as Ridascreen™ test kit (R-Biopharm, Darmstadt, Germany) for PSP toxins, DSP-check kit (UBE Industries, Tokyo, Japan) and Rougier Biotech ELISA (Montreal, Quebec, Canada) for DSP toxins, ADDA ELISA kit for MCs and NOD (Abraxis, Warminster, PA, USA), and Cigua-Check for CTX toxins (Oceanit Test System, Hawaii, USA) [10,13,26,53,54]. More methods are being developed toward commercial ELISA kits in detecting various algal toxins. For example, immunizing and plate coating antigens were prepared by derivatizing YTXs and conjugation to protein. The polyclonal antibodies have a broad specificity for many of the known YTX analogues [55].

Method Name	Toxins	Matrix	Detection Level	Detection Level Type	Bias	Precision	Spiking Level	Method Source
cELISA	DA	Sea water	10 ng/L	MDL	N/A	N/A	N/A	[16]
cdELISA	MC-LR	Algal culture	10 µg/L	MDL	83.7% Rec	9.9% (RSD)	10–500 µg/L	[53]
cdELISA	MC-LR	Drinking water	6 ng/L	MDL	86–116%	N/A	0.01–0.1 µg/L	[50]
cidELISA	MCs and nodularins	Raw water, drinking water	0.02–0.07 µg/L	LOQ	N/A	<<20% (RSD)	N/A	[45]
ELISA	MC-LR	Groundwater and surface water	0.1–0.15 µg/L / 0.2 µg/L	MDL / LOQ	80–150% Rec	10% (RSD)	0.04–0.05 µg/L	[48]
TRFIA	MC-LR	Water	0.01–10 µg/L	QR	N/A	1.6–12.2%	N/A	[52]

Table 3: Detection limit, accuracy and precision of selected bioassays capable of detecting trace levels of primary algal toxins; cELISA = competitive ELISA; cdELISA = competitive direct ELISA; cidELISA = competitive indirect ELISA; TRFIA = ELISA-like time-resolved fluorescence immunoassay; MC = microcystin; MC-LR = microcystin-leucine and arginine; DA = domoic acid; MDL = method detection limit; LOQ = limit of quantitation; QR = quantitative range; Rec = recovery; N/A = not available; RSD = relative standard deviation.

Cell-based assays employ live mammalian cells, which are based on cellular response that incorporates both binding to the receptors and subsequent intracellular signaling responses of the cell. Cellular assays rely on a number of end-points, including morphological change, or more commonly cytotoxicity and reporter gene activation [10]. A rainbow trout gill cytotoxicity assay (RCA) detected lake water toxicity of multiple toxins, but was unable to reproduce toxicity following exposure to toxin or noxious compound standards [56]. This bioassay was insensitive to toxins and only sensitive to noxious compounds at concentrations exceeding reported environmental averages (EC50 ≥10 µM). The toxicity was also confounded by other bio- and abio-factors such as taxa, growth stage, location (intracellular more toxic than extracellular), and iron status. An *in vitro* rat hippocampal slice preparation was used as a means of rapidly and specifically detecting the marine algal toxins STX, PbTx, and DA through toxin-specific electrophysiological signatures [57]. Field test demonstrated that this slice preparation reliably detected STX in a linearly responsive fashion at toxin concentrations of 25–200 nM, and tests of naturally contaminated shellfish confirmed the utility of this assay as a screening method for PSP. Another high sensitivity bioassay of PSP and ASP at the nM level was developed based on the fluorimetric detection of $[Ca^{2+}]$ in rat cortical primary cultures under the electrical field stimulation [58]. The haemolysis of sheep erythrocytes was used as a rapid and sensitive method to detect palytoxin in water samples, a good correlation was found between haemolytic assays and the total toxin content measured through HR-LC/MS [59]. Cell-based mouse neuroblastoma assay (MNA) performed favorably over the mouse bioassay because of its higher sensitivity and less time (4 hr vs. 48 hr) [60]. Similarly, red drum erythrocytes were used to detect hemolytic toxin of crude algal extracts from the Gulf of Mexico [61].

Based on the detection of housekeeping genes in toxigenic algae and the genes related with the synthesis of their toxins, molecular probe technique is another field witnessed a significant progress with the rapidly increased GenBank database in the last decade [25,26]. A detailed account of molecular methods for the detection of cyanotoxins in environmental samples is beyond the scope of this review, but can be found in [26]. The molecular probes replacing the traditional microscope are often the antibodies or a short segment of DNA that are specific for the HAB species of interest [1]. Oligonucleotide probes have been employed to identify HAB species using short, synthetic DNA that selectively binds to sequences specific to a target organism. Their use in targeting rRNA in HAB species has been approached in several ways, including whole-cell hybridization, sandwich hybridization and polymerase chain reaction (PCR)-based methods [62]. Hybridization of target rRNA from HAB cells to immobilized probes on the microspheres was visualized using Cy3-labeled secondary probes in a sandwich-type assay format, and a detection limit of 5 cells for all target organisms were determined within 45 minutes [62]. Microarray, PCR and more recently quantitative real-time polymerase chain reaction (qPCR, first-

commercialized in 1997) have become the emerging techniques for the detection and quantification of low concentration microorganisms including toxin-producing algae [26,63]. Using most-probable-number PCR (MPN-PCR) and five distinct bont gene-specific primers, the abundance of *Clostridium botulinum* in algal mats was quantified and the type of botulism neurotoxin (bont) genes associated with this organism was determined [64]. In a study on the cyanobacteria abundance and MC toxin in a shallow lake in Vancouver, WA, USA, qPCR was shown to be useful in probing toxin-producing gene (*mcy*E) from a cyanobacteria with low abundance, complementary to traditional methods with microscopical counts, ELISA and PCR results [65]. The traditional PCR-based methods, although they are capable of detecting DNA/RNA at low concentrations, their precision may be compromised due to a number of factors such as reagent depletion, completion of amplicons with primers, and the loss of polymerase activity as the number of amplification cycle increases [63].

Recent Development in Instrumental Analysis of Algal Toxins

Chemical instrumental analysis has its superior capability in detecting trace levels of toxins in comparison with other traditional non-chemical methods. For example, in a surveillance testing of algal toxins in shellfish from Scottish waters, LC-MS detected the presence of 63% of the shellfish analyzed, in contrast to 24% using the Jellett Rapid Test and only 5% based on mouse bioassay [66].

Most of the early methods in the 1980s employed HPLC (e.g., [67]) for the detection of algal toxins in both water and shellfish samples, because most algal toxins have UV-absorbing chromophores (e.g., a maximum UV absorption at 238 nm for most MCs and NODs [26]). Typically, algal and shellfish samples are extracted with methanol followed by C18 reverse phase HPLC equipped with UV-diode array detector (DAD) [17,68]. The HPLC method, however, can be interfered by other UV-absorbing chemicals present in sample matrix. In labs without a mass spectrometer or in cases when confirmation analysis is not the goal, the UV or fluorescence detectors can still be used for algal toxin analysis which demand less sensitive and selective determination particularly in simple matrix such as drinking water or cleaned tissue extract such as shellfish samples [17,69]. Derivation will improve the detection by enhancing UV absorption or fluorescence emission signals of the parent algal toxins. For example, DA collected from SPE was derivatized by 4-fluoro-7-7-nitro-2,1,3-benzoxadiazole [70]. This derivatized product is subject to sensitive fluorimetric HPLC quantification, analyte recovery, repeatability and detection limit achieved 89%, 6.2% and 120 ng-DA/L, respectively (Table 3). There were also other studies aimed at improved or novel HPLC methods including the use of amperometric HPLC [71], capillary electrophoresis (CE) and capillary electrochromatography (CEC) for the analysis of DSP, ASP, and MCs [72].

Structural studies entail the extensive use of both NMR and mass spectrometry. Mass spectrometric is superior in acquiring low detection limit up to ng/L or lower (Table 4). Apart from its superior quantitation, mass spectrometry is capable of definite structural identification. In particular, the rapid technical progress in LC-MS on the MS front has offered the unprecedented capability in detecting more toxins at the much lower concentrations. The tandem MS (MS/MS) developed approximately 15 years ago significantly improved our traditional MS methods with two stages of mass analysis – one to pre-select an ion and the second to analyze fragments induced. With less reliance on the chromatographic separation, the use of LS-MS/MS also facilitates the tedious sample preparation and clean-up. LC-MS techniques offer multidimensional resolution of complex mixtures allowing distinguishing compounds in overlapping chromatographic peaks. The triple quadrupole (TQ) with atmospheric-pressure ionization sources (API) has been used for routine trace organic contaminant analysis in many research labs. The TQ mass spectrometers isolate a selected ion and to collisionaly induce fragmentation, thus eliminating any potential interferences from the sample matrix, mobile and stationary phases. Tandem MS is ideal for the simultaneous analysis of concurrent presence of multiple toxins in water and shellfish [66], including the measurement of covalently bound toxins (the form that is assimilated into the food chain) [17] which was unlikely to be differentiated by ELISA and traditional HPLC. LC-MS/MS has therefore become the norm and the standard method for algal toxin for both quantitative and qualitative measurement – providing extremely low detection limit and unequivocal and definite structural information to search for new toxins. LC-MS/MS has allowed the detection of spirolider (a cyclic imine toxin), 20-methyl spirolide G in Norwegian shellfish and planktons samples [73]. Combined with the use of chemical degradation and derivatization, MS/MS was able to detect several toxic peptides from blue-green algae at the nanomole level, including two additional toxins that were thought to belong to a family of seven-residue cyclic peptides, a cyclic imine toxin, having the general structure cyclo-D-Ala-L-Xaa-erythro-,B-methyl-D-isoaspartic acid-L-Yaa-Adda-D-isoglutamic acid-N-methyldehydroalanine, where Xaa and Yaa represent variable amino acids of the L configuration and Adda is 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid [74].

More recently, quadrupole ion trap (QIT) and hybrid quadrupole/time-of-flight (TOF) instruments have extended the possibilities of structural identification. TOF enables accurate molecular weight (MW) to be determined, allowing for exact MW match suitable for non-target analysis. In light of the most LC-MS requirement for extensive extraction and clean-up for complicated matrices, the use of laser ablation electrospray ionization (LAESI) with MS/MS could further eliminate sample extraction or clean-up so that DA in mussel tissue homogenates can be directly detected with a detection limit of 1 mg/kg, and recovery of 103–125% [75]. Combined with the use of NMR, many new algal toxins have been identified and their structures have been characterized in the recent years. For example, LC-MS3 was performed on an LCQ Deca ion trap mass spectrometer fitted with an ESI interface and coupled to HPLC-photo-diode array detector (PDA). Two new pectenotoxins, 36S-PTX-12 and 36R-PTX-12 in Dinophysis spp, occurred as a pair of equilibrating diasteroisomers that were different from PTX-2 [76]. With the use of alkaline hydrolysis, several groups of new conjugates of okadaic acid (OA) and dinophysisoxins-2 (DTX2) in seawater were identified [77]. A C8-dio ester, a C9-dio ester, and new C8-triol ester of OA were characterized using QIT with multiple stages of mass spectrometry (HPLC-MS^2, MS^3, and MS^4) in combination with various derivatization procedures. Using collision-induced dissociation / post-source decay matrix-assisted laser desorption / ionization-time-of-flight mass spectrometry (CID/PSD MALDI-TOF), 13 MCs were identified, including three new variants of microcystins [46]. Unlike LC-MS, however, MALDI-TOF-MS directly analyzes dried and solid microbial samples but it cannot be used for quantitation and it requires skillful experts and expensive equipment [25,26].

Method Name	Toxins	Matrix	Detection Level	Detection Level Type	Bias	Precision	Spiking Level	Ref
MRM-LC-MS/MS	MCs / CYL	Lake water	2–9 ng/L / 0.3 µg/L	LOQ	93–103% Rec	8% (RSD)	50–500 ng/L	[79]
LC-MS/SIM	MC	Sea water	0.1 µg/L	MDL	N/A	N/A	N/A	[15]
LC-HRMS	Ovatoxin; palytoxin	Sea aerosol	1.6–3.13 µg/L / 3.13–6.25 µg/L	LOD / LOQ	75% Rec	N/A	N/A	[80]
MRM-LC-MS/MS	DA	Sea water	1.2 ng	MDL	N/A	N/A	N/A	[16]
LC-API/MS	OA / DTX-1, PbTx-2, Pbtx-3, DA	Phytoplankton extract	1 /1.3 / 5 / 6 / 15 ng	LOD	104.5% Rec	4.2% (RSD) , 5% (SD)	N/A	[28]
LC-MS/MS /(ESI-MRM)	BSXs; KBTs	Sea water, algal culture	2–5 µg/L /5–200 µg/L	LOQ / linear range	70% Rec	20%	N/A	[81]
LC-ESI-MS	STX, DA, anatoxin-a, NOD, MCs, OA, DTX-1	Phytoplankton extract	0.5–1 ng / 1 – 50 ng	LOD / linear range	96–114% Rec	3.9–7.1% (RSD)	N/A	[29]
HILIC-MS/MS	STX	Algal samples	3 µg/L / 11 µg/L	LOD / LLOQ	99.9% Rec	N/A	10 µg/L	[82]
MRM-LC-MS/MS	DA	Sea water	30 ng/L	MDL	90% Rec	5%	N/A	[83]
MRM-LC-MS/MS	MC-RR and conjugates	Fish plasma and bile extract	6–12 ng/L / 15–22.5 ng/L	LOD / LOQ	81–94% Rec	2–11% (RSD)	0.02 µg/L	[84]
MRM-LC-MS/MS	MCs, NOD	Lake water	2 ng/L	MDL	70–114% Rec	20% (RED)	N/A	[85]
SPE-HPLC-FLD	DA	Sea water	120 ng/L	MDL	89% Rec	6.20%	N/A	[70]
LDTD-APCI-MS/MS	MCs	Lake water	0.1 / 0.9 µg/L	LOD / LOQ	103% Rec	15%	N/A	[86]

Table 4: Detection limit, accuracy and precision of instrumental methods for the detection of trace levels of selected algal toxins; APCI = atmospheric pressure chemical ionization; API = atmospheric pressure ionization; ESI = electrospray ionization; FLD = fluorescence detector; HILIC = hydrophilic interaction liquid chromatography; HRMS = high resolution mass spectrometry; LDTD = laser diode thermal desorption; MRM = multi-reaction monitoring mode; SPE = solid phase extraction; BSXs = brevisulcatic acids; CYL = cylindrospermopsin; DA = domoic acid; DTX-1 = dinophysistoxin 1; KBTs = brevisulcenals; MC-RR = microcystin-arginine and arginine; NOD = nodularin; OA = okadaic acid; PbTx-2 = brevetoxin 2; PbTx-3 = brevetoxin 3; STX = saxitoxin; LOD = limit of detection; LOQ = limit of quantitation; LLOQ = lower limit of quantitation; MDL = method detection limit; N/A = not available; RSD = relative standard variation; SD = standard deviation.

Table 4 also includes the GC-based mass spectrometry in algal toxin analysis. Under the GC operating condition, peptide bonds in proteinaceous material can be cleaved. Although GC-MS or GC-MS/MS are rarely used for high molecular weight algal toxins without derivation, it was reported for their potential in direct detection of a thermally stable hepatotoxin CYN (5 ppm detection threshold) based on the presence of diagnostic ions using conventional pyrolysis (Py-GC/MS) and thermally-assisted hydrolysis and methylation (TCh-GC/MS) [78]. The GC method has the disadvantage of the production of a variety of secondary by-products.

In Situ monitoring of Harmful Algal Blooms and Algal Toxins

Beyond the traditional visual confirmation of water discoloration, fish kills, and laborious cell counts, new technologies for bloom monitoring and tracking span a wide range from the large scale using satellite remote sensing to the smallest scale of "molecular probes" [1,79-87]. These new technologies stem from the need for real- or near real-time simultaneous detection of HAB species and their toxins such that surface water and coastal resource managers can promptly mitigate their economic, ecological, and environmental impacts, including providing the timely warning of approaching HABs [33,88]. Some of the bioassays and instrumental methods discussed previously can be adapted into the tools for in situ real-time monitoring. For example, with a reported detection limit of 8.8 cells per mL of *Microcystis* spp., qPCR holds promise as a valuable quantification tool in identifying the blooming sources and establishing the proportion of toxic and non-toxic genotypes within a cyanobacterial bloom [89]. A multiplex qPCR approach was developed to sensitively and specifically detect, differentiate and estimate potentially toxic *Anabaena, Microcystis* and *Planktothrix* genotype compositions in Missisquoi Bay, Quebec, Canada [90]. Using a remote liquid handling robot and

qPCR thermocycler, a similar multiplex-tandem PCR allowed semi-automated and simultaneous detection of toxigenic cyanobaterial blooms [91]. Such high throughout biological approaches, termed as "omics" (genomics, protomics, and metabolomics) technologies [92] will help identify biomarkers and provide a timely and reliable biomonitoring approach in the event of HABs.

The onboard Environmental Sample Processor (ESP, http://www.mbari.org/esp) is such a successful example by integrating the remote detection of both HAB species and their toxins in marine water [33]. The ESP is a robotic electromechanical/fluidic system that employs molecular diagnostic tests (DNA probe arrays) to concurrently detect HAB species as well as algal toxin DA with a detection limit of ng/L within 2-3 h using a competitive ELISA onboard ESP.

Albeit less preferable, an alternative strategy is intensive sample collection and preservation through remotely programmed control followed by later laboratory analysis. This is suitable for those observing networks for the HABs that occur in waters of, for example, the U.S. Integrated Ocean Observing System (http://www.ioos.noaa.gov). Formalin-preserved whole water phytoplankton samples were collected at the offshore samplers moored in Willapa Bay, Washington, which was stored for later analysis of DA by ELISA. Monitoring data from 2002 to 2006 using these preserved samples correlated well with the data obtained from two adjacent beaches [88].

Sensor technologies are particularly attractive for monitoring purpose because of the *in situ* applications and real- or near-real-time data acquisition. Gawley et al. [93] synthesized eleven anthracylmethyl crown ethers and found excellent fluorescence enhancement (10-20%) at STX concentration of 5 μM, which is very close to the detection limit by the mouse bioassay. Ding and Mutharasan [94] achieved a detection limit of 1 ng/L using a 1 mm×3 mm sensoring device called piezoelectric-excited millimeter-sized cantilever for rapid and sensitive detection of MC-LR. In this new device, MC-LR (antigen) binds specifically to an antibody immobilized on a cantilever sensor, the effective mass of the cantilever increases and alters the sensor resonant frequency. The frequency decreases proportional to the toxin concentration. A neuronal network biosensor (NNB) was examined and achieved 0.031 and 0.33 nM detection limits for two marine neurotoxins STX and PbTx-3 spiked in seawater-based medium, respectively [95]. This NNB relied on cultured mammalian neurons (from embryonic mice) grown over microelectrode arrays, where the inherent bioelectrical activity of the network can be monitored noninvasively [95]. Sensors can be made attractive portable device, such as the portable surface plasmon resonance biosensor system for the detection of domoic acid. Antibodies were used to develop competition- and displacement-based assays using a portable six-channel SPR. This portable device was able to reach a detection limit of 3 μg/L (10 nM) and quantifiable range of 4–60 μg/L (13–200 nM) for DA. The method correlates well with the detection of DA in concentrated algal extracts or high dissolved levels in seawater [96].

Satellites remote sensoring has been used to track HABs in many occasions, including the Gulf of Mexico to detect blooms with chlorophyll signature. Many algal accessory pigments (chlorophylls, carotenoids, and phycobiliprotein) are taxonomically significant, their vibrational spectroscopy as a mean of pigment detection in algae is also attractive because tedious pigment extraction and separation is no longer needed. Following visible light excitation of algal pigment, there was some success of using Raman spectra to characterize algae at the class level [97], but selective excitation of algal toxin in the whole cell to differentiate toxic versus non-toxic algal species has not been succeeded to date. The satellite data may be limited by cloud cover, lack of detection below one optical depth, and revisit frequently, all of which can lead to extended period without data. These shortcomings can be overcame by the use of an autonomous underwater vehicle (AUV) platform that support an optical phytoplankton discriminator (OPD) [87]. Using a Remote Environmental Monitoring UnitS (REMUS) AUV with an OPD deployed on the west of Florida coast, this autonomous platform along with remote sensing data, provide an early warning and monitoring system to reduce the HAB impact.

Summary and Future Perspectives

The detection of algal toxins and monitoring of causative harmful algal blooms are of paramount importance from the economic, environmental and public health perspectives. Existing methods including mouse bioassays and commercially available ELISA kits are not sufficient to meet the goals of various research and monitoring efforts. Extensive work has been done in the past decade in search for a fast, sensitive, selective, and inexpensive biological approach with toxicological relevance of algal toxins, in combination with a chemical instrumental method (typically LC-MS based) for a more sensitive quantitation and definite structural confirmation. Recent research has led to a wide range of technology advancement and innovations in the analysis of algal toxins for various applications – spanning from new passive sampling device using SPATT specifically for algal toxins, new ELISA kits with lower detection limit for more toxins, new molecular probing tools to identify causative microorganism and genes, quick and screening tools and sensor devices for field testing, new mass spectrometric applications geared toward the discovery of new algal toxins and their structural identities, and the large-scale HAB monitoring using remote sensing. Clearly, there is the need for inexpensive but reliable bioassays, chemical methods or integrated bio-analytical methods like any other emerging contaminants for regulatory and non-regulatory monitoring. However, such methods do not seem to be at hand to meet the various needs of monitoring purposes. More reliable methods capable of detecting multiple algal toxins for the early warning and routine HAB monitoring programs worldwide are envisioned. New toxins and their congeners continue to be discovered with the use of application of new mass spectrometry at their lower detection limit to better understand the environmental fate of algal toxins. Molecular-based methodologies will provide major development in the control measures of toxins and causative HABs in the future. Further research is warranted toward the development of regulatory as well as field methods using various biological and chemical sensors.

List of Acronyms

As a quick reference, a table of acronyms of toxins, analytical techniques and other terms mentioned in this review is provided in Table 5.

ANTX	Anatoxins
APCI	atmospheric pressure chemical ionization
API	atmospheric pressure ionization
ASP	amnestic shellfish poisoning
AUV	autonomous underwater vehicle

AZAs	azaspiracids
BSXs	brevisulcatic acids
CCL	Contaminant Candidate List
cdELISA	competitive direct ELISA
CDP	computationally designed polymer
CE	capillary electrophoresis
CEC	capillary electrochromatography
CFP	ciguatera shellfish poisoning
cidELISA	competitive indirect ELISA
CID	collision-induced dissociation
CTX	ciguatoxin
CYL	cylindrospermopsin
DA	domoic acids
DAD	diode array detector
EC_{50}	median effective concentration
ELISA	enzyme-linked immunosorbent assay
ESI	electrospray ionization
ESP	Environmental Sample Processor
FLD	fluorescence detector
GD	gymnodimine
HABs	harmful algal blooms
HILIC	hydrophilic interaction liquid chromatography
HPLC	high-performance liquid chromatography
HRMS	high resolution mass spectrometry
KBTs	brevisulcenals
Kow	octanol - water partition coefficient
LC-ESI-MS	liquid chromatography-electrospray ionization-mass spectrometry
LC-MS	liquid chromatography-mass spectrometry
LD_{50}	half lethal dose
LDTD	laser diode thermal desorption
LOD	limit of detection
LOQ	limit of quantitation
LLOQ	lower limit of quantitation
MAbs	monoclonal antibodies
MC-LR	microcystin-leucine and arginine
MC-RR	microcystin-arginine and arginine
MC-YR	microcystin-tyrosine and arginine
MC-WR	microcystin-tryptophan and arginine
MCs	microcystins
MDL	method detection limit
MNA	mouse neuroblastoma assay
MRM	multi-reaction monitoring mode
MW	molecular weight
N/A	not available
NMR	nuclear magnetic resonance
NNB	neuronal network biosensor
NOD	nodularins
NSP	neurotoxic shellfish poisoning
OA	okadaic acid
OPD	optical phytoplankton discriminator
PbTx	brevetoxin
PDA	photo-diode array detector
POCIS	polar organic chemical integrative samplers
PSD MALDI	post-source decay matrix-assisted laser desorption / ionization
PSP	paralytic shellfish poisoning
PTX-2	pectenotoxin-2-seco acid
QIT	quadrupole ion trap
qPCR	quantitative polymerase chain reaction
QR	quantitative range
RCA	rainbow trout gill cytotoxicity assay
Rec	recovery
REMUS	Remote Environmental Monitoring UnitS
RSD	relative standard deviation
RSV	relative standard variation
SD	standard deviation
SPATT	solid-phase adsorption toxin tracking
SPE	solid phase extraction
SPMD	semi-permeable membrane device
STX	saxitoxin
TCh-GC/MS	thermally-assisted hydrolysis and methylation gas chromatography-mass spectrometry
TOF	time-of-flight
TRFIA	time-resolved fluorescence immunoassay
TQ	triple quadrupole
WHO	World Health Organization

YTXs	yessotoxins

Table 5: List of Acronyms

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