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A Global Analysis of the Relationship between Concentrations of Microcystins in Water and Fish

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Cyanobacteria, the primary bloom-forming organisms in fresh water, elicit a spectrum of problems in lentic systems. The most immediate concern for people and animals are cyanobacterial toxins, which have been detected at variable concentrations in water and fish around the world. Cyanotoxins can transfer through food webs, potentially increasing the risk of exposure to people who eat fish from affected waters, yet little is known about how cyanotoxins fluctuate in wild fish tissues. We collated existing studies on cyanotoxins in fish and fresh water from lakes around the world into a global dataset to test the hypothesis that cyanotoxin concentrations in fish increase with water toxin concentrations. We limited our quantitative analysis to microcystins because data on other cyanotoxins in fish were sparse, but we provided a qualitative summary of other cyanotoxins reported in wild, freshwater fish tissues. We found a positive relationship between intracellular microcystin in water samples and microcystin in fish tissues that had been analyzed by assay methods (enzyme-linked immunosorbent assay and protein phosphatase inhibition assay). We expected microcystin to be found in increasingly higher concentrations from carnivorous to omnivorous to planktivorous fishes. We found, however, that omnivores generally had the highest tissue microcystin concentrations. Additionally, we found contrasting results for the level of microcystin in different tissue types depending on the toxin analysis method. Because microcystin and other cyanotoxins have the potential to impact public health, our results underline the current need for comprehensive and uniform detection methods for the analysis of cyanotoxins in complex matrices.

Keywords: cyanobacteria, harmful algal blooms, cyanotoxins, freshwater, fish, human health

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INTRODUCTION

Freshwater harmful algal blooms (HABs) are generally caused by cyanobacteria (Lopez et al., 2008). Large-scale blooms are considered unsightly and may cause foul odors (Watson et al., 2016), and many cyanobacteria may also produce toxic secondary metabolites (Wiegand and Pflugmacher, 2005). The negative effects of cyanobacterial toxins (cyanotoxins) have been observed for over a century (Francis, 1878) and, in terms of impacts to humans, include interruptions to municipal water supplies, closures of recreational sites, and illness (Griffiths and Saker, 2003; Bullerjahn et al., 2016; Svirčev et al., 2017).

Consumption of fish from waters affected by cyanobacteria blooms is an important route of human exposure to cyanotoxins (Ibelings and Chorus, 2007; Wilson et al., 2008; Peng et al., 2010; Poste et al., 2011). As much as 40% of global finfish production comes from fresh water (Lynch et al., 2016), and cyanotoxins have been detected in freshwater fish from around the world (Smith et al., 2008; Lee et al., 2017). Previous research has demonstrated that cyanotoxins move through food webs (Karjalainen et al., 2005; Smith and Haney, 2006; El Ghazali et al., 2010; Lance et al., 2014). Biomagnification of cyanotoxins in aquatic food webs is debated but may depend on the types of toxins and their chemical characteristics. The majority of studies have focused on the heptapeptides microcystins, which have been shown to accumulate in some fish tissues (Lance et al., 2014), primarily the liver, and in zooplankton (Hauser-Davis et al., 2015). Some studies have also shown trophic transfer of microcystins (Sotton et al., 2014), however, studies of biomagnification included only a limited number of observations from nature (e.g., Ibelings and Havens, 2008; Kozlowsky-Suzuki et al., 2012). Field studies are critical to uncover patterns that may not be detected in the laboratory setting, due to uncertainties in translating laboratory derived estimates of bioconcentration factors to those in the uncontrolled environment (Mackay et al., 2016).

The factors that influence cyanotoxin concentrations in fish likely include the concentration of the specific toxin(s) in the environment in combination with the period of exposure and fish metabolic processes (Jia et al., 2014; Gurbuz et al., 2016). Further, different cyanotoxin quantification methods could also introduce variability that may artificially enhance or inhibit detection of patterns in nature if toxin concentrations are over- or underestimated. One way to understand cyanotoxin concentrations in wild fish tissues is to determine how environmental exposure may influence toxin accumulation in the fish. For example, Amé et al. (2010) found a significant positive correlation between the dissolved microcystin in the water of Los Padres Lake (Argentina) and the microcystin concentration in the liver of the planktivore *Odontesthes bonariensis*, while intracellular microcystin was not correlated. Other studies have found high concentrations of cyanotoxins in fish tissues from systems with high concentrations in the water (Amrani et al., 2014; Jiao et al., 2014; Nchabeleng et al., 2014). Conversely, negative correlations have also been observed (Wilson et al., 2008; Bruno et al., 2009; Zhang et al., 2013a). While a consistent relationship is not evident among individual studies, a growing number of field studies have measured cyanotoxins in fish across a range of trophic levels and tissue types.

We used published data on cyanotoxin concentrations in fish and fresh water to better understand observed concentrations of cyanotoxins in wild fish. We gathered data from field studies which reported cyanotoxin concentrations in lentic, freshwater systems to examine cyanotoxin accumulation in freshwater fish across the globe. Our objectives were to (1) determine if cyanotoxin concentrations in fish are reflective of levels of cyanotoxins in the water in which the fish reside, and (2) identify any differences in the accumulation of toxins among tissue types and fish feeding mode (carnivorous, omnivorous, planktivorous). We hypothesized that on a global scale, cyanotoxin concentrations in fish tissues are positively correlated to cyanotoxin concentrations in the water. We expected a test at the global scale to reveal broad patterns in the way cyanotoxins accumulate in fish and to identify feeding groups that may be prone to higher accumulation of toxins. We further hypothesized that planktivorous fishes will have higher cyanotoxin concentrations than fish of other feeding modes because they may directly consume potentially toxic cyanobacteria or toxic zooplankton. High concentrations of microcystins have been previously reported in zooplankton (Ferrão-Filho and Kozlowsky-Suzuki, 2011). Zooplankton may take up dissolved toxins from the water (Karjalainen et al., 2005) and ingest toxins directly from cyanobacteria (Wilson et al., 2006). Additionally, fish that feed on potentially toxigenic cyanobacteria, have a direct path of cyanotoxin exposure that is presumably insignificant or nonexistent for carnivorous fish. Among the tissues, the concentrations of cyanotoxins, specifically microcystins, are often reported to be higher in liver, kidney, and intestine relative to concentrations in the muscle (Mekebri et al., 2009; Amrani et al., 2014; Ni et al., 2017). We expected the assembled data on fish tissues to parallel these observations.

MATERIALS AND METHODS

To test our hypotheses, we conducted an extensive literature search to collect data from field studies in the primary and gray literature reporting concentrations of cyanotoxins in water and fish. Online searches through Web of Science, Google Scholar, and Academic Search Premier were conducted using relevant search terms. Key terms, "cyanotoxins" or "cyanobacteria toxins" and "fish," in combination with other words, such as "harmful algal bloom," "blue-green algae," "lake," "freshwater," and the names of several cyanotoxins most commonly reported in the scientific literature (microcystin, nodularin, cylindrospermopsin, anatoxin, saxitoxin, and β-methylamino-L-alanine) were used to create a list of field studies measuring cyanotoxins in fish and fresh water concurrently. Truncation and wildcard symbols were used to expand the search results and allow variations in the

TABLE 1 | The six cyanotoxin types represented in the literature that matched our search criteria are presented here with the number of studies, fish tissue types, and fish species sampled for each cyanotoxin.

Toxin	No. studies	No. tissues types	No. species
MC	50	20	67
BMAA	4	3	28
DABA	2	3	8
ATX	3	3	12
CYN	2	1	10
STX	4	1	11

The number of studies shown do not add to 58 (the total number of studies) because some studies tested for multiple toxins, thus are represented more than once. Cyanotoxin acronyms are as follows: MC, microcystins, BMAA, β-N-methylamino-L-alanine, DABA, 2,4-diaminobutyric acid dihydrochloride, ATX, anatoxin-a, CYN, STX, cylindrospermopsin, and saxitoxin.

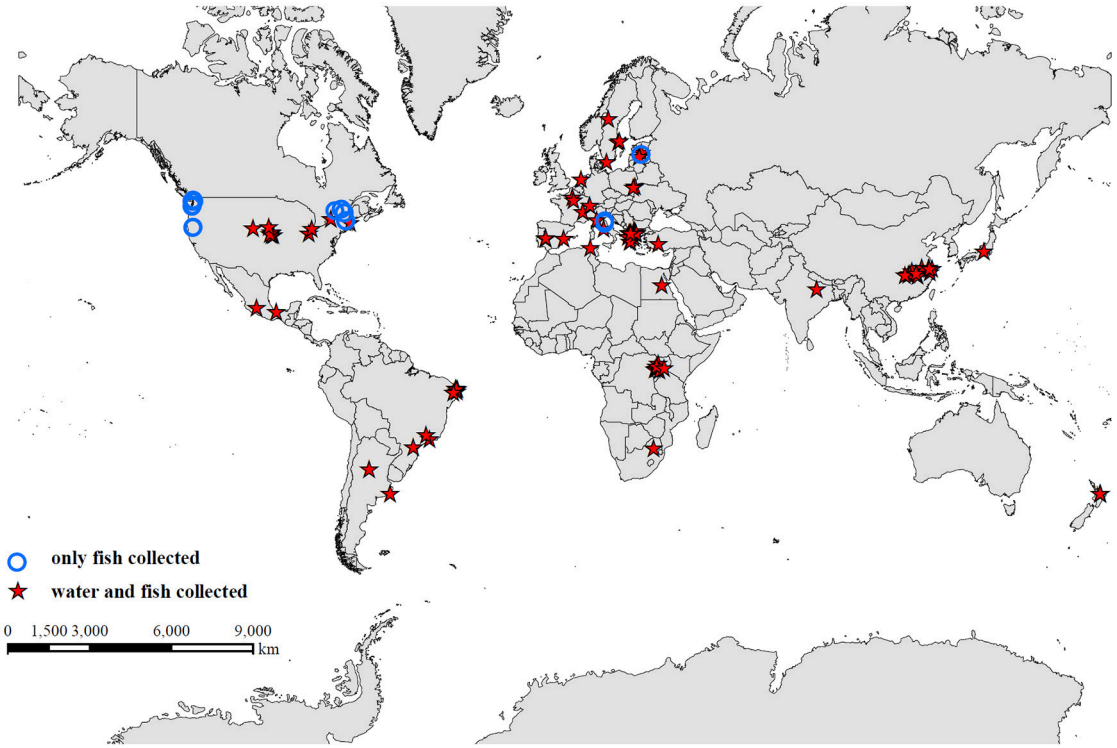

FIGURE 1 | Global distribution of 99 freshwater systems, including lakes, ponds, and reservoirs, from studies used in the data synthesis. Studies that collected fish and water samples concurrently are identified by a red star, and studies that collected only fish samples are indicated by a blue circle.

format or spelling of different terms (for example, "microcystins" or "microcystin"). Search options were set to return results from all text associated with the source, including title, key words, the body of the article, and references. Research with paired observations of cyanotoxins in fresh water and fish were targeted and the search was limited to studies from lentic systems. Studies that did not provide water toxin concentrations, but reported toxin concentrations in fish were also included for comparisons of toxin levels among tissue types and fish feeding mode. The references of each study were thoroughly searched for additional sources.

From each study matching our search criteria, cyanotoxin data were gathered from text, tables, and figures. Cyanotoxin concentration values were extracted from figures using WebPlotDigitizer version 3.8[1] Non-detects or concentrations below the limit of detection were recorded as a concentration of zero. When concentrations were presented as a range, the midpoint was used. The methods used for quantification of cyanotoxins in water and fish tissues were also documented. We found several studies that analyzed cyanotoxins in fish stocked in cage or net enclosures in lakes. Data from such studies were excluded. Studies that provided insufficient detail regarding sample collection, processing or analyses were also excluded. The number of extraction and analysis methods used to identify cyanotoxins in tissues are variable, and extraction efficiencies can be poor (Ernst et al., 2005; Smith and Boyer, 2009). To control for variation in extraction efficiencies, statistical analyses were limited to data from studies that reported tissue extraction efficiencies >50% or used methods (such as homogenization with acidified methanol extraction) where previously reported recoveries were above this threshold.

As cyanotoxin data were reported in different units across studies, concentrations were first converted to μg/g or μg/L and grouped according to the fraction of the toxin measured in the water sample (intracellular, extracellular, and total, which was the sum of the intra- and extracellular toxins). Extracellular toxins were always reported in volumetric units (e.g., ng/L), but total and intracellular toxins were documented in both units per volume and units per biomass (e.g., μg/g), and usually as dry weight. Measurements of cyanobacterial biomass at the time of sampling, if available, were used as conversion factors to convert water toxin concentrations from μg/g to μg/L. Two studies presented intracellular toxin concentrations in fresh weight, so a factor of 10 (ratio of wet/dry weight) was used to obtain the dry weight equivalent (Messineo et al., 2009). Fish toxin concentrations were also reported in both units of dry and fresh weight (DW and FW, respectively). A factor of 0.31 (Poste et al., 2011) was used to convert fresh weight values to dry weight (Equation 1). Ibelings et al. (2005) provided cyanotoxin concentrations for smelt (*Osmerus eperlanus*), ruffe (*Gymnocephalus cernua*), and perch (*Perca fluviatilis*) livers in units of ash-free dry weight (AFDW). Estimates of percent fish

[1]http://arohatgi.info/WebPlotDigitizer.

TABLE 2 | Descriptive statistics for concentrations (μg/g DW) of each cyanotoxin class in wild, freshwater fish tissues, including the sample size (*n*), minimum value reported (min), first and third quartiles (Q1 and Q3, correspondingly), median, and the maximum (max) and mean values.

Toxin	Tissue	*n**	Min	Q1	Median	Q3	Max	Mean
BMAA	Muscle	55	0	0.38	0.85	3.93	35.91	3.55
	Brain	5	0	0.01	0.02	0.03	1.39	0.29
	Liver	1	4.13	–	–	–	–	–
DABA	Muscle	19	0	0.08	0.25	0.45	1.70	0.38
	Brain	1	0.03	–	–	–	–	–
	Liver	1	0	–	–	–	–	–
ATX	Muscle	39	0	0	0	25.50	63.48	12.71
	Gill	1	56.45	–	–	–	–	–
	Liver	23	0	0.74	6.10	19.60	48.29	11.85
CYN	Viscera	2	0.00194	–	–	–	0.00871	0.00532
	Muscle	11	0.00029	0.00063	0.00136	0.00260	0.00407	0.00168
	Ovary	1	0.00023	–	–	–	–	–
STX	Muscle	20	0	0.0010	0.0023	0.0526	0.1548	0.0281
MC	Muscle	1,035	0	0	0.0014	0.0387	3.2700	0.0753
	Liver	554	0	0	0.0089	0.2865	375.3000	5.3920
	Hepatopancreas	15	0.3392	0.4297	0.6066	0.7477	1.6180	0.7110
	Intestine	77	0	0.0408	0.1357	0.6474	7.4420	0.5669
	Gill	13	0	0.0006	0.0081	0.0341	0.1290	0.0231
	Gonad	2	0.0015	–	–	–	0.0269	0.0142
	Kidney	93	0	0.0253	0.1100	0.5025	14.1400	0.6752
	Ovary	18	0.0010	0.0123	0.0195	0.0723	0.1665	0.0454
	Brain	42	0	0.0098	0.2327	0.8757	2.0710	0.4704
	Blood	9	0.6169	0.7599	4.2600	11.7400	46.9800	11.1400
	Gut	31	0.0009	0.1041	0.2620	0.5520	2.6700	0.4367
	Roe	1	0.3100	–	–	–	–	–
	Spleen	16	0	0	0	0.0629	2.0600	0.2140
	Gallbladder	16	0	0.0065	0.0441	0.0900	0.2273	0.0674
	Whole	25	0.0032	0.0307	0.0461	0.1168	0.6942	0.0982
	Viscera	8	0.0218	0.0258	0.0884	1.3260	8.8580	1.5920
	Belly flap	29	0.0155	0.0672	0.1858	0.2926	0.9785	0.2283
	Bile	5	0	0	3.1200	8.4800	22.8600	6.8920
	Skin	1	0.0245	–	–	–	–	–
	Heart	16	0	0.0146	0.0245	0.0333	0.0876	0.0324

**n is the number of data points rather than number of individual fish because multiple fish were pooled in some analyses.*

liver ash reported in the literature were used to convert ash-free dry weight to dry weight. Łuczynska et al. (2016) found 1.66 ($n = 7$) and 1.36 ($n = 6$) percent ash in the livers of wild *P. fluviatilis* from two lakes in Poland. Using the average ash content in *P. fluviatilis* from both lakes (1.51% ash), we assumed 100 g of dry liver tissue contained 1.51 g of ash, or 0.0151 g ash per gram of dried fish liver. The ash-free mass of the tissue ($M_{AFDW} = 0.9849$ g or 1 g dry tissue minus 0.0151 g ash) was used as a conversion factor for *P. fluviatilis* (Equation 2). Due to limited published data on percent ash in *O. eperlanus* and *G. cernua* liver tissue, a conversion factor was approximated from the average percent ash across a range of species, including *P. fluviatilis* (Łuczynska et al., 2016), *Esox lucius*, *Salmo fario*, *Cyprinus carpio*, *Lota vulgaris* (Atwater, 1891), and *Macquaria australasica* (Sheikh-Eldin et al., 1995). The resulting M_{AFDW} for *O. eperlanus* and *G. cernua* was 0.9764 g (Equation 2).

$$\left(\frac{\mu g \text{ toxin}}{g \text{ FW}}\right) \times \left(\frac{1 \text{ g FW}}{0.31 \text{ g DW}}\right) \quad (1)$$

$$\left(\frac{\mu g \text{ toxin}}{g \text{ AFDW}}\right) \times \left(\frac{M_{AFWD}}{1 \text{ g DW}}\right) \quad (2)$$

All fish toxin concentration values were paired with a corresponding water toxin value from the respective system.

When necessary, water toxin concentrations were averaged across the same time period and sample sites as the fish collection. The data did not conform to any parametric assumptions (normality, homoscedasticity) and sample sizes were sometimes low for subsets of the data. Consequently, we used non-parametric bootstraps to conduct our analyses. Data are available online (Flores et al., 2018).

We used Analysis of Covariance (ANCOVA) to test our hypothesis that cyanotoxin concentrations in fish were positively related to cyanotoxin concentrations in the water, and to also test for differences in tissue toxin concentrations among tissue types and fish feeding modes. Subsets of the data were created based on cyanotoxin class (microcystins, saxitoxin, etc.), cyanotoxin quantification method, and water sample type (intra- or extracellular and total). All statistical analyses were conducted separately on each resulting subset of the data. Separation of the data based on the fraction of toxin in the water allowed us to test for differences in accumulation assuming that different exposure routes may exist depending on the state of the toxin (i.e., in the cyanobacteria cell, dissolved in the water, or combined exposure). Data obtained using the enzyme-linked immunosorbent (ELISA) or activity-based (e.g., protein phosphatase inhibition) assays were analyzed separately from chromatographic analytical techniques because the assay

TABLE 3 | The number of studies that used the indicated analysis method.

	MC	BMAA	DABA	ATX	CYN	STX
LC-MS	7					
LC-MS/MS	10	2	1			
UPLC-MS/MS		1				
GC-MS	1					
ELISA*	25				2	2
PP2A inhibition assay	2					
LDTD-APCI-HRMS	1					
HPLC-UV	2					
HPLC-FD		1	1	3		2
HPLC-PDA	2					

The method acronyms are as follows: LC-MS, liquid chromatography-mass spectrometry; LC-MS/MS, liquid chromatography tandem mass spectrometry; UPLC-MS/MS, ultra-performance liquid chromatography tandem mass spectrometry; GC-MS, gas chromatography-mass spectrometry; ELISA, enzyme-linked immunosorbent assay; PP2A, protein phosphatase type 2A inhibition assay; LDTD-APCI-HRMS, laser thermal desorption-atmospheric pressure chemical ionization-high-resolution mass spectrometry; HPLC-UV, high pressure liquid chromatography with ultra violet; HPLC-FD, fluorescence; HPLC-PDA, photodiode array detection.

**ELISA for MC, CYN, and STX were specific for each toxin and reactive with multiple congeners.*

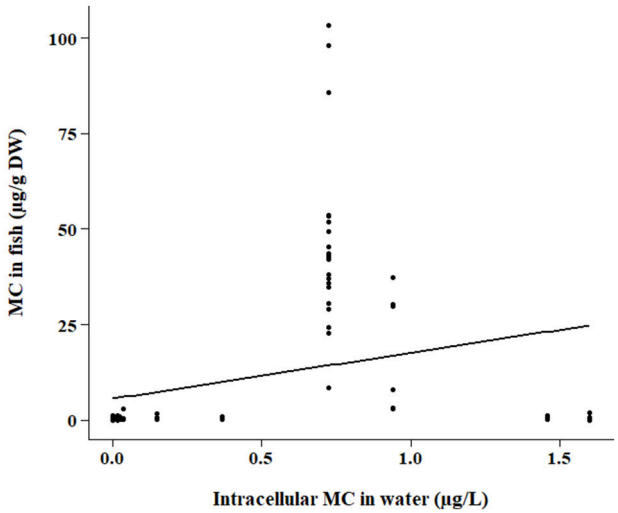

FIGURE 3 | Same as **Figure 2** except samples were quantified by the PP2A method.

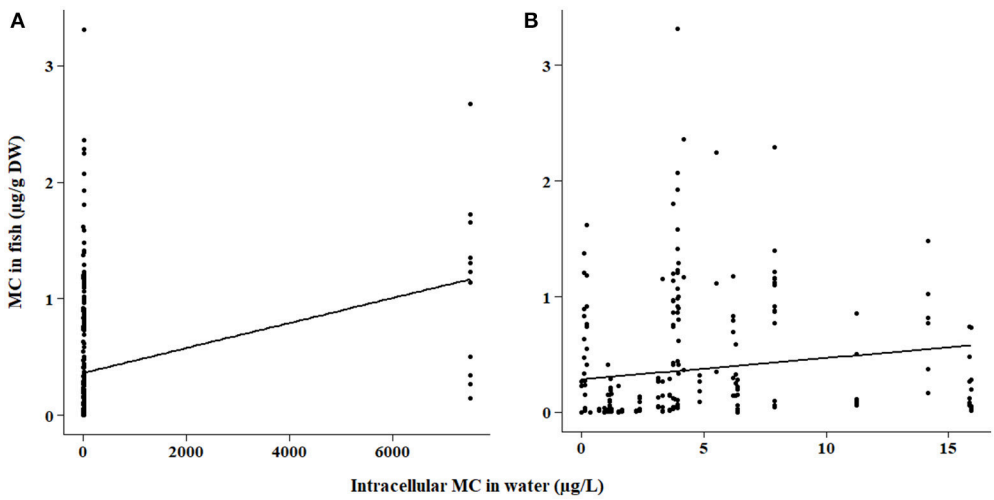

FIGURE 2 | (A) Microcystin (MC) in fish tissues as a function of intracellular MC concentration in the water for samples quantified by the ELISA method. Fish tissues include all tissue types and fish feeding modes. **(B)** Same data as **(A)** but with focus on intracellular MC concentrations below 20 µg/L.

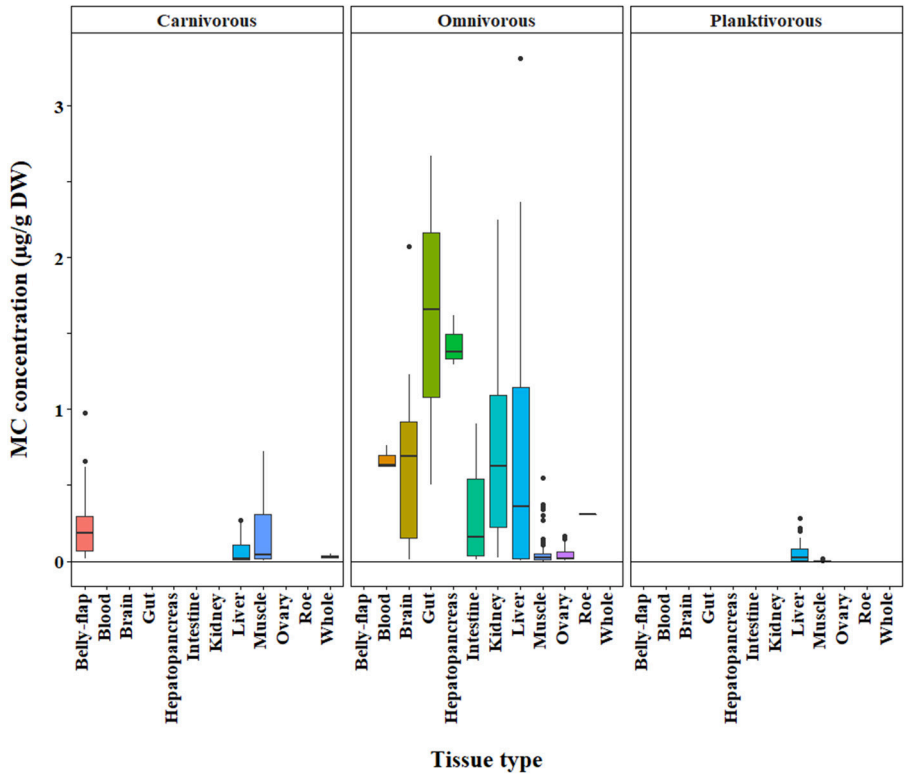

FIGURE 4 | Microcystin (MC) concentrations in fish grouped by trophic level (carnivore, omnivore, planktivore) across a range of tissue types. Concentrations were quantified by the ELISA method. The raw data are shown.

and analytical approaches differ substantially in toxin variants targeted and in the principle of detection; results from the two method types do not always corroborate each other. For example, all ELISA data we obtained for microcystins used a polyclonal anti-body that cross-reacts with most microcystin congeners that have an intact ADDA residue. Conversely, data we obtained from studies using chromatographic methods for microcystin only allow for identification and quantification of toxins for which reference standards exist. The difference can be an issue, for example in microcystins, where many congeners are known but few analytical standards exist (Hu et al., 2017). For each data subset previously described, we generated a bootstrap sample by randomly selecting n paired observations (fish and water with associated tissue and feeding modes) from the original data (method 1), with replacement, where n was the number of observations in the data subset. The F-value of the covariate (water), independent variable (tissue type) and their interaction was calculated from this bootstrap sample. This process was repeated $B = 10{,}000$ times to create a bootstrap distribution of F-values for each variable. We calculated the 95th percentiles of the bootstrap distributions to compare the observed F-values from the ANCOVA of the original data. The observed F-value was significant if it fell above the 95th percentile of the bootstrap distribution of F-values. The process was repeated by randomly selecting $B = 10{,}000$ samples of the original data, but further randomizing the data so that fish samples were randomly assigned a water sample, tissue type, and feeding mode (method 2). In this way, we tested if the observed F-values from the model were significantly different from a distribution of randomly generated, and randomly sampled relationships. When the interaction effect was significant, we conducted a bootstrap two-way Analysis of Variance (ANOVA) on the residuals to remove the effect of the covariate (water toxin concentration) and tested for differences in toxin concentration among tissue types and fish feeding modes. In all other cases (when the interaction effect was not significant), a two-way ANOVA was used on the bootstrap data to test for toxin differences among tissues and feeding modes. In cases where only one fish feeding mode was available, only differences between tissues were tested (one-way ANOVA). Linear models were all assessed by comparing the F-value of the original data to the distribution of bootstrap F-values. Significant results were followed by pairwise comparisons of tissues and feeding modes. We generated 10,000 bootstrap samples using the same sampling method as was used for the significant ANOVAs. For each bootstrap sample we calculated the difference in means for each pairwise comparison and created a distribution of the difference. We calculated the difference for the same pairwise comparisons in the original data and compared the difference to each respective distribution of differences created in the bootstrap. Significance was determined if the difference of the original data was above the 97.5th or below the 2.5th percentiles of the distribution of differences. All

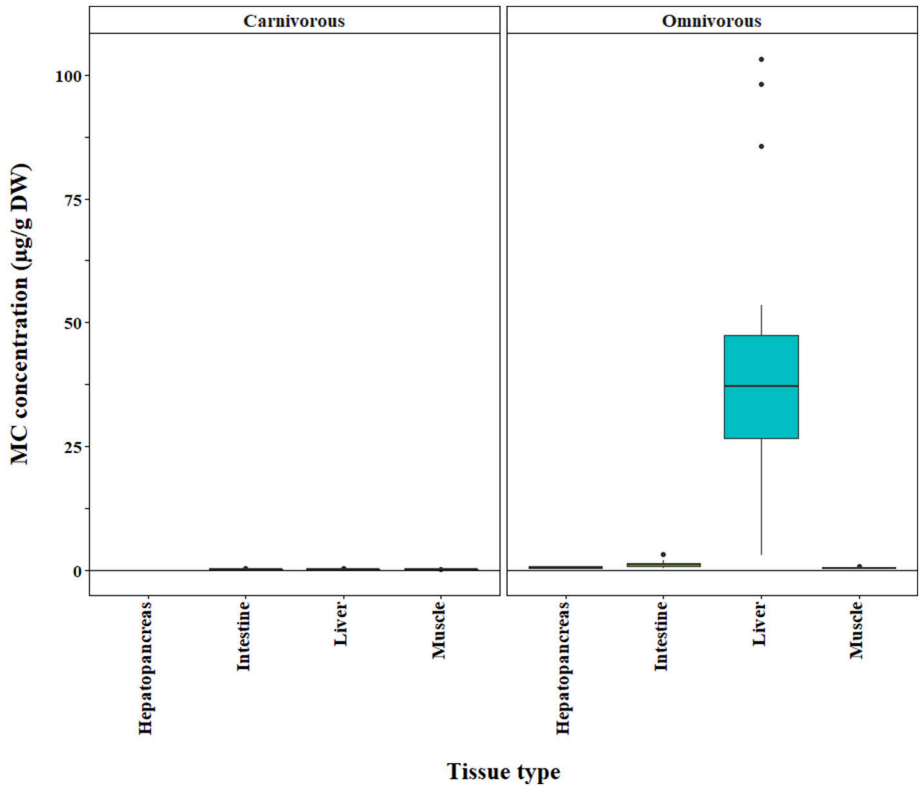

FIGURE 5 | Microcystin (MC) concentrations in tissues of carnivorous and omnivorous fishes based on PP2A method. The raw data are shown.

statistical analyses were conducted in RStudio version 1.0.136 (R Core Team, 2016).

RESULTS AND DISCUSSION

A total of 115 studies were examined from the primary and gray literature, of which 58 freshwater field studies (Supplementary Table 1) met our search criteria and were selected for the present analysis. Of these 58 studies, nearly 90% were on microcystins (MC), while the remainder included β-*N*-methylamino-L-alanine (BMAA), its isomer, 2,4-diaminobutyric acid dihydrochloride (DABA), anatoxin-*a* (ATX), cylindrospermopsin (CYN), and saxitoxin (STX; **Table 1**). The selected studies provided concentrations of cyanotoxins in fish tissues alone or paired with water samples that were collected during the same period as the fish sampling. The studies spanned 99 freshwater systems, including lakes, ponds and reservoirs, across 23 different countries (**Figure 1**).

Cyanotoxin concentrations in water samples were highly variable. Water toxin concentrations ranged from undetected to over 7,000 µg/L in one case (intracellular MCs; dissolved MCs reached 13 µg/L). A water concentration of intracellular CYN was only reported in one study at 423.5 µg/g DW. Neurotoxins found in waters reached 25.3 µg/L BMAA, 21.1 µg/L DABA, 24.2 µg/L STX, and 35.0 µg/L ATX. Cyanotoxin concentrations in fish tissues were also highly variable (**Table 2**). Ten different types of analysis methods were used across all studies to analyze cyanotoxins in tissues (**Table 3**). Microcystins had the highest diversity of species and tissues tested for cyanotoxins (**Tables 1**, **2**), and the greatest number of quantification methods (**Table 3**). The highest toxin concentration was MC in the liver of a planktivorous smelt (*O. eperlanus*) at 375.3 µg/g DW (**Table 2**).

Due to the limited data for the other toxin classes, quantitative analyses could only be conducted on MCs. An analysis of MC congeners individually was not possible due to the limited number of studies providing congener-specific data in wild fish. Many studies (43%) used the ADDA-specific ELISA method for quantification of MCs (**Table 3**), which does not allow for identification of individual congeners. In addition, some studies which used analytical methods reported the sum of the different congeners detected (MCs or total MCs) or as "MC-LR equivalents."

The bootstrap analyses did not indicate any significant relationships using bootstrap sampling method 1. However, sampling method 2 revealed positive relationships within the ELISA and PP2A (protein phosphatase type 2 inhibition assay) subsets. Intracellular MC concentration in the water was positively correlated with fish tissue MC concentration detected by ELISA ($p < 0.0001$; **Figure 2**) and PP2A ($p = 0.0006$; **Figure 3**). The high variability in MC concentrations in fish tissues may be due to effects from different MC congeners present in the data, or to differences in accumulation between

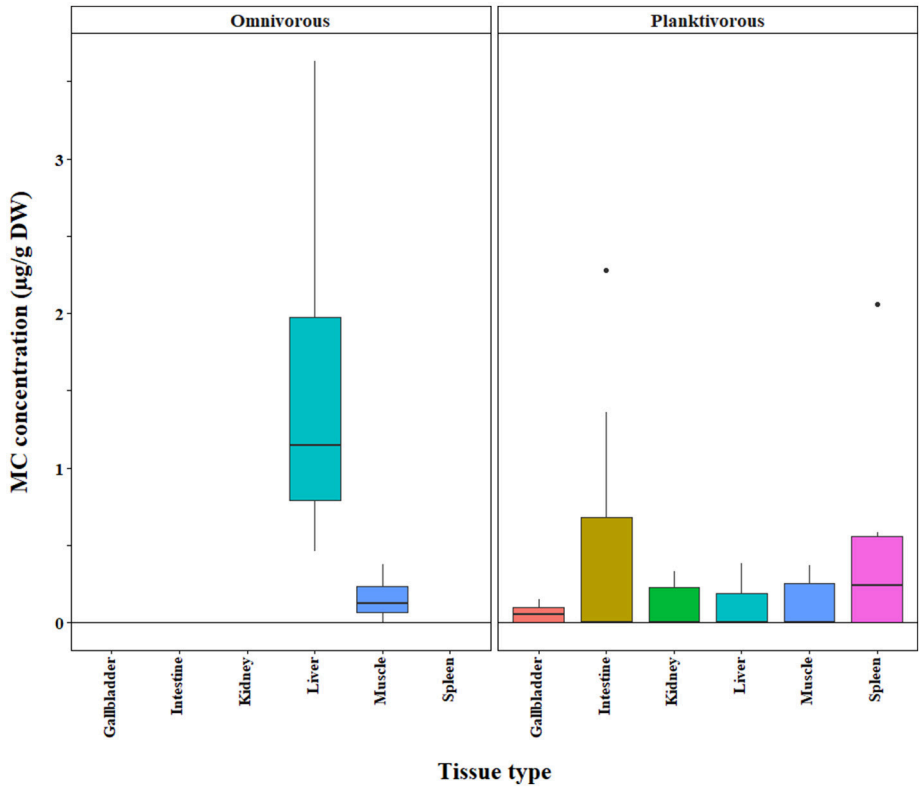

FIGURE 6 | Microcystin (MC) concentrations in the tissues of omnivorous and planktivorous fish tissues quantified by LC-MS. A subset of the original, raw data is shown for which significant differences between tissue types and feeding groups were detected in a bootstrap analysis of the residuals.

tissue types or fish of different feeding modes. For example, the higher range of concentrations in the tissues of fish analyzed with PP2A (**Figure 3**) compared to ELISA (**Figure 2**) could be due to presence of other protein phosphatase inhibitors in the tissues. MC-LR recovery in the livers of rainbow trout (*Oncorhynchus mykiss*) using different detection methods were higher in the protein phosphatase assay (protein phosphatase type 1) compared to ELISA or HPLC (Ernst et al., 2005). Further, 30% more MCs were found in the muscle of tilapia (*Oreochromis niloticus* and *Tilapia rendalli*) with a type 2 protein phosphatase inhibition assay than the ELISA (Deblois et al., 2008). In both of these cases, the higher concentrations detected with the protein phosphatase inhibition assay were likely attributed to other non-MC compounds with phosphatase inhibiting activity (Ernst et al., 2005; Deblois et al., 2008). Pesticides, including endothall, cantharidin, and other algal metabolites such as some anabaenopeptins and oscillamides, occur in aquatic environments and have also been shown to inhibit protein phosphatases (Erdodi et al., 1995; Sano et al., 2001; Gkelis et al., 2006).

We found a significant interaction effect between MC in fish tissues and MCs in the water analyzed by LC-MS and LC-MS/MS ($p = 0.0025$ and $p = 0.0129$, respectively). The LC-MS and LC-MS/MS subsets were further tested for differences in MC accumulation between tissue types and forage modes in a two-way ANOVA of the residuals, followed by pairwise comparisons. The LC-MS data subset by extracellular MCs in the water were significantly different in tissue MC concentrations ($p < 0.0001$), but the intracellular MC subset was not. Tissues were significantly different in toxin concentration for LC-MS/MS ($p < 0.0001$; intracellular MC subset). We also found differences in tissue types analyzed by other methods, however, the results were conflicting and will be summarized separately below.

We found that omnivores generally had higher MC concentrations than carnivores and planktivores across different MC analysis methods (ELISA, $p < 0.0001$; PP2A—only omnivores and carnivores tested due to a lack of planktivore data, $p = 0.0001$; LC-MS—only omnivores and planktivores tested due to limited carnivore data, $p = 0.003$; **Figures 4–6**, respectively). Planktivores were not significantly different than carnivores when sufficient data allowed us to test them together. However, data on planktivores ($n = 126$) were less abundant than for carnivores ($n = 313$) and omnivores ($n = 767$).

The bootstrap analysis also indicated that MC concentrations were significantly different between tissue types, but the results differed depending on the analysis method. For example, liver MC concentrations were greater than muscle when measured by GC-MS ($p = 0.0023$; **Figure 7**), PP2A ($p = 0.0001$; **Figure 5**), and LC-MS ($p = 0.0023$; **Figure 6**), but not when measured by ELISA (**Figure 4**) and LC-MS/MS (**Figure 8**). We compared tissues common to all MC analysis methods where significant differences were detected between the tissues (ELISA, PP2A, LC-MS,

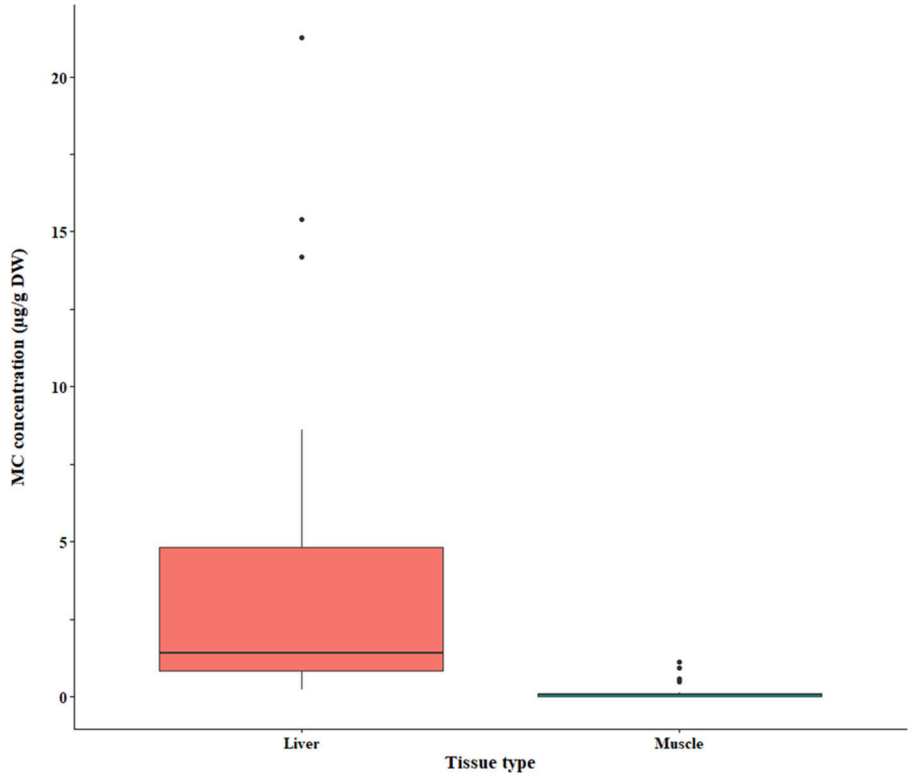

FIGURE 7 | Microcystin (MC) concentrations in fish liver and muscle quantified by the GC-MS (MMPB) method. The raw data are shown.

LC-MS/MS, GC-MS; **Table 4**). GC-MS was only used to measure liver and muscle, so no further comparisons could be made for that method. Other than the liver and muscle, no differences were detected between tissue MC content by LC-MS. For LC-MS/MS, kidney samples had the highest MC concentrations while other tissues were not significantly different. The PP2A subset yielded liver as the tissue with the highest MC content, but other tissues analyzed by this method were also not significantly different from one another. For ELISA, gut, hepatopancreas, and brain ranked above the other tissues by MC concentrations, and the remaining tissues were not significantly different.

To our knowledge, our synthesis is the most comprehensive representation of information available on cyanotoxins in wild, freshwater fish. We found that MCs compose the majority of studies on cyanotoxins in fish, a theme that parallels the field of cyanotoxin research. More than half of the primary literature addresses MC, relative to other types of cyanotoxins, and the areas of environmental and human health are understudied (Merel et al., 2013). The limited data for other classes of cyanotoxins in fish and water prevented us from conducting statistical tests on toxins other than MCs. Using bootstraps analyses, we found evidence of a positive correlation between intracellular MC in the water and MC in the tissues of fish, which suggests that increases in MC concentrations in lentic systems may result in higher MC concentrations in fish. MCs are often found at higher concentrations in intracellular form than extracellular, unless at the end of a bloom cycle (Cazenave et al., 2005). Fish and other organisms that feed on cyanobacteria may directly ingest MC. Upon bloom senescence, intracellular toxins may be released into the water. Systems with periods of higher intracellular MC concentrations, may be followed by periods of subsequent high extracellular MC concentration, which may directly expose aquatic biota to toxins. The dominant route of exposure to fish is unknown, but trophic transfer, and direct transfer via ingestion of cells and immersion in MC-contaminated water have been demonstrated in laboratory experiments (Soares et al., 2004; Smith and Haney, 2006; Sieroslawska et al., 2012). Contrary to our expectations, omnivores were the group with the highest cyanotoxin concentrations. Omnivorous fish can be exposed to toxins through almost any possible route, from immersion in a "toxic bath" of dissolved toxins in the water, to ingestion of plants, animals, or other potentially toxic matter. In any case, MCs are unlikely to cross cell membranes freely, but rather transported by organic transporting polypeptides as in human tissues (Fischer et al., 2010). Thus, the distribution of these receptors in fish tissues will dictate the most common exposure route. The number of potential exposure routes for omnivorous fish may help explain their high degree of toxin concentrations relative to other feeding modes. More data on wild fish of other feeding modes, especially planktivores, would help better define this relationship. Furthermore, tissue MC concentrations were difficult to compare due to the number of different methods used to analyze toxin concentrations, and the variability in tissue

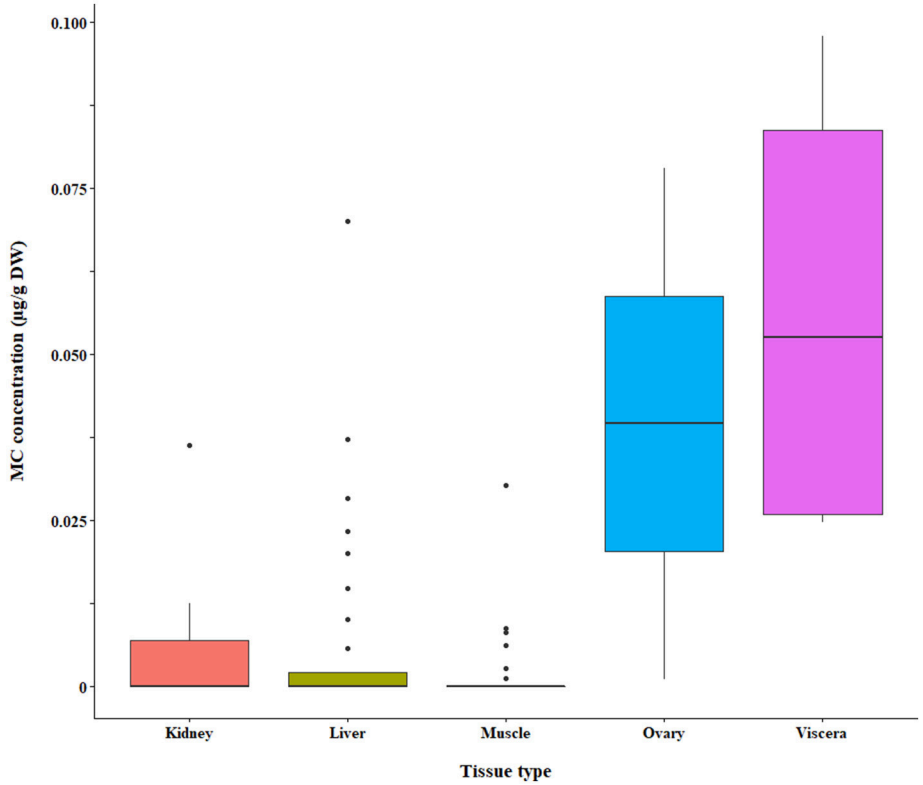

FIGURE 8 | Microcystin (MC) in fish tissues quantified by LC-MS/MS. A subset of the raw data is shown for which significant differences between tissue types were detected by bootstrap analysis of the residuals.

types tested by each method. Evidence for previously detected relationships, such as the greater concentrations of MC in liver than muscle, are supported, however relationships between other tissue types are not as clear and may depend on which methods are used for MC detection.

The analytical method used is likely extremely important in influencing the observed toxin concentration in fish. Different concentrations of MCs from the same sample have been previously detected with different analysis methods (e.g., Gkelis et al., 2006; Hardy et al., 2015). Comparison of MC concentrations in different organisms is difficult due to the variety of methods employed, protein binding affinity, and differences in pharmacokinetics in different organisms (Adamovskỳ et al., 2007; Zhang et al., 2013b). Analysis methods may be more comparable if they have similar extraction efficiencies, but many studies do not report this critical information. Out of the studies we analyzed, less than half conducted spike-recovery tests and reported extraction efficiencies. Isotopically labeled standards for select cyanotoxins have only recently become available commercially (e.g., Cambridge Isotope Laboratories, Inc.) to control for variability in extraction efficiencies. To make data more comparable in the absence of standardized analysis methods, future studies should conduct spike recovery tests to support the efficacy of the methods or use surrogate standards to estimate extraction efficiency. Our results emphasize the need for increased efforts to standardize cyanotoxin extractions from tissues and analytical methods (Smith and Boyer, 2009). Furthermore, only a handful of field studies on cyanotoxins other than MCs in freshwater systems exist, so relatively little is known regarding accumulation and presence of other cyanotoxins in wild fish. Cyanobacteria produce hundreds of secondary metabolites, many of which have been detected in natural systems (Rohrlack and Utkilen, 2007; Beversdorf et al., 2017). Co-occurrence of multiple cyanotoxins in wild fish have also been detected (Pawlik-Skowronska et al., 2012; Al-Sammak et al., 2014)—field studies on toxins other than MC are necessary to determine the risk of exposure to other bioactive products produced by cyanobacteria.

Our global synthesis includes the majority of studies that have been published on cyanotoxins in wild, freshwater fish. Much of what we know about cyanotoxin concentrations in fish comes from laboratory experiments and exposure studies. However, the growing number of field studies which analyze cyanotoxins in seafood is attributed to an equally growing concern for the transfer of toxins from food sources to people (Lee et al., 2017). We found toxin concentrations were highly variable across tissue types and fish feeding modes. Indeed, some of this variability may be attributed to the numerous methods that are used to quantify cyanotoxins. One of the major limitations of the present study was the large proportion of data coming from ELISA, and a general lack of congener-specific data. Due to matrix effects

TABLE 4 | Microcystin (MC) quantification methods for which significant differences in MC concentrations between tissue types were detected by bootstrap analyses.

	ELISA	PP2A	LC-MS	LC-MS/MS	GC-MS
Muscle	■	■	■	■	■
Intestine	■	■	■		
Liver	■	■	■	■	■
Kidney	■		■	■	
Ovary	■			■	
Brain	■				
Hepatopancreas	■	■			
Blood	■				
Gut	■				

Tissue types common across methods are indicated by a black box.

and cross-reactivity of ELISA with MC metabolites, ELISA can be considered a semi-quantitative method (Kopp et al., 2013; Schmidt et al., 2013). All currently available analysis methods have pros and cons (Foss and Aubel, 2015), and which method is used often depends on the purpose for testing. For quantitative analysis in fish tissues, however, LC-MS/MS appears to offer the greatest combination of sensitivity and specificity for congener—specific analysis (Kopp et al., 2013; Preece et al., 2015).

MCs covalently bind extensively to proteins (MacKintosh et al., 1995), and thus protein-bound MC fractions are important for understanding MC dynamics in fish tissues. The wide majority of MC analyses in wild fish have not used methods that can detect tissue-bound MCs, even though much of the total microcystin concentration can be bound to the tissues (Williams et al., 1997a,b; Lance et al., 2010; Greer et al., 2017). Covalently bound toxins may not be biologically available to consumers and/or unable to inhibit protein phosphatases, but proteolytic enzymes in the digestive system could free them from the tissues (Smith et al., 2010). The binding of MCs to protein phosphatases may, to some extent, be reversible (Miles et al., 2016). The consequences of the irreversible binding of MC to protein phosphatases is not well understood (Ibelings and Chorus, 2007; Smith et al., 2010), and conventional extraction methods may not be efficient for quantification of the protein-bound fraction of MCs in biological samples (Neffling et al., 2010). Covalently bound MCs are not considered toxic, but if they can be released from proteins under certain conditions, the covalently bound fraction may act as a secondary toxic pool whereby additional toxins can be released back into circulation of the organism (Miles et al., 2016). The 2-methyl-3-methoxy-4-phenylbutyric acid (MMPB) method, targets all MCs with an intact native ADDA residue and in most cases targets both protein and non-protein bound MCs. As such, this method in combination with LC-MS/MS may provide one analytical method for quantification of the total toxic MC pool in fish tissues. This would offer both the specificity and sensitivity of standard mass spectrometry methods and the ability to target all MCs with an intact native ADDA residue. The MMPB method has been significantly improved and standardized for water samples (Foss and Aubel, 2015), but further work is needed for complex matrices such as fish tissues.

Information about the accumulation of cyanotoxins in one fish species from different lakes or multiple species from the same lake is needed to understand and prevent the possible exposure of humans through consumption (Peng et al., 2010). Our large-scale analysis of cyanotoxins in fish and fresh water informs efforts to understand harmful cyanobacterial bloom impacts on globally important areas such as fisheries, human health and natural resource management. In addition, a global dataset on cyanotoxins in water and fish will streamline future research efforts by providing a baseline for research and management organizations to compare regional levels of cyanotoxin concentrations in water or fish to those from other locales. Because climate change and continued anthropogenic eutrophication are expected to increase the occurrence and severity of HABs (O'Neil et al., 2012), understanding cyanotoxin dynamics in fish and fresh water are becoming increasingly important for human health. Progress in this research area is currently hindered by analytical limitations, such as the lack of standardized extraction procedures and analytical standards for tissues (Hu et al., 2017). The analysis provided here shows that current and future research efforts should focus on standardized extraction and analysis methods for cyanotoxin in fish tissues as an important human exposure route. In addition, data on the occurrence of other cyanotoxins besides MCs in fish tissues are needed.

AUTHOR CONTRIBUTIONS

NF: Conducted data mining, the statistical analysis, and manuscript writing; TM: Provided guidance on cyanotoxin analysis methods in the literature, statistical analysis, and comments on the manuscript; JS: Developed the idea for the paper and contributed guidance on the methods, statistical analysis and manuscript writing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2018.00030/full#supplementary-material

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