pH dependent octanol–water partitioning coefficients of microcystin congeners

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ABSTRACT

Hazardous algal blooms can generate toxic compounds with significant health impacts for exposed communities. The ubiquitous class of algal toxins known as microcystins exhibits significant heterogeneity in its peptide structure, which has been minimally studied, given the significant impact this has on hydrophobicity, acid/base character and related environmental fate and health effects. Octanol–water partition coefficients for the microcystin congeners MCLR, MCRR, MCLY, MCLF, and MCLA were calculated over an environmentally and physiologically relevant pH range. Microcystin-LR log(K_{ow}) partition coefficient values were found to be consistent with previously established literature values, 1.67 to −1.41 between pH 1 and 8. Microcystin RR was found to be pH insensitive with a log (K_{ow}) of −0.7. The remaining congeners exhibit similar pH dependence as MCLR, with systematic increases in hydrophobicity driven by the introduction of more hydrophobic residues to their variable amino acid region. The variation in pH dependent hydrophobicity suggests increased propensity for bioaccumulation and alternate environmental fates for differing microcystin forms, requiring further investigation.

Key words | algal blooms, algal toxin, cyanobacteria, microcystin, partition coefficient

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INTRODUCTION

Microcystins (MC) are a class of cyclic heptapeptides produced by fresh water cyanobacteria (blue-green algae), primarily those in the genus *Microcystis*, with worldwide occurrence (Dietrich & Hoeger 2005). Cyanobacteria are photosynthetic organisms that constitute an important component of varied aquatic ecosystems where they are important primary producers and also play key roles in nitrogen fixation. Under suitable environmental conditions, cyanobacteria populations may undergo explosive growth and toxin-producing species will form large, hazardous algal blooms (HABs), which are increasing in frequency as a result of anthropogenic eutrophication and climate change (Hallegraeff 1993; Paerl *et al.* 2001; O'Neil *et al.* 2012).

While multiple toxins are produced by cyanobacterial blooms, microcystins are among the most commonly reported hepatotoxic compounds found in surface water (Chorus & Bartram 1999; Sivonen & Jones 1999; D'Anglada & Strong 2015; Loftin *et al.* 2016). Microcystins are selectively absorbed into liver cells by organic anion transport polypeptides and inhibit protein phosphatases (Campos & Vasconcelos 2010; Pereira *et al.* 2013), resulting in liver cell death, dysfunction, and, if sufficient exposure has occurred, lethality (Briand *et al.* 2003; Lone *et al.* 2015; Bengis *et al.* 2016). The microcystin class shares a common peptide structure containing multiple non-standard amino acids and two variable amino acid moieties (Figure 1), resulting in dozens of distinct forms with different physiochemical properties.

doi: 10.2166/wh.2018.257

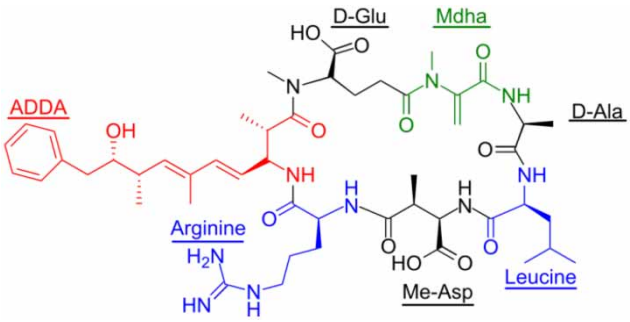

Figure 1 | Structure of microcystin-LR with highlighted structural elements. The non-standard amino acid ADDA (red) is responsible for much of microcystin's hydrophobicity; Mdha (green) contains the covalent binding site for phosphatase inhibition. Residues highlighted in blue are variable between congeners. Please refer to the online version of this paper to see this figure in color: http://dx.doi.org/10.2166/wh.2018.257.

Toxicity is known to vary greatly between congeners as a result of their pH dependent solubility and bioavailability (Stoner *et al.* 1989; Gupta *et al.* 2003), and environmental fate is closely tied to both environmental pH and microcystin hydrophobicity (Pérez & Aga 2005; Schmidt *et al.* 2014).

Microcystin contamination is predominantly found as the MCLR peptide form, and most analytical examination of microcystin class behaviors is based on this species due to its prevalence and known intraperitoneal toxicity (Gupta *et al.* 2003). However, blooms are highly variable in their toxin content and frequently contain a plurality of toxic congeners rather than a dominant species (del Campo & Ouahid 2010; Bouhaddada *et al.* 2016). The constituent amino acids of the conserved microcystin backbone are balanced between the highly hydrophobic amino acid, ADDA [(2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-(4*E*),(6*E*)-dienoic acid], and the polarity of the glutamic and aspartic acids (Figure 1). Thus, the nature of the variable amino acids can substantially influence the overall hydrophobicity of the peptide and its response to pH.

Understanding the pH dependent hydrophobicity of microcystins is of substantial value in modeling the ultimate environmental fate of released microcystin species, and in the bioavailability of the toxins once exposed. Cyanobacteria flourish in mildly alkaline conditions (López-Archilla *et al.* 2004; Lehman 2007), providing one pH range for microcystin release and exposure. Microcystin experiences a series of pH environments during the oral exposure pathway; pH is low (1–4) in the lower stomach but gradually decreases in acidity to a pH of ~6 in the duodenum and pH >7 by the terminal ileum (Fallingborg 1999). This has some implications for the enterohepatic recirculation of microcystin toxins. Hepatocytes are the primary target of microcystin toxins and MCLR is known to penetrate via the multispecific hepatic bile acid transport system (Eriksson *et al.* 1990), due to its high water solubility at physiological pH. The uptake availability of alternate congeners is highly related to their gastrointestinal availability across the gut pH range, as well as their hydrophobicity at physiologically neutral pH, where more hydrophobic species are more readily transported through enterohepatic recirculation.

Previous efforts have been made to determine octanol–water partitioning coefficients (K_{ow}) for MCLR, with ranges of 2.18 to −1.76 at pH = 1 to 10 (de Maagd *et al.* 1999), 1.63 to −1.56 at pH = 1 to 12 (Liang *et al.* 2011), and as high as 4.2 at pH = 7 having been reported (Rivasseau *et al.* 1998). However, most congener forms have not previously been reported over the wide range of potentially relevant pH values. In order to examine the influence of pH on the K_{ow} of alternate microcystin congeners we measured the values over a wide pH range for five major microcystin variants, including MCLR. The selected congeners – MCLR, MCRR, MCLY, MCLF, and MCLA – include the most important microcystin toxins (Butler *et al.* 2009).

MATERIALS AND METHODS

Materials were sourced from Sigma-Aldrich unless otherwise specified and were used as received. Microcystin standards were obtained as follows: MCLA and MCRR from Beagle Bioproducts Inc. (Columbus, OH); MCLR from Greenwater Laboratories (Palatka, FL); MCLF and MCLY from Enzo Life Sciences (Farmingdale, NY).

Stock solutions of the microcystins MCLR, MCRR, MCLY, MCLF, and MCLA were prepared in 50:50 methanol:water to a concentration of 1 mg/mL. Phosphate buffers were prepared at pH = 2.0, 5.0, 6.0, 7.0, and 8.0 to a concentration of 100 mM. Buffer at pH = 1.0 was prepared using HCl/KCl. All buffers were ionic strength adjusted using KCl. For each microcystin, aliquots were spiked into

5.0 mL of pH buffers 1.0–8.0 (Figure 2). An additional back extraction control was prepared by directly spiking microcystin into 1 mL of octanol. Samples and back extraction controls were prepared in triplicate. One milliliter of octanol was added to each aqueous solution and the two phases were shaken for 48 hours at 25 °C. After equilibrium was reached, the samples were centrifuged at 100 g for 20 minutes to ensure complete phase separation.

Seven hundred microliters were removed from each octanol phase using a syringe and the interfacial region was discarded. From the aqueous layer, 4.7 mL of water was retained for analysis and pH adjusted with 1 mL of 400 mM, pH = 7.0 phosphate buffer. The removed octanol phase was back extracted with 5 mL of NaOH at pH = 12.0. A 4.7 mL fraction of the basic phase was removed and pH adjusted as the aqueous fraction. The octanol-only samples were prepared similarly to the removed octanol fractions.

The phase extracts were prepared for liquid chromatography–mass spectrometry (LC-MS) analysis following a modified version of EPA method 544. In brief, samples were concentrated onto preconditioned Oasis HLB SPE cartridges, eluted in 5 mL of methanol, and evaporated under dry nitrogen stream to 1 mL final volumes. A 100 μL aliquot was diluted with 300 μL of 2 mM ammonium formate and transferred to an HPLC vial for LC-MS quantification.

LC-MS-TOF analysis was performed using an Agilent 1100 series HPLC equipped with an Eclipse Plus C8 column (2.1 × 50 mm, 3.5 μm; Agilent) interfaced to an Agilent 6210 series LC-TOF system in negative mode. Microcystin samples were injected with 5 μL volume and separated using a binary mobile phase gradient consisting of 0.4 mM ammonium formate in 95:5 deionized water: methanol (mobile phase A) and 95:5 methanol:deionized water (mobile phase B). The initial mobile phase (25% B) was ramped to 80% B over 5 minutes, then to 100% over 2 minutes followed by a 3-minute hold at 100% B and a 4-minute post time equilibration at the starting conditions. Microcystin concentrations were determined by integration of the [M-H]-species using seven-point external calibration curves prepared from the initial stock solutions for each MC variant. Measured concentrations in the octanol phase were adjusted for back extraction efficiency based on the measured values from the octanol-only control samples, and partitioning coefficient values were calculated for each peptide and pH combination. Data processing was carried out in R and plots prepared with ggplot2.

RESULTS AND DISCUSSION

Values for pH dependent octanol–water partitioning coefficients (D_{ow}, Table 1) over the pH ranges measured were plotted and polynomial fit with 95% confidence intervals (Figure 3). Recovery percentages from the octanol phase ranged from 75 to 101% (Table 2).

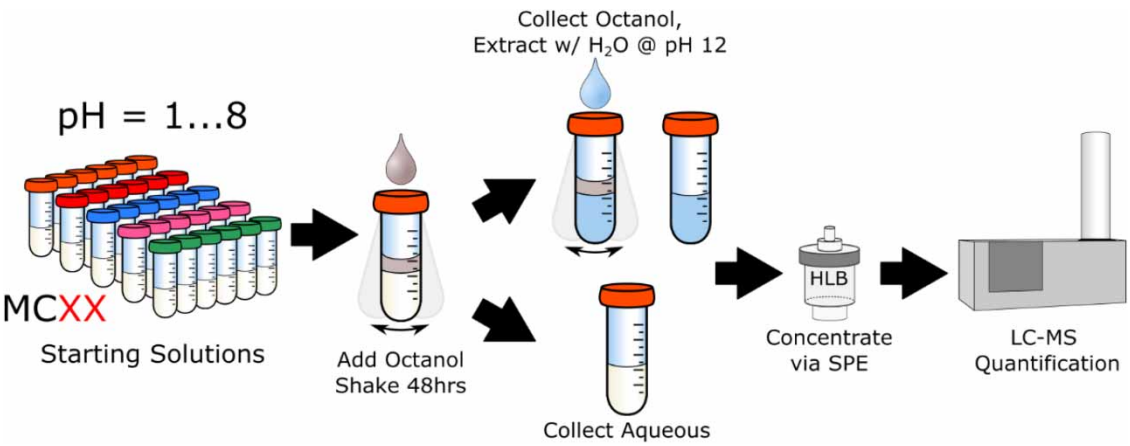

Figure 2 | Workflow overview of K_{ow} determination. Solutions of microcystin standards were partitioned with octanol for 48 hours, and the phases separated. Microcystin was recovered from the octanol fraction via back extraction with pH 12 water. Following solvent exchange to aqueous phases all microcystin fractions were concentrated via SPE and quantified by LC-MS.

Table 1 | $Log_{10}(D_{OW})$ measurements for microcystin congeners under pH buffered conditions with standard deviations ($n = 9$)

	Buffer pH					
	1	2	5	6	7	8
MCLA	1.30 ± 0.06	1.14 ± 0.07	0.41 ± 0.08	0.67 ± 0.04	0.16 ± 0.05	0.19 ± 0.15
MCLF	3.10 ± 0.10	2.75 ± 0.12	1.76 ± 0.06	0.65 ± 0.06	0.06 ± 0.13	−0.16 ± 0.13
MCLR	1.67 ± 0.09	1.43 ± 0.14	−0.64 ± 0.19	−0.78 ± 0.06	−1.2 ± 0.2	−1.41 ± 0.2
MCLY	3.50 ± 0.20	2.23 ± 0.02	1.09 ± 0.03	0.12 ± 0.17	−0.65 ± 0.09	−0.8 ± 0.3
MCRR	−0.71 ± 0.05	−0.72 ± 0.05	−0.69 ± 0.03	−0.68 ± 0.06	−0.72 ± 0.05	−0.65 ± 0.06

The D_{OW} curves for each species are predominantly driven by the identity of the variable amino acid residues and their hydrophobicity and K_a values. MCRR possesses an overabundance of charged sites throughout the pH range measured, and consequently is completely insensitive to pH changes ($p = 0.10$, ANOVA) exhibiting a flat, hydrophilic preference. MCLR shows the largest dependence on pH, with a $\log(D_{OW})$ that shifts from −1.41 to 1.67 as the pH decreases, which is consistent with previously reported values (Liang *et al.* 2011). The remaining curves show inflection around the pK_a for the glutamic and aspartic acid residues ($pK_a \sim 4$) as expected, and possess a maximum octanol affinity when minimally charged (low pH). In the absence of a variable residue containing a charged site, congeners possessing hydrophobic amino acids are uniformly shifted towards higher hydrophobicity as expected. For example, the systematic difference between MCLF and MCLY almost exactly matches the difference in

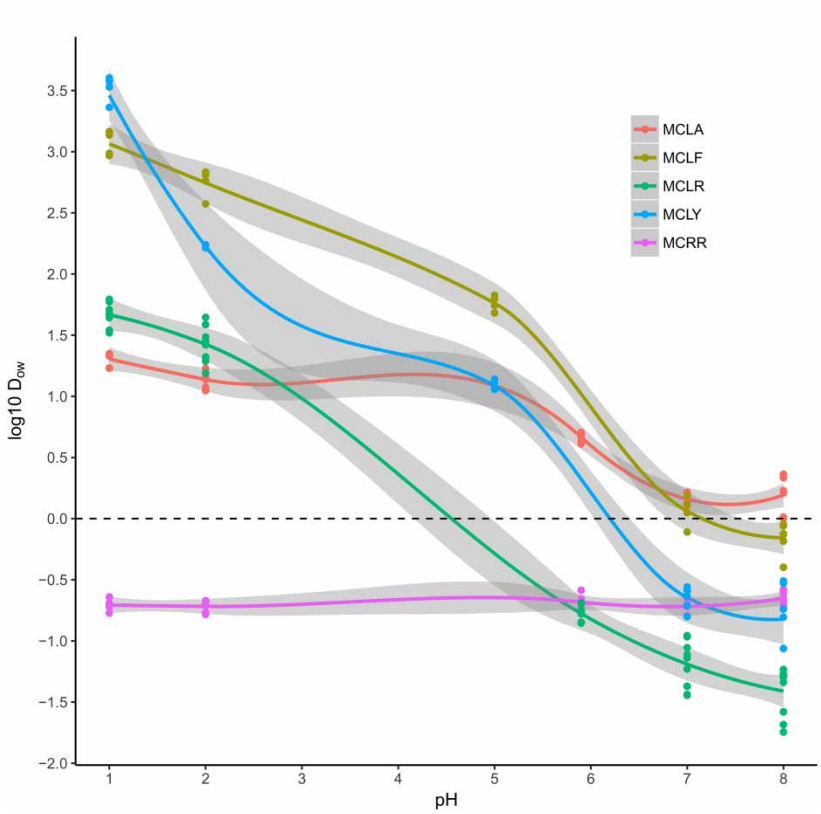

Figure 3 | pH dependent D_{ow} for various microcystin species with LOESS curves and 95% confidence intervals.

Table 2 | Efficiency measurements for the recovery of various microcystin species from octanol with a pH = 12.0 aqueous back extraction

	Spiked MC (μg)	Measured (μg)	Std dev	Back extraction efficiency (%)
MCLA	25	17.6	3.5	87.8
MCLF	25	15.0	5.0	75.1
MCLR	50	50.5	7.2	101.1
MCLY	25	17.9	2.4	89.3
MCRR	50	43.5	5.0	87.1

Wimley-White whole-residue hydrophobicity of phenylalanine and tyrosine in isolation (Wimley *et al.* 1996).

All the measured microcystin congeners appear at least mildly hydrophilic under expected cyanobacterial growth conditions (neutral to mild alkalinity) but show marked increases in hydrophobicity driven by their variable amino acid residues when exposed to acidic conditions, such as those likely to be encountered during ingestion. Microcystin-LR has a proposed abiotic fate involving binding to soil and suspended particulates (Schmidt *et al.* 2014), but the increased hydrophilicity of many MC congeners under typical cyanobacterial growth conditions would suggest that this route may be less efficient for those forms and that they possess longer aqueous residence times. Moreover, the observed hydrophobicity at gut pH allows for more substantial bioaccumulation and biouptake than would have been predicted from congener measurements in neutral or basic aqueous systems. This is consistent with reported bioaccumulation for microcystins (Ibelings & Chorus 2007), which would not have been predicted from neutral D_{ow} measurements, and indicates that some congeners may have a significantly higher potential for bioaccumulation than MCLR alone.

The relative hydrophobicity of microcystin congeners has also been hypothesized to determine the relative toxicities, with congeners showing greater hydrophobicity being more toxic. Likely this is the result of the cellular uptake mechanism and/or passage through the gut. It has been shown that MCLR, MCRR, MCLW, and MCLF are all equally inhibitory to protein phosphatases in cell free systems but their toxicity in cell cultures varies considerably, with the more hydrophobic MCLW and MCLF being more toxic than MCLR, while MCLR is likewise more toxic than MCRR (Fischer *et al.* 2010). In water containing eight microcystin congeners (MCLR, 7-dm-MCLR, MCYR, MCRR, 7-dm-MCRR, MCLY, MCLW, and MCLF) MCLW and MCLF accounted for 10% of the microcystins present but were estimated to have contributed 45% of the total toxicity (Faassen & Lürling 2013).

CONCLUSIONS

$Log_{10}(D_{OW})$ values for five microcystins were calculated across a range of pH values from 1 to 8. Values calculated for MCLR closely matched previously established literature values, while values for congeners with different variable amino acids track very closely with theoretical estimates based on individual amino acid hydrophobicities. Notably, MCRR does not demonstrate pH dependent solubility, as other forms did, likely due to the inclusion of multiple charged side chains preventing the formation of net neutral species in the measured pH range. The relatively higher measured hydrophobicity of the other alternate forms suggests a greater potential for bioaccumulation, especially via oral exposure routes. This is consistent with observations of microcystin congener toxicity in available literature. The D_{OW} values calculated should provide a useful basis for modeling of environmental and biological fate of alternative microcystin forms, which may make up a sizeable fraction of toxic bloom products and are thus far understudied.

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First received 2 November 2017; accepted in revised form 26 February 2018. Available online 3 April 2018