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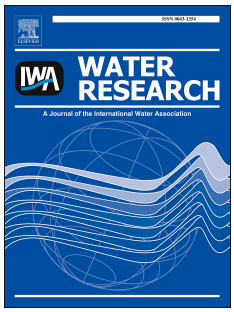

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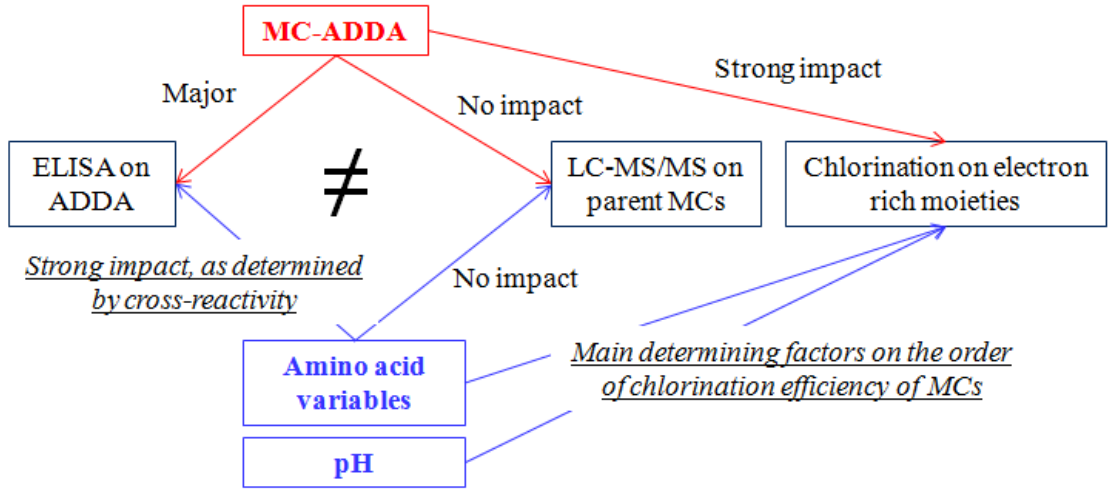
Graphical Abstract



Varied influence of microcystin structural difference on ELISA cross-reactivity and chlorination efficiency of congener mixtures

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Abstract

Enzyme-linked immunosorbent assay (ELISA) is an antibody-based analytical method that has been widely applied in water treatment utilities for the screening of toxic cyanobacteria metabolites such as microcystins (MCs). However, it is unknown how the minor structural difference of MCs may impact their measurement and chlorination kinetics via ELISA method. It was found in this study that, regardless of experimental conditions (n=21), there was no MC-YR or MC-LY residual, while different removal rates of other MCs were observed (MC-RR > MC-LR > MC-LA ~ MC-LF) as measured by liquid chromatography tandem mass spectrometry (LC-MS/MS), which was consistent with the relative reactivity of the amino acid variables with free chlorine. The removal of total MCs was generally lower as measured by ELISA than by LC-MS/MS. By incorporating both analytical results, existence of ADDA-containing byproducts or byproducts that had a higher sensitivity toward the ELISA kit was demonstrated, after excluding the contribution of the cross-reactivity of the parent MCs. It should be noted, however, that the cross-reactivities of MCs could be influenced not only by MC congeners, but also by other conditions such as mixtures and the applied ELISA kit.

Keywords: ELISA; LC-MS/MS; chlorine; microcystin; mixture; cross-reactivity

1. Introduction

Cyanobacteria are among the most ancient organisms on earth and have been found to be highly adaptive to various environmental conditions (Catherine *et al.* 2013, de la Cruz *et al.* 2013, Makhalanyane *et al.* 2015, Merel *et al.* 2013, Schopf 2006). Their increasing occurrence in fresh water sources has induced significant research interest and public concern, because certain cyanobacteria species are capable of producing toxic metabolites known as cyanotoxins or cyanobacterial toxins (Catherine *et al.* 2013, de la Cruz *et al.* 2013, Makhalanyane *et al.* 2015, Merel *et al.* 2013). In order to secure safe drinking water, there is a need for a timely and successful detection of these toxins before the application of an appropriate cyanobacteria/cyanotoxin treatment process (He *et al.* 2016, Merel *et al.* 2013).

Antibodies isolated against a specific toxin or a specific group of toxins have been considered as the most promising screening method for cyanotoxins (McElhiney and Lawton 2005). The enzyme-linked immunosorbent assay (ELISA) is one of such methods that have been widely applied, due to its cost efficiency per sample, minimum sample processing and fast throughput (Sangolkar *et al.* 2006). The USEPA has recommended ELISA for water treatment utilities as a primary analytical tool for the quantification of total microcystins (MCs) in raw and treated water (USEPA 2015). The commonly used Abraxis Microcystins/Nodularin-ADDA ELISA test kit is an indirect competitive ELISA kit. Its detection mechanism is primarily through the competition between the ADDA functional groups in the toxins and the immobilized microcystins-protein analogue for the binding sites of the polyclonal sheep antibodies in solution. However, MC has been reported to have more than 150 congeners (Samdal *et al.* 2014), with widely varying ELISA sensitivity and cross-reactivity (Rapala *et al.* 2002, Sangolkar *et al.* 2006). Further, general water quality parameters such as natural organic matter may have an

unpredictable impact on the ELISA readings (Brun *et al.* 2005). Proper quality assurance (QA) procedures can be used to assess the presence and degree of the interference, but such QA samples are often not included in a standard protocol. These differing sensitivities and interference can cause significant overestimation (typically) or underestimation (less frequently) of the ELISA reading relative to the concentration of known species quantified by liquid chromatography (Mountfort *et al.* 2005).

Free chlorine has varied degrees of reactivity with organic compounds ranging from < 0.1 – 10^9 M^{-1} s^{-1}, with the most reactive sites being amines, reduced sulfur moieties and activated aromatic systems (Deborde and Von Gunten 2008). Since chlorination of peptide bonds is generally slow (Ho *et al.* 2006), it is the terminal or side amino group that determines overall reactivity of the peptides (Hureiki *et al.* 1994). Therefore, for the peptide MCs, the different reaction rates with chlorine are probably due to the difference in their amino acid variables. Information on how minor structural changes within the MC congeners can affect the reaction rates of MCs with oxidants has yet been far from comprehensive (Acero *et al.* 2005, He *et al.* 2015, Ho *et al.* 2006, Rodríguez *et al.* 2007). Since chemical oxidation of MCs aims to transform toxic parent MCs without necessarily a mineralization, there could be a large number of oxidation byproducts with the intact ADDA functional group (Mash and Wittkorn 2016, Rosenblum *et al.* 2017, Zhang *et al.* 2016), interfering with the ELISA analysis.

In this study, free chlorine was selected as a model compound to degrade six UCMR4 MCs. The main objectives were (1) to investigate the impact of MC structural difference on the chlorination kinetics of MC mixtures; (2) to examine its influence on ELISA cross-reactivity and subsequently ELISA measurement; (3) to study the correlation between the liquid chromatography tandem mass spectrometry (LC-MS/MS) and ELISA results in both raw and

chlorinated samples; and (4) to evaluate the potential role of cross-reactivity in addition to the commonly known contribution of oxidation byproducts on the degradation kinetics, as interpreted by ELISA.

2. Materials and Methods

2.1.Natural water collection

Three water samples, from Finger Lakes (FL, Waterloo, NY, on 07/25/2016), Grand Lake St. Marys (GLSM, Celina, OH, on 07/26/2016), and Lake Mead (LM, Henderson, NV, on 07/27/2016), were collected in 10 L cubitainers and transported in an iced cooler. Upon receiving, samples were filtered through a glass microfiber filter (GMF, 1.5 µm, Whatman®, Marlborough, MA USA) by a vacuum pump. From a preliminary screening, no MCs (MC-LA, -LF, -LR, -LY, -RR, and -YR, structures of which are shown in Figure 1) or cylindrospermopsin (CYN) which are included in UCMR4 (the fourth Unregulated Contaminant Monitoring Rule, (USEPA 2016)) were detected by LC-MS/MS. However, the ELISA test for the filtered GLSM sample showed a total MC concentration of 3.40±0.22 µg/L. The results are consistent with the historically predominant demethylated-MCs in Ohio inland lakes (personal communication with Ohio EPA staff). More information on the quality of GLSM can be found in Text S1, Figure S1, in Supplementary Information (SI).

2.2.Materials

Cyanotoxins used for the experiments were purchased in powder form from Enzo Life Sciences (MC-LA (ALX-350-096-C100), MC-LF (ALX-350-081-C100), MC-LR (ALX-350-012-C500), MC-LY (ALX-350-148-C100), MC-RR (ALX-350-043-C500), MC-YR (ALX-350-

044-C100), and CYN (ALX-350-149-C100), Farmingdale, NY USA). The stock solutions were prepared by mixing the chemicals as received with 4 mL deionized water (DI). The cyanotoxins for making LC-MS/MS calibration curves were also purchased from Enzo Life Sciences (*i.e.*, MC-LA (ALX-350-096-C025), MC-LF (ALX-350-081-C025), MC-LR (ALX-350-012-C050), MC-LY (ALX-350-148-C025), MC-RR (ALX-350-043-C050), MC-YR (ALX-350-044-C025), and CYN (ALX-350-149-C025)). Instead of DI, they were dissolved in methanol in our lab. ELISA kits (Microcystins/Nodularins (ADDA), PN 520011OH) were purchased from Abraxis (Warminster, PA USA). The 5.6% liquid sodium hypochlorite (NaOCl) was purchased from Fisher Scientific (Waltham, MA USA) and used to prepare the stock solutions.

2.3.Experimental procedures

The main experimental design is shown in Table S1. Samples were generally spiked with six MC congeners plus CYN, except stated otherwise. The initial concentrations, determined against LC-MS/MS calibration curves using a separate batch of stock solutions from the same manufacturer, were considered as true initial toxins concentrations. The experimental conditions involved a varied water matrix (GLSM, LM, and FL, the DOC of which were 7.8, 2.9 and 2.9 mg/L, respectively), oxidant dose (low (L), medium (M), and high (H), representing $[Cl_2]_0$:DOC_0 mass ratio of 0.5, 1, and 2, respectively), pH (6, 8, and 10), and temperature (T, 10, 20±2 and 30 ^{0}C). The experiments were performed as batch processes using 250 mL amber glass bottles, containing a sample volume of 150 mL. Water pH was adjusted using H_2SO_4 and NaOH, and measured using a pH meter (Accumet® AP110, Fisher Scientific, Waltham, MA USA). The 20±2 ^{0}C experiments were conducted at room temperature in a well circulated lab. The 10 ^{0}C and 30 ^{0}C conditions were controlled by a chiller and water bath, respectively. Sodium

thiosulfate was added after 20 min treatment to quench any residual oxidant. Chlorinated samples were injected into LC-MS/MS without any post treatment. Dilutions were conducted whereas necessary for the ELISA analysis based on the LC-MS/MS results.

2.4.Analytical methods

DOC was measured using a TOC analyzer (Shimadzu, Columbia, MD USA) according to Standard Methods 5310B. ELISA analysis was conducted following U.S. EPA Method 546 by using the cyanotoxin automated assay system (CAAS, Model 2900, Abraxis, Inc., Warminster, PA USA). An LC-MS/MS method, which has been reported previously in detail for the detection of common cyanotoxins (Wert *et al.* 2014), was used for the identification and quantification of the cyanotoxins. MS/MS analysis was performed using both negative and positive electrospray ionization (ESI), with MC-RR and -YR quantified by the (+) mode while all the others by the (-) mode. Each analyte was monitored by a quantitation transition and at least one additional confirmation product ion. The method reporting limit (MRL) was determined to be 0.5 µg/L (Wert *et al.* 2014).

3. Results and discussion

3.1.Oxidant kinetics in MC mixtures determined by LC-MS/MS

As shown in Figure 2 and Figure S2, compared to other variables such as temperature, oxidant dose, and the background matrix, chlorination of MCs was influenced more significantly by water pH, following generally pH 6 > pH 8 > pH 10. For pH 6, all chlorination experimental series showed a complete removal of total MCs, excepted for one data point, which was by using GLSM that had a high DOC level and was treated with $[Cl_2]_0$:DOC_0 mass ratio of 1. In general,

the acidic form of HOCl (pK_a = 7.5, (Acero *et al.* 2005)) is the most effective at oxidizing organic compounds. However, higher pH leads to a predominance of OCl^- ion and the deprotonation of certain functional groups on a target species, thereby impacting chlorination reactivity. As mentioned above, the phenolic acid and the phenonate have distinctive rate constants with chlorine, and thus the negative effect of high pH may be offset for MCs having tyrosine (with the side chain pK_a of 10.07 (Thorson *et al.* 1995)). Other amino acid variables (without any pK_a except for a value of 12.48 for arginine (Guan *et al.* 2015)), the status of which are not influenced by experimental pH, may be more influenced by the speciation of chlorine. The observed chlorination degradation efficiency of MCs in this study, *i.e.*, pH 6 > pH 8 > pH 10, was therefore consistent with the hypochlorous acid species being more reactive than the hypochlorite ion.

Regardless of the experimental conditions, for the chlorinated samples (n=21), there was no MC-YR or MC-LY residual (indicating fast reaction); while different extents of degradation on the other MCs (MC-RR > MC-LR > MC-LA ~ MC-LF) were observed (indicating slower reactions). The results suggested the influence of minor structural difference within MC congeners on the chlorination kinetics.

Among the amino acid variables in MCs, the activated aromatic compound, *i.e.*, tyrosine (Y), has the highest reactivity, with a second-order rate constant of 0.36 M^{-1} s^{-1} for the phenol at the acidic pH and $2.19x10^4$ M^{-1} s^{-1} for the phenolate at alkaline pH conditions (Ho *et al.* 2006). Arginine similar compound, ethyl guandine (as a terminal amine group), has a rate constant of 19 M^{-1} s^{-1} at pH 7.2 (Deborde and Von Gunten 2008). Both these two amino acids have a higher reactivity with chlorine than the ADDA group, which is commonly represented by the sorbic acid, *i.e.*, 2.3 M^{-1} s^{-1}, at pH 7.2 (Ho *et al.* 2006). The L-phenylalanine (F), on the other hand, is

not activated and is thus expected to have a low reactivity with chlorine (Hureiki *et al.* 1994). Lastly, leucine (L) and alanine (A) have minimum reactivity with chlorine (Ho *et al.* 2006, Hureiki *et al.* 1994). It could thus be expected that the reactivity of MCs with FC follows MC-YR > MC-LY > MC-RR > MC-LR > MC-LF ~ MC-LA, which is consistent with our results. In fact, though comparable apparent and second-order rate constants for the reaction of MC-LR and MC-RR with chlorine have been reported by Acero (2005) at pH 6.3 and 7.9 (Acero *et al.* 2005), a different study by Ho (2006) showed higher chlorination efficiency of MC-RR than MC-LR in two different natural water samples, as suggested by their apparent second-order rate constants, at pH 6.3 and 7.9 (Ho *et al.* 2006). The authors also showed an order of MC-YR > MC-RR > MC-LR ≥ MC-LA (Ho *et al.* 2006).

Influence of MC minor structural difference on the oxidation kinetics was systematically examined and discussed in this section. With results on MC-LA, -LR, -RR, and -YR consistent with literature data (Acero *et al.* 2005, Ho *et al.* 2006), and the well validation using MC-LF and -LY, chlorination of other MCs (>150 congeners) could be similarly predicted.

3.2.Chlorination kinetics determined by ELISA – contribution of byproducts?

As shown in Figure 3, there was a relatively lower removal rate of total MCs calculated by using ELISA results than by using LC-MS/MS results. In the other words, the ELISA to LC-MS/MS ratio for the initial sample was lower than the ratio for the treated sample. Such an observation could be expected to result from the existence of ADDA-containing byproducts for ELISA detection (Rosenblum *et al.* 2017). However, considering two facts, (1) MC congeners are known to have different ELISA cross-reactivity, and (2) chlorine shows different reactivity with MCs, there could be an increase in the overall ELISA to LC-MS/MS ratio due to the slower

192 removal of higher ELISA cross-reactivity MCs in the congener mixtures. Therefore, for the MC
193 congener mixtures, presence of ADDA-containing byproducts cannot be regarded as the only
194 contributing factor to the lower removal rate as calculated by ELISA. This hypothesis will be
195 interpreted and demonstrated below.
196
197 3.2.1. Higher measured ELISA to measured LC-MS/MS ratio
198 As shown in Table 1, the total MCs measured by ELISA for the ten control samples were
199 approximately 1.5 times higher than the LC-MS/MS results (n=10). The higher ratios have been
200 reported by other researchers (Foss and Aubel 2015, Rapala *et al.* 2002). Foss and Aubel (2015)
201 showed 65.95±23.11% (n=22) as the average percentage of LC-MS/MS compared to Abraxis
202 ELISA analysis for natural water samples collected from different sources, which equaled to an
203 ELISA to LC-MS/MS ratio of 1.72±0.66 (Foss and Aubel 2015). However, the correlation is
204 cyanobacteria species dependent. Lei (2004) showed a ratio ranging from 0.16 for the MCs
205 isolated from *M. aeruginosa* 526 to 1.20 for the MCs isolated from *M. aeruginosa vi*, with an
206 overall average of 0.94±0.43 (n=6) (Lei *et al.* 2004). The observations may be due to the
207 different mixtures of MC congeners, because different MCs have different ELISA cross-
208 reactivities, *i.e.*, different binding efficiency with the antibodies that are raised typically against
209 MC-LR (McElhiney and Lawton 2005, Zeck *et al.* 2001). Efforts were thus taken to estimate the
210 cross-reactivities of the six UCMR4 MCs. If successful, the measured ELISA should be
211 comparable to the predicted ELISA which is equal to Σ (cross-reactivity × actual variant
212 concentration measured by LC-MS/MS), in the control samples.
213 Higher ELISA to LC-MS/MS ratios were also observed in the treated samples regardless
214 of the experimental conditions, *i.e.*, 1.66 (R^2 = 0.93, Figure 4). After considering the cross-

reactivity, whether the elevated ratios as compared to the control were attributed to the presence of ADDA-containing byproducts could be justified.

3.2.2. Estimation of ELISA cross-reactivity for individual MCs in DI water

An individual MC at four different concentration levels was prepared in DI water. The measured ELISA to measured LC-MS/MS ratios were calculated. The ratio for MC-LR was 0.94±0.11. After this ratio for MC-LR was assigned as 1.00, the cross-reactivity, defined as the average of the measured ELISA to measured LC-MS/MS ratios, was estimated as 2.17±0.42, 0.94±0.02, 2.05±0.16, 0.62±0.11, and 0.94±0.13, respectively, for MC-LA, LF, LY, RR and YR (n=4), as shown in Table 1. Alternatively, the cross-reactivity, estimated using the slope of the linear regression, was determined to be 2.42, 0.94, 2.07, 0.84, and 1.08, respectively, for MC-LA, LF, LY, RR and YR, with the corresponding slopes to be 2.18 (R^2 = 0.99), 0.85 (R^2 = 1.00), 1.86 (R^2 = 0.99), 0.76 (R^2 = 0.98), and 0.97 (R^2 = 0.96), as shown in Table 1, Figure 5 and Figure S4. These values are generally consistent with the reported literature data by Loftin *et al.* (2010), except for MC-LA for which Loftin reported 1.12 (Loftin *et al.* 2010). The high cross-reactivity for MC-LA was consistent with the most recent studies, where the steric structure of microcystin was indicated to influence the binding efficiency and thus led to a higher cross-reactivity of MC-LA as compared to MC-LR (Guo *et al.* 2017, Rochelle 2015). Therefore, ELISA that uses MC-LR as the standard would not show the true concentrations of the other MCs.

When using the calculated values in this section, the predicted ELISA to measured ELISA ratio for the mentioned ten control samples was 0.68±0.08 (using average value, (2)-2, Table 1), and 0.77±0.08 (using linear regression slope, (3)-2, Table 1), both sets of values under-

predicted the ELISA results. One possible reason was that those numbers were determined using individual MC spikes, while the reactivity for an individual MC may behave differently to that present in a multiple congener system. However, when an extra set of experiments was done by mixing the six MCs in DI, CRW (pH = 8), WL (pH = 8), and LM (pH = 8) water samples, the ratio turned out to be 1.44±0.25 (using average value), and 1.56±0.27 (using linear regression slope), both over estimating the ELISA results. One other reason was the use of a different ELISA lot for this series of tests. Fluctuation in the immune response in the animals during the cultivation of the antibodies may lead to such viability in the antibody mixtures and thus different cross-reactivities (McElhiney and Lawton 2005).

3.2.3. Estimation of ELISA cross-reactivities in MC mixtures using Solver in Excel

Solver in Excel was applied for the initial ten control samples to estimate the cross-reactivities of the MCs. The objective in Solver parameters was set as "measured ELISA = Σ (cross-reactivity × actual variant concentration measured by LC-MS/MS)", by changing the cross-reactivities of the MCs, the initial values of which were assigned as 1.00. The results were found to be 1.19±0.06, 1.20±0.05, 1.21±0.08, 1.98±0.32, and 1.47±0.12, respectively, for MC-LA, LF, LY, RR and YR (n=10), as shown in Table 1 and Figure 5 (indicated as "This study (Solver-calculated, Abraxis)"). This set of cross-reactivities predicted well the ELISA results with the predicted ELISA to measured ELISA ratio of 1.01±0.10. Raw data reported by Foss and Aubel (2015), using Abraxis ELISA (PN 520011), were recalculated also by Solver (Foss and Aubel 2015). All numbers below the MRL were eliminated from the calculation. The results are shown in Figure 5 (indicated as "Foss and Aubel 2015 (Solver-calculated, Abraxis)"). A value of 1.12±0.06 (n=2) was observed for MC-LA, comparable to the value reported by Loftin (2010)

and by this study using Solver estimation (Loftin *et al.* 2010). Consistent with this study, the high cross-reactivity values of 1.93±0.76 (n=13) and 1.38±0.53 (n=19) were found for MC-RR and MC-YR, respectively.

Apparently, the steric hindrance is not the only factor that influences the binding efficiency. Rapala (2002) proposed the potential contribution of the hydrophobicity of the MCs (Rapala *et al.* 2002). The relative hydrophobicity of the free amino acids was found to be 41, 100, 97, -14 and 63, for alanine (A), phenylalanine (F), leucine (L), arginine (R), and tyrosine (Y), respectively (Monera *et al.* 1995). The sum for LA, LF, LR, LY, RR, and YR is thus 138, 197, 83, 160, -28, and 49, respectively. The much higher cross-reactivities for MC-RR and MC-YR may therefore be due to their higher hydrophobicity as compared to MC-LR. Since not a single set of cross-reactivity data shown in Figure 5 is consistent with those hydrophobicity numbers, it may be speculated that steric hindrance, hydrophobicity, differences in ELISA production lots such as types of antibodies, immunized species, immunogens, MC standard sources, MC producing species, and even analytical procedures have all contributed to the inconsistency in the reported cross-reactivities of MCs (Lei *et al.* 2004, Rapala *et al.* 2002, Rochelle 2015, Zeck *et al.* 2001). The ELISA reading range may be one of the influencing factors, as shown by higher numbers predicted using the ELISA results between 1.66 – 2.39 µg/L than those between 3.26 – 3.70 µg/L (Table S2).

Both Fisher *et al.* (2001) and Mountfort *et al.* (2005) used the ELISA plates prepared in their labs and showed a different set of cross-reactivities (Figure 5) (Fischer *et al.* 2001, Mountfort *et al.* 2005). Using MC-LR isolated by a different laboratory, a high ratio of 1.64±0.14 (n=2) was observed as compared to the standards purchased from Calbiochem and the ELISA from Strategic Diagnostics Inc. (Rapala *et al.* 2002). Different MC material lots could

again give a different ratio, *e.g.*, 0.66±0.02 (n=2) vs 0.50 (n=1) for MC-RR, which were much lower than the MC-RR isolated from *Anabaena* strain 90 with a ratio of 1.66±0.04 (n=2, estimated using Solver) (Rapala *et al.* 2002). Gurbuz *et al.* (2009) followed the method described in Metcalf *et al.* (2000). Instead of using polyclonal antibodies, monoclonal antibodies were used to prepare the ELISA (Gurbuz *et al.* 2009, Metcalf *et al.* 2000). Since monoclonal antibody-based ELISA, though sensitive and highly reproducible, is highly congener specific (McElhiney and Lawton 2005), the recalculated cross-reactivities using their reported HPLC and ELISA numbers showed a high viability as represented by the error bars in Figure 5, which may suggest the inappropriateness of using the monoclonal ELISA for the screening of total MCs in natural water (Gurbuz *et al.* 2009). In fact, when Zeck *et al.* (2001) used a monoclonal antibody (clone (MC10E7)) for the ELISA test, their results showed highly diverse cross-reactivities with the percentage cross-reactivity ranging from 134 for the [D-Asp^3]MC-RR to less than 10^{-4} for MC-LA, LF and LY (Zeck *et al.* 2001).

Since Abraxis ADDA ELISA has been commercially available and commonly applied, it is meaningful to further evaluate the cross-reactivities of the MCs and the stability of the test kit. A more carefully designed test should be conducted in order to obtain the true numbers. With the vast existence of different MC congeners, the contribution and the influence of different amino acid variables should also be systematically assessed. Cross reactivity was evaluated based on a limited concentration range for each of the MC congeners. The reported cross reactivity factors may also change (linearly or non-linearly) as the MC concentrations changes. Differences in the cross reactivity over a wider range of concentrations, and whether the reported cross-reactivity values can be extrapolated to other studies, are not a part of this study and should be evaluated in future work.

307

308 3.2.4. Chlorination kinetics determined by ELISA – contribution of byproducts and cross-
309 reactivity

310 As discussed above, chlorine reacts preferably with MC-YR (a cross-reactivity of
311 1.47±0.12) and MC-LY (a cross-reactivity of 1.21±0.08) (Acero *et al.* 2005, Ho *et al.* 2006).
312 MC-RR is such a compound that has a medium reactivity with chlorine and a high ELISA cross-
313 reactivity (1.98±0.32). Its residual in the MC mixtures (MC-RR to total MCs ratio, detected by
314 LC-MS/MS) was around 0.37±0.02 in the ten control samples. For the treated samples, the ratio
315 varied from 0.19 to 0.58, depending on the treatment conditions. The higher measured ELISA to
316 LC-MS/MS ratio could therefore be attributed to the elevated concentration of MC-RR in the
317 treated samples. Using the estimated cross-reactivities determined by Solver in this study, the
318 predicted ELISA that represented only the parent MCs were found to be lower than the measured
319 ELISA, as shown in Figure 6, demonstrating the existence of ADDA-containing byproducts or
320 byproducts that had a higher cross-reactivity. However, without ruling out the contribution of the
321 cross-reactivity of parent MCs in the treated samples, it was less convincing by attributing
322 directly the elevated ELISA to LC-MS/MS ratio to the existence of ADDA-containing
323 byproducts.

324

325 **4. Conclusions**

326 This study compared two commonly applied analytical methods, ELISA and LC-MS/MS,
327 for the detection of microcystins with and without chlorination. For the chlorinated samples
328 (n=21), there was no MC-YR or MC-LY residual, regardless of the experimental conditions;
329 while different extends of degradation on the other MCs (MC-RR > MC-LR > MC-LA ~ MC-

LF) were observed, which was consistent with the relative reactivity of the amino acid variables with free chlorine. The chlorination efficiency followed apparently pH 6 > pH 8 > pH 10, highly dependent on the speciation of the oxidant rather than the side chains, the speciation of which was not impacted by the studied pH except for tyrosine. Other variables such as temperature, oxidant dose, and the background matrix, though not as significant, also contributed to the removal of MCs. The cross-reactivities of the six UCMR4 MCs were estimated using two different methods, *i.e.*, (1) direct calculation of ELISA to LC-MS/MS ratio for the individual MC standard in DI water; and (2) an estimation by Solver in Excel for the MC mixtures in different water matrices, showing an inconsistency on the cross-reactivities determined in this study by using the same ELISA kit but a different lot and by mixing different MCs, as compared to the numbers reported in literature. A systematic experimental design should be conducted to provide a more robust set of cross-reactivity data. Results in this study also demonstrated the existence of ADDA-containing byproducts or byproducts that had a higher cross-reactivity, by ruling out the contribution of the cross-reactivity of the parent MCs.

This work has several implications for water utilities monitoring and treating for MCs. First, ELISA results may produce apparently higher concentrations than LC-MS/MS results, depending on cross-reactivity of congeners, and in cases where congeners such as demethylated MC-LR exist and are detectable by ELISA but are not quantified by LC-MS/MS in the absence of a standard. Second, when using ELISA to measure concentrations of MCs in finished water, ADDA-containing post-chlorination byproducts may produce an elevated ELISA result as compared to LC-MS/MS analysis, leading to potential false positive results requiring public notification. Third, oxidation of MCs is variable by congener type and water quality conditions,

thus utilities would be well-served to identify which congeners dominate in their source water to better inform decision making on tailored treatment approaches.

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363 **Captions**

364 **Figures**

365 Figure 1. Structures of MCs. A = Alanine; F = Phenylalanine; L = Leucine; R = Arginine; Y =
366 Tyrosine.

367 Figure 2. Degradation of MCs: influence of congeners, pH and oxidant type. The order of the x-
368 axis was arranged based firstly on the initial pH and secondly on the total MCs as
369 measured by LC-MS/MS. GLSM = Grand Lake St. Marys; FL = Finger Lakes; LM =
370 Lake Mead; L, M, and H in oxidant dose = low, medium, and high, corresponding to an
371 $[Cl_2]_0$:DOC_0 mass ratio of 0.5, 1 and 2, respectively; T = temperature (^{0}C).

372 Figure 3. Comparison of chlorination kinetics, C/C_0 by ELISA vs C/C_0 by LC-MS/MS. Dash line
373 has a slope of 1.0.

374 Figure 4. Measured ELISA vs measured LC-MS/MS in chlorinated samples.

375 Figure 5. Inconsistency in ELISA cross-reactivity. Numbers for Foss and Aubel (2015) and
376 Gurbuz *et al.* (2009) were recalculated by using the reported raw data and the Solver in
377 Excel. Fisher *et al.* (2001) used lab prepared ELISA plates coated with OVA-ADDA-
378 hemiglutaryl conjugate; Mountfort *et al.* (2005) used lab prepared ELISA plates with
379 modified coating following Fisher *et al.* (2001), with the numbers recalculated from the
380 ratios among PP-2A, ELISA, and LC-MS; Gurbuz *et al.* (2009) used lab prepared ELISA
381 with monoclonal antiserum (Alexis 804-320) and goat anti-rabbit IgG-HRP, according to
382 Metcalf *et al.* (2000); Loftin *et al.* (2010) and Rochelle (2015) used Abraxis
383 Microcystins-ADDA ELISA; Foss and Aubel (2015) used Abraxis Microcystins-ADDA
384 ELISA (PN 520011); this study used Abraxis Microcystins-ADDA ELISA (PN
385 520011OH, purchased at two different times) (Fischer *et al.* 2001, Foss and Aubel 2015,
386 Gurbuz *et al.* 2009, Loftin *et al.* 2010, Metcalf *et al.* 2000, Mountfort *et al.* 2005,
387 Rochelle 2015).

388 Figure 6. Measured ELISA vs predicted ELISA using the Solver estimated cross-reactivity. Dash
389 line has a slope of 1.0.

390

391 **Tables**

392 Table 1. Estimation of cross-reactivity. (1) by using the Solver in Excel and the ten control
393 samples in this study; and (2) & (3) in a separated experiment by spiking four different
394 levels of individual MC in DI water, with a different lot of ELISA kit used in this case.
395 The cross-reactivity of MC-LR (X_{LR}) was assigned as 1.00. The predicted and the
396 measured ELISA results in the last column are based upon the same ten control samples.
397 The average values are expressed as "mean ± standard deviation". Concentration unit is
398 in µg/L.

399

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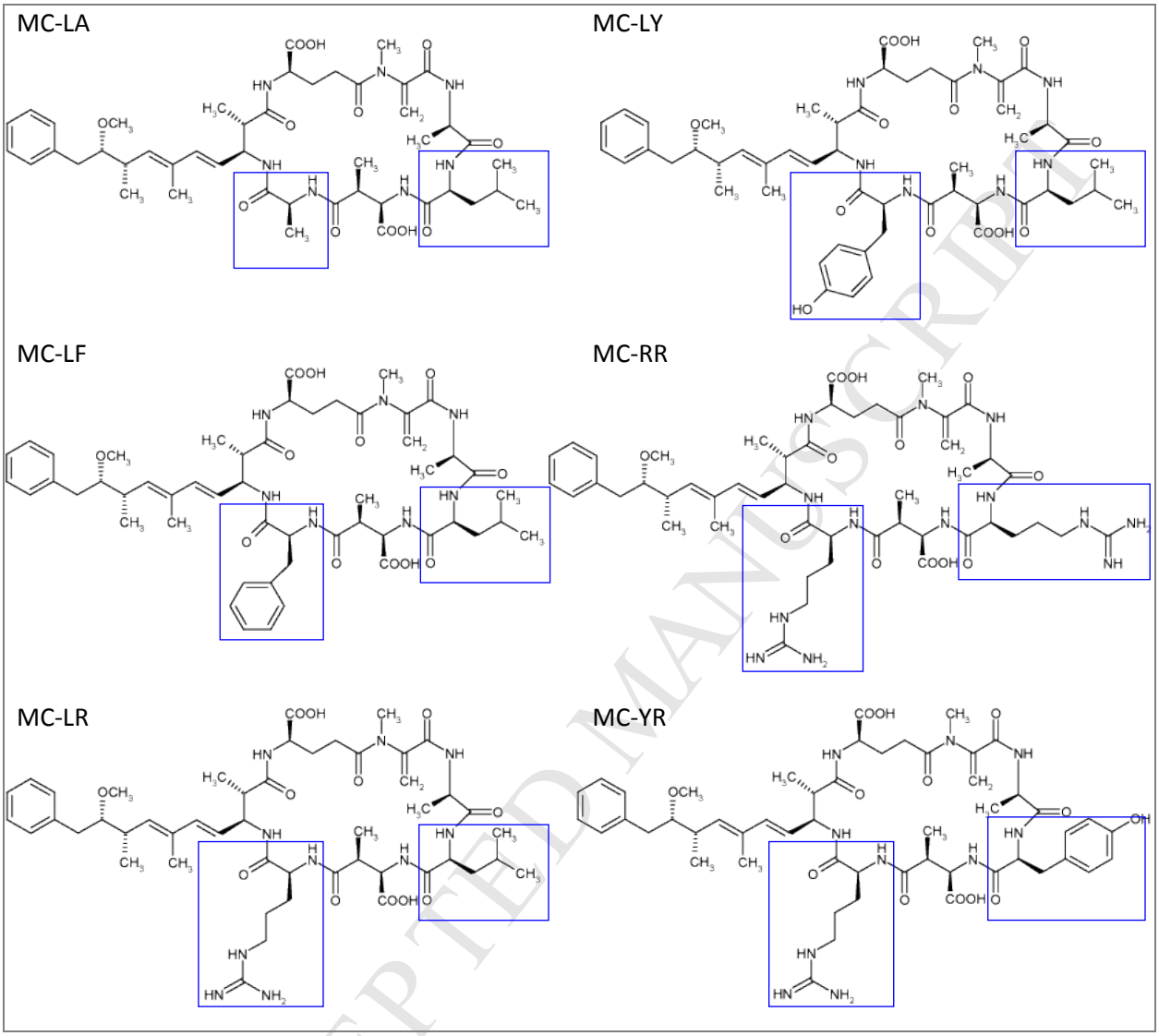
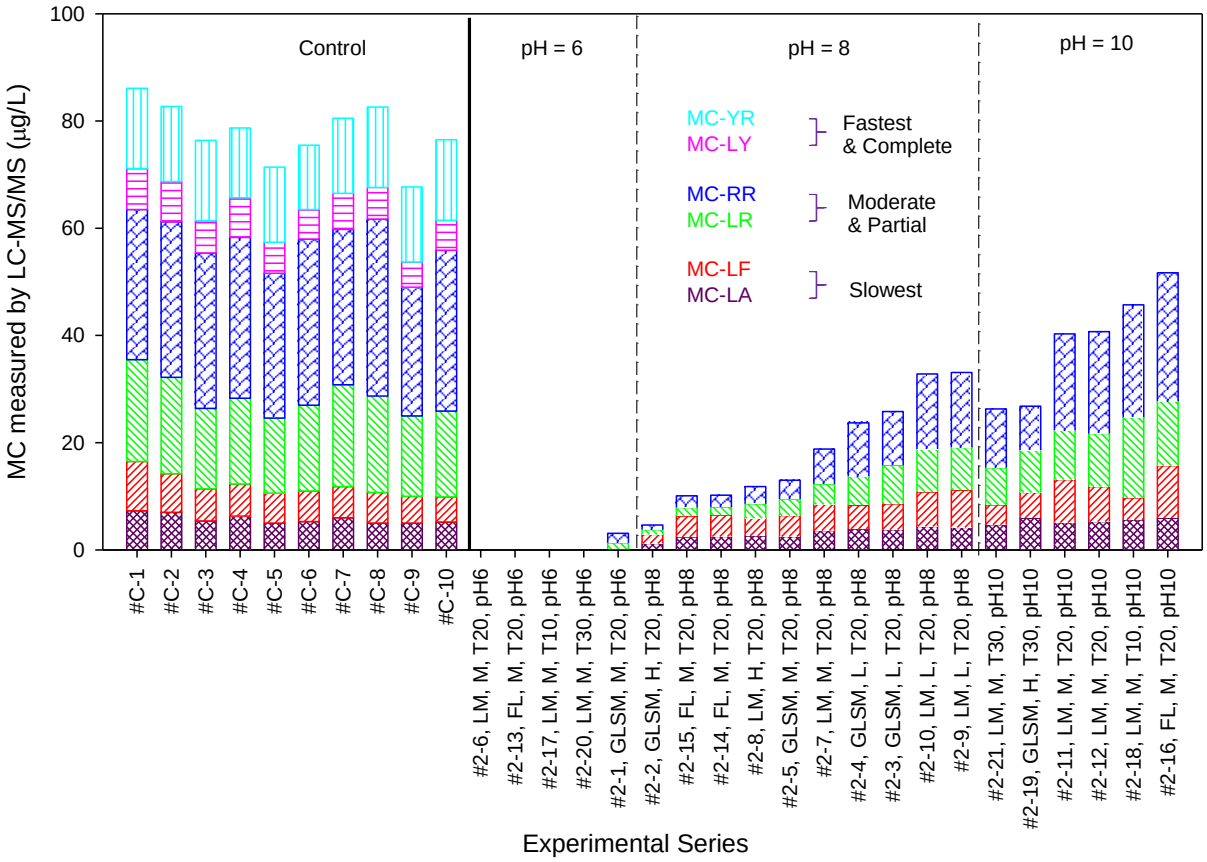
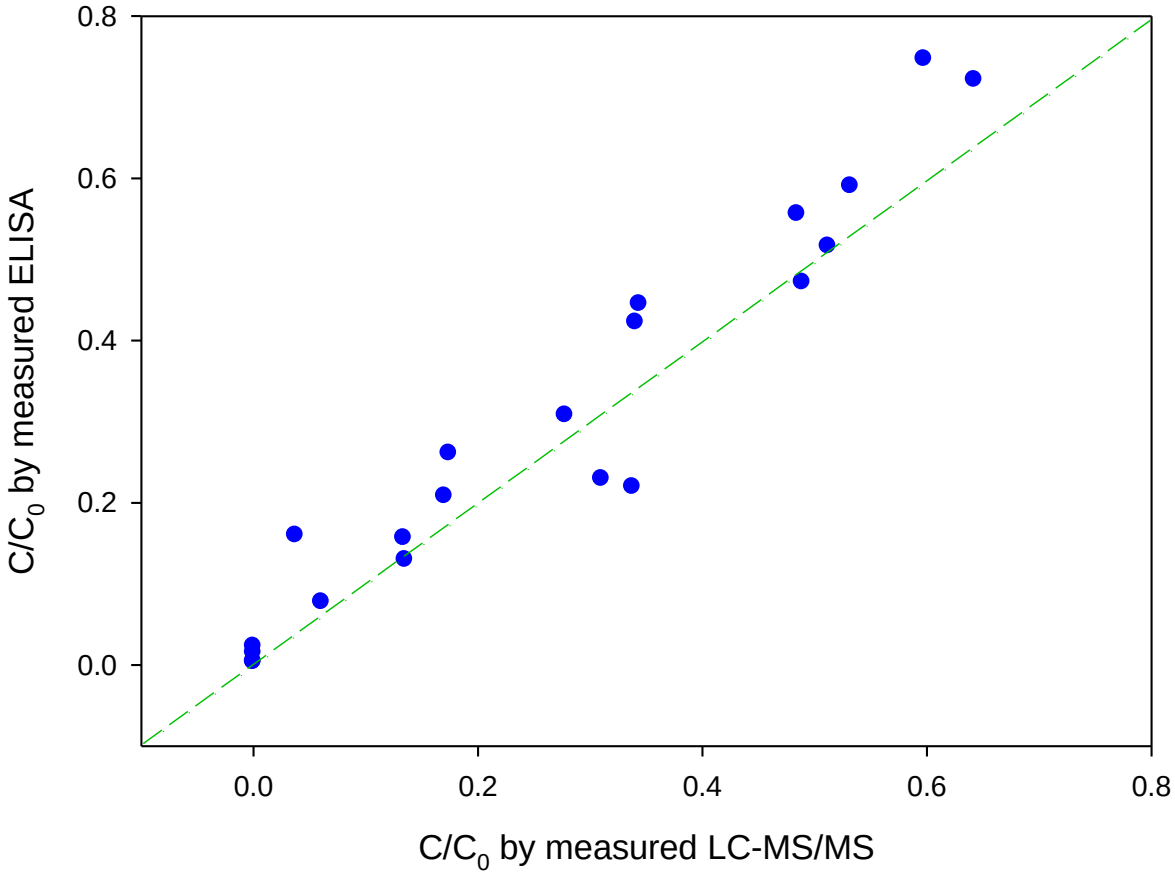
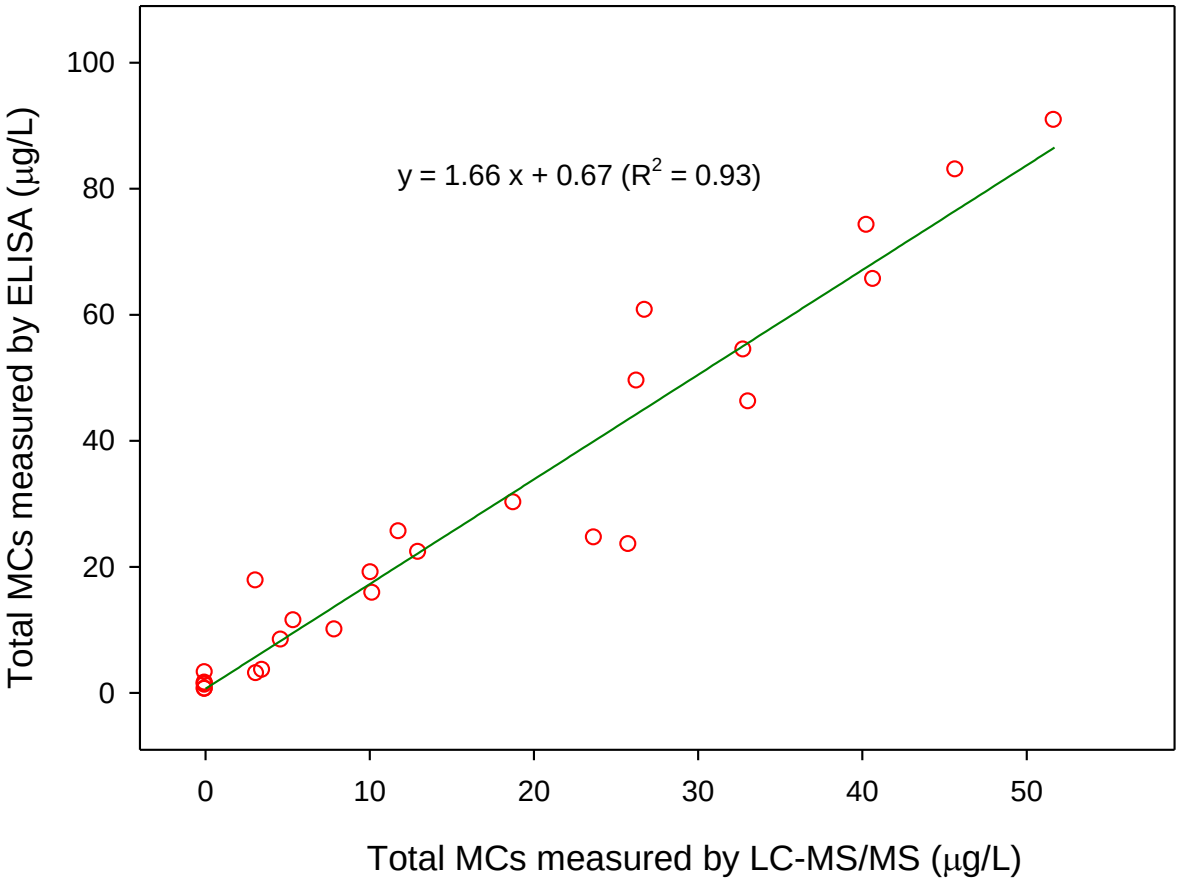
495

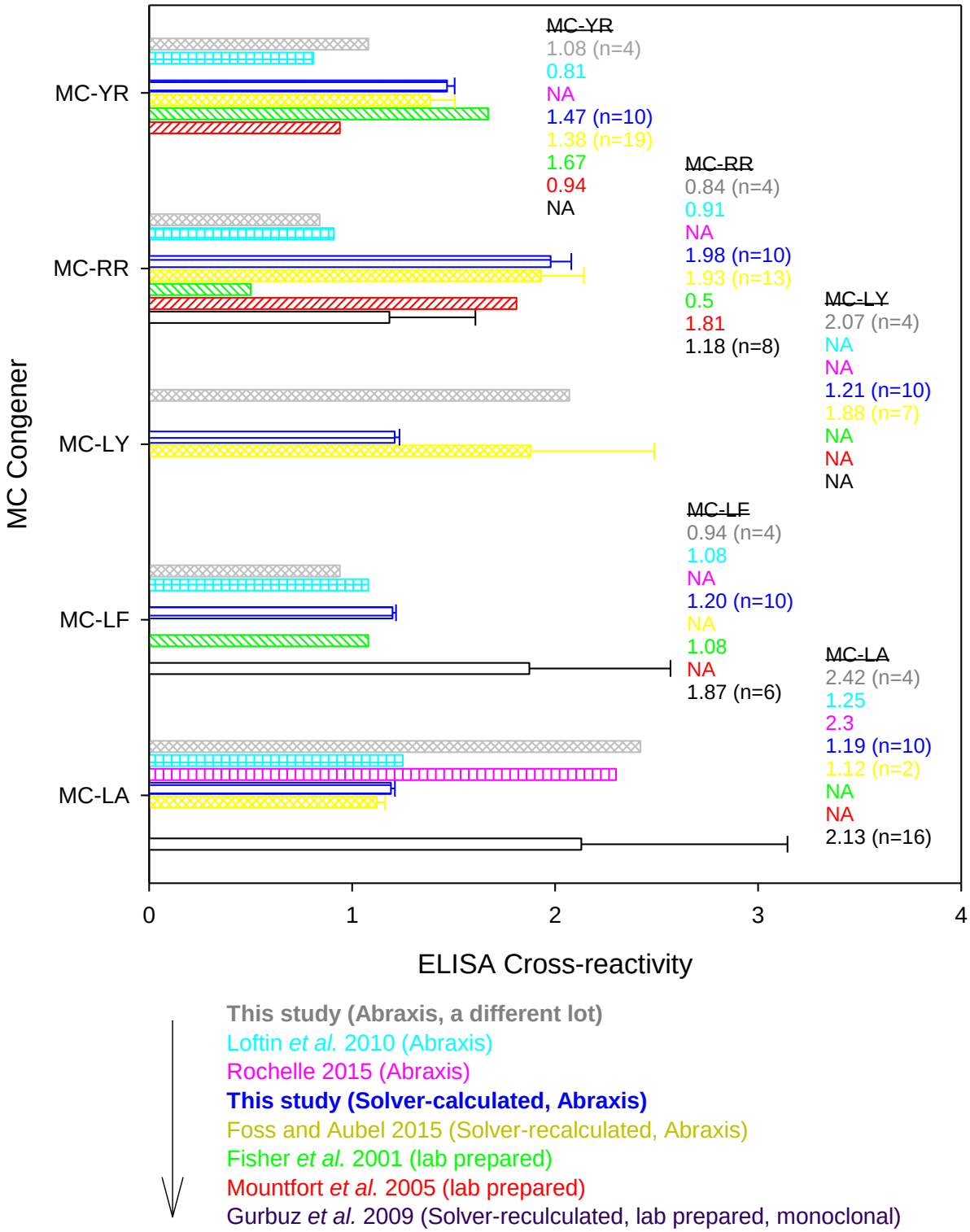
LC-MS/MS								ELISA	ELISA/LC MSMS Ratio	Predicted to measured ELISA Ratio
CYN	MC-LA	MC-LF	MC-LR	MC-LY	MC-RR	MC-YR	Total	Total		
Average concentration for the ten control samples										
7.86± 0.49	5.75± 0.86	6.09± 1.28	16.60 ±1.78	6.27± 0.98	29.00 ±2.40	14.10 ±0.99	77.81 ±5.54	116.76±15.29	1.50±0.16	
(1) Estimated cross-reactivity by Solver using the ten control samples										
	1.19± 0.06	1.20± 0.05	1.00	1.21± 0.08	1.98± 0.32	1.47± 0.12				1.01±0.10
Using a different lot of ELISA kit, individual MC in DI water										
(2)-1 Average ratio of measured ELISA to LC-MS/MS										
	2.04± 0.39	0.88± 0.02	0.94± 0.11	1.92± 0.15	0.59± 0.10	0.88± 0.12				0.64±0.07
(2)-2 Estimated cross-reactivity using average ratio (X_{LR} = 1)										
	2.17± 0.42	0.94± 0.02	1.00	2.05± 0.16	0.62± 0.11	0.94± 0.13				0.68±0.08
(3)-1 Slope of the linear regression for measured ELISA vs LC-MS/MS										
	2.18	0.85	0.90	1.86	0.76	0.97				0.69±0.08
R^2	0.99	1.00	0.96	0.99	0.98	0.96				
(3)-2 Estimated cross-reactivity using slope of the linear regression (X_{LR} = 1)										
	2.42	0.94	1.00	2.07	0.84	1.08				0.77±0.08

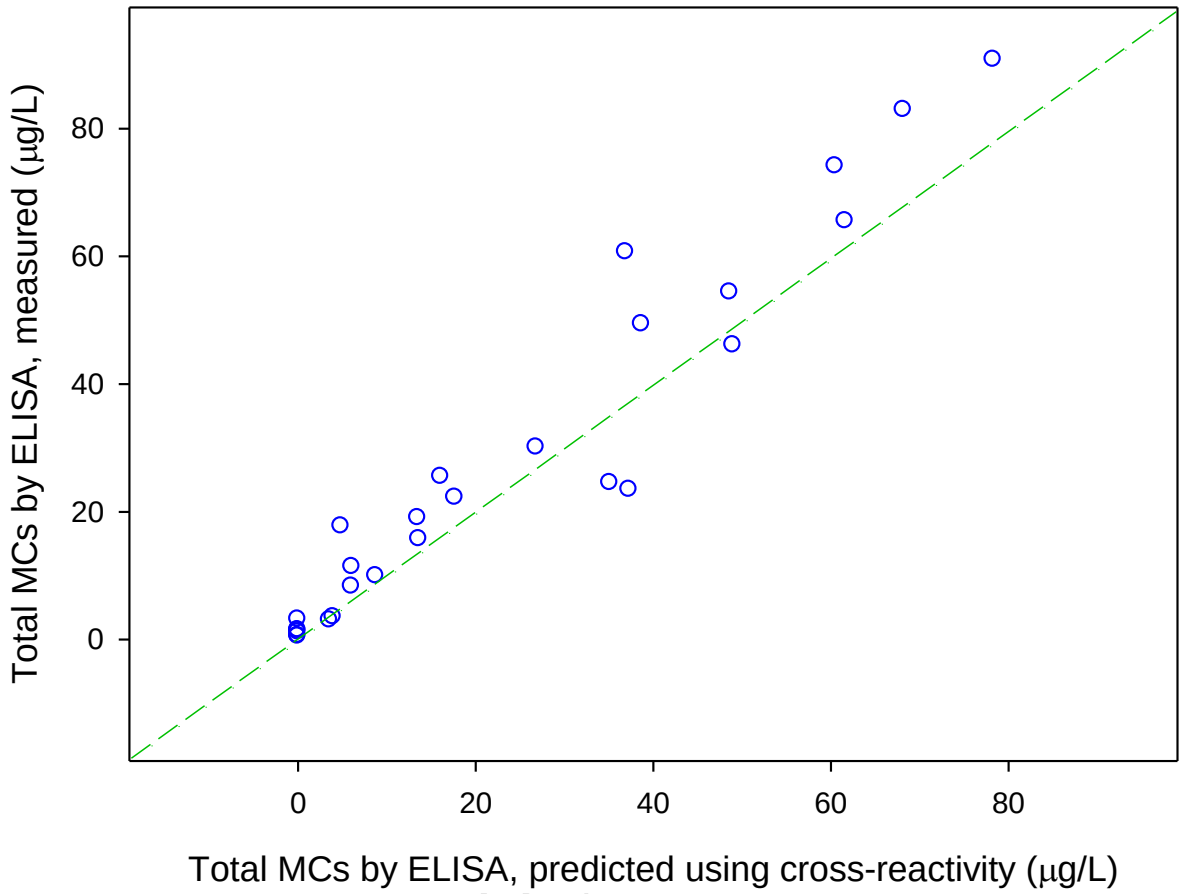





0.8
0.6
0.4
0.2
0.0
C/C0 by measured ELISA
0.0
0.2
0.4
0.6
0.8
C/C0 by measured LC-MS/MS


y = 1.66 x + 0.67 (R2 = 0.93)
Total MCs measured by ELISA (µg/L)
Total MCs measured by LC-MS/MS (µg/L)
0
20
40
60
80
100
0
10
20
30
40
50


MC-YR
MC-RR
MC-LY
MC-LF
MC-LA
MC Congener
0
1
2
3
4
ELISA Cross-reactivity
~~MC-YR~~
1.08 (n=4)
0.81
NA
1.47 (n=10)
1.38 (n=19)
1.67
0.94
NA
~~MC-RR~~
0.84 (n=4)
0.91
NA
1.98 (n=10)
1.93 (n=13)
0.5
1.81
1.18 (n=8)
~~MC-LY~~
2.07 (n=4)
NA
NA
1.21 (n=10)
1.88 (n=7)
NA
NA
NA
~~MC-LF~~
0.94 (n=4)
1.08
NA
1.20 (n=10)
NA
1.08
NA
1.87 (n=6)
~~MC-LA~~
2.42 (n=4)
1.25
2.3
1.19 (n=10)
1.12 (n=2)
NA
NA
2.13 (n=16)
This study (Abraxis, a different lot)
Loftin et al. 2010 (Abraxis)
Rochelle 2015 (Abraxis)
This study (Solver-calculated, Abraxis)
Foss and Aubel 2015 (Solver-recalculated, Abraxis)
Fisher et al. 2001 (lab prepared)
Mountfort et al. 2005 (lab prepared)
Gurbuz et al. 2009 (Solver-reculculated, lab prepared, monoclonal)


Total MCs by ELISA, measured (µg/L)
0
20
40
60
80
0
20
40
60
80
Total MCs by ELISA, predicted using cross-reactivity (µg/L)

Highlights

- Amino acid variables and pH have a strong impact on HOCl kinetics of MC mixtures.
- Cross-reactivity of MCs is estimated showing different sensitivity toward ELISA.
- Inconsistency exists in the cross-reactivity of MCs in this study and literature.
- Higher removal kinetics of total MCs is shown by LC-MS/MS than by ELISA.
- Both byproducts and cross-reactivity contribute to the sustained ELISA.