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Effect of chlorination on the protein phosphatase inhibition activity for several microcystins

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ABSTRACT

Microcystins are of particular concern due to their toxicity to both humans and animals and may be the most prominent cyanotoxin observed in freshwater. Although a number of studies have investigated the fate of microcystins and other algal toxins through drinking water treatment facilities, measurement of their potential for toxic activity after chlorination, a popular form of treatment in the United States, has not been investigated. In this study, six microcystin variants are subjected to chlorine oxidation. The degradation of each microcystin variant is measured by liquid chromatography/mass spectrometry simultaneously with protein phosphatase inhibition (PPI) response over reaction time with chlorine. Results show that inhibition is dependent on the incorporated amino acid residues, their placement within the microcystin structure, as well as pH. This pH dependence may have practical implications to such activities such as drinking water treatment when the pH is usually adjusted to around 8. Namely, at this pH, even with chlorine addition for disinfection, PPI activity may not be totally eliminated even when the initial MYCs are eliminated.

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1. Introduction

Eutrophication of water bodies from increases in nutrient influx and warming water temperatures have contributed to a decline in water quality (Cousino et al., 2015; Vasconcelos, 2015). The occurrence of algal blooms, which have become a source of concern for both recreational and drinking water use, can thrive under such conditions. In addition to causing aesthetic or operational difficulties in management of drinking water treatment facilities, a number of algal species can generate toxic compounds which can adversely affect both human and animal health. The increased occurrence of HABs has been well documented in the literature, and one consequence of this is a proliferation of incidents in which microcystins enter drinking water treatment. Such incidents have been well documented, including the “Do Not Drink” advisory in Toledo in 2014 (Jetoo et al., 2015). Among the most predominant cyanotoxins are microcystins, nodularins, anatoxins, saxitoxins and lipopolysaccharides.

Microcystins (MYCs) are of particular concern due to their toxicity to both humans and animals and can be the most prominent cyanotoxin observed in freshwater. MYCs are a group of hepatotoxins that comprises over 85 known variants following a common structural basis – a cyclic heptapeptide scaffold including two modified amino acids, 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4(E),6(E)-decadienoic acid, ADDA, and methyldehydroalanine, MDHA (Fig. 1). Within this framework, two amino acids are at the primary locations for structural diversity (referred as the X- or Z-positions), from which the naming scheme is established. Microcystin-LR (MYC-LR), for example, has a leucine and arginine at these two positions. In freshwater environments, MYCs can be generated by several algal species including *Microcystis*, *Planktothrix*, *Anabaena*, *Aphanizomenon*, *Nostoc*, *Cylindrospermopsis* and *Umezakia*.

Acute exposure to MYCs has been shown to induce severe liver and kidney damage and serious cellular damage due to oxidative stress. (Duy et al., 2000; Slatkin et al., 1983). Evidence of these potential health effects has been summarized in several epidemiological studies (Carmichael et al., 2001; Azevedo et al., 2002; Yuan et al., 2006; Jochimsen et al., 1998; Zhou et al., 2002). In addition to liver and kidney damage, chronic exposure to MYCs may also promote tumor growth (carcinogenicity) and gastroenteritis

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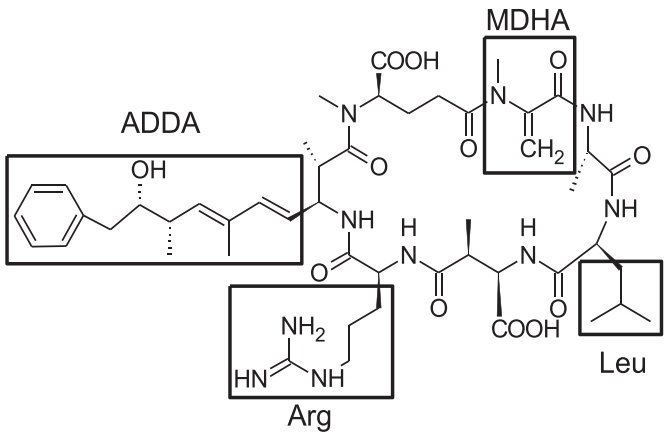

Fig. 1. Chemical structure of Microcystin-LR, one many structurally related cyclic heptapeptides comprising the microcystin family. Common features to the group include the ADDA (3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyl-4(E), 6(E)-decadienoic acid) and methyldehydroalanine (MDHA) modified amino acids. Both the leucine (Leu) and arginine (Arg) residues are sites of structural diversity among the family of compounds, referred to as positions X and Z, respectively.

(Falconer, 1991; Grosse et al., 2006; Luděk et al., 2009; Yu, 1995; Zilberg, 1966).

The toxicity of MYC-LR involves the inhibition to serine/threonine protein phosphatases (Campos and Vasconcelos, 2010). It has been shown a covalent linkage is formed between the MDHA residue of the microcystin and the Cys-273 of PP-1c (Dawson and Holmes, 1999). In addition, there is also a strong hydrophobic interaction between PPA2A and the ADDA moiety of the MYC (Xing et al., 2006; Sarma, 2012). This interaction becomes very important in the case where the MDHA moiety is methylated/demethylated (i.e. MYC-HtyR), inhibiting covalent bonding between the MYC and the phosphatases (Sarma, 2012).

Due to its suspected toxicity, MYC-LR has been included in the Drinking Water Contaminant Candidate List 4 (USEPA, 2015a). The World Health Organization has set a drinking water Provisional Guideline Value (PGV) for 1.0 μg/L (for total microcystin-LR, free plus cell-bound) (WHO, 2004). While many countries have adopted the WHO PGV as their own guidance or regulatory value, several countries have set their own regulatory values. However, the adverse toxicological effect for each MYC variant are not equivalent (hence the use of MYC-LR concentration equivalents). A discussion of the use of "concentration equivalents" verses "toxicity equivalents" for relating microcystins to microcystin-LR is given in Chorus and Bartram (2005).

Because ingestion of MYCs is a potential exposure route, an improved understanding of the fate of microcystins through and following drinking water treatment helps fulfill an important public health goal. A number of studies have investigated the fate of microcystins and other algal toxins through drinking water treatment facilities. An adequate summary can be found in Hoeger et al. (2005) and AWWARF (2001). Ozone, chlorine, and chlorine dioxide administered at typical drinking water concentrations are able to oxidize MYC-LR with adequate contact time (Wert et al., 2014). Rodríguez et al. (2007) concluded the reaction efficiency of conventional oxidants with MYC-LR proceeds in the order of ozone > permanganate > chlorine > chlorine dioxide.

The goal of this study is to investigate protein phosphatase inhibition (PPI) response resulting from the chlorination of MYCs. There currently exists an insufficient understanding of the toxicological implications of MYC chlorination. This has many important implications. First, because free chlorine residuals are applied in United States drinking water distribution systems to prevent microbial regrowth, the presence of the free chlorine residual may help provide additional protection from MYCs that enter the distribution system. Second, as is the case with other types of contaminants, it should not be assumed that oxidation of MYC results in a decrease in toxicity. However, Tsuji et al. (1997) found the toxicity of the MYC-LR chlorination by-products were effectively negligible using several toxicological measurements, including a PPI assay. Third, the reactivity of HOCl can vary due to such water quality characteristics as pH or temperature. The pH has a profound influence on the oxidation potential of chlorine, where the reactivity decreases with increasing pH ($pK_H = 7.54$ at 25 °C). In addition, the presence of an ionizable group substituted into the MYCs variants may influence reactivity (Morris, 1978). As shown in Table 1, the primary amino acids with pK_a values in the relevant range are arginine at 12.0 and tyrosine at 10.1 (Pattison and Davies, 2001).

2. Material and methods

2.1. Chemicals and reagents

MYC-LA, -LF, -LR, -LY, -RR, and -YR were purchased from Enzo Life Sciences (Farmingdale, NY USA). Acetonitrile, methanol, and formic acid were of LC-MS or greater grade, and liquid sodium hypochlorite was obtained from Sigma-Aldrich (St. Louis, MO USA). Ultrapure reagent water (RW) was obtained using a Millipore Advantage A10 ultrapure water system (Billerica, MA USA). Sodium ascorbate was used for quenching the reaction (Spectrum, Gardena, CA USA).

2.2. Experimental procedure

The determination of reaction kinetic variables was performed in individual aqueous solutions containing a phosphate buffer (10 mM) prepared at pH 6.5 or 7.5 or a borate buffer (25 mM) at pH 8.5. A 5 μg/L MYC solution was prepared at a given pH, and the reaction was initiated by the addition of hypochlorite at a concentration of 4 mg Cl_2/L and vigorously shaken. This chlorine dose represents the maximum free chlorine concentration permitted in a US drinking water distribution system (USEPA, 2015b) and at least a 1000-fold excess (by mass) versus the initial MYC concentration. All reactions were performed in the dark at room temperature (20–25 °C) for up to 80 min. A 1.6 mL sample aliquot was taken at specified time intervals and quenched with 30 μL of 17.5 mM sodium ascorbate and shaken vigorously. Samples were placed at 4 °C prior to analysis.

2.3. Analytical methods

Free chlorine was measured using Hach DPD Method 10102 on a Hach DR2500 UV–Vis spectrophotometer (Loveland, CO USA). Solution pH was measured using a Hach SensION 156 pH meter. The MYC concentration was determined using a Thermo Scientific Ultimate 3000 ultra high-pressure liquid chromatographic (UHPLC) system coupled to a Thermo Scientific LTQ XL/Orbitrap Discovery, which combines a linear ion trap MS with an Orbitrap Fourier transform high-resolution mass spectrometer (FTMS). All analytes were introduced by electrospray ionization (ESI) with nitrogen (99.999%) used as the nebulizing gas. Each were detected by the FTMS detector operated at a mass resolution of 7500 in full scan mode with a mass range between 900 and 1350 Da. The automatic gain control target value was set to 4×10^5, with a maximum injection time of 1050 ms.

Chromatographic separation was performed following online solid-phase extraction (SPE). A sample volume of 1200 μL was

Table 1
Target MYCs and their associate properties. The pK_a of the residual amino acid groups were taken from Pattison and Davies, 2001.

Microcystin	Formula	Monoisotopic Mass-M_{mi} (Da)	$[M + H]^+$ (Da)	First Variable residue (X)	Second Variable residue (Z)	pK_a (Ionizable groups in Variable residue
MYC- LA	$C_{46}H_{67}N_7O_{12}$	909.485	910.492	Leucine	Alanine	N/A
MYC- LF	$C_{52}H_{71}N_7O_{12}$	985.516	986.523	Leucine	Phenylalanine	N/A
MYC- LR	$C_{49}H_{74}N_{10}O_{12}$	994.549	995.556	Leucine	Arginine	12.0
MYC- LY	$C_{52}H_{71}N_7O_{13}$	1001.511	1002.518	Leucine	Tyrosine	10.1
MYC- RR	$C_{49}H_{75}N_{13}O_{12}$	1037.566	519.792[a]	Arginine	Arginine	12.0, 12.0
MYC- YR	$C_{52}H_{72}N_{10}O_{13}$	1044.528	1045.535	Tyrosine	Arginine	10.1, 12.0

N/A denotes that the variable residues at the X and Y position have no ionizable functional groups.
[a] Denotes compound where the mass species observed is doubly charged, $[M + H]^{2+}$.

injected onto a Waters XBridge C18 SPE column (10 μm, 2.1 × 30 mm, Milford MA) using 100% DI water. The sample was then back-eluded from the SPE column onto a Thermo Hypersil Gold aQ analytical column (1.9 μm, 2.1 × 50 mm, Waltham, MA USA) in series with a Hypersil Gold PFP analytical column (1.9 μm, 2.1 × 50 mm) operated at 30 °C. Separation was achieved with the following eluents: (A) 0.02% Formic acid in RW, (B) Methanol, (C) Acetonitrile and (D) 0.4% Formic Acid in RW. The chromatographic solvent mixture was varied based on the analyte.

Protein phosphatase inhibition (PPI) was measured using a Zeulab Microcystest PPI assay (Life Sciences Advanced Technologies, St. Petersburg, FL). The manufacturer's directions for the assay were used. In summary, 50 μl of sample/standard were added to each well. A phosphatase solution was added at 70 μl, followed by 90 μl of a chromogenic solution. The plate was incubated at 37 °C for 30 min. A quenching agent (70 μl) was then added. The plate was then measured on an Awareness Technology Chromate 7300 Microplate Reader (Palm City, FL). Standards (0.25–2.5 μg/L) consisting of Microcystin-LR were provided by the manufacturer. Samples were diluted with DI water if the sample measured above the upper standard (2.5 μg/L). Standard curve was measured in duplicate and spike samples were utilized as needed.

3. Results

3.1. Chlorination of Microcystin-RR and -LR

Both MYC-LR and -RR share the same arginine residue at the Z position but differ at the X position with a leucine or arginine residue, respectively. Whereas a leucine residue is inherently non-reactive, the guanidine functional group on the arginine residue is known to be chlorine reactive (Pattison and Davies, 2001; Yildiz et al., 1998). Due to the arginine moieties present, a relatively fast reaction was observed for MYC-RR. Essentially no MYC-RR remains after 45 min irrespective of pH. The pseudo first-order rate constant (k'), when an excess molar concentration of chlorine is used, can be written as follows:

$$\ln\left(\frac{[MYC]}{[MYC]_0}\right) = -kt \quad (1)$$

A plot of ln [MYC-RR]/[MYC-RR]$_0$ verses time (Fig. 2) resulted in a decrease in measured k' values as the pH increased from 6.5 to 8.5 (Fig. 2). This would be in accordance with the reduced oxidative potential of the predominant chlorine specie ([HOCl]/[OCl^-]) (Morris, 1978).

Similar k' values were observed for MYC-LR, Fig. 2. Acero et al. (2005) also found similar rates of chlorine oxidation between MYC-RR and -LR. Previous studies also found that decreasing pH directly resulted in a faster reaction (Tsuji et al., 1997; Yang et al., 2014). However, the pH dependence was somewhat different where the reaction at pH 7.5 was faster than that found for pH 6.5 and 8.5. The PPI activity results showed a similar pH trend. Unlike these previous studies where a molar ratio between MYC-LR to chlorine was 4.3:1 (lowest MYC-LR concentration) and 50:1, respectively, a 10^5 M excess was used in the present study. These conditions appropriately simulate realistic drinking water treatment conditions, where the active chlorine residual would be in a much greater excess relative to MYC. The inconsistency observed in pH dependency between studies could not be reconciled due to the significant differences in relative oxidation potential.

The response measured by the PPI assay parallels that of the measured MYC-RR concentration, with a decrease in the rate of deactivation at higher pH. In Fig. 3, a plot of relative concentration, ln([MYC-RR]/[MYC-RR]$_0$), is shown in correlation with relative PPI response ln([MYC]/[MYC]$_0$). The deactivation of the PPI activity strongly correlates with the loss of the parent compound at pH 8.5 and only deviates very slightly from the ideal slope of 1.0 (expected if there is a 1:1 relationship between rate of response) at lower pH, Table 2. A 1:1 correlation between activity deactivation and the remaining MYC-LR concentration was also observed for each pH. This indicates no significant PPI activity is exhibited by the transformation by-products of either MYC-RR or -LR. In the absence of the original MYC, a complete deactivation of inhibitive activity is observed. A similar observation for MYC-LR was seen by Tsuji et al. (1997) and Senogles-Derham et al. (2003).

3.2. Chlorination of Microcystin-LY and -YR

MYC-LY and -YR contain the tyrosine residue which, due to the phenolic group, is readily reactive with chlorine. Because MYC-YR contains both the arginine and tyrosine residues, it should inherently be more reactive in the presence of chlorine. The k' measured for MYC-YR loss is indeed greater than all the MYCs studied, Fig. 4. No traceable amount of MYC-YR is observed after 4 min at both pH 7.5 and 8.5, while a somewhat slower reaction at pH 6.5 is observed. MYC-LY degraded at a rate slower than that of MYC-YR, but generally faster than observed for MYC-RR or -LR. The rate of reaction for MYC-LY and -YR increased with increasing pH, Fig. 4. Gallard and von Gunten (2002) similarly found the kinetic rate of chlorine with phenols was greatest at between pH 8.0 and 9.0 revealing the influence of speciation from both hypochlorite and phenol. Fig. 4 also shows that pseudo first order conditions are not appropriate for describing the oxidation of MYC-YR. The plot of ln([MYC]/[MYC]$_0$) verses time at pH 6.5 shows a curvilinear response. An initial fast reaction ($k'_{Initial}$ = 0.77 min^{-1}) is followed by a slower one (k'_{slow} = 0.0348), consistent when two competing reactions are occurring simultaneously. This would be appropriate given the fact there are two reactive moieties on MYC-YR both exerting a chlorine demand.

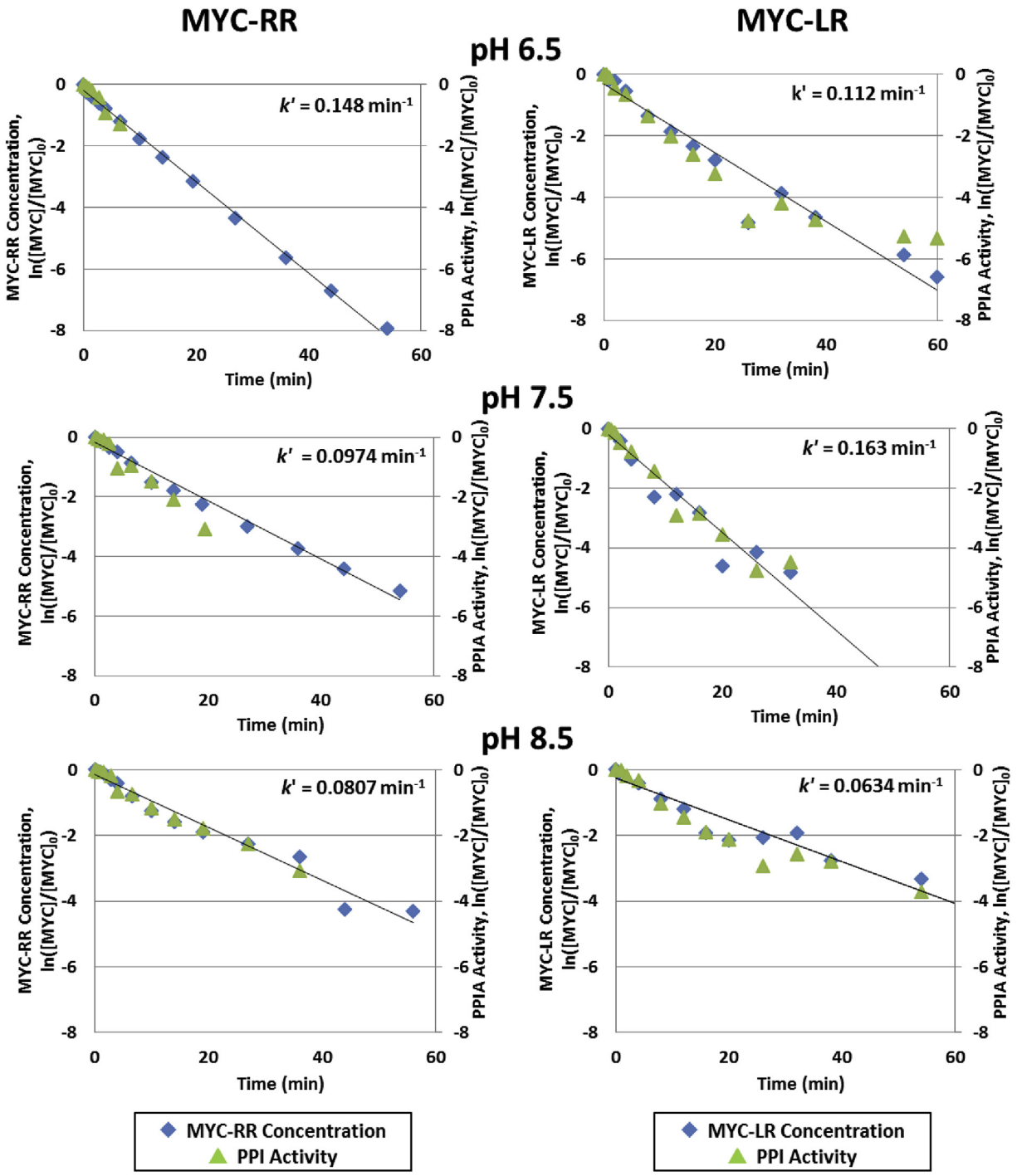

Fig. 2. Plot of relative concentration (Left Axis) and relative PPI response (Right Axis) verses time for the reaction of MYC-RR (Left) and MYC-LR (Right) with chlorine.

In contrast to the observed loss of the parent the rate at which PPI deactivation occurs is significantly slower for both MYC-LY and -YR, Fig. 5. Both show that the difference in measured deactivation and measured MYC loss is greater at higher pH. For MYC-LY at pH 6.5, the deactivation correlates initially with concentration loss, but then deviates as the react progresses. This trend is not observed at higher pHs. The observation of PPI activity even after the disappearance of the parent MYC can be explained by the formation of PPI active by-products. It is likely the variety of by-products formed differs by pH due to the influence of both the speciation of chlorine and the ionizable amino acid residue.

Table 2 compares the measured PPI activity of the mixture over time verses the measured concentration of the parent compound (both as a function of $\ln([MYC]/[MYC_0])$). For MYC-LY and -YR, the slope of the curve, being less than 1.0, demonstrates an activity response that is not due to the parent alone but in combination with transformation by-products. Each shows this effect is greater at higher pH, suggesting the fraction of by-products expressing PPI activity or their relative potency is more significant at higher pH relative to the remaining MYC.

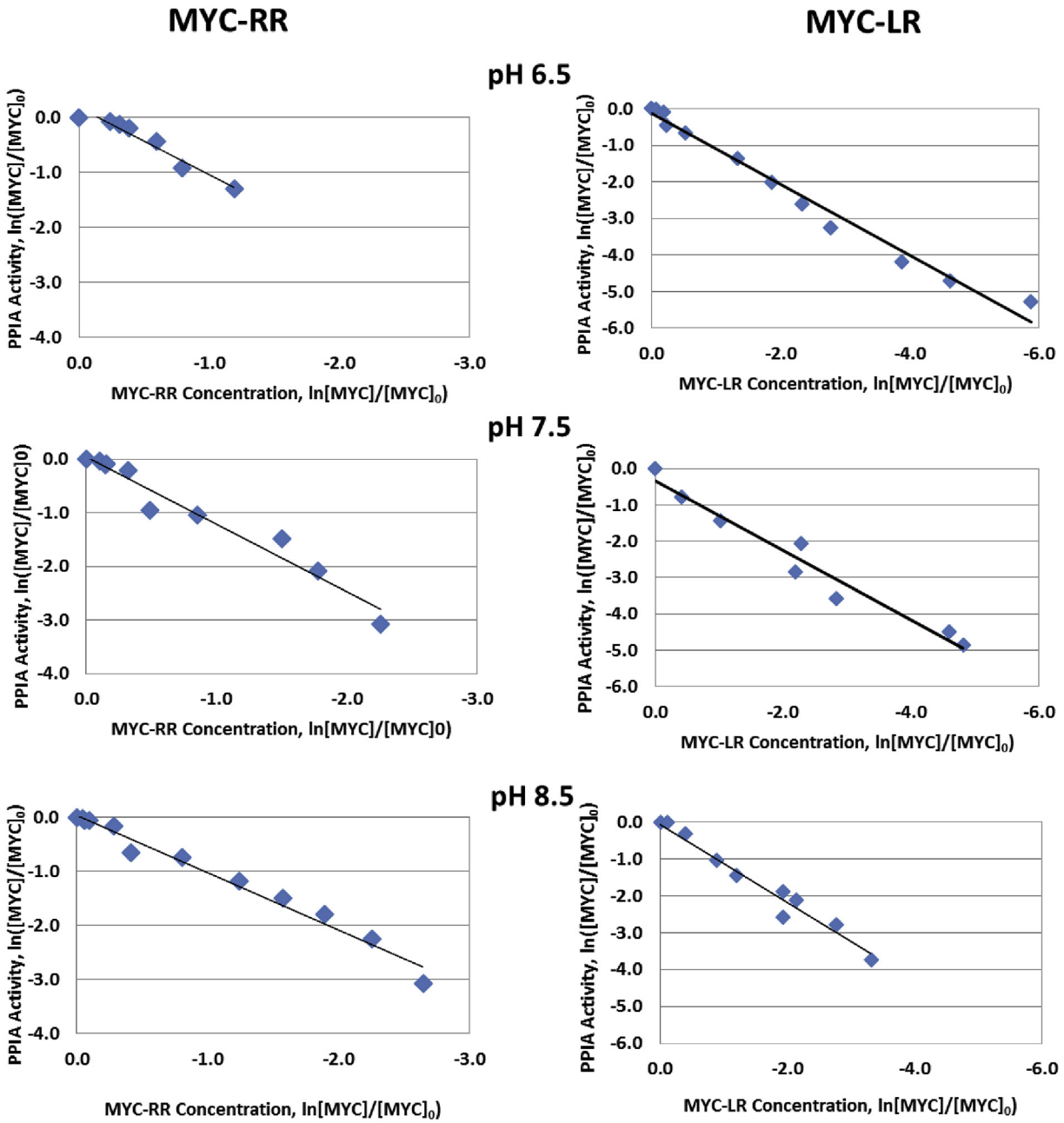

Fig. 3. Correlation of relative residual concentrations, ln([MYC]/[MYC_0]), after chlorination of MYC-RR (Left) and MYC-LR (Right) versus the relative PPI activity response, ln([MYC]/[MYC_0]), at pH 6.5, 7.5 and 8.5.

Table 2
Correlation of relative concentrations, ln([MYC]/$MYC]_0$), after chlorination of each microcystin verses relative PPI activity response, ln([MYC]/$MYC]_0$), at pH 6.5, 7.5 and 8.5. S represents the Standard Error of the Regression.

MYC	6.5				7.5				8.5			
	Slope	Int.	R^2	S	Slope	Int.	R^2	S	Slope	Int.	R^2	S
MYC-RR	1.22	0.18	0.94	0.13	1.26	0.049	0.96	0.24	1.06	0.037	0.98	0.15
MYC-LR	0.97	0.14	0.98	0.15	0.96	0.33	0.97	0.25	1.06	0.062	0.97	0.37
MYC-LA	1.72	0.035	0.94	0.23	3.20	0.41	0.91	0.25	2.99	0.084	0.96	0.08
MYC-LF	18.03	0.04	0.85	0.37	10.11	0.15	0.962	0.24	10.77	0.17	0.88	0.68
MYC-LY	0.71	0.34	0.93	0.20	0.55	0.024	0.98	0.18	0.22	0.066	0.97	0.06
MYC-YR	0.63	0.50	0.91	0.39	0.23	0.011	0.99	0.04	0.047	0.021	0.94	0.04

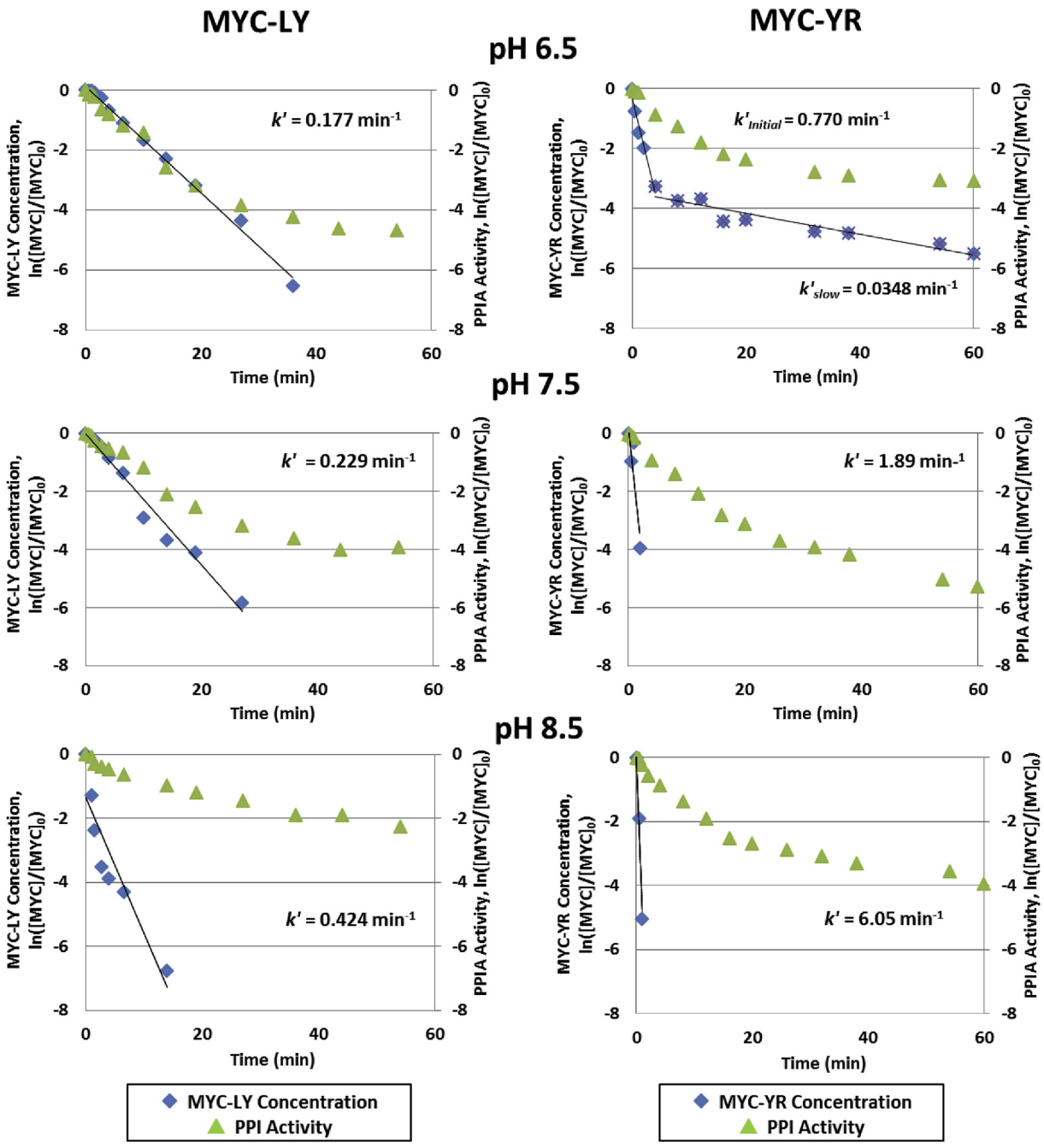

Fig. 4. Plot of relative concentration (Left Axis) and relative PPI response (Right Axis) verses time for the reaction of MYC-LY (Left) and MYC-YR (Right) with chlorine.

3.3. Chlorination of Microcystin-LA and -LF

MYC-LA and -LF share the same leucine residue at the X position but differ at the Z position with alanine or phenylalanine residues, respectively. Neither MYC is expected to be especially reactive in the presence of chlorine, there are no reactive functionalities associated with these residues (Pattison and Davies, 2001). Accordingly, any observable reaction is likely to be due to the non-variable reactive sites in the microcystin skeleton, of which the ADDA, and to a lesser extent, MDHA moieties are the most likely candidates for reactivity. Relative to MYC-RR and -LR, the measured k' for both MYC-LA and -LF are significantly slower (Fig. 6). MYC-LF is nearly non-reactive at pH 6.5 and only slightly increases in reactivity at higher pH. The reaction with MYC-LA slows significantly with increasing pH, consistent with the change in $HOCl/OCl^-$ speciation.

Measured PPI activity response is pH dependent for both MYC-LA and -LF (Fig. 6). However, the rate of deactivation is significantly faster than can be attributed to the measured loss of the parent compound, Table 2. The measured inhibition shows a nonlinear pseudo first-order response. A fast initial loss in activity is observed, but then slows significantly over time. As the reaction proceeds over time, additional deactivation of PPI activity for both MYCs tends to parallel the loss of the measured parent concentration.

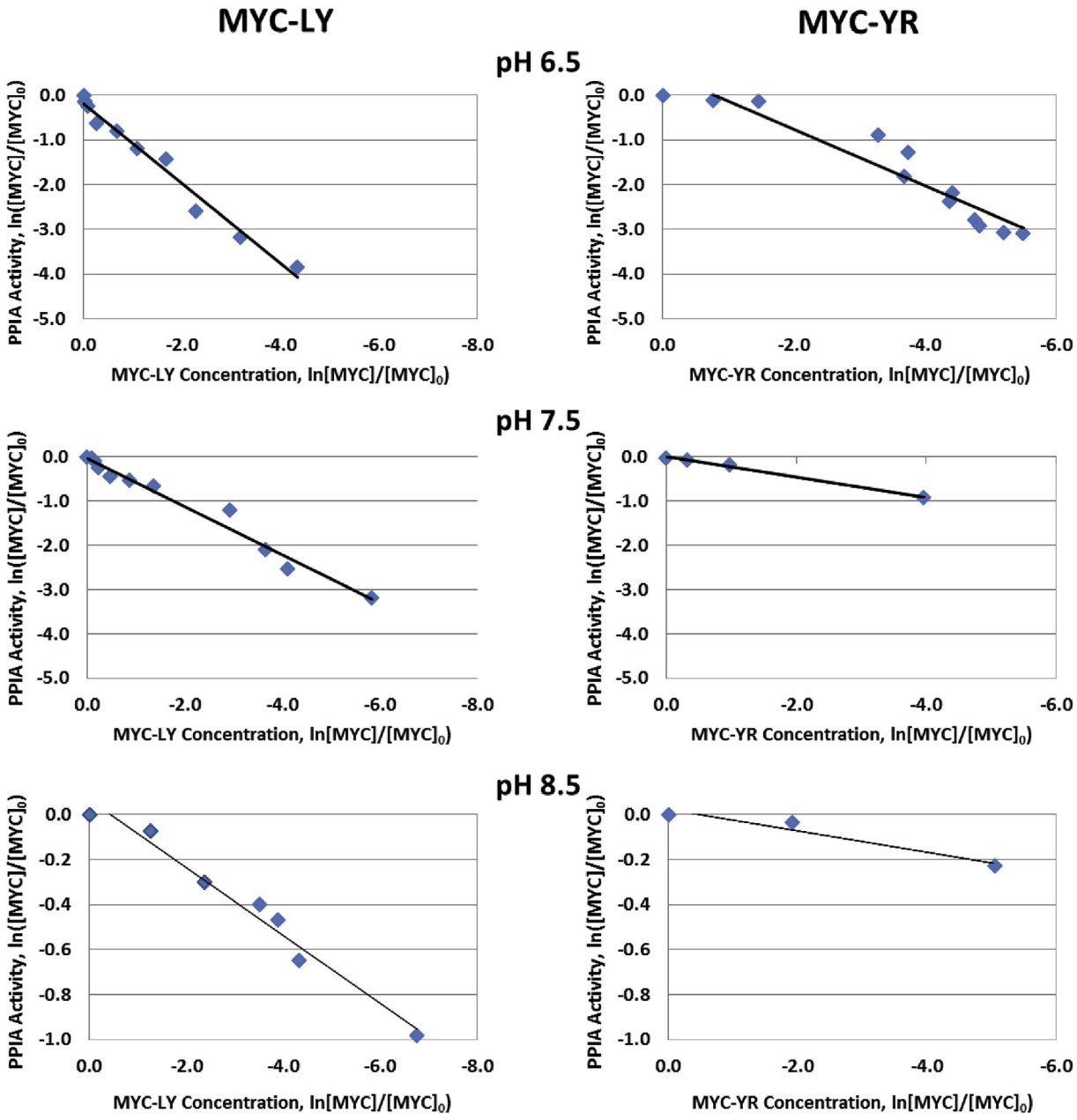

Fig. 5. Correlation of relative residual concentrations, ln([MYC]/[MYC_0]), after chlorination of MYC-LY (Left) and MYC-YR (Right) versus the relative PPI activity response, ln([MYC]/[MYC_0]), at pH 6.5, 7.5 and 8.5.

4. Discussion

The microcystin variants used in this study were chosen to observe the influence of the variable residues on both chlorination reactivity and PPI activity over time. Arginine and tyrosine residues are expected to be reactive in the presence of chlorine, whereas leucine, alanine and phenylalanine are non-reactive.

Unlike MYC-RR where two arginine groups are substituted at the X- and Z-positions, the arginine residue resides only at the Z-position in MYC-LR. Replacement of the leucine moiety at the X-position with arginine does not result in an increase in reaction rate, nor alter the measured PPI response. As such, total PPI activity measured during the reaction in both cases is due to the remaining parent compound. Because the major active inhibition site on MYCs is due to the ADDA sub-structure, the proximity of the Z-position arginine to the ADDA essentially disrupts PPI expression during chlorination and a deactivation of PPI activity. Both observations suggest the reactive site is limited to the area on MYC-RR and -LR in proximity to the ADDA moiety, whether the point of oxidant attack is at the arginine itself or favorably influences the reactivity of the double bond of the ADDA moiety due to its proximity and its highly basic characteristic (Hammerl and Klapötke, 2005).

Relative to MYC-LR, the substitution of the leucine residue at the X-position with tyrosine as with MYC-YR not only results in an increase in the rate of reaction but the presence of PPI active transformation by-products. Similarly, replacing the Z-position arginine with tyrosine in MYC-LY dramatically increases the reaction rate as well as decreases the rate of deactivation in the measured PPI activity. The initial oxidative attack and oxidative substitution is likely on the phenolic group (Gallard and von

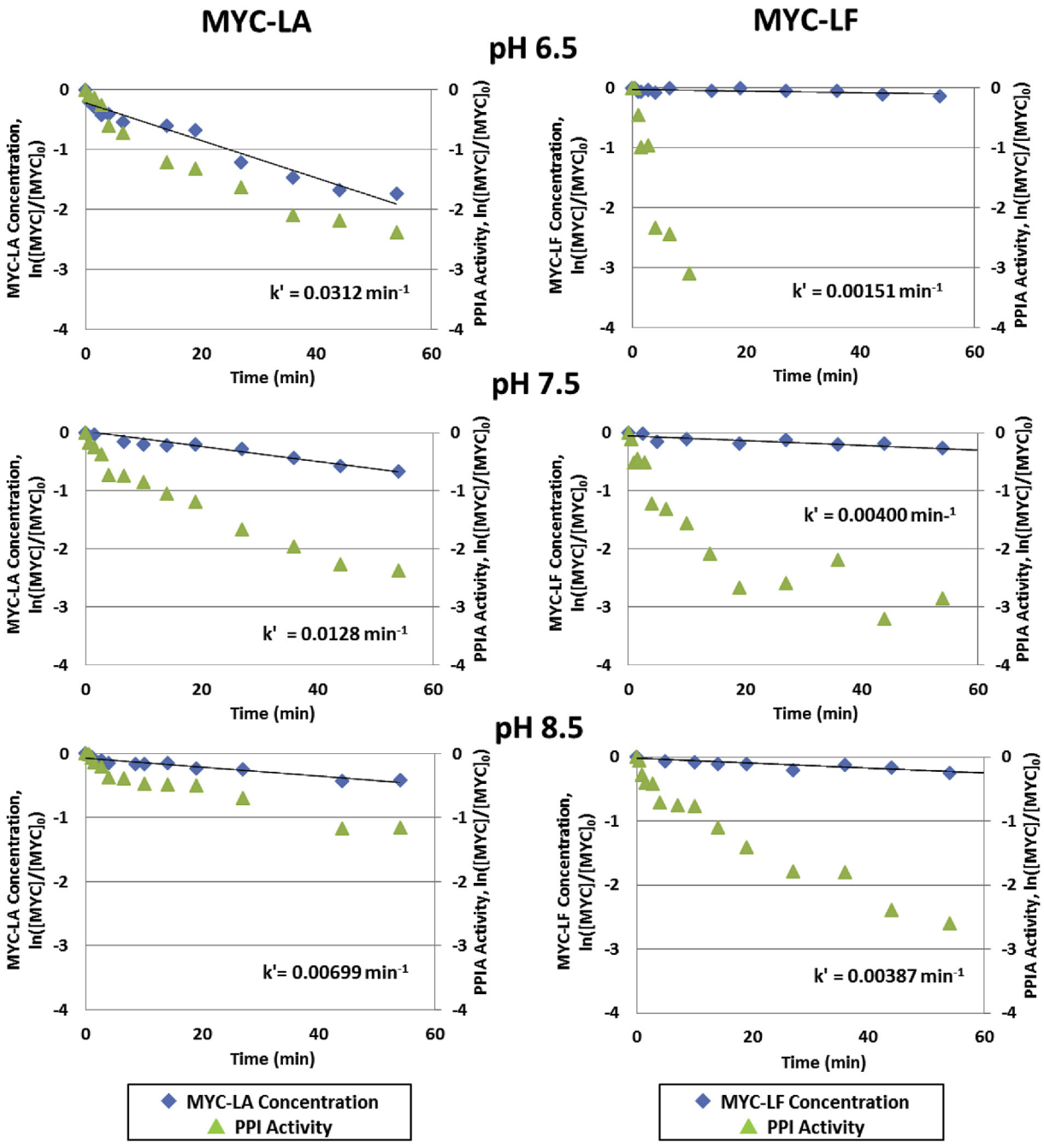

Fig. 6. Plot of relative concentration (Left Axis) and relative PPI response (Right Axis) verses time for the reaction of MYC-LA (Left) and MYC-LF (Right) with chlorine.

Gunten, 2002) of the tyrosine. Because the tyrosine is at the X-position and should have no appreciable influence upon the ADDA moiety, the initial by-products themselves should be expected to express PPI activity, as was observed in the study. Over time, competing reactions on the parent and further reactions to these initial transitional by-products result in additional by-products trending to show little to no inhibitive activity resulting a gradual decrease in the overall measured PPI activity.

MYC-LY demonstrates the influence of substituting a tyrosine residue at the Z-position. At pH 6.5, where the reaction rate of the phenolic group is expected to decrease, the initial transformation by-products essentially express no PPI activity. As the reaction progresses, the reaction with the phenolic group becomes more important. This results in a gradual delinearization of the plot of relative concentration verses relative PPI response, Fig. 4. After the initial chlorine demand is satisfied, the overall influence of observed by-products express inhibitive response in the assay relative to the remaining concentration of MYC-LY. At higher pH, where the influence of the phenolic reaction is much more significant and substitution onto the phenolic group is more favorable, the initial by-products exhibit inhibitive activity. With additional contact time, the influence of the additional reactions increases, such as that expected at the ADDA double bond, resulting in the production by-products with lower or no PPI activity response.

As demonstrated by MYC-LA and -LF, where no reactive residues are present in the X- or Z-position in the MYC, any observable

chlorine oxidation is likely to be due to the non-variable reactive sites in the microcystin skeleton, of which the ADDA moiety is the most likely candidate for reactivity (Tsuji et al., 1997). The PPI assay response for both MYC-LA and -LF does not demonstrate a direct correlation with the concentration measure of parent (Fig. 7), but rather a greater rate of deactivation in PPI assay response relative to parent concentration.

Since oxidation is expected to be along the conjugated diene of the ADDA moiety, it is postulated that interconversion through a short-lived chloronium ion, is responsible for the observed differences in measured response between the PPI assay and the remaining measured MYC concentration (An and Carmichael, 1994; Carey, 1992). An and Carmichael (1994) found that the stereoisomeric configuration of the methyl group on ADDA at C-6 is significant in determining the expression of inhibition response. This methyl group is required to be in the (E) stereoisomeric form to express inhibition. The other stereoisomer, the (Z) form, exhibits no characteristic inhibition. Briefly, the chloronium ion would be stabilized due to resonance along the double bond and allow for rotational freedom. If this is followed by nucleophilic elimination of the chlorine, restoration of the diene would result. In this case, based on the observations found in this study, the formation of the (Z) stereoisomer would be favored which would no longer exhibit inhibition. Unfortunately, there is no definitive separation of isomeric peaks in the chromatograph. Previous studies have suggested that in addition to dihydromicrocystin, the main by-product of the chlorination of MYCs and one that demonstrates no significant toxicity under several measures, other stereoisomers may also be formed (Acero et al., 2005; Tsuji et al., 1997). It is possible that some of the unknown products seen in these previous studies, where mass spectroscopy was not used for identification, are stereoisomers of the original MYC.

5. Conclusion

Microcystins readily react with chlorine so long as there is an

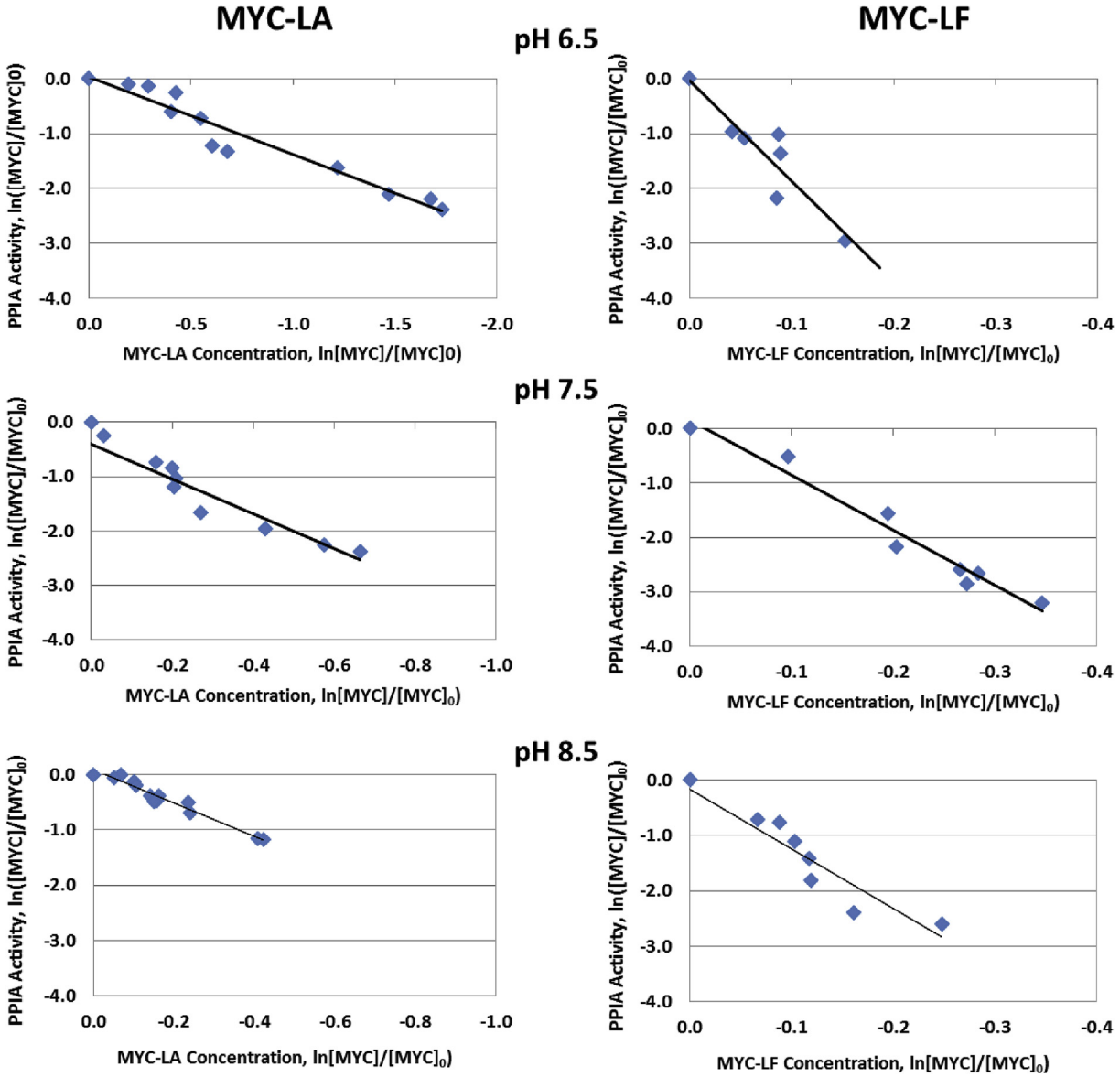

Fig. 7. Correlation of relative residual concentrations, ln([MYC]/[MYC0]), after chlorination of MYC-LA (Left) and MYC-LF (Right) versus the relative PPI activity response, ln([MYC]/[MYC0]), at pH 6.5, 7.5 and 8.5.

ionizable residue that exists at one or both variable positions. Results show that PPI activity is dependent on both the amino acid residues as well as pH. In the case of MYC-RR and -LR, there is no noticeable contribution to the PPI activity as a result of the formation of its by-products. This differs for MYC-YR and -LY, where the influence of the tyrosine residue tends to produce transformation by-product intermediates that retain a measure of activity. In all cases, the initial intermediates appear to be relatively short-lived, eventually shifting the mixture of by-products proportionally to those with less to no observed inhibition. In the absence of any ionizable residues, the apparent measured concentration of the parent MYC degrades at a much slower rate than the decline in the measured inhibition.

The results presented may have practical implications to such activities such as drinking water treatment when the pH is usually adjusted to around 8.0 or so. Namely, at this pH, even with chlorine addition for disinfection, PPI activity may not be totally eliminated even when the initial MYCs no longer measurable.

Disclaimer

The views expressed in this paper are those of the authors and do not necessarily reflect the views or policies of the U.S. Environmental Protection Agency.

References

American Water Works Association Research Foundation (AWWARF), 2001. Assessment of Blue-green Algal Toxins in Raw and Finished Drinking Water. American Water Works Association Research Foundation and American Water Works Association, Denver, CO.

An, J., Carmichael, W.W., 1994. Use of a colorimetric protein phosphatase inhibition for the study of microcystins and nodularins. Toxicon 32, 1495–1507.

Acero, J., Rodriguez, E., Meriluoto, J., 2005. Kinetics of reactions between chlorine and the cyanobacterial toxins microcystins. Water Res. 39, 1628–1638.

Azevedo, S.M., Carmichael, W.W., Jochimsen, E.M., Rinehart, K.L., Lau, S., Shaw, G.R., Eaglesham, G.K., 2002. Human intoxication by microcystins during renal dialysis treatment in Caruaru-Brazil. Toxicology 181–182, 441–446.

Campos, A., Vasconcelos, A., 2010. Molecular mechanisms of microcystin toxicity in animal cells. Int. J. Mol. Sci. 11, 268–287.

Carey, F.A., 1992. Organic Chemistry, second ed. McGraw and Hill, New York.

Carmichael, W.W., Azevedo, S.M., An, J.S., Molica, R.J., Jochimsen, E.M., Lau, S., Rinehart, K.L., Shaw, G.R., Eaglesham, G.K., 2001. Human fatalities from cyanobacteria: chemical and biological evidence for cyanotoxins. Environ. Health Perspect. 109, 663–668.

Chorus, I., Bartram, J., 2005. Cyanobacteria in Water: A Guide to their Public Health Consequences, Monitoring and Management. E&FN Spon, London.

Cousino, L.K., Becker, R.H., Zmijewshl, K.A., 2015. Modeling the effects of climate change on water, sediment and nutrient yields from the Maumee River watershed. J. Hydrol. Reg. Stud. 4 (Part B), 762–775.

Dawson, J.F., Holmes, C.F., 1999. Molecular mechanisms underlying inhibition of protein phosphatases by marine toxins. Front. Biosci. 4, 646–658.

Duy, T.N., Lam, P.K., Shaw, G.R., Connell, D.W., 2000. Review Toxicology and risk assessment of freshwater cyanobacterial (blue-green algal) toxins in water. Rev. Environ. Contam. Toxicol. 163, 113–185.

Falconer, I.R., 1991. Tumor promotion and liver injury caused by oral consumption of cyanobacteria. Environ. Toxic. Water 6, 177–184.

Gallard, H., von Gunten, U., 2002. Chlorination of phenols: kinetics and formation of chloroform. Environ. Sci. Technol. 36, 884–890.

Grosse, Y., Baan, R., Straif, K., Secretan, B., El Ghissassi, F., Cogliano, V., 2006. Carcinogenicity of nitrate, nitrite, and cyanobacterial peptide toxins. Lancet. Oncol. 7, 628–629.

Hammerl, A., Klapötke, T.M., 2005. Nitrogen: Inorganic Chemistry: Encyclopedia of Inorganic Chemistry. In: King, R.B. (Ed.), second ed. Wiley, Hoboken, NJ. USA, pp. 55–58.

Hoeger, S.J., Hitzfeld, B.C., Dietricha, D.R., 1995. Occurrence and elimination of cyanobacterial toxins in drinking water treatment plants. Toxicol. Appl. Pharm. 203, 231–242.

Jetoo, S., Grover, V.I., Krantzberh, G., 2015. The Toledo drinking water advisory: suggested application of the water safety planning approach. Sustainability 7, 9787–9808.

Jochimsen, E.M., Carmichael, W.W., An, J.S., Cardo, D.M., Cookson, S.T., Holmes, C.E., Antunes, M.B., de Melo Filho, D.A., Lyra, T.M., Barreto, V.S., Azevedo, S.M., Jarvis, W.R., 1998. Liver failure and death after exposure to microcystins at a hemodialysis center in Brazil. N. Engl. J. Med. 338, 873–878.

Luděk, B., Babica, P., Maršálek, B., 2009. Toxins produced in cyanobacterial water blooms – toxicity and risks. Interdiscip. Toxicol. 2, 36–41.

Morris, J.C., 1978. The chemistry of aqueous chlorine in relation to water chlorination. In: Jooley, R.L. (Ed.), Water Chlorination: Environmental Impact and Health Effects, vol. 1. Ann Arbor Science Publishers, Ann Arbor, Michigan, pp. 21–34.

Pattison, D.I., Davies, M.J., 2001. Absolute rate constants for the reaction of hypochlorous acid with protein side chains and peptide bonds. Chem. Res. Toxicol. 14, 1453–1464.

Rodríguez, E., Onstad, G.D., Kull, T.P.J., Metcalf, J.S., Acero, J.L., von Gunten, U., 2007. Oxidative elimination of cyanotoxins: comparison of ozone, chlorine, chlorine dioxide and permanganate. Water Res. 41, 3381–3393.

Sarma, T.A., 2012. Handbook of Cyanobacteria. CRC Press, Boca Raton, FL USA.

Senogles-Derham, P.-J., Seawright, A., Shaw, G., Wickrami-singh, W., Shahin, M., 2003. Toxicological aspects of treatment to remove cyanobacterial toxins from drinking water determined using the heterozygous P53 transgenic mouse model. Toxicon 41, 979–988.

Slatkin, D.N., Stoner, R.D., Adams, W.H., Kycia, J.H., Siegelman, H.W., 1983. Atypical pulmonary thrombosis caused by a toxic cyanobacterial peptide. Science 220, 1383–1385.

Tsuji, K., Watanuki, T., Kondo, F., Watanabe, M.F., Nakazawa, H., Suzuki, M., Uchida, H., Harada, K., 1997. Stability of microcystins from cyanobacteria–IV. Effect of chlorination on decomposition. Toxicon 35, 1033–1041.

U.S. Environmental Protection Agency (USEPA), 2015a. Federal Register. EPA-HQ-OW-2012-0217 80: 6076–6084.

U.S. Environmental Protection Agency (USEPA), 2015b. http://water.epa.gov/drink/contaminants/#Disinfectants.

Vasconcelos, V., 2015. Global Changes and the new challenges in the research on cyanotoxin risk evaluation. Limnetica 34, 149–158.

Wert, E.C., Korak, J.A., Trenholm, R.A., Rosario-Ortiz, F.L., 2014. Effect of oxidant exposure on the release of intracellular microcystin, MIB, and geosmin from three cyanobacteria species. Water Res. 52, 251–259.

World Health Organization (WHO), 2004. Guidelines for Drinking-water Quality, third ed., vol. 1. World Health Organization, Geneva, p. 515. Recommendations.

Xing, X., Xu, Y., Chen, Y., Jeffrey, P.D., Chao, Y., Lin, Z., Li, Z., Strack, S., Stock, J.B., Shi, Y., 2006. Structure of protein phosphatase 2A core enzyme bound to tumor-inducing toxins. Cell 127, 341–353.

Yang, F., Zhou, Y., Sun, R., Wei, H., Li, Y., Yin, L., Pu, Y., 2014. Biodegradation of microcystin-LR and-RR by a novel microcystin-degrading bacterium isolated from Lake Taihu. Biodegradation 25, 447–457.

Yildiz, G., Demiryürek, A.T., Sahin-Erdemli, I., Kanzik, I., 1998. Comparison of antioxidant activities of aminoguanidine, methylguanidine and guanidine by luminol-enhanced chemiluminescence. Br. J. Pharmacol. 124, 905–910.

Yu, S.Z., 1995. Review Primary prevention of hepatocellular carcinoma. J. Gastroenterol. Hepatol. 10, 674–682.

Yuan, M., Carmichael, W.W., Hilborn, E.D., 2006. Microcystin analysis in human sera and liver from human fatalities in Caruaru, Brazil. Toxicon 48, 627–640.

Zhou, L., Yu, H., Chen, K., 2002. Relationship between microcystin in drinking water and colorectal cancer. Biomed. Environ. Sci. 15, 166–171.

Zilberg, B., 1966. Gastroenteritis in Salisbury European children - a five-year study. Cent. Afr. J. Med. 12, 164–168.