CRC for Water Quality
and Treatment

WATER SERVICES ASSOCIATION
of Australia

Chlorination of Saxitoxins

Research Stages 1 to 3

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Foreword

This report has arisen from a Water Services Association of Australia (WSAA) funded project on chlorination of water to remove the cyanobacterial toxins, the saxitoxins (paralytic shellfish poisons).

The research was commissioned by WSAA in order to investigate methods for the removal of these toxins in drinking water and was undertaken in three separate stages. The results of these three discrete investigations are reported in this document as Stages 1 to 3.

Stage 1 of this research was published in June 2001 as WSAA Research Report No. 208.

The authors wish to thank the referees for this report, Professor Paul Lam, City University of Hong Kong and Professor Paolo Ricci, University of San Francisco, for their thorough critique of this manuscript, and their useful suggestions for improvement.

Abbreviations used in this paper

AWQC	Australian Water Quality Centre
C	C-toxin
dcSTX	Decarbamoyl saxitoxin
DOC	Dissolved organic carbon
GTX	Gonyautoxin
HPLC	High performance liquid chromatography
HPLC-MS/MS	High performance liquid chromatography – tandem mass spectrometry
LD50	Lethal dose to 50% of the test animals
NHMRC	National Health and Medical Research Council
NRCET	National Research Centre for Environmental Toxicology
PSP	Paralytic shellfish poison
STX	Saxitoxin

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Executive Summary

Saxitoxins (paralytic shellfish poisons, PSPs) in water can be removed to varying degrees by chlorination. The order of ease of removal of the saxitoxins was saxitoxin (STX) > GTX3 ~ C2 > C1 > GTX2. The effectiveness is very pH dependent, with a high removal possible at pH 9 provided a residual of 0.5 mg/L free chlorine is present after 30 minutes contact time. Removal appears not to be greatly affected by chlorine dose in that doses leading to a higher residual after 30 minutes do not remove substantially more toxins. Removal as a function of pH is not linear with the degree of removal increasing rapidly at around pH 7.5. The form of the chlorinating agent, ie, whether gaseous chlorine or sodium hypochlorite, had no effect on saxitoxins removal when the effect of pH was taken into account. Chlorination without pH adjustment may be feasible if less than complete removal of saxitoxins is required, ie, removal to meet a health advisory level only is required. This will of course depend on the toxic composition and concentrations.

The more effective removal at higher pH was unexpected as chlorine is known to be a weaker oxidant under these conditions. However, this has been ascribed to the toxin molecules being present in an unprotonated form at higher pH and more susceptible to oxidation.

Given the nature of the effect of pH on removal, more work was undertaken on toxin removal in the pH range 7.5 - 9 in order to better determine optimum removal conditions. In addition, removal from cellular material was also investigated and the toxicity of a chlorinated sample was determined using the mouse bioassay. The latter assay was undertaken to determine if any of the oxidation products were toxic in the same manner as the parent compounds.

With the lower detection limits obtained in Stage 2 of the study, more precise removal efficiencies at the higher pH levels could be obtained. STX removal of over 95% was obtained at pH levels 7.5 and above with semi-purified material. However, the other saxitoxins were more recalcitrant and a pH of 9 was required to effect >90% removal. Toxins in cell free extracts or in the presence of cellular extracts were removed to approximately the same, or slightly lower degree in the case of cell free extracts, at a given pH with a similar chlorine residual after 30 minutes contact time. The overall results from the range of experiments carried out indicate that a pH of 9 is required to remove > 90% of saxitoxins using chlorine when a residual of around 0.5 mg/L is present after 30 minutes contact time.

GTX5 (B1) and decarbamoylsaxitoxin (dcSTX) were also included in this Stage 2 study by NRCET for the first time. These toxins had been identified previously in some toxic *A. circinalis* (Negri *et al.*, 1995; Velzeboer *et al.*, 1998, 2000) but were not always quantified. Their removal was found to be equal to or better than that of STX. In the Stage 2 work, slightly higher removal efficiencies of C1 compared with C2 were obtained at the higher pH levels (>7) compared with those obtained in Stage 1. The ease of removal at these higher pH levels was GTX5 (B1) ~ dcSTX > STX > GTX3 ~ C2 ~ C1 > GTX2. Only slightly higher removal efficiencies were obtained at the higher chlorine dose of 20 mg/L, a result that had been found in Stage 1.

This research has demonstrated the kinetics of degradation of saxitoxins at pH's both in the alkaline range (9.0) and in the neutral range (around 7.5). Degradation was shown to follow second order kinetics and depended on concentration, with maximum degradation occurring immediately after chlorine dosing.

To more closely simulate the conditions operating in most treatment plants, chlorination was also undertaken using purified toxins spiked into low DOC water and an initial chlorine dose of approximately 2mg/L. Water was sourced from a reservoir used to supply water for drinking purposes to the northern suburbs of Brisbane.

Executive Summary
continued

The raw water was at a pH of 7.7 and had a chlorine demand of 2.1mg/L over 30 minutes. For the chlorination experiments the water was filtered to remove particulate matter and purified saxitoxins were added to produce water containing appreciable levels of saxitoxins (C1,2 approx 30µg/L; GTX2,3 approx 20µg/L; STX approx 20µg/L) but with low chlorine demand (less than 2µg/L). Chlorinations were performed on water containing purified STX, GTX,3 and C1,2 individually and as mixtures of all toxins. Interestingly, enhanced degradation of GTX2,3 was demonstrated in the mixture compared with GTX2,3 alone. Slightly enhanced degradation of the other toxins investigated was also demonstrated in the mixture of all toxins.

Of most importance to the success of chlorination to remove saxitoxins is the requirement to elevate pH for optimum degradation. The relationship between enhanced degradation and elevated pH was still maintained at low initial chlorine doses but was not as apparent as with phase 1 and 2 research conducted at higher DOC and initial chlorine doses. The main factor for this was the fact that while GTX2,3 demonstrated significantly enhanced degradation at pH 9.0 compared with 7.5, the C toxins and STX did not. The fact that STX degrades to a significant extent at pH 7.5 was demonstrated at higher initial chlorine doses but the research did not demonstrate the same with the C toxins.

One key finding of the research was the fact that chlorination to degrade saxitoxins when low DOC water and correspondingly low chlorine doses (2mg/L) were used, was at least as efficient as higher initial chlorine doses (up to 20mg/L) in higher DOC water. As with higher initial doses of chlorine, dosing with chlorine at an initial concentration of 2mg/L produced second order degradation kinetics where the removal of saxitoxins was fastest in the first few minutes after chlorination. This is an important aspect of the research findings since it signifies that elevation of pH when chlorinating in water treatment plants will remove most of the saxitoxins in the first 5 or 10 minutes. When considered in terms of return for effort, a diminishing return over longer periods of time will be experienced.

Australian *Anabaena circinalis* predominately produces C toxins, but these are the least toxic of the saxitoxins. Expression of the results of degradation on a saxitoxin toxic equivalent basis has permitted an estimation of the removal efficiency for toxicity in addition to removal of individual toxins. Although saxitoxins are removed in that the parent compounds are not detected by chemical analysis, there is no indication as to the nature of the oxidation products. To determine if they have any residual toxicity, toxicity testing was undertaken using mouse bioassay. This demonstrated that chlorination removed the acute toxicity of the samples. No information however exists to determine if any byproducts of chlorination exist which may exert some form of chronic toxicity.

Introduction

Aims

Stage 1 Investigations

To determine the efficiency of chlorine in both gaseous form and as hypochlorite, to degrade a range of saxitoxins under differing pH values.

Stage 2 Investigations

To determine the destruction of the saxitoxins in detail in the pH range 7.5 – 9, using cell-free extract and cellular material from *A. circinalis* in addition to semipurified toxins.

Stage 3 Investigations

To evaluate the kinetics of degradation of saxitoxins that occur in *Anabaena circinalis* (C-toxins, gonyautoxins and saxitoxins) with DOC and chlorine dose levels that were relevant to natural source waters.

Background

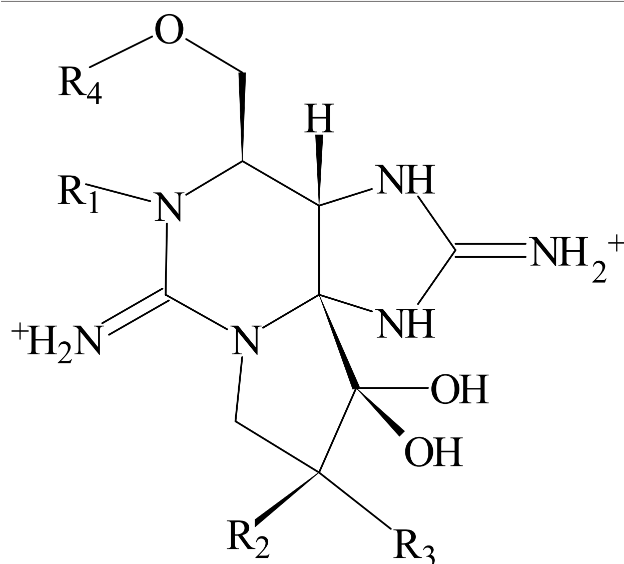
Cyanobacteria (blue-green algae) occur worldwide in both fresh and marine waters (Carmichael, 1997). These organisms can produce chemicals with intense taste and odour properties, as well as toxins. Blooms of these organisms appear to be increasing in frequency as a result of increasing nutrient enrichment of water bodies. Their toxins are of interest to the water industry as during a bloom, the potential exists for the toxins to be distributed in reticulated water. Water treatment processes which can remove toxins are therefore of considerable importance to the water industry.

Cyanobacteria can produce toxins of various classes with differing toxic properties (Codd *et al.*, 1999). Perhaps the most frequently encountered are the microcystins, hepatotoxic cyclic heptapeptides produced most commonly by species of *Microcystis* but also by other genera. *Microcystis aeruginosa* is a common species found in both hemispheres with blooms frequently being toxic (Codd, 1995). Not all blooms of a particular species are toxic, nor is toxicity necessarily constant throughout a particular bloom or over a particular time period (Fastner *et al.*, 1999). Microcystins are readily oxidised by chemicals commonly used for disinfection, eg, chlorine (Nicholson *et al.*, 1994; Tsuji *et al.*, 1997) and ozone (Rositano *et al.*, 1998b). Thus their removal by oxidation during the water treatment disinfection process can be readily achieved.

Another common bloom-forming cyanobacterial genus is *Anabaena*. Blooms of species of this genus are also relatively common worldwide. In the northern hemisphere, the common species is *A. flos-aquae* which produces primarily the neurotoxic alkaloid, anatoxin-a (Carmichael, 1997). In Australia, blooms are generally *A. circinalis* which, when neurotoxic, have been shown to contain the saxitoxins (Humpage *et al.*, 1994; Jones and Negri. 1997; Velzeboer *et al.*, 1998). To date, this is the only neurotoxic cyanobacterium yet found in Australia.

Introduction
Continued

Figure 1. Structures of the Saxitoxins (Paralytic Shellfish Poisons)(PSPs). Toxicity data from Oshima *et al.* (1995)



	R1	R2	R3	Net Charge	Relative Toxicity
R4=CONH$_2$ (CARBAMATE TOXINS)					
STX	H	H	H	+2	1
neoSTX	OH	H	H	+2	0.924
GTX1	OH	H	OSO_3^-	+1	0.994
GTX2	H	H	OSO_3^-	+1	0.359
GTX3	H	OSO_3^-	H	+1	0.638
GTX4	OH	OSO_3^-	H	+1	0.726
R4 = CONHSO$_3^-$ (N-SULFOCARBAMOYL (SULFAMATE) TOXINS)					
GTX5 (B1)	H	H	H	+1	0.064
GTX6 (B2)	OH	H	H	+1	-
C1 (epiGTX8)	H	H	OSO_3^-	0	0.006
C2 (GTX8)	H	OSO_3^-	H	0	0.096
C3	OH	H	OSO_3^-	0	0.013
C4	OH	OSO_3^-	H	0	0.058
R4=H (DECARBAMOYL TOXINS)					
dcSTX	H	H	H	+2	0.513
dcneoSTX	OH	H	H	+2	-
dcGTX1	OH	H	OSO_3^-	+1	-
dcGTX2	H	H	OSO_3^-	+1	0.651
dcGTX3	H	OSO_3^-	H	+1	0.754
dcGTX4	OH	OSO_3^-	H	+1	-

The saxitoxins are widely produced by dinoflagellates in the marine environment where they are known as paralytic shellfish poisons (PSPs), and shellfish feeding on these organisms can themselves become toxic and hazardous if consumed. Human fatalities have been recorded from the consumption of shellfish contaminated in this way (Kao, 1993). Poisoning incidents usually coincide with the sudden proliferation of these organisms to produce visible blooms, the so-called "red tides" (Anderson, 1994). Members of this group of toxins have also been recorded from other freshwater cyanobacteria, namely *Aphanizomenon flos-aquae* (Ikawa *et al.*, 1985), *Lyngbya wollei* (Carmichael *et al.*, 1997) and *Cylindrospermopsis raciborskii* (Lagos *et al.*, 1999).

The saxitoxins are a relatively complex class of 18 compounds with widely differing toxicities which can be divided into three groups as shown in Figure 1. They can also be divided into three groups based on the net charge of the molecule under acidic conditions (Shimizu, 1988; Hall *et al.*, 1990) (Figure 1). This grouping comprises the saxitoxins (saxitoxin (STX), neosaxitoxin (neoSTX) and decarbamoyl derivatives) (charge +2;), the gonyautoxins (GTXs) including decarbamoyl derivatives (charge +1) and C toxins (charge 0). Only the N-non-hydroxylated toxins have to date been found in Australian *A. circinalis*, ie, C1 and C2, GTX2 and GTX3, saxitoxin itself, and the decarbamoyl derivatives of GTX2, GTX3 and saxitoxin. Toxin profiles appear to be reasonably dominated by the less-toxic C toxins (Negri *et al.*, 1997; Velzeboer *et al.*, 1998).

The widespread occurrence of saxitoxins then makes them a very important class of cyanobacterial toxins for the Australian water industry. Their removal during water treatment is of particular interest.

Chlorination is widely used for disinfection in the water industry and has already been shown to be effective for destroying microcystins and cylindrospermopsin (Nicholson *et al.*, 1994; Tsuji *et al.*, 1997; Senogles *et al.*, 2000). If chlorine were also effective in destroying saxitoxins, then this would offer a very simple and convenient solution as the infrastructure for chlorination is in place in most water distribution systems. This study was therefore carried out to determine the effectiveness of chlorination for destroying saxitoxins. The outcome should be readily transferable to the water industry in terms of chlorination conditions which might be employed to destroy saxitoxins, ie, the "chlorine removal envelope".

The research was undertaken in three stages. The first demonstrated that chlorine has the capacity to degrade saxitoxins depending on pH. The second phase investigated more closely the pH range between 7 and 9 and additionally determined the kinetics of saxitoxin degradation. The third phase conducted degradation trials in low DOC water with purified saxitoxins and low initial chlorine doses (2mg/L) and showed that the efficiency of chlorine degradation of saxitoxins was not dependent on high initial doses of chlorine. The three phases are reported sequentially in separate sections of this report.

Chapter 1. Stage 1 Investigations

Degradation of saxitoxins by chlorination using gaseous chlorine, sodium hypochlorite over a range of pHs

1.1 Research Objectives

The experimental protocol was developed to satisfy the following criteria.

- The experiments would be performed at a sufficiently high toxin concentration that samples do not need preconcentration of toxins using extraction procedures before analysis. This will overcome any problems of errors introduced by the extraction/concentration step. Concentration by rotary evaporation would be acceptable if required.
- "Environmentally relevant" concentrations would be used if possible. Then both absolute and relative removals can be used in the assessment of the suitability of chlorine for destroying saxitoxins. A concentration of around 50 µg/L for the individual C toxins, the toxins predominating in toxic *A. circinalis*, has been suggested as representative of a reasonably heavy bloom. However, material available for this study comprised frozen scum collected from Coolmunda Dam, Queensland, during a bloom. Since it is not feasible to isolate the individual saxitoxins for study, it was proposed to semi-purify the material in order to remove cellular material which would contribute to the consumption of chlorine during the chlorination experiments. This process would result in an extract with concentrations of the individual toxins in a ratio similar to the original material.

  The experiments would be carried out on one dilution of the extract only. Multiple dilutions necessary to get each individual toxins around a particular concentration would not be carried out as this substantially increases the number of experiments.

  Given the toxin profile of the study material, it was estimated that spiking water samples to produce concentrations of the C toxins around 50 µg/L would result in the concentration of saxitoxin itself being too low to be accurately determined by the analytical procedure. Therefore a compromise was reached where it was decided to spike water samples such that higher concentrations of the C toxins were produced. Given the toxin profile of this material (Velzeboer *et al.*, 1998), the following toxin concentrations should be attainable with a single spike of the semi-purified extract of the frozen scum material:

Toxin	Concentration (µg/L)
C1	100
C2	210
GTX2	13
GTX3	44
Saxitoxin	17

- Both gaseous chlorine and sodium hypochlorite as sources of chlorine would be investigated. Although theoretically there should be no difference in speciation of chlorine from these two sources at the same pH, it would be wise to eliminate any possible variation in results due to the chlorine source.
- The experiments would be performed in buffered high-purity water to determine the effect of pH on toxin removal.
- Experiments would also be carried out with toxins spiked into real waters. These would consist of waters from both Adelaide and Brisbane.
- Toxin removal would be determined under conditions which result in a chlorine residual of 0.5 mg/L after 30 minutes. This reflects the conditions under which disinfection using chlorine is often carried out, and is also consistent with the conditions under which the chlorination of microcystins has been investigated. However, if chlorine doses sufficient to produce this residual are not sufficient to achieve adequate removal, higher doses will need to be examined.
- In practice, chlorine concentration (residual) and contact time will determine removal. Therefore measurements of degradation on a time basis to study kinetics of degradation would be undertaken on a limited number of samples.

In addition, cell-free extracts of cultures and live material should also be investigated at a later stage as there could there be a different result with live material, *cf*, permanganate and *Microcystis*. In this example it has been shown that intracellular toxin is not readily accessible to the oxidant, and therefore removal is not as effective as with the dissolved toxin.

Experiments were carried out at the Australian Water Quality Centre (AWQC), Adelaide and the National Research Centre for Environmental Toxicology (NRCET), Brisbane. Since both organisations used slightly different methodologies, each is discussed separately where relevant. The aims of the study were not fully realised at both organisations due to time constraints, in particular experiments on buffered samples were not carried out at AWQC due to problems associated with obtaining a suitable buffer which did not interfere with the toxin analysis method being utilised by that organisation.

Stage 1 - Degradation of saxitoxins by chlorination using gaseous chlorine, sodium hypochlorite over a range of pHs
Continued

1.2 Materials and Methods

1.2.1 Chemicals and Reagents

High purity water was obtained from a Milli-Q system (Millipore Corporation, USA). Chemicals were laboratory and analytical grades from various suppliers.

1.2.2 Source of Saxitoxins

Scum material collected during a bloom of toxic *A. circinalis* in Coolmunda Dam, Warwick, Queensland in May 1997 and kept frozen was used as the source of the saxitoxins. This highly toxic material had previously been shown to have the following toxin content (Velzeboer *et al.*, 1998):

Toxin	**Concentration (µg/g dry wt. cellular material)**
C1	1030
C2	2140
GTX2	130
GTX3	440
dcGTX2	present*
dcGTX3	present*
saxitoxin	170

* not quantified due to unavailability of standards

The total toxin content was 3910 µg/g dry wt. cellular material or 720 µg/g as saxitoxin equivalents.

1.2.3 Semi-purification of Toxins

Extraction

The frozen scum material was thawed, then frozen and thawed a further twice to lyse the cyanobacterial cells. The sample was centrifuged at 10000 rpm for 30 min at 4°C, the supernatant removed and 0.05M acetic acid added to the cell pellet (0.6 mL per mL of original scum volume). Following sonication of the pellet in the aqueous acetic acid solution, centrifugation was again carried out at 10000 rpm for 30 min at 4°C. The supernatants were combined and concentrated to approximately one-third of their original volume by rotary evaporation at 40°C.

Protein Precipitation

To the concentrated extract, an equal volume of cold 95% ethanol was added and the solution allowed to stand on ice for 1 hour. It was then centrifuged at 10000 rpm for 30 min at 4°C, the supernatant removed and concentrated to approximately one quarter of its original volume by rotary evaporation at 40°C. This removed most of the ethanol. The concentrated extract was the filtered through a glass-fibre filter (GF/C).

Pigment Removal

Pigment removal was carried out using C18 column chromatography. The C18 packing (200g per 500 mL concentrated extract; Waters bulk packing material, 55-105 µm) was suspended in methanol and the slurry poured into a glass chromatography column (10 cm diameter) and allowed to settle to produce a bed of approximately 5 cm depth. The methanol was drained to the surface of the packing and 500 mL aliquots of 80%, 60%, 40% and 20% methanol sequentially added with draining to the surface of the column packing between additions. The packing was finally washed with water (1 L).

The concentrated extract was added to the column and allowed to drain to the surface of the packing with any eluate being collected. The column was then eluted with water (1 L) and 0.05M acetic acid (500 mL) with the eluate being collected each time. The eluates were combined and concentrated to a workable volume (as small as possible without being too viscous) by rotary evaporation. The concentrations of toxins in a typical extract (mg/L) were:

Toxin	**Concentration**	**Concentration (STXequiv)**	**Toxicity(%)**
C1	104	0.62	3.5
C2	39	3.74	21.2
GTX2	14.5	5.20	29.5
GTX3	5.55	3.54	20.1
saxitoxin	4.55	4.55	25.8

The extract also contained dcGTX2 and dcGTX3, but these were not quantified due to lack of standards. Toxins were recovered in proportions only roughly similar to those in the original material. This suggests incomplete recovery of some toxins, as well as possible interconversion of the epimers (C1 and C2, and GTX2 and GTX3) during the purification/concentration process.

Column Cleanup

The C18 column material was cleaned of extracted pigments by sequentially washing with 500 mL aliquots of 20%, 40%, 60% and 80% methanol. The column was then washed with methanol until the washings were colourless, and the C18 packing material stored under methanol.

Stage 1 - Degradation of saxitoxins by chlorination using gaseous chlorine, sodium hypochlorite over a range of pHs
Continued

1.2.4 Spiking Protocol

Initial experiments at AWQC were carried out by spiking water samples (125 mL) with 500 µL of the semi-purified, concentrated extract. However, the chlorine demand of these samples was relatively high, thereby leading to difficulties in obtaining low chlorine residuals after 30 minutes contact time with high precision. As a result, in later experiments, the extract was spiked at half this level, ie, 250 µL, and the analytical procedure adjusted accordingly.

1.2.5 Water Samples

AWQC

The Adelaide reservoir water used in this study was collected from Hope Valley reservoir on 16 December 1999. It was stored at 4°C and before use filtered through a 0.45µm membrane (Gelman Supor-450). The pH was adjusted with either sodium hydroxide or phosphoric acid solutions.

NRCET

Raw water was collected from North Pine Dam Queensland. This water was drawn off at 15 metres water depth and was used as is, ie, without filtration. The pH of this water was 7.5 and the dissolved organic carbon content was 4.9 mg/L.

1.2.6 Buffered Water Samples

The effect of pH on toxin removal was investigated at NRCET using buffered high purity water at pH 5, 7 and 9. These waters were obtained by adjusting the pH of an 0.1M phosphate buffer prepared from disodium hydrogen phosphate (7.99 g/L) and potassium dihydrogen phosphate (5.95 g/L) with sodium hydroxide or phosphoric acid solutions to the desired levels. The evaluation of buffers is described in Appendix 2.

1.2.7 Chlorine Dosing

Gaseous chlorine

Gaseous chlorine was dosed as chlorine water prepared by sparging chlorine gas through high purity water cooled on ice for 2 min. The resultant chlorine water was stored at 4°C in the dark.

Hypochlorite

In order to obtain a chlorine solution with a chlorine level similar to the chlorine water, stock sodium hypochlorite was diluted 1 in 10 using high purity water. The resultant solution (approximately 1500 mg/L chlorine) was stored at 4°C in the dark.

Standardisation of chlorine solutions

In order to determine the volume of chlorine water or hypochlorite required for specific chlorine doses, the chlorine content of the stock solutions was determined on a daily basis.

AWQC

Chlorine levels were determined by FAS titration with DPD as indicator according to AWQC method AWQC T0136-03.

NRCET

Free residual chlorine was measured using a HACH-2000 spectrophotometer, with DPD powder pillows in a 25 ml volume according to the manufacturer's specifications.

Dosing

The chlorine demand of the waters being tested was determined by dosing at various levels of chlorine and measuring the chlorine residual after 30 min. Dosing was then carried out at a level necessary to produce a residual of 0.5 mg/L free chlorine after 30 min. The pH was also monitored before dosing and at the end of the contact period. For a toxin spike of 250 µL, chlorine doses of around 0.75 mL of the stock solutions were required.

The specific procedure was as follows.

AWQC

The toxin extract (250 µL) was added to approximately 50 mL sample in a hypervial and the solutions mixed. The vial was filled almost to the top with sample, the chlorine dose added, the vial then completely filled with sample, sealed and the contents mixed. The sample volume of 125 mL was required to enable both chlorine residual and toxin measurements to be carried out. After 30 min in the dark the vial was uncapped and the analyses carried out. For the chlorine demand experiments, a 100 mL aliquot was removed for chlorine determination. For the toxin degradation experiments, a 25 mL aliquot was removed and the remaining chlorine quenched prior to analysis for the saxitoxins. The remaining 100 mL in the vial was transferred to a titration flask and the chlorine residual determined. The pH was determined before and after addition of the toxin spike and the chlorine solution on duplicate samples. Blanks of undosed spike toxins were also carried out.

NRCET

A similar procedure to that used at AWQC was employed, except that the experiments were carried out on 125 mL of sample in 250 mL Schott bottles, and 25 mL aliquots were removed for toxin analysis and 25 mL for residual chlorine determination.

Stage 1 - Degradation of saxitoxins by chlorination using gaseous chlorine, sodium hypochlorite over a range of pHs

Continued

Kinetics of degradation was examined as a time course using gaseous chlorine only. Samples were collected at 0, 5, 10, 15, 20, 30, 40, 50, 60, and 120 minutes after chlorination for toxin and free chlorine analysis. The chlorine dose used was that which in earlier experiments on toxin degradation gave a residual of 0.5 mg/L after 30 minutes contact time. Raw water (no pH adjustment) and buffer solution pH 7 were the only samples tested. A final sample volume of 650 ml was used in a sealed 1 L Schott bottle.

Quenching of residual chlorine

AWQC

Residual chlorine was quenched after 30 min contact time in a sample aliquot taken for toxin analysis by the addition of sodium thiosulphate solution. A stock solution was prepared by dissolving sodium thiosulphate pentahydrate at the rate 0.27 g per mL of water and storing at 4°C. For quenching, 40 µL of the stock solution was added to 25 mL of sample. Other quenching agents were examined prior to selecting sodium thiosulphate (see Appendix 1).

NRCET

Residual chlorine was quenched by the addition of a sodium sulphite solution (40 µL) to 25 mL of sample. The sodium sulphite solution was prepared at a concentration of 0.14 g/mL and stored at 4°C.

1.2.8 Determination of Chlorine Concentrations

AWQC

The method used for the standardisation of the chlorine solutions and the determination of chlorine residuals was based on Australian Water Quality Centre internal method AWQC T0012-03. This procedure utilises titration of 100 mL samples against FAS with DPD as indicator. All reagents were prepared according to the method except the analytical buffer which was prepared with all components at 5 times the concentrations listed in the method. This was necessary so that the buffering capacity was sufficient to overcome the buffering capacity of the samples, especially the buffered samples. The ionic strength of the buffer in the buffered samples needed to be relatively high in order for there to be sufficient capacity to counter the effect of the relatively high chlorine doses required.

NRCET

Chlorine residuals were determined as described earlier.

1.2.9 Analysis for Saxitoxins

AWQC

Analysis was carried out by the standard AWQC procedure using HPLC separation of the toxins, post-column derivatisation and fluorescence detection (Rositano *et al.*, 1998a). This method follows that of Oshima *et al.* (1989) very closely and requires three separate analytical runs to determine the range of saxitoxins. In this study samples were analysed directly without concentration (C toxins) or initially concentrated by a factor of around 10 by rotary evaporation for determination of saxitoxin and the GTX toxins. It was found that the concentration step for saxitoxin and the GTX toxins could be avoided if the HPLC injection volume was increased to 150 µL when analysing for these compounds. This could be achieved without any deterioration in analytical performance, eg, peak shapes were still excellent with no broadening or tailing. Detection limits using this approach were

Toxin	Detection Limit (µg/L)	Injection Volume (µL)
C1	20	10
C2	10	10
GTX2	2	150
GTX3	1	150
Saxitoxin	2	150

NRCET

At NRCET/QHSS toxins were analysed by pre-column oxidation HPLC with fluorescence detection, using the method QHSS EMM-042 (based on Lawrence *et al.*, 1995). A 100 µL sample following quenching of residual chlorine was mixed with 250 µL of 1 M sodium hydroxide and 25 µL of 10% hydrogen peroxide and allowed to stand for three minutes. The addition of 25 µL glacial acetic acid neutralized the solution. Samples were then analysed on an Alltima C18 (150 x 4.6 cm, Alltech, Australia) HPLC column with a gradient of 0 to 10% acetonitrile in an ammonium formate buffer. The fluorescent oxidation products were detected using a band fluorescence HPLC detector at an excitation wavelength of 330 nm and emission wavelength of 390 nm.

The oxidation product of saxitoxin elutes as a single peak. The oxidation products of the gonyautoxins (GTXs) co-elute, as do the oxidation products of the C toxins. Peak areas were compared to a saxitoxin standard and a mixed GTX standard (GTX2 and GTX3). No C toxin standard was available and results for these compounds are reported as a percentage of peak area compared to the non-chlorinated control samples. Limits of detection were as follows:

Saxitoxin	0.1 µg/L
GTXs	0.5 µg/L
C toxin	0.2% area of the original peak area before chlorination.

Stage 1 - Degradation of saxitoxins by chlorination using gaseous chlorine, sodium hypochlorite over a range of pHs
Continued

1.3 Results

1.3.1 Initial Experiments with High Level Spikes – Buffered High Purity Water

Initial experiments with buffered waters at AWQC utilised a spike of 500 µL semi-purified extract in 125 mL water sample. Chlorine doses required to obtain a residual of 0.5 mg/L free chlorine after 30 minutes were relatively high (around 35 mg/L), thereby necessitating the use of a phosphate buffer with an ionic strength of 0.1M (Appendix 2). At these high buffer strengths, HPLC analysis with the larger injection volumes (150 µL) necessary for quantification of the GTX toxins gave rise to such poor chromatography that the peaks were too broad and poorly shaped for quantification to be carried out. The phosphate buffer at this concentration therefore interfered in the analysis and this work could not be pursued.

Later work where the toxin spike volume and consequently the chlorine dose were halved with toxins still being able to be determined accurately with sufficient sensitivity suggested that the buffer ionic strength could be reduced under these conditions with the buffering capacity still being maintained. The reduced buffer ionic strength and its effect on buffering capacity have still to be examined in detail.

1.3.2 Buffering Capacity

Some preliminary experiments on the effect of spiking toxins and dosing chlorine under the experimental conditions adopted in this study on the pH of real waters was undertaken. This was carried out to determine if these reservoir waters had any buffering capacity when their pH levels were adjusted with acid or base. The results are tabulated in Appendix 3 for the two reservoir waters used, and indicate the large changes resulting from spiking and dosing, even with the lower volume toxin spikes and consequent lower chlorine dose rates. Thus pH must be carefully monitored during these experiments with the pH following chlorine dosing being the actual pH at which the reaction is occurring. This pH following chlorine dosing was taken as the pH of the saxitoxin removal process.

1.3.3 Toxin Removal

Buffered High Purity Water (NRCET)

Experiments at NRCET were carried out to examine the effect of a chlorine dose sufficient to provide a residual of around 0.5 mg/L after 30 minutes contact time, but also to observe any effect with elevated chlorine doses with 30 minutes contact time might have on toxin degradation. Experiments were run in duplicate and the average free chlorine residuals using both sodium hypochlorite and gaseous chlorine together with toxin removals are listed in Appendix 4. Similar chlorine doses were required using either hypochlorite or gaseous chlorine to obtain similar chlorine residuals. In general chlorine doses of approximately 15 mg/L hypochlorite and 17.5 mg/L gaseous chlorine were required to give a free chlorine residual of 0.5 mg/L after 30 minutes

It was clear from these results that pH has a significant effect on the degradation rate. At pH 5 only around 80% of STX was removed whereas at both pH 7 and 9 removal was around 95%. Greater influence of pH on GTX and C toxins removal was observed. Removal of GTXs was only around 15% at pH 5, increasing to around 25% at pH 7 and up to around 70% removal at pH 9. At pH 5 approximately 20% of the C toxins were removed, with approximately 30% and 80% removal at pH 7 and 9 respectively. Overall the increase in removal when the pH was increased from 5 to 7 was much less than the removal observed when the pH was in creased from 7 to 9. The chlorine dose had very little effect on toxin removal. Higher doses leading to higher residuals (around 1 - 2.5 mg/L rather than 0.5 mg/L) after 30 minutes contact produced only marginally greater toxin removals. STX was the toxin most easily removed, followed by the C toxins and then the GTX toxins.

Reservoir Waters (AWQC and NRCET)

Experiments were carried at both organisations out to examine the effect of a chlorine dose sufficient to provide a residual of around 0.5 mg/L after 30 minutes contact time on the removal of saxitoxins from natural (reservoir) waters. These were carried out with both gaseous chlorine and sodium hypochlorite. These were also carried out at the natural pH of the water as well as on the water following pH adjustment to 5, 7 and 9. As indicated previously, the relatively high chlorine doses necessary to produce the required residual under the designated experimental conditions resulted in large pH changes. Consequently it was important to measure pH following the addition of the chlorine, and use this value as the pH at which the saxitoxins were removed, and this was done at both organisations (Appendix 3). The results are tabulated in Appendices 5 and 6, and graphed in Figures 1.1 – 1.5. For the purpose of graphing, where toxin was not detected after chlorination, removal was nominally recorded as 95%. Curves presented in these figures are included only as an aid for visual interpretation of degradation trends.

Stage 1 - Degradation of saxitoxins by chlorination using gaseous chlorine, sodium hypochlorite over a range of pHs
Continued

Figure 1.1: Removal of C1 toxin as a function of pH

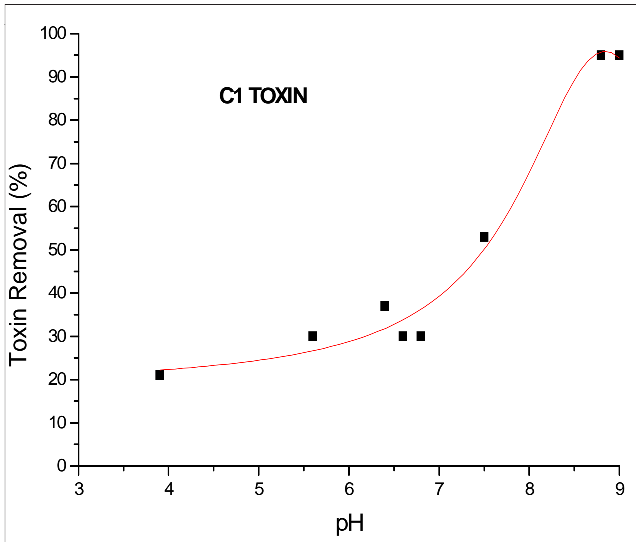


Figure 1.4: Removal of GTX3 toxin as a function of pH

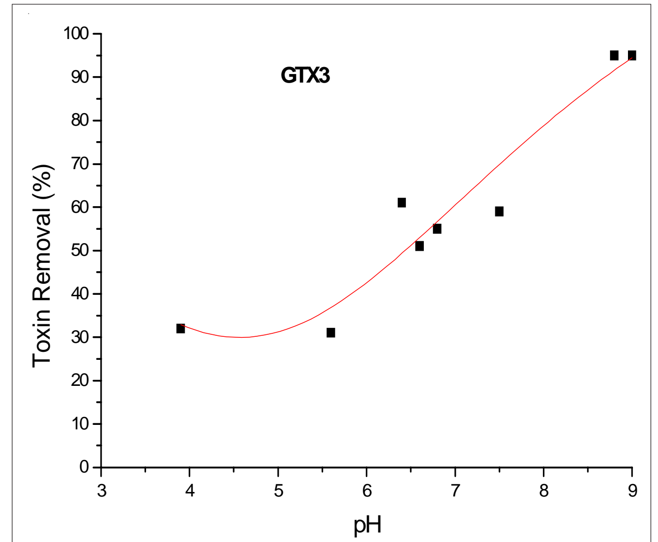


Figure 1.2: Removal of C2 toxin as a function of pH

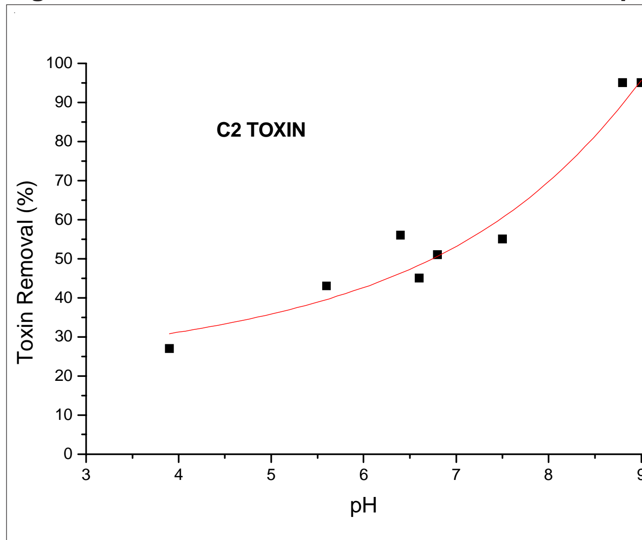


Figure 1.5: Removal of saxitoxin as a function of pH

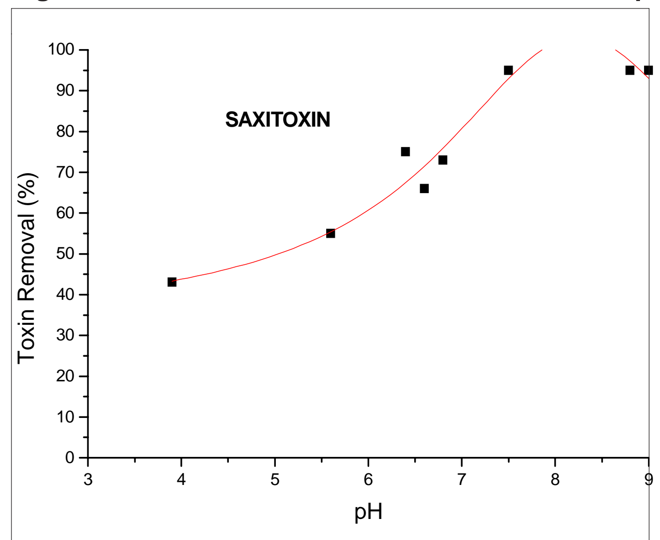
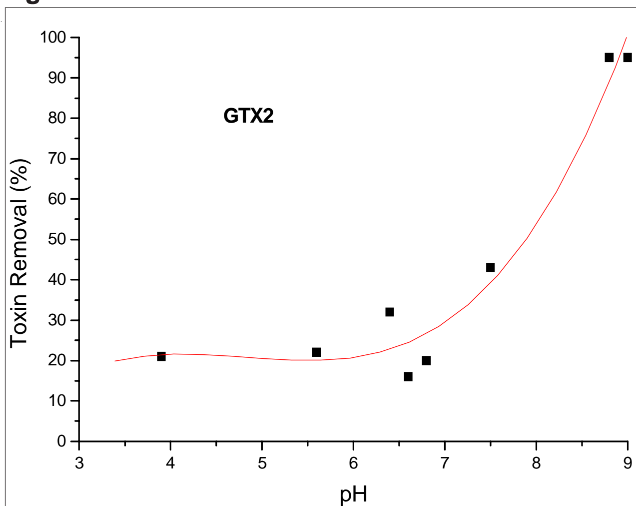


Figure 1.3: Removal of GTX2 toxin as a function of pH



Stage 1 - Degradation of saxitoxins by chlorination using gaseous chlorine, sodium hypochlorite over a range of pHs
Continued

1.4 Discussion

As with the buffered high purity water results, there was a marked effect of pH on toxin removal. Removal was more effective at higher pH values (9) compared with lower pH values (5 and 7). Again the increase in removal when the pH was increased from 5 to 7 was much less than the removal observed when the pH was in creased from 7 to 9.

With the NRCET results, removal using gaseous chlorine was much less than that obtained using sodium hypochlorite at the same pH. The reason for these discrepancies is not clear. The measured pH values after treatment with gaseous chlorine appear much higher than expected. If these pH values were in reality much lower, then a poorer removal would be expected. Removals obtained by NRCET were also much lower than those obtained by AWQC at the same pH for both gaseous chlorine and sodium hypochlorite. The difference with gaseous chlorine may be partly due to a pH effect discussed above. This difference may have been also partly due to some bias in the NRCET analytical procedure as the removals for the reservoir water were significantly less than those obtained for the buffered high purity water samples at the same pH.

The results obtained by AWQC indicate high removal at pH 9. The removal of GTX2 can be more accurately determined at high removal efficiencies as the analytical detection limit is lower. Given that GTX2 which is the most recalcitrant of the saxitoxins to removal is very high, removal of the other toxins is probably much higher than suggested by the data. The order of ease of removal of the saxitoxins was STX > GTX3 ~ C2 > C1 > GTX2. The form of the chlorinating agent, ie, whether gaseous chlorine or sodium hypochlorite, had no effect on toxin removal when the effect of pH was taken into account.

The more effective removal of STXs at higher pH was somewhat surprising. The effectiveness of chlorine as an oxidising agent is greater at lower pH. This is due to the predominance of hypochlorous acid at low pH over hypochlorite which has a lower oxidation potential (Glaze, 1990). Thus less effective oxidation at higher pH is expected. However the saxitoxins are molecules with a purine alkaloid nucleus which contains nitrogen atoms. These nitrogen atoms will be protonated to a degree which depends on pH. At higher pH these atoms will be unprotonated and it may be that these molecules are much more susceptible to oxidation, even by hypochlorite which is a weaker oxidant compared with hypochlorous acid. This finding does, however, reflect the effect that pH has on the sensitivity of detection of these saxitoxins in analytical procedures using post-column oxidation. Sensitivity which depends on the oxidation of the toxins to a fluorescent derivative increases as the pH increases from 6.5 (Sullivan *et al.*, 1985). This indicates that oxidation, at least to form fluorescent products, is more efficient as the pH increases.

There was no evidence of high pH values alone effecting removal of the toxins. STX which is the most readily removed of the toxins was found at much the same concentration in all control samples, irrespective of the pH. Control samples were samples carried through the whole experimental process but without chlorine being added.

1.4.1 Kinetic Study of Toxin Removal (NRCET)

The kinetics of toxin removal was investigated by determining removal as a function of time with gaseous chlorine and both buffered high purity water and reservoir samples. The results are shown in Appendix 7. The results obtained compare well with results obtained in previous experiments at the same pH. Again toxin removal in the raw reservoir water was less than that obtained at a similar pH with buffered water samples. A kinetic examination indicates that the majority of toxin degradation occurs within the first 5 minutes. There was further toxin removal after 30 minutes contact time. Allowing the chlorine contact time to run for up to 24 hours did result in some further removal of GTX and C toxins in both the buffered and raw water samples which was surprising given that the chlorine was almost totally consumed after 30 minutes.

1.5 Summary and Conclusions for Stage One

Saxitoxins in water can be removed by chlorination. The effectiveness is very pH dependent, with a high removal possible at pH 9 under conditions where a residual of around 0.5 mg/L free chlorine is present after 30 minutes contact time. Removal appears not to be greatly affected by chlorine dose in that doses leading to a higher residual after 30 minutes do not remove substantially more toxins. Removal as a function of pH is not linear with the degree of removal starting to increase rapidly at around pH 7.5. The order of ease of removal of the saxitoxins was STX > GTX3 ~ C2 > C1 > GTX2. The more effective removal at higher pH was unexpected as chlorine is known to be a weaker oxidant under these conditions. However, this may be due to the toxin molecule being present in a form at higher pH which is more susceptible to oxidation. The form of the chlorinating agent, ie, whether gaseous chlorine or sodium hypochlorite, had no effect on toxin removal when the pH was taken into account.

1.6 Recommendations for Stage One

The pH dependence of toxin removal, together with the rapidly increasing effectiveness as the pH increases above 7.5, indicates that further studies should be done in the pH range 7.5 - 9 to determine optimum conditions for removal of toxins. If effective removal at a pH closer to 7.5 can be demonstrated, this will make it more feasible for chlorination to be used in practice for toxin removal as the degree of pH adjustment necessary for most waters will be much reduced. For many waters pH might not require adjustment at all, especially as chlorine doses in practice even to produce a residual of 0.5 mg/L free chlorine after 30 minutes contact time are usually sufficiently low that the pH of the water is not greatly changed.

Determining removal where the individual toxins are measured is desirable although more time consuming as it produces more rigorous results. However, the method for determining individual toxins as employed by AWQC in these studies did not have a sufficiently low detection limit to allow an accurate determination of toxin removal once removals reached around the 80% level. Thus the analytical conditions should be investigated to improve sensitivity by a factor of at least 5. If this can be achieved, removal efficiencies of greater than 95% should be able to be measured.

Further work is required to determine toxin removal in the presence of cellular material, and with live material, to determine whether there are any confounding factors under these conditions which much more closely reflect the real-world situation. This should be carried out at the optimum pH conditions.

Although saxitoxins are removed in that the parent compounds are not detected by chemical analysis, there is no indication as to the nature of the oxidation products. To determine if they have any residual toxicity, a sample which has had the toxins successfully removed by chlorine as indicated by chemical analysis should be tested by mouse or cell receptor bio-assay.

Chapter 2. Stage 2 Investigations

Degradation of saxitoxins by chlorination at elevated pHs

2.1 Introduction

The experimental protocol was described in detail in report of Stage 1 of these investigations.

Again experiments were carried out at the Australian Water Quality Centre (AWQC), Adelaide and the National Research Centre for Environmental Toxicology (NRCET), Brisbane. Since both organisations used slightly different methodologies, each is discussed separately where relevant.

The experimental procedures were as described in the Stage 1 report with some minor differences. The detection limit for the saxitoxins analysis at AWQC was improved by increasing the instrument sensitivity resulting in higher removal efficiencies being able to be more accurately determined. The emphasis was on determining the destruction of the saxitoxins in some detail in the pH range 7.5 – 9, as well as some work directly with cell-free extract and cellular material from *A.circinalis*.

In Stage 2, gaseous chlorine only was used at AWQC as previous results had shown, as expected, no difference in the behaviour of gaseous chlorine *versus* sodium hypochlorite for a given pH. Since in Stage 1 the chlorine dose resulting in a residual of 0.5 mg/L free chlorine after 30 minutes contact time was close to 17 mg/L, all experiments in Stage 2 at AWQC were carried out at this dose. In addition, experiments were also carried out with higher doses of 20 mg/L for semi-purified material.

Two doses were used with cell-free extract and cellular material to produce chlorine residuals of the order of 0.5 mg/L. In these experiments (NRCET), sodium hypochlorite was used as the chlorinating agent.

2.2 Materials and Methods

2.2.1 Toxin material

A freeze-dried sample of *Anabaena circinalis*, sourced from Coolmunda Dam QLD, (1997 bloom) was supplied by AWQC. This was also the source of the semi-purified material.

2.2.2 Raw Water

A raw water sample from Lake Samsonvale (North Pine dam) was used in experiments with cell-free extract and cellular material. This was collected from 0.5 m depth near the southern arm next to NRCET test site (GPS co-ordinates of site are 27 deg 17.260 S, 152 deg 54.627 E) on 25/01/02. This has shown a chlorine demand of 2.0 mg/L. This water (stored at 4ºC) was used in all experiments without any filtration. Water from Hope Valley reservoir was again used by AWQC for experiments with semi-purified material, including the samples generated for toxicity testing.

2.2.3 Chlorination of Dam Water Containing Cell Free Extract

To Coolmunda freeze-dried material (250 mg) was added water (30 mL) and the suspension was sonicated for 30 minutes. This was then centrifuged and the supernatant liquid separated. The pellet was re-extracted with 50 mM acetic acid (2 x 30 mL). With each extraction the sample was sonicated for 15 minutes. All extractions were then combined and ultracentrifuged for 1 hour at 35 000 rpm at 4ºC. The supernatant liquid was carefully separated and lyophilised to reduce volume (final volume adjusted to 10.0 mL). Preliminary extraction processes have shown that two extractions of 50 mM acetic acid will release all the toxin. This material then contained the saxitoxins plus other soluble co-extractives from *A. circinalis*.

All chlorinations at NRCET were done with NaOCl. The experiments were conducted in 50 mL tubes in duplicate. To 50 ml of North Pine raw water, was added the above toxin extract (500 µL). The pH was adjusted with 100 mM KOH and NaOCl solution added to give a Cl_2 concentration of 7.2 mg/L. The solution pH was measured. The samples were stirred in the dark for 30 minutes. At this stage a portion of the sample (15 mL) was neutralised with a $Na_2S_2O_3$ solution to remove excess chlorine. The

Stage 2 - Degradation of saxitoxins by chlorination at elevated pHs
Continued

residual chlorine was measured using Cl_2 powder pillows. These values varied from 0.3 to 0.85 mg/L. A portion of the neutralized solution (10.0 mL) was then carefully lyophilised to reduce the volume. The final volume was adjusted to 1.0 mL and toxin concentrations were determined by HPLC as described in the Stage 1 report.

2.2.4 Chlorination of Dam Water Containing Toxins in the Presence of Cellular Material

The chlorination experiments were performed in 50 mL tubes in duplicate. Coolmunda *A. circinalis* (100 mg) was sonicated in the presence of 50mM acetic acid (40 mL). This suspension (2mL) was added to 48 mL of North Pine raw water, the pH adjusted with 100 mM KOH and NaOCl solution added to give a Cl_2 concentration of 20.0 mg/L. The pH was then measured. The samples were then stirred in the dark for 30 minutes. A portion of the test sample (15 mL) was neutralised with $Na_2S_2O_3$ after the 30 minute period. The residual chlorine of the remaining sample was measured using Cl_2 powder pillows. These were measured to be 0.5 to 1.1 mg/L. A portion of the neutralized sample (10.0 mL) was then carefully lyophilised and the final volume adjusted to 1.0 mL and toxin concentrations were determined by HPLC as described in the Stage 1 report.

2.2.5 Kinetics of chlorination of saxitoxins at pH 9.2

Saxitoxin material was used as previously described and was added to raw dam water in the form of a cellular suspension. The pH of the study was 9.2 and sodium hypochlorite was used as oxidant. The chlorine dose was 20mg/L and the chlorine demand of the dam water was 2mg/L. The chlorine residual was 1.6mg/L after 30 minutes. All results are the mean of duplicate determinations.

2.2.6 Mouse bioassay.

The mouse bioassay was carried out by MedVet Science, Adelaide, South Australia with mice of approximately 20 g in weight. A water sample (Hope Valley Reservoir) was spiked with a larger dose (2.5 mL) of semi-purified toxin material, chlorinated to give a concentration of 20 mg/L chlorine under conditions where a final pH of 9 was aimed for after 30 minutes contact time. However, the larger dose of semi-purified toxin caused a greater decrease in pH than expected and the samples after addition of the chlorine were adjusted to pH 9 with sodium hydroxide solution. A larger dose of semi-purified was required due to the unavailability of the semi-purified material used in other experiments. The only semi-purified material available contained the C toxins at concentrations similar to the original material, but the other toxins were at concentrations approximately 10 times less. Therefore to generate samples amenable to analysis by the procedures used here, the spiked volume needed to be 10 times more to compensate for the reduced concentrations. The higher dose was also necessary to produce solutions which, following a reasonable degree of concentration, were acutely toxic to mice. Higher concentration factors would have also produced samples with high dissolved salts concentrations which may have been, by themselves, lethal to mice.

The pH after 30 minutes contact time was only slightly lower. The chlorine residual and toxin concentration were determined as described except that a 50 mL aliquot was used for the chlorine determination. A 40 mL aliquot was concentrated to 2.5 mL by rotary evaporation and 1 mL injected intraperitoneally into a mouse. A negative control (Hope Valley water treated with chlorine and the chlorine quenched with sodium thiosulphate) and positive controls (Hope Valley water spiked with toxins with and without sodium thiosulphate) were also carried out. All controls were concentrated in the same manner as samples (rotary evaporation of 40 mL aliquots to 2.5 mL prior to intraperitoneal injection). Controls and samples were tested in duplicate. Toxin concentrations were not monitored by HPLC in these samples except for the C toxins which were monitored as an indicator of toxin removal. Based on the known LD50 of the saxitoxins and the concentration in the semi-purified material used for spiking, the amount of toxin in 1 mL injected into a mouse following concentration would be about twice the lethal dose.

Stage 2 - Degradation of saxitoxins by chlorination at elevated pHs
Continued

2.3 Results

2.3.1 Chlorination of semi-purified extracts

By running the instrumentation at higher sensitivity, lower detection limits were achieved which resulted in higher removal efficiencies being more accurately determined. The results for the 17 and 20 mg/L dose rates are shown in Figures 2.1 – 2.5 and Appendices 1 and 2. Also included in the plots are the data obtained in Stage 1.

Removals with these higher doses were only slightly higher than those obtained with a 17.0 mg/L dose rate. After 30 minutes contact time, higher chlorine residuals were recorded as expected. However, the additional residuals were less than the additional chlorine added, suggesting that the chlorine demand is not fully satisfied in 30 minutes. The high chlorine demand in these experiments was due to the high concentration of extracts which was necessary to obtain toxin concentrations which could be readily measured. In a treatment situation it is likely that the chlorine demand would be lower than in these experiments. Effective removal should therefore be achieved with lower chlorine doses.

In the Stage 2 work, slightly higher removal efficiencies of C1 compared with C2 were obtained at the higher pH levels (>7) compared with those obtained in Stage 1. The ease of removal at these higher pH levels was STX > GTX3 ~ C2 ~ C1 > GTX2. Only slightly higher removal efficiencies were obtained at the higher chlorine dose of 20 mg/L, a result that had been found in Stage 1.

With the lower detection limits obtained in this Stage 2 study, more precise removal efficiencies at the higher pH levels could be obtained. STX removal of over 95% was obtained at pH levels above 8. With a higher chlorine dose of 20 mg/L, >95% removal could be obtained at a pH as low as 7.5. However, the other saxitoxins were more recalcitrant and a pH of 9 was required to effect >90% removal of these compounds.

2.3.2 Chlorination of cell-free extract and cellular material

The results of chlorination of a cell-free extract and *A. circinalis* cellular material, including percentage removal of toxins, chlorine dose and chlorine residual at a range of pH values from 7.5 to 9.6 are tabulated in Appendix 10. These data are graphed in Figures 2.6 and 2.7.

Figure 2.1: Removal of C1 toxin as a function of pH

Note: The straight lines presented in Figures 2.1 to 2.5 represent linearised visual fits of the data to demonstrate the lowest pH at which >90% removal of toxin occurs. Curves over the complete pH range are included only for visual interpretation of degradation trends.

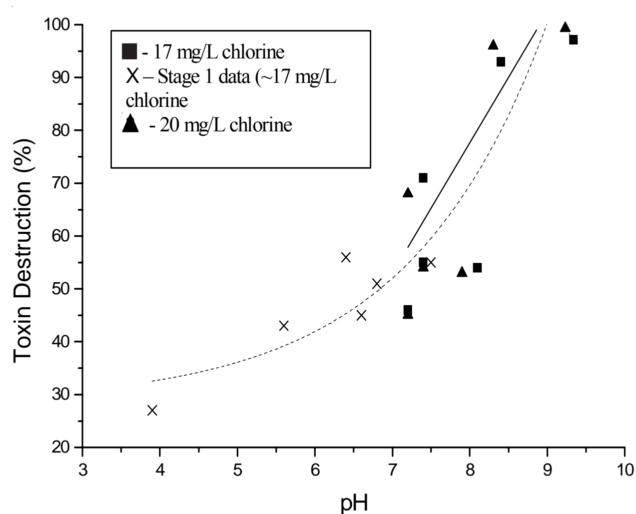
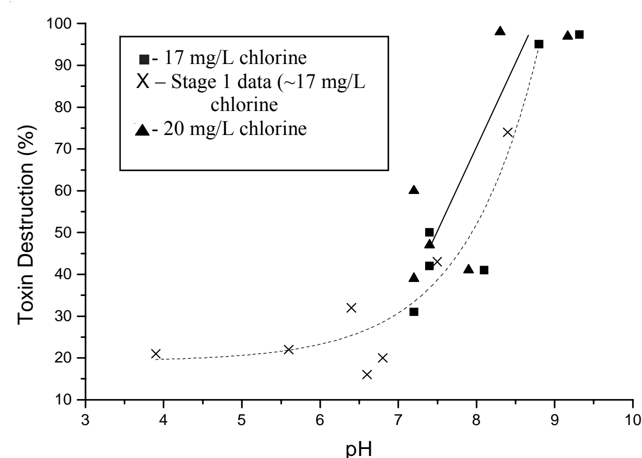


Figure 2.2: Removal of C2 toxin as a function of pH



Stage 2 - Degradation of saxitoxins by chlorination at elevated pHs
Continued

Figure 2.3: Removal of GTX2 toxin as a function of pH

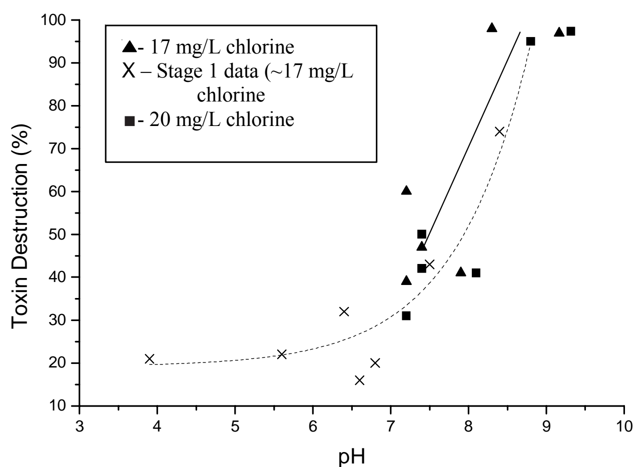


Figure 2.4: Removal of GTX3 toxin as a function of pH

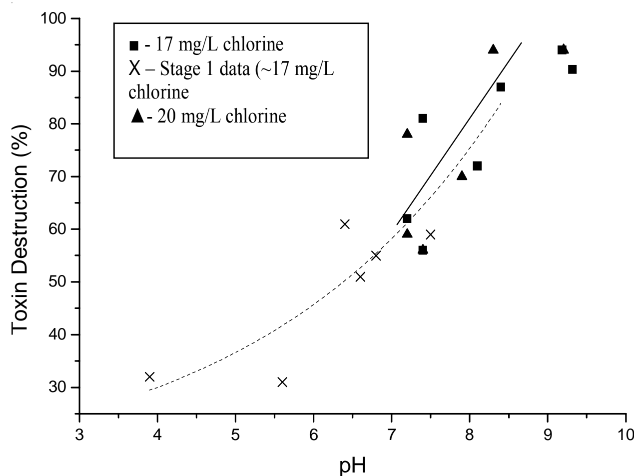


Figure 2.5: Removal of saxitoxin as a function of pH

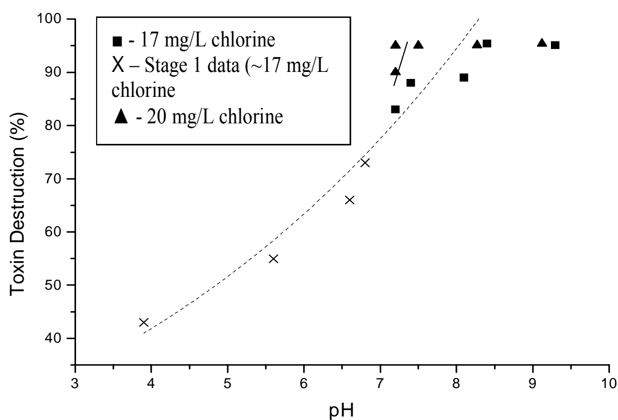


Figure 2.6. Degradation of saxitoxins in *A. circinalis* cell-free extract. Toxin B1 is also known as GTX5.

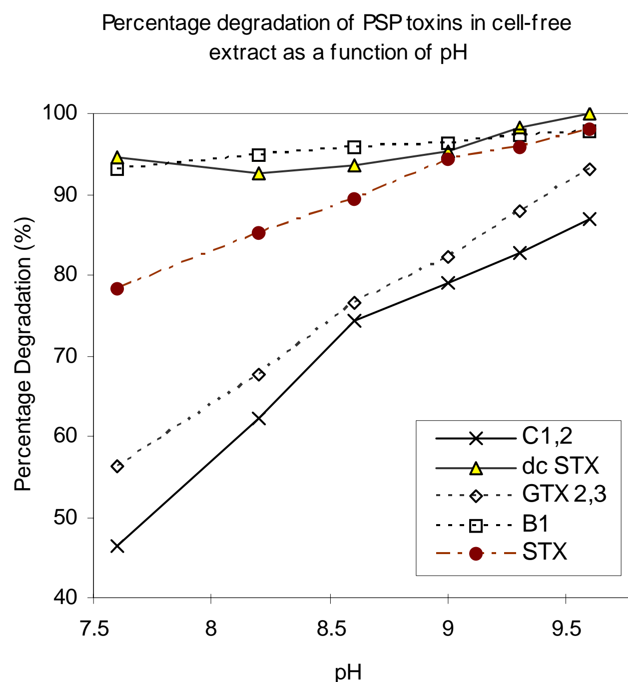
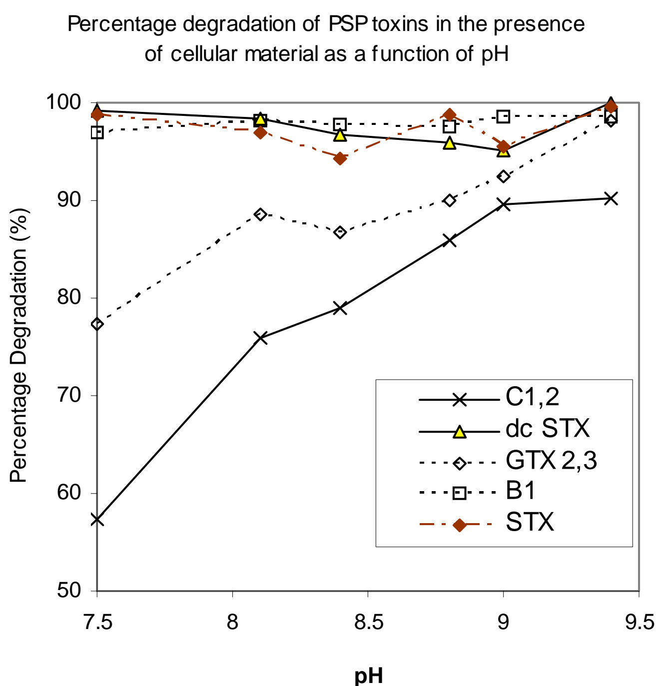


Figure 2.7. Degradation of saxitoxins in *A. circinalis* cellular material. Toxin B1 is also known as GTX5.



Stage 2 - Degradation of saxitoxins by chlorination at elevated pHs
Continued

2.3.3 Kinetics of chlorination of saxitoxins at pH 9.2.

Table 2.1 presents results of the chlorination with respect to time. These results are also plotted with respect to time in the accompanying figures as are the reciprocals of the toxin concentrations which are essentially linear indicating a second order removal of the toxins. The plots of toxin concentration versus time show an exponential decay with most removal of toxins in the first few minutes of chlorination. Figures 2.8 to 2.13 also show calculated values for removal of toxins using second order decay kinetics.

Table 2.1. Removal of saxitoxins with respect to time after chlorination.

Time after chlorination (mins)	$C_1 + C_2$ conc. (µg/L)	STX conc. (µg/L)	GTX_2 + GTX_3 conc. (µg/L)
0	129	1.5	20.2
1	86	0.49	8.2
2	67	0.38	6.4
5	42	0.27	3.5
10	30	0.19	2.4
15	29	0.15	2.1
20	23	0.08	1.2
30	18.4	0.05	0.9
45	14.7	0.04	0.8
60	11.9	0.03	0.7

Figure 2.8. Degradation kinetics of carbamoyl toxins. (predicted concentration based on second order kinetics).

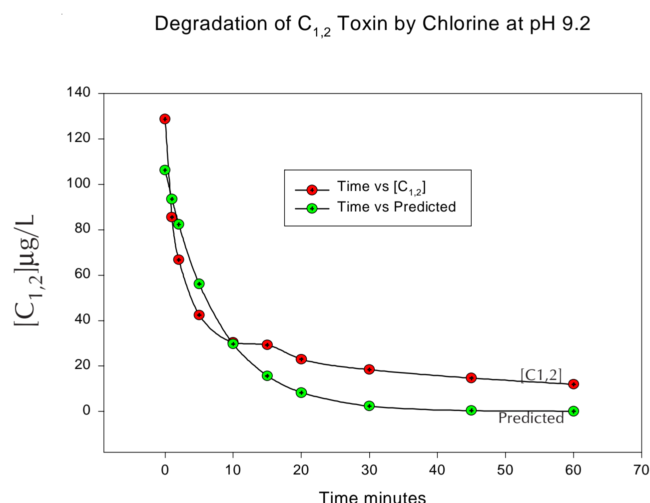


Figure 2.9. Second order kinetic plot for carbamoyl toxins.

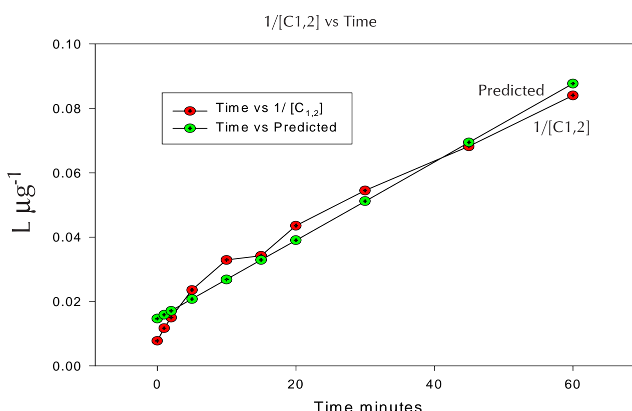


Figure 2.10. Degradation kinetics of saxitoxin. (predicted concentration based on second order kinetics)

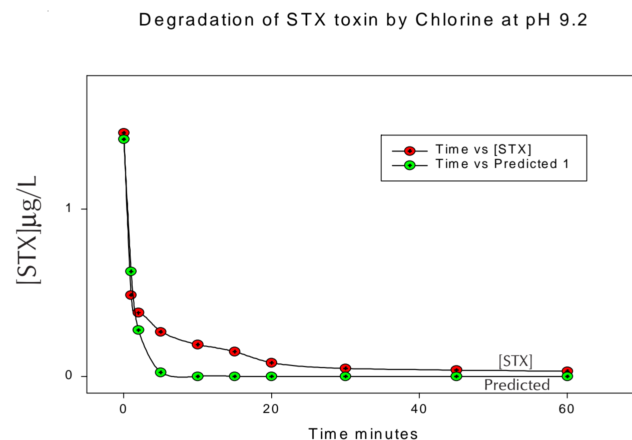
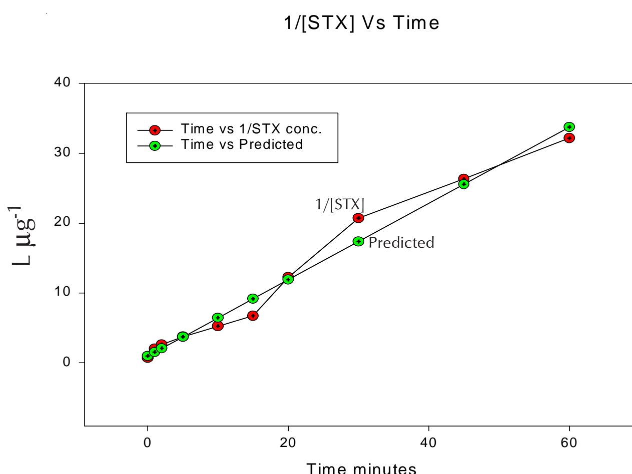


Figure 2.11. Second order kinetic plot for saxitoxin



Stage 2 - Degradation of saxitoxins by chlorination at elevated pHs

Continued

Figure 2.12. Degradation kinetics of gonyautoxins 2 and 3. (predicted concentration based on second order kinetics)

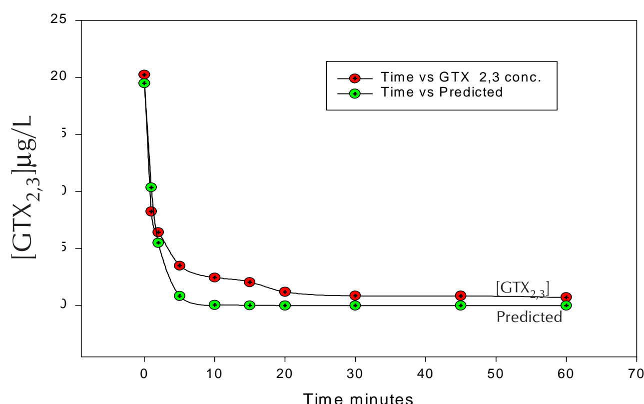
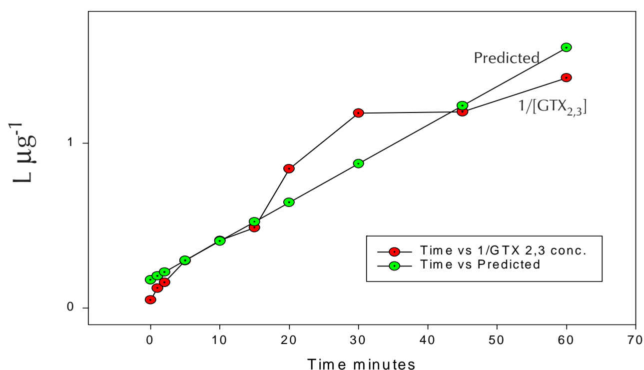


Figure 2.13. Second order kinetics for gonyautoxins 2 and 3

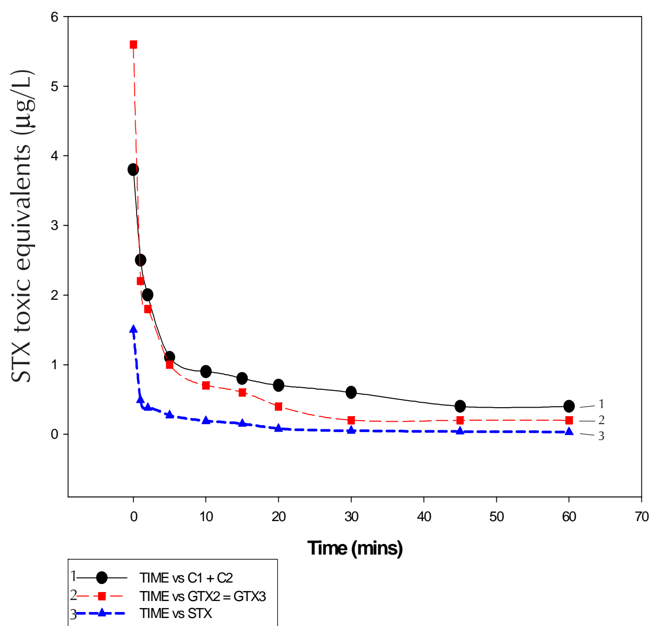


Note: In figures 2.8 to 2.13, the curves shown for degradation of individual toxins are included only for visual interpretation of degradation trends. Predicted degradation curves are also included and are calculated from second order kinetics. The lines in the second order kinetic plots (figures 2.9, 2.11 and 2.13 are calculated using equations for second order rate processes.

2.3.4 Expression of kinetic study results as saxitoxin toxic equivalents

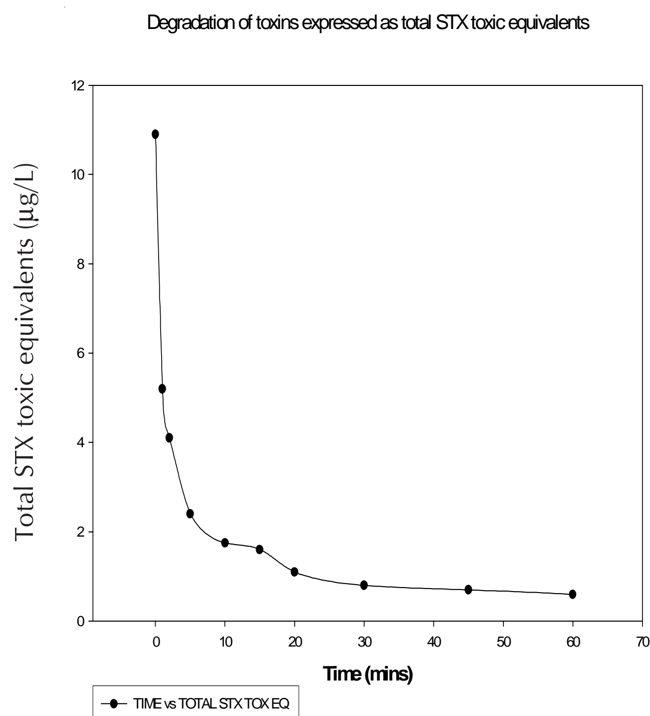
It is of importance to determine the kinetics of removal of saxitoxins based on saxitoxin toxic equivalents. This was achieved by normalizing mouse unit toxicity of the individual saxitoxins to saxitoxin (STX) as unity. Saxitoxin equivalent concentrations were then determined by multiplying the concentrations of individual saxitoxins by the toxicity relative to saxitoxin. In the kinetics study, the C toxins and GTX toxins were investigated as groups rather than C1, C2, GTX2 and GTX3 separately. The data from the stage 2 chlorination of individual toxins was used to estimate the proportion of individual toxins in groups and also the removal ratios of individual toxins in the groups based on toxicity equivalents. Figure 2.14 provides details of the degradation of the toxin groups expressed as saxitoxin equivalents. Figure 2.15 presents details of degradation of total toxins (summation of all saxitoxins present) expressed as saxitoxin equivalents.

Figure 2.14. Kinetics of degradation of saxitoxin classes at pH 9.0 expressed as saxitoxin toxic equivalents.



Stage 2 - Degradation of saxitoxins by chlorination at elevated pHs
Continued

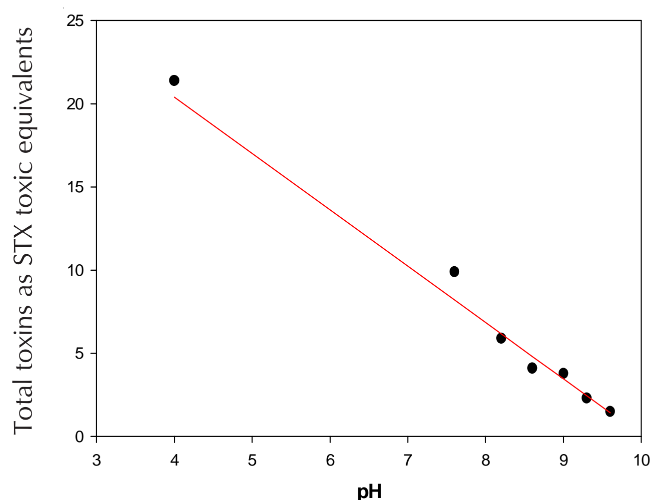
Figure 2.15. Kinetics of degradation of total toxins at pH 9.0 expressed as saxitoxin toxic equivalents



2.3.5 Expression of results of stage 2 chlorination study as saxitoxin toxic equivalents.

The data produced by NRCET from the study on the effect of pH on saxitoxins degradation has been expressed in terms of saxitoxin equivalents of total toxins and plotted against pH for both the semipurified toxin and cellular material experiments. These data are shown in Figures 2.16 and 2.17.

Figure 2.16. Destruction of total saxitoxins in semipurified form, expressed as saxitoxin toxic equivalents with pH. (Note that the data point at pH 4 represents total STX equivalent concentration before chlorination and the value of 4 was arbitrarily chosen to display trends)



Stage 2 - Degradation of saxitoxins by chlorination at elevated pHs
Continued

Figure 2.17. Destruction of total saxitoxins as cellular material, expressed as saxitoxin toxic equivalents with pH (Note that the data pointat pH 4 represents total STX equivalent concentration before chlorination and the value of 4 was arbitrarily chosen to display trends)

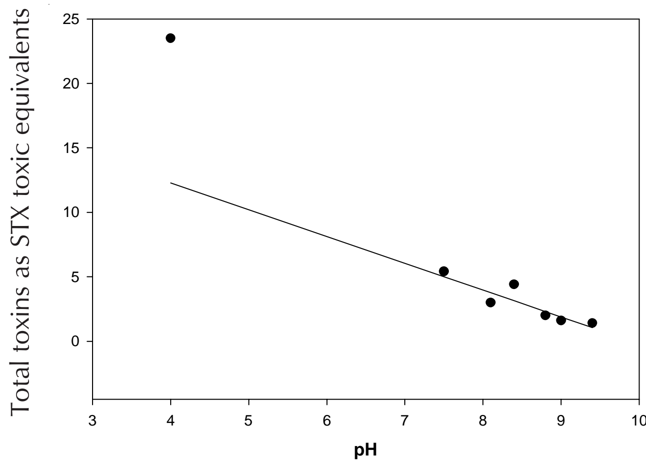
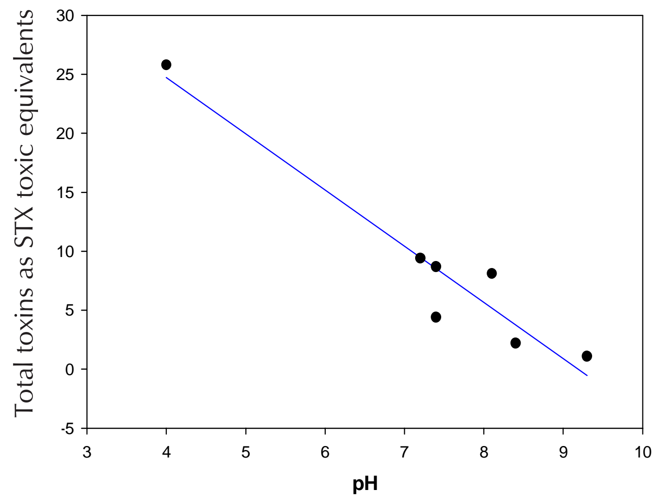


Figure 2.18. Destruction of total saxitoxins in semipurified material in Hope Valley water dosed at 17mg/L chlorine, expressed as saxitoxin toxic equivalents with pH (Note that the data point at pH 4 represents total STX equivalent concentration before chlorination and the value of 4 was arbitrarily chosen to display trends)



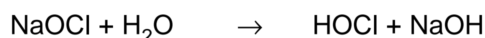
The data from AWQC in stage 2 (for chlorine dose 17mg/L, producing residuals 0.2 to 0.5mg/L) has been evaluated in terms of total saxitoxin toxic equivalents and are presented in Figure 2.18.

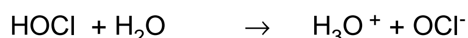
Stage 2 - Degradation of saxitoxins by chlorination at elevated pHs
Continued

2.4 Discussion

The behaviour of toxins in the cell-free extract and in the presence of cellular material was similar to that of the semi-purified material, ie, removal increased as the pH was increased. Removals at a particular pH were similar to, or slightly lower than in the case of cell free extracts, those obtained with semi-purified material although this may not be readily apparent from the graphs due to the different scales used. GTX5 (B1) and dcSTX were also included in this Stage 2 study and their removal was found to be equal to or better than that of STX.

The chlorine source used in the experiments with cell-free and cellular material was sodium hypochlorite which in water dissociates according to:


$$NaOCl + H_2O \rightarrow HOCl + NaOH$$


$$HOCl + H_2O \rightarrow H_3O^+ + OCl^-$$

$$NaOH \rightarrow Na^+ + OH^-$$

Originating from a strong base (NaOH) and a weak acid (HOCl), the degree of dissociation of NaOCl is pH dependent. At pH 7.5, hypochlorous acid and hypochlorite ion are in equal proportion. At the solution pHs used in this study, the proportion of hypochlorous acid and hypochlorite ion are as follows:

pH 7.0	75 : 25
pH 7.5	50 : 50
pH 9.0	3 : 97

It was clear from the results that the greatest degradation of saxitoxins occurred at high pH. Since hypochlorous acid is known to be a stronger oxidant than the hypochlorite ion, the pH effect appears to be more important for the degree of protonation of the saxitoxins and its analogues. The general saxitoxin structure has two guanidinium nitrogens of different pKa values. There are also distinct charge differences between the three classes of saxitoxin (ie. STX, GTX and C toxins) in the samples studied which may also influence the degree of protonation at different pH levels which in turn may effect the oxidation process. Essentially at higher pH, the degree of protonation of the guanidinium ions is reduced therefore making the toxins more susceptible to attack at these sites. Saxitoxin itself has two free guanidinium ions whereas other classes of saxitoxins have one or both of these ions sulphated which may confer a degree of protection against oxidative attack.

The results demonstrate that chlorine degrades saxitoxins at high pH according to a second order rate process. This equates to more rapid destruction initially at higher toxin concentrations with the majority of toxin (and saxitoxin toxic equivalents) being removed before 5 minutes after dosing. In addition, it can be observed when evaluating the results of stage 2 from both NRCET and AWQC that removal efficiency of total toxins (expressed as saxitoxin equivalents) follows a linear relationship with pH with highest efficiency being demonstrated at pH 9 or above.

The results of the mouse bioassay experiments are shown in Appendix 4. The C toxins only were monitored by HPLC as an indicator of removal of toxins in general. This and previous work has shown that the C toxins are removed at rates similar to the other toxins (GTX 2 and 3, and saxitoxin). In the positive controls, the C toxin concentrations remained constant while in the samples treated with chlorine, complete removal was observed indicating complete removal of all toxins. Toxin removal as determined by HPLC was accompanied by removal of acute neurotoxicity. Therefore the saxitoxins are not converted to highly acutely toxic products. It would appear, then, from these results that the products of chlorination are relatively benign.

2.5 Summary and Conclusions for Stage Two

Saxitoxins in water can be removed by chlorination but the effectiveness is very pH dependent. Removal as a function of pH is not linear with the degree of removal starting to increase rapidly at around pH 7.5. Removal appears not to be greatly affected by chlorine dose in that doses leading to a higher residual after 30 minutes do not remove substantially more toxins. Removal as a function of pH was not greatly affected by whether the toxins were present in a semi-purified form, as cellular extracts or present with cellular material provided that sufficient chlorine was used to achieve a residual of 0.5 mg/L after 30 minutes contact time. Cellular extracts did appear to give consistently slightly lower removals however. GTX5 (B1) and dcSTX were also included in this Stage 2 study and their removal was found to be equal to or better than that of STX. The overall results from the range of experiments carried out indicate that, to ensure a high level (> 90%) of removal of all saxitoxins produced by Australian strains of *Anabaena circinalis*, ie, the non-hydroxylated toxins, a pH of 9 is required under conditions where a residual of around 0.5 mg/L free chlorine is present after 30 minutes contact time.

The order of ease of removal of the saxitoxins was GTX5 (B1) ~ dcSTX > STX > GTX3 ~ C2 ~ C1 > GTX2 at the higher pH levels.

Because a second order rate process was shown to control the degradation of saxitoxins, this means that the majority of the degradation occurs in the first few minutes after the start of chlorine dosing and that after an hour only a small extent of additional degradation would be expected to occur.

Removal of toxins is accompanied by the disappearance of acute neurotoxicity suggesting that the products of chlorination are relatively benign.

In the experiments with semi-purified material, the high concentration of the extracts resulted in a high demand and correspondingly high chlorine doses. In a natural bloom the chlorine demand would be expected to be lower and the required residual achieved with chlorine doses less than those utilised here and probably similar to normal operations.

2.6 Recommendations for Stage Two.

To ensure a high level (> 90%) of removal of all saxitoxins produced by Australian strains of *Anabaena circinalis*, ie, the non-hydroxylated toxins, a pH of 9 is required under conditions where a residual of around 0.5 mg/L free chlorine is present after 30 minutes contact time. Water filtration plants will therefore need to operate under this relatively severe pH regime to ensure removal of saxitoxins during blooms of *A. circinalis*. If less removal is required, then operation at lower pH levels may be possible. This will depend on initial toxin concentrations and the final concentrations required. Because of the kinetics of degradation of saxitoxins with chlorine, any adjustment of pH to higher levels would not need to be for extended time periods.

Chapter 3. Stage 3 Investigations

Kinetics of Chlorination of Saxitoxins at Chlorine Doses Relevant to Source Waters

3.1 Introduction

It was proposed to evaluate the kinetics of degradation of saxitoxins that occur in *Anabaena circinalis* (C-toxins, gonyautoxins and saxitoxins) with DOC and chlorine dose levels that were more relevant to natural source waters than those used in stages 1 and 2 of this research. The research was conducted on natural reservoir water that has been spiked with purified saxitoxins instead of the use of semipurified toxin material that imparted a high chlorine demand to the test waters. This ensured that the DOC levels of the spiked water were less than 5mg/L, similar to most reservoir waters. The initial chlorine dose should be less than 5mg/L. A number of time points between zero and 60 minutes were used to produce the kinetic data and all studies were performed in duplicate. Results on the kinetics of degradation with chlorine were produced for the groups of saxitoxins (C-toxins, GTXs and STXs) individually as purified toxins and for a mixture of these toxins. All chlorinations were performed at pH 7.5 and pH 9.0. Results are also be interpreted and reported in terms of saxitoxin toxic equivalents to give a better indication of removal of toxicity from the treated waters.

3.2 Materials and Methods

3.2.1 Toxin Purification

The toxins used in this phase of the degradation studies (C1, C2, GTX2, GTX3, STX) were isolated and purified from freeze-dried cellular material of *Anabaena circinalis* sourced from Coolmunda Dam as used in the previous phases of this investigation. Toxins were purified initially on a BioGel P2 column using 100 mM acetic acid as the eluent. The column was run at a flow rate of 1 ml/min and fractions (5 ml) were collected. The individual toxin fractions were identified by mass spectrometry. Similar fractions were pooled, lyophilized and dissolved in milliQ water. These were then separated on a cationic exchange resin Bio-Rex 70 using an acetic acid gradient (0 – 2.5 M). The column was run at a flow of 2.0 ml/min. Toxin fractions were identified by HPLC-mass spectrometry and quantified by prechromatographic oxidation/HPLC. These (single component fractions of purity > 90%) were used in chlorination experiments individually or as mixtures of known composition. Toxin standards for use in quantitative estimations were purchased from the National Research Council in Canada as certified reference standards with the exception of the C toxins, which were not commercially available. Purified C toxins produced in this work were used as primary standards.

3.2.2 Processing of Water

The water used in the experiments was collected from North Pine Dam, which is source water for Brisbane's northern regions water supply. The pH of the water was 7.7 and had a chlorine demand of 2.1 mg/L (over 30 minute period). The water used in the study was allowed to stand overnight for particulate matter to settle, decanted and stirred in an open beaker (5L) for 2 hours, then filtered using glass fibre filters (0.7 micron). In a blank study, the chlorine concentration (2.0 mg/L) did not change significantly over a 30 minute period.

Stage 3 - Kinetics of Chlorination of Saxitoxins at Chlorine Doses Relevant to Source Waters
Continued

3.2.3 Chlorination

Chlorination was undertaken on source water spiked with purified saxitoxins both individually and in mixtures. The chlorination was performed using chlorine water. This was prepared by passing chlorine gas through (for 5 minutes) MilliQ water (250 ml) contained in a 500 ml Schott bottle kept in ice. The resultant straw coloured solution was kept refrigerated at $4^{0}C$ and the chlorine content was determined using HACH powder pillows by serial dilution just prior to use.

To a 300 ml glass beaker containing 250 ml of water and a magnetic stir bar, a known amount of toxin (approximately 5 –8 µg was added, to give a final toxin content of 20 – 32 µg/L) which would be representative of the maximum level of saxitoxins likely to be encountered in source waters featuring low chlorine demand when a bloom of *Anabaena circinalis* had occurred. The pH was adjusted using 100 mM NaOH solution. A subsample (1 ml) was taken at this point. A known amount of Cl_2 water was then added to the solution to give a final $[Cl_2]$ of 2.0 mg/L. The time measurement was started at this point. At predetermined time intervals, a 10.0 ml sample was withdrawn and added to a 50 ml tube containing 10 µL of $Na_2S_2O_3$ (1.0 M) to quench the excess Cl_2. During the experiment, the pH was maintained by the addition of a small amount of 50 mM NaOH. The samples removed at predetermined time intervals were carefully lyophilized, final volume adjusted to 1.0 ml with milliQ water and toxin concentration was determined by precolumn oxidation and HPLC-MS/MS. The Cl_2 concentration was also measured at predetermined time intervals by withdrawing 10.0 ml of reaction medium and diluting to 25 ml. The Cl_2 measurement was done using HACH powder pillows. Throughout the experiment the solution was stirred by means of a magnetic stirrer.

The results tabulated are the average of duplicate experiments.

3.3 Results

Table 3.1 gives results for residual free chlorine levels in source water containing the various purified saxitoxins at 30mins and 60mins after dosing with chlorine at 2mg/L.

Table 3.1. Chlorine Levels after 30 and 60 minutes

Toxin	pH	Initial Chlorine Dose	Chlorine level after 30 min	Chlorine level after 60 min
GTX 2,3	7.5	2.0	0.8	0.6
GTX 2,3	9.0	2.0	0.9	0.7
C1,2	7.5	2.0	1.2	1.0
C1,2	9.0	2.0	1.2	0.9
STX	7.5	2.0	1.5	1.5
STX	9.0	2.1	1.8	1.7
Toxin Mixture	7.5	2.0	0.9	0.7
Toxin Mixture	9.0	2.0	0.9	0.6

The kinetic degradation data for the C toxins, GTXs and saxitoxin are presented in Table 3.2. This table gives concentration/time data and percent decomposition at times to 60mins. As with previous phases of the study, it can be observed that increased degradation occurs with elevated pH. Additionally in this phase of the study featuring lower chlorine demand, it was observed that the efficiency of degradation of C1 and C2 toxins is higher than previously determined in test waters with higher levels of DOC.

Stage 3 - Kinetics of Chlorination of Saxitoxins at Chlorine Doses Relevant to Source Waters *Continued*

Table 3.2. Individual Toxin Degradation Data

Time min	[C 1,2] µg/L pH 7.5	% decom	[GTX2,3] µg/L pH 7.5	% decom	[STX] µg/L pH 7.5	% decom	[C 1,2] µg/L pH 9.0	% decom	[GTX2,3] µg/L pH 9.0	% decom	[STX] µg/L pH 9.0	% decom
0.0	27.1	0	20.0	0	35.6	0	28.7	0	20.4	0	19.8	0
0.5	19.8	27	15.8	21	-	-	25.1	13	16.7	18	1.8	91
1.0	15.2	44	-	-	11.6	67	20.0	30	13.7	33	1.7	91
2.0	13.0	52	14.6	27	27.5	23	15.2	47	11.8	42	-	-
5.0	13.2	**51%**	15.2	**24%**	17.4	**51%**	12.8	**55%**	9.5	**53%**	1.2	**94%**
10.0	9.9	63	18.5	7	17.0	52	14.2	50.1	7.2	65	1.1	94
15.0	8.7	68	15.3	23	17.8	50	11.4	60	6.5	68	0.9	95
20.0	10.1	63	17.1	14	15.0	58	9.0	69	5.8	72	1.1	94
30.0	8.9	**67%**	15.4	**23%**	13.0	**63%**	7.3	**75%**	4.6	**77%**	1.1	**94%**
45.0	7.3	73	15.6	22	8.9	75	5.3	82	3.7	82	0.7	96
60.0	6.9	**75%**	14.0	**30%**	7.9	**78%**	2.0	**93%**	4.0	**80%**	0.5	**98%**

Degradation data for the mixed toxins at both pHs is presented in Table 3.3. In this set of experiments it was shown that degradation efficiency of the GTXs was improved compared with chlorination of these toxins individually. The reason for this difference is not obvious unless there is some degree of catalytic activity from the presence of the other toxins or their possible degradation products.

Table 3.3 Degradation Data for the Mixed Toxins

Time min	[C 1,2] µg/L pH 7.5	% decom	[GTX2,3] µg/L pH 7.5	% decom	[STX] µg/L pH 7.5	% decom	[C 1,2] µg/L pH 9.0	% decom	[GTX2,3] µg/L pH 9.0	% decom	[STX] µg/L pH 9.0	% decom
0.0	37.45	0	18.95	0	19.70	0	32.00	0	20.40	0	18.20	0
0.5	29.15	22	16.35	14	-	-	31.40	2	9.30	54	1.40	92
1.0	26.10	30	14.30	25	2.00	90	30.10	6	8.60	58	0.80	96
2.0	21.05	44	12.65	33	2.00	90	24.55	23	6.70	67	0.90	95
5.0	17.15	**54%**	11.30	**40%**	2.70	**86%**	17.95	**44%**	3.15	**85%**	0.50	**97%**
10.0	15.75	58	9.30	51	2.30	88	14.85	54	2.20	89	0.30	98
15.0	17.10	54	8.75	54	2.00	90	11.80	63	2.65	87	0.60	97
20.0	12.65	66	7.05	63	0.80	96	8.10	75	1.40	93	0.30	98
30.0	11.80	**68%**	5.10	**73%**	0.60	**97%**	9.30	**71%**	1.05	**95%**	0.20	**99%**
45.0	8.90	76	5.90	69	-	-	3.70	88	0.92	95	-	-
60.0	7.60	**80%**	4.85	**74%**	0.60	**97%**	2.00	**94%**	0.94	**95%**	0.26	**99%**

The degradation of toxins is represented graphically in Figures 3.1 to 3.8

Stage 3 - Kinetics of Chlorination of Saxitoxins at Chlorine Doses Relevant to Source Waters *Continued*

Figure 3.1. Decomposition of C1,2 toxins with time at pH 7.5 and residual chlorine levels over time.

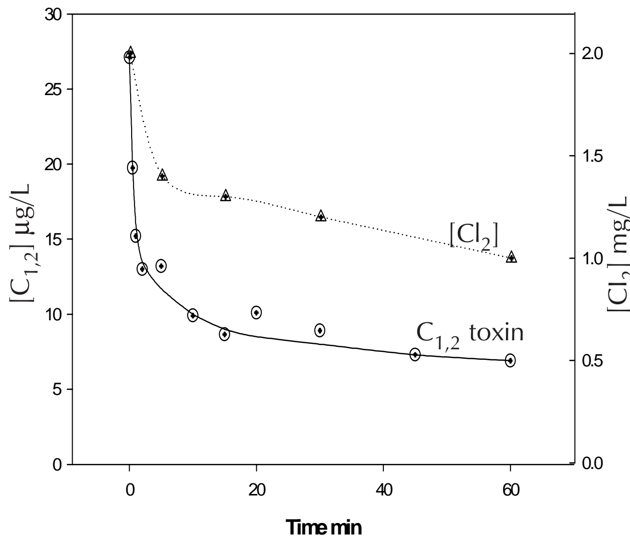


Figure 3.3. Degradation of STX toxin with time at pH 7.5 and free chlorine levels

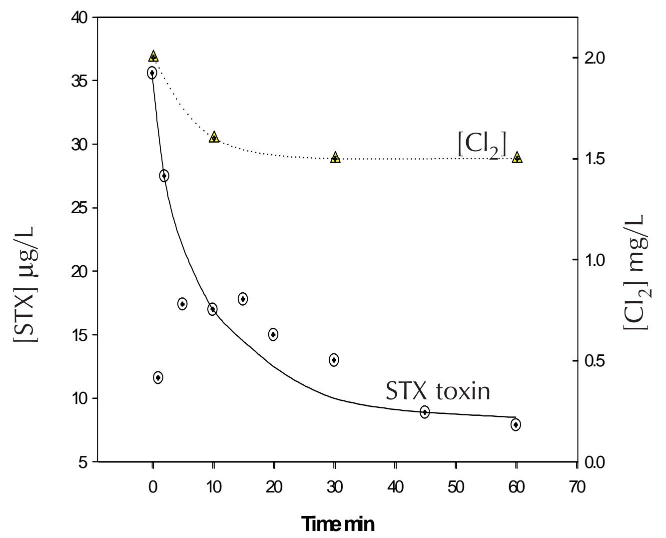


Figure 3.2. Degradation of GTX2,3 toxins with time at pH 7.5 and free chlorine levels

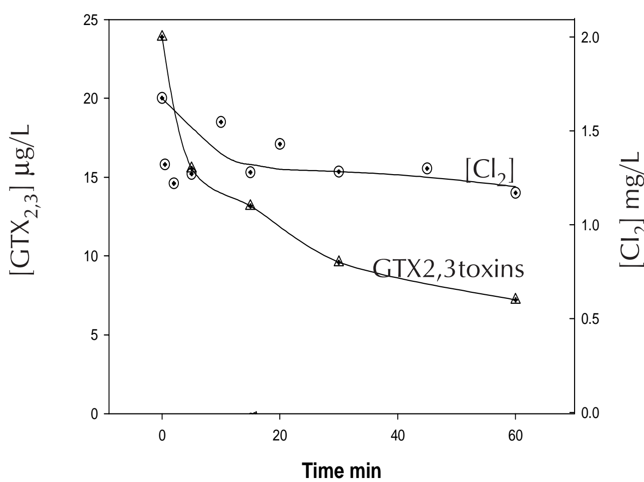
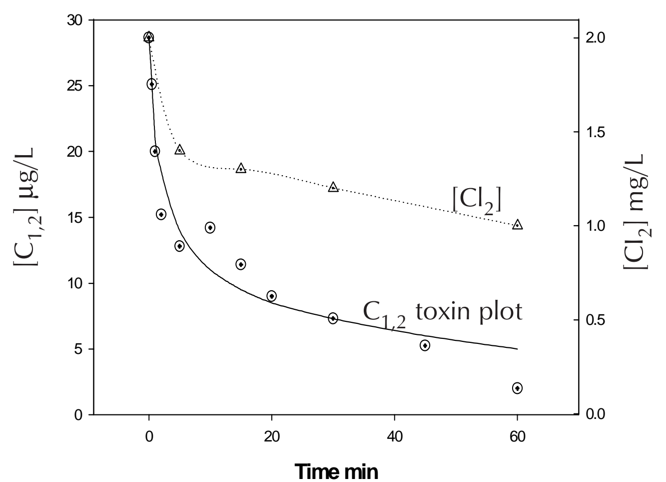


Figure 3.4. Degradation of C1,2 toxins over time at pH 9.0 and free chlorine levels



Stage 3 - Kinetics of Chlorination of Saxitoxins at Chlorine Doses Relevant to Source Waters *Continued*

Figure 3.5. Degradation of GTX2,3 toxins over time at pH 9.0 and free chlorine levels

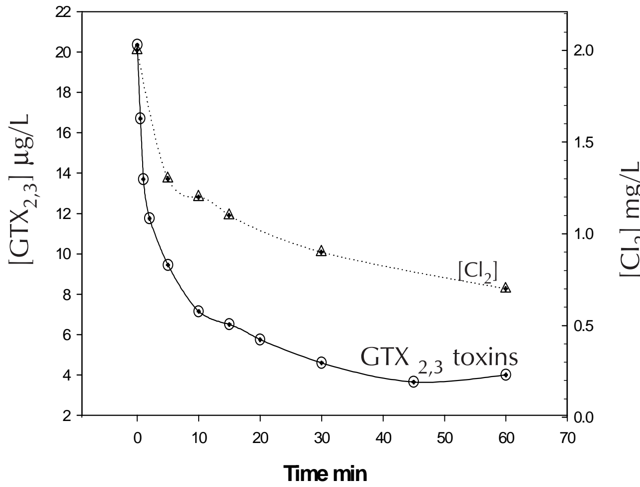


Figure 3.6. Degradation of STX over time at pH 9.0 and free chlorine levels

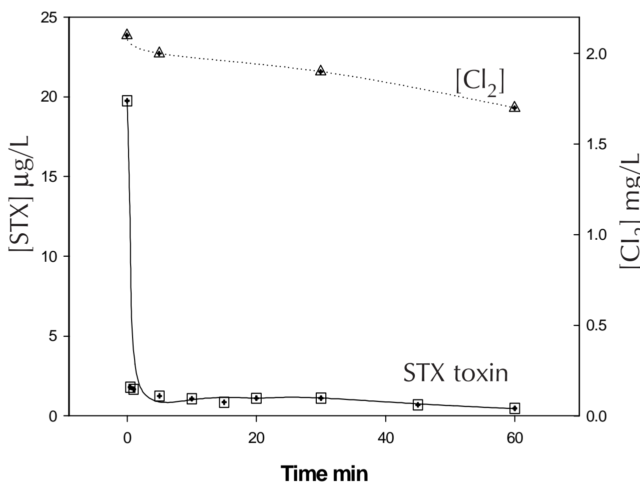


Figure 3.7. Degradation of the mixture of saxitoxins over time at pH 7.5 and free chlorine levels

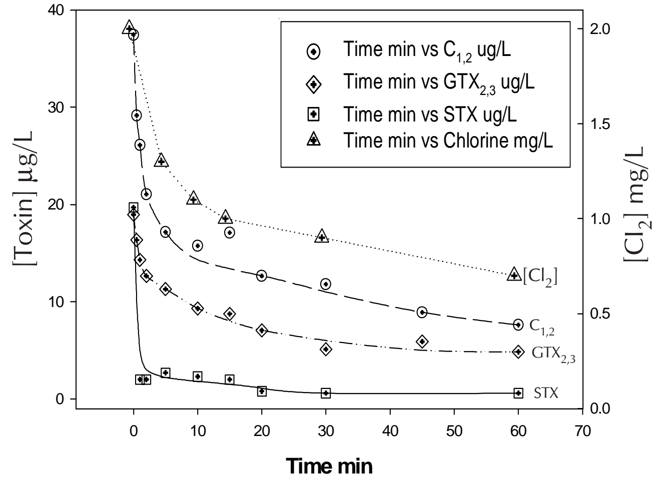
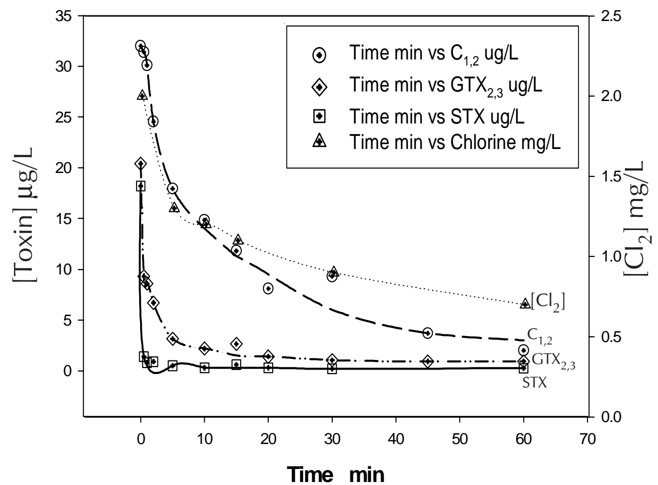


Figure 3.8. Degradation of the mixture of saxitoxins over time at pH 9.0 and free chlorine levels



Stage 3 - Kinetics of Chlorination of Saxitoxins at Chlorine Doses Relevant to Source Waters *Continued*

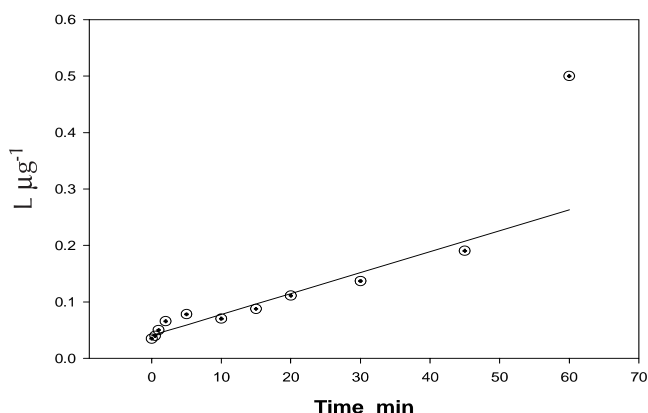
3.4 Discussion

When the organic background is low, as in the case of some source waters and pre-treated and filtered water (but not disinfected) a 2.0 mg/L chlorine dose was sufficient to remove the majority of saxitoxins toxins at pH 9.0. With an individual toxin concentration set to about 30 µg/L as in the case of a severe toxic A*nabena circinalis* bloom, at pH 9.0, about 50% of $C_{1,2}$ and $GTX_{2,3}$ toxins will degrade within 5 minutes with a chlorine dose of 2 mg/L. During the same time period over 90% of STX has decomposed. After 30 minutes over 75% $C_{1,2}$ and $GTX_{2,3}$ toxins have decomposed, whereas STX decomposition was already complete.

When a mixture of toxins were chlorinated (Table 3.3) at pH 9.0, over 97% STX and over 85% GTX2,3 toxins decomposed within the first 5 minutes whereas only 44% C1,2 toxins decomposed. After 30 minutes, the decomposition had increased to over 95% for STX and GTX2,3 and over 70% for C1,2 toxins.

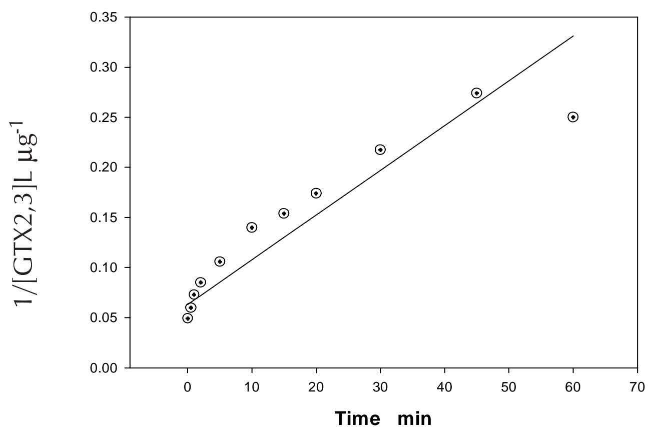
Chlorination of an organic compound is a complicated process, and it is possible that several parallel reactions taking place at the same time. This will complicate the kinetic studies due to several different reactions happening simultaneously, but at different rates. However, at pH 9.0 and above, the bulk of the chlorination reaction with most of the saxitoxins studied follows second order kinetics when excess chlorine is present. When *Time Vs Toxin concentrations* were plotted, (Figures 3.1- 3.8) the line joining the data points in most cases represents the general trend of decomposition. These plots were further analysed to obtain the order of reaction. The plots of *Time Vs Reciprocal of saxitoxin concentration*, in an ideal second order situation represent a straight line. In most cases at high pH, the bulk of the reaction (> 70%) can be explained this way by a second order process. The straight lines shown (Figures 3.9 – 3.11) for individual toxin degradation represent the lines of best fit for the calculated second order reactions.

Figure 3.9 Second order rate plot for decomposition of C1,2 toxins.



Note: The point representing 60 minutes is an outlier and was disregarded in calculating the line of best fit for the second order rate process.

Figure 3.10. Second order rate plot for decomposition of GTX 2,3 toxins



Stage 3 - Kinetics of Chlorination of Saxitoxins at Chlorine Doses Relevant to Source Waters *Continued*

Figure 3.11. Second order rate plot for decomposition of STX toxin

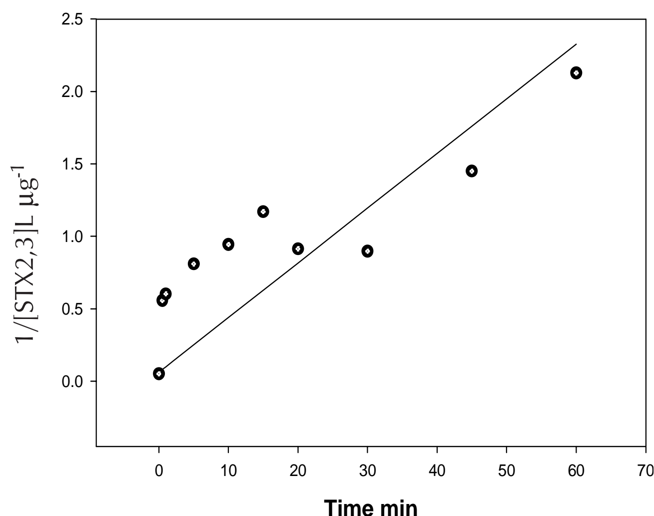


Table 3.4. Removal of toxicity at pH 7.5 for mixed toxins

Time (mins)	Toxicity as Saxitoxin toxic equivalents (concentration x relative toxicity)			
	STX	C1, C2	GTX2, GTX3	Total Toxicity
0	5.5	7.0	7.4	19.9
0.5	-	5.2	7.0	12.8
1	0.6	3.9	5.3	9.8
2	0.7	3.4	4.9	9.0
5	1.0	3.4	4.5	9.9
10	0.7	2.6	3.7	7.0
15	0.7	2.3	3.3	6.3
20	0.5	2.6	2.9	6.0
30	0.5	2.3	2.9	5.7
60	0.3	1.8	2.9	5.0

Note1. Toxicity expressed as concentration x toxicity relative to saxitoxin

Note 2. Kinetic data from stage 3 was applied to the toxin composition used in the stage 2 study.

3.4.1 Expression of phase 3 kinetic study results as saxitoxin toxic equivalents.

As with the results of previous phases of this study it is important to express the results as saxitoxin toxic equivalents in order to better determine the removal of toxicity due to saxitoxins from drinking waters. As previously, this was achieved by multiplying the concentrations of individual saxitoxins by their toxicity relative to saxitoxin (STX). In the stage 3 study the concentrations of STX, C1,2 and GTX2,3 spiked into the water were of the order of 20 to 30µg/L. This concentration range was chosen so that it was possible to accurately measure the degradation of the toxins. This ratio of toxins however, is not representative of that normally produced by *Anabaena circinalis* where the C toxins dominate. To represent removal of toxicity from water impacted by a large toxic bloom, the toxin composition used in the stage 2 investigation was used for simulation purposes. The kinetic data for toxin degradation in stage 3 was applied to the following concentrations of toxins: C1, 210µg/L; C2, 80µg/L; GTX2, 30µg/L; GTX3, 10µg/L; STX 10µg/L. Using the kinetics determined in stage 3, the removal of toxicity with time at pH 7.5 and 9.0 is presented in Tables 3.4 and 3.5.

Table 3.5. Removal of toxicity at pH 9.0 for mixed toxins.

TIME (mins)	Toxicity as Saxitoxin toxic equivalents (concentration x relative toxicity)			
	STX	C1, C2	GTX2, GTX3	Total Toxicity
0	5.5	7	7.4	19.9
0.5	0.6	6.2	6.0	12.8
1	0.6	5.0	4.9	10.5
2	0.6	3.7	4.3	8.6
5	0.6	3.2	3.4	7.2
10	0.3	3.5	2.5	6.3
15	0.4	3.0	2.3	5.7
20	0.4	2.0	2.2	4.6
30	0.4	1.8	1.8	4.0
45	-	1.2	1.4	2.9
60	0.2	0.5	1.8	2.5

Note1. Toxicity expressed as concentration x toxicity relative to saxitoxin

Note 2. Kinetic data from stage 3 was applied to the toxin composition used in the stage 2 study.

Considering removal of toxicity with time, Table 3.6 shows that approximately 50% of the toxicity is removed after one minute, approximately 70% after 10 mins and approximately 80% after 30mins at pH 9.0.

Stage 3 - Kinetics of Chlorination of Saxitoxins at Chlorine Doses Relevant to Source Waters
Continued

Table 3.6. Efficiency of removal of toxicity due to mixed saxitoxins with respect to time.

Time (mins)	Percent removal of total toxicity	
	PH 7.5	PH 9.0
0	0	0
0.5	36	36
1	51	47
2	55	57
5	50	64
10	65	68
15	68	71
20	70	77
30	71	80
60	75	87

The results for removal of saxitoxin toxicity by chlorination at both pH 9.0 and 7.5 using chlorine doses of 2mg/L are graphically represented in Figures 3.12 and 3.13.

Figure 3.12. Graphical representation of the removal of saxitoxin toxicity from water by chlorination at a dose of 2mg/L and at a pH of 9.0.

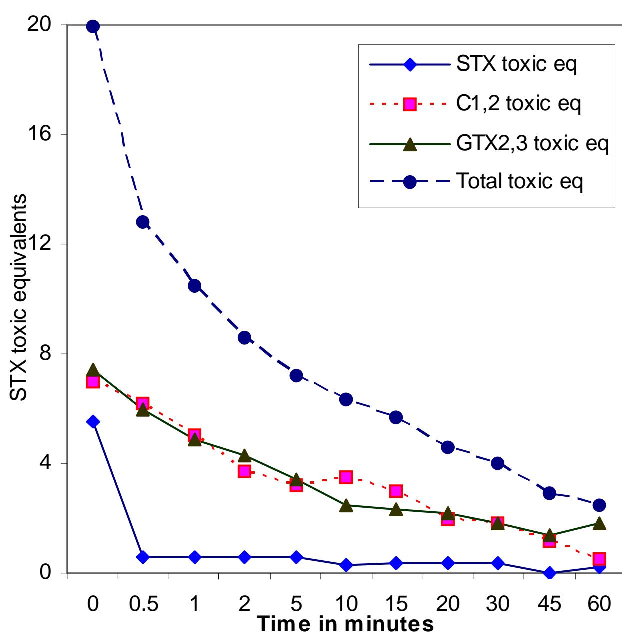
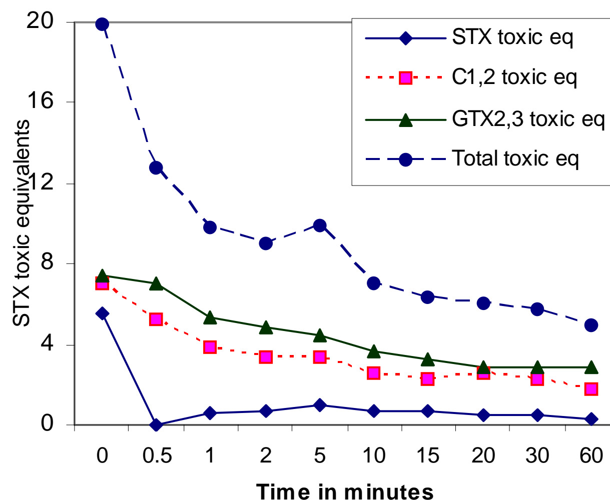


Figure 3.13. Graphical representation of the removal of saxitoxin toxicity from water by chlorination at a dose of 2mg/L and at a pH of 7.5.



3.5 Summary and Conclusions for Stage Three

Chlorination of saxitoxins present in low DOC water using chlorine doses around 2mg/L are as effective as using high initial doses of chlorine in high DOC water. This demonstrates that a high initial dose of chlorine is not necessary for degradation of saxitoxins and is not related to the efficiency of degradation. Additionally it is apparent that chlorine does not selectively oxidize saxitoxins at high chlorine doses, but as expected, oxidizes saxitoxins plus organic matter according to its ease of oxidation. Providing that there is residual free chlorine, oxidation of saxitoxins will occur and efficiency is controlled by solution pH.

Second order degradation rate processes have been shown to operate for all toxins investigated. Second order degradation processes imply that degradation depends on concentration with greatest degradation occurring in the period immediately following chlorine dosing.

Higher degradation efficiency of saxitoxins was demonstrated in stage 3 where test waters had lower DOC and correspondingly chlorine doses than those used in stages 1 and 2. It is however recommended that caution be applied in interpretation of results, as use of other source waters may not achieve the degradation efficiencies demonstrated here. It would be prudent to assume that efficiencies of degradation of saxitoxins in waters by chlorination would lie within the range of results demonstrated in stages 1, 2 and 3 of the research.

Acute toxicity of saxitoxins was shown to be removed by treatment with chlorine, although it is unknown if any byproducts are formed that may exert chronic toxicity endpoints.

Chapter 4. Overall conclusions and recommendations

We have demonstrated that under appropriate conditions saxitoxins can be removed by chlorination although the efficiency is dependent on pH and the structures of the individual toxins.

It has been shown in this research that as long as there is a free chlorine residual of around 0.5mg/L, degradation will be promoted and that increasing the free chlorine residual does not appear to affect toxin removal. Increased efficiency of removal occurs at elevated pH with a pH of 9.0 being the highest tested and the most effective. Removal of toxins at acid pHs was not particularly effective and significant removal was only demonstrated at pH 7.5 and above.

In all cases removal was most effective for saxitoxin which is also the most toxic analogue. Saxitoxin also carries a double charge (on the guanidinium ions of the molecule) while the gonyautoxins (GTXs) are singly charged and the carbamate toxins (C toxins) are uncharged at neutral pH.

The guanidinum ions appear to be the site of chlorine attack and at lower pH these sites are protonated and therefore more protected from oxidative attack by chlorine. Conversely at high pH the guanidinium ions are not protonated and are therefore available for oxidation by chlorine. The degree of degradation was mainly influenced by the pH and the saxitoxin structures.

The source of chlorine (gaseous or hypochlorite) was shown not to influence the degree of degradation.

The kinetic studies of degradation of saxitoxins with chlorine revealed that the degradation of saxitoxins at pH of 7.5 or greater follows a second order rate reaction. This relates to more rapid saxitoxin degradation in the first 5 or 10 minutes after dosing with slower removal of the remaining saxitoxins after this time.

The research has demonstrated that the form of the toxins, whether in semipurified form, cellular extracts or cellular material did not affect the degree of degradation as long as a free chlorine residual (of 0.5mg/L) was present after 30 minutes of oxidation. Additional saxitoxins, GTX5 (B1) and dcSTX were evaluated for degradation and their removal was found to be similar to that of STX. The semipurified toxins, cellular extracts and cyanobacterial cellular material all required high initial chlorine doses and the chlorine demand would normally be lower than that used here.

To account for any potential differences in toxin removal efficiency caused by the high initial chlorine dose, further experiments were run using purified toxins spiked in filtered raw water and treated with chlorine at an initial chlorine dose of 2mg/L. The results demonstrated that degradation of toxins in low chlorine demand water was equal to, if not better than the degree of removal obtained when using high initial chlorine doses (up to 20mg/L).

Chlorination has been demonstrated to useful in the removal of saxitoxins from water under optimum conditions, and this has been confirmed by mouse bioassay which confirmed removal of acute toxicity. It is not known however, whether chlorination results in the formation of byproducts with chronic toxicity. Studies of the kinetics of degradation of saxitoxins using initial chlorine doses of 2mg/L has revealed that a second order rate process is still operating, implying that the majority of removal occurs in the first 5 to 10 minutes after chlorination is initiated.

The research results have been expressed in terms of saxitoxin toxic equivalents, a term which is more relevant to producing safe drinking water. This enables a prediction of the "toxicity" of treated water which is a simpler and more health relevant estimation of saxitoxins remaining than estimating concentrations of individual saxitoxins which vary considerably in terms of their toxicity. Given that most blooms of *Anabaena circinalis* would result in much lower concentrations than those removed in these studies, it should be possible to remove these toxins by chlorination at pH 9.0 for 30 minutes to levels below the proposed NHMRC health alert level of 2µg/L saxitoxin toxic equivalents.

Chapter 5. Acknowledgements

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Appendix I

Quenching of Residual Chlorine Prior to Toxin Analysis

It has been shown that chemicals used to quench residual chlorine prior to analysis can have detrimental effects on the analyte of interest. A common quenching agent, sodium sulphite, has been shown to destroy some disinfection by-products resulting from the reaction of chlorine with naturally occurring organic matter (Croue and Reckhow, 1989; Bauman and Stenstrom, 1989). Microcystins can also be destroyed by some of these chemicals if used in high concentrations to quench residual oxidants (Rositano and Nicholson, unpublished results). Consequently the effect of various quenching agents on the various saxitoxins was investigated.

The concentrated semi-purified extract was spiked into high purity water and to aliquots of this solution, the quenching agents sodium sulphite, sodium thiosulphate, ascorbic acid and ammonium chloride added to produce concentrations of 100 mg/L. None of the quenching agents appeared to degrade the saxitoxins. On the basis of these results, sodium thiosulphate was chosen as the quenching agent to be used in further experiments at AWQC. It was also tested at a higher concentration (0.28 g/L) on toxins spiked into Hope Valley water. The toxin extract was spiked into the water (25 mL) to give concentrations identical to those in the dosing experiments. Sodium thiosulphate (40 μL) was added as described in the "Experimental" section. This higher concentration also had no effect on the saxitoxins. Samples at NRCET were quenched using either sodium sulphite or sodium thiosulphate.

Appendix 2

Buffer Capacity Experiments

In order to determine the effect of pH on the capacity of chlorine to remove saxitoxins, it was necessary to develop buffered systems which were capable on maintaining pH during both spiking with the semi-purified toxin (contains low levels of acetic acid), and dosing with chlorine, either chlorine water or sodium hypochlorite. Initial attempts with buffers such as Tris (tris(hydroxymethyl) ammonium chloride) indicated that this compound had a significant chlorine demand itself. The use of phosphate buffers was then investigated, at 0.01 and 0.1M concentrations. pH 5 and 9 are at the outer limits of traditional phosphate buffers so it was considered that dosing at these pH values might result in an unacceptable pH shift.

0.1M phosphate buffered waters were prepared from disodium hydrogen phosphate (7.99 g/L) and potassium dihydrogen phosphate (5.95 g/L) with sodium hydroxide or phosphoric acid solutions added to bring the pH to the desired levels. 0.01M buffered waters were prepared using one-tenth of these concentrations. The results are shown in the following table.

Buffer capacity results with differing ionic strength phosphate buffers at different pH values

Initial pH	pH after spiking (500 μL extract)	pH after dosing	pH 30 min after dosing
0.01M phosphate buffer, chlorine dose 26 mg/L (chlorine water)			
7.0	7.0	6.9	6.8
9.1	8.6	8.0	7.9
0.1M phosphate buffer, chlorine dose 33 mg/L (chlorine water)			
5.0	5.0	4.9	5.0*
7.0	7.0	7.0	7.0*
9.0	8.9	8.7	8.7*

* average of 3 replicates

The results indicate the 0.01M phosphate buffer maintains the pH reasonably well at pH 7 when chlorine water is dosed at 26 mg/L, which is slightly less than the dosed required in the toxin degradation experiments. However, it does not have the capacity to maintain the pH at 9. With 0.1M phosphate buffer, the pH was maintained at pH 5 and 7 when chlorine was dosed at 33 mg/L, the dose required to produce a residual of 0.5 mg/L after 30 min contact time. At pH 9, there was a slight reduction in pH which was still considered acceptable.

Appendix 3

Effect of Toxin Spiking and Chlorine Dosing on the pH of Reservoir Water

Initial pH	pH after spiking (500 mL extract)	pH after dosing	pH 30 min after dosing
Hope Valley Reservoir, SA			
Toxin spike 500μL, chlorine dose 38.7 mg/L (gaseous)			
5.0*	4.9	3.7	3.6
7.0*	6.9	6.3	6.3
9.0*	8.2	6.7	6.8
8.2 (natural pH)	7.8	6.8	
Toxin spike 250 μL, chlorine dose 17 mg/L (gaseous)			
8.2 (natural pH)	7.8	6.8	
Toxin spike 250 μL, chlorine dose 17 mg/L (hypochlorite)			
8.2 (natural pH)	7.8	8.8	

* pH adjusted with acid or base; starting pH 8.1. All pH values are average of 3 replicates.

Initial pH	pH after spiking (500 mL extract)	pH after dosing	pH 30 min after dosing
North Pine Dam, Queensland			
Toxin spike 250 μL, chlorine dose 16.5 - 19 mg/L (gaseous)			
5.0*	5.1	5.2	
7.0*	7.1	7.2	
9.0*	8.4	8.7	
7.9 (natural pH)	8.1	7.8	
Toxin spike 250 μL, chlorine dose 14 - 15 mg/L (hypochlorite)			
5.0*	5.1	5.7	
7.0*	7.2	7.5	
9.0*	8.2	8.9	

* pH adjusted with acid or base. Average of 3 replicates.

Appendix 4

Removal of Saxitoxins in Buffered High Purity Water Following Chlorination, Contact Time 30 min - NRCET Results

pH	Chlorine dose (mg/L)	Free chlorine residual (mg/L)	Toxin Removal (%)		
			C Toxins	GTXs	Saxitoxin
Sodium hypochlorite					
5	16	0.6	17	17	80
	19	2.2	18	11	79
7	15	0.3	34	26	94
	19	2.2	39	30	95
9	14	0.5	82	77	96
	18	2.5	85	86	97
Gaseous chlorine					
5	16	0.4	20	13	76
	20	2.2	29	20	78
7	17	0.3	25	22	94
	21	1.2	37	28	96
9	16	0.2	77	64	95
	20	1.3	79	74	95

Appendix 5

Removal of Saxitoxins in Raw Water Following Chlorination, Contact Time 30 min - NRCET Results

pH	Chlorine dose (mg/L)	Free chlorine residual (mg/L)	Toxin Removal (%)		
			C Toxins	GTXs	Saxitoxin
Sodium hypochlorite					
5	16	0.6	17	17	80
	19	2.2	18	11	79
7	15	0.3	34	26	94
	19	2.2	39	30	95
9	14	0.5	82	77	96
	18	2.5	85	86	97
Gaseous chlorine					
5	16	0.4	20	13	76
	20	2.2	29	20	78
7	17	0.3	25	22	94
	21	1.2	37	28	96
9	16	0.2	77	64	95
	20	1.3	79	74	95

Appendix 6

Saxitoxins Removal Following Chlorination in Hope Valley Reservoir Water. Contact Time 30 min - Toxin Spike = 250 µL. AWQC Results

	pH after chlorina tion	Free chlorine residual mg/L	Toxin				
			C1	**C2**	**GTX2**	**GTX3**	**Saxitoxin**
Gaseous chlorine: Intitial pH natural (8.2), chlorine dose 17.0 mg/L							
Control conc (µg/L)			144 (18)	71 (4)	26.7 (2.5)	11.5 (1.5)	9.9 (0.1)
Conc after 30 min (µg/L)	6.8	0.51	101 (9)	35 (4)	21.3 (0.7)	5.2 (0.4)	2.7 (0.6)
Removal (µg/L))			43 (27)	36 (8)	5.4 (3.2)	6.3 (1.9)	7.2 (0.7)
Removal (%)			30	51	20	55	73
Gaseous chlorine: Initial pH natural (8.2) ** 500 µL spike, chlorine dose 32.0 mg/L**							
Control conc (µg/L)			482 (18)	175 (3)	56.4 (2.7)	20.4 (0.4)	26.8 (1.5)
Conc after 30 min (µg/L)	6.8	0.40	337 (44)	109 (12)	49.6 (3.2)	13.8 (0.9)	14.1 (1.7)
Removal (µg/L)			145 (62)	66 (15)	6.8 (5.9)	6.6 (1.3)	12.7 (3.2)
Removal (%)			30	38	12	32	47
Sodium hypochlorite: Initial pH natural (8.2), chlorine dose 15.0 mg/L							
Control conc (µg/L)			138 (26)	66 (0)	26.9 (4.1)	11.0 (0.1)	9.3 (0.9)
Conc after 30 min (µg/L)	8.8	0.63	<20	<10	1.4 (0.4)	<2	<2
Removal (µg/L)			>118	>56	25.5 (4.5)	>9.0	>7.3
Removal (%)			>86	>85	95	>82	>78
Gaseous chlorine: Initial pH 5.4, chlorine dose 16.0 mg/L							
Control conc (µg/L)			175 (22)	62 (8)	24.3 (0.3)	8.1 (0.3)	11.1 (0.4)
Conc after 30 min (µg/L)	3.9	0.22	139 (13)	45 (5)	19.3 (2.1)	5.5 (0.5)	6.3 (0.2)
Removal (µg/L)			36 (35)	17 (13)	5.0 (2.4)	2.6 (0.8)	4.8 (0.6)
Removal (%)			21	27	21	32	43
Gaseous chlorine: Initial pH 7.1, chlorine dose 16.5 mg/L							
Control conc (µg/L)			154 (24)	56 (10)	26.8 (0.9)	10.5 (0.6)	10.3 (0.1)
Conc after 30 min (µg/L)	6.6	0.41	108 (13)	31 (4)	22.6 (2.5)	5.1 (0.5)	3.5 (0.8)
Removal (µg/L)			46 (37)	25 (15)	4.2 (3.4)	5.4 (1.1)	6.8 (0.9)
Removal (%)			30	45	16	51	66
Gaseous chlorine: Initial pH 9.0, chlorine dose 18.5 mg/L							
Control conc (µg/L)			113 (6)	64 (3)	32.2 (0.3)	12.5 (0.3)	8.0 (0.4)
Conc after 30 min (µg/L)	6.4	0.56	71 (2)	28 (3)	21.9 (0.7)	4.9 (0.4)	<2
Removal (µg/L)			42 (8)	36 (6)	10.3 (1.0)	7.6 (0.7)	>6.0
Removal (%)			37	56	32	61	>75
Sodium hypochlorite: Initial pH 5.0, chlorine dose 16.5 mg/L							
Control conc (µg/L)			142 (13)	51 (5)	35.1 (0.3)	10.8 (0.3)	8.7 (0.1)
Conc after 30 min (µg/L)	5.6	0.44	99 (16)	29 (4)	27.5 (1.7)	7.5).5)	3.9 (0.4)
Removal (µg/L)			43 (29)	22 (9)	7.6 (2.0)	3.3 (0.8)	4.8 (0.5)
			30	43	22	31	55

Average of 2 replicates (controls) or 3 replicates (conc after 30 minutes). Number in brackets is standard deviation.

Appendix 7

Removal of Saxitoxins With Time in Buffer (pH 7) and Raw Water Following Chlorination - NRCET Results

Time (min)	Free chlorine residual (mg/L)	Toxin Removal (%)		
		C Toxins	GTXs	Saxitoxin
Buffer pH 7 - chlorine (gaseous) dose 15 mg/L				
5	0.9	14	10	84
10	0.6	19	12	90
15	0.5	23	15	83
20	0.4	27	16	94
30	0.2	29	17	95
40	0.2	32	19	96
50	0.1	34	19	96
60	0.1	38	21	96
120	0.1	42	24	97
24 hr	-	49	25	97
Raw water pH 7.5 - chlorine (gaseous) dose 19 mg/L				
5	1.0	3	1	35
10	0.7	5	3	37
15	0.5	7	3	40
20	0.4	9	4	41
30	0.3	11	6	41
40	0.3	12	7	42
50	0.2	14	9	42
60	0.2	16	10	43
120	0.2	31	21	44
19 hr	-	41	25	65

Appendix 8

Saxitoxins Removal Following Chlorination in Hope Valley Reservoir Water. AWQC Results (chlorine dose 17mg/L)

	pH after chlorination	Free chlorine residual mg/L	Toxin				
			C1	C2	GTX2	GTX3	Saxitoxin
Initial pH 9.5							
Control conc (μg/L)	7.2	0.48	204	67	19.2	9.1	10.1
Conc after 30 min (μg/L)			94	36	13.3	3.5	1.7
Removal (μg/L))			110	31	5.9	5.6	8.4
Removal (%)			54	46	31	62	83
Initial pH 9.0							
Control conc (μg/L)	7.4	0.26	199	74	22.7	8.5	10.7
Conc after 30 min (μg/L)			88	33	13.1	3.7	1.3
Removal (μg/L)			111	41	9.6	4.8	9.4
Removal (%)			56	55	42	56	88
Initial pH 9.5							
Control conc (μg/L)	7.4	0.26	196	66	18.6	9.9	9.8
Conc after 30 min (μg/L)			68	19	9.3	1.9	<0.5
Removal (μg/L)			128	47	9.3	8.0	>9.3
Removal (%)			65	71	50	81	>95
Initial pH 9.6							
Control conc (μg/L)	8.1	0.40	204	72	21.7	11.3	9.8
Conc after 30 min (μg/L)			80	33	12.7	3.2	1.1
Removal (μg/L)			124	39	9.0	8.1	8.7
Removal (%)			61	54	41	72	89
Initial pH 9.7							
Control conc (μg/L)	8.4	0.21	217	74	20.7	11.8	10.4
Conc after 30 min (μg/L)			36	5	5.4	1.5	<0.5
Removal (μg/L)			181	69	15.3	10.3	>9.9
Removal (%)			83	93	74	87	>95
Initial pH 10.1							
Control conc (μg/L)	9.3	0.52	187	67	15.3	12.1	9.2
Conc after 30 min (μg/L)			20	2	<0.5	1.2	<0.5
Removal (μg/L)			167	65	>14.8	10.9	>8.7
Removal (%)			89	97	>97	90	>95

All concentrations average of duplicate experiments

Contact Time = 30 min. Toxin Spike = 250 μL.
Chlorine Dose 17 mg/L.

Appendix 9

Saxitoxins Removal Following Chlorination in Hope Valley Reservoir Water. AWQC Results (chlorine dose 20mg/L)

	pH after chlorination	Free chlorine residual (mg/L)	Toxin				
			C1	C2	GTX2	GTX3	STX
Initial pH 9.5							
Control conc (µg/L)			204	67	19.2	9.1	10.1
Conc after 30 min (µg/L)	7.2	1.7	84	37	11.7	3.7	1.0
Removal (µg/L)			120	30	7.5	5.4	9.1
Removal (%)			59	45	39	59	90
Initial pH 9.6							
Control conc (µg/L)			196	66	18.6	9.9	9.8
Conc after 30 min (µg/L)	7.2	1.8	60	21	7.4	2.2	<0.5
Removal (µg/L)			136	45	11.2	7.7	>9.3
Removal (%)			69	68	60	78	>95
Initial pH 9.0							
Control conc (µg/L)			199	74	22.7	8.5	10.7
Conc after 30 min (µg/L)	7.4	1.3	90	34	12.0	3.7	0.5
Removal (µg/L)			109	40	10.7	4.8	10.2
Removal (%)			55	54	47	56	95
Initial pH 9.6							
Control conc (µg/L)			204	72	21.7	11.3	9.8
Conc after 30 min (µg/L)	7.9	1.2	87	34	12.9	3.4	0.7
Removal (µg/L)			117	38	8.8	7.9	9.1
Removal (%)			57	53	41	70	93
Initial pH 9.7							
Control conc (µg/L)			217	74	20.7	11.8	10.4
Conc after 30 min (µg/L)	8.3	1.4	19	3	<0.5	0.7	<0.5
Removal (µg/L)			198	71	>20.2	11.1	>9.9
Removal (%)			91	96	>98	94	>95
Initial pH 10.1							
Control conc (µg/L)			187	67	15.3	12.1	9.2
Conc after 30 min (µg/L)	9.2	2.2	13	1	<0.5	0.7	<0.5
Removal (µg/L)			174	66	>14.8	11.4	>8.7
Removal (%)			93	99	>97	94	>95

All concentrations average of duplicate experiments

Contact Time = 30 min. Toxin Spike = 250 µL.
Chlorine Dose 20 mg/L.

Appendix 10

Results of Chlorination of Saxitoxins in Cell-free Extract and Cellular Suspensions of *A. circinalis.* (NRCET Results)

Chlorination of saxitoxins in cell-free extract from *A. circinalis.* Note that the pH presented in the table refers to the pH after the addition of NaOCl.

Sample	C1, C2 µg/L (%removed)	dcSTX µg/L (%removed)	GTX2, 3 µg/L (%removed)	GTX5 (B1) µg/L (%removed)	STX µg/L (%removed)	Initial Cl_2 Dose mg/L	Residual Cl_2 Level mg/L
Initial Toxin Concentration	226	0.55	26.0	17.2	5.5		
pH 7.6 After Chlorination	121 (46)	0.03 (95)	11.4 (58)	1.2 (93)	1.2 (78)	7.20	0.28
pH 8.2 After Chlorination	85 (62)	0.04 (93)	8.4 (69)	0.9 (95)	0.8 (85)	7.20	0.29
pH 8.6 After Chlorination	58 (74)	0.04 (93)	6.1 (77)	0.7 (96)	0.6 (89)	7.20	0.31
pH 9.0 After Chlorination	47 (79)	0.03 (93)	4.6 (85)	0.6 (96)	0.3 (95)	7.20	0.35
pH 9.3 After Chlorination	39 (83)	0.01 (98)	3.1 (88)	0.4 (98)	0.2 (96)	7.20	0.85
pH 9.6 After Chlorination	29 (87)	0.00 (100)	1.7 (92)	0.4 (98)	0.1 (98)	7.20	0.58

Appendix 10
Continued

Chlorination of saxitoxins in cellular suspension of *A. circinalis*. Note that the pH presented in the table refers to the pH after the addition of NaOCl

Sample	C1, C2 µg/L (%removed)	dcSTX µg/L (%removed)	GTX2, 3 µg/L (%removed)	GTX5 (B1) µg/L (%removed)	STX µg/L (%removed)	Initial Cl_2 Dose mg/L	Residual Cl_2 Level mg/L
Initial Toxin Concentration	265	0.63	29	20	5.7		
pH 7.5 After Chlorination	113 (57)	0.01 (98)	6.6 (77)	0.6 (97)	0.07 (99)	20.00	0.50
pH 8.1 After Chlorination	63 (76)	0.01 (98)	3.3 (89)	0.4 (98)	0.18 (97)	20.00	0.60
pH 8.4 After Chlorination	60 (77)	0.02 (97)	3.8 (87)	0.4 (98)	0.33 (94)	20.00	0.50
pH 8.8 After Chlorination	37 (86)	0.03 (95)	2.9 (90)	0.5 (97)	0.07 (99)	20.00	0.56
pH 9.0 After Chlorination	27 (90)	0.03 (95)	2.2 (92)	0.3 (99)	0.25 (96)	20.00	0.79
pH 9.4 After Chlorination	39 (85)	0.00 (100)	0.5 (98)	0.3 (99)	0.03 (100)	20.00	1.11

Appendix 11

Mouse Bioassay Results

Sample	Result
Negative control[2]	-, -
Positive control with thiosulphate[3]	+, +
Positive control without thiosulphate[4]	+, +
Treated sample[5]	-, -

- = non toxic; + = toxic

1. An aliquot (40 mL) of all samples concentrated to 2.5 mL by rotary evaporation prior to toxicity testing. All mice approximately 20 g in weight with 1.0 mL injected intraperitoneally. Mice which died did so within 8 – 16 minutes with evidence of a neurotoxic effect. All initial sample volumes 125 mL.
2. Water sample dosed to give 20 mg/L chlorine and quenched after 30 min with sodium thiosulphate (200 µL)
3. Water sample spiked with toxin (2.5 mL) followed by sodium thiosulphate addition (200 µL) after 30 min
4. Water sample spiked with toxin (2.5 mL) only
5. Water sample spiked with toxin (2.5 mL), dosed with chlorine to give a concentration of 20 mg/L followed by quenching with sodium thiosulphate (120 µL) after 30 min. pH after 30 min contact = 8.8; chlorine residual = 2.8 mg/L.

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