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ARTICLE *in* ENVIRONMENTAL SCIENCE AND TECHNOLOGY · MAY 2001

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Environ. Sci. Technol. **2001**, *35,* 1445−1451

Radioreceptor Assays for Sensitive Detection and Quantitation of Saxitoxin and Its Analogues from Strains of the Freshwater Cyanobacterium, *Anabaena circinalis*

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Toxic freshwater cyanobacteria can contaminate water supplies and adversely effect humans, agricultural livestock, and wildlife. Toxicity is strain-specific so morphological observations alone cannot predict the hazard level. Two microtiter plate based bioassays have emerged for measuring saxitoxin (STX) and its derivatives, commonly found in the freshwater cyanobacteria *Anabaena* and *Aphanizomenon*. They use radioactively labeled STX binding by sodium channels, STX's pharmacological target, or an unrelated protein, saxiphilin. These bioassays were challenged with extracts of toxic and nontoxic strains of *Anabaena circinalis,* and the results were compared with HPLC analysis. Both radioreceptor assays had detection limits of 2 μg STX equivalents (STXeq)/L, which is below the concentration proposed for a health alert, namely 3 μg STXeq/L. In all cases, statistically significant correlations existed between all toxicity measurements of the same extracts with the methods used herein. Sodium channel and saxiphilin assays however predicted less toxicity relative to HPLC analysis. The only exception to this was the equivalency observed between saxiphilin measurement and HPLC quantitation corrected for mammalian toxicity. Saxiphilin assay predicted toxicity in one strain was 3 orders of magnitude more than by sodium channel assay, and no STX was detected by HPLC. Lack of acetylcholinesterase inhibition showed this bioactivity was not anatoxin-a(S), a toxin also produced by this *A. circinalis* with some resemblance to the region of STX bound by saxiphilin. Presence of anatoxin-a(S) was predicted for another strain by this same acetylcholinesterase assay that, if confirmed by chemical analysis, would be the first report of anatoxin-a(S) in an Australian cyanobacterium.

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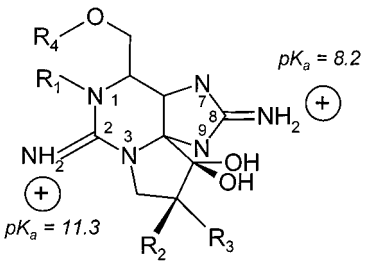
Introduction

Water is one of our most critical resources, necessary not just for drinking but for agriculture. Its contamination therefore can affect not just humans but agricultural livestock as well as wildlife that drinks the water or lives in this environment. As the world's population grows, there is mounting pressure on water supplies and the quality thereof. A water quality management issue that has emerged in recent times are blooms of toxic freshwater algae that may be consumed inadvertently or may toxify the water supplies directly (*1*). These algae are generally cyanobacteria that may produce hepatotoxins such as microcystin and nodularin as well as the neurotoxic anatoxins and saxitoxins. Consequently, the World Health Organization has incorporated tolerable levels for several of these toxins in their drinking water guidelines. At present, such universal limits are not agreed upon for the saxitoxins, but 3 μg/L of saxitoxin equivalents has been proposed as the freshwater concentration at which a health alert must be instituted (*2*). Australia is a continent where this problem is magnified because of the paucity of rainfall making water conservation and management a critical national issue. It is not a problem restricted to Australia however with saxitoxin-producing cyanobacterial blooms being reported in Portugal (*3*), Brazil (*4*), Italy (*5*), and the U.S.A. (*6*).

Saxitoxin (Figure 1; STX) blocks passage of sodium across the excitable cell membrane of nerves and muscle by occlusion of the voltage gated sodium (Na) channel (*7, 8*). This prevents the conduction of an action potential, paralyzing the intoxicated victims, sometimes to the point of death. In the freshwater environment, it can be synthesized by *Anabaena circinalis* (*9*), *Aphanizomenon flos-aquae* (*10*), *Cylindrospermopsis raciborski* (*4*), *Lyngbya wollei* (*11*), and *Planktothrix* sp. FP1 (*12*). In fact, one of the world's largest cyanobacterial blooms involved *Anabaena circinalis* along 1000 km of one of Australia's major river systems reaching cell concentrations of almost 1,000,000 cells/mL (*13*). Mouse bioassay of extracts from this chain of blooms indicated the presence of a neurotoxic factor, probably STX or a chemical relative (*14*). Receptor bioassays are the new tools for detection and quantitation of STX and its naturally occurring analogues, referred to here as the saxitoxins (STXs). One such assay uses the pharmacological receptor for STX, the voltage gated sodium channel, which has been shown to closely mimic overall mammalian toxicity when used to test molluscan shellfish contaminated by STX from marine microalgae (*15*). A second binding assay utilizes a new receptor for saxitoxin called saxiphilin for which a physiological function has yet to be elucidated (*16, 17*). Saxiphilin is a highly specific receptor for STXs, unrelated to the sodium channel but is instead a member of the transferrin family of proteins, better known for their function of iron transport. Unlike the sodium channel used for STX assays, there is no mammalian isoform of saxiphilin.

More than one STX can occur in toxic algae and bioassay data is the result of the cumulative effect of the different members of the family of toxins, all with different potencies. Chemical confirmation of individual STXs can be achieved by comparison with standards after high performance liquid chromatography (HPLC) separation (*18*). This generates toxin quantities in absolute terms, which can then be converted to mammalian toxicity based upon conversion factors for each toxin, but only for those for which this value has been, or can be, determined. A drawback of this technique then is that it is only useful for toxicity measurement where toxin standards exist, and new and biologically active STXs will

10.1021/es001575z CCC: $20.00
Published on Web 03/03/2001



	R_1	R_2	R_3	R_4
STX	H	H	H	$CONH_2$
dcSTX	H	H	H	H
C1	H	H	OSO_3^-	$CONHSO_3^-$
C2	H	OSO_3^-	H	$CONHSO_3^-$
GTX2	H	H	OSO_3^-	$CONH_2$
GTX3	H	OSO_3^-	H	$CONH_2$
GTX5	H	H	H	$CONHSO_3^-$
dcGTX2	H	H	OSO_3^-	H
dcGTX3	H	H	OSO_3^-	H

FIGURE 1. Structures of STXs in extracts of *Anabaena circinalis* in this study.

not be easily identified and quantitated. Biological assays therefore remain the premier method for detection of bioactive STXs. To manage these toxic freshwater algae and their toxins, techniques must be entrained which enable sensitive detection and quantitation. Herein, we compare the Na channel and saxiphilin radioreceptor assays with HPLC for their ability to detect and quantify STXs from the cyanobacterium *Anabaena circinalis*, a major freshwater management problem in Australia and a model for similar nuisance water-borne microorganisms in other countries.

Materials and Methods

1. Reagents. All buffers and general chemicals were from Sigma (St. Louis, MO), and water was deionized (≈18 MΩ) with a Barnstead system (Barnstead, IA). Saxiphilin from the centipede *Ethmostigmus rubripes* was prepared as reported previously (*17*) and stored at −80 °C until use. Likewise, rat brain synaptosomes (*19*) were stored at −80 °C until use. Tritiated saxitoxin ([^{3}H]STX) was purchased from Amersham Pharmacia Biotech (UK). Saxitoxin (STX; Calbiochem, San Diego, CA) was diluted in 1 mM citrate buffer (pH 5.0). Toxin standards for HPLC analysis of STXs were kindly donated by Prof. Y. Oshima and Dr. H. Onodera of Tohoku University, Japan.

2. *Anabaena circinalis* Culture and Extraction. Cultures were originally isolated from the Australian regions listed in Table 1 and grown in 50 mL of Jaworsky's medium (JM) (*20*). Cells were harvested during late logarithmic growth phase. Levels of contaminating heterotrophic bacteria from *A. circinalis* cultures were reduced by filtration through a 3.0 μm filter (Millipore, NSW, Australia) followed by washing with an equal volume of JM. Cells were resuspended in 1 mL of JM and transferred to a 1.5 mL tube. Samples were centrifuged at 12,000 rpm for 5 min, and the supernatant was discarded. Cell pellets were resuspended in 1 mL of Milli-Q water, to preserve the native toxin composition and mimic the medium in which these toxins would be released in nature, and sonicated for 2 min on ice using a Branson Sonifier model 450 (Branson Ultrasonics Corporation, Danbury, CT) set at 80% duty cycle with a power output of 160 W. A 15 μL sample was removed and checked on a slide under a microscope to assess for complete sonication. Prior to assay, cellular debris were removed from the extracts by centrifugation.

3. Radioreceptor Assays for Saxitoxins. The saxiphilin microtiter plate [^{3}H]STX binding assay was conducted as previously described (*17*, *21*). The assay requires presoaking of Multiscreen 96-well GF/B microtiter filtration plates (Millipore, Sydney, Australia) with 0.3% (w/v) polyethylimine for at least 1 h prior to the addition of reagents. All reactions were performed in a total volume of 150 μL containing 20 mM MOPS−NaOH (pH 7.4), 200 mM NaCl, 1 nM [^{3}H]STX, and 150 μg crude centipede saxiphilin, as measured by total protein. Experiments were allowed to equilibrate at room temperature (~25 °C) for 90 min prior to aspiration through the filters. Wells were rinsed three times with 180 μL of ice cold water. It should be noted that the tritium in [^{3}H]STX is derived from an exchange labeling procedure, and it may back-exchange with the water in assay buffers. It has been shown however that at the temperature at which the assay is conducted, only 20% of the tritium label is lost after 40 h (*22*), and it is therefore highly unlikely that this effect would have any impact upon this assay. Filters were punched from the filtration plate into a flexible 96-well scintillation counting plate (EG & G Wallac, Turku, Finland, cat. No. 1450-401) after which 200 μL of Optiphase Supermix scintillant (EG & G Wallac, Turku, Finland) was added to each filter, the plate was sealed and vortexed for 5−10 min, and the filters were allowed to equilibrate to the scintillant for several hours prior to counting in a Microbeta plate scintillation counter (EG & G Wallac, Turku, Finland). In each assay, control samples containing 5 μM STX were used to determine the level of background radioactivity. Values were then normalized to 100% using control samples containing no competitor.

Inhibition of [^{3}H]STX binding to rat brain sodium channels was measured using a modification of a reported microtiter plate method (*15*). Assay conditions were varied so that there was a total volume of 150 μL of 100 mM MOPS−NaOH (pH 7.4), 100 mM choline chloride, 1 nM [^{3}H]STX, and 26 μg protein of rat brain synaptosomes. Experiments were equilibrated at 4 °C for 60 min prior to aspiration through the filters and rinsing filters three times with 180 μL of an ice cold solution of 100 mM MOPS−NaOH (pH 7.4) with 100 mM choline chloride. Filters were removed from the Multiscreen GF/C 96-well microtiter filtration plates (Millipore, NSW, Australia) and counted in precisely the same manner described for the saxiphilin assay.

4. Primary Screening and Determination of STX Concentrations. Samples were screened for inhibition of [^{3}H]STX binding by incubating duplicate samples of 10 μL of each sample. For reasons elaborated below, after the primary screening of the extracts, those samples which inhibited [^{3}H]STX binding by greater than 80% were diluted to achieve an inhibitory value between 20 and 80%. The nature of a sigmoid curve means that subtle variation in inhibition outside this range translates into inordinately large uncertainty in derived STX equivalents (STXeq) concentrations. If dilution was necessary, extracts were retested in a series of 2-, 5-, 10-, 50-, 100-, 500-, and 1000-fold dilutions which invariably resulted in at least two different inhibitory values between 20 and 80% inhibition. To calibrate the radioreceptor assays for calculation of STXeq in test samples, a competition curve was conducted using unlabeled STX for each receptor. These competition curves were fit with the following equation: percent [^{3}H]STX bound = $100*([STX]^n/([STX]^n + IC_{50}^n))$, where IC_{50} is concentration of STX which causes 50% inhibition, and n is slope of the curve, using the curve-fitting routine of Sigmaplot (SPSS, Chicago, IL). For back calculation from an inhibition value to a concentration of STX, the above equation can be converted to the form $((100\text{-}F)/F)^{1/n}*IC_{50}$ where F = percent bound of [^{3}H]STX relative to controls in the presence of extracts, and n and IC_{50} were the Hill slope and concentration of STX which causes 50% inhibition in the control curves, respectively. From the percent inhibition values derived from titration of the samples as described above, a value for STXeq in nM is obtained which is not corrected for the different affinities of STX analogues for the

TABLE 1. *Anabaena circinalis* Strain Details and Cell Counts of Material Extracted for Analysis along with the Percentage Inhibition of Radioreceptor Assays in Primary Screen as Detailed within the Materials and Methods, Section 4

sample no.	strain designation	origin	no. of cells extracted ($\times 10^5$)	% inhibition	
				Saxiphilin	Na channel
1	AWQC[a] 118A	Booligal, Lachlan River, NSW	240	−10	6.4
2	AWQC 118C	Booligal, Lachlan River, NSW	150	96.7	102.9
3	AWQC 131C	Lake Cargelligo, NSW	340	97	97.9
4	AWQC 134C	Corowa, Murray River, NSW	160	95.8	98.0
5	AWQC 148C	Farm Dam, Fish Creek, VIC	160	97	64.1
6	AWQC 150A	Burrinjuck Dam, NSW	230	96.9	96.3
7	AWQC 173A	Bourke, Darling River, NSW	21	94.6	89.6
8	AWQC 279B	Chaffey Dam, NSW	36	93.9	90.7
9	AWQC 306A	Solomon Dam, Palm Island QLD	460	−4	5.9
10	AWQC 307C	Bundaberg, Burnett River, QLD	260	12.3	−4.9
11	AWQC 310F	Farm Dam, Millawa, VIC	380	16.7	−6.4
12	AWQC 323B	Clydesdale Lake, Perth WA	440	95.7	94.0
13	AWQC 332H	Pukallus Weir, Yarraman, QLD	290	−30.1	−7.7
14	AWT[b] 204A	Darling River, NSW.	640	95.6	97.7

[a] Australian Water Quality Centre Culture Collection. [b] Australian Water Technologies, North Ryde, NSW, Australia.

respective receptors. The final toxicity measurement is the average of the values derived from the multiple inhibitory values between 20 and 80% inhibition obtained in the titration of unknown samples described above.

5. HPLC Analysis for Saxitoxins. Extracts were acidified to 0.05 M acetic acid to provide a stable chemical environment for STXs and filtered through a 10,000 MW cutoff centrifugal filter (Millipore, NSW, Australia). HPLC analyses for the individual STXs were performed by toxin separation with a Waters 600 HPLC, combined with a Pickering PCX 5100 Postcolumn Reactor using 5 μm, 250 × 4.6 mm Alltima ODS column (Alltech, IL) at a flow rate of 0.8 mL/min (*18*, *23*). Fluorescent toxin derivatives were detected using a Linear LC305 spectrofluorometric detector with excitation at 330 nm and emission at 390 nm. HPLC toxin profiles were converted to absolute toxin values by multiplying the concentration of each toxin in an extract by its toxicity relative to STX (*18*) and summing to obtain the total extract toxicity in STXeq. Toxin standards were kindly donated by Professor Y. Oshima and Dr. H. Onodera of Tohoku University, Japan.

6. Confirmation of Mammalian Neurotoxicity. Extracts of several strains were injected intraperitoneally into each of 2 mice weighing 17−23 g. Neurotoxicity was defined as the death of one or both mice within 30 min by respiratory arrest as evidenced by convulsions.

7. Correlation Analyses between Toxicity Measurements. Spearman's correlation coefficients, and the statistical significance thereof, from correlation analyses between toxicity measurements yielded by the different methods used here were calculated with SPSS for Windows, Ver 9.0 (*24*).

8. Assay for Acetylcholinesterase Inhibition. A protocol for measuring acetylcholinesterase activity (*25*) was adapted to test the extracts for the presence of anatoxin-a(s). In short this involved incubation of duplicate 20 μL aliquots of *A. circinalis* extracts in the presence of 4 mU acetylcholinesterase from electric eel (Sigma cat. No. C2888) in the presence of 625 μM acteylthiocholine and 420 μM Ellman's reagent in 50 mM phosphate buffer (pH 7.5). Kinetic readings were taken at 15 s intervals at 405 nm on a Spectra spectrophotometric plate reader (EG & G Wallac, Turku, Finland) governed by the Biolise software for Windows (ver. 2.01). Inhibition was calculated as the change in initial reaction velocities of the enzyme in the presence and absence of the test solutions after subtraction of background absorbance. Screening for acetylcholinesterase inhibition was conducted twice with the same samples.

Results

Fourteen isolates from various regions of Australia (Table 1) were grown for extraction. A number of these are known STX producers (*9*). Each strain demonstrated different vigor in terms of growth with some generating up to 20 times more biomass than other strains during the same growth period. In the primary screen for bioactivity, 9 of the 14 extracts inhibited binding of [^{3}H]STX to both the Na channel and saxiphilin (Table 1). In all cases, except for sample 5, the presence of STXs was confirmed by HPLC analysis (Table 2). C1 and C2 dominated the toxin profiles with lesser amounts of GTX3, GTX2, dcGTX3, dcGTX2, dcGTX5, and STX also detected. The isolate from Clydesdale Lake contained an unusual toxin profile which was dominated by GTX5 and a smaller proportion of STX. No N_1-hydroxy analogues were detected. The lack of detected activity in samples 1, 9, 10, 11, and 13 was unrelated to the amount of biomass as several of these were the most dense and vigorous of the cultures (Table 1). Of those extracts that inhibited the radioreceptor assays in the primary screen, all inhibited the assays almost completely, necessitating dilution of extracts for quantitation using the calibration curves. Extracts of strains 2, 4, 5, 6, and 7 were tested by intraperitoneal injection into mice, with all of them eliciting lethal neurotoxicity.

The calibration curve for saxiphilin possessed an IC_{50} for unlabeled STX of 1.1 nM with a Hill slope of 1.1 (Figure 2A). For the Na channel, the calibration curve possessed an IC_{50} for unlabeled STX of 1.1 nM with a Hill slope of 1.0 (Figure 2B). From these values, the equation to derive STXeq from the extract inhibition values reduced to $((100\text{-}F)/F)^{0.9}$*1.1 nM for saxiphilin and ((100-F)/F)*1.1 nM for the Na channel. An example of the calculation using this formula is when an extract causes 50% inhibition. For saxiphilin, this results in an STXeq value of 1 nM for saxiphilin and 1.1 nM for the Na channel assay. For an extract that causes 75% inhibition, one obtains an STXeq value of 3.0 nM from the saxiphilin assay and 3.3 nM for the Na channel assay.

Figure 3 shows the correlation between the Na channel and saxiphilin assay quantitations for the extracts deemed to contain STXs from the primary screen. As alluded to above, there was little agreement between the two assays for one particular sample, namely strain 5, down to the detection limit of approximately 1−5 μg/L. Peaks in HPLC chromatograms were verified by comparison of retention times and fluorescence emission maxima with standards, the disappearance of peaks by eliminating postcolumn oxidation, and spiking experiments with standards. Several HPLC analyses are depicted in Figure 4. In fact, HPLC analysis could not

TABLE 2. Individual STXs Composition of Culture Extracts from HPLC Analysis[a]

sample	C1	C2	dcGTX3	GTX5	dcGTX2	GTX3	GTX2	dcSTX	STX	total (μg/L)	total (μg STXeq/L)
1	0	0	0	0	0	0	0	0	0	0	0
2	31	19	10	0	8	12	12	7	1	4.10	1.29
3	45	19	4	0	3	7	9	11	2	5.68	1.25
4	50	33	1	0	2	5	7	0.3	1	3.51	0.44
5	0	0	0	0	0	0	0	0	0	0	0
6	28	17	4	0	7	11	16	14	2	4.10	1.30
7	46	30	0.7	0	1	11	11	0	2	0.51	0.09
8	51	37	0.3	0	1	5	5	0	0.5	1.62	0.17
9	0	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0	0
11	0	0	0	0	0	0	0	0	0	0	0
12	0	0	0	93	0	0	0	0	8	1.21	0.16
13	0	0	0	0	0	0	0	0	0	0	0
14	49	19	4	0	10	5	10	2	0.4	7.45	1.49

[a] The values for individual toxin analogues are given as mol %. STXeq/L were calculated using conversion factors for each toxin derived from toxicology studies as described in Materials and Methods.

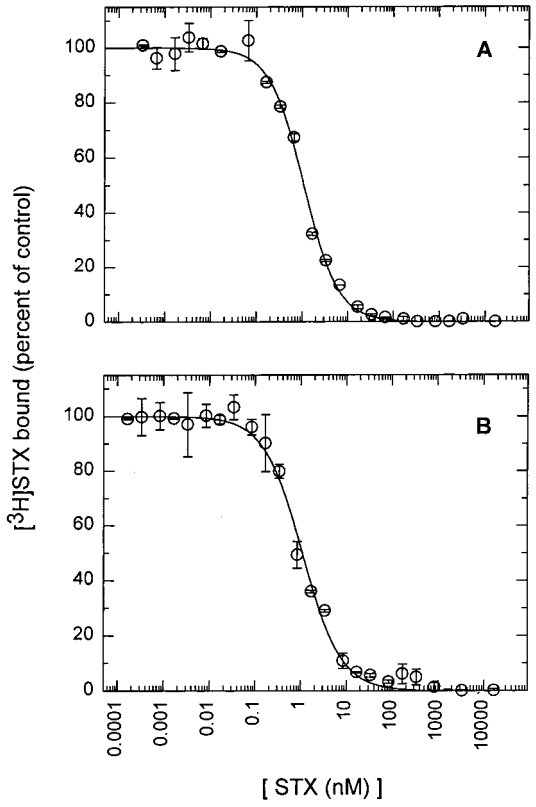


FIGURE 2. Calibration curves using unlabeled STX versus [^{3}H]STX binding by *E. rubripes* saxiphilin (A) and rat brain sodium channel (B). Data points are the mean and standard errors of duplicate measurements.

detect any STX-like peaks in strain 5 (Figure 4C). Its exclusion from the comparison between the two radioreceptor assays results in a correlation of $r = 0.83$ ($p < 0.05$). Despite these two receptors having different sensitivities for different members of the STX family (*7, 16, 17*), the relative consistency in the toxin composition of the extracts (Table 2) would explain the comparatively good correlation between the two methods (Figure 3). There was, however, a ratio of 1.9 between the two methods, as measured by the slope of the regression line, with saxiphilin regularly showing a greater response than the Na channel. Within assay variability, day-to-day reproducibility and reliability in the hands of different experimenters of these radioreceptor assays have previously been reported (*15, 21*).

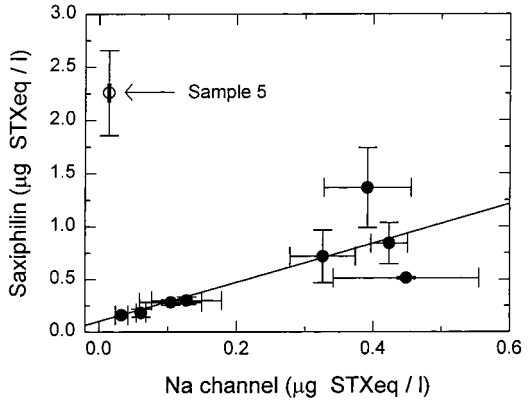


FIGURE 3. Correlation between cyanobacteria extract STXeq values determined with the rat brain sodium channel and saxiphilin radioreceptor assays ($r = 0.83$, $p < 0.05$). Sample 5, which exhibited unusual bioactivity results, is highlighted and excluded from the correlation analysis. The regression line is fit to the equation $y = 1.9x + 0.1$.

The correlation between the Na channel quantitation and HPLC analysis without considering mammalian toxicity (Figure 5A) was high ($r = 0.96$, $p < 0.01$). Again, excluding sample 5 from the comparison of saxiphilin and the same HPLC derived quantities of total STXs concentrations (Figure 5B), a correlation is obtained of $r = 0.95$ ($p < 0.01$), and the ratio between the two methods was 5.1 with HPLC again detecting a higher concentration of toxin. If one converts the total concentration of STXs as measured by HPLC to STXeq by taking into consideration mammalian toxicity (Figure 6), then the correlation with the Na channel assay is $r = 0.94$ ($p < 0.01$) (Figure 6A). The ratio between the two methods is reduced such that the bioassay predicted 4 times less toxicity. With the saxiphilin assay (Figure 4B), r remains 0.96 ($p < 0.01$) with HPLC measured toxicity, but with a ratio of 1.5 with HPLC once again detecting more toxicity (Figure 6B).

The unexplained bioactivity in strain 5 in the saxiphilin assay prompted an examination of other algal-derived agents that one may hypothesize could interfere with STX binding to saxiphilin. Anatoxin-a(s) is an organophosphate produced by some members of the *Anabaena* genus (*26, 27*) that bears a structural resemblance to the 7,8,9 guanidino group of STX essential for binding to saxiphilin (*28*). This toxin can be readily assayed for by measuring acetylcholinesterase inhibition activity. Using this method, no anatoxin-a(S) like activity was detected in strain 5, with only strain 3 significantly inhibiting acetylcholinesterase (Table 3).

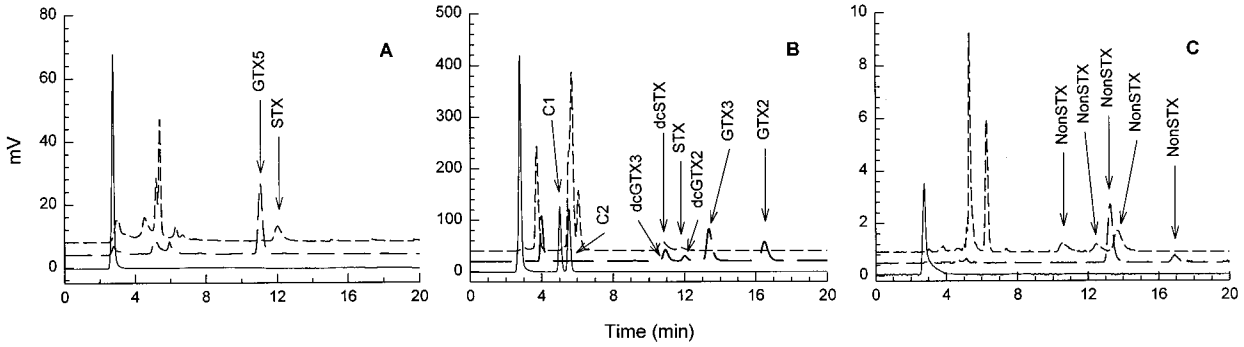


FIGURE 4. Several examples of HPLC analysis of STXs from (a) sample 12, (b) sample 3, and (c) sample 5. Three classes of PSTs are analyzed using three separate conditions (C-toxins, solid line; gonyautoxins, long dash; saxitoxins, short dash) as described in Materials and Methods. Peaks that correspond with standard STXs are labeled using abbreviations from Figure 1. Of interest is the lack of such peaks in sample 5, with those peaks observed being distinguished from STXs as described in the text. Note that HPLC traces for the different analyses in each figure have been offset to allow clear depiction.

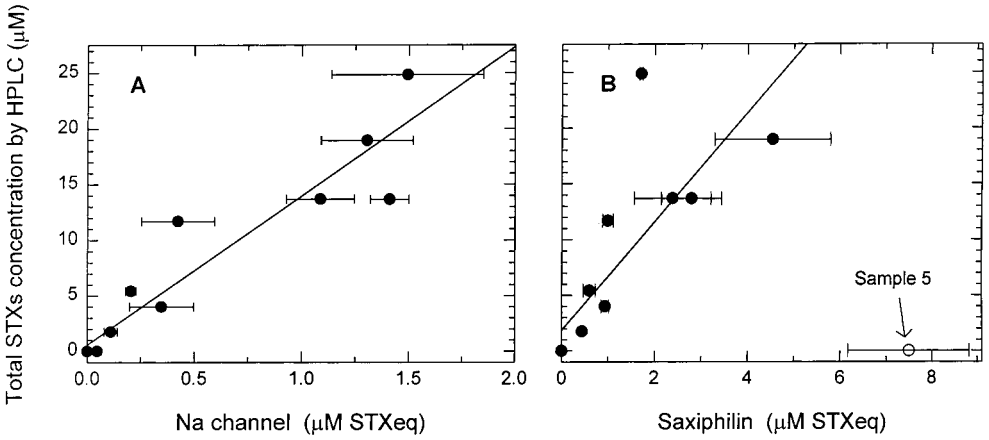


FIGURE 5. Correlation between the total concentration of STXs in cyanobacteria extracts determined by HPLC analysis and (a) rat brain Na channel ($r = 0.96$, $p < 0.01$) and (b) saxiphilin ($r = 0.95$, $p < 0.01$) radioreceptor assays. In (b), the value of sample 5, which exhibited unusual bioactivity results, is highlighted and is excluded from the correlation analysis. The regression line for (a) is fit to the equation $y = 13.4x + 0.6$, while for (b) the regression line is $y = 5.1x + 1.7$.

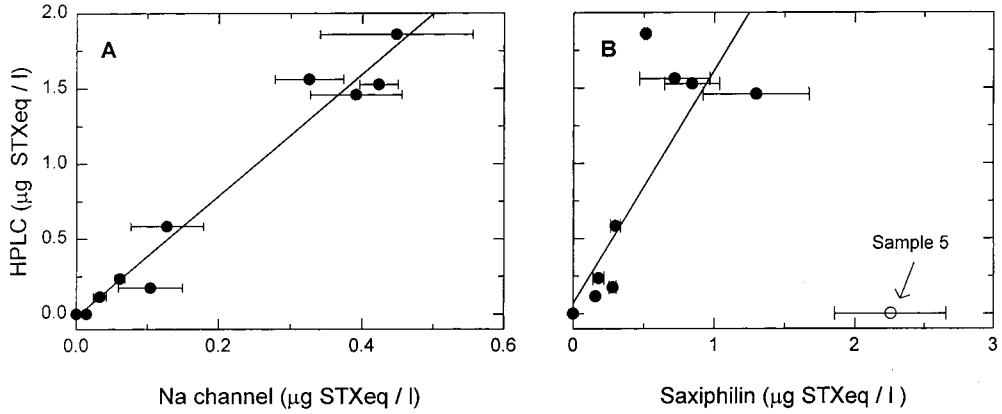


FIGURE 6. Correlation between the STXeq in cyanobacteria extracts determined by HPLC analysis and conversion based upon mammalian toxicity factors and (a) rat brain Na channel ($r = 0.94$, $p < 0.01$) and (b) saxiphilin ($r = 0.96$, $p < 0.05$) radioreceptor assays. In (b), the value of sample 5, which exhibited unusual bioactivity results, is highlighted and is excluded from the correlation analysis. The regression line for (a) is fit to the equation $y = 4.0x$, while for (b) the regression line is $y = 1.5x + 0.07$.

Discussion

Anabaena circinalis has a record of manufacturing STXs (*9*), a major concern because of the potential for contamination of drinking water. The diversity of bloom-forming freshwater cyanobacteria being found to produce these toxins is increasing in number (*3*−*6*). Such contaminated water may have detrimental effects upon humans, rural livestock, and native wildlife. In Australia, *Anabaena circinalis* is known to have caused the death of sheep, and STXs were the likely lethal agent (*29*). Earlier reports of livestock mortality are also likely to have resulted from neurotoxic *A. circinalis* blooms (*14, 30*). Monitoring for these noxious algae is therefore becoming a priority for water management authorities around the world, and the mere presence of the alga is not a reliable indicator of toxicity. As previous

TABLE 3. Inhibition of Acetylcholinesterase by Extracts of *A. circinalis* Strains from Two Separate Experiments[a]

sample no.	% inhibition screen 1	% inhibition screen 2	sample no.	% inhibition screen 1	% inhibition screen 2
1	31.6, 20.4	33.1, 24.1	8	−1.8, −0.9	2.2, 0.7
2	4.9, 5.9	9.4, 5.3	9	5.3, −2.9	4.1, 2.8
3	65.0, 71.6	67.4, 73.4	10	25.2, 12.8	25.6, 22.2
4	3.6, 2.3	0.9, 7.3	11	2.5, −6.3	−4.4, 0.2
5	−1.3, −4.1	3.1, 5.4	12	2.5, 1.6	3.3, 4.8
6	11.1, 7.9	19.9, 14.0	13	0.0, 0.6	−1.3, −1.2
7	0.6, 4.9	0.9, 6.4	14	7.2, 6.8	9.0, 5.8

[a] Numbers given are individual measurements of each duplicate from the two separate screening experiments.

investigations have shown, toxin production by *A. circinalis* is strain dependent (*9, 31, 32*).

The various strains of *A. circinalis* used in this study manufactured STXs on very different scales ranging from no toxin production to just over 450 ng STXs per million cells (as measured by HPLC in strain 8 and taking into account cell numbers extracted). Reported densities of *A. circinalis* blooms in Australia have reached 940,000 cells/mL (*13*). If such blooms include strain 8 and it synthesizes the toxins to the same level as found here in culture, then it would generate a potential toxin load of 423 μg STXs/L of water. This is well above the sensitivity limit of the radioreceptor assays used in the present study. For instance, if one adopts a conservative limit of 30% inhibition as a value that is true inhibition and not related to artifactual or biological variation, this translates to detection of 0.47 nM STXeq in both the saxiphilin and Na channel assays. In this study, 10 μL of samples was assayed in a final assay volume of 150 μL giving a dilution factor of 15. Therefore the stock [STXeq] at this inhibition value would be 7.1 nM STXeq in both assays. This converts to 2.1 μg/L STXeq, a value slightly below the concentration proposed for health alerts for the STXs (*2*). Increased sensitivity can be obtained by increasing the amount of sample assayed. The detection limit for HPLC was 0.5 to 5 μg/L using a 20 μL injection volume, depending on the sensitivity for the individual toxins. Obviously, such limits can be improved upon by increasing the amount injected. The detection limit of the mouse bioassay is 0.18 μg STX/mL or 180 μg STX/L (*33*), a limit that would necessitate sample concentration for the mouse bioassay to be of comparable utility as HPLC and radioreceptor assays for water quality testing targetting STXs.

Confirmation of individual STXs after bioassay is often achieved by HPLC analysis. A single value for a test sample is obtained by conversion of individual toxin amounts to STX equivalents using factors derived from toxicity studies with each toxin upon mammals. For instance, the C-toxins are mildly toxic to mammals compared to STX itself and would therefore contribute little to overall toxicity of a complex mixture in mammals. More broadly, however, it is unknown what the effect of these toxins is upon agricultural livestock and invertebrate and vertebrate wildlife that may encounter contaminated water, and these conversion factors may be irrelevant when one assesses the overall impact of these cyanobacterial toxins. Bioassays do not differentiate between different toxins and provide only a single value for overall biological activity resulting from the cumulative effect of all of the related toxins. To gauge the reliability of the bioassays used here by comparison to the finer analysis provided by HPLC, toxicity data from HPLC can be in both absolute terms which ignores the different potencies of the toxins or by using conversion factors based upon the biological efficacy of the individual toxins. Such conversion factors for the STXs have only been generated for mammalian toxicity by virtue of mouse lethality. Both radioreceptor assays produced toxicity measurements significantly correlated with HPLC analysis whether these mammalian toxicity conversion factors were used or not. Both of these receptor assays are in a microtiter plate format, and they therefore possess all the virtues this provides, namely rapidity and ease of sample handling. This, along with the quality of their toxicity measurement, makes them a potentially powerful tool for water quality management.

Despite the quality of the correlation with HPLC, the Na channel assay consistently underestimated the amount of toxin present. Although mammalian toxicity factors are in fact invalid for comparing HPLC analysis with saxiphilin, the quantities determined for algal extracts closely agreed with HPLC derived toxicity values when they had been corrected for mammalian toxicity without under- or overestimating the amount of toxin present. An improved correlation between saxiphilin and HPLC analysis would be expected if conversion factors could be generated for each of the STXs in this assay. This underestimation of total toxin content by radioreceptor assays may be because all of the toxin molecules in a test sample are never bound by the biological receptors. For instance, the conditions necessary for valid interpretation of receptor binding data require that the number of binding sites available must be no more than 20% of the concentration of the available ligand (*34*). At worst then, over 80% of the toxin in a test sample is unbound with the amount bound reflecting the ratio of radiolabeled and unlabeled ligands. To further complicate the matter, if the unlabeled competitor has an affinity unequal to that of the radioligand, then the percent bound does not reflect the absolute ratio of unlabeled and radiolabeled ligands but some variation thereof relative to the difference in ligand affinities.

In *Anabaena circinalis*, the relative proportions of the various STXs are usually conserved between different toxic strains and dominated by the sulfated STXs, C1, and C2 (*9, 31*). We found here an unusual toxin profile dominated by gonyautoxin-5 in an extract of a Western Australian strain (sample 12). Despite this difference, toxicity measurement with the radioreceptor assays for this sample correlated well with HPLC quantitation. Of concern is the unknown bioactivity observed for strain 5 in the saxiphilin assay. Saxiphilin has been widely challenged with many compounds and conditions to gauge its susceptibility to artifactual results (*16, 21, 28*). It has been found to be highly specific for STXs, and no organic compound has been found that disassociates STX from the protein. Salts and acidity also have little effect on this assay. Exhaustive analysis using HPLC did not reveal any additional fluorescent peaks which might correspond to new or unknown STXs in this extract, yet an extract of this strain caused death of mice from neurotoxicity as evidenced by respiratory arrest and convulsions. This strain is being examined in much greater detail to identify the factor generating this saxiphilin activity and whether it may in fact be a new toxin, a disturbing possibility for water management authorities, or a compound nontoxic to mammals that can interfere with this assay. If this activity translates into toxicity, then a major concern is that HPLC analysis or Na channel based bioassays which encompass the radioreceptor assay used herein, cell viability assays and mouse lethality assay, are unable to detect this bioactivity, and hence a potentially dangerous water contaminant may go undetected with dire consequences.

Of a more regional concern is the observation of acetylcholinesterase inhibitory activity associated with strain 3. No anatoxin-a(S) like activity has ever been reported for Australian cyanobacteria, and if the activity observed by us is indeed anatoxin-a(S), or a like compound, then this presents a new risk for Australian water consumption in that it may result in intoxication. Chemical analyses are necessary

to confirm the identity of this biological activity.

Strains of *A. circinalis* in laboratory culture either produce no toxins or are highly toxic (*31*), indicating the wider variation in toxicity of field samples is primarily due to environmental factors. Without any taxonomic clues to lethality it is safest to assume a mildly toxic bloom could transform into a highly toxic event under different environmental conditions or if the dominance by particular strains shift within a bloom. For effective water supply management, biological observations must therefore be married with toxicological methods to prevent unnecessary prohibitions based solely on presence of potentially toxic algae. In the future, more accurate detection of toxigenic strains (*31*, *32*) and/or the genetic basis for STX biosynthesis may also afford better prebloom water quality management.

These two receptor assays described in this paper provide sensitive and new tools for detection of toxicity in neurotoxic cyanobacterial blooms. These receptor assays can be quite cost-effective. For instance, at today's prices, the specialized microtiter plates used for the assays cost less than U.S. $15 per plate, a cost that can be reduced with bulk purchase. The combined cost of all of the other reagents such as buffers, salts, and [^{3}H]STX is no more than U.S. $10 per plate. All up, this means that a single microtiter plate plus reagents costs less than U.S. $25 with which one can generate 96 data points. Depending on the number of positive and negative controls and replicates of test samples, toxicity values for test samples can cost U.S. $2−4 in consumables. Coupling this cost-effectiveness with their high throughput capability by virtue of their microtiter plate format, greater sampling rates are possible of many more locations within a water body. This can provide water quality managers with a more comprehensive profile within the whole water body that will account for toxicity variation throughout a bloom. The fast and reliable screening and quantitation offered by receptor assays can be used to quickly determine if water supplies have been contaminated by STXs to dangerous levels.

Acknowledgments

This is publication number 1044 of the Australian Institute of Marine Science. B.A.N. was funded by the Cooperative Research Centre for Water Quality and Treatment and the Australian Research Council.

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Received for review August 10, 2000. Revised manuscript received November 27, 2000. Accepted November 30, 2000.

ES001575Z