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Spatial Variability in Anatoxin Production and Development of a Molecular Screening Tool for Anatoxin Producing Benthic Cyanobacteria

RESEARCH BASED SOLUTIONS

Spatial Variability In Anatoxin Production And Development Of A Molecular Screening Tool For Anatoxin Producing Benthic Cyanobacteria

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EXECUTIVE SUMMARY

Proliferations of benthic *Phormidium* sp. have become increasingly prevalent in New Zealand rivers. Associated anatoxin (a neurotoxin produced by cyanobacteria) production has resulted in numerous animal deaths and health warnings. Marked differences in the presence and concentrations of anatoxins in *Phormidium* mats have been observed both within and between rivers. This variability has raised concerns on best practices for collecting samples used to make risk assessments. Additionally, the cost associated with toxin testing often limits the number of samples that can be tested. This study had two objectives:

- To assess the variability of anatoxins within sampling sites with the aim of establishing a methodology for collecting representative samples for use in risk assessments.
- To use a PCR-based approach to determine if the presence of a *ks2* PCR-product (which has been shown to be putatively involved in the biosynthetic pathway of anatoxin production), correlates with the presence of anatoxins in cultures and environmental samples, and to assess the applicability of using this method to screen environmental samples for the potential to produce anatoxins.

Anatoxin variability was investigated by collecting samples from seven New Zealand rivers; 15 samples were collected from a 10 × 10 m grid in each river. Anatoxin concentrations were determined using liquid chromatography-mass spectrometry (LC-MS). No anatoxins were detected in samples from two of the sites and at one site anatoxins were detected in all 15 samples. At three sites both toxic and non-toxic samples co-occurred, sometimes within 1 m of each other. Based on binomial probabilities and the percentage of samples that contained anatoxins in this study, we recommend that 10 samples are collected at a sampling site to accurately assess the presence and concentrations of anatoxins.

Recent molecular-based methods may provide cost-effective approaches for screening samples for anatoxin production potential. We used primers designed to amplify a *ks2* sequence to assess a PCR-based approach. Detection of the *ks2* PCR product successfully distinguished anatoxin- and non-anatoxin-producing *Phormidium* strains from our culture collection. Results from environmental samples were more variable with the two methods in congruence in only 58% of samples. The *ks2* PCR product was detected in 30% of samples when no anatoxins were detected using LC-MS. In 10% of samples no *ks2* product was detected when anatoxins were detected using LC-MS (*i.e.* false negatives). To confirm the presence of anatoxins and to determine their concentrations, samples testing positive by PCR should be followed up using a chemical-based method, *e.g.* LC-MS. The false negatives accounted for only a small percentage of the samples and could have been due to the presence of other anatoxin-producing cyanobacteria that are not detected with the primer set used. Further investigation is required. The detection of *ks2* PCR-products, even when no anatoxins were detected, may result in this method being a useful early warning tool. The PCR-based method used requires limited sample preparation and relatively low capital expenditure, and therefore has the potential to be used as a cost-effective method to screen multiple samples for anatoxin production potential.

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1. INTRODUCTION

Benthic, mat-forming cyanobacteria are widespread throughout New Zealand rivers (Biggs & Kilroy 2000); the most common genus in New Zealand is *Phormidium*. During stable flow conditions, *Phormidium* can proliferate, forming expansive black/brown leathery mats across large expanses of river substrate.

Risks to human health and impacts to aquatic ecosystems are largely unknown, however, multiple dog poisonings/deaths since 1999 in Southland (1999 and 2000), Wellington (1999 and 2005-08), Napier (2004), Nelson (2008-09), Tasman (2009), Bay of Plenty (2007-08), West Coast (2008-09) and Canterbury (2007-09), have been associated with contact with benthic cyanobacteria in rivers (Hamill 2001; Wood *et al*. 2007b). In most instances the dog deaths were associated with mats of *Phormidium* and the presence of the neurotoxins anatoxin-a (ATX) and homoanatoxin-a (HTX). Over the summer low flows of 2007-08 and 2008-09, significant coverage of the riverbeds in the Manawatu and Horowhenua areas by *Phormidium* mats resulted in strong community interest and concern for the health impacts to river users and animals.

Limited monitoring to date has shown that toxin-producing species can be found extensively throughout a region but the presence of toxins is highly variable *(e.g.* cyanobacterial mats in six out of 10 rivers surveyed in Canterbury contained detectable levels of anatoxins, however, in the Horizons Region extensive mat coverage throughout large catchment areas showed no positive anatoxin results). Unlike for planktonic cyanobacterial blooms, published information on toxic benthic species is scarce and thus guidance on best practices for sampling and managing benthic cyanobacterial proliferations is limited. Consequently, Regional Councils have been 'closing' rivers on the basis of a very limited understanding of risks and not adequately identifying rivers that are unsafe for users or aquatic ecosystem health.

Recent research undertaken in New Zealand (Heath *et al*. 2010) has resulted in the isolation and culturing of multiple strains of *Ph. autumnale* sourced from rivers throughout New Zealand. Heath *et al.* (2010) showed that both toxic and non-toxic genotypes co-occur in benthic cyanobacterial proliferations; a result further exemplified by an in-depth study of *Phormidium* in the Hutt River (Lower Hutt, New Zealand) where anatoxin concentrations varied markedly both spatially and temporally (Heath, Wood and Ryan unpublished data). This research also found no correlation between anatoxin concentrations and the percentage of the river substrate that cyanobacterial mats occupy (Heath, Wood, Ryan unpublished data). One of the aims of this study was to assess variability in the presence of anatoxins within sampling sites, with the goal of establishing a methodology for collecting representative benthic cyanobacterial samples for use in risk assessments.

Traditional methods (*e.g.* microscope/macroscopic assessments) used for risk assessments do not allow distinction between toxic and non-toxic strains. Additionally, current methods for cyanotoxin detection (*e.g.* LC-MS) are relatively expensive and thus impractical for large-scale surveillance or routine monitoring programmes. In contrast, advances in molecular biology

have allowed the development of tools that allow specific, sensitive and rapid identification of problematic species and/or genes involved in toxin production. Recently a research group in France published findings of a gene (*ks2*) that appears to be linked to anatoxin production in benthic cyanobacteria (Cadel *et al.* 2009). This has the potential to allow development of a rapid, sensitive, and cost effective molecular-based method for screening benthic cyanobacterial samples for anatoxin-production potential. The second aim of this study was to use a PCR-based approach to determine if the presence of *ks2* PCR-product correlates with detection of ATX and HTX, and to assess the applicability of using PCR to screen environmental samples for *ks2* and thus anatoxin-production potential. Both cultures and environmental samples of cyanobacteria were screened.

2. METHODS

2.1. Cyanobacterial cultures

Twenty-seven cyanobacterial strains (Table 1), isolated as part of previous projects (Wood *et al.* 2007a; Wood *et al.* 2008; Heath *et al.* 2010), were screened to determine if the presence of the *ks2* gene fragment correlated with detection of anatoxins. ATX has been detected previously in a strain of *Aphanizomenon issatchenkoi* (CYN16) and two strains of *Ph. autumnale* (CYN49 and CYN53; Wood *et al.* 2007a; Heath *et al.* 2010). Although ATX and HTX were initially detected in *Ph. autumnale* strain CYN49, these compounds were undetectable in subsequent testing of sub-cultured samples of the strain (Wood *et al.* 2008). Frozen material from the initial collection of CYN49, which tested positive for HTX and ATX, as well as the subsequent sub-cultured strain which tested negative for HTX and ATX, were included in this study.

Table 1. Culture code, species identification, isolation location, and presence or absence of anatoxins and the *ks2* fragment in all cultures used in this study.
ATX = anatoxin-a, HTX = homoanatoxin-a.

Code	Species	Location	Toxin	*ks2*
VUW3	*Phormidium autumnale*	Wainuiomata River	-	-
VUW4	*Ph. autumnale*	Hutt River	-	-
VUW5	*Ph. autumnale*	Waingongoro River	-	-
VUW6	*Pseudanabaena* sp.	Wainuiomata River	-	-
VUW7	*Ph. autumnale*	Wainuiomata river	-	-
VUW8	*Ph. autumnale*	Akatarawa River	-	-
VUW9	*Ph. autumnale*	Hutt River	-	-
VUW10	*Ph. autumnale*	Wainuiomata river	-	-
VUW11	*Ph. autumnale*	Pembroke Road	-	-
VUW12	*Ph. autumnale*	Wainuiomata River	-	-
VUW14	*Ph. autumnale*	Hutt River	-	-
VUW15	*Pseudanabaena* sp.	Whakatiki River	-	-
VUW16	*Ph. autumnale*	Pelorous River	-	-
VUW 17	*Ph. autumnale*	Mangatinoka Stream	-	-
VUW18	*Ph. autumnale*	Makarewa River	-	-
VUW19	*Ph. autumnale*	Mangaroa River	-	-
VUW20	*Ph. autumnale*	Rangataiki River	-	-
VUW22	*Ph. autumnale*	Waimana River	-	-
CYN38	*Ph. murrayi*	Red Hills Tarn	-	-
CYN39	*Ph. murrayi*	Red Hills Tarn	-	-
CYN47	*Ph. autumnale*	Ashley River	-	-
CYN48	*Ph. autumnale*	Ashley Rriver	-	-
CYN49	*Ph. autumnale*	Hutt River	-	-
CYN49*	*Ph. autumnale*	Hutt River	ATX and HTX	+
CYN53	*Ph. autumnale*	Rangataiki River	ATX	+
CYN 55	*Ph. autumnale*	Roding River	-	-
CYN16	*Aphanizomenon issatchenkoi*	Lake Hakanoa	ATX	-

* Frozen archived material from when this culture was positive for ATX/HTX

2.2. Environmental samples

Benthic cyanobacterial mats were collected from seven New Zealand rivers between January and March 2009 (Table 1). With the exception of Mangatarere Stream, anatoxins had been detected at each of the seven sites on previous occasions (Wood *et al.* 2007; Heath *et al.* 2010; Wood unpublished data). At each river a 10 × 10 m grid was set up in a riffle (a shallow region of a river where the surface is broken into ripples or waves by totally or partially submerged obstructions, Figure 1). Cyanobacterial mat coverage was measured in five 1 m^2 quadrats randomly positioned within each grid. Random numbers were used to select fifteen sampling points within grids (Figure 2). At each sampling point cyanobacterial samples were collected by scraping a mat from one rock into a sterile Falcon tube (15 mL). Where no mats were present at a sampling point, the closest upstream mat was selected for sampling. Samples

were frozen (-20°C) for later molecular and toxin analyses. Sub-samples (5 mL) were preserved using Lugol's Iodine for morphological (to genus level) identification.

Table 2. Sampling sites, locations and dates, and percentage of river substrate covered with cyanobacterial mats.

Sampling Site	Location	Sampling Date	Percent coverage
North Island			
Hutt River	41°11'29"S, 174°55'55"E	23 January 2009	30
Mangatarere Stream	41°03'20"S, 175°29'51"E	16 March 2009	40
Mangaroa River	40°05'38"S, 175°07'35"E	17 March 2009	80
Waipoua River	40°55'21"S, 175°38'26"E	15 March 2009	30
South Island			
Ashley River	41°17'00"S, 172°32'46"E	24 March 2009	20
Maitai River	41°16'16"S, 173°18'86"E	20 March 2009	15
Waimea River	41°18'41"S, 173°07'41"E	4 April 2009	20

Figure 1. Photo showing 10 × 10 m grid in a riffle in the Waipoua River, Masterton.

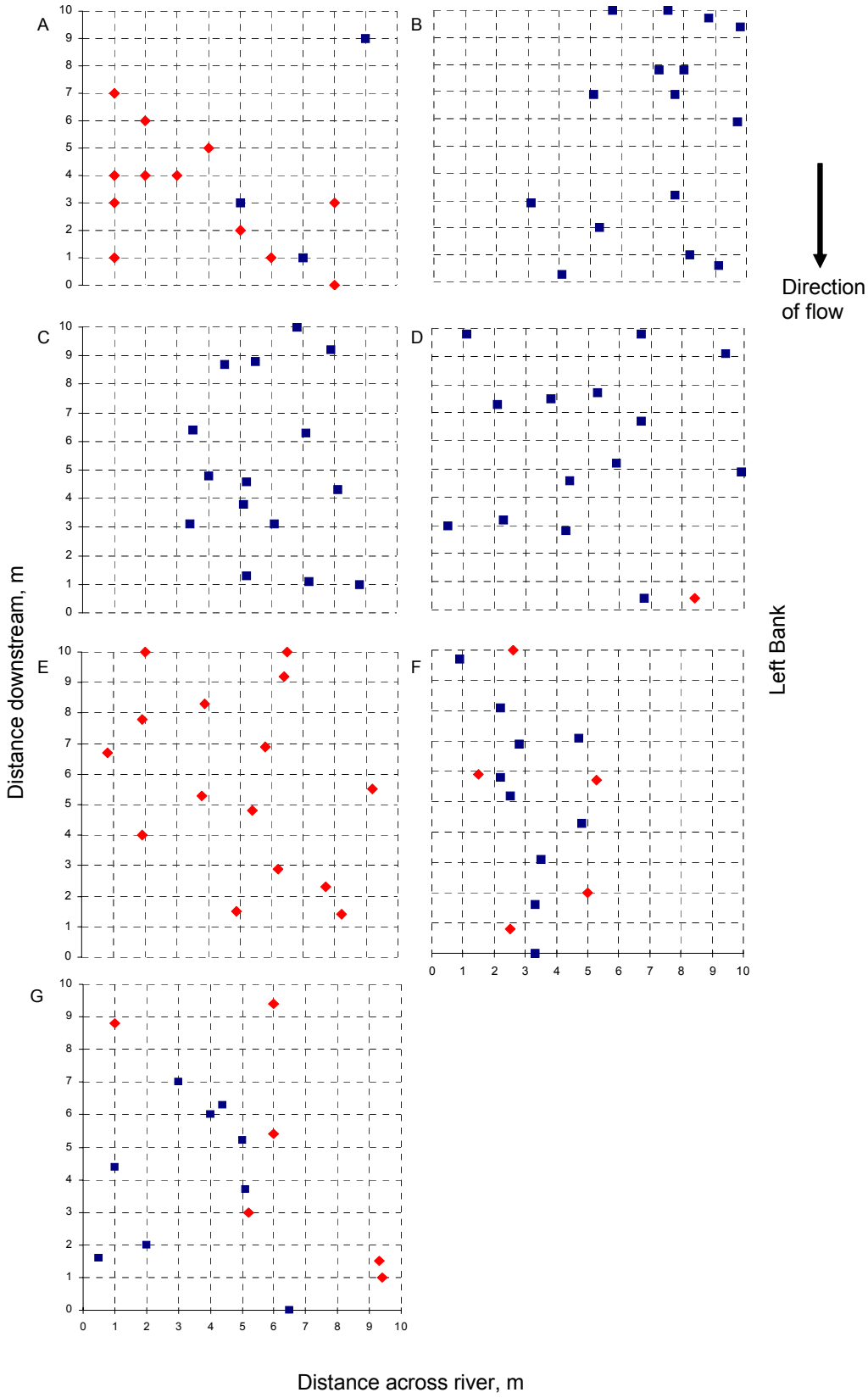

Figure 2. Location of randomly selected sampling points within each 10 × 10 m grid. A, Hutt River; B, Mangatarere Stream; C, Mangaroa River; D, Waipoua River; E, Ashley River: F, Maitai River and G, Waimea River. ◆ anatoxins detected, ■ no anatoxins detected.

2.3. Morphological identification of benthic mat samples

The dominant cyanobacterium in each mat was identified by microscopy (BX51, Olympus, Wellington, New Zealand).

2.4. Chemical detection of anatoxins in cultures and benthic mats

Sub-samples of all cyanobacterial mats and cultures were lyophilized (freeze-dried) and then extracted (100 mg) with 10 mL of MilliQ water containing 0.1% formic acid. Samples were sonicated (15 min) and centrifuged (4000 × *g* for 10 min). The supernatant was collected (15 mL Falcon tube) and a second extraction was undertaken on the pellet using 5 mL of MilliQ water containing 0.1% formic acid. The supernatants were combined and an aliquot of the supernatant was analysed directly for ATX, HTX and their degradation products dihydroanatoxin-a (dhATX), dihydrohomoanatoxin-a (dhHTX), epoxyanatoxin-a (epoxyATX) and epoxyhomoanatoxin-a (epoxyHTX) using Liquid Chromatography Mass Spectrometry (LC-MS). Anatoxins were separated by LC (Waters Corp., MA, USA) using a 50 × 1.0 mm Acquity BEH-C18 (1.7 µm) column (Waters Corp., MA). The mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile) were used at a flow of 0.3 mL min^{-1}, isocratic for 1 minute at 100% A followed by a gradient to 50% B over 2 minutes. Injection volume was 5 µL. The Quattro Premier XE mass spectrometer (Waters-Micromass, Manchester) was operated in ESI+ mode with capillary voltage 0.5 kV, desolvation gas 900 L h^{-1}, 400°C, cone gas 200 L h^{-1} and cone voltage 25 V. Quantitative analysis was by multiple reaction monitoring (MRM) using MS-MS channels set up for ATX (166.15 > 149.1; Rt 1.0 min), HTX (180.2 > 163.15; Rt ca. 1.9 min), dhATX (168.1 > 56; Rt 0.9 min), dhHTX (182.1 > 57; Rt ca. 1.9 min), epoxyATX (182.1 > 98) and epoxyHTX (196.1 > 140; Rt ca. 1.9 min). The instrument was calibrated with dilutions in 0.1% formic acid of authentic standards of ATX (A.G. Scientific, CA).

2.5. Isolation of DNA, *ks2* PCR and analysis

Six samples from each site were selected for PCR-based *ks2* analysis (Table 3). These samples were selected to provide a cross-section of toxic and non-toxic (where available) samples from each site. DNA from each selected environmental sample and from all 27 laboratory cultures was extracted from approximately 0.05 g of lyophilized material using the Power Soil™ kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA) according to the manufacturer's protocol.

Cultures and environmental samples were screened for the presence of the *ks2* fragment using a 25 µL reaction mixture containing approximately 30 ng of DNA, 480 nM of the primers given in Cadel-Six *et al.* (2009; Geneworks, Australia), 200 µM dNTPs (Roche Diagnostics), 1 × *Taq* PCR buffer (Invitrogen), 1 U of Platinum *Taq* DNA polymerase (Invitrogen), 2.5 mM $MgCl_2$ (Invitrogen) and 2.0 µg non-acetylated bovine serum albumin (Sigma). PCR reactions were run on an iCycler thermal cycler (Biorad, USA) with the following conditions:

94°C for 2 min followed by 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 1 minute, repeated for 35 cycles with a final extension at 72°C for 7 minutes. Bands were sized on a 1% agarose gel stained with ethidium bromide. The *ks2* fragment is approximately 420 bp. The PCR product from CYN53 was purified using a High Pure PCR product purification kit (Roche Diagnostics) and sequenced bi-directionally using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). A step-by-step protocol on how to perform this PCR is given in Appendix 1.

2.6. Statistical analysis of toxin data

Toxin data from sites that tested positive for anatoxins were pooled and the percentage of anatoxin occurrence in these samples calculated. Using binomial probabilities the number of samples required to detect anatoxins with 95%, 99% and 100% confidence was determined. Additionally, the numbers of samples required to detect anatoxins with 95% and 99% confidence at a site with a prior determined percentage of anatoxins was modelled.

3. RESULTS

3.1. Benthic cyanobacterial coverage and identification

The highest cyanobacterial percentage cover was observed at the Mangaroa River site (80%, Table 1). This was markedly higher than other sites where coverage ranged from 15% to 40% (Table 2). The dominant cyanobacterium (identified by microscopy) in all samples was *Phormidium* (most likely *Phomidiumautumnale*).

3.2. Anatoxin detection in benthic mats

Anatoxins were detected at five of the seven sites (Figure 2 and Table 3). The Ashley River was the only site in which all fifteen samples contained anatoxins. In contrast, only one sample in the Waipoua River site contained anatoxins (Figure 2 and Table 3). At sites where both toxic and non-toxic samples were present (Hutt, Waipoua, Maitai and Waimea), no obvious correlation between sample location and anatoxin detection was found (Figure 2). Anatoxins were not detected in any samples from the Mangaroa River or Mangatarere Stream.

The composition and concentrations of anatoxins varied among sites. Twelve of the 15 Hutt River samples contained dhATX with maximal concentrations of 5.39 mg kg^{-1}. Low levels of HTX and dhATX were also detected in three of these samples (Table 3). In the Ashley River, despite no ATX being detected, all samples contained dhATX (0.39 - 8.89 mg kg^{-1}) and lesser amounts of HTX and dhHTX. The Maitai River samples contained low levels of dhHTX (< 0.2 mg kg^{-1}), with no parent compound detected, whereas the Waimea River samples

contained low levels of HTX (< 0.5 mg kg^{-1}) with no degradation compounds detected (Table 3). No epoxy-degradation products were detected.

3.3. *ks2* PCR results

Strain CYN53 and the frozen archived material of CYN49 gave positive *ks2* PCR products (Table 3). The sequence from the CYN53 PCR product (GU363536) shared a 94% similarity with the *ks2* sequence reported by Cadel-Six *et al*. (2009, FJ477836) from an *Oscillatoria* sp. Sequencing was undertaken to confirm that the PCR had amplified the correct PCR product. Strain CYN53 was then used as a positive control for all subsequent PCR undertaken on the environmental samples.

The results of the *ks2* PCR correlated with the LC-MS analysis for 58% of environmental samples screened (Table 3). The *ks2* PCR product was detected in 30% of samples when no anatoxins were detected. In 10% of samples no *ks2* product was detected when anatoxins were detected using LC-MS (*i.e.* false negatives, Table 3).

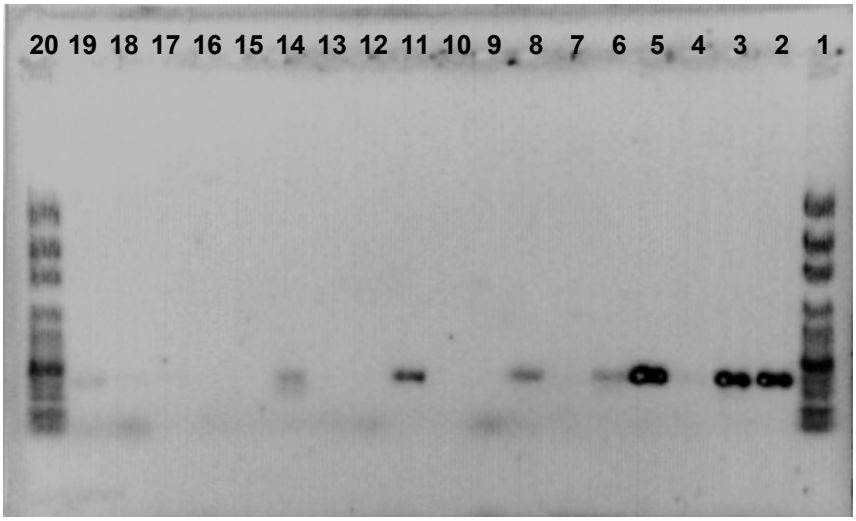

Figure 3. Photo showing gel used to screen *ks2* PCR-products (~420 bp).
1, 100 bp ladder; 2-7, Ashley River Site 1-6; 8-13, Mangaroa Site 1-6; 14-19 Waimea River Sites 3, 4, 6, 9, 10, 11; 20, 100 bp ladder.

Table 3. Concentrations of anatoxins and presence or absence of the *ks2* PCR product in environmental samples collected from seven New Zealand rivers. Only six samples at each site were screened for *ks2*, shading highlights false negatives. All toxin results are given as mg kg^{-1} of lyophilized weight. ATX = anatoxin-a, HTX = homoanatoxin-a, dhATX = dihydroanatoxin-a, dhHTX, = dihydrohomoanatoxin-a, nd = not detected, nt = not tested.

	Hutt River						Waipoua River						Ashley River						Maitai River					
	ATX	**HTX**	**dhATX**	**dhHTX**	**TOTAL**	***ks2***	**ATX**	**HTX**	**dhATX**	**dhHTX**	**TOTAL**	***ks2***	**ATX**	**HTX**	**dhATX**	**dhHTX**	**TOTAL**	***ks2***	**ATX**	**HTX**	**dhATX**	**dhHTX**	**TOTAL**	**ks2**
1	nd	nd	0.38	nd	**0.38**	+	nd	nd	nd	nd	nd	nt	nd	1.02	4.45	0.63	**6.09**	+	nd	nd	nd	0.08	**0.08**	+
2	nd	nd	1.40	nd	**1.40**	nt	nd	nd	nd	nd	nd	nt	nd	0.95	3.85	1.12	**5.92**	+	nd	nd	nd	nd		-
3	nd	nd	nd	nd		+	nd	nd	nd	nd	nd	nt	nd	0.49	2.01	0.64	**3.14**	+	nd	nd	nd	0.06	**0.06**	nt
4	nd	nd	1.77	nd	**1.77**	nt	nd	nd	nd	nd	nd	nt	nd	0.68	2.52	0.37	**3.57**	+	nd	nd	nd	nd		nt
5	nd	nd	0.19	nd	**0.19**	+	nd	nd	nd	nd	nd	nt	nd	1.13	4.47	0.71	**6.31**	+	nd	nd	nd	nd		+
6	nd	nd	5.39	0.24	**5.64**	+	nd	nd	nd	nd	nd	nt	nd	1.29	8.89	2.63	**12.81**	-	nd	nd	nd	nd		nt
7	nd	nd	nd	nd		+	nd	nd	nd	nd	nd	nt	nd	0.18	0.39	0.00	**0.56**	nt	nd	nd	nd	nd		+
8	nd	nd	1.13	nd	**1.13**	nt	nd	nd	nd	nd	nd	nt	nd	1.02	4.02	0.92	**5.96**	nt	nd	nd	nd	nd		nt
9	nd	nd	3.18	nd	**3.18**	-	nd	nd	nd	nd	nd	nt	nd	1.29	4.47	0.82	**6.59**	nt	nd	nd	nd	0.08	**0.08**	nt
10	nd	0.07	1.06	nd	**1.13**	nt	nd	nd	nd	nd	nd	-	nd	0.65	3.49	1.31	**5.46**	nt	nd	nd	nd	nd		nt
11	nd	0.08	0.31	nd	**0.39**	nt	nd	nd	nd	nd	nd	+	nd	0.95	4.75	1.02	**6.72**	nt	nd	nd	nd	nd		nt
12	nd	nd	0.37	nd	**0.37**	nt	nd	nd	nd	nd	nd	+	nd	0.71	2.82	0.26	**3.79**	nt	nd	nd	nd	0.17	**0.17**	nt
13	nd	nd	nd	nd		nt	nd	nd	nd	nd	nd	-	nd	0.80	2.40	0.17	**3.38**	nt	nd	nd	nd	0.07	**0.07**	-
14	nd	nd	0.44	nd	**0.44**	nt	nd	nd	nd	nd	nd	+	nd	1.45	5.51	0.78	**7.74**	nt	nd	nd	nd	nd		-
15	nd	nd	0.38	nd	**0.38**	nt	nd	0.87	1.55	nd	**2.42**	+	nd	0.37	1.30	0.08	**1.75**	nt	nd	nd	nd	nd		nt
Ave.		**0.07**	**1.33**	**0.24**	**1.37**			**0.87**	**1.55**		**2.42**			**0.87**	**3.69**	**0.76**	**5.32**					**0.09**	**0.09**	
Max.		**0.08**	**5.39**	**0.24**	**5.64**			**0.87**	**1.55**		**2.42**			**1.45**	**8.89**	**2.63**	**12.81**					**0.17**	**0.17**	

Table 3. continued

	Waimea River						Mangaroa					Mangatarere				
	ATX	**HTX**	**dhATX**	**dhHTX**	**TOTAL**	**ks2**	**ATX**	**HTX**	**dhATX**	**dhHTX**	***ks2***	**ATX**	**HTX**	**dhATX**	**dhHTX**	***ks2***
1	nd	0.09	nd	nd	**0.09**	nt	nd	nd	nd	nd	+	nd	nd	nd	nd	-
2	nd	0.13	nd	nd	**0.13**	nt	nd	nd	nd	nd	-	nd	nd	nd	nd	+
3	nd	nd	nd	nd		+	nd	nd	nd	nd	-	nd	nd	nd	nd	-
4	nd	0.21	nd	nd	**0.21**	-	nd	nd	nd	nd	-	nd	nd	nd	nd	-
5	nd	nd	nd	nd		-	nd	nd	nd	nd	+	nd	nd	nd	nd	-
6	nd	nd	nd	nd		nt	nd	nd	nd	nd	-	nd	nd	nd	nd	+
7	nd	nd	nd	nd		nt	nd	nd	nd	nd	nt	nd	nd	nd	nd	nt
8	nd	nd	nd	nd		nt	nd	nd	nd	nd	nt	nd	nd	nd	nd	nt
9	nd	0.26	nd	nd	**0.26**	+	nd	nd	nd	nd	nt	nd	nd	nd	nd	nt
10	nd	nd	nd	nd		+	nd	nd	nd	nd	nt	nd	nd	nd	nd	nt
11	nd	0.47	nd	nd	**0.47**	+	nd	nd	nd	nd	nt	nd	nd	nd	nd	nt
12	nd	nd	nd	nd		nt	nd	nd	nd	nd	nt	nd	nd	nd	nd	nt
13	nd	0.22	nd	nd	**0.22**	nt	nd	nd	nd	nd	nt	nd	nd	nd	nd	nt
14	nd	nd	nd	nd		nt	nd	nd	nd	nd	nt	nd	nd	nd	nd	nt
15	nd	nd	nd	nd		nt	nd	nd	nd	nd	nt	nd	nd	nd	nd	nt
Ave.		**0.23**			**0.23**											
Max.		**0.47**			**0.47**											

3.4. Statistical analysis to determine optimal number of samples

When the data from the five locations that contained positive results were pooled, 65% of the samples were positive for anatoxins. Using binomial probabilities, the number of samples required to detect anatoxins was predicted to be three samples for 95% probability, five samples for 99% probability and 10 samples for 100% (Figure 4). Binomial probability curves were plotted to predict the number of sampling points that would need to be sampled to ensure collection of a positive sample given prior knowledge of anatoxin occurrence at a site (Figure 4). For example, if the percentage of samples containing anatoxins was known to be 40%, six sampling points would need to be sampled to be 95% percent confident of detecting anatoxins and 10 sampling points to be 99% confident.

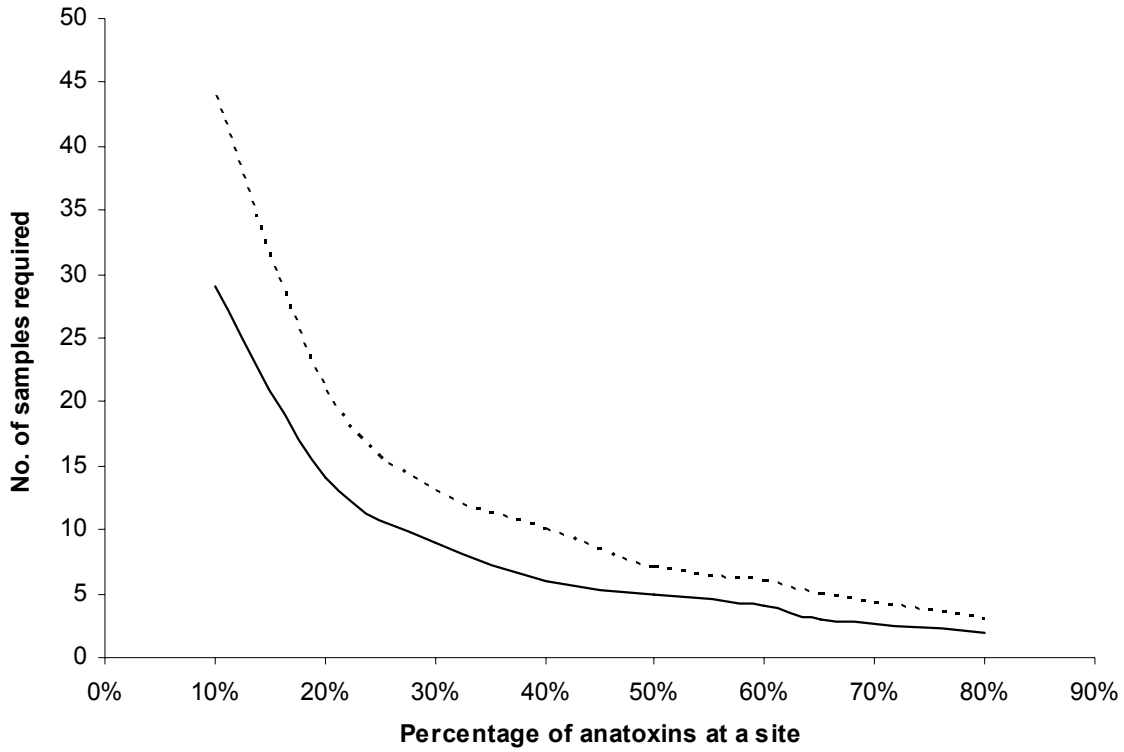

Figure 4. Binomial probability curves showing the predicted number of samples that should be collected to ensure detection of anatoxins with 95% (——) and 99% (------) confidence.
Use of these curves requires a predetermined knowledge of the percentage of samples containing anatoxins at a site.

4. DISCUSSION

Our previous research has shown that anatoxin production varies spatially and temporally within river systems (Heath, Wood and Ryan unpublished data), however, to devise robust sampling practices investigation of the anatoxin variability within a sampling site was necessary. The results of this study showed fine-scale spatial variability of anatoxins within 10 × 10 m grids. Of seven sites sampled, the Ashley River site was the only site where all samples contained detectable levels of anatoxins. At the three sites where both toxic and non-toxic samples co-occurred, mats less than 1 m apart varied in anatoxin content. Physiochemical parameters (*i.e.* light, temperature, flow, nutrient concentrations) do not vary over these distances in the river systems investigated, therefore a plausible explanation for observed differences in anatoxin levels is the co-occurrence of toxic and non-toxic strains at the same site. In addition to spatial variability, temporal variability of toxin production was also demonstrated. Previously, high concentrations of anatoxins (max. dhATX 535 mg kg^{-1}, ATX 2 mg kg^{-1}, 13 dhHTX 59 mg kg^{-1}, HTX 23 mg kg^{-1}, 13 February 2008) were detected at the Mangaroa site (Heath, Wood and Ryan unpublished data). However, in the present study no anatoxins were detected in samples collected from the Mangaroa site.

Anatoxin-a was detected in three of the cyanobacterial cultures tested but was not detected in any of the environmental samples collected. Degradation products were not detected in the cultures; in contrast, dihydro-degradation products were common in the environmental samples, often at concentrations higher than the parent compounds. Anatoxin-a degrades readily, especially in sunlight and at high pH, whereas the dihydro-degradation product is more stable (Smith & Lewis 1987). At two locations (Matai and Hutt rivers), dihydro-degradation products were detected in samples without any detectable levels of the parent compounds. The high incidence of finding anatoxin degradation products in the absence of parent compounds is of high importance since degradation products are not monitored in routine toxin analysis of cyanobacterial samples in New Zealand. Although anatoxin degradation products are considered non-toxic (Smith & Lewis 1987), they may be useful indicators of toxin-producing potential, previous toxic proliferations or nearby toxic mats. No epoxy-degradation products were detected in this study. Trace levels of similar compounds were detected; however the levels were too low for confirmatory work to be undertaken.

The high cost of cyanotoxin testing using chemical methods makes analysis of multiple samples unaffordable for health authorities. The results presented in this study indicate that collecting single samples from a site is unlikely to provide an accurate assessment of the presence of anatoxin at that location. Based on the percentage of samples that contained anatoxins in this study we recommend that 10 samples be collected from each site. The 10 samples could then be pooled and a single analysis undertaken on the pooled sample. Alternatively, an initial 10 × 10 m grid assessment, as described in this study, could be undertaken to determine the percentage occurrence of anatoxins at a site and the binomial probability curves given in Figure 4 used to predict the number of samples required. Samples collected for toxin analysis should be stored chilled (5°C) or frozen (-20°C) and in the dark, and shipped on ice to the analytical laboratory overnight. Further information on benthic

sample collection is available in the recently released New Zealand guidelines for cyanobacteria in recreational fresh waters (Ministry for the Environment and Ministry of Health 2009).

Genes encoding enzymes involved in the biosynthetic pathways of ATX and HTX in *Oscillatoria* have now been identified (Cadel-Six *et al*. 2009; Méjean *et al.* 2009). We used primers designed to amplify the *ks2* sequence (Cadel-Six *et al.* 2009) to assess the potential of using a PCR-based approach to screen cultures and environmental samples for the potential to produce ATX and HTX. The method developed worked successfully for all cultured *Phormidium* strains analysed. The frozen archived material of CYN49 which, when initially isolated produced ATX and HTX, contained the *ks2* sequence, whereas the fresh non-anatoxin producing isolate did not. Strain CYN49 may have lost the *ks2* gene and thus the ability to produce anatoxins through the sub-culturing process used to maintain isolates in culture. No *ks2* PCR-product was detected in the *Aph. issatchenkoi* strain (CYN16), suggesting ATX may be produced via a different biosynthetic pathway or that this species has a differing gene arrangement.

The results of the PCR-based method and the LC-MS analyses were in congruence in 58% of the environmental samples; however there were examples where the results of the two methods differed. PCR is an extremely sensitive technique, often surpassing the sensitivity of chemical detection methods. False positives can be explained by two possibilities. 1) Anatoxins are present in the samples but at levels below the detection limits of LC-MS. 2) The genes for anatoxin production are present but the cyanobacteria are not actively producing anatoxins. The four samples which were positive for anatoxins via LC-MS but in which no *ks2* product was detected could have resulted from; sequence variability within this region in *P.autumnale* or the presence of other anatoxin-producing cyanobacteria in the sample that produce anatoxin via a different gene sequence, as was the case with CYN16. Further investigation and the development of additional molecular markers is required to resolve these issues.

The PCR-based method requires limited sample preparation and relatively low capital expenditure and therefore has the potential to be used as a cost-effective method to screen multiple samples for the potential to produce anatoxins. The detection of *ks2* PCR-products when no anatoxins were detected via LC-MS indicates that the PCR method may be a useful early warning tool. To confirm the actual presence of anatoxins and their concentrations, samples that test positive for the *ks2* gene by PCR should be followed up using a chemical-based method, such as LC-MS. The *ks2* PCR-product was detected in samples from all seven rivers visited, suggesting that toxic genotypes are present in many rivers and that the concentrations of anatoxins produced is mostly controlled by the proportional abundance of the anatoxin-producing genotypes, *i.e.* when samples are dominated by toxic genotypes, anatoxin concentrations will be high. Identification of the variables, (*i.e.* temperature, light, nutrients) that trigger toxic genotypes to out-compete non-toxic genotypes or vice-versa, requires further research. Further information on variables that regulate anatoxin production may lead to the development of predictive models that will improve the management of benthic cyanobacteria.

Our recent research suggests the presence of cytotoxic compounds affecting mammalian cells in multiple *Phormidium* species collected around New Zealand (Wood, Froscio and Campbell, unpublished data). Therefore, we recommend that health warnings should not rely solely on the presence of known cyanotoxins and that the percentage cover of benthic mats within a river should also be considered. Additionally, under certain environmental conditions (*e.g.* prolonged periods of low and stable flow), or as mats become thicker (and bubbles of oxygen become entrapped within the mats), cyanobacteria detach from the substrate and may accumulate along river edges. Cyanobacterial accumulations along river edges results in higher risk to human and animal health due to the increased probability of river users coming into contact with cyanobacterial material. This knowledge, in concert with the data presented in this and previous studies (Wood *et al.* 2007a; Heath *et al.* 2010), has been used as the basis for the development of guidelines for monitoring benthic cyanobacteria in New Zealand (Ministry for the Environment and Ministry of Health 2009). A three-tier alert level framework that uses cyanobacterial abundance and the occurrence of mats visibly detaching from the substrate to determine the alert level status has been developed. Cyanotoxin analysis is recommended to further define potential health risks. The PCR-based technique described in this report could be incorporated into routine monitoring programmes to enable screening of large numbers of samples for anatoxin-production potential.

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7. APPENDICES

Appendix 1. Protocol for *ks2* PCR.

The following protocol describes the setup of a PCR reaction for detection of the *ks2* fragment. Volumes need to be adjusted depending on the number of samples to be analysed.

1. Prepare the Master Mix according to Table 4. Multiply the volumes by the number of samples to be analysed + 3. For example, if 5 samples are to be analysed, the volumes in Table 4 are to be multiplied by 8 to allow for the 5 samples, a positive and a negative control, and one extra volume to allow for pipetting. All Master Mix components should be kept on ice.
2. Place the appropriate number of 0.5 mL PCR tubes into a tube rack. If available, use an ice rack chilled to -5°C.
3. Aliquot 22 µL of Master Mix into each PCR tube.
4. Add 3 µL of DNA at 5 ng μL^{-1} (sample/positive control) or MilliQ water (negative control).
5. Seal caps tightly with caps.
6. Vortex for 5 s to mix.
7. Centrifuge briefly to collect any droplets to the bottom of the tubes.
8. Position the PCR tubes in the PCR machine. If possible avoid using the wells at the edges of the machine.
9. Perform PCR using the following conditions: 94°C for 2 min followed by 35 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 1 min, and then a final extension at 72°C for 7 min.
10. Prepare a 1% agarose gel in 1 x TAE (see recipe below).
11. Mix samples with a gel loading buffer.
12. Load 5 uL of a 1 kp DNA ladder to the first well (volume may vary with type of ladder used).
13. Load 10-20 uL (depending on gel type) of sample to each well.
14. Run gel at 70 V for 120 min (optimal run time and voltage will vary with different systems).
15. Stain gel with ethidium bromide (~ 20 mins).
16. Visualize gel of a UV documentation system. The *ks2* fragment is approximately 420 bp.

Table 4. Master Mix composition for PCR-detection of the *ks2* fragment for a single 25 µL reaction.

Master Mix Reagents	Volume (µL) for single 25 µL reaction
$MgCl_2$ (50 mM)	1.25
10 × PCR buffer (-$MgCl_2$)	2.50
dNTPs (10 mM)	0.50
Non-acetylated BSA (5 mg/mL)	5.00
Forward Primer ATXF* (10 µM)	1.20
Reverse Primer ATXR* (10 µM)	1.20
TAQ (5 U/µL)	0.10
MilliQ Water	10.25
DNA (5 ng/µL) or Milli Q water (- CTRL)	3.00
TOTAL VOLUME	25.00

*Primer sequences:
ATXF - CGCAAATCGATG CTCACTTA, ATXR – CCACTGGCTCCATCTTGATT

50X TAE recipe

1. Add the following to 900ml distilled H_2O
    - 242 g Tris base
    - 57.1 mL Glacial acetic acid
    - 18.6 g EDTA
2. Adjust volume to 1 L with additional distilled H_2O

To make a 1X working stock of TAE take add 20 ml of 50X TAE to 980 mL of distilled water.