RESEARCH ARTICLE

CYANO RT-Microarray: A Novel Tool to Detect Gene Expression in Cyanobacteria

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ABSTRACT

EU µAQUA made early warning systems for freshwater pathogens and toxins. Barcodes for each cyanobacterial toxin gene published before 2011 were designed and used in a microarray format to capture messenger RNA (mRNA) to detect toxin gene expression at early stages of bloom development. A reverse transcriptase (RT) microarray was developed to detect toxin expression, which had low expression levels. Probes immobilized on the microarray slide captured the mRNA and were extended directly on the microarray. RT extension incorporated fluorescently labeled oligonucleotides to ensure a high signal detected by the microarray scanner. The CYANO RT microarray was laboratory tested with known toxic cyanobacteria and field-tested. Hybridizations without RT extensions were barely detectable in the cultured strains. However, with RT extensions, hybridized mRNA was easily detected. Field samples were equally successful and consistent with companion studies from the same sites using HPLC/(MS-MS) (High Performance Liquid Chromatography/Mass Spec). In some cases, amplified expression produced a signal when that toxin was not detected using chemical means. The RT microarray may be more sensitive than HPLC/MS-MS. Further studies are needed to determine if the RT-microarray is detecting a very low expression of the toxin genes and, hence, more sensitive as an early warning system predicting toxin potential.

KEYWORDS: Reverse transcriptase; Gene expression; Cyanobacterial toxins; Microarray

INTRODUCTION

Cyanobacteria are oxygenic phototrophs that produce a variety of toxins, many of which are cyclic peptides; these are hepatotoxins, or neurotoxic alkaloids [1] and pose a serious health threat to drinking water worldwide [2,3]. Hepatotoxins include microcystins, nodularins, and cylindrospermopsins, whereas neurotoxins include anatoxin-A and saxitoxins. Genera typically known to produce these toxins are indicated in Table 1.

Table 1 Summary of the literature [23-68] used to assemble and modify the probes designed for the toxin array. Pathways published after 2011 were not included in this version of the microarray. The cyanobacterial genera typically associated with each toxin are indicated.

Saxitoxin	Aeruginosin	Microcystin	Cylindropsermopsin	Nodularin	Phycocyanin	Gas Vesicles	House Keeping Genes
Anabaena, *Aphanizomenon*, *Lyngbya*, *Cylindrospermopsis*	*Planthothrix* *Microcystis*, *Nodularia*	*Microcystis*, *Anabaena*, *Planktothrix (Oscillatoria)*, *Nostoc*, *Dolichospermum*, *Hapalosiphon*, *Anabaenopsis*, *Aphanizomenon flos-aquae*	*Cylindrospermopsis*, *Aphanizomenon*, *Anabaena* *Raphidiopsis*, *Umezakia*	*Nodularia*			
Al-Tebrineh et al., 2010	Ballot et al., 2010	Briand et al., 2009	Ballot et al., 2010	Jonasson et al., 2008	Ballot et al., 2010	Becker et al., 2005	Iteman et al., 2000
Fathalli et al., 2011	Fergusson et al., 2000	Fathalli et al., 2011	Rasmussen et al., 2008	Jungblut & Neilan 2006			Neilan et al., 1997
Kellmann et al., 2008	Gugger et al., 2002	Ginn et al., 2010	Schembri et al., 2001	Koskenniemi et al., 2007			Nübel et al., 1997
	Halinen et al., 2008	Hisbergues et al., 2003	Valério et al., 2005	Lyra et al., 2005			Rudi et al., 1998, 20(
	Henson et al., 2002	Jungblut & Neilan 2006	Wilson et al., 2000	Moffitt & Neilan 2001			Schoenhuber et al., 1999
	Ishida et al., 2007	Kaebernick et al., 2000		Moffitt et al., 2001			Svenning et al., 2004
	Mihali et al., 2009	Noguchi et al., 2009					
	Rajaniemi et al., 2005	Nonneman & Zimba 2002					
		Ostermaier & Kurmayer 2009, 2010					
		Ouahid et al., 2005					
		Rantala et al., 2004, 2008					
		Sipari et al., 2010					
		Tillett et al., 2001					
		Tooming-Klunderud et al., 2008					
		Vaitomaa et al., 2003					
		Valerio et al. 2005, 2009					

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Dermotoxins, a third class of toxins, are produced by *Lyngbya, Schizothrix*, and *Oscillatoria* [1]. Some cytotoxins are also known [4]. Nutrient availability and other abiotic factors can affect toxin production [5,6]. Different strains of the same species can be toxic or non-toxic, making morphological species identification unreliable for predicting toxins or toxin potential [7]. Functions for the toxins are speculative. They are considered secondary metabolites [1]. Only in a few cases (microcystins and saxitoxins) were the complete pathways known at the time of the design of this microarray [4,8-10]. Anatoxin pathways were published after the toxin array was tested and are not included on this version of the toxin array.

RT-PCR methods have been developed for various toxin genes in various pathways. It has been suggested that these methods could detect cryptic cyanobacterial species, i.e., those capable or potentially producing toxic blooms so that water bodies at potential risk for cyanobacterial toxic blooms could be identified early in the season [11]. However, PCR methods can be biased and subject to inhibition from natural products in the field samples as well as from reaction conditions [12]. If the PCR is inhibited, then positive results can be missed. Our method only uses the RT enzyme once the mRNA has been hybridized to the array (no enzymes used up to this point) and all impurities washed away before the RT enzyme is applied to the microarray.

In keeping with the general objectives of the European Union (EU) µAQUA project to make early warning systems for freshwater pathogens, two microarrays were designed:

- A phylochip to detect pathogenic species in freshwaters, which included cyanobacteria along with other bacteria and protozoa.
- A microarray to detect the messenger RNA (mRNA) from the cyanobacterial toxin genes.

The latter microarray, to detect the expression of the toxin genes, is a new type of microarray and is described in detail here. Probes (barcodes) for each cyanobacterial toxin gene from available publications, prior to 2011, of molecularly characterized cyanotoxin gene pathways (Table 1) were designed and used in a microarray format to capture the mRNA for these genes. The pathway for anatoxin was published after the microarray was designed and tested and is not included here, although these toxins were detected in the two field sites by the chemical methods compared here to the toxin array. The barcodes include 3 coding regions in the aeruginosin pathway, 35 coding regions in the microcystin pathway, 12 coding regions in the cylindrospermopsin *cpr/aoa* gene cluster pathway, 2 coding regions in the nodularin pathway, 3 coding regions in the saxitoxin pathway, and for controls: 6 coding regions in the phycocyanin pathway, 1 coding region in the gas vesicle pathway, and 7 coding regions involved in housekeeping gene pathways.

Initially, the signals obtained from the hybridizations were so low that they could barely be distinguished above the background signal and so a method was developed to extend the probes (barcodes) using reverse transcriptase directly on the microarray by incorporating fluorescently labeled oligonucleotides as it was extended. This is essentially the same reaction that is performed to generate complementary DNA (cDNA) only it was performed directly on the microarray to produce an enhanced signal that enabled the mRNA to be detected from a variety of cyanotoxin genes. This novel tool is a measure of potential toxigenicity in cyanobacteria and could possibly be used to predict toxic cyanobacterial blooms or to identify the potential of any water body for cyanobacterial blooms, especially if used routinely as a monitoring tool and used in parallel with a species array [13-16, 21] to compare the species present with their potential toxicity. Initial tests are presented here to show its feasibility and field tests from one sampling day from the Netherlands are reviewed [16] and compared to field samples taken for two years in France.

MATERIALS AND METHODS

Environmental sites

The toxin gene array was laboratory tested using pure cultures of cyanobacterial species known to be toxic or nontoxic and field-tested in two countries (the Netherlands and France, Table 2). In these two countries, aliquots of the same sample were tested for toxins using standard methods in companion papers [16-18]. In the Netherlands, six water bodies were sampled once in the summer of 2015, and in France, Canet Lagoon (Table 2) was sampled monthly from August 2011 to May 2013. The Dutch sites LA1–LA3 are along an inland waterway and sites LA4–LA6 are small lakes; all have known cyanobacterial blooms each year, primarily from *Dolichospermum, Microcystis*, and *Aphanizomenon*. The *Anabaena* probes recognize *Dolichospermum*, which was formerly placed in *Anabaena*. The Canet site was selected in the µAQUA project as a representative brackish water site in this region of France, and its cyanobacterial community was unknown at the time of sampling.

Table 2 Location of the sampling sites from the six Netherlands lakes (LA) and Canet Lagoon, France (CA).

Code	Location	Latitude	Longitude
LA1	Nuldernauw Harbor	52°26′	5°47′
LA2	Nuldernauw	52°26′	5°49′
LA3	Wolderwijd Zoetermeerse	52°33′	5°55′
LA4	Plas	52°08′	4°51′
LA5	De Put De Grote	52°08′	4°51′
LA6	Plas	52°05′	4°32′
CA	Canet Lagoon,	42°66′	3°03′

Filtration for RNA extraction

A total of 50 L was collected from the French site and 4 L from the Dutch sites sampled at each time, concentrated using the Hemoflow hollow fiber filter (Hemoflow HF80S, Fresenius, Bad Homberg, Germany), and back-flushed with 1 L solution (1 L milliQ-H_2O, phosphate buffered saline (0.01 M), 5 mL Tween 80 solution (0.5%), 100 mg Sodium Hexametaphosphate (0.01%), 1 mL Antifoam B emulsion) to yield a 1 L concentrate. Two hundred milliliters of the concentrated eluate were sequentially filtered through the eight different filters of decreasing pore size (20 µm, 10 µm, 5 µm, 2 µm, 0.8 µm, 0.45 µm, 0.1 µm, and 0.025 µm filters, Millipore, Billerica, MA, USA) and placed into 1 mL of Tri-Reagent (Sigma Aldrich, St. Louis, MO, USA) [13], then stored frozen at −80 °C for further analysis and extraction for downstream analysis by the species and the toxin array. The hollow fibre filters are very easy to use, making the filtration of 50 liters attainable in 30 minutes or less. This is an obvious advantage over the standard filtration of one liter or less using standard Millipore filtration methods. Such a large volume is needed for the extraction of total RNA for the species microarray and for sufficient mRNA typical of low expression of toxic genes. For small water bodies or rivers a 50-liter sample should be more than adequate to characterize the water body but for larger water bodies multiples of the 50-liter samples could be easily taken and filtered in the same day.

RNA extractions

All filters were pooled and extracted with Tri-Reagent following the protocol in Lewis [19] as modified in Kegel et al. [19] with the following additional modifications for cyanobacterial extractions from Yilmaz [20]. Briefly, after addition of the RNA extraction control (500,000 *Dunaliella* cells) and 250 µL of 0.5 mm ZR BashingBeads to the pooled samples, samples were bead-beaten twice at maximum speed for 1 min. Then, 0.2 M Tris (pH 5.0), 0.02 M ethylenediaminetetraacetic acid (EDTA) (pH 8.0), and 1% sodium dodecyl sulfate (SDS) buffer were added and well mixed followed by the addition of 0.75 M NH_4 acetate and 1% potassium ethyl xanthogenate (Sigma, St. Louis, MO, USA) and incubation at 65°C for

15 min. The following procedure was then performed as described in Kegel et al. [19].

Probe design

A microarray for pathogenic freshwater species was developed and has been tested elsewhere within the EU µAQUA project [14-17, 22]. A companion microarray for toxin pathway genes was also developed and this method and preliminary field tests of this array are presented here. For the toxin array, probes were designed to target the microcystin synthetase gene cluster, including ten genes (*mcy*A to J), aeruginosin synthetase gene clusters (*aer*), saxitoxin biosynthesis gene cluster (*sxt*), and the *aoa/cyr* clusters putatively involved in cylindrospermopsin biosynthesis (Table 1). These probes were spotted onto the toxin array to capture the mRNA for these toxin genes. Toxin pathways published after 2011 were not included. Probes were tested for specificity using various cyanobacterial species including *Cylindrospermopsis raciborskii*, *Aphanizomenon* PCC 7905, *Microcystis* sp. BC 84/1, *Microcystis aeruginosa* CCAP 1450/8, *Planktothrix agardhii* strains 34, 126, and 137, *Planktothrix rubescens* strains 34, 137, and 9316, and *Dolichospermum* sp. (formerly *Anabaena* sp.). All probe sequences are patent pending.

Microarray hybridization for the gene expression array

The microarrays used for the toxin gene expression detection were spotted with 83 probes, including 3 positive controls for hybridization quality, 3 negative controls for absence of non-specific hybridization, 7 positive controls for RNA extraction efficiency, and 70 probes encompassing different coding regions of genes involved in toxin biosynthesis and housekeeping gene pathways as follows: 3 coding regions in the aeruginosin pathway, 35 coding regions in the microcystin pathway, 12 coding regions involved in the cylindrospermopsin toxin pathway, 2 coding regions involved in the nodularin pathway, 6 coding regions involved in the phycocyanin pathway, 1 coding region involved in the gas vesicle pathway, 3 coding regions involved in the saxitoxin pathway, and 7 coding regions involved in housekeeping genes pathways. The non-toxin genes were added to the microarray as controls for cyanobacterial populations. These probes were modified for microarray use from the references [23-68] in Table 1 according to each toxin pathway.

Hybridization of the mRNA to the toxin array

Each microarray slide contained two arrays with eight replicates for each probe. Hybridizations of each sample were performed on different slides, thus producing a pseudo-replicate. Considering two arrays per sample, each probe is, therefore, represented by 16 spots, and the signal for the 16 spots was averaged. One mL of the mixture obtained from the pooled filters previously stored in TRI-reagent at −80°C degree plus an internal extraction quality control (Lambda DNA) was processed for total RNA extraction using TRIzol® Reagent according to the patented MIDTAL procedure (patent WO2015008011 A1). RNA quality and purity (260/280 ratio: 1.8–2.2 and 260/230 ratio: 1.8–2.3) were measured by NanoDrop® Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The integrity and size distribution of total RNA was checked with a 2100 Bioanalyzer (Agilent, Technologies Inc. Santa Clara, CA, USA). One microgram of total RNA extracted from field samples was labeled and purified using a Platinum Bright 647 Infrared Nucleic Acid kit (Leica Biosystem, Nussloch GmbH, Nußloch, Germany) according to the manufacturer's instructions. The degree of labeling (DoL) was determined by measuring concentration and incorporating the dye using a NanoDrop® Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Samples with DoL values between 1.0 and3.0 were processed to hybridization. Labeled RNAs were fragmented by adding 1/10 volume of Fragmentation Buffer (salt buffer) (100 mM $ZnCl_2$ in 100 mM Tris-HCl, pH 7.0) and incubated for 15 min at 70°C and immediately chilled down on ice to room temperature. The reaction was stopped by adding 1/10 volume of 0.5 M EDTA, pH=8 to the sample. Microarray hybridizations were performed following optimized procedures based on protocols pub-lished in Kegel et al. [13]. Briefly, labeled field samples (1 µg RNA) were mixed with (2 ×) hybridization buffer containing 3 µL Poly-dA (1 µM) and 10 ng TBP-control made up to a final volume of 30 µL. Poly-dA was added to block the poly-T spacer on the probe and TBP was the TATA box gene fragment added as the positive hybridization control [15]. The labeled RNA was then denatured for 5 min at 95°C. After denaturation, the samples were placed on ice, and 7.5 µL of 4X KREA block (background blocker from Leica Biosystems, Nussloch GmbH, Nußloch, Germany) were added. The hybridization mixture was equally distributed to each array covered with lifter-slips (cover slips with raised edges) and cleaned with ethanol (LifterSlips, Erie Scientific, VWR International, Radnor, PA, USA). Slides were placed into SCIENION's sciHYBCHAMBER that maintained a moist environment to avoid evaporation, and hybridizations were carried out for 1 h at 65°C using a water bath. To remove unhybridized RNA, the slides were successively washed with three washing steps with increasing buffer stringency under agitation and in the dark to protect the fluorophore. The first buffer (2 × saline-sodium citrate (SSC)/10 mM EDTA/0.05% SDS) and the second buffer (0.5 × SSC/10 mM EDTA) washings were performed at room temperature for 10 min, whereas the final most stringent wash (0.2 × SSC/10 mM EDTA) was performed at 50°C.

The signals from the two replicate hybridization arrays are regressed one against the other in order to obtain the Cy5 RNA hybridization and signal amplification efficiency. The closer R^2 is to 1, the more efficient and reliable the hybridization was between the two blocks. Values under 75% were repeated, if necessary (Table 3).

Table 3 R^2 of the correlation/comparison of replicate hybridizations of Canet-Saint Nazaire samples for each sampling date over the two-year period. Examples of the replicate hybridization regression curves are shown in Fig. 1.

Sample date	R^2
05-07-2011	ND
09-08-2011	ND
26-09-2011	0.98
24-10-2011	0.94
07-03-2012	0.99
03-04-2012	0.92
09-05-2012	0.97
08-06-2012	0.99
03-07-2012	0.94
07-08-2012	0.98
07-08-2012	ND
18-09-2012	0.99
16-10-2012	0.93
19-11-2012	0.96
28-03-2013	0.97
18-04-2013	0.99
16-05-2013	0.9

RT extension of mRNA bound to microarray

After the third wash of the microarray, lifter-slips were put back onto each array and retro-transcription was performed using the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). Three washings should ensure that any potential inhibitors of the RT enzyme would be removed. The reaction was performed following the manufacturer's instructions with the addition of Cy5dCTP to the master mix in a final volume of 30 µL, and the mixture was incubated for 45 min at 50°C. Slides were washed successively with the washing buffers 1, 2, 3 as described above.

Microarray analysis

GPR Files exported from the Genepix scanner were loaded into the GPR analyzer program [69] for analysis of the replicate hybridizations. Results were exported from the GPR analyzer program into Excel and normalized with the signal from the buffer. Some Excel files were imported into PermutMatrix, which is freely available software that allows a heat map representation of microarray data (http://www.atgc-montpellier.fr/permut matrix/).

UPLC/MS-MS/microspheres

All of the chemical analyses shown in this paper are detailed in Greer et al. [17,18]. The results in those papers and those from the Dutch lakes [16] have been reproduced here for comparison with the toxin array from Canet Lagoon.

RESULTS

Hybridization quality and experimental replication

The hybridization and signal amplification quality of each sample was checked by regression scatter plotting the SN (signal to noise) ratio of each hybridization replicate. The average curve and the R^2 were calculated for each sample and any sample showing an R^2 of less than 0.75 was reexamined to see why the correlations were not over 0.90, was discarded and redone. Examples of a good and a poorer hybridization scatter plots are shown in Fig. 1. Of the nineteen samples taken over the two-year period, only four were not processed for analysis because of poor RNA quality. All R^2 values were over 0.9 (Table 3).

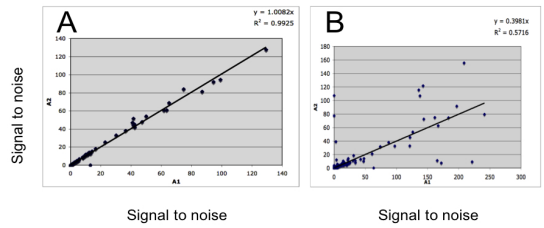

Figure 1: Examples of typical scatter plots of the signal to noise (SN) ratio of replicate 1 (**A1**) versus replicate 2 (**A2**) for any sample, A represents a good hybridization vs. a poorer one in B. Examination of the raw signals on the right revealed a difference in intensity for the same probes.

Laboratory tests

Figure 2 shows a comparison of before and after reverse transcriptase (RT) amplification of the captured mRNA for the toxin genes isolated from cultures of two different cyanobacterial species. It is immediately obvious that the RT amplification has greatly increased the signal intensity to a level at which it could be detected by the microarray scanner (Fig. 2). Among the pathways studied, several were housekeeping genes specific or typical of cyanobacteria, which were added to the microarray as positive controls. These included genes for gas vesicle proteins and for phycocynanin. Although these control probes are highlighted between the two species in Fig. 2, it is clear that the mRNA captured by the microarray is different between the two species because of the different toxins that are produced by the two species tested. Figure 3 shows the % difference in the signal intensity for the genes on the microarray for mcyA, mcyB and mcyC from *A. flosaquae* before and after reverse transcription directly on the microarray (Fig. 3).

Field tests

The Dutch lakes

Six shallow water bodies were sampled once during the summer of 2013 (Table 4) [16]. The extraction of RNA from the sample taken at LA 3 did not yield sufficiently high quality RNA for both the species array and the toxin array to be performed, so only the hybridization for the species array was performed [16]. In the Van der Waal study [16], toxins were also measured by a standard method (UPLC) and only anatoxin-A and some microcystins were above the detection level (Figs. 4A and 4B). In all of lakes tested with the toxin array, expression of the toxin genes was recorded (Fig. 4C) but not for all toxin pathways. The toxin profiles in the interconnected waterways LA1-LA2 were more similar than to those of the isolated lakes.

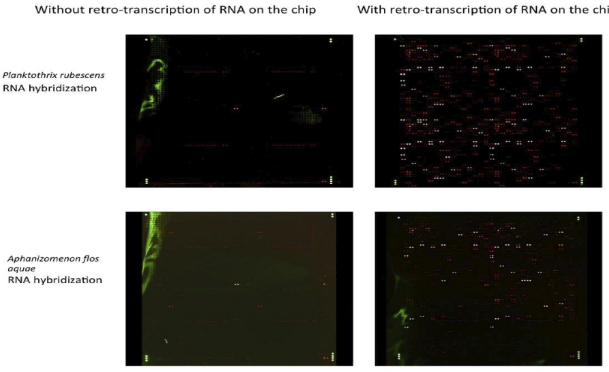

Figure 2: Comparison of microarray fluorescence signal intensities for the recognition of toxin genes from *Planktothrix rubescens* (strains BC9316; 137 and 34) and *Aphanizomenon flos-aquae* (Strain PCC 7905) before (left panel) and after (right panel) reverse transcription of the RNA hybridized to the probe. During complementary DNA (cDNA) synthesis based on the probe extension, fluorescent oligonucleotides are incorporated to produce the signal seen in the microarray on the right. Red dots are saturated signals. Reproduced from Kegel et al. [19] with permission from Perspectives in Phycology, Schweizerbart, Germany, https://www.schweizerbart.de/journals/pip/.

The *mcy*A, B, E, G, and J genes, and the *Aoa*B, *Aoa*C, and saxitoxin genes were actively expressed as compared to other genes in the other toxin pathways on the microarray. Saxitoxin was not detected by the traditional methods of UPLC [17], but its mRNA was detected in the toxin array. The highlighted probes mainly concerned *Aphanizomenon*, *Anabaena* (represented in the Dutch water bodies as *Dolichospermum*), and *Microcystis* toxin genes and were present in all sites but with relatively higher signals in site LA4 and LA5 [17]. In the microcystin pathway, *mcy*C and *mcy*D were barely detectable in the gene expression array, but *mcy*A and *mcy*E displayed very high signals (Fig. 4C). Genes *mcy*A–E are required to produce the toxins, whereas *mcy*G–J are involved in toxin modification, i.e., tailoring genes. Thus, there was a good correlation between toxins recorded either by the toxin array or by traditional methods and the species array [17].

The French Coastal Lagoon

Canet Saint Nazaire exhibits a wide annual variation in salinity (9 to 28 PSU average range) according to Casabianca [70]. No obvious cyanobacterial blooms were observed at the Canet Saint Nazaire site during the sampling period. Strong signals were obtained for some toxin pathways, emphasizing the toxic potential of the cyanobacteria sampled throughout the sampling period, being stronger in 2011 than in 2012-13 (Fig. 5). The level of housekeeping gene expression was also moderate to low, no more than 10 times above the background level, which likely indicates relatively inactive cells (Fig. 5C) or a low number of cells. This point emphasizes the need to use this tool as a monitoring device to capture and correctly interpret the population status of the cyanobacterial community. Strong signals were obtained for some toxin pathways, emphasizing the toxic potential of the cyanobacteria sampled throughout the sampling period, being stronger in 2011 than in 2012-13 (Fig. 5).

The level of housekeeping gene expression was also moderate to low, no more than 10 times above the background level, which likely

indicates relatively inactive cells (Fig. 5C) or a low number of cells. Continual monitoring would enable the correct interpretation of the signal.

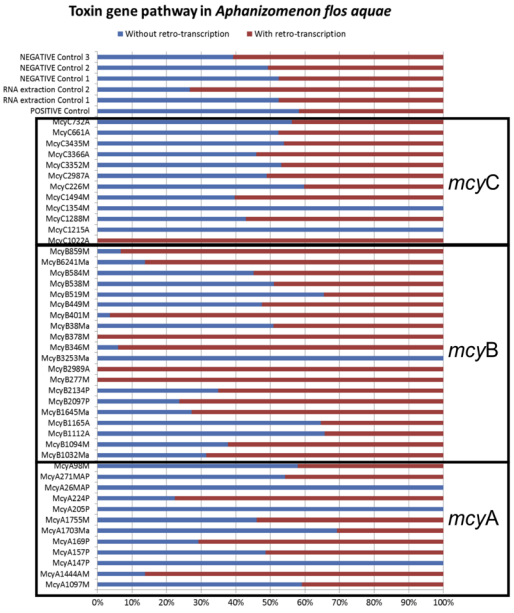

Figure 3: Graphic representation of the signal intensity of the fluorescent signal of the toxin transcript hybridization before and after extension of the transcript by retro-transcription from multiple probes for three gene s from the microcystin pathway as shown in Fig. 2. Redrawn from Kegel et al. [19].

Nodularin is a toxin produced by *Nodularia* using the *Nda*A–I gene cluster. The nodularin pathway was found to be continuously slightly active during all of the sampling campaign showing no peaks in abundance (Fig. 5, Table 4). *Nodularia spumigena*, the causative species producing this toxin, was identified by the ribosomal RNA (rRNA) species microarray but also isolated into culture in the spring and autumn periods (unpublished).

Aeruginosin can be produced by *Nodularia*, *Microcystis* and *Planktothrix*. The three genes on the toxin array were expressed in similar low quantities throughout the sampling period (Fig. 5, Table 4) and could have been produced by *Nodularia* because of its isolation during the sampling period [18].

Cylindrospermopsin is produced by numerous cyanobacteria including *Cylindrospermopsis*, *Anabaena*, *Oscillatoria*, *Raphidiopsis curvata*, *Umezakia natans*, and *Aphanizomenon*. This toxin is produced by two gene clusters: *cyr*A–O and/or *aoa*A–C. The cylindrospermopsin toxin pathway did not show any *cyr* gene expression except for one sample collected on 9 August 2011 (Table 4). However, the *Aoa* gene cluster showed a high expression level for the first period of the sample collection spanning from August 2011 to August 2012 (Fig. 5, Table 4). UPLC/MS-MS found small traces of the toxin in two samples in the spring of 2013.

Saxitoxin is encoded by the *sxt*A–Z genes and is found in *Anabaena circinalis, Aphanizomenon flos-aquae, Aphanizomenon grazile, Cylindrospermopsis raciborskii*, and *Lyngbya wollei*. Expression of genes coding for this neurotoxin was found in the Canet Saint Nazaire samples with particularly high signals in the first sampling year (Fig.5, Table 4). This neurotoxin was not measured in the accompanying UPLC/MS-MS detection studies [18].

Anatoxins were consistently detected by the microsphere or Luminex method, but less so by Ultra-Performance Liquid Chromatography (UPLC)-tandem mass spectrometry (MS-MS) [17,18]. On three occasions, they were above 5 mg/L, but this pathway is not on the present version of the microarray (Table 2). These toxins were detected in Oct of 2011 and in the spring of 2012 and 2013.

Genes for microcystin synthesis and modification were highly expressed especially in the first sampling year. No microcystin toxins were detected by chemical means [17,18]. The highest gene expression of the microcystin biosynthesis pathway involving the *Mcy*A–J gene cluster were the microcystin A, B and E genes, but the other microcystin genes required to produce the toxins were present only at expression level throughout the sampling period, supporting the potential toxicity of *Microcystis*, *Anabaena*, and *Aphanizomenon* (Fig. 5, Table 4) who were found by the species array (Medlin unpublished).

These gene expression patterns tended to support the toxin detection by chemical means in that in no case were the toxins confirmed by the chemical detection of the toxins themselves by UPLC/MS-MS nor by the microsphere methods [17,18] that were not present on the toxin array. This excludes the detection of anatoxins, whose pathway was published after the toxin array was designed and spotted. In the Canet samples, the chemical analyses using UPLC-MS/MS [17,18] detected small amounts of H2-ATX- a and some traces of H2-homo-ATX-a in 3 samples, whereas significant levels of both were detected in all samples using the more sensitive microsphere or Luminex technology. Microcystins were not detected in seawater and brackish water samples as compared to freshwater samples and it was concluded that the UPLC/MS-MS detection suffered from a matrix effect in seawater and brackish water samples (i.e., interference from solutes in the field sample that cannot be removed during the extraction process) as compared to Luminex results [17,18].

A Euclidean distance tree was constructed from the signal intensities (Fig. 5B). The two outliers correspond to the two dates with the strongest microcystin signals. After that the sampling dates tended to cluster by seasons or sequential dates, with the samples from 2011 in two clusters followed by the 2012-2013 samples.

DISCUSSION

The presence of cyanobacteria in aquatic environments leads to the potential risk of cyanotoxin contamination of the environment and to public health. The early detection of cellular growth and bloom monitoring can be measured by cell counts or other means of molecular detection. Measurements of cell densities or pigment contents allow both, but these methods are not sufficiently accurate to predict actual cyanobacterial risk. Even detection by PCR or qPCR may not be a reliable indicator of cyanobacterial toxin risk [71]. Our novel microarray allows for the detection of very low expression of mRNA from cyanobacterial toxin genes by amplifying that signal using RT directly on the microarray. This is the first attempt to use RT directly in a microarray format. A reverse transcriptase reaction can be performed directly on the microarray to extend the mRNAs bound to the capture barcodes on the microarray so that they produce a signal that can be read by the laser. The variation in signal intensity would suggest that the signal intensity is at least semi-quantitative, but this remains to be fully tested. This effectively extends the use of a microarray to detect genes that are expressed in low quantities and provides a new tool for early warning of the toxic potential of any water body. Preliminary field tests suggest that it is more sensitive than the chemical tests, because, in the field tests, there were samples in which cells were detected by microscopic counts [16] where the toxin array detected gene expression of the toxins, but there was no detection by chemical means. This method has an advantage over qPCR methods in that all genes can be detected simultaneously, and the isolation of the mRNA prior to the RT amplification of the signal does not involve any enzymes. qPCR would only detect the presence of toxin genes but not if they were being actively expressed. Natural PCR inhibitors are known to affect positive results [69-72]. Cost comparisons to HPLC are unknown.

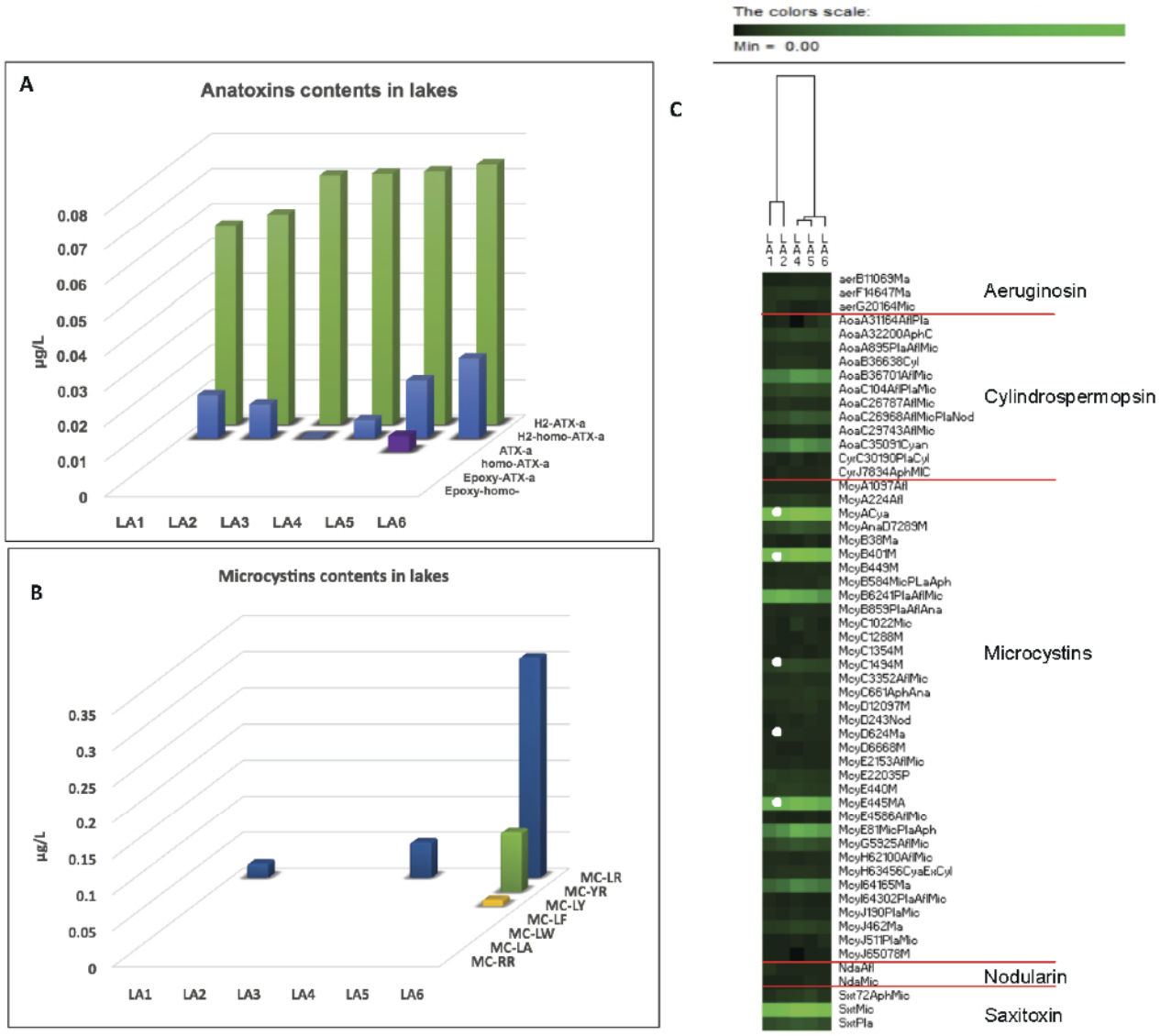

Figure 4: Comparison of HPLC/tandem mass spectrometry (MS-MS) measurements of anatoxin (**A**) and microcystin (**B**) toxins with the heatmap (**C**) of amplified signals of the expression pathways of all genes on the microarray across the Dutch lakes, LA3 was not hybridized because of low mRNA quantity; redrawn from [16]. White dots in (**C**) denote the genes necessary to make microcystin, whereas others are tailoring genes.

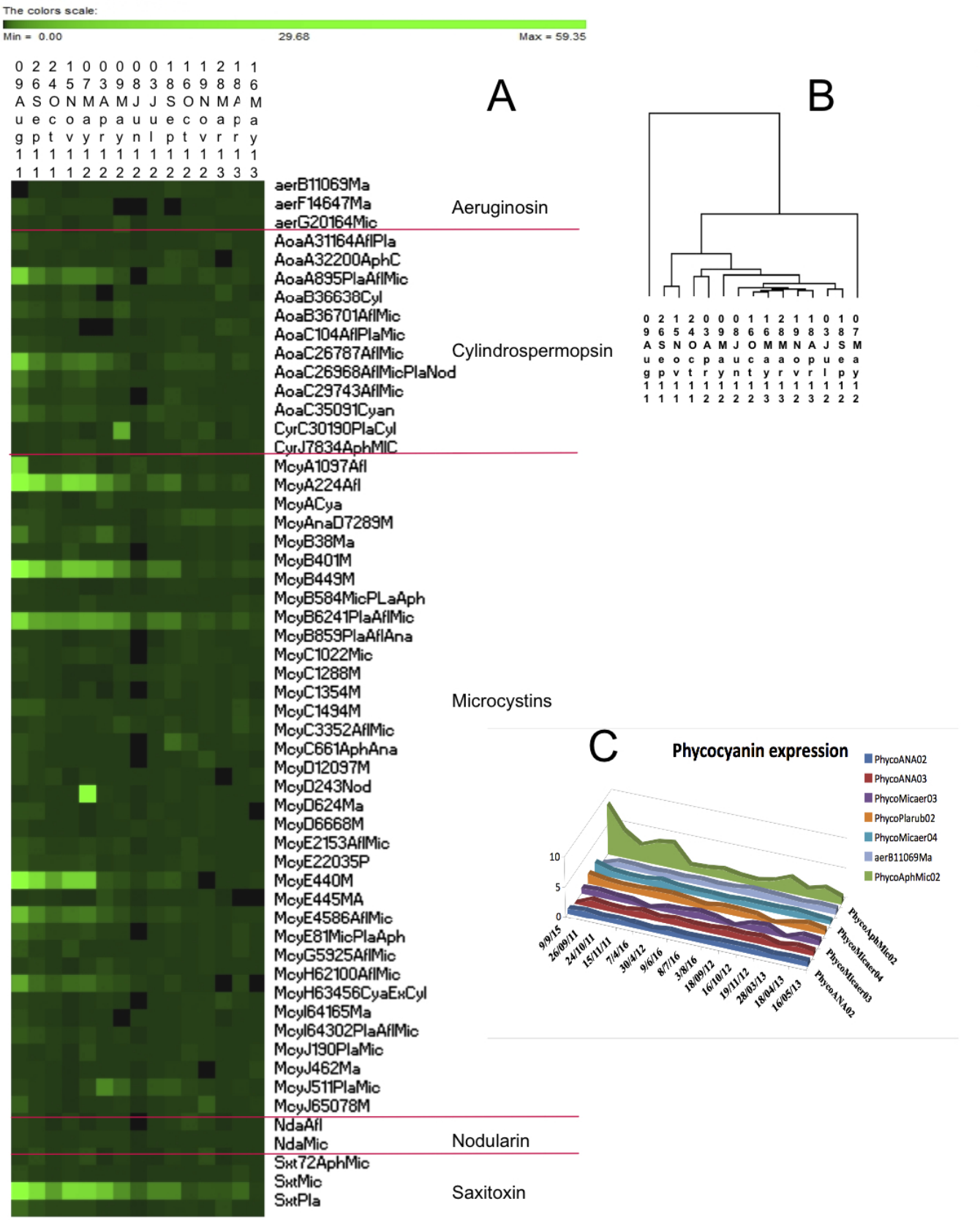

Figure 5: (A) Heatmap of gene of amplified signals of the expression pathways of all genes on the microarray from Canet Lagoon for two years sampling. (B) Euclidean distance clustering of the gene expression in A. (C) Expression of housekeeping genes for the sampling period.

Table 4. Comparison of microsphere data (M) [17] and UPLC-MS/MS data (H) [17,18] with reverse transcriptase (RT) gene expression from Canet Lagoon. Gene expression values over 1.5 are in bold. nt=not tested. For the two cylindrospermopsin pathways, the chemical detection is reported in both pathways although it cannot distinguish the two pathways.

Date	Anatoxin Aeruginosin		Microcystin A-E genes	Microcystin H,I,J genes	Cylindrospermopsin *cyr*A–O genes	Cylindrospermopsin *aoa*A–C genes	Nodularin	Saxitoxin	Phycocyanin	Housekeeping
09/08/11	nt	**RT**	**RT**	**RT**	**RT**	**RT**	RT	**RT**	**RT**	**RT**
26/09/11	nt	**RT**	**RT**	RT	RT	**RT**	RT	**RT**	**RT**	**RT**
24/10/11	M, H	RT	**RT**	RT	RT	RT	RT	**RT**	**RT**	RT
15/11/11	M	**RT**	**RT**	RT	RT	**RT**	RT	**RT**	**RT**	RT
07/03/12	M	**RT**	**RT**	RT	RT	**RT**	RT	**RT**	**RT**	RT
03/04/12	M, H	RT	**RT**	RT	RT	RT	RT	**RT**	RT	RT
09/05/12	M	**RT**	**RT**	**RT**	RT	**RT**	RT	**RT**	RT	RT
08/06/12	M, H	RT	**RT**	RT	RT	RT	RT	RT	RT	RT
03/07/12	M	RT	**RT**	RT	RT	RT	RT	**RT**	RT	RT
18/09/12	M	RT	**RT**	**RT**	RT	RT	RT	RT	RT	RT
16/10/12	M	RT	**RT**	RT	RT	RT	RT	RT	RT	RT
19/11/12	M	RT	**RT**	RT	RT	RT	**RT**	RT	RT	RT
28/03/13	M, H	RT	**RT**	RT	H, RT	H, RT	RT	RT	RT	RT
18/04/13	M, H	RT	**RT**	RT	H, RT	H, RT	**RT**	**RT**	RT	RT
16/05/13	M, H	RT	**RT**	RT	RT	RT	RT	RT	RT	RT

For the Netherlands samples taken on a single day in the summer of 2015, the toxin array results were consistent with the species microarray and cell count results, which showed the presence of the corresponding toxin producer [16] but represent only a snapshot in time. The toxin array detected all toxins detected by UPLC/MS-MS [17,18], except for the anatoxins, which are not on the toxin array. In *Microcystis*, cell counts were high in some lakes where the toxin array showed low expression and in *Aphanizomenon,* gene expression was high where no cells were seen. In that study, low viability was used to explain the first discrepancy, whereas greater sample volumes (20L) as compared to 1 mL were used to explain the second anomaly between cell counts and gene expression.

Notably in the gene cluster for microcystins, *Mcy*C and *Mcy*D were expressed in lower amounts, and their microarray signal was just above the detection level in all of the Dutch lakes. This would suggest either that the population had finished producing its toxins and was becoming senescent, or that these gene products are needed in a lower concentration than the other components of the gene cluster to make the active toxin; both of these explanations are supported by studies on the genetic basis of toxin production [7]. In contrast, anatoxins must have been highly expressed because high amounts of toxins were detected by UPCA/MS-MS, but cell numbers inferred from the microarray species array were notably lower than the actual cell numbers counted [16]. One interpretation of this could be that there was a subset of the *Anabaena* population that was actively growing and producing the toxins, or that the population was dying and that toxin production increased as the cells became senescent.

Gene expression for one gene in the saxitoxin cluster was detected, but no cells capable of producing that toxin were detected, nor were the toxin itself detected chemically. More genes would be needed on the array for each of the genes present for this toxin.

For the Canet Lagoon, no cell counts were made, and only a comparison between chemical analyses and microarray analysis is possible. Several genes in the cylindrospermopsin pathway were detected by the array, but no toxins were detected with either the microspheres or trace amounts were detected with the UPLC methods [17]. Nodularin gene expression was consistent also with the detection of the causative species and its detection on the species array (unpublished), but the toxin was not detected in the two samples measured by UPLC in the companion study made by Greer et al. [17]. Low expression of the genes was detected by the microarray. Relatively high expression of saxitoxin was not confirmed by UPLC/MS- MS tests [17]. These observations would suggest that the toxin array is more sensitive and can detect toxin potential before it can be detected chemically.

The toxin chip is only qualitative but reveals the expression of genes involved in each toxin biosynthesis pathway tested and, in some cases, revealed them before they were detected by chemical means. The toxin chip can serve as a potential early warning system in any monitoring study, which qPCR could only show that species with toxin genes are present. It is clear from these preliminary studies that the toxin array shows great potential. A more in-depth study should be made, making a direct comparison of the gene expression array with toxin measurements by standard methods (UPLC, etc.) with laboratory cultures so that limits of detection and quantification (LOD; LOQ) can be determined. Also, a time series study should be performed in any water body to determine how much in advance of the chemical detection the microarray can detect gene expression of the toxins. The toxin array should be studied in more detail with more toxic cultures and concomitant chemical analysis to determine if the array can detect the expression of the genes before they can be detected chemically. Should this prove to be the case, then the toxin array is more sensitive and could provide an earlier detection of the toxic potential of any water body in any monitoring scheme.

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