Evaluation of a Norwegian-developed ELISA to determine microcystin concentrations in fresh water

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Abstract

Cyanobacteria are known for their extensive and highly visible blooms in rivers or dams in Africa. One of the most important cyanobacteria is *Microcystis aeruginosa* which can synthesise various microcystins that may affect the health of humans and animals. Accurate and efficient detection of microcystins in water is thus important for public and veterinary health. Two enzyme-linked immunosorbent assays (ELISA), a commercially-available ELISA kit (Abraxis) and a newly-developed Norwegian ELISA (putatively cheaper and more robust) were used to detect microcystins in fresh water in South Africa. Water samples were collected monthly at two sites, the Hartbeespoort Dam and a crocodile breeding dam. Extremely high microcystin concentrations (exceeding 360 µg L^{-1}) were detected in the Hartbeespoort Dam during January 2015, whereas the microcystin concentrations in the crocodile breeding dam peaked during March–April 2015. Both ELISAs were positively correlated when analysing water samples 'as is' and following resin adsorption and methanol extraction. However, following resin adsorption and methanol extraction of the water samples, the correlation between the two assays was much stronger. These results suggests that the two ELISAs provide comparable results. If the Norwegian-developed ELISA can be packaged and made available as a user-friendly kit, it could be used successfully in surveillance programmes to monitor microcystin concentrations in fresh water bodies in Africa.

Keywords: correlation, cyanobacteria, ELISA, fresh water, microcystins

INTRODUCTION

Cyanobacteria or blue green algae are known for their extensive and highly visible blooms in rivers or dams in Africa (Ndlela *et al.* 2016). The cyanobacteria synthesise various cyano-toxins that can affect the health of humans as well as terrestrial and aquatic animals (Carmichael 1992; Malbrouck & Kesemont 2006; Bengis *et al.* 2016). The universal occurrence of toxic cyanobacteria as well as concerns about contamination and potential consequences of exposure to cyanotoxins in recreational and potable water have enforced surveillance programmes. In South Africa, about 80 dams were monitored between October 2002 and September 2003 and the results revealed that the Hartbeespoort Dam had high levels of eutrophication (Department of Water Affairs and Forestry 2000). One of the most important cyanobacteria in the Hartbeespoort Dam is *Microcystis aeruginosa* which can synthesise various microcystin congeners ranging in toxicity with microcystin-LR (MC-LR) being one of the most toxic (Van Ginkel 2003; Ballot *et al.* 2014; Ndlela *et al.* 2016). Masango *et al.* (2008) determined the microcystin-LR (MC-LR) congener-independent microcystin concentration expressed in terms of MC-LR in the Hartbeespoort Dam during the winter and summer of 2005–2006, using a commercial ELISA assay (Abraxis Micro-cystins-ADDA ELISA kit) and reported high microcystin concentrations in both seasons.

The cyanotoxins are not destroyed by boiling water (Harada 1996) and not all drinking water purification plants make use of expensive sophisticated purification steps that can remove cyanotoxins (Momba *et al.* 2004). The monitoring of microcystin concentrations in surveillance programmes in fresh water bodies in Africa is thus crucial but, often due to a shortage of funds and skilled manpower, expensive monitoring methodology cannot be employed. It is therefore important to develop a cost-effective, easy-to-use, robust, but also sensitive and reliable, monitoring tool for the detection of these toxins in fresh water. In 2009, Rundberget and co-workers reported the use of toxin adsorbent discs to monitor algal toxins. A resin, contained in a thin layer in the disc, to increase the surface area, adsorbed the toxins in the water. The authors concluded that this adsorbent disc was cheap to produce, easy to deploy, and a convenient method to enhance the collection of the cyanotoxins in water bodies (Rundberget *et al.* 2009). Comprehensive research studies on the extraction and detection of microcystins have led to a number of methods, such as protein phosphatase inhibition assays (Lambert *et al.* 1994), enzyme-linked immunosorbent assays (ELISA) (Fischer *et al.* 2001; Samdal *et al.* 2014), liquid chromatography (McElhiney & Lawton 2005) and polymerase chain reaction (Pedro *et al.* 2013) to determine microcystin concentrations in water samples. However, some of these methodologies require expensive equipment, highly skilled personnel and have a narrow specificity depending on the availability of standards and/or cannot distinguish between very toxic microcystins and less toxic microcystin metabolites.

Samdal *et al.* (2014) reported the development of a very sensitive ELISA with a wide cross-reactivity with microcystin variants and nodularins that could possibly be used in developing countries as a more cost-effective monitoring tool compared to currently-available commercial ELISAs (Samdal, personal communication, 2013). It should be noted that both the Norwegian and commercially available Abraxis ELISAs are indirect competitive ELISAs that will detect microcystins as well as nodularins (Samdal *et al.* 2014), but different approaches were used in their development. The Abraxis ELISA detects the ADDA (4E,6E-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid) amino acid present in the majority of the heptapeptide microcystins and the pentapeptide nodularins. Cross-reactivity

with the following cyanotoxins was established: MC-LR; -RR; -YR; -LW; -LF; 3-desmethyl-MC-LR; 3-desmethyl-MC-RR and nodularin (Fischer *et al.* 2001). For the Norwegian ELISA, instead of using the ADDA moiety of cyanobacterial toxins as immunogen, a mixture of equivalent quantities of MCs (MC-LA; -LF; -LW; -YR and -WR) conjugated to bovine serum albumin was used (Samdal *et al.* 2014). The objective was to recognise the general cyclic peptide structure of microcystins and not specifically the ADDA moiety in microcystins and their less toxic metabolites. Cross-reactivity with the following cyanotoxins was demonstrated: MC-LR; -RR; -YR; -LA; -LF; -LY; -LW; [Dha7]MC-LR; [Dhb7]MC-RR and nodularin-R (Samdal *et al.* 2014). The aim of this study was to compare the sensitivity and accuracy of this newly-developed Norwegian ELISA with the commercially-available Abraxis ELISA kit to detect microcystins in surface water.

METHODS

Water samples

Sub-surface water samples (5 L) were collected every month (August 2014–September 2015) from two collection sites, the Hartbeespoort Dam (S 25° 44.904′; E 27° 50.149′) and a commercial crocodile farm's breeding dam, 17 km downstream of the Hartebeespoort Dam, and transported to the laboratory in a cooler box with ice bricks. The water samples were subjected to three consecutive freeze (−20°C)/thaw cycles and sonicated for 5 min to rupture the cyanobacterial cells to release the intracellular microcystins. Following sonication, particles were removed by filtration through a nylon net (90-µm mesh size).

Microcystin extraction

To extract the microcystins, 3 g of Dialon HP-20 resin (Sigma-Aldrich) was activated as previously described (Singo *et al.* 2017), mixed with 1 L of the water sample and continuously shaken for 19 h on a linear shaker (Model 202, Labotec). The resin water mixture was then transferred to a 25 mL Varian Bond-elute reservoir fitted with non-absorbent cotton wool plug. The resin that settled at the bottom was washed free of salts using 30–50 mL ultrapure water (Milli-Q50 France). A plunger from a 20-mL syringe was used to remove excess water from the column. The adsorbed microcystin was removed from the resin by gently mixing the resin with 10 mL methanol in the column and leaving it for 15 min to stand before eluting the column at a slow rate of 0.5–1 drops s^{-1}. The methanol (10 mL) extraction step was repeated before finally flushing the column with an additional 3 mL methanol to remove any remaining microcystins. The methanol extract was transferred to screw cap glass tubes, the final volume recorded and adjusted to 23 mL before storing at −20 °C until further analysis.

Enzyme linked immunosorbent assay (ELISA)

Abraxis microcystin ELISA
The assay, packaged as a kit, was performed following the manufacturer's instructions provided on the package insert (Abraxis Microcystins-ADDA ELISA kit information sheet). The kit contained one microtiter plate consisting of 12 removable strips of eight wells. The strips were pre-coated with the known antigen. All the reagents were supplied ready to use.

Analyses of the water samples ('as is') and the resin-adsorbed methanol-extracted water samples were conducted in duplicate. The methanol-extracted samples were diluted with the sample diluent provided to ensure that the concentration of the methanol did not exceed 5% to avoid false positive results. Absorbance was read at 450 nm using a microplate reader (Biotek Synergy HT, Software GEN 5.1) within 15 min after the addition of the 'stop solution' to quench the reaction. The total incubation time of the three major incubation steps was 150 min. Abraxis ELISA data were analysed using the four-parameter logistic fitting with Excel Solver for Microcystin ELISA Version 20060924 (ELISA Software) supplied with the Abraxis kit.

Norwegian-developed microcystin ELISA
Unlike the Abraxis microcystin ELISA, the assay was not packaged as a kit. The plate-coating antigen [Asp3]MC-RY, the primary antibody (raised in sheep), a few pre-coated plates for trial studies and the Standard Operating Procedure (SOP) were supplied by the developers. Additional microplates and all other reagents as per SOP were obtained from commercial suppliers. The commercial reagents and plates were not ready for immediate use and the plates had to be coated and buffers prepared before each assay. The user had to adjust the incubation conditions (i.e. reagent concentrations) to compensate for reagent batch differences.

An ELISA plate was pre-coated overnight with the coating antigen as supplied. Analyses of the water samples ('as is') and the resin-adsorbed methanol-extracted water samples were conducted in duplicate. Sample dilutions of 1.2× and 10× for water and methanol extracts, respectively, were incorporated into the first step of the assay to prevent matrix interferences. The extracts were diluted to contain less than 10% methanol. Sample analysis was performed as described by the SOP (Norwegian Veterinary Institute, Oslo, Norway). Absorbance was read at 450 nm using a microplate reader (Biotek Synergy HT, Software GEN 5.1) within 15 min after the addition of the 'stop solution' (10% H_2SO_4) to quench the reaction. The total incubation time of the three major incubation steps was 195 min. The Norwegian ELISA data were analysed with the software programme provided by the Norwegian Veterinary Institute in Oslo, Norway.

Liquid chromatography–high resolution mass spectrometry

Details of the chemicals and equipment were described previously by Singo *et al.* 2017.

Preparation of the calibration curve
A combined standard containing MC-LR, MC-RR and MC-YR was prepared in methanol and diluted to obtain a calibration range between 500 and 62.5 µg L^{-1}. Spiked water samples were analysed by liquid chromatography–high resolution mass spectrometry (LC-HRMS) to determine the percentage recovery of the three microcystin variants.

Analysis of water samples
The methanol-extracted water samples were evaporated under nitrogen and dissolved in 200 µL methanol. An aliquot of each sample (20 µL) was diluted to total volume of 520 µL and analysed. Samples that exceeded the calibration range were diluted further and analysed.

High performance liquid chromatograph conditions

The high performance liquid chromatography consisted of a gradient pump coupled to a C_{18} analytical column. The mobile phase was a gradient of water to acetonitrile, both containing 5 mM ammonium acetate and 1.0% formic acid. A linear gradient was used.

High resolution mass spectrometer conditions

Mass spectrometric analysis employed a Thermo Fisher QExactive HRMS (Thermo Fisher, USA) controlled by Xcalibur software. The scan range was from 100 to 1,100 *m/z* units with a resolution of 140,000 in positive mode. Quantitative analyses were performed using Xcalibur Quanbrowser software.

Statistical analyses

The microcystin concentrations by month at two sites (the Hartbeespoort Dam and a crocodile breeding dam downstream thereof and supplied by the Hartbeespoort Dam), were captured. The sample Pearson product-moment correlation coefficient (*r*) between pairs of microcystin analyses was determined using robust Bayesian parameter estimation (Kruschke 2013) in R (R Core Team 2012) and JAGS (Plummer 2003). The paired microcystin analyses compared ELISAs (Norwegian versus Abraxis, with or without extraction), extraction method (water 'as is' versus resin-adsorbed methanol-extracted, within assays), and compared LC-HRMS to ELISA analyses (Abraxis and Norwegian). All paired measurements were matched by site and month. To compare the two water collection sites, the Bayesian equivalent of a paired sample *t*-test was conducted, following Kruschke (2013). Microcystin data approximately followed a lognormal distribution, thus data were log-transformed using log(x + 0.01) where appropriate. Effect sizes and group means are reported as back-transformed values, using exp(y)-0.01.

RESULTS AND DISCUSSION

Initially, during the months of August–October 2014, the microcystin concentrations, as determined by both ELISAs, were higher in the crocodile breeding dam than in the Hartbeespoort Dam (Table 1). This, however, changed from December 2014 when the microcystin concentrations in the Hartbeespoort Dam were generally higher. Microcystin concentrations at the Hartbeespoort Dam peaked during the summer months (December–January), which coincided with warmer climatic conditions and rainfall with subsequent run-off of nutrients into the dam. The high microcystin concentrations detected in the Hartbeespoort Dam are not surprising, as the dam has been renowned for its poor water quality since the mid-20th century (Allanson & Gieskes 1961). Masango *et al.* (2008) also reported higher microcystin concentrations during the summer compared to the winter, with the winter levels 1,000 times higher than the prescribed World Health Organisation (WHO) guideline value of 1 μg L^{-1} (1 ppb) for total microcystin for drinking water (WHO 2011). The highest microcystin concentrations were detected later in the season at the crocodile breeding dam, during March–April 2015 (Table 1).

Table 1 | Microcystin concentrations expressed as $\mu g\,l^{-1}$ in the 1 L water sample for the Norwegian ELISA, Abraxis ELISA and the LC-HRMS analysis

| Month | Water analysed 'as is' | | | | Water analysed following resin adsorption and methanol extraction | | | | | |
| | Hartbeespoort Dam | | Crocodile breeding dam | | Hartbeespoort Dam | | | Crocodile breeding dam | | |
	Norwegian[a]	Abraxis[b]	Norwegian	Abraxis	Norwegian	Abraxis	LC-HRMS[c]	Norwegian	Abraxis	LC-HRMS
Aug 2014	<0.04	0.14	0.19	4.18	0.12	0.15	0.01	2.59	2.92	0.65
Sept 2014	3.85	0.19	0.19	7.56	0.06	0.2	0.01	3.78	4.15	0.59
Oct 2014	<0.04	1.80	0.17	8.51	0.86	1.31	0.18	0.47	1.56	0.12
Nov 2014	0.19	1.07	<0.04	1.98	0.17	0.29	0.11	0.09	0.40	<LOD
Dec 2014	18.65	83.75	<0.04	1.09	473	43.71	86.35	0.07	0.24	0.01
Jan 2015	6.8	422.07	2.15	9.26	>1,950	362.76	368.79	5.53	3.25	2.62
Feb 2015	2.02	5.89	1.14	4.34	8.44	7.51	3.53	9.35	7.66	5.08
Mar 2015	12.92	36.23	7.83	45.61	0.47	114.42	76.86	15.79	13.92	8.27
Apr 2015	7.23	8.07	7.25	150.72	4.0	11.89	1.24	9.2	9.74	4.09

[a]LOD = 0.04 $\mu g\,l^{-1}$.
[b]LOD = 0.10 $\mu g\,l^{-1}$.
[c]LOD = 0.002 $\mu g\,l^{-1}$.

Table 2 | Results of Bayesian *t* tests on paired differences in microcystin concentrations in resin-adsorbed methanol-extracted water samples and water analysed 'as is' by the Abraxis ELISA (μg L^{-1}), the Norwegian ELISA (μg L^{-1}), and LC-HRMS (μg L^{-1})

Method	Paired difference (Hartbeespoort Dam minus Crocodile breeding dam)		
	μ	HPDI[a]	Probability of $\mu > 0$
LC-HRMS[b]	15.44	−22.94–93.35	60.9%
Abraxis (water 'as is')	10.66	−69.56–118.02	49.8%
Abraxis (resin/methanol extraction)	16.21	−28.93–94.78	62.8%
Norwegian (water 'as is')	2.75	−1.24–7.46	92.4%
Norwegian (resin/methanol extraction)	−2.35	−9.28–4.58	15.3%

Water samples were collected from August 2014–September 2015 at the Hartbeespoort Dam and at a commercial crocodile farm's breeding dam 17 km downstream of the Hartebeespoort Dam.
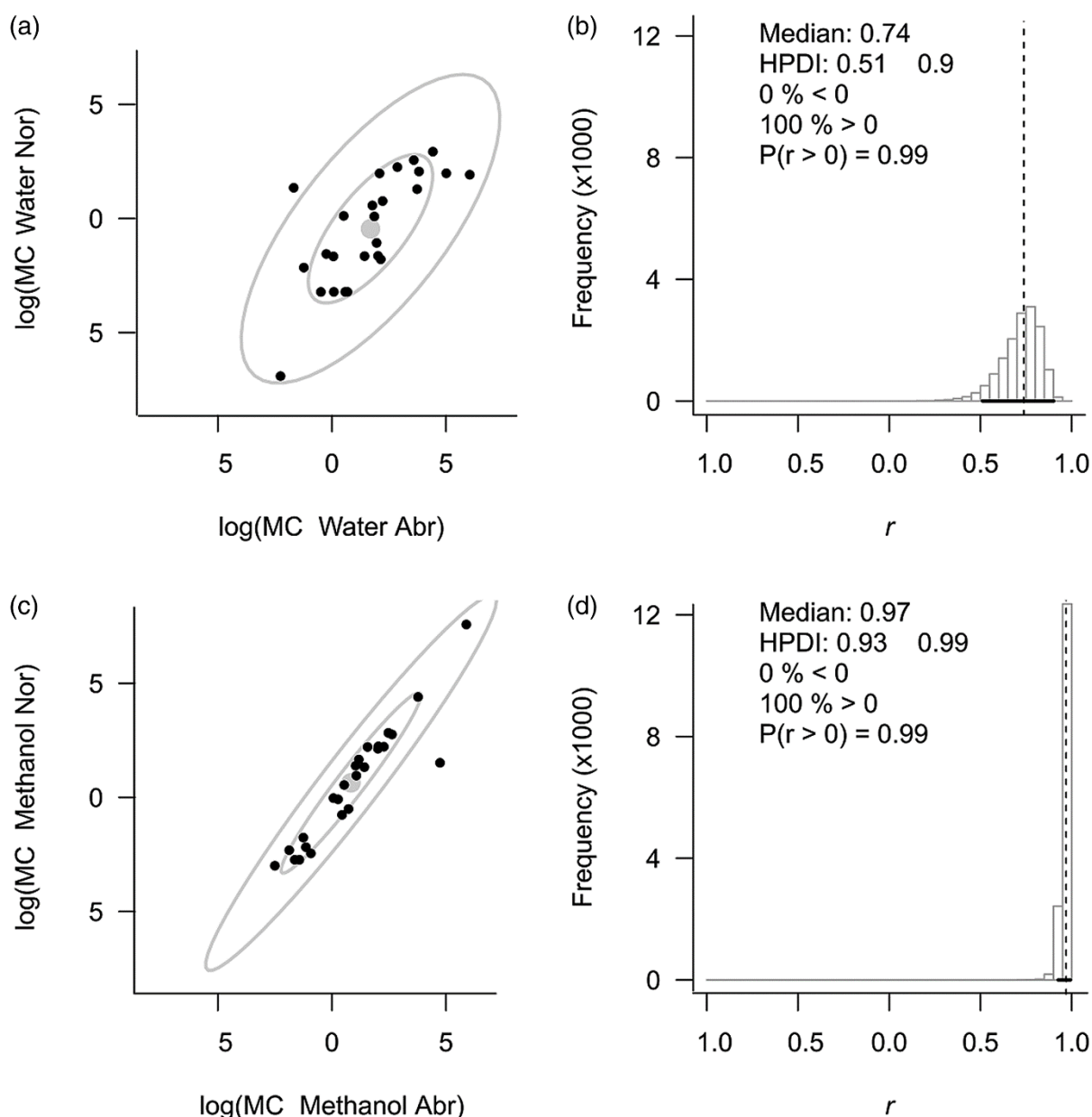[a]HPDI = Highest posterior density interval (Bayesian credible interval).
[b]LC-HRMS = only MC-LR, MC-RR and MC-YR.

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Monthly microcystin concentrations at the two sites (Table 1) appeared to differ between the Norwegian and Abraxis ELISAs. In general, the microcystin concentrations of the water samples analysed 'as is' with the Abraxis ELISA were higher than the Norwegian ELISA. However, following resin adsorption and methanol extraction, the sensitivity of the Norwegian ELISA improved and the microcystin concentrations were similar to those measured with the Abraxis ELISA (Table 1). LC-HRMS analysis of spiked water samples with three MC congeners (i.e. MC-LR; -RR and -YR) indicated poor recoveries. Percentage microcystin recovery ranged from 37–68%. Nonetheless, when statistically analysed across the entire sampling period, the microcystin concentrations between the two collection sites did not differ. The Bayesian credible intervals (highest posterior density intervals) on the paired differences between sites from each month covered zero for all methods used (Table 2). The sampling effort could be increased in a future study to detect a possible temporal shift in peak microcystin concentrations which is suggested anecdotally by the monthly samples.
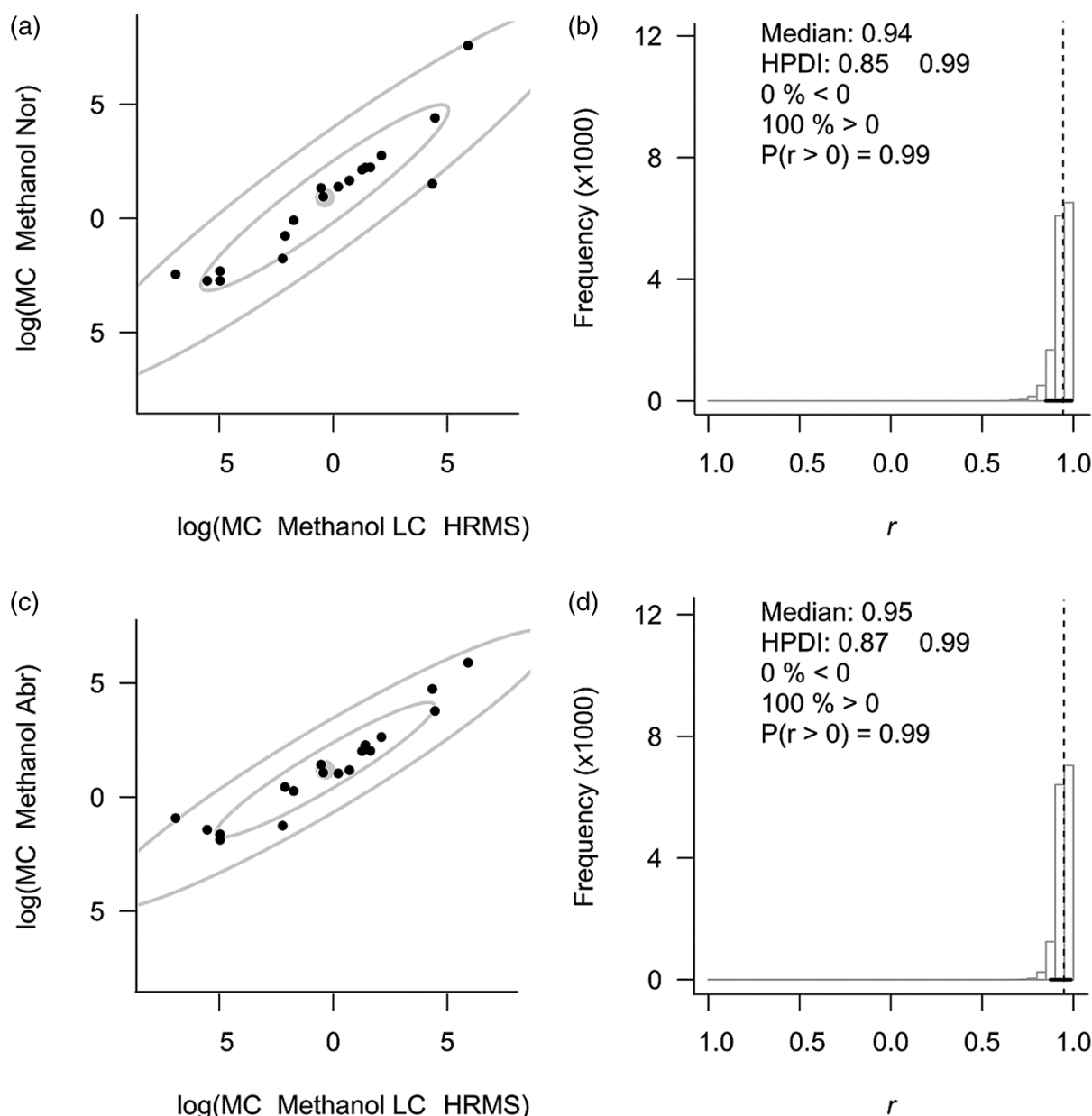
The two ELISAs produced log-transformed microcystin concentrations that were positively correlated (Figure 1(a) and 1(c)), however, the correlation was much weaker when comparing the two ELISAs using water 'as is' (Figure 1(a)). Where the microcystin concentrations were very low in water samples, the buffers which were added to avoid the matrix effect when using the Norwegian ELISA diluted the samples even further. When the frozen and thawed water samples were adsorbed to a resin and extracted with methanol, the microcystin concentrations as determined with both ELISAs were highly positively correlated, (Figure 1(c)), which suggests that the two ELISAs provided comparable results. Test interferences, caused by matrix effects, cannot be completely excluded due to the high variability of compounds that may be present in water samples. To prevent the interference of matrix effects in the Norwegian ELISA a 1.2× dilution of the water samples were performed, but the small dilution factor probably cannot explain the weaker correlation (Figure 1(a)). To avoid matrix effects all samples containing methanol were diluted to contain less than 5% and 10% methanol for the Abraxis and Norwegian ELISAs, respectively. Although the methanol extracts were diluted more than twice for the Abraxis ELISA compared to the Norwegian ELISA, it did not seem to have an effect on the positive correlation of the results between the two assays (Figure 1(c)).
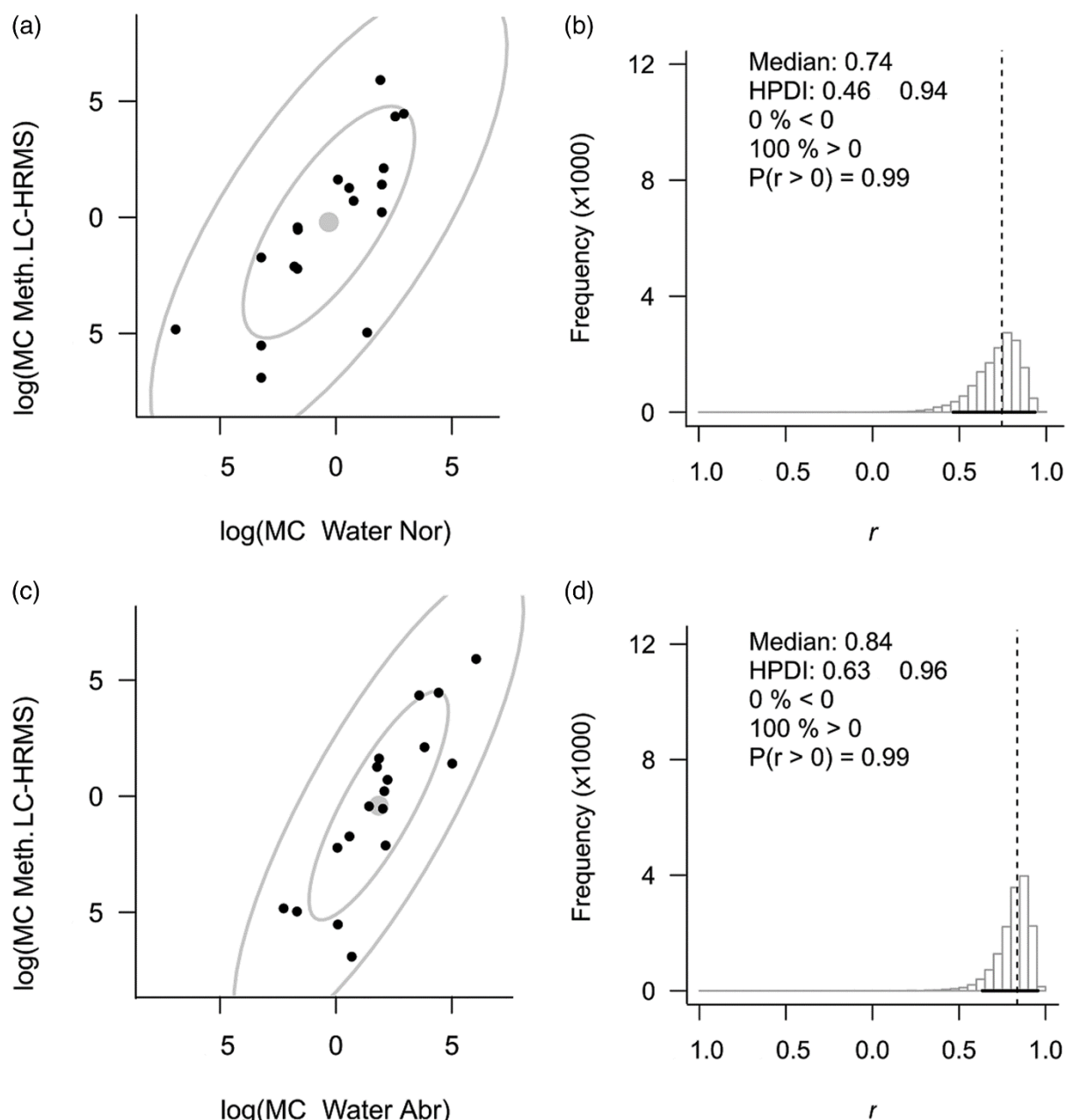
Figure 1. Correlation between log-transformed microcystin (MC) concentrations measured using the Abraxis (Abr) and Norwegian (Nor) ELISAs. Water used 'as is' (a and b), and resin adsorption/methanol extraction (c and d). (a and c) Model fit of the estimated bivariate *t*-distribution (ellipses covering 50% and 95% of the distribution) relative to the log-transformed raw data. (b and d) Bayesian posterior distribution on the sample Pearson product-moment correlation coefficient (*r*). The highest posterior density interval (HPDI) is depicted with the thick horizontal line (b and d).

For all resin-adsorbed methanol-extracted samples, the log-transformed LC-HRMS microcystin concentrations and the log-transformed Abraxis or Norwegian ELISA microcystin concentrations were also highly (positively) correlated (Figure 2(a) and 2(c)). If the LC-HRMS analysis provides a comparative standard, then both ELISA methods (Norwegian and Abraxis) performed very well relative to the LC-HRMS results.
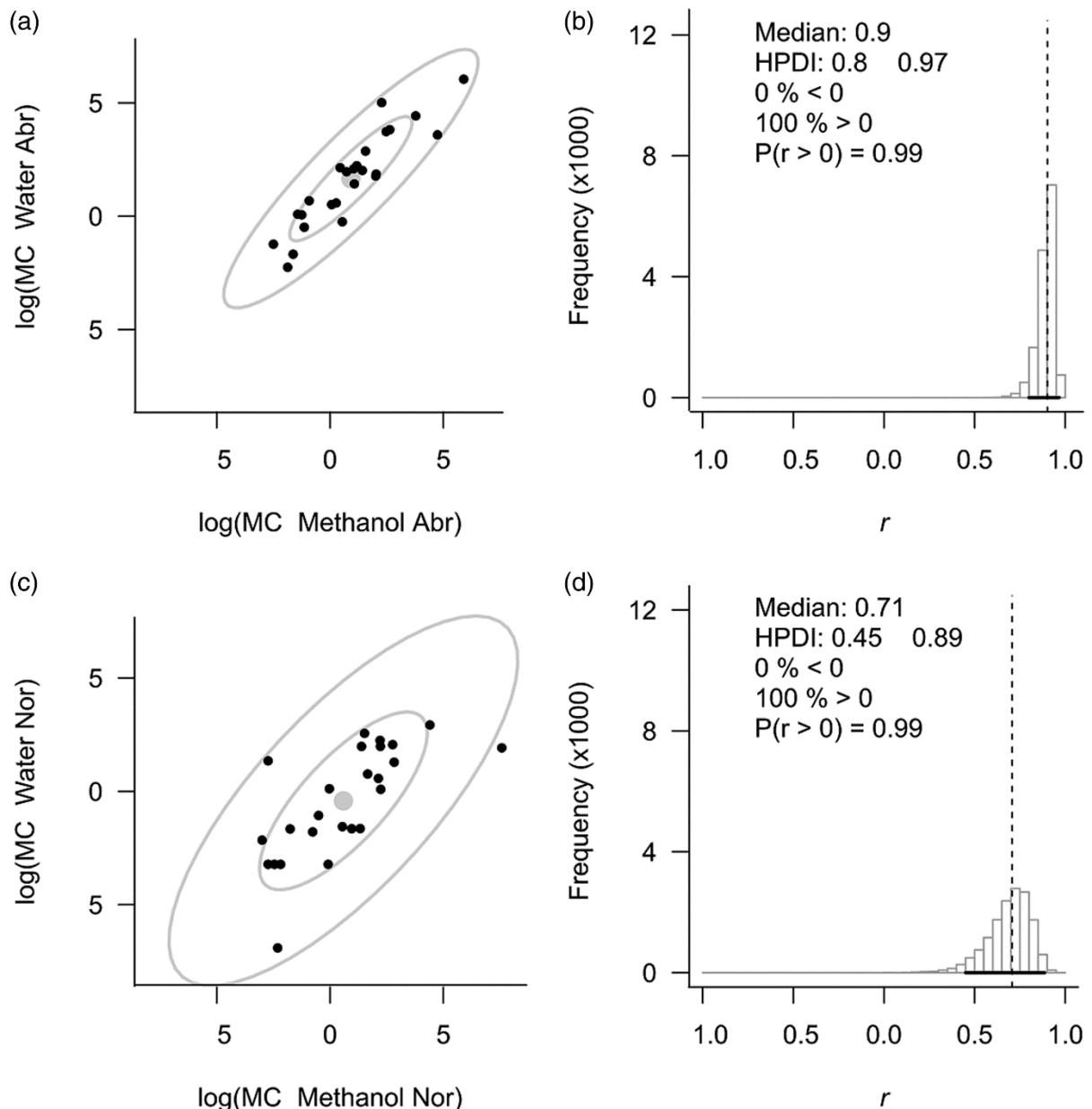
Figure 2. Correlation of log-transformed microcystin (MC) concentrations determined after resin adsorption/methanol extraction between (a and b) Norwegian ELISA (Nor) and liquid chromatography-high resolution mass spectrometry (LC-HRMS) and between (c and d) Abraxis ELISA (Abr) and LC-HRMS. (a and c) Model fit of the estimated bivariate *t*-distribution (ellipses covering 50% and 95% of the distribution) relative to the log-transformed raw data. (b and d) Bayesian posterior distribution on the sample Pearson product-moment correlation coefficient (*r*). The highest posterior density interval (HPDI) is depicted with the thick horizontal line (b and d).

When comparing water analysed 'as is' with both the Abraxis and Norwegian ELISAs and water analysed following resin adsorption and methanol extraction with LC-HRMS, the correlation was much weaker, but still positive (Figure 3(a) and 3(c)). Within a given ELISA (Abraxis or Norwegian), the correlation of microcystin concentrations between water used 'as is' and water after resin adsorption/methanol extraction was strong (positive) for the Abraxis ELISA (Figure 4(a)), but considerably weaker for the Norwegian ELISA (Figure 4(c)). It appears as though analysing water samples after adsorption to a resin followed by methanol extraction is more reliable than analysing water 'as is' in both ELISAs.

Figure 3. Correlation between water analysed 'as is' with the two ELISA methods, the Norwegian ELISA (a and b) and the Abraxis ELISA (c and d), and liquid chromatography-high resolution mass spectrometry (LC-HRMS). (a and c) Model fit of the estimated bivariate *t*-distribution (ellipses covering 50% and 95% of the distribution) relative to the log-transformed raw data. (b and d) Bayesian posterior distribution on the sample Pearson product-moment correlation coefficient (r). The highest posterior density interval (HPDI) is depicted with the thick horizontal line (b and d).

Figure 4. Correlation between water analysed 'as is' and resin-adsorbed methanol-extracted log-transformed microcystin (MC) concentrations measured using (a and b) Abraxis ELISA (Abr) and (c and d) Norwegian ELISA (Nor). (a and c) Model fit of the estimated bivariate *t*-distribution (ellipses covering 50% and 95% of the distribution) relative to the log-transformed raw data. (b and d) Bayesian posterior distribution on the sample Pearson product-moment correlation coefficient (*r*). The highest posterior density interval (HPDI) is depicted with the thick horizontal line (b and d).

Pedro (2013) assessed microcystin concentrations in fresh water reservoirs in Mozambique using the Abraxis ELISA kit and concluded that although it is a good screening tool to determine microcystin concentrations in fresh water bodies, the Abraxis ELISA kit is expensive. Kits imported from the USA and Europe are perceived to be expensive by researchers in African countries, because of the poor exchange rates of the African currencies and import duties. Additional factors contributing to the costs are the impor-tation of small numbers of kits directly from international manufacturers due to the absence of distributors in Africa and using a kit partially when only a few samples need to be tested. On this point, the Abraxis kit offers an advantage as a subset of the microtitre plate can be used when individual removable strips covering eight wells each are removed. These

could be used separately allowing the analysis of a few samples on different occasions, provided that a standard curve is included for each run.

The Norwegian researchers' aim was to provide a more affordable microcystin ELISA to African countries for surveillance purposes (Samdal, personal communication, 2014). If the robustness of the two assays is compared, the Norwegian ELISA was more robust in terms of sensitivity and methanol extract matrix effects. However, in terms of water matrix effects, the Abraxis ELISA was more robust and the reference range, measuring microcystin concentrations, is wider.

CONCLUSIONS

The Norwegian ELISA gave comparable results and may provide a cheaper, rapid and robust alternative method to quantify low microcystin concentrations. If the Norwegian ELISA can be simplified and supplied as an inexpensive kit it could probably be used in surveillance programmes to monitor microcystin concentrations in fresh water bodies in Africa.

ACKNOWLEDGEMENTS

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