New Jersey Drinking Water Quality Institute Testing Subcommittee

Report on the Development of a Practical Quantitation Level for Microcystin in Drinking Water

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Overall Recommendation

This report presents the Drinking Water Quality Institute (DWQI) Testing Subcommittee's evaluation of analytical method availability and sensitivity for detection of the cyanotoxin microcystin in finished drinking water and the recommendation for an analytical Practical Quantitation Level (PQL) for the cyanotoxin microcystin in finished drinking water. A PQL is the minimum concentration at which a contaminant can be reliably quantitated within acceptable limits of uncertainty. This report will be used in conjunction with the information generated by the Health Effects Subcommittee and Treatment Subcommittee in recommending drinking water standards for cyanotoxins.

Several approaches were used by the Testing Subcommittee to derive a PQL for microcystin, and the resulting PQLs from those approaches were considered in the final determination of the PQL. The value of 0.3 μg/L (microgram per liter) was recommended as the PQL by the Testing Subcommittee. The background and the specific approaches used to derive the PQL are presented below.

Executive Summary

The New Jersey Department of Environmental Protection Division of Science and Research (NJDEP-DSR) recommended drinking water guidance values for microcystin of 0.07 µg/L for children less than 6 years of age and 0.3 µg/L for older individuals (NJDEP 2021). The drinking water guidance values recommended by NJDEP-DSR are more stringent than the drinking water Health Advisories (HAs; non-enforceable guidance values for contaminants not regulated with a National Primary Drinking Water Regulation) established in 2015 by the United States Environmental Protection Agency (USEPA) for microcystin of 0.3 µg/L for bottle fed infants and children less than 6 years of age and a higher value of 1.6 µg/L for older individuals (USEPA 2015a).

In December 2021, the Drinking Water Quality Institute (DWQI) began evaluating cyanotoxins, including microcystin and several others (DWQI 2022). In October 2022, the DWQI Health Effects Subcommittee released a memorandum summarizing its review of the Reference Doses and drinking water guidance values developed by NJDEP-DSR for microcystins and three other cyanotoxins, cylindrospermopsin, anatoxin-a, and saxitoxin (NJDOH 2022). The Health Effects Subcommittee supported the use of NJDEP-DSR's drinking water guidance for the four aforementioned cyanotoxins.

The Testing Subcommittee evaluated existing certified analytical methods for detecting the cyanotoxin microcystin, including USEPA Method 546: Determination of Total Microcystins and Nodularins in Drinking Water and Ambient Water by Adda Enzyme-Linked Immunosorbent Assay, and whether these methods could support the Health Effects Subcommittee recommendation of 0.07 of µg/L for microcystins (Zaffiro et al 2016).

The Testing Subcommittee reviewed a two-part scientific evaluation conducted by NJDEP into the testing of microcystin with an enzyme linked immunosorbent assay (ELISA) using the ADDA-OH kit, a biological assay which was validated for UCMR4 as USEPA Method 546. This performance data was used to validate an (at the time of the experiment) unapproved modification to Method 546, the Streptavidin Enhanced Sensitivity (SAES) assay. Data from this validation study was used in support to determine the PQL. A round robin analysis was conducted with partner laboratories which were conducting cyanotoxin monitoring in drinking water systems using the data obtained during the validation study. Based on the results generated from the round robin study, and from the minimum reporting levels (MRLs) obtained from the performance data obtained from labs certified for EPA 546 a PQL of 0.3 µg/L was recommended.

It was recommended that any result ≥ 0.3 µg/L for total microcystins in finished drinking water be followed up with confirmation by USEPA Method 544: Determination of Microcystins and Nodularin in Drinking Water by SPE and LC-MS/MS Detection (Shoemaker et al 2015). Additionally, any modifications to USEPA Method 544, such as analysis of 3-methoxy-2-methyl-4-phenylbutyric acid (MMPB), an oxidation product of microcystin, are not approved modifications at this time, per USEPA, and may result in bias detections.

While this report primarily addresses microcystin through USEPA Method 544 and Method 546, the DWQI Health Effects Subcommittee recommended health-based drinking water guidelines for three additional cyanotoxins which were not evaluated as part of the Testing Subcommittee's review or NJDEP's round robin study. Cylindrospermopsin and anatoxin-A have a USEPA approved methodology, USEPA Method 545: Determination of Cylindrospermopsin and Anatoxin-a in Drinking Water by Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC/ESI-MS/MS; USEPA 2015b), as well as an existing ELISA analytical methodology (ELISA; USEPA 2016). The last cyanotoxin, saxitoxin, does not have an USEPA approved methodology but does have an existing ELISA analytic methodology with extensive usage in the food industry for shellfish testing. The NJDEP-DSR recommended drinking water guidance values for cylindrospermopsin, anatoxin-a, and saxitoxin in finished drinking water are within the range of quantification for the ELISA methodology for these toxins. However, additional research is needed for the development of a PQL for these cyanotoxins, due to the limited number of laboratories performing these methods.

Analytical Methods

Two laboratory methods are available for the detection and quantification of microcystin, a toxin produced by some cyanobacteria in freshwater: a biological assay known as enzyme linked immunosorbent assay (ELISA; i.e., USEPA Method 546), and an analytical chemical method using liquid chromatography with tandem mass spectrometry (LC- MS/MS), such as USEPA Method 544:Determination of Microcystins and Nodularin in Drinking Water by SPE and LC-MS/MS Detection.

It is important to note that while USEPA Method 546 is a method which detects total microcystins, USEPA Method 544 detects only six of the several hundred microcystins that are known to exist plus nodularin and therefore cannot be used to quantitate total microcystins. During a bloom event where microcystin is being produced, the specific congener(s) may change, being influenced by both biotic and abiotic factors. While USEPA Method 544 is more precise, it is more limited in scope. This is why USEPA recommends Method 544 for confirmation testing but not as a first-round screening method.

As part of the fourth iteration of the Unregulated Contaminant Monitoring Rule (UCMR4), the USEPA required 800 randomly selected small public surface water and ground water under the direct influence of surface water systems (total served populations of $\leq$ 10,000) and all large public surface water and ground water under the direct influence of surface water systems (total population served >10,001) to monitor for a list of ten cyanotoxins for a four-month period from March 2018 through November 2020 (USEPA 2016). Prior to the start of UCMR4 monitoring, validated analytical methods for determining and quantifying cyanotoxin levels in ambient and finished drinking water were developed and released by the USEPA.

Based on data from numerous sources, microcystins appear to be the most commonly produced cyanotoxin in ambient waters (Bláha et al., 2009). Microcystins are a group of >200

congeners, many of which appear to be metabolic or transformative (natural or otherwise) products of other microcystin congeners (Bouaïcha et al., 2019) of varying toxicity and stability. Microcystin-LR (for the amino acids leucine [L] and arginine [R]) appears to be the most commonly produced and detected (Catherine et al., 2017) of the >200 microcystin congeners. Because of structural similarity of the microcystin congeners, a region that is present in all congeners known as 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (ADDA) is generally targeted and analyzed to determine the total microcystin concentration in a sample. Additionally, because of structural homology with microcystins, another cyanotoxin known as nodularin is also detected when using this analysis.

EPA Method 546 is a biochemical assay for the quantification and detection of total microcystin in either ambient (surface) water or finished drinking water. The method lists a MRL of 0.3 µg/L and can often be performed in a laboratory setting with existing equipment, resulting in less capital investment than is needed for other methods. Generally, it takes only a few hours from sample collection to data generation.

Laboratories that conduct USEPA Method 546 can assemble their own biological assay or purchase a complete kit (ADDA-OH) from a commercial vendor. The kit, at a minimum, must contain standards for development of a standard curve needed for each run, test blanks, and additional verification material to ensure that microcystin is not being falsely or inaccurately detected in samples. The ADDA-OH kit, which was USEPA Environmental Technology Verification approved and validated initially by Ohio USEPA, is a total microcystin detection assay which targets the ADDA region on the microcystin molecule. USEPA Method 546 outlines how to perform the steps required to set up this assay using individual components.

EPA Method 544 is an analytical chemistry-based analysis method that requires more capital investment and preparation time for analysis. It is also more limited in terms of the specific congeners that it detects, which are six congeners of microcystin and nodularin (seven total cyanotoxins). While the USEPA Method 544 MRL is orders of magnitude lower than for USEPA Method 546, with the ability to quantify down to between 0.003 µg/L and 0.02 µg/L (Shoemaker et al 2015), there are also limitations to use of the method. Compared to USEPA Method 546, the analysis and preparation times for samples are longer, and Method 544 requires more handling considerations than Method 546, as will be discussed later in this report. USEPA Method 544 is also more costly to perform, and very few laboratories currently have this testing capability.

UCMR4 required a phased approach for microcystin analysis in which samples were initially screened using USEPA Method 546, and any sample that exceeded the MRL of 0.3 µg/L was then analyzed with USEPA Method 544 for confirmation (USEPA 2016). There are limitations to the validity of this approach, since results from USEPA Method 546 reflect the total concentration of all congeners, including the metabolic and transformative products, while results from USEPA Method 544 reflect the concentrations of only six congeners.

During the period of review for the development of this Testing Subcommittee report, it was discovered that some New Jersey utilities were using an ADDA test kit during routine surveillance monitoring that utilizes a variation of detection chemistry (i.e., Streptavidin enhanced sensitivity [SAES]) kit) that was not an approved modification to USEPA Method 546 at the time. The SAES kit has higher sensitivity and was eventually validated by USEPA in USEPA Method 546 to have a lower MRL of 0.1 µg/L than the ADDA-OH kit MRL of 0.3 µg/L. USEPA has now confirmed that the SAES test kit is an allowed modification to USEPA Method 546 (personal communication with USEPA). Data Sources for PQL Determination for USEPA Method 546 (ADDA-OH).

NJDEP has traditionally used the PQL as the practical analytical data quantitation limit for individual parameters that it regulates. To determine the PQL, the MDL, low-point on the calibration curve, and reporting limit intralaboratory data from a minimum of 5 analytical laboratories (certified laboratories if a promulgated method is approved by the NJDEP/DSR Office of Quality Assurance) are pooled. Since the MDL is not calculated as described in the 40 CFR Appendix B to Part 136 - Definition and Procedure for the Determination of the Method Detection Limit - Revision 2 (ECFR 2024) as part of the method performance procedures in USEPA Method 546, only the MRL calculation and other quality control measures are reported and summarized in this document.

MRLs from NJDEP certified laboratories

NJDEP's Office of Quality Assurance (OQA) currently certifies the four (4) external laboratories shown in Table 1 below for drinking water (3 labs) and/or non-potable water (2 labs) analyses by USEPA Method 546 as well as the NJDEP Bureau of Freshwater and Biological Monitoring (BFBM) laboratory. It should be noted that these are not the same laboratories that participated in the round robin study previously discussed for the part two of the analysis of the microcystin validation study. Each of the four external laboratories was contacted by phone to request information on their current MRLs. As shown in Table 1, MRLs for drinking water analysis were provided by four labs, and they were consistent with the USEPA UCMR4 MRL for Method 546 of 0.3 µg/L.

Table 1: Minimum Reporting Levels (MRLs) for USEPA Method 546 from External Laboratories Certified by NJDEP OQA

Lab Number	Lab Name	Contact Name	Lab Number	Matrix	Minimum Reporting Limit (MRL) [mg/L]
07105	NEW JERSEY CENTER FOR WATER SCIENCE AND TECH	TSUNGTA HSU	973-655-3711	Non-Potable Water	0.3
11896	NJDEP - ENVIRONMENTAL MONITORING LABORATORY	VICTOR PORETTI	609-292-0427	Drinking Water and Non-Potable Water	0.3
CA008	EUROFINS EATON ANALYTICAL, LLC - POMONA	YOON CHA	626-386-1188	Drinking Water	0.3

PA059	PA DEP BUREAU OF LABORATORIES	DR. PAMELA HIGGINS	717-346-8618	Non-Potable Water	0.5

Statistical Analysis of USEPA Method 546 Data

Performance data for USEPA Method 546 was available from two laboratories that are certified for this method by NJDEP OQA, the New Jersey Center for Water Science and Tech laboratory and the NJDEP BFBM laboratory, as discussed below.

Performance data was provided by the New Jersey Center For Water Science and Tech Laboratory, which determined the MRLs from duplicate analysis of five samples using the MRL calculation specified in Method 546. The results of that experiment are indicated in Table 2.

Table 2: Minimum Reporting Limit (MRL) Determination for Method 546 ADDA-OH at the New Jersey Center for Water Science and Tech Laboratory (Lab Number 07105)

	Dup1	Dup2	AVG[Reported]
	[µg /L]	[µg /L]	[µg /L]
MRL1	0.295	0.277	0.3
MRL2	0.293	0.248	0.3
MRL3	0.285	0.282	0.3
MRL4	0.177	0.255	0.2
MRL5	0.279	0.334	0.3

In addition, duplicate analysis across the calibration range showed percent relative standard deviations of 5.3% to 15.8%, indicating good repeatability of the results as shown in Table 3.

Table 3: Percent Relative Standard Deviation (%RSD) of Duplicate Analysis of Calibration Standards for Method 546 at the New Jersey Center for Water Science and Tech Laboratory (Lab Number 07105)

546	Expected Concentrations	Dup1	Dup2	
		Concentration (µg/L)	Concentration (µg/L)	%RSD
STD1	0.15	0.134	0.107	15.8
STD2	0.4	0.446	0.393	8.9
STD3	1.0	0.91	1.067	11.2
STD4	2.0	1.902	2.05	5.3

Evaluation of Performance Data from Certified Laboratories

Data for Method 546 from the NJDEP BFBM laboratory was also evaluated. A series of seven (7) replicate analyses at two different concentrations, 0.3 µg/L and 0.5 µg/L, was conducted by the BFBM lab. A statistical analysis of these data was then conducted using the online R statistical program for the "Bootstrap Estimate of a Confidence Interval of the Mean" calculation. The results of the generated normal distribution are shown in Figure 1 and Table 4 below indicating that these quantification values are not biased or skewed and that the upper confidence limit of the MRL is 0.32 µg/L, which supports a PQL of 0.3 µg/L.

Table 4: R Statistical Program Output for Estimate of the Confidence Interval of the Minimum Reporting Level (MRL) for USEPA Method 546 ADDA-OH from the NJDEP BFBM Laboratory

LCL	XBAR	UCL	CONF	NREP
µg/L	µg/L	µg/L		
0.21	0.27	0.32	95.1	2000

Abbreviations; LCL = Lower Confidence Level

XBAR = Mean value estimate

UCL = Upper Confidence Level, *This UCL is used to verify the proposed PQL of 0.3 µg/L using the information presented above.

CONF = 95.1 % Confidence Level indicates that 95.1% of the certified laboratories can achieve a MRL of 0.3 µg/L.

NREP = The number of iterations used to create the normal distribution in Figure 1

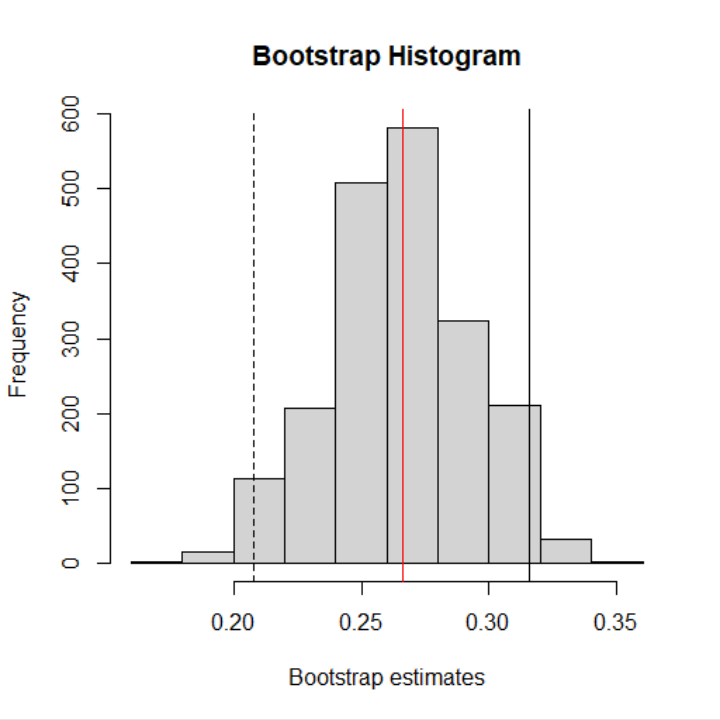

Figure 1: Bootstrap Estimate of a Confidence Interval of the Mean Distribution of the Minimum Reporting Level (MRL) for Method 546 from the NJDEP BFBM Laboratory

Information from the National Environmental Methods Index (NEMI)

An additional data source for PQL determination is the National Environmental Methods Index (NEMI), https://www.nemi.gov/methods/method_summary/13256/. Method 546 is listed in NEMI with an applicable concentration range of 0.3 mg/L to 2.2 mg/L which is consistent with the current MRLs from the phone survey. The determinations of the MRLs are acceptable substitutes for PQL calculations for parameters of interest due to the intralaboratory consistency in the national and New Jersey certified laboratory community.

Evaluation of Gold Standard (formerly Abraxis) ADDA SAES Method

As discussed above, the SAES kit is a more sensitive method for total microcystins than the ADDA-OH kit. Table 5 below shows performance data for the SAES method from the Gold Standard (formerly Abraxis) laboratory for three standard reference concentrations of total microcystins, 0.05, 0.15, and 0.40 µg/L. The standard reference material (SRM) performance data from the vendor shows acceptable % RSD for the low-level calibration at these concentrations, as shown in Table 5. This analysis was performed after the NJDEP lab studies described above and with data further below (appendix).

Table 5: Data provided by Gold Standard (formerly Abraxis) on Percent Relative Standard Deviation (%RSD) in Analysis of Standard Reference Material for the SAES Method at Three Total Microcystins Concentrations

SRM	[Reported concentration	%RSD
[µg/L]	[µg/L]	
0.05	0.043	10.6
0.15	0.166	7.2
0.40	0.408	1.4

A series of three (3) intralaboratory performance evaluations were conducted by Dr. Rob Newby, DSR Research Scientist 1 (microbiology) using the Gold Standard (formerly Abraxis) SAES ELISA low level method for total microcystins at the DSR laboratory. Calibration standards (0.07, 0.1, and 0.3 µg/L) were run in duplicate in each of the three evaluations. The raw data for the calibration standard samples and the quality control samples are shown in Table 6.

In brief, samples were prepared using tap water which was quenched of disinfection residual by the use of 100 mg/L sodium thiosulfate (Sigma Aldrich, Burlington, MA). These samples were spiked using a known concentration of microcystin-LR (Gold Standard Diagnostics, Warminster, PA) to the SRM concentrations.

Table 6: SAES ELISA Total Microcystins Intralaboratory Analysis (Raw Data) Performed by NJDEP

Expected Concentration [µg/L]	Measured Concentration [µg/L]	Batch Run
0.07	0.07	SAES1
0.07	0.05	SAES1
0.07	0.08	SAES2
0.07	0.07	SAES2
0.07	0.08	SAES3
0.07	0.04	SAES3
0.1	0.10	SAES1
0.1	0.10	SAES1
0.1	0.12	SAES2
0.1	0.11	SAES2
0.1	0.12	SAES3
0.1	0.15	SAES3
0.3	0.30	SAES1
0.3	0.31	SAES1
0.3	0.26	SAES2
0.3	0.33	SAES2
0.3	0.43	SAES3
0.3	0.24	SAES3
LFB 1*	0.57	SAES2
LFB 1	0.47	SAES2
LFB 1	0.58	SAES1
LFB 1	1.41	SAES3
LFB 1	1.44	SAES3
LFB 1	0.56	SAES1
LFB 2	0.37	SAES2
LFB 2	0.42	SAES2
LFB 2	0.56	SAES1
LFMSD1*	1.30	SAES1
LFMSD1	1.40	SAES1
Low CV*	0.32	SAES1
Low CV	0.26	SAES1
LRB*	0.01	SAES1
LRB	0.01	SAES1
LRB2	0.54	SAES1
QCS*	0.52	SAES1
QCS	0.54	SAES1
QCS	0.66	SAES2
QCS	0.62	SAES2
QCS	0.55	SAES3

QCS	0.55	SAES3

Abbreviations;
*LFB – Laboratory Fortified Blank
*LFMS/D – Laboratory Fortified Matrix Spike, Duplicate
*Low-CV – Low-Range Calibration Verification
*LRB – Laboratory Reagent Blank
*QCS - Quality Control Standard

Based on these data the percent relative deviation (%RSD) was calculated at 0.07, 0.10, and 0.30 µg/L as 25.3%, 16.0% and 21.4%, respectively. The percent relative standard deviation values at these low levels were much higher than for the Method 546 (ADDA-OH) data provided by the New Jersey Center for Water Science and Tech Laboratory, which ranged from 5.3% to 15.8% RSD (Table 3).

The online statistical package R was utilized to simulate a Bootstrap Estimate of a Confidence Interval of the Mean analysis of the MRL for the ADDA SAES ELISA method data shown in Table 6. This evaluation was done due to the lack of intralaboratory data for this new low level analysis methodology. A uniform normal distribution was generated from 2000 iterations of the bootstrap method (Figure 2). The results of the MRL determination for the Microcystins-ADDA SAES ELISA method are shown in Table 7.

Table 7: R Statistical Program Output for Estimate of the Confidence Interval for the MRL for the ADDA SAES Method

LCL	XBAR	UCL	CONF	NREP
µg/L	µg/L	µg/L	%	
0.31	0.44	0.58	95.05	2000

Abbreviations;

LCL = Lower Confidence Level

XBAR = Mean value estimate

UCL = Upper Confidence Level,

CONF = 95.05 % Confidence Level indicates that 95.05% of the certified laboratories can achieve a PQL of 0.3 µg/L or above that level by the distribution generated.

NREP = The number of iterations used to create the normal distribution in Figure 2 below

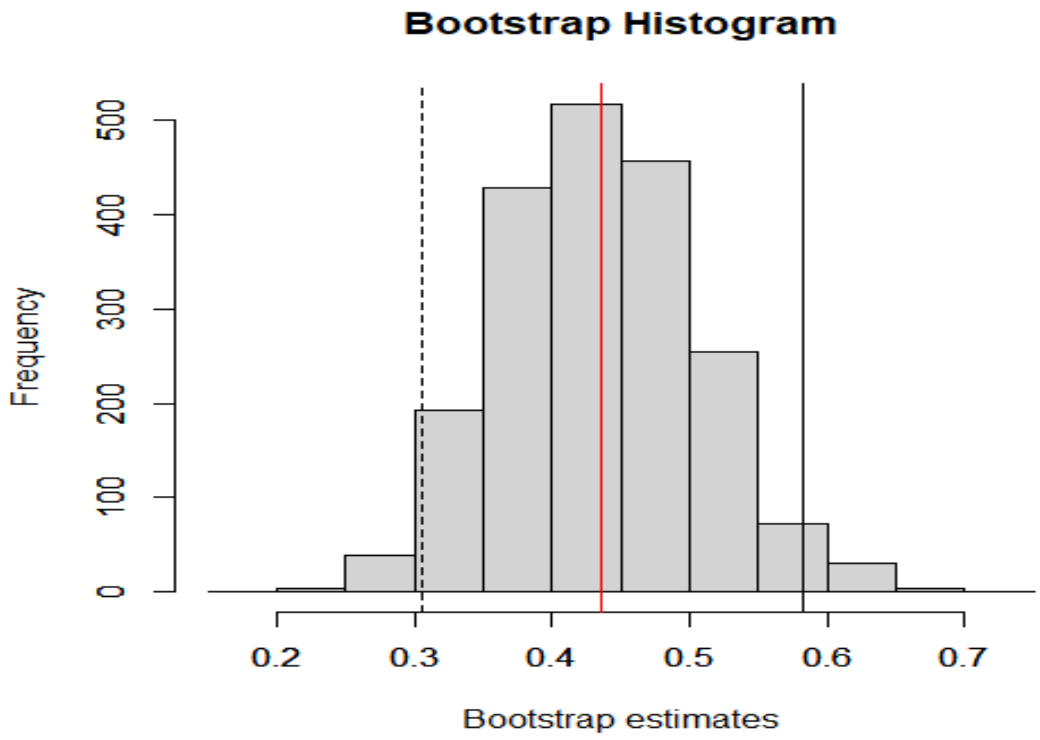

Figure 2: Bootstrap Estimate of a Confidence Interval of the Mean Distribution of the Reporting Level for the ADDA SAES Method

Although the calibration standard range was 0.07 µg/L to 0.3 µg/L and the laboratory fortified blank (LFB) and quality control (QC) standards ranged from 0.37 µg/L to 1.44 µg/L, the distribution estimated a lower confidence level of 95.05% for quantitation (i.e., the MRL) of 0.31 µg/L (rounded to two digits), which is above the two lower levels used for calibration standards, 0.07 µg/L and 0.10 µg/L. This was due to the relatively high %RSD levels that were calculated for the analysis of the six calibration standards at each level. The calculation of the PQL is based on the UCL of the analysis performed (0.58 µg/L). Due to a low abundance of laboratory data (under 5 participating laboratories), this statistical estimation approach is used for determination of PQLs only in situations where there is a lack of 5 or more laboratories that can provide MDL, low-point calibration, and reporting level data.

In addition, the median value was 0.33 µg/L and the mean value was 0.42 µg/L from the experimental data, indicating that the data were biased low.

NJDEP Validation Study and Intralaboratory Study (Round Robin)

NJDEP conducted a two-part scientific evaluation of the testing of microcystin with ELISA using kits with (i.e., SAES kit) and without (i.e., ADDA-OH kit) the SAES modification, with the ADDA-OH kit being validated for UCMR4 as USEPA Method 546. At the time of the investigation, the USEPA had not accepted the SAES kit as a valid modification to EPA 546 and lower set points (below the MRL of 0.3 µg/L) had not been validated (personal communication, USEPA). NJDEP BFBM/DSR initially conducted a validation study (part 1) of the SAES assay at concentrations of interest. The performance of this assay/kit was compared to USEPA 544 (part 3).

Additionally, the first part of this evaluation investigated the sensitivity of the SAES kit, which reported a detection limit of 0.016 µg/L**.** The study determined that the kit could not consistently and reliably support quantification, or even detection, of microcystins at the Health Effects Subcommittee recommended level of 0.07 µg/L.

During the first part of the evaluation the precision and accuracy of each kit (ADDA-OH and SAES) at selected ranges (0, 0.07, 0.1, 0.3, and 1 µg/L) which were determined to be important to the study were tested in ten (10) technical replicates. Data from this study was used in subsequent analysis to validate the performance of the kits at the selected ranges as well as for the PQL determination. Technical data is presented in Appendix A of this report.

As part of the validation of the SAES kit for its use in place of the ADDA-OH kit for general ELISA use for microcystin detection, a comparability study (part 3) was conducted where samples were run on the SAES kit then duplicate analysis was performed on samples using EPA 544.

The secondary purpose of the investigation was to determine if laboratories in New Jersey, which were conducting cyanotoxin monitoring in finished drinking water, could detect cyanotoxins at known concentrations in spiked samples with good precision using the microcystin kit they were currently using (ADDA-OH or SAES). The study was conducted during a round robin style sampling blitz with three external volunteer participating laboratories and the data from the NJDEP lab, for a total of four laboratories. Technical data is submitted in Appendix A of this report.

The results of the interlaboratory study were used in the development of a PQL of 0.3 µg/L, as described below. The recommendation takes into consideration the ability of the laboratory to adequately and reliably quantify the level of cyanotoxins, as well as the performance variations within the participating laboratories due to interferences that may occur at the lower levels.

Recommendations

Data from the validation study (part 1) and from labs certified by NJDEP OQA for USEPA Method 546 were utilized to perform a bootstrap analysis to help in determining the PQL. The method employed uses a pooled method detection limit (MDL), low point calibration curves, and reporting limit data from the certified laboratories. The report determines a PQL of 0.3 µg/L for microcystin.

Therefore, due to limitations with current detection capability of microcystin in finished drinking water, a PQL value of 0.3 µg/L is recommended. While both NJDEP and USEPA were able to confirm independently that the SAES kit chemistry can detect to 0.1 µg/L, the kit performance does not do so reliably, with labs within the round robin study not capable of detecting the value, compared to values at 0.3 µg/L (Appendix A, Table 13-14)

Additionally, because biological assays are not perfect and subject to interference, it is recommended that any detection below the reporting level be followed up with confirmation with USEPA 544. It should be noted that this process is not perfect because USEPA 544 is limited in the scope of congeners it will detect and quantify, as well as by limited laboratory capacity for the testing.

Some commercial laboratories report the use of a method that analyzes for 3-methoxy-2-methyl-4-phenylbutyric acid (MMPB), an oxidation product of the ADDA moiety (Standard Methods 10110, Proposed, 2018), with LC/MS/MS as an alternative to USEPA Method 544. The Testing Subcommittee has not evaluated this method as part of this review, and it remains an invalid modification of USEPA Method 544. This method would need to be validated, similar to the study reported here, as the USEPA has highlighted concerns with the use of the MMPB in finished water (especially in chlorinated water systems) due to the potential of bias on the high side due to interference from disinfection/oxidation byproducts (Rosenblum et al, 2017).

While this report primarily addresses microcystin through USEPA Methods 544 and 546, the DWQI Health Effects Subcommittee made recommended drinking water guidelines for three additional cyanotoxins that were not evaluated in the Testing Subcommittee's review and NJDEP's round robin study. Cylindrospermopsin and anatoxin-A have a USEPA approved methodology, USEPA Method 545: Determination of Cylindrospermopsin and Anatoxin-a in Drinking Water by Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC/ESI-MS/MS, USEPA, April 2015b) as well as an existing ELISA analytical methodology (Gold Standard Diagnostics, Product No. 522011; 520060) which is able to detect within the NJDEP's guidance values for finished drinking water. The last cyanotoxin, saxitoxin, does not have a USEPA approved methodology but does have an existing ELISA analytic methodology that is extensively used in the food industry for shellfish testing (Gold Standard Diagnostics, Product No. 52255b). The Health Effects Subcommittee recommendations for all three of these toxins are detectable within the range of quantification for the ELISA methodology. However, additional research is needed for the

development of a PQL for these cyanotoxins, due to the limited number of laboratories performing these methods.

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APPENDIX A: Kit Validation and Intralaboratory Study (Round Robin)

Background

Since testing limitations existed at the time when NJDEP-DSR recommended a drinking water guidance of 0.07 µg/L for microcystins and USEPA was validating the SAES test kit, the following studies were conducted:

1) A comparison of the validated USEPA 546 kit (ADDA Testing Kit-520011OH) and the SAES kit for analysis of known concentrations of microcystin in various matrices of water conducted in the NJDEP BFBM and DSR laboratories.
2) A round robin style test in which samples with known concentrations of microcystin in lab grade water were sent to New Jersey laboratories for analysis. Performance data were evaluated based on the kit (ADDA-OH or SAES) used for analysis.
3) A comparison of results from the SAES kit to the USEPA Method 544 which was analyzed in the NJDEP Pesticides Laboratory for analysis of known concentrations of microcystin in spiked drinking water samples; once a lower level is known based on data from 1) & 2).

In the study that compared results of the ADDA-OH and SAES test kits in the NJDEP laboratories (Part 1), samples of lot certified microcystin-LR (Gold Standard Diagnostics, Warminster, PA) at the selected concentrations were prepared in either quenched tap water (Trenton Water Works) or lab grade sterile deionized water (18.2 MΩ·cm). This was repeated for 10 technical replicates, with lysing or filtering of the samples performed per EPA 546 specifications. Each sample batch was prepared in bulk and analysis was performed from these bulk prep samples. Aliquots were made from this bulk solution and frozen solid at -20ºC. This was done to maximize the likelihood of detection and to maximize the chance of detection at the spiked concentrations, while reducing the likelihood of degradation. All test kits were from the same lot to minimize lot to lot variability. Samples were not run on the same day or in the same runs to ensure that machine bias for reporting was not a factor in the results.

Results of this study are shown in Figures 3 and 4 and Tables 9 through 13, and a summary of these results was presented to the Drinking Water Quality Institute in December 2021. Table 9 presents the data used to generate the calibration curve for the ADDA-OH test kit shown in Figure 3. The values shown in Table 9 represent data generated after the completion of the ELISA reaction. A 4-parameter log curve is generally recommended to generate the standard curve for this reaction, and the equation is shown in this table. In compliance with NJDEP protocols, the ELISA standards are analyzed with each run, regardless of identical lot information. Thus, each ELISA curve generated by NJDEP by the standards run on that ELISA plate with those plate conditions.

Bulk Spike Sample Preparation

Samples were prepared in bulk to minimize errors due to small volume pipetting as well to minimize any issues with sample degradation due to compound instability. To test potential interference within a drinking water matrix, and since the analysis would be performed using drinking water, samples were prepared in bulk using dechlorinated tap water which was provided from the utility to the laboratory (Trenton Water Works). At the time of testing no microcystins were detected in the finished drinking water product (Table 6, sample ID: LFSMD).

Additionally, to ensure that any potential organic or inorganic inhibitors were not causing sample loss, a sample was prepared using lab grade sterile deionized water (18.2 MΩ·cm). Samples were prepared using a stock of microcystin-LR (Gold Standard Diagnostics, Warminster, PA), and aliquoted into glass amber tubes. These tubes were frozen solid at -20°C and thawed as needed for analysis. Table 8 below provides the breakdown of the spiked samples and the source water used for their preparation.

Table 8. Sample concentration and water source for preparation.

Sample & Concentration	Source
0.07 µg/L	Quenched Tap Water
0.1 µg/L	Quenched Tap Water
0.3 µg/L	Quenched Tap Water
1 µg/L	Lab Grade Water
2 µg/L	Quenched Tap Water

All standards used in the preparation of the ELISA curves were used as is from the ELISA kits (ADDA-OH – 520011OH; SAES – 520011SAES) without modification per the manufacturer specifications.

Table 9. Calibration curve data for ADDA-OH Testing Kit in the NJDEP BFBM/DSR Lab

Standards (µg/L)	Optical Density (averaged)	Predicted values	Residual Squares	B/Bo*	Std Dev	%CV
0.000	1.408	1.413	2.21E-05		0.006	0.402
0.150	1.156	1.140	2.55E-04	0.821	0.021	1.835
0.400	0.830	0.848	3.07E-04	0.589	0.011	1.363
1.000	0.549	0.554	2.29E-05	0.390	0.000	0.000
2.000	0.396	0.359	1.35E-03	0.281	0.019	4.827

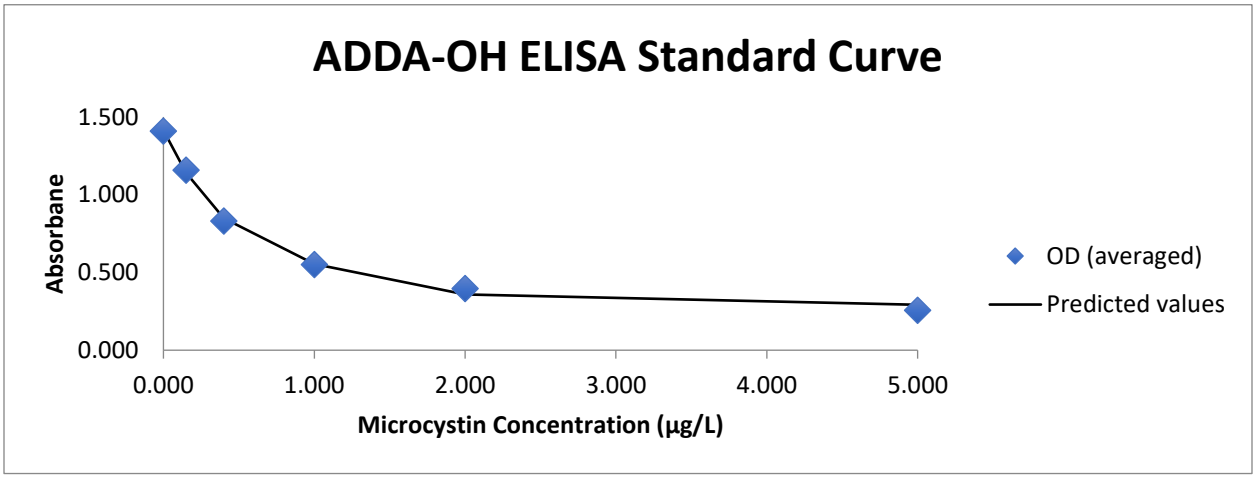
5.000	0.256	0.282	6.62E-04	0.182	0.004	1.657
Sum Of Squares			2.62E-03			

Parameter (Y=(A-D)/(1+(X/C)^B)+D)			**R-Squared**
A	1.413	(Max.)	0.99741
B	1.127	(Slope)	
C	0.447	(IC_{50})	
D	0.207	(Min.)	

*B/B_0 is the ratio of binding, a common control used to optimize the range of the curve.

Figure 3 presents the standard ELISA Log_{10} curve and the expected values associated with the ELISA when performing the ADDA-OH chemistry in samples prepared in lab grade water. The points represent the average of duplicate samples. Numerical values will differ based on the binding efficiency of the antibody in the specific test lot and other biotic and abiotic factors, and per USEPA 546, variation is allowed within acceptable ranges.

Figure 3. ELISA Log_{10} calibration curve for ADDA-OH Testing Kit in the NJDEP BFBM/DSR lab



The concentrations of spiked microcystin-LR selected for use in the round robin study were 0.07, 0.1, 0.3, and 2 µg/L in quenched tap water, and 1 µg/L in Milli-Q (ultrapure) water. Ten individual analyses of these concentrations with the ADDA-OH test were performed by the NJDEP BFBM/DSR lab. Data from one analysis was randomly selected for presentation in Table

10, with the average of the 10 runs presented in Table 13. The lab reagent blank (LRB) is as an in-assay way to provide a clean matrix to determine if erroneous signal detection is occurring due to contamination of wells or due to handling, and results for this sample should be as close to zero as possible. A QCS sample, i.e. a spiked sample of known microcystin concentration, is provided by the vendor who manufactures the kit as required by USEPA Method 546. The QCS sample is used to determine the reaction's "robustness." This can be likened to a "positive control" sample as the QCS has a defined and acceptable range where too low or too high means the curve that was generated from the standards is possibly suspect. LCRC (low calibration range check) is utilized to verify the accuracy of the curve at or near the reporting limit.

Table 10. Results of a single run of the ADDA-OH Testing Kit in the NJDEP BFBM/DSR lab for samples prepared in lab grade water at the concentrations used in the round robin study

Sample (µg/L)/Control	Absorbances	Std Dev	%CV	Sample Concentration (µg/L)	Sample Concentration Avg (µg/L)	Comments
LRB	1.309			0.055		LESS than Standard 1 (0.15 µg/L), outside of quantifiable range
LRB	1.269	0.028	2.194	0.076	0.065	
QCS	0.691			0.637		
QCS	0.694	0.002	0.306	0.631	0.634	
LCRC	0.855			0.391		
LCRC	0.830	0.018	2.098	0.421	0.406	
0.07	1.267			0.077		LESS than Standard 1 (0.15 µg/L), , outside of quantifiable range
0.07	1.263	0.003	0.224	0.079	0.078	
0.1	1.185			0.123		LESS than Standard 1 (0.15 µg/L), , outside of quantifiable range
0.1	1.180	0.004	0.299	0.126	0.124	
0.3	0.918			0.324		
0.3	0.912	0.004	0.464	0.330	0.327	
2	0.395			2.003		
2	0.395	0.000	0.000	2.003	2.003	
1 in Milli-Q water	0.555			0.996		
1 in Milli-Q water	0.542	0.009	1.676	1.044	1.020	

Acronym Definitions:
LRB – Laboratory Reagent Blank
QCS – Quality Control Sample
LCRC – Low Calibration Range Check

Table 11 presents the data used to generate the calibration curve for the SAES test kit shown in Figure 2. Note that the range of calibration standards (provided with the kit) for the SAES test kit is 0.05-5 µg/L as compared to 0.15-5 ug/L for the ADDA-OH test kit (Table 9). The values shown in Table 11 represent the data generated after the completion of the ELISA reaction. A 4-parameter log curve is generally recommended to generate the standard curve for this reaction, and the equation is shown in this table. Figure 2 shows the standard ELISA Log_{10} curve and expected values associated with the ELISA when performing the SAES chemistry. At the time when this assay was being performed, this kit was not yet an accepted modification to USEPA 546 and this work was being performed to ensure that this detection chemistry could meet or exceed the MRL of the ADDA-OH kit. As indicated above, numerical results will differ among analyses based on the binding efficiency of the antibody in the specific test kit and other biotic and abiotic factors. The points represented are the average of duplicate samples. Per USEPA 546, variation is allowed within acceptable ranges. In compliance with NJDEP protocols, the ELISA standards are analyzed with each run, regardless of identical lot information. Thus, each ELISA curve generated by NJDEP by the standards run on that ELISA plate with those plate conditions.

Table 11. Calibration curve data for microcystin samples prepared using the SAES test kit in the NJDEP BFBM/DSR lab

Standards (ug/L)	OD (averaged)	Predicted values	Residual Squares	B/Bo	Std Dev	%CV
0.000	2.074	2.080	4.88E-05		0.012	0.580
0.050	1.713	1.680	1.09E-03	0.826	0.052	3.014
0.150	1.400	1.420	4.04E-04	0.675	0.006	0.404
0.400	1.096	1.132	1.26E-03	0.529	0.035	3.226
1.500	0.788	0.736	2.63E-03	0.380	0.009	1.167
5.000	0.432	0.453	4.66E-04	0.208	0.004	0.819
Sum Of Squares			5.89E-03			

Parameter (Y=(A-D)/(1+(X/C)^B)+D)			R-Squared
A	2.080	(Max.)	0.99677
B	0.614	(Slope)	
C	0.484	(IC_{50})	

D	0.065	(Min.)

*B/B_0 is the ratio of binding, a common control used to optimize the range of the curve.

Figure 4. ELISA Log_{10} calibration curve for microcystin samples using the SAES test kit in the NJDEP BMBM/DSR lab

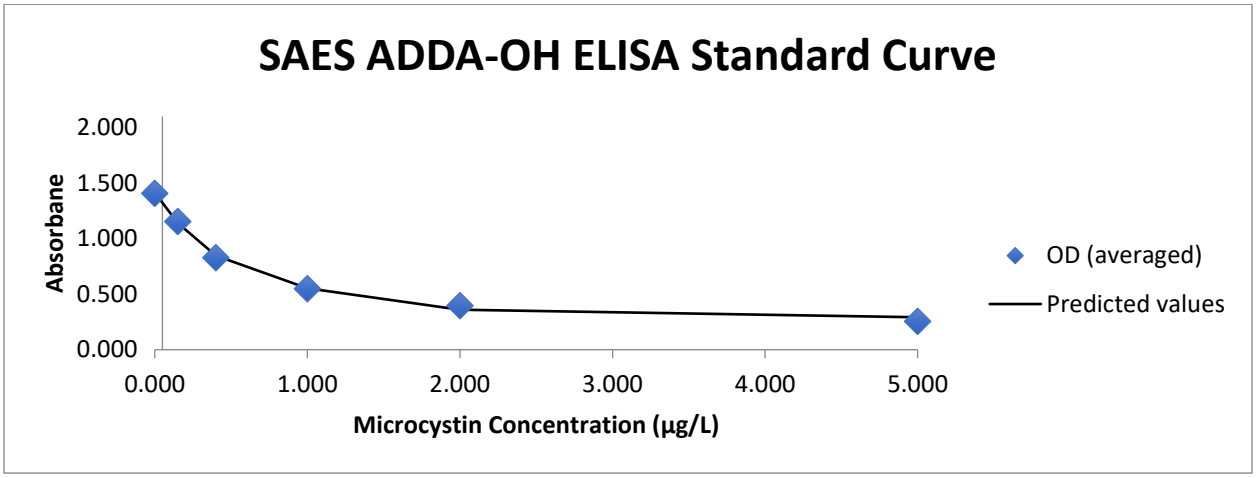


Table 12 shows the computation of the values of a single select run of the five concentrations used in the round robin study (discussed in this report) with the SAES chemistry. This run was randomly selected out of the 10 runs that NJDEP had conducted. Both LRB and QCS were included in the test. The SAES kit does not use an LCRC (Low Calibration Range Check) control; however, one may be required at some point in the future. A second "blank" containing just deionized water was used to keep well length consistent for reporting.

As mentioned above, the concentrations of the spiked microcystin-LR NJDEP used for the round robin were 0.07, 0.1, 0.3, and 2 µg/L in quenched finished drinking water, and 1 µg/L in Milli-Q water. Out of 10 individual analyses with the SAES kit, data from one analysis was selected and presented in Table 12, with the average of the 10 runs presented later in Table 13.

Table 12. Results of a single run in the NJDEP lab of the SAES kit chemistry comparison study of the five concentrations used for the round robin

Sample (µg/L)/Control	**Absorbances**	**Std Dev**	**%CV**	**Sample Concentration (µg/L)**	**Sample Concentration Avg (µg/L)**	**Comments**
LRB	1.986			0.004		LESS than Standard 1, outside of quantifiable range (0.05 µg/L)
LRB	1.922	0.045	2.316	0.009	0.006	
QCS	0.978			0.658		
QCS	0.995	0.012	1.219	0.622	0.640	

0.07	1.560			0.087		
0.07	1.554	0.004	0.272	0.089	0.088	
0.1	1.580			0.080		
0.1	1.594	0.010	0.624	0.075	0.077	
0.3	1.306			0.224		
0.3	1.313	0.005	0.378	0.219	0.222	
2	0.662			1.982		
2	0.646	0.011	1.730	2.110	2.046	
1 in Milli-Q water	0.819			1.119		
1 in Milli-Q water	0.756	0.045	5.657	1.397	1.258	
Blank	2.151			Invalid		LESS than Standard 1, outside of quantifiable range (0.05 µg/L)
Blank	2.139	0.008	0.396	Invalid	Invalid	

Acronym Definition:
LRB- Laboratory Reagent Blank
QCS- Quality Control Sample

Table 13 is the average of the 10 independent runs for the ADDA-OH and the SAES kit as performed by the NJDEP BFBM/DSR lab for the samples prepared for the round robin. The expected value for the samples is listed as well as the actual recovery value. In brief, samples were prepared using dechlorinated tap water which was first verified to not contain microcystin or exhibit abnormal interference (data not shown). Once established, microcystin was spiked to the dechlorinated finished drinking water to a final concentration of 0.07, 0.1, 0.3, and 2 µg/L in the water. Prepped spiked samples were then frozen and thawed three times and filtered through into glass amber vials as if it was actual samples as per USEPA 546. This was done to measure sample loss or background interference from filters, which has been noted from numerous sources. In addition, since organic degradation of microcystin has been noted in USEPA 546, a 1 µg/L in deionized water sample (MilliQ) was prepared to reduce the chance of degradation from the sample matrix.

Table 13. Average results of 10 runs of the ADDA-OH and SAES kits in the NJDEP BFBM/DSR laboratory for the concentrations used in the interlaboratory comparison study

ADDA Kit Chemistry						
Spiked concentrations	0.07 µg/L	0.1 µg/L	0.3 µg/L	2 .0 µg/L	1.0 µg/L in Q	QCS (0.6 µg/L)
Expected concentrations	0.07 µg/L	0.1 µg/L	0.3 µg/L	2.0 µg/L	1.0 µg/L in Milli-Q water	0.6 µg/L
Measured concentrations	0.078 µg/L	0.124 µg/L	0.327 µg/L	2.003 µg/L	1.02 µg/L	0.634 µg/L
% recovery	**111.43%**	**124.00%**	**109.00%**	**100.15%**	**102.00%**	**105.67%**

SAES Kit Chemistry						
	0.07 µg/L	0.1 µg/L	0.3 µg/L	2.0 µg/L	1.0 µg/L in Q	QCS (0.6 µg/L)
Expected concentrations	0.07 µg/L	0.1 µg/L	0.3 µg/L	2.0 µg/L	1.0 µg/L in Milli-Q water	0.6 µg/L
Measured concentrations	0.088 µg/L	0.077 µg/L	0.222 µg/L	2.046 µg/L	1.258 µg/L	0.64 µg/L
% recovery	**125.71%**	**77.00%**	**74.00%**	**102.30%**	**125.80%**	**106.67%**

Only three volunteer New Jersey laboratories responded to the NJDEP's recruitment for the round robin study. Each lab was given samples in the summer of 2021. The laboratories could decide which test kit(s) they would use, and they submitted that information to NJDEP along with their results.

The results of the round robin study, which included the NJDEP BFBM/DSR laboratory and three volunteer New Jersey drinking water utility, are presented in Table 14 below. Samples were prepared The NJDEP BFBM/DSR laboratory and one volunteer laboratory, Lab 3, provided data for both the ADDA-OH and the SAES kits for comparison. One of the 10 runs of each test performed by the NJDEP laboratory was selected for comparison, instead of the average since the participating volunteer laboratories only performed one run of the test(s) that they used. Other than the analytical results and the kit information, no additional data was provided to NJDEP by the volunteer labs. While each laboratory stated that the ELISA runs met the accepted QA/QC criteria, it is not known if the runs were run to USEPA 546 specifications with QA/QC in USEPA 546 valid or acceptable. Results were considered acceptable to the kit manufacture specification (personal communication NJDEP and laboratories). Samples were provided to the labs in 15mL frozen aliquots from the bulk sample preparation that was previously stated in Table 1. Labs were instructed to process the samples as they normally would with a water sample, except with the freeze-thaw cycles; since the tubes were already frozen. Laboratories were instructed not to omit filtration.

Table 14. Results of the round robin study from the NJDEP laboratory and three volunteer laboratories..

Lab	Kit	Expected concentration	Measured concentration (µg/L)	Recovery
NJDEP BFBM/DSR	ADDA	0.07 (µg/L)	0.068 µg/L	**97.14%**
NJDEP BFBM/DSR	SAES		0.088 µg/L	**125.71%**
1	ADDA		0.101 µg/L	**144.29%**
2	SAES		0.065 µg/L	**92.86%**
3A	ADDA		<DL	
3B	SAES		0.049 µg/L	**70.00%**

Lab	Kit	Expected concentration (µg/L)	Measured concentration (µg/L)	Recovery
NJDEP BFBM/DSR	ADDA	0.10 µg/L	0.082 µg/L	**82.00%**
NJDEP BFBM/DSR	SAES		0.104 µg/L	**104.00%**
1	ADDA		0.134 µg/L	**134.00%**
2	SAES		0.108 µg/L	**108.00%**
3A	ADDA		<DL	
3B	SAES		0.053 µg/L	**53.00%**

Lab	Kit	Expected concentration (µg/L)	Measured concentration (µg/L)	Recovery
NJDEP BFBM/DSR	ADDA	0.30 µg/L	0.328 µg/L	**109.33%**
NJDEP BFBM/DSR	SAES		0.304 µg/L	**101.33%**
1	ADDA		0.339 µg/L	**113.00%**
2	SAES		0.262 µg/L	**87.33%**
3A	ADDA		<DL	
3B	SAES		0.215 µg/L	**71.67%**

Lab	Kit	Expected concentration (µg/L)	Measured concentration (µg/L)	Recovery
NJDEP BFBM/DSR	ADDA	2.0 µg/L	1.875 µg/L	**93.75%**
NJDEP BFBM/DSR	SAES		2.049 µg/L	**102.45%**
1	ADDA		1.954 µg/L	**97.70%**
2	SAES		2.098 µg/L	**104.90%**
3A	ADDA		1.595 µg/L	**79.75%**
3B	SAES		1.431 µg/L	**71.55%**

Lab	Kit	Expected concentration (µg/L)	Measured concentration (µg/L)	Recovery
NJDEP BFBM/DSR	ADDA	1.0 µg/L *	1.004 µg/L	**100.40%**
NJDEP BFBM/DSR	SAES		0.955 µg/L	**95.50%**
1	ADDA		1.029 µg/L	**102.90%**

2	SAES		0.94 µg/L	**94.00%**
3A	ADDA		0.503 µg/L	**50.30%**
3B	SAES		0.842 µg/L	**84.20%**

Acronym Definition:
DL- Detection Limit
* Sample prepared in MilliQ (lab grade) Water

Data Analysis of Part 1 and 2

Generally, the SAES kit performed better than the ADDA-OH kit at lower levels with higher recovery (Table 13, Table 14). The NJDEP laboratory data (Table 13) are the average of 10 technical replicates over multiple curves each with a paired coefficient of variation (CV) under 15%, (CV data not shown, one run shown for ADDA-OH in Table 2 and for SAES in Table 12). However, taken into context, these data do not represent data from samples in real world environments, as the study was designed to measure kit performance and evaluate the lowest level detection possible under best performance conditions. Additionally, in order to extrapolate to below 0.1 µg/L in ADDA-OH kits, data must manually be exported and graphed, which requires some technical knowledge of working with the dataset. While theoretically possible to "see" data below the 0.1 µg/L point in ADDA-OH, the accuracy of that data will always be suspect. If following the phased approached used in USEPA UCMR-4 with a cutoff below 0.3 µg/L which was used in UCMR4, subsequent sampling and follow-up with USEPA 544 will be triggered at low levels where the data are uncertain. Samples are allowed no more than a paired CV of 15% and standards are allowed no more than a 10% paired CV while performing USEPA 546, which means samples on the curve are not going to be exact concentrations; rather approximations of the value based on the fit of the curve. While the SAES kit has a much lower limit of detection (0.016 µg/L), based on our experience, the kit generally errs in reporting higher values for low concentration samples, but is more accurate at concentrations above the midpoint of the standard curve. An example of this is provided in Table 13

Data Analysis Part 3

Moving past the round robin was the second objective of the study which was to validate the values detected with the SAES kit with USEPA 544. Use of USEPA 544 would be important for determination accuracy of the low range detection below the validated MRL of USEPA 546 (0.3 µg/L) using the traditional ADDA-OH assay kit.

Table 15 shows the results of analysis with the SAES kit with samples either frozen and filtered before analysis or spiked in right before the analysis. The purpose of this evaluation was to determine if there was bias or loss in recovery with the microcystin related to how the sample was processed (i.e., freezing and filtering) and the enhanced sensitivity of the SAES kit. Since the kit lacks calibration standard at the low (<0.07 µg/L) end of the curve, which is the recommended NJDEP Health Advisory guidance value, a concentration near that point may be marked as non-detect or be masked by interference rather than show as a true positive. De Detections at this level (0.07 µg/L) with the SAES kit highlight that under ideal conditions sample recovery of this value

is possible. It is noted and has been reported before that slight increases in concentration are observed after processing, a result which is not significant nor unexpected.

Table 16 shows the data from USEPA 544 analyzed from the duplicated sample processing for the SAES kit at the concentrations shown in Table 15. Samples were concurrently prepared and analyzed using both methods to validate the low limit of detection of the kit.

Table 17 highlights a direct comparison of USEPA 544 (LC-MS/MS) and the SAES Kit based on samples prepared in a finished drinking water matrix. Concentrations presented in Tables 15-17 were selected based on data from the intralaboratory method (0.07 µg/L, 0.10 µg/L, and 0.30 µg/L microcystin).

Table 15. Recovery values/spiking values in SAES kit when microcystin is added to finished drinking water before processing (freezing and filtering) versus adding directly before run.

		Expected concentration (µg/L)	Measured Concentration (µg/L)	Recovery
Added before processing	MSQ-0.07	0.07	0.09	128%
	MSQ-0.1	0.10	0.13	134%
	MSQ-0.3	0.30	0.38	129%
Added before running	MSQ-0.07-Spike	0.07	0.08	112%
	MSQ-0.1-Spike	0.10	0.14	137%
	MSQ-0.3-Spike	0.30	0.36	121%

Table 16. Sample validation run of USEPA 544 (LC-MS/MS) for microcystin concentrations (µg/L) used in Part 1 for validation of SAES ELISA Method.

Sample ID	Nod-R	MC-YR	MC-RR	MC-LR	MC-LA	MC-LY	MC-LF	Detection concentration
MSQ-FDW-0	nd	nd	nd	0	nd	nd	nd	µg/L
MSQ-FDW-0.07	nd	nd	nd	0.083	nd	nd	nd	
MSQ-FDW-0.1	nd	nd	nd	0.130	nd	nd	nd	
MSQ-FDW-0.3	nd	nd	nd	0.390	nd	nd	nd	

All units are in µg /L
Acronym Definitions:
Nod-R – Nodularin R
MC-YR – Microcystin-YR
MC-RR – Microcystin-RR
MC-LR – Microcystin-LR
MC-LA – Microcystin-LA
MC-LY – Microcystin-LY
MC-LF – Microcystin-LF
ND – Non-detect

Table 17. Comparison of values between the expected and actual detection of the SAES kit (using USEPA 546) and USEPA Method 544, based on the mean of two samples in a spiked drinking water matrix.

Spiked Concentration	Method Results EPA 546 (µg/L)	Method Results EPA 544 (µg/L)	EPA 546/Expected	EPA544/Expected	(EPA546/EPA 544)
0.07 (µg/L)	0.09 (µg/L)	0.083 (µg/L)	128.60%	118.60%	108.40%
0.1 (µg/L)	0.124 (µg/L)	0.13 (µg/L)	124.00%	130.00%	95.40%
0.3 (µg/L)	0.389 (µg/L)	0.39 (µg/L)	129.70%	130.00%	99.70%
0 (µg/L)	0 (µg/L)	0 (µg/L)	N/A	N/A	N/A

Discussion

Figure 3 and Figure 4 are the Log_{10} plots of the ADDA-OH and SAES ELISA reactions with reaction controls. The data were exported and plotted using a 4-parameter solver equation from Abraxis Eurofins. The solved equation for each log transformed curve is provided in Table 9 and Table 11 respectively. Exported data is the only way that the points falling outside the limit of detection for ADDA-OH kits can be observed. The data showed that 0.07 µg/L could not be detected using the ADDA-OH kit. However, since 0.07 µg/L falls below the detection limit for the kit (0.1 µg/L) , the automated software analysis will flag the samples as below the lowest standard (0.15 µg/L) and thus a flag as a "non-detect". It is not common practice to export data manually and view raw data or generate plots like those displayed in Figure 9 or 10.

Cumulative NJDEP BFBM/DSR data are presented in Table 13, with the data from the three volunteer laboratories that participated in the round robin study presented in Table 14. Microcystin ELISA is run in duplicate samples so data from labs 1, 2, and 3 are presented as the average of duplicate samples, whereas the NJDEP BFM/DSR data are the average of the 10 runs. Lab 3 provided data from both ADDA-OH and SAES, and their data were presented as 3A -for ADDA-OH and 3B for SAES. All labs received the same spiked samples which were frozen at -20^{o}C and were asked to process them no longer than 30 days after sample receipt. Samples did not have to be run concurrently, which allowed the labs to fit samples as space allowed. Labs were asked to process the samples like they normally would (including filtration) but asked to exclude the additional freeze-lyse step.

Data from the second part of the round robin highlighted interesting trends associated with samples given to partner drinking water laboratories of known concentrations of microcystin spiked samples. The ADDA-OH kit performed the worst in our hands at lower concentrations at or below 0.1 µg/L, with the data being the most erratic (Table 14). As stated above, with having to manually export data to see values below 0.1, generally the data would often "bounce" below 0.1 µg/L and made percent recovery lower than expected (Table 13). Compared to the SAES kit which generally in our observations is reporting concentrations higher

than expected (Table 13, Table 15); these higher values still were correctly identified as "detections". Since various factors could affect detection, it is possible that human or machine errors are to blame for the higher detected values.

Based on the data in Table 14 and USEPA allowing the SAES kit as an approved modification to Method 546, the third part of the Round Robin consisted of verifying low-level detections with the SAES kit (i.e., close to the recommended NJDEP-DSR guidance value of 0.07 µg/L and the USEPA Health Advisory level of 0.3 µg/L). Since the SAES kit had provided acceptable results at 0.1 µg/L in the NJDEP BFBM/DSR lab and Lab 2 in the Round Robin study (Table 14), we selected 0, 0.07, 0.1, and 0.3µg/L as the comparison samples for the SAES kit and USEPA 544. Finished drinking water was collected from a water source where cyanobacteria blooms had previously occurred (New Jersey Water Supply Authority, Manasquan Reservoir, MSQ).

Since USEPA 546 and USEPA 544 require different dichlorination (quenching) methods, a large volume (1L) of finished drinking water from this source water with known blooms was collected and split into two separate containers containing the appropriate quenching agents. Samples were confirmed not to contain microcystin by testing the water with USEPA Method 546 (data not shown). Once confirmation was performed, samples were aliquoted from the stock quenched water and spiked with concentrations of microcystin-LR (Gold Standard Diagnostics, Warminster, PA). Spiked samples were refrigerated and sent to the NJDEP pesticides laboratory and analyzed using USEPA Method 544 within 48 hours. Concurrently, samples were processed according to USEPA Method 546, and frozen and thawed three times before being passed through a glass fiber filter and transferred to a fraction tube for analysis using the SAES kit. Finished drinking water that was not spiked, but was frozen and thawed, was then spiked with all three concentrations and run in parallel to measure any inhibitory effects from the drinking water itself or loss from filtering. Data from validation of USEPA Method 544 is presented in Table 16, and data from USEPA Method 546 for comparison is in Table 15. Sample spikes contained only microcystin-LR so no other congener detections are expected, as confirmed in Table 16. A comparison between the two tests is shown in Table 17. While differences are expected given the nature of the testing, the validation of detection using EPA 544 highlights that in this testing, since only microcystin-LR was used there was little variation in analysis between EPA 546 and 544. It is unlikely that in a real-world scenario that these differences would be as low, as congener bias and selection would likely skew the data. In this scenario since the spike samples contained only microcystin-LR and the study was designed to validate the performance the SAES kit using EPA 546; EPA 544 was used to ensure that detections with the SAES kit were confirmed detections and not due to any reactions of compounds inside the finished drinking water matrix. Generally, a good agreement was observed between the two analysis performed SAES and USEPA 544. The limitation of this analysis is that only one congener of microcystin (microcystin-LR) was used, and USEPA 544 is calibrated to six congeners of microcystin (including microcystin-LR) and one homologous structure, nodularin (listed out in Table 16), there remains the possibility that the ELISA method could report a detection of a congener not included in USEPA 544.

Consideration of Use of Lower Calibration Standards in ADDA-OH Test

During the presentation of this data to the Testing Subcommittee, a suggestion was made to consider using alternative calibration standards which would allow a higher resolution within the concentration range of interest to NJDEP. Currently the calibration standards for ADDA-OH ELISA, kit are 0, 0.15, 0.4, 1.0, 2.0, and 5.0 µg/L, and for the SAES kit the standards are 0, 0.05, 0.15, 0.4, 1.5, and 5 µg/L. Other ELISA kits are available which detect microcystin-LR specifically, rather than total microcystin, use a more compressed standard regimen and have lower detection limits since the antibody used for microcystin-LR is specific for that congener and has very poor cross reactivity with other congeners. The calibration standards for these microcystin-LR assays are typically 0, 0.05, 0.1, 0.2, 0.4, and 2 µg/L. However, the use of a range of standards within the concentrations of interest to NJDEP with the broader antibody for the ADDA group (i.e., the ADDA-OH kit) would not lead to increased resolution at lower concentrations, given the cross reactivity among congeners and binding efficiency values associated with the antibody. Since NJDEP is not proposing a health advisory level specifically for microcystin-LR and instead for total microcystin congeners, the approach of using an alternate set of standards with the ADDA-OH detection methodology would not increase resolution on the lower end of the concentration curve.