

DEVELOPMENT OF A PROTOCOL: MONITORING FOR MICROCYSTINS BY ELISA AT LAKE MANATEE

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INTRODUCTION

Lake Manatee Reservoir is an earthen impoundment of the Manatee River formed in 1967 near Bradenton. It covers about 729 hectares (1800 acres) when full and extends nearly 9.7 kilometers (6 miles) upstream from the dam. It has an average depth of 3.9 meters (12.8 feet) with a relatively shallow sloping shoreline in most places. Farmland, orange groves, woods, scrub, and homesites occupy the land that surrounds the lake. The area around Lake Manatee is becoming a popular place to live, despite the fact that it is more than 16.1 kilometers (10 miles) from I-75 and 24.2 kilometers (15 miles) from downtown Bradenton. There is a state park along a portion of the southwestern shoreline that provides a beach, a boat ramp, dock, hiking, and camping facilities for visitors. It is not unusual to see cows, alligators, and turtles sunning themselves near the banks all around the lake. Anhinga, herons, wood storks, and egrets flock to its shallows and shores.

Lake Manatee serves as the drinking water source of about 2/3 of the water consumed by nearly 300,000 people in Manatee and Sarasota Counties. It is a eutrophic lake with concentrations of nutrients entering the lake in runoff from neighboring orange groves, farmlands, home lawn fertilization, and cow pastures as well as naturally occurring phosphate. The lake has periodic blue green algae blooms of *Anabaena*, *Oscillatoria*, and *Microcystis*, mostly occurring between April and September. Certain species of these cyanobacteria have been known in other parts of the world to produce microcystins; a group of hepatotoxins that may produce liver anomalies, including hemorrhage and other systemic upsets that are sometimes lethal in both animals and people.

The World Health Organization (WHO) considers 1.0 part per billion (ppb) of microcystins a dangerous level. Microcystins is also on the EPA's "Drinking Water Candidate Contaminant List" (DWCCCL) which includes contaminants being considered for regulation under the Safe Drinking Water Act.

A recent survey of surface water bodies in Florida indicated trace levels of microcystins present in Lake Manatee during a severe cyanobacterial bloom. Further investigation of cyanotoxins at the water treatment plant was determined to be prudent, with a monitoring program that included microcystins. Operations needed a protocol that would give sufficient lead time, should there be any microcystin contamination, that there would be time to perform remediation before the toxins reached 1.0 ppb. The protocol also had to be compatible with the existing Taste and Odor (T&O) Monitoring Program, another cyanobacteria related detection plan.

The water treatment plant, as part of its quality assurance program, has a taste and odor monitoring program that monitors for 2-Methyl isoborneol and geosmin, odorants sometimes produced by *Anabaena* and *Oscillatoria*. In conjunction with the odorant monitoring program, an algae collection and monitoring plan is in place that surveys the

whole lake shoreline on a twice weekly basis during the “algae season” and once or twice per month in the off time. It was also used as a collection vehicle for the samples that were to be analyzed for the microcystins toxins.

There were several analytical methods appropriate for the analysis of microcystin in water. The two that seemed most suitable were enzyme linked immunosorbent assay (ELISA) and high performance liquid chromatography/mass spectrometry (HPLC/MS). It was decided to start with the less complex, more easily used and less expensive of the two, ELISA, until the necessary budgeting and training matters could be implemented for the HPLC/MS equipment.

The Envirologix© ELISA kits and a Stat Fax 303 microstrip reader were purchased. A sampling plan was developed and sample-handling procedures were examined. The more appropriate of two ELISA methods to use for our specific situation was decided upon.

Holding times and handling methods were scrutinized. A set of spiked samples was created to be used as ongoing analytes to be measured each analysis date. An aliquot of each was to be assayed, the recoveries noted, then the samples were to be stored in the refrigerator until the next analysis date, when they were re-examined. This procedure was started in November 2001 and will continue until the samples are completely used.

The ELISA was performed in various trials to give us the optimal performance for our laboratory needs. Sensitivity was of prime importance, as the lowest possible detection limit was desired. The analyst’s operating conditions were scrutinized and sample matrix influence was also taken into consideration. A quality control protocol was developed to determine the precision and reliability of the test.

It was decided that the increased sensitivity method would be used to monitor and detect any microcystins that might occur in the lake water. It was found that the ELISA method is reliable enough to detect them at concentrations about 10 times less than the WHO MCL (Maximum Contaminant Level). Detection at lower levels, without a concentration step, was found to be unreliable.

SAMPLING PLAN

The T&O sampling plan was developed to anticipate the occurrence of large concentrations of 2-MIB and geosmin in the lake water. The plan was used to reduce the possibility of having an uncontrollable algae bloom, which may cause a “taste and odor event”.

The lake was divided into 15 discrete areas from the dam at the western end to Verna Bethany Bridge at the northeastern end. The samples are collected for the algae count in water no deeper than three to four feet and as close to shore as possible. Composite samples are collected in each area, 500 milliliters total, collected in 50-milliliter aliquots. Two hundred milliliter sub samples are gravity filtered through sand in a Sedgwick-Rafter funnel and resuspended in 20 milliliters of filtrate. The resuspended samples are microscopically examined and counted on a Sedgwick-rafter counting cell (SM10200).

A portion of the composite sample is reserved for microcystins analysis in 4-milliliter vials with zero headspace. The microcystins are relatively heavy molecules, each having a

molecular weight around 1000. They should be kept cool and be handled gently with no unnecessary agitation.

Using the algae counts as an indication of the possible presence of microcystins, samples were selected for analysis. Since we were not sure where in the lake the microcystins might be found we used several selection criteria. First, if some *Microcystis* were recognized in the algae count; the sample from the area where they were found would be tested for microcystins. Next, a couple of samples would be chosen from an area where other cyanobacteria, *Anabaena* or *Oscillatoria*, were found. Always assuming that all of the cyanobacteria have not been found and to rule out the absence of microcystins due to competitive disadvantage, samples from several areas that had no cyanobacteria were chosen. A total of 10 samples plus QC were analyzed at one time.

METHOD DEVELOPMENT

Equipment

An Enviroligix[®] Microcystin Plate Kit (#EP022), was used to perform the **ELISA** analysis. It was chosen because it was relatively inexpensive, required a relatively small sample size, and had shown to be fairly reliable in published studies. In this assay, microcystin toxin in the samples reacts with a limited number of antibody binding sites located on the bottom of small wells on a microtiter plate. After a wash step the results are visualized with a color development step. The plates are scanned by a spectrophotometer and the concentrations of toxin are quantitated by comparing optical densities to those of an established calibration curve.

Micropipettes were used in varying volumes. It is important to check the calibration before each use by measuring a known aliquot of water on a balance accurate enough to measure to the nearest tenth of a milligram.

A microstrip reader, **Stat Fax 303 spectrophotometer**, was purchased to read the optical densities of each sample-containing well. The spectrophotometer's software automatically calculates the concentration in each well from a previously run calibration curve. It has the advantage of being able to store calibration curves and analysis methods so that a curve performed several weeks previously can be used in the quantification of later samples having the same lot numbers for the reagents and check standards as those used in the analysis of the calibration curve.

Testing Its Capability

On March 20, 2001 the first set of samples was analyzed. For the next six months the regular sensitivity method, which tested samples with concentrations between 0.17 and 1.60 ppb, was used. The negative control calibrator was assigned a value of 0.09 ppb. The sample aliquots were analyzed only once per test and the analysis was performed almost every week using samples collected during the weekly algae sampling. Several check standards at a concentration of 0.50 ppb were analyzed along with the lake samples to get a feel for the accuracy of the method. Then, regular check standard analysis, at the same concentration, was performed with each set of environmental samples. All of the lake and finished water sample results were less than 0.17 ppb. After six months it was determined that a lower detection level would be advantageous.

The manufacturer's increased sensitivity method was used to look at smaller concentrations, between 0.01 and 0.45 ppb, the negative control being assigned a value of 0.01 ppb. Method detection limit studies were performed using duplicate sample analysis and two kinds of samples. Aliquots of Milli-Q water were spiked with the non-toxic calibrator included in the kit and other aliquots were spiked with microcystins-LR toxin. It was determined that the method detection limit (MDL) was 0.035 ppb and that a practical quantitation limit (PQL) was 0.105 ppb (see Table 1).

Using this increased sensitivity calibration method, the samples and QC samples were also analyzed in duplicate. Using duplicate analysis of the sample aliquots was decided upon to insure a more accurate result.

Date Analyzed	True Value	Instrument value diluted calibrators	Microcystin-LR Standards
10/2/01	0.15ppb	0.17	
10/8/01	0.15ppb	0.14	0.39
10/10/01	0.15ppb	0.17	0.42
10/10/01	0.15ppb	0.16	0.45
10/10/01	0.15ppb	0.16	0.47
10/11/01	0.15ppb	0.15	0.37
10/11/01	0.15ppb	0.15	0.41
10/11/01	0.15ppb		0.41
Mean	0.15ppb	0.157	0.417
Standard Dev		0.0111270	0.0340168
% RSD		7.08	8.15
MDL		0.035	0.107
PQL		0.105	0.320

Table 1. Method Detection Limit (MDL) and Practical Quantitation Limit(PQL) for Increased Sensitivity Method using the dilute calibrators included in the kit and standards made using an independently produced microcystin-LR standard.

Developing a QC Protocol

Although the kit instructions do not include instructions to analyze Quality Control samples, it was decided that doing so would help give an indication of the precision and accuracy of the method. Quality Control samples are analyzed to demonstrate to any observer that the method is working and to what degree it is working. It is assumed that if the recoveries are acceptable for a check standard, then the recoveries for the samples are the same or similar if there are no sample interferences or contaminants. A negative control, or blank is analyzed to demonstrate that there is probably no contamination of the reagents or wells.

Using the original analytical protocol, 0.50 ppb check standards were analyzed with every set of samples analyzed. The mean recovery for twenty-one check standards analyzed over a period of six months was 88.3% with a 22.2% RSD (Table 2). It should be noted (see Table 1a) that the first two kits used had much higher RSD's, 30.6% and 29.6%. Better data was produced using calibration curves #6 and #7, having RSD's 14% and 17.1%. This could be related to the greater experience with the method.

When the increased sensitivity method was used, 0.15 ppb check standards were analyzed with each set of samples. Analyzing each sample and check standard in duplicate, the mean recovery of twenty-five check standards was 101.6% with 15.6% RSD (Table 3). When this method was used a more consistent calibration was used. A single calibration curve was used for all kits having the same lot numbers for reagents and standards. When the recoveries decreased significantly or a new set of lot numbers was started, a new curve was created. The decreased RSD's for calibration curves #8 and #11 may be due to the use of a consistent

curve and the increased number of samples analyzed per curve.

Date	% Recovery
3/28/01	120.0
4/3/01	60.0
4/10/01	80.0
4/24/01	80.0
5/1/01	60.0
5/15/01	80.0
5/24/01	120.0
5/30/01	60.0
5/30/01	80.0
6/5/01	100.0
6/21/01	108.0
6/26/01	118.0
7/6/01	84.0
7/10/01	104.0
7/13/01	88.0
7/17/01	78.0
7/27/01	90.0
7/30/01	86.0
8/8/01	114.0
8/20/01	76.0
9/4/01	68.0
mean	88.3
Standard Dev	19.6
% RSD	22.2
n=21	

Table 2. Check standard percent recovery using the standard ELISA method. The Calibration Range is from 0.09 parts per billion to 1.60 parts per billion. Standards, having the concentration 0.50 ppb, were analyzed singly. Table includes data collected using seven different calibration curves created with standards from four different lot numbers.

Calibration #4 using kit #4

Date	% Recovery
3/28/01	120.0
4/3/01	60.0
4/10/01	80.0
4/24/01	80.0
5/1/01	60.0
mean	80.0
Standard Dev	24.5
% RSD	30.6
n=5	

Calibration #6 using kit #6

Date	% Recovery
6/5/01	100.0
6/21/01	108.0
6/26/01	118.0
7/6/01	84.0
mean	102.5
Standard Dev	14.4
% RSD	14.0
n=4	

Kit #5
new calibration for each test

Date	% Recovery
5/15/01	80.0
5/24/01	120.0
5/30/01	60.0
5/30/01	80.0
mean	85.0
Standard Dev	25.2
% RSD	29.6
n=4	

Calibration #7
standards from several kits

Date	% Recovery
7/10/01	104.0
7/13/01	88.0
7/17/01	78.0
7/27/01	90.0
7/30/01	86.0
8/8/01	114.0
8/20/01	76.0
9/4/01	68.0
mean	88.0
Standard Dev	15.0
% RSD	17.1
n=8	

Table 2a: Check standard percent recovery for the standard ELISA method grouped according to calibration curve. Calibration range is from 0.09 parts per billion to 1.60 parts per billion. The data in each of the boxes was collected using standards and reagents having the same lot numbers.

Date	% Recovery
9/25/01	106.7
10/2/01	113.3
10/8/01	93.3
10/10/01	113.3
10/10/01	106.7
10/10/01	106.7
10/11/01	100.0
10/11/01	100.0
10/11/01	81.2
10/23/01	100.0
11/5/01	73.0
11/29/01	126.7
12/5/01	106.7
12/11/01	126.7
12/12/01	93.3
12/18/01	92.8
12/18/01	100.0
1/11/02	120.0
1/30/02	80.0
2/22/02	66.7
2/27/02	106.2
3/15/02	93.8
3/19/02	125.0
3/26/02	106.2
mean	101.6
Standard Dev	15.8
% RSD	15.6
n=25	

Table3. Check standard percent recovery for the increased sensitivity ELISA method. The calibration range is from 0.01 parts per billion to 0.45 parts per billion. Standards, having the concentration 0.15 ppb, were analyzed in duplicate. Table includes data collected using four different calibration curves created with standards and reagents from four different lot numbers.

Calibration #8
standards from several kits

Date	% Recovery
9/25/01	106.7
10/2/01	113.3
10/8/01	93.3
10/10/01	113.3
10/10/01	106.7
10/10/01	106.7
10/11/01	100.0
10/11/01	100.0
10/11/01	81.2
mean	102.4
Standard Dev	10.2
% RSD	10.0
n=9	

Calibration #11
standards from several kits

Date	% Recovery
12/5/01	106.7
12/11/01	126.7
12/12/01	93.3
12/18/01	92.8
12/18/01	100.0
1/11/02	120.0
2/27/02	106.2
3/15/02	93.8
3/19/02	125.0
3/26/02	106.2
mean	107.1
Standard Dev	12.9
% RSD	12.0
n=10	

Calibration #9 using Kit #9

Date	% Recovery
10/23/01	100.0
11/5/01	73.0
mean	86.5
Standard Dev	19.1
% RSD	22.1
n=2	

Calibration #12 using Kit #12

Date	% Recovery
1/30/02	80.0
2/22/02	66.7
mean	73.4
Standard Dev	9.4
%RSD	12.8
n=2	

Table 3a Percents recovery for the increased sensitivity ELISA method.

The calibration range is from 0.01 parts per billion to 0.45 parts per billion.
The data in each of the boxes was collected using standards and reagents having the same lot numbers on the same/single calibration curve.

Determining Holding Times Using Decomposition Standards

An attempt was made to determine sample holding times for samples stored in a laboratory refrigerator.

A set of standards were prepared and stored to examine sample behavior over time. Two different matrices were used to examine the possible matrix influence on sample decomposition.

Three standards were prepared using microcystins-LR as the source of the toxin. Four milliliter aliquots of raw lake water and ultra pure deionized water (Milli-Q) were spiked. The raw water standards were prepared at two concentrations, 1.0 ppb and 0.16 ppb. The milli-Q standard was prepared at a concentration of 0.16 ppb. Although the lower concentrations are near the PQL, they were chosen because they most nearly simulated the concentrations that might be found in the lake environment.

The standards were stored in the dark at $5^{\circ}\pm 1^{\circ}$ C from November 29, 2001 to the present. They were only removed from the refrigerator to remove sample portions for analysis, and then returned immediately after aliquots were removed.

An aliquot of each standard was analyzed weekly or twice per month, over a four-month period. The 1.0 ppb standard was analyzed by diluting it to one half its original concentration. When the data (Figure 1) were examined from that period, it was concluded that all of the samples could be stored for at least one month without significant change in their concentrations. It was noted that the concentration of microcystin in the 1.0 ppb raw water sample seemed to increase rather than deteriorate. However, the standard deviation and the %RSD are close to the increased sensitivity data standard deviation and %RSD, perhaps indicating that there was no physical change to the standard.

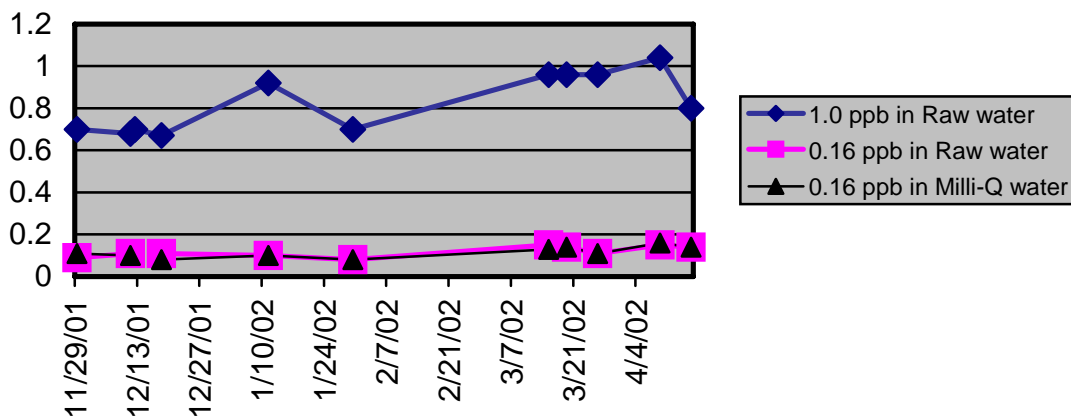


Figure 1. Microcystins Decomposition Data (for raw data see Table 4).

Table 4. Raw data from Decomposition test for holding time determination.

Cal Curve #	Date Analyzed	Raw Water @		Milli-Q @
		1.0 ppb	0.16 ppb	0.16ppb
10	11/29/01	0.70	0.09	0.11
11	12/11/01	0.68	0.11	0.10
11	12/12/01	0.70		
11	12/18/01	0.67	0.11	0.08
11	1/11/02	0.92	0.10	0.10
12	1/30/02	0.70	0.08	0.08
11	3/15/02	0.96	0.15	0.13
11	3/19/02	0.96	0.14	0.14
11	3/26/02	0.96	0.11	0.11
11	4/9/02	1.04	0.15	0.16
11	4/16/02	0.80	0.14	0.14
	mean	0.83	0.12	0.11
	Standard Dev	0.15	0.03	0.03
	% RSD	18.1	22.1	23.9
	% Recovery	82.9	72.2	70.1

Comparison of Microcystin Standards

At the time the Method detection limit studies were performed, two kinds of samples were analyzed using the duplicate sample analysis of the increased sensitivity method. Aliquots of Milli-Q water were spiked at 0.15 ppb with the non-toxic calibrator included in the kit and other Milli-Q standards of the same calculated concentration were prepared using microcystins-LR toxin from an independent source.

Comparison of the data from the sets of seven individual standards shows that the LR standards read nearly three times higher than the kit calibrators (Table 1). The precision data were comparable for the two sets of data (7.1% RSD, 8.2% RSD). The microcystins standards are not universally available and the ones that are available are not always a certified concentration. The kit literature suggests that the LR would read somewhat higher, however, the discrepancy may also be explained by the lab using a standard that was not certified, thus creating a solution of LR that is more concentrated than its calculated value.

ANALYST'S NOTES

The analyst's precision and accuracy in measuring and delivering the trace volumes of reagents and strict attention to timing is crucial to the quality of the data. If these factors are not tightly maintained, the value of the results will be suspect. An analyst experienced in the use of microliter pipettes, paying attention to details should be able to generate similar precision and accuracy results on a routine basis. Note the first few sets of standards in Table 1a. The higher RSD's may reflect the analyst's inexperience with the method.

Since the sample size is so small, fifty microliters, every little bit counts. Splashing or spillage could lose half the sample with one small slip. The wells should be kept dust free when stored and should not have smudges on the outside or unidentifiable particles on the inside at any time during the analysis. After the wash solution step and before the addition of the substrate, the wells should be checked for anything that might obstruct light passage through the well and the medium it will hold.

Check pipettes for correct calibration every time the analysis is run. Small changes in the calibration of the measuring devices can make a big difference in the outcome of the analysis.

The best results were achieved when the procedure is followed with manic consistency. Timing of each addition is crucial. Consistency of measurement is a must. The same amount should be added in the same sample order taking the same amount of time each time the analysis is performed. Each time the assay is performed it should be consistent with the procedure performed on the calibration curve to be used, so that when the quantitation is performed the curve will match the sample procedure to the minute.

CONCLUSIONS ABOUT THE USEFULNESS OF THE PROTOCOL

The cost of implementing the protocol, in time and effort is modest when compared to more sophisticated instrumental analysis.

It has little impact on the programs already in place. The sampling procedure is the same as that for the algae monitoring program therefore there is little additional cost, time, or effort needed for the additional *Microcystis*/microcystins sampling. The sampling and subsequent microscopic examinations are as good as the technician performing the procedures. When the sampling is done with care and the count is done according to the SOP, the procedure is very effective at finding cyanobacteria that are present.

The analysis by ELISA takes 1½ hours of the analyst's time and a small amount of training. The kit cost, depending on how frequently the tests are performed, may be significant.

The data are useful when used as a presence/absence indicator. The recovery data, at 101.6% on average, seems to indicate that the method can tell when the toxin is present. However, the precision data collected over the year for both the general method and the increased sensitivity method (22.2 % RSD and 15.6% RSD) indicates that the ELISA analysis may not always give accurate quantitation data for samples

having concentrations less than 0.2 ppb. It should be noted that the mean recoveries and RSD's vary from kit to kit and lot number to lot number (See Tables 1, 1a, 2, 2a and 3). A very obvious example can be seen in the graph of the decomposition standard data for January 30, 2002. The kit is adequate for identifying the toxin at mid range concentrations, but not as accurate or reliable as could be desired for quantitation.

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