Total Microcystins and Nodularins
Standard Operating Procedure

1. Scope and Application
   
   1.1 Analytes
   Total Microcystins and Nodularins
   
   1.2 Reporting Limit
   0.3 μg/L as Microcystin-LR (MC-LR)
   
   1.3 Applicable Matrices
   This method is used to determine the concentration of total microcystins and nodularins (both intracellular and extracellular) in non-potable freshwaters.
   
   1.4 Dynamic Range
   The analytical range for the determination of total microcystins and nodularins is from 0.30 μg/L to 5.0 μg/L. Sample concentrations below the reporting limit are flagged “<” in the Laboratory Information Management System (LIMS). Sample concentrations that exceed the upper range limit must be diluted and reanalyzed.
   
   1.5 Regulatory Information

2. Summary of Procedure
   Ambient water samples undergo three freeze/thaw cycles in order to lyse cyanobacterial cells and ensure all microcystins and nodularins are present in the dissolved phase. Lysates are then filtered and analyzed using an indirect competitive Enzyme-Linked Immunosorbent Assay (ELISA) procedure, based on the congener-independent detection of the ADDA amino acid side chain present in most microcystin and nodularin congeners. The endpoint of the ELISA procedure is the colorimetric determination of total microcystins/nodularins concentration, which is inversely proportional to sample absorbance. A four-parameter logistic regression model is used for calibration. The assay “exhibits very good cross-reactivity with all cyanobacterial cyclic peptide toxin congeners tested to date” (Eurofins Abraxis, 2021). See the ELISA Kit User Guide (kept on file in the laboratory with the SOP) for details.
3. Safety Issues

3.1 Chemical Hazards
A. Gloves, safety glasses, and lab coats should be worn when preparing and performing this analysis.
B. The microcystin-LR found in the standards is a hepatotoxin. The stop solution contains sulfuric acid.
C. Dry ice should be handled using insulated gloves.
D. The 95% ethanol solution should be handled using 8 mil nitrile gloves.
E. For proper handling techniques for specific chemicals, consult the appropriate Safety Data Sheets (SDS).

4. Sample Preservation, Containers, Processing and Analysis Times

4.1 Sample Preservation
A. Samples should be chilled upon collection and kept at or below 10°C. Sample temperatures are checked with an NIST-traceable infrared thermometer upon arrival at the laboratory.
B. If the sample temperature is above 10°C due to a short sample transit time, and the ice in the cooler is still frozen, samples do not need to be flagged. Otherwise, samples received outside the acceptable temperature range should be noted on the login sheet, and sample results flagged with a “W” data flag in LIMS.
C. Gently mix each sample by inversion. Using a micropipette, transfer a 10 mL aliquot of each sample to a 40-mL amber glass lysing vial. The original sample container and lysing vial should be labeled with the Sample ID. The original sample container and lysing vial are then transferred to the freezer for storage until sample preparation begins.

4.2 Containers
A. Samples are collected in unbaked 250-mL amber glass bottles, with PTFE (polytetrafluoroethylene) lined plastic caps.
B. Sample bottles should be filled with no more than 100 mL of sample water and stored horizontally in the freezer. Minimum acceptable sample volume is 10 mL.
C. Results for samples collected in the incorrect container type are flagged with a “C” data flag in LIMS.

4.3 Processing and Analysis Times
A. Sample Processing: 14 days
B. Lab Analysis: 14 days
C. LIMS Entry: 1 week
D. Data are assigned an “H” data flag in the LIMS if the EPA-mandated holding time of 14 days is not met. See EPA 546, Section 8.6 for further details.
5. Reagents, Standards, and Equipment

5.1 Reagent General Information

All reagents are commercially purchased, reagent grade or better, and should be stored in their original container. Date and initial reagent bottles when received and when opened. Note expiration date, if any.

5.2 Reagents

A. Eurofins Abraxis Microcystins/Nodularins (ADDA) ELISA Kit, PN 520011. Kits are shipped chilled and are refrigerated immediately upon arrival at the laboratory. The top of each kit is initialed and dated, both when received and when opened for the first time. Reagents from one kit lot should not be used with reagents or standards from another kit lot. The exception to this rule is the wash buffer; wash buffers from one kit lot can be used with wash buffer from another kit lot, or with wash buffer purchased separately. All kit reagents should be kept refrigerated (2-8°C). All kits include:
1. Sample Diluent
2. Anti-Microcystins Antibody Solution
3. Anti-Sheep Immunoglobulin G (IgG)/Horseradish Peroxidase (HRP) Conjugate Solution
4. Color/Substrate Solution (Tetramethylbenzidine, TMB)
5. Stop Solution (Sulfuric Acid Solution)
6. Wash Buffer, 5x Concentrate (can also be purchased separately, Eurofins Abraxis, PN 300005).

B. Other Reagents
1. 1 N Hydrochloric Acid Solution
2. 70% v/v Isopropanol
3. Methanol, ACS Grade
4. Dry Ice Pellets
5. Ethanol, 190 Proof

5.3 Standards

A. Eurofins Abraxis Microcystins/Nodularins (ADDA) ELISA Kit, PN 520011. Standards from one kit lot should not be used with standards or reagents from another kit lot. All kit standards should be kept refrigerated (2-8°C). Kit standards include:
1. Microcystin Standard 0, 0.00 μg/L
2. Microcystin Standard 1, 0.15 μg/L
3. Microcystin Standard 2, 0.40 μg/L
4. Microcystin Standard 3, 1.00 μg/L
5. Microcystin Standard 4, 2.00 μg/L
6. Microcystin Standard 5, 5.00 μg/L
B. The following standards are part of the Eurofins Abraxis Method 546 Accessory Pack, PN 520013. All accessory pack standards should be kept refrigerated (2-8°C).
   1. Microcystin-LR Low Calibration Verification (Low-CV) Standard, 0.3 μg/L
   2. Microcystin-LR Spiking Solution, μg/L (Can also be purchased as a separate item, PN 300702)
   3. Microcystin-LR Quality Control Sample (QCS), 0.6 μg/L

5.4 Equipment
   A. Water Bath
   B. EVA (Ethylene-Vinyl Acetate) Ice Container
   C. Disposable Syringes, polypropylene barrels with polyethylene plungers
   D. 25 mm glass fiber syringe filters, 0.45 μm
   E. Disposable polystyrene beakers
   F. 4-mL amber glass vials
   G. 40-mL amber glass vials

6. Laboratory Performance
   A. An analytical batch is defined as all calibration standards, environmental samples, and batch quality control samples run together on a single 96-well plate. Every calibration standard, batch quality control sample, and environmental sample is analyzed in two separate wells (well replicates) on each plate.
   B. The Laboratory Reagent Blank (LRB) is comprised of Milli-Q water and is used to ensure that samples or standards have not been subject to contamination during preparation or analysis. The LRB is processed through the lysing and filtration process in the same way as a field sample.
   C. The Low Calibration Verification (Low-CV) is used to ensure the accuracy of the calibration curve near the MRL. The Low-CV is purchased as part of the Eurofins Abraxis Method 546 Accessory Pack. It has a concentration of 0.30 μg/L and does not undergo the lysis/filtration step.
   D. The Laboratory Fortified Blank (LFB) is used to ensure method performance in the absence of a sample matrix. The LFB is fortified with the Microcystin-LR Spiking Solution at the EC₅₀, which is the concentration at the inflection point of the calibration curve (0.60 μg/L). Two separate LFBs are processed through the lysing and filtration process.
   E. The Laboratory Fortified Sample Matrix (LFSM) and Laboratory Fortified Sample Matrix Duplicate (LFSMD) are used to ensure method performance in the presence of a sample matrix. The LFSM and LFSMD are fortified with the Microcystin-LR Spiking Solution at the 1.00 μg/L
level and are each processed through the lysing and filtration process separately.

F. A Quality Control Sample (QCS) is analyzed with each analytical batch for quality assurance purposes. The QCS is purchased as part of the Eurofins Abraxis Method 546 Accessory Pack, originates from a different source than the calibration standards, and has a concentration of 0.60 μg/L. The QCS does not undergo the lysis/filtration step.

7. QC Procedure

7.1 Calibration Requirements

A. The calibration model used for the analysis is a four-parameter logistic regression model. Sample absorbance is related to the concentration of total microcystins and nodularins in the sample using the following equation:

\[ y = \frac{(a - d)}{1 + \left( \frac{x}{c} \right)^b} + d \]

where \( a \) is the absorbance at the bottom plateau of the calibration curve, \( b \) is the slope-related term at the inflection point of the calibration curve, \( c \) is the concentration at the inflection point (EC\(_{50}\)) of the calibration curve, and \( d \) is the absorbance at the top plateau of the calibration curve, \( x \) is the sample concentration, and \( y \) is the sample absorbance.

B. A new calibration is performed each time a new analytical batch is analyzed. The square of the correlation coefficient \( r^2 \) of the calibration curve must be ≥ 0.98; if \( r^2 \) is < 0.98, the results of the analytical batch are invalid, and the batch must be reanalyzed.

7.2 Well Replicate Requirements

A. For each set of calibration standard well replicates, the percent coefficient of variation (%CV) of the absorbance values is determined using the formula below. The %CV must be ≤10%. One pair of well replicates can exceed this 10% CV limit, if the %CV is ≤15%. If the %CV criteria are not met, the results of the analytical batch are invalid, and the batch must be reanalyzed.

\[ \%CV = \frac{\text{Standard Deviation of Absorances}}{\text{Mean Absorbance}} \times 100\% \]

B. For each set of batch quality control sample well replicates, the percent coefficient of variation (%CV) of the absorbance values is determined using the equation in section 7.2.A. The %CV must be ≤15%. If the %CV criteria are not met for the Low-CV, LRB, LFB, or QCS, the results of the analytical batch are invalid, and the batch must be reanalyzed. If the %CV criteria are not met for the LFSM and/or LFSMD, the LFSM/LFSMD results are invalid, and the 20 or fewer samples that are
analyzed between the invalid LFSM/LFSMD pair and the next LFSM/LFSMD pair or the end of the batch must be reanalyzed.

C. For each set of environmental sample well replicates, the percent coefficient of variation (%CV) of the absorbance values is determined using the equation in section 7.2.A. For environmental samples, the %CV must be ≤15%. If the %CV criteria are not met, the results for the sample in question are invalid and the sample must be reanalyzed. If the sample in question was used for the LFSM/LFSMD, the LFSM/LFSMD results are invalid, and the 20 or fewer samples that are analyzed between the invalid LFSM/LFSMD pair and the next LFSM/LFSMD pair or the end of the batch must be reanalyzed.

7.3 Batch Quality Control Requirements

A. Two LRBs, both derived from the same lysate, are run per analytical batch. The concentration measured in both LRBs must be less than half the MRL (<0.15 μg/L), or else any samples with results above the MRL are invalid and must be reanalyzed.

B. The measured concentration in the Low-CV must be ≥50% of the true value (≥0.15 μg/L) and ≤150% of the true value (≤0.45 μg/L). If the concentration falls outside this range, the results of the analytical batch are invalid, and the batch must be reanalyzed.

C. The percent recovery for both LFBs must be ≥60% (≥0.60 μg/L) and ≤140% (≤1.40 μg/L) of the fortified concentration. If the percent recovery of either (or both) LFBs falls outside this range, the results of the analytical batch are invalid, and the batch must be reanalyzed. Percent recovery is calculated using the following equation:

\[
%\text{Recovery} = \frac{\text{Measured Concentration}}{\text{Fortified Concentration}} \times 100
\]

D. If the analytical batch contains 20 or fewer samples, one LFSM/LFSMD is required. If the analytical batch contains more than 20 samples, an additional LFSM/LFSMD pair is required. Over time, vary the sampling sites used for the LFSM/LFSMD.

E. If the measured concentration of the LFSM and/or LFSMD is >5.00 μg/L (highest calibration standard) or the fortification level (1.00 μg/L) is not at least twice the concentration in the parent sample, the LFSM/LFSMD results are considered unusable and are discarded.

F. The mean percent recovery of the LFSM and LFSMD is calculated using the following equation:

\[
\text{Mean % Recovery} = \frac{(A - B)}{C} \times 100\%
\]
where $A$ is the mean measured concentration of the LFSM and LFSMD, $B$ is the concentration of the unfortified parent sample, and $C$ is the fortification concentration. If the concentration of the unfortified parent sample falls below the MRL, it should still be used in the calculation. The mean percent recovery should be $\geq 60\%$ and $\leq 140\%$. If the mean percent recovery falls outside this range, the parent sample should be flagged as “suspect-matrix” using the “M” flag in LIMS.

G. To evaluate the precision of measurement between the LFSM and LFSMD, Relative Percent Difference (RPD) is calculated using the equation below, where both LFSM and LFSMD are the measured concentrations of each respective fortified sample. The RPD should be $\leq 40\%$. If the RPD is $>40\%$, the parent sample should be flagged as suspect-matrix using the “M” flag in the LIMS.

\[
RPD = \frac{|LFSMD - LFSM|}{(LFSMD + LFSM)/2} \times 100\%
\]

H. The measured concentration in the QCS must be $\geq 70\%$ of the true value ($\geq 0.42 \mu g/L$) and $\leq 130\%$ of the true value ($\leq 0.78 \mu g/L$). If the measured concentration falls outside this range, results of the analytical batch are invalid, and the batch must be reanalyzed. If the QCS concentration is outside of the acceptable range on the reanalysis, troubleshoot the instrument.

I. Quality control acceptance criteria are summarized in the table below.

<table>
<thead>
<tr>
<th>Quality Control Requirement</th>
<th>Acceptance Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibration Well Replicates</td>
<td>%CV for absorbance values $\leq 10%$, one pair may be $\leq 15%$. $r^2 \geq 0.98$.</td>
</tr>
<tr>
<td>Environmental and QC Sample Replicates</td>
<td>%CV for absorbance values $\leq 15%$.</td>
</tr>
<tr>
<td>Laboratory Reagent Blank (LRB)</td>
<td>Concentration in both LRBs $&lt;\text{one-half the MRL (}&lt;0.15 \mu g/L$).</td>
</tr>
<tr>
<td>Low Calibration Verification (Low-CV)</td>
<td>Percent recovery must be $\geq 50%$ and $\leq 150%$ of the true value.</td>
</tr>
<tr>
<td>Laboratory Fortified Blank (LFB)</td>
<td>Percent recovery for both LFBs must be $\geq 60%$ and $\leq 140%$ of the fortified concentration.</td>
</tr>
<tr>
<td>Laboratory Fortified Sample Matrix (LFSM/LFSMD)</td>
<td>Mean percent recovery of pair must be $\geq 60%$ and $\leq 140%$, and relative percent difference (RPD) must be $\leq 40%$. Fortification level (1.00 $\mu g/L$) must be at least twice the concentration in the parent sample.</td>
</tr>
</tbody>
</table>
8. Chemical Analysis Procedure

8.1 Instrumentation

Awareness Technology/Eurofins Abraxis ChemWell 2925 Cyanotoxin Automated Assay System (CAAS)
Eurofins Abraxis Manager Software, Version 6.4.1.1139

8.2 Sample Preparation/Lysis Procedure

A. Retrieve the lysing vials for the samples to be analyzed and the 250-mL amber sample bottle(s) for the sample(s) being used for the LFSM/LFSMD from the freezer. Remove the Method 546 Accessory Pack from the refrigerator.

B. If analysis will be performed during the same workday, remove the Eurofins Abraxis Microcystins/Nodularins ELISA kit from the refrigerator. Allow kit components to warm to room temperature before use.

C. Fill out the lysing bench sheet (can be printed from CAAS\Lysing Logs) with the Sample IDs to be lysed. Indicate Total Microcystins/Nodularins by filling the Analyte(s) column with an “M”. If samples were collected in the incorrect container type, the “Proper Presv?” and “Adjustments Needed” columns should be marked as “No” and “Moved to Amber Glass,” respectively.

D. If samples were previously prepared for cylindrospermopsins analysis, the filtrates used for that analysis can also be used for the microcystins/nodularins analysis; a second lysing procedure is not needed. However, if a sample’s pH was adjusted during the cylindrospermopsins sampling procedure, a new lysate must be prepared from a sample bottle that was not pH-adjusted.

E. Fill water bath with deionized water. Power on, adjust the setpoint to 35°C, and allow the temperature to stabilize before placing any samples in the bath.

F. Transfer the lysing vials and sample bottles to the water bath and allow all containers to completely thaw.

G. Once thawing is complete, remove each vial or 250-mL amber sample bottle from the bath, and gently invert several times to mix.

H. Prepare the LRB by pipetting 10 mL of Milli-Q water into a lysing vial.

I. Prepare the LFBs by adding approximately 5 mL of Milli-Q water into 10-mL volumetric flasks. Fortify by adding 120 μL of Microcystin-LR Spiking Solution to each flask. Bring the flasks to full volume with Milli-Q water, cap, mix well, and transfer to a lysing vial.

J. Prepare the LFSM/LFSMDs by adding approximately 5 mL of sample water into 10-mL volumetric flasks. Fortify by adding 200 μL of Microcystin-LR Quality Control Sample (QCS) Percent recovery must be ≥70% and ≤130% of the true value.
Spiking Solution to each flask. Bring the flask to full volume with sample water, cap, mix well, and transfer to a lysing vial.

K. Record the lot numbers and expiration dates of the spiking solution and the accessory pack on the lysing bench sheet.

L. Place the 250-mL amber sample bottles used to prepare the LFSM/LFSMD back into the freezer.

M. Prepare a dry ice/ethanol bath by adding dry ice to the EVA ice container, followed by enough 95% ethanol so that the liquid level is below the caps of the 250-mL amber sample bottles and lysing vials. The dry ice/ethanol bath should be used in a fume hood. Record the lot number of the ethanol used on the lysing bench sheet. Alternatively, a freezer may be used in place of the dry ice/ethanol bath.

N. Environmental samples should undergo only 3 freeze/thaw cycles to ensure consistency of results between samples. Thawing frozen samples upon arrival at the laboratory or removing a sample from the freezer and allowing it to thaw to obtain a sample aliquot are both counted as one freeze/thaw cycle. If a sample has already undergone 3 freeze/thaw cycles, it is ready to be filtered and can be set aside until all other samples have been lysed.

O. The LRB and LFBs, which are prepared directly from Milli-Q water and have not been stored frozen, must undergo 3 freeze/thaw cycles.

P. LFSMs and LFSMDs must also undergo 3 freeze/thaw cycles. Freeze/thaw cycles completed before spiking count toward the total. For example, if the sample bottle for the parent sample has already undergone 1 freeze/thaw cycle, only 2 more freeze/thaw cycles are needed.

Q. For the samples that require additional freeze/thaw cycles, place the lysing vials into the ethanol/dry ice bath and allow to freeze completely. Alternatively, if using a laboratory freezer, place the lysing vials inside the freezer in a horizontal position and allow to freeze completely. Once frozen, transfer the lysing vials to the water bath and allow to thaw completely.

R. For each environmental or QC sample lysed, record the number of freeze-thaw cycles performed in each container (250-mL sample bottle or lysing vial) on the lysing bench sheet.

S. Once the final thaw is completed, decant each lysing vial into a disposable polystyrene beaker. Filter each using a disposable syringe and 0.45 μm filter. Use 5 mL of sample water to rinse the filter, discarding the rinsate. After rinsing the filter, filter approximately 3 mL of sample water into a 4-mL amber vial labeled with the Sample ID. Record the syringe and filter lot numbers on the lysing bench sheet.

T. If a sample is likely to have a concentration greater than the highest calibration standard (5.00 μg/L), it should be diluted before analysis.
Dilutions should be performed using filtered lysate such that the measured concentration is as close as possible to the concentration at the EC$_{50}$ of the calibration curve (~0.60 μg/L, but varies from batch to batch). Either Milli-Q water or the Sample Diluent included with the ELISA kit may be used as the diluent.

U. Dilutions should be performed in Class A volumetric flasks. If sample volume is insufficient for the use of a volumetric flask, dilutions may be performed by pipetting the appropriate volumes of filtered lysate and diluent into a 4-mL amber vial and inverting several times to mix well.

V. If the samples will not be analyzed the same day, the lysates should be stored in the freezer until ready for analysis.

W. All volumetric flasks used during the preparation process should be washed with laboratory detergent, tap water, deionized water, ACS grade methanol, and Milli-Q water. Allow flasks to air dry after washing.

8.3 Pre-Analysis CAAS Startup

A. If the Eurofins Abraxis Microcystins/Nodularins ELISA kit, Method 546 Accessory Pack, and sample lysates are not at room temperature, remove from refrigerator or freezer and allow to warm to room temperature.

B. Power on the CAAS using the large dipswitch on the instrument’s back panel. Open the Abraxis Manager software. If the software initialization fails due to low pressure in the pressurized wash and rinse bottles, check the bottle seals and try again. If these steps do not solve the problem, or another type of initialization failure occurs, troubleshoot the instrument.

C. Empty both instrument waste containers.

D. Empty the prime rinse bottle and pressurized wash bottles; rinse and refill with Milli-Q water.

E. Prepare the Wash Buffer by diluting Wash Buffer concentrate up to 500 mL (1 bottle) or 1000 mL (2 bottles) in an appropriately sized volumetric flask. Fill the pressurized wash bottle.

F. From the Routines menu in Abraxis Manager, select Start of Day. The CAAS will perform several preventative maintenance actions. If the probe, syringes, or wash head appear to be malfunctioning, repeat the Start of Day procedure. If this does not resolve the issue, troubleshoot the individual component using the Wash Probe, Prime Syringes, Prime Washer – Wash Bottle, or Prime Washer – Rinse Bottle options. Repeat.

G. From the Routines menu in Abraxis Manager, select Wash Probe. After the probe wash is complete, repeat. If the probe is aligning correctly with the wash, proceed with the pre-run maintenance. Otherwise, perform the alignment procedure (Utilities -> Alignment -> Probe vs. Wash Cup).
H. From the Routines menu in Abraxis Manager, select **Prime Syringes**. After the priming is complete, **repeat**. If large bubbles remain in the syringes after priming, remove the prime water feed line from the rinse bottle and place into a small beaker of 70% isopropanol. Prime the syringes until no bubbles remain. Once bubbles are removed, transfer the prime water feed line to a small beaker of Milli-Q water and prime the syringes two more times to remove isopropanol from the system. Once this rinse is complete, place the prime water feed line back into the rinse bottle and prime once more.

I. From the Routines menu in Abraxis Manager, select **Prime Washer – Wash Bottle**. After the priming is complete, **repeat**. If the streams of liquid from the wash head are not straight down or contain air bubbles, prime the wash head two-three more times to see if any potential clog is removed. If not corrected, use a thin piece of copper wire to clean the clogged nozzle(s), and then perform the Prime Washer step **twice more**.

J. From the Routines menu in Abraxis Manager, select **Prime Washer – Rinse Bottle**. After the priming is complete, **repeat**. Verify the streams of liquid from the wash head are straight down and do not contain air bubbles. If the streams are not straight down or contain air bubbles perform the troubleshooting steps mentioned above in the **Prime Washer – Wash Bottle** section.

**8.4 Sample Analysis on the CAAS**

A. Eurofins Abraxis kit lot numbers and expiration dates, as well as lot numbers and expiration dates for kit reagents and standards, must be recorded in the lab notebook. If a particular lot has been used for analysis previously, indicate this information in the lab notebook. If a new bottle of isopropanol or 1 N HCl is opened, record the lot number, date opened and expiration date in the lab notebook.

B. In Abraxis Manager, select the **Sample** tab in the lower portion of the program window.

C. In the lower left corner of the window, select **Add Numerical ID**. Type “QCS” and click OK. Then type in the Sample ID of the sample used to prepare the first LFSM/LFSMD, followed by the first 20 samples. If there are multiple samples with Sample IDs all in consecutive order, enter the total number of samples in the Total field and the software will automatically populate Sample IDs.

D. If there are more than 20 samples in the batch, enter the Sample ID of the 21st sample (used to prepare the second LFSM/LFSMD), followed by “LFSM 2”, LFSMD 2” and the remaining samples. After all Sample IDs have been entered, enter “LFB 2” and “LRB 2” to include the second pair of LFBs and LRBs at the end of plate.
E. If there are 20 or fewer samples in the batch, after all Sample IDs have been entered, enter “LFB 2” and “LRB 2” to include the second pair of LFBs and LRBs at the end of plate.

F. After all samples have been entered, check the **Select All** checkbox in the lower right-hand corner of the screen.

G. Select **MICROCYSTINS ADDA 546** from the list of tests in the center of the screen and click **Add Test**. This will populate the Work List on the right-hand side of the screen. Ensure that the boxes next to each sample are checked and the number of replicates on each sample is set to “2”.

H. Click the **Calibrate** button on the bottom righthand side of the screen. A dialog box stating “Calibration tests are prepared” will appear. Click OK to close the dialog box.

I. Once the dialog box is closed, click the **Request** button at the bottom right-hand corner of the screen. A dialog box will appear. Ensure that **Reset Rack 1**, **Reset Rack 2**, and **Reset Plate** are checked and click OK to close the dialog box.

J. At the prompt, enter the lot number of the test kit being used for the analysis. Press OK to close the window.

K. The analysis schedule will appear, click OK to accept the schedule and close the dialog box.

L. The layout tab shows where to place each standard, reagent, and sample, as well as how many wells in the well plate will be used for analysis. To move a standard, reagent, or sample to a different place on the rack, click on the standard, reagent, or sample of interest and drag it to a different position. Note that each standard, reagent, or sample can be moved to another place on their respective rack but cannot be moved from one rack to the other. A map of the well plate layout can be found below. Note that LFSM 1 and LFSMD 1 are prepared from Sample 1; LFSM 2 and LFSMD 2 (if needed) are prepared from Sample 21.

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<tbody>
<tr>
<td>A</td>
<td>Std 0</td>
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<td>Sample 1</td>
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<td>Sample 12</td>
<td>Sample 16</td>
<td>Sample 20</td>
<td>Sample 22</td>
<td>Sample 26</td>
<td>Sample 30</td>
<td>LRB 2</td>
</tr>
<tr>
<td>H</td>
<td>Std 3</td>
<td>Low-CV</td>
<td>QCS</td>
<td>Sample 4</td>
<td>Sample 8</td>
<td>Sample 12</td>
<td>Sample 16</td>
<td>Sample 20</td>
<td>Sample 22</td>
<td>Sample 26</td>
<td>Sample 30</td>
<td>LRB 2</td>
</tr>
</tbody>
</table>

Author: Joshua Rosen    SME: Jennifer Graham    QA Officer: Noel Deyette    WSC Director: Gary Wall
M. Remove the caps from each vial, ensuring there are no bubbles of liquid at the tops of the vials. If there is any liquid present around the rim of a vial, remove using a lab wipe. If needed, use small colored stickers on the vial lids to ensure the correct cap is placed on each container after analysis is complete. Place each standard, reagent or sample in the appropriate position in the racks and ensure that the racks are properly aligned with the pins inside the CAAS.

N. Add the test strips from the test kit to the well plate, pressing each strip in firmly to ensure the wells are flush with the plate. If there are any partially full rows on the well plate, use uncoated “dummy” wells to fill the unused places.

O. Place the well plate into the instrument and click Start Run in the lower right-hand corner of the program window. Click OK in the dialog box that pops up.

P. Once the run begins, the instrument will check the volume of liquid in each standard and reagent vial to ensure there is enough liquid to complete the analysis. If a vial does not have enough liquid to complete the analysis, the instrument will notify the user to add more volume. Once more volume is added, click Check Volumes. The instrument will recheck the volume of liquid in the vial and proceed with the analysis.

Q. Once the analysis is completed, a dialog box stating “Tests are all finished” will appear. Press OK to close the dialog box.

8.5 Report Generation

A. Click on the Calibration tab in the lower portion of the Abraxis Manager window and select Microcystins ADDA 546. Adjust the width of the columns so all data are clearly visible, and press Print Preview.

B. Click Print in the top left-hand corner of the screen. Print the report to PDF to \Caas\Calibration Curves\Microcystin and save in the format YYYY-MM-DD. If another file already has this name, add a “_2” to the file name. Print a paper copy.

C. Click the Test List tab at the bottom of the Abraxis Manager window. Then, check the Select All box on the left-hand bottom corner of the screen, and click the Accept button next to the Select All checkbox.

D. Click the Report Tab. Check the box next to the date and time of the run to select all results. Adjust the width of the columns so all data are clearly visible, and press Print Preview.

E. Click Print in the top left-hand corner of the screen. Print the report to PDF to \Caas\Sample Reports\Microcystin and save in the format YYYY-MM-DD. If another file already has this name, add a “_2” to the file name. Print a paper copy.
F. To export the data for entry into LIMS, click **Export**. Save the data as YYYY-MM-DD.xls in the following directory: \CAAS\YYYY\MC\. If another file already has this name, add a “_2” to the file name. After saving the file, change the file extension from .xls to .txt.

### 8.6 Post-Analysis Shutdown

A. Remove rack 1 and rack 2 from the CAAS. Recap all reagents and standards (except for the 1 N HCl solution) and place them back into test kit boxes and refrigerate. Sample lysate vials should be recapped and returned to the freezer. Return Rack 1 (with open container of 1 N HCl in position 1) and Rack 2 to the CAAS.

B. Dump the contents of the sample wells into a sink and flush with copious amounts of water. Discard the used sample wells, except for any “dummy” wells, which can be reused.

C. From the Routines menu in Abraxis Manager, select **End of Day**. The software will prompt the user to check for the presence of the 1 N HCl container; press OK to proceed. The CAAS will perform several preventative maintenance tasks. If the probe, syringes, or wash head appear to be malfunctioning, repeat the End of Day procedure. If this does not resolve the issue, troubleshoot the individual component using the Wash Probe, Prime Syringes, Prime Washer options.

D. Once the End of Day Routine is complete, power down the CAAS and exit the Abraxis Manager software.

### 8.7 Maintenance

A. If the Probe-Z drive starts to make squeaking or grinding noises, and/or fails to move down fully while pipetting liquid into sample wells, oil the drive screw using the oil provided by the manufacturer.

B. If the alignment between the sample probe and other components (Racks, Wash Cup, Well Plate) is incorrect, perform the alignment procedure as described in the instrument user manual.

C. If the alignment between the wash head and the well plate is incorrect, perform the alignment procedure as described in the instrument user manual.

D. Check all motor timing parameters on a quarterly basis using the procedure described in the instrument user manual. If motor timing is out of specification, oil the motor as needed.

E. Any maintenance performed must be recorded in the lab notebook.

### 8.8 Data Processing and LIMS Entry

A. Before LIMS data entry, check that batch QC is within the acceptable range by using the QC spreadsheet located at \CAAS\QC Spreadsheet\MC\MC Master Spreadsheet. Save as a new file to the same directory using the following format: YYYY-MM-DD. If another file already has this name, add a “_2” to the file name. Enter the specified
information for the Eurofins Abraxis Method 546 Accessory Pack into the designated fields on the spreadsheet.

B. Enter the requested values into the spreadsheet, which will automatically compare the entered value to the acceptance criteria summarized in Section 7.3.I and display PASS/FAIL or YES/NO, depending on the specific metric. If the QC data are acceptable, data can be prepared for review and import to LIMS. If the QC data are unacceptable, the batch will need to be reanalyzed. Print a copy of the spreadsheet.

C. For batches with acceptable QC, prepare the data for entry into LIMS by opening the .txt file created in step 8.5.F using Microsoft Excel. Delete all columns except those with the headers “NAME” and “CONC_MEAN.” Change the headers on the two data columns from NAME and CONC_MEAN to Sample_ID and Concentration, respectively.

D. Delete all rows that contain results for the calibration standards, as well as any rows containing samples or QC that will not be reported (e.g., failed dilutions, unusable LFSM/LFSMDs). To ensure proper LIMS import, the Sample_ID cell for each environmental sample should only contain the Sample ID; delete any additional characters. Format the cells in the Concentration column as “Number”, with 3 decimal places.

E. Highlight all data in both columns, except the headers. Select the Data tab, and under Data Tools, click Remove Duplicates. In the dialog box, make sure both columns are selected and click OK. A dialog box will appear to indicate that the duplicate rows have been removed. Click OK.

F. If any dilutions were made, multiply the measured concentration by the dilution factor to obtain the actual concentration. Replace the measured concentration with the actual concentration.

G. If multiple dilutions are made to one sample, the dilution with a measured concentration closest to the EC<sub>50</sub> concentration should be selected for reporting to maximize measurement accuracy.

H. Save the data as a .csv file to \CAAS\YYYY\MC\ using the following format: YYYY-MM-DD. If another file already has this name, append a “_2” to the file name. Print a copy of the file as well.

I. Before handing the data off to a secondary data reviewer, fill out a batch cover sheet (paper copies available in the laboratory) as well as an ELISA Data Review Sheet (available for printing at \CAAS\Data Review Sheet\ELISA Checklist.xlsx).

J. Assemble all printed material into a data packet, following the page order below:
   i. Batch Cover Sheet
   ii. ELISA Data Review Sheet
   iii. QC Spreadsheet
   iv. Lysing Log(s)
K. If one or more samples in the batch were previously lysed for cylindrospermopsin, include a copy of that lysing log in the data packet. Copied lysing logs are marked with a red “COPY” stamp. The batch in which the original log resides should also be noted on the copy.

L. A secondary data reviewer must review the entire data packet and sign off on the ELISA Data Review Sheet to confirm that data are acceptable for reporting. After the data have been approved by the secondary data reviewer, it may be imported into LIMS.

M. On the LIMS server, double click on the Watershed LIMS icon.

N. Click Import Data.

O. Under the Import drop down, choose Water Cyanotoxins.

P. Choose and open the desired .csv file.

Q. Choose Client, Analysis, Units, type in Test Date, and choose Analyst.

R. Exclude and/or edit any as data necessary.

S. Click Client ID to Sample No.

T. Click Check Analysis.

U. Click Check RL Flag.

V. For each sample, add any other applicable flags (C, M, W) to the flag column. Do not enter the “H” holding time flag; the LIMS will add this automatically based on the difference between the sampling and analysis dates.

W. If a sample concentration is less than the reporting limit (0.30 μg/L), but greater than or equal to the concentration of the lowest non-zero standard (0.15 μg/L), apply an “N” flag.

X. Note that the National Water Information System (NWIS) will only accept the first three data flags assigned in the LIMS. If a sample has more than three data flags (excluding “<“), “C”, “H”, and “N” flags should be listed before “M” and “W” flags.

Y. Click Set Data.

Z. Investigate problems for data that did not transfer or are duplicated.

9. Calculations and Data Reporting

A. Data are output and stored in the LIMS in units of micrograms per liter, (μg/L).

B. Data are exported to NWIS from LIMS using the NWIS parameter code 89011 and the NWIS method code IMM34. Data exported from NWIS is rounded to two significant figures.
10. Archiving
   A. Data files are backed-up daily by an automated back-up program. Paper copies of the runs are filed and retained indefinitely. The laboratory LIMS system is backed up daily by an automated back-up program.
   B. Samples and sample lysates are stored frozen until analyses for all requested cyanotoxins are completed and the data have been reviewed and approved. Sample lysates are retained indefinitely.

11. References

12. Key Words
   ELISA, Enzyme-Linked Immunosorbent Assay, water analysis, chemical analysis, microcystins, nodularins, freshwater.
13. Revision Record

The SOP will be revised and approved as changes are required. A review will be performed no later than every two years from the last approval date and the SOP revised if necessary.

<table>
<thead>
<tr>
<th>Revision</th>
<th>Date</th>
<th>Responsible Person</th>
<th>Description of Change</th>
</tr>
</thead>
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<tr>
<td>1.0</td>
<td>06/11/2020</td>
<td>Joshua Rosen</td>
<td>First version of SOP.</td>
</tr>
<tr>
<td>1.1</td>
<td>03/16/2021</td>
<td>Joshua Rosen</td>
<td>Added data export from software, import into LIMS, data flags, sample dilutions, and lysing bench sheet. Updated lysing procedure, report generation/printing, and archiving. Numerous minor edits for grammar, clarity, and flow.</td>
</tr>
<tr>
<td>1.2</td>
<td>02/09/2022</td>
<td>Joshua Rosen</td>
<td>Made changes to fonts, corrected misc. grammatical errors. Clarified QC requirements for LFSM/LFSMD. Updated sample prep, data reporting and LIMS entry sections to reflect actual workflow. Added NWIS method code.</td>
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