



Select Cyanobacterial Toxins by Direct Aqueous Injection UPLC/MS/MS

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## 1. Title

Select Cyanobacterial Toxins by Direct Aqueous Injection UPLC/MS/MS.

## 2. References

Oehrle, S., Southwell, B., Westrick, J. Detection of various freshwater cyanobacterial toxins using ultra-performance liquid chromatography tandem mass spectroscopy. *Toxicon*. May 2010; 55 (5), 965-972.

Graham, J., Loftin, K., Meyer, M., Ziegler A. Cyanotoxin Mixtures and Taste-and-Odor Compounds in Cyanobacterial Blooms from the Midwestern United States. *Environmental Science & Technology*. 2010, 44 (19), 7361-7368.

Yen, HK., Lin, TF., Liao, PC. Simultaneous detection of nine cyanotoxins in drinking water using dual solid-phase extraction liquid chromatography-mass spectrometry. *Toxicon*. August 2011; 58 (2), 209-218.

Draper, W. et al. Optimizing LC-MS-MS determination of microcystin toxins in natural water and drinking water supplies. *Analytical Methods*, 2013, 5 (23), 6796-6806.

Draper, W., Xu, D., Perera, SK. Electrolyte-Induced Ionization Suppression and Microcystin Toxins: Ammonium Formate Suppresses Sodium Replacement Ions and Enhances Protiated and Ammoniated Ions for Improved Specificity in Quantitative LC-MS-MS. *Anal. Chem*. 2009, 81 (10), 4153-4160.

Operator Manual, Waters Xevo TQD.

## 3. Scope and Principle of the Analysis

This is an ultra-performance liquid chromatographic (UPLC) tandem mass spectroscopy (MS/MS) method applicable to the determination of certain algal toxins in raw and finished drinking water. The following compounds are determined using this method:

Analyte	Chemical Abstract Services Registry Number
Anatoxin	64285-06-9
Cylindrospermopsin	143545-90-8
Microcystin-LF	154037-70-4
Microcystin-LR	101043-37-2
Microcystin-LR (desmethylated)	120011-66-7
Microcystin-LW	157622-02-1

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Microcystin-LY	123304-10-9
Microcystin-RR	111755-37-4
Microcystin-RR desmethylated)	131022-02-1
Microcystin-WR	138234-58-9
Microcystin-YR	101064-48-6
Nodularin	118399-22-7

In order to measure the total amount of toxins present, bacterial cells are lysed by subjecting the water samples to three (3) freeze/thaw cycles. Each sample is then filtered and placed in a suitable auto-sampler vial for analysis. UPLC tandem mass spectroscopy analyses are performed with a Waters Acquity H-Class LC/MS system (Waters Corporation, USA). The system has a TQD detector which is a tandem quadrupole MS/MS, which monitors a specific precursor ion and fragment (or product) ion for each analyte.

## 4. Interferences

Interferences in the method can be due to contaminants in solvents, reagents, glassware and other processing apparatus. The instrument system is highly sensitive and requires the utmost care to prevent system contamination. In particular, nothing that has been in contact with soaps/detergents or used in methods using sodium reagents should be used. Additional sources of contamination have not been identified for this method; nevertheless, the following procedures are implemented to minimize the risk of contamination.

### 4.1. Glassware Cleaning

Glassware is cleaned by washing with DI. Washed glassware is then rinsed with LC/MS-grade solvent such as methanol or acetonitrile. Glassware is allowed to dry under a fume hood. It is then capped with aluminum foil and stored.

### 4.2. Sample Bottle Cleaning

Only new sample bottles from the manufacturer should be used for this method. Bottles that have been washed in the washroom must not be used.

### 4.3. Use of High Purity Reagents and Solvents

The use of ultra-high purity reagents and solvents helps to minimize interference problems. All the reagents, solvents and solutions used in this method are classified as UPLC, LC/MS-grade, or equivalent by their respective manufacturers. Refer to [Section 7](#) for a list of reagents used in this method.

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#### **4.4. Analysis of Laboratory Reagent Blanks**

Lab Reagent Blanks (LRBs) are defined as reagent blanks that are analyzed in the same exact manner as samples. LRBs are indicators of the presence of contaminants in the laboratory environment, the reagents, or the apparatus. All reagents and apparatus must be routinely demonstrated to be free from interferences under the conditions of the analysis by running laboratory reagent blanks as described in [Section 10.4](#).

### **5. Safety Issues**

MSDS sheets are on file in the laboratory for reference to any safety issues concerning the chemicals used in this method.

Methanol and acetonitrile are both flammable and pose a risk of poisoning by inhalation or swallowing. Avoid breathing vapors, and contact with skin and eyes. These reagents should be handled in a fume hood with proper personal protective equipment. Mobile phase wastes for the UPLC are collected during the analysis in 5-gallon polypropylene containers. When full, containers are stored in the Hazardous Waste Storage area and manifested according to Laboratory policy.

### **6. Apparatus and Equipment**

#### **6.1. Sample Bottles**

Grab Sample Bottles—250mL polypropylene screw cap bottles.

#### **6.2. Glassware and Apparatus**

- Volumetric flasks: various sizes.
- Graduated cylinders: 500mL, 10mL.
- Volumetric pipettes: various capacities.
- Micro Syringes: various sizes.
- Disposable syringes: 3mL Luer Lock sterile, Latex and Silicon oil free (Fisher Cat. # 14-817-27, 100/pkg.).

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- 0.2 um, 17mm PVDF Syringe Filters: Filters volumes <10mL (National Scientific Cat. # F2513-6, 100/pk) (Fisher Cat. # 03-377-155).
- Sample Vials: 8mL amber glass vials with Teflon-lined caps (National Scientific, Cat. # B7800-3A, 200 vials and caps per case).
- Analytical Balances: Mettler Models AE 24 and PM600.
- Auto-sampler Vials: LCMS Certified Amber with pre-slit screw-top caps (Waters Corp., Cat. # 600000669CV).
- Vortex-Genie Shaker.

### 6.3. Instrumentation and Operating Conditions

#### Waters H-Class UPLC

Instrument Description:

Xevo TQD UPLC/MS/MS System:  
Acquity H-Class UPLC with Dual-Column Compartments

UPLC Columns:

Waters Acquity UPLC HSS T3 2.1 x 100mm, 1.8um.

Operating Conditions

Mobile Phase: Water/Acetonitrile  
Channel A = 100% Water with 0.1% Formic Acid  
Channel B = 100% Acetonitrile with 0.1% Formic Acid  
Flow Rate: = 0.45 mL/min  
Temperature: Column Temp: 40°C  
Sample Injection Volume: 25µL

Gradient Program:

Time	Equil.	0.8	9.8	10.81	12	14
% A	98	98	30	10	98	98
% B	2	2	70	90	2	2

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## 7. Reagents, Standards and Solutions

### 7.1. Reagents

- Methanol: Optima LC/MS Grade (Fisher Scientific, Cat. # A456-4 or equivalent).
- Acetonitrile: Optima LC/MS Grade (Fisher Scientific, Cat. # A955-4 or equivalent).
- DI Water: Obtained from a Millipore MilliQ Integral water system (Final Resistivity = 18 megohms or greater).
- Formic Acid: Optima LC/MS Grade (Fisher Scientific, Cat. # A117-50 or equivalent).
- Ammonium Formate: Optima LC/MS Grade (Fisher Scientific, Cat. # A115-50 or equivalent).

### 7.2. Certified Stock Standards

All certified standards are purchased from reputable vendors and stored according to manufacturer instructions until ready for use. Equivalent solutions may be obtained from any approved vendor although currently there are limited options available.

The following standards are purchased from Enzo Life Sciences, Inc. 10 Executive Blvd, Farmingdale, NY 11735.

- Anatoxin:** Cat. # BML-C118. 1mg.
- Cylindrospermopsin:** Cat. # ALX-350-149. 25µg.
- Microcystin-LA:** Cat. # ALX-350-096. 25µg.
- Microcystin-LF:** Cat. # ALX-350-081. 25µg.
- Microcystin-LR:** Cat. # ALX-350-012. 50µg.
- Microcystin-LR (desmethylated):** Cat. # ALX-350-173. 25µg.
- Microcystin-LW:** Cat. # ALX-350-080. 25µg.
- Microcystin-LY:** Cat. # ALX-350-148. 25µg.
- Microcystin-RR:** Cat. # ALX-350-043. 50µg.
- Microcystin-RR (desmethylated):** Cat. # ALX-350-168. 25µg.
- Microcystin-WR:** Cat. # ALX-350-167. 25µg.
- Microcystin-YR:** Cat. # ALX-350-044. 25µg.
- Nodularin:** Cat. # ALX-350-061. 50µg.

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The following standards are purchased from Sigma Aldrich. 3050 Spruce Street, Saint Louis, MO 63103.

**[D-Ala2]-Leucine enkephalin:** Cat. # E5008. 5mg.

**L-Phenylalanine:** Cat. # P2126. 100g.

**Monuron (PESTANAL):** Cat. # 36174. 100mg.

As the stock standards are received as neat powders, they must be dissolved in the proper solution. Follow manufacturer recommendations regarding appropriate solutions. Use a Vortex-Genie to ensure proper mixing.

### 7.3. Preparation of Working Standards

All solutions described in this section are stored in a freezer at  $<0^{\circ}\text{C}$  and protected from light. Expiration dates are two months from preparation or the vendor expiration for the certified standard, whichever comes first. It has been noted that some standards will exhibit signs of degradation prior to the listed expiration dates. Any standards showing evidence of decreased performance must be discarded and new standards prepared. Due to this rapid degradation and the cost of the stock standards, it is recommended that only one or two milliliters of standard be prepared at a time. Standards and reagents **MUST NOT** be prepared in the Organics Laboratory hoods due to sodium contamination issues.

As the standard materials are stored in a freezer, time must be allowed for the standards to come to room temperature before preparation. Mixing standard materials prior to use is also recommended.

#### **Leucine enkephalin and Monuron Standards**

Leucine enkephalin and monuron stock standards must be diluted to a usable concentration. Perform a series of serial dilutions in 1mL methanol until the proper concentrations (2.5 $\mu\text{g}/\text{mL}$  for Leucine enkephalin and 1 $\mu\text{g}/\text{mL}$  for monuron), are achieved. Be sure to record the standard in the appropriate standard logbook and to label the vial appropriately (See [Section 7.4](#)).

#### **Anatoxin and Cylindrospermopsin Standards**

Both anatoxin and cylindrospermopsin working standards have a concentration of 1 $\mu\text{g}/\text{mL}$  in 5% methanol solution with 0.1% formic acid. Prepare fresh 5% methanol solution each time standards are made (500 $\mu\text{L}$  methanol and 10 $\mu\text{L}$  formic acid brought to 10mL final volume with DI). Transfer the appropriate volume of stock standards to the methanol solution, vortex to mix and label the vial.

#### **Microcystins and Nodularin Mix Standard**

Prepare 1mL of a 1 $\mu\text{g}/\text{mL}$  mix standard by transferring the appropriate volumes of all ten microcystins and nodularin to an auto-sampler vial and bring to volume with methanol. Vortex to mix and label the vial.



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## 7.4. Standard Labeling, Record Keeping and Replacement

### Labeling

Each individual standard solution (calibration and all control solutions) is labeled with the date of preparation, unique laboratory identifier, concentration and contents of the solution, and the initials of the analyst who prepared the solution.

### Record Keeping

A notebook is kept for recording the date of preparation of standard calibration solutions, method of preparation, identifying number, and expiration date. This notebook is kept in the Organics Laboratory (Room 313) or the Food Chemistry Laboratory (Room 204).

### Replacement

Stock standard solutions are to be replaced sooner than the two-month expiration date if comparison with laboratory fortified blanks, or QC samples indicate a problem.

### Expired Stock Standards

Expired stock standards are **NOT** used for preparation of calibration solutions or for any other control solution. Expired stock standards and solutions are discarded into appropriate hazardous waste containers. When filled, containers are stored in the Hazardous Waste Storage area and manifested according to Laboratory policy.

## 8. Sample Collection, Preservation and Handling

The laboratory staff prepares bottle orders for all sampling events. One (1) 250mL polypropylene screw cap bottle is used for each sample. Upon receipt in the laboratory, samples are stored at <math>0^{\circ}\text{C}</math> in a laboratory freezer.

An example of a sampling bottle label:

RI Department of Health Laboratory ( ) Legal      HAB  
Sample # \_\_\_\_\_ Date/Time Collection \_\_\_\_\_  
Collection Point: \_\_\_\_\_  
Collector: \_\_\_\_\_ Program: \_\_\_\_\_  
Preservation Added: N/A





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## 9. Calibration and Standardization

In this method, the analytical system is calibrated via external standards. In this technique, four calibration standards are prepared for each analyte of interest. The lowest standard represents analyte concentrations near their respective detection limits, but below their reporting limits. The remaining standards bracket the analyte concentrations expected to be seen in the samples.

### 9.1. Preparation of Calibration Standards

Add approximately 1mL of fresh 5% methanol solution with 0.1% formic acid to an auto-sampler vial. Spike 20 $\mu$ L each of the 1 $\mu$ g/mL anatoxin, cylindrospermopsin, monuron and microcystin/nodularin mix standards and bring to a final volume of 2mL with the methanol solution for a final concentration of 10 $\mu$ g/L. Vortex to mix and label as the 10 $\mu$ g/L Calibration Standard.

Dilute the 10 $\mu$ g/L Calibration Standard with 5% methanol solution with 0.1% formic acid to make three additional calibration standards at 5, 1, and 0.5  $\mu$ g/L. Vortex to mix and label appropriately. It is recommended that calibration standards should be analyzed as soon as possible following preparation. If this is not feasible, then the standards should be stored in the freezer until analysis.

### 9.2. Calibration Standard Analysis

Starting with the standard of lowest concentration (0.5  $\mu$ g/L), analyze each calibration standard according to [Section 11](#) and tabulate peak area vs. the concentration of each standard. Use the LS/MS data analysis software (TargetLynx) to prepare a linear calibration curve for each target analyte and the surrogate. The coefficient of determination ( $R^2$ ) must be equal to or greater than 0.98.

Calibration curves are run before each set of samples, however an existing calibration curve may be used if it is verified before any samples are analyzed. Existing curves are verified by analyzing continuing calibration verification standards (CCV). In order to be deemed passing, analyte responses in the CCV must be at least 70% of the expected value. If the CCV does not pass, a new calibration curve must be prepared and analyzed.



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## 10. Quality Control

The laboratory performs initial demonstration of capability (IDC) and method detection limit (MDL) studies, as part of initial quality control studies. Data quality is evaluated and documented by continued analysis of a number of different quality control (QC) measures (see below). The laboratory maintains records to document the quality of data that is generated. Data quality checks are compared with established performance criteria to determine if the results of analyses meet the performance characteristic of the method.

Minimum quality control requirements for this method include:

- Continuing Calibration Verification Standards (CCVs)
- MRL Check Standard
- Initial Demonstration of Capability (IDC)
- MDL Determination (MDL)
- Laboratory Reagent Blanks (LRB)
- Laboratory Fortified Sample Matrix (LFM)
- Surrogate Standard (SS)
- External Quality Control Sample (QCS)

### 10.1. Continuing Calibration Verification / MRL Check Standards (CCVs)

CCVs are calibration standards analyzed every eight hours to substantiate the continued accuracy of the analytical system. A MRL check standard is a low level standard, analyzed with each batch of samples, used to verify the accuracy of the calibration curve at the MRL. As continued verification at the MRL is a priority for this analysis, a calibration standard at 1.0 µg/L is used as a joint MRL check and CCV standard.

The measured concentration for the CCVs must be at least 70% of the expected value for all analytes. Recoveries below 70% indicate a loss in sensitivity of the system, requiring reanalysis of all samples back to the last passing CCV. Recoveries greater than 150% indicate recalibration for accurate quantitation is necessary for any samples containing the analyte in question. A new calibration curve must be generated and the samples must be re-analyzed with this curve ([Section 9](#)).

CCV standards are, at a minimum, run at the beginning and at the end of the sample analysis. It is recommended that additional CCVs be analyzed intermittently throughout lengthy analytical sequences. As previously mentioned in Section 9.2, once an acceptable calibration curve has been established, CCVs may be used to verify the working calibration curve instead of creating a new curve to run with the samples.



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## 10.2. Initial Demonstration of Capability (IDC)

Initial demonstrations of capability are performed to verify and document that laboratory procedures are capable of meeting performance criteria as outlined in the method. Individual analysts must demonstrate proficiency with the analytical techniques prior to generating data for environmental samples.

### Preparation and Analysis

Prepare four (4) individual samples of a 1.0 µg/L concentration of analyte mix by spiking 100µL of a 10µg/L Calibration Standard into 900µL of 5% methanol solution with 0.1% formic acid and analyze according to the procedure in [Section 11](#).

### Data Evaluation

For each analyte, the recovery value for all four samples must fall in the range of ± 20%, and the %RSD of all four values must be 20% or less. For those compounds that meet the acceptance criteria, performance is judged acceptable and sample analysis may begin. For those compounds that fail these criteria, this procedure must be repeated using four fresh samples until satisfactory performance has been demonstrated.

IDC standards must be repeated each time the system hardware or analytical conditions are modified.

## 10.3. MDL Determination (MDL)

Principle of the MDL Calculation: The method detection limit (MDL) is defined as the minimum concentration of a substance that can be identified, measured and reported with 99% confidence that the analyte concentration is greater than zero and determined from analysis of a sample in a given matrix containing analyte.

### Preparation and Analysis

Prepare seven (7) individual samples of a 1.0 µg/L concentration of analyte mix by spiking 100µL of a 10µg/L Calibration Standard into 900µL of 5% methanol solution with 0.1% formic acid and analyze according to the procedure in [Section 11](#).

### Data Evaluation

Calculate the average concentration in µg/L, and the standard deviation of the concentrations for each analyte. For each analyte, the average concentration must be between 70% and 130% of the true value and the calculated MDL must be less than the reporting limit.

$$\text{MDL} = 3.143 * \text{standard deviation}$$

(Where 3.143 = t value for 99% confidence level and 7 trials)



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#### **10.4. Laboratory Reagent Blanks (LRB)**

The LRB is an aliquot of reagent water that is treated exactly as a sample. The LRB is used to determine if method analytes or other interferences are present in the laboratory environment, reagents, or other apparatus.

##### **Preparation and Analysis**

LRBs are prepared by adding 10 $\mu$ L of 1 $\mu$ g/mL monuron standard to 5mL of DI water. One LRB is prepared with every batch of samples. Analyze according to the procedure outlined in [Section 11](#).

##### **Data Evaluation**

The concentration of any analyte detected in the LRB should not exceed the MDL of that analyte. If the concentration of any analyte in the LRB exceeds the MDL, samples containing that analyte must be reanalyzed after the contamination has been found. If the concentration of any analyte in the LRB exceeds the MDL, but is not present in any samples, then the samples do not have to be reanalyzed. In this instance, details will be included in a report narrative.

#### **10.5. Laboratory Fortified Sample Matrix (LFM)**

LFM samples are aliquots of client-supplied environmental samples that are spiked with target compounds and carried through every aspect of the procedure, from preparation to analysis. Matrix spike samples are analyzed to evaluate the effect of the sample matrix on the accuracy of the analytical procedure. The background concentrations of the analytes in the sample matrix must be determined in a separate aliquot, and the measured values in the LFM corrected for background concentrations.

LFM samples are fortified to contain a 5 $\mu$ g/L solution and are analyzed at a frequency of 5% of samples analyzed, or at least 1 per batch, whichever is greater.

##### **Preparation and Analysis**

The LFM is prepared by adding 25 $\mu$ L of 1 $\mu$ g/mL microcystin mix standard and 10 $\mu$ L of 1 $\mu$ g/mL monuron standard to 5mL of a field sample. Analyze with the associated field sample according to [Section 11](#).

##### **Data Evaluation**

Calculate the percent recovery, P of the concentration for each analyte, after correcting the analytical result, X, from the fortified sample for the background concentration, b, measured in the unfortified sample, i.e.,:

$$P = 100 (X - b) / \text{fortifying concentration of } 5\mu\text{g/L}$$



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The recoveries for the LFM should be  $\pm 35\%$ . If the recovery of any such analyte falls outside the designated range, and the laboratory performance for that analyte is shown to be in control, the recovery problem encountered with the dosed sample is judged to be matrix related, not system related. The result for that analyte in the unfortified sample is labeled suspect/matrix to inform the data user that the results are suspect due to matrix effects.

#### **10.6. Surrogate Standard (SS)**

The surrogate (monuron) standard is used to monitor method performance with each sample by determining approximately how much recovery (or loss) may have occurred during analysis. The surrogate solution is added to each sample and taken through the entire sample preparation and analysis procedure. The expected concentration in each sample is  $2.00\mu\text{g/L}$ .

##### **Preparation and Analysis**

Add  $10\mu\text{L}$  of the SS to  $5\text{mL}$  aliquots of all QC samples (LRB, LFM) and field samples prior to filtration and analysis.

##### **Data Evaluation**

Acceptable recovery for the surrogate standard is  $\pm 30\%$  of  $2.00\mu\text{g/L}$ . Samples that fall outside this range must be reported as suspect due to unacceptable surrogate recovery.

#### **10.7. Control Charts**

Control charts are used to demonstrate that the analytical process is in statistical control, and to detect any trends or bias. If trends are detected, they are corrected before an “out of control” situation occurs. Control charts may be generated within the laboratory LIMS.



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**10.8. Frequency and Limits of Acceptability**

QC Standard	Description	Frequency	Acceptance Criteria	Corrective Action
Continuing Calibration Verification (CCV)	Standard Solution @ 1.0 ug/L.	Beginning and end of analytical run.	≥ 70% of true value	Repeat. Prepare new standard. Prepare a new calibration curve.
Initial Demonstration of Capability	Four replicates of 1.0 µg/L fortified reagent blank.	Upon implementation of method or after changes such as change in column or equipment.	± 20 % of fortified amount; RSD of 20% or less.	Repeat with 4 samples until performance is satisfactory.  Sample analysis cannot begin until acceptance criteria are attained.
MDL	Seven replicates of 1.0 µg/L fortified reagent blanks.	Annually or if there are major changes in columns or equipment.	Must be less than the reporting limit.	Repeat the analysis using 7 samples over several days.
Laboratory Reagent Blank (LRB)	Reagent water that is treated exactly as the sample. It is used to determine if there are interfering peaks.	Before processing samples during the implementation process and before each set of samples is run on the system	Method analytes cannot be present at more than the detection limit.	Determine and eliminate source of contamination.
Laboratory Fortified Sample Matrix (LFM)	Assesses analyte recovery. Fortification = 5.0 ppb	5% of the routine samples or one sample per set, whichever is greater.	Percent Recovery in the range of 65-135%.	If the recovery is out of range but the QCS and the LFB are in control then the recovery problem is due to a matrix effect and should be noted on the analysis report.
Surrogate Standard: Monuron	2ug/L Monitors method performance.	Added to all field and QC samples.	70-130% Recovery	Samples that fail criteria must be reported as suspect due to unacceptable surrogate recovery.

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## 11. Analytical Procedure

### 11.1. Sample Lysing Procedure

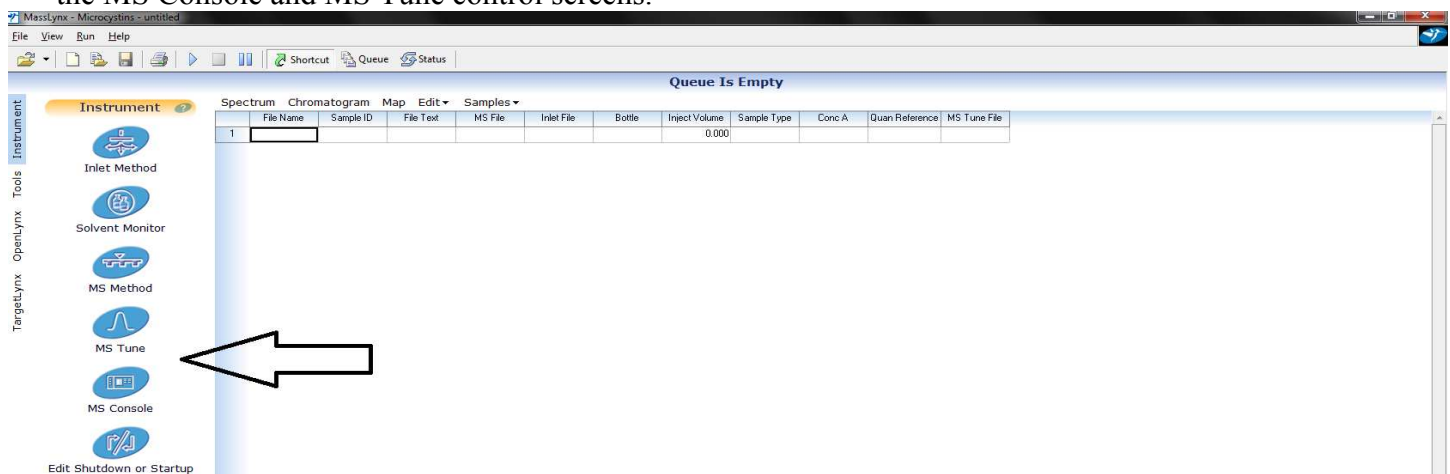
For this method, bacterial cells must be lysed to release any toxins present within their cells. This is accomplished by repeated freezing and thawing of the water samples.

- Upon arrival at the laboratory samples are placed in a freezer. Samples must then be thawed completely and refrozen two more times for a total of three (3) freeze thaw cycles. The final thaw should occur just prior to sample analysis. Once the samples have been thawed, they are ready for filtering and analysis.

### 11.2. LC/MS System Initialization

Prior to running samples, the system must be brought online. As the initialization period takes some time, the system should be brought online before filtering samples.

- Begin by making sure the LC solvent reservoirs A and B have sufficient volume of their mobile phases (DI and LCMS grade acetonitrile both with 0.1% formic acid). If the system has not been operational for some of time (several weeks), discard any remaining solvents, rinse and refill with new mobile phase. Mobile phases **MUST NOT** be prepared in the Organics Laboratory hoods due to sodium contamination issues.
- Also check to see that the Seal Wash reservoir has sufficient reagent (35% methanol, 35% acetonitrile, 30% DI). The wash runs continuously when the LC has power, so will empty faster than expected.
- Open the MassLynx system control software on the computer (it is on the desktop). Once loaded, open the MS Console and MS Tune control screens.

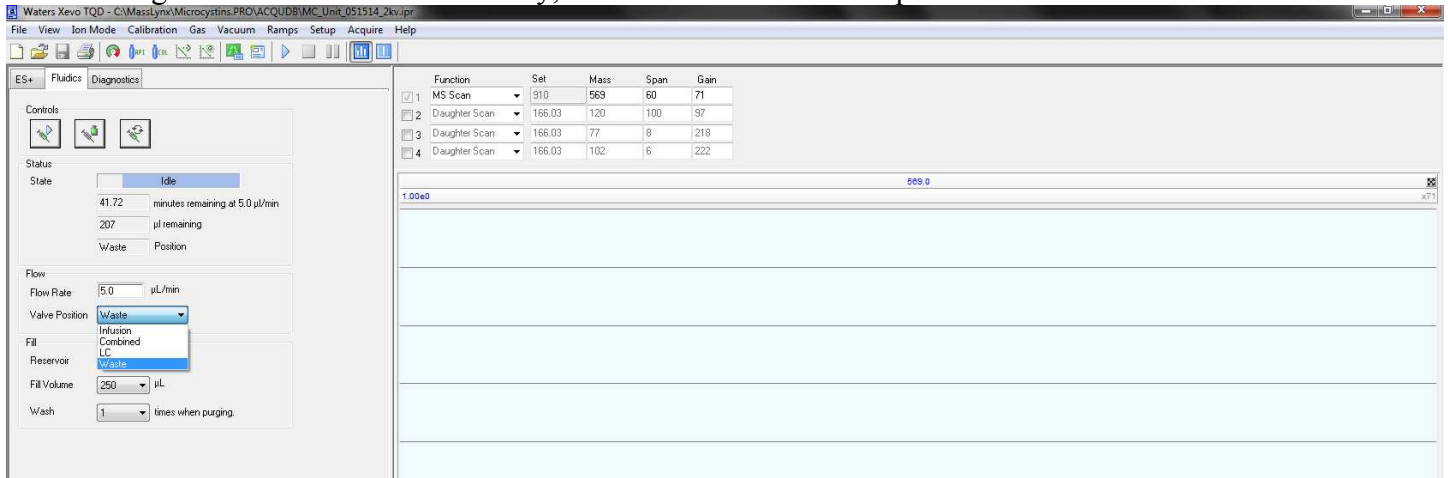


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- Go to the MS Tune screen and check to see that the MS fluidics valve is set to Waste. Do this by selecting the fluidics tab. If not already, select Waste from the drop down menu.



- Go to the MS Console screen. The MS Console screen shows an overview of the entire system and allows for control of each component. Turn on the mass spectrometer (XEVO TQD) by first turning on the collision gas, the API gas and finally the heaters by selecting the operate button. The gas flows and temperatures will default to the last run MS Tune file. When new methods are run, the associated Tune files will update the MS conditions to their appropriate settings.



- Select the Quaternary Solvent Manager (QSM) control page and prime the solvents (channels A and B) for three minutes (Control/Prime Solvents). The system will prime channel A for three minutes and then

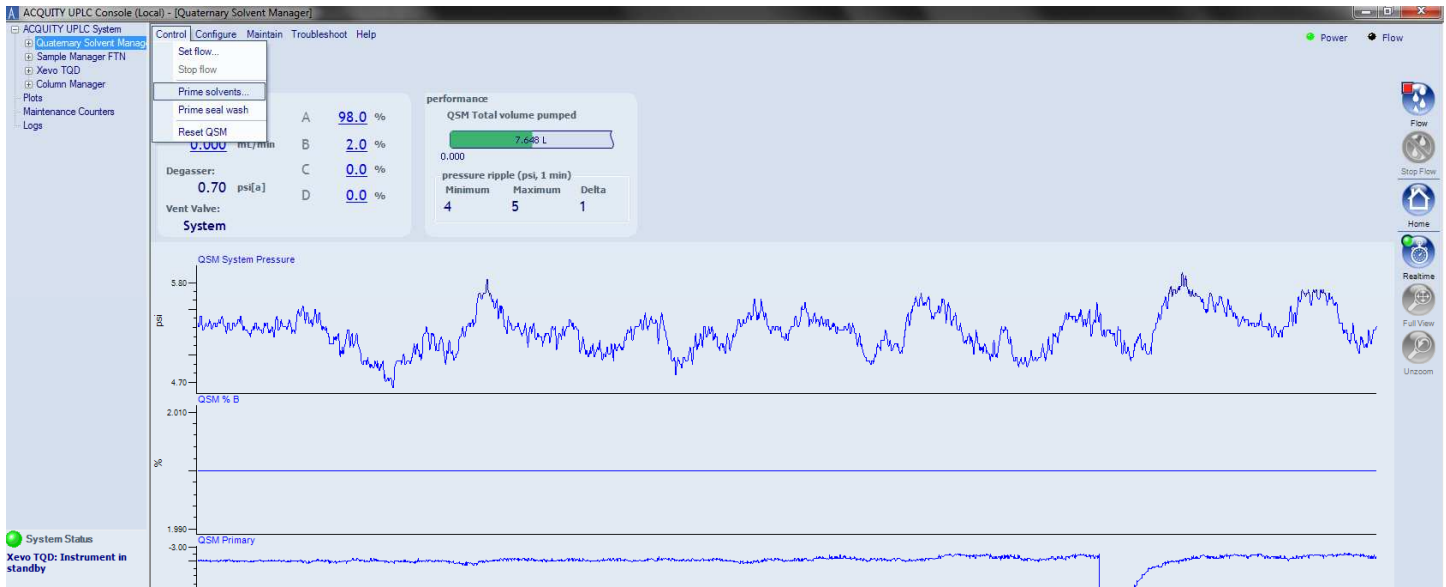
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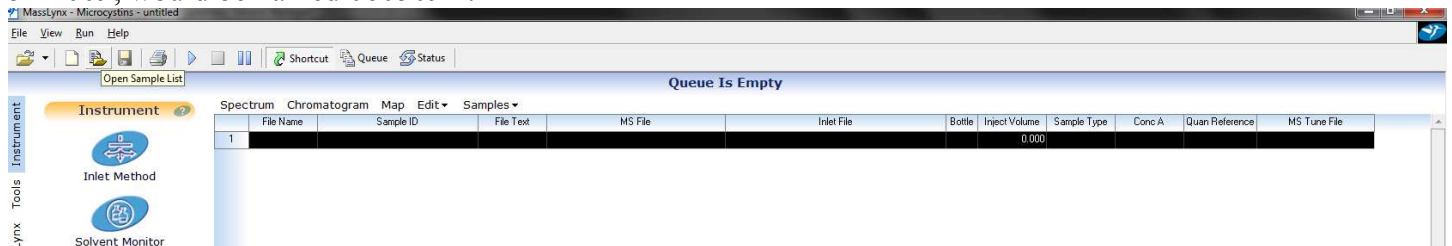
channel B. Make sure the solvents are flowing to waste properly through the waste tubing on the bottom front of the system. While the solvents are priming, prime the seal wash (Control/Prime seal wash). If you can see bubbles moving in the line (open the QSM door to see the lines) then the lines are primed and you can turn off the seal wash prime. Unlike the solvents, the seal wash prime must be manually turned off.



- Once the solvents have been primed. Turn on the LC flow (Control/Set Flow) to match the initial gradient conditions of 98% A at 0.45mL/minute. Allow the system time to settle. Once the pressure ripple delta (shown in the center of the QSM page above) is below 20, the system is ready to begin a run.
- While the system settles, go to the Sample Manager control page and prime the syringe (Control/Prime Syringe). Prime the syringe for 10-15 cycles.

### 11.3. Sample List Setup

Creating sample lists (or sequences) in the Masslynx software allows for the automated analysis of samples.. To load a previous sequence as a template, go to the Masslynx instrument page and click on the open sample list icon. Then load a recent sequence. Sequences are named by date, so the first sequence analyzed on June 5<sup>th</sup> 2009, would be named 060509A.

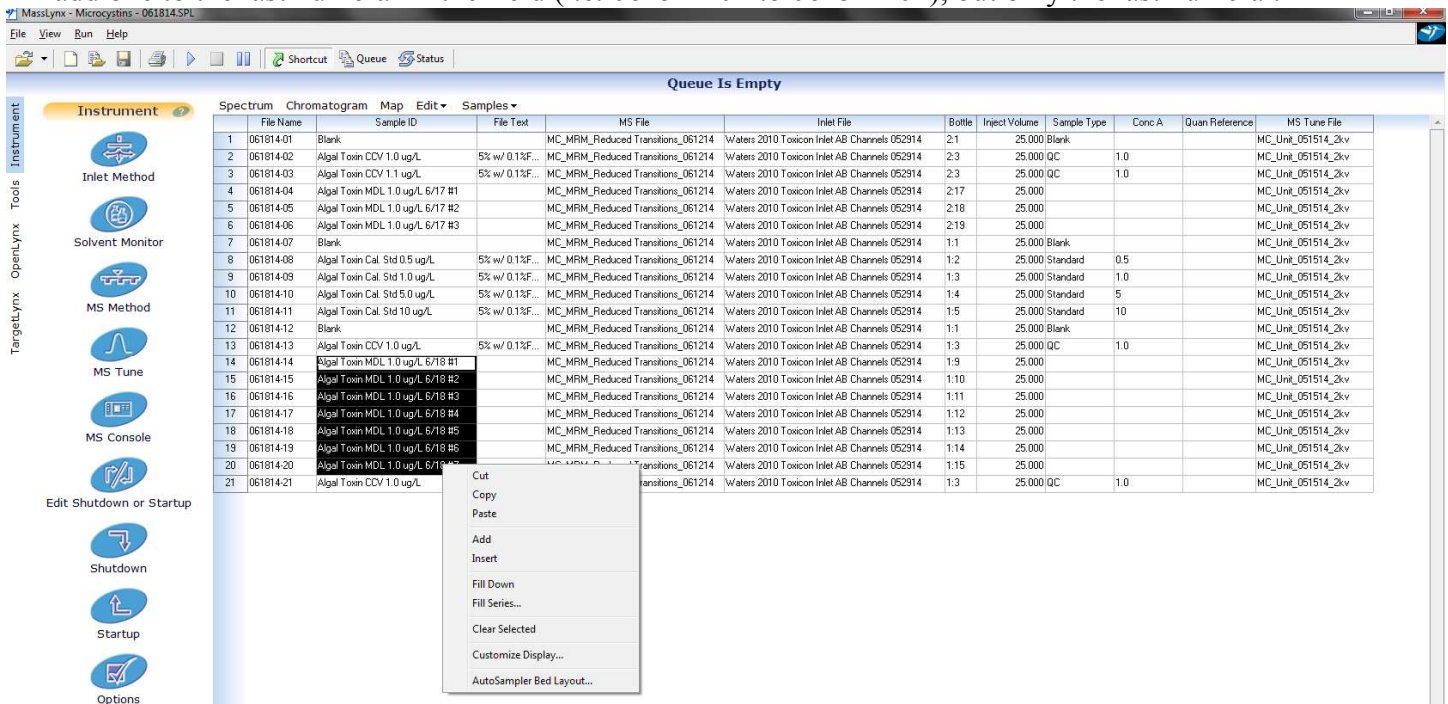


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- Fill out the table to run the calibration standards, QC and field samples in the order desired. Each sample at minimum needs a file name (generally the date with a numeric suffix i.e. 062514-01), Sample ID, MS File, Inlet File, Bottle Location, Injection Volume and MS Tune File.
  - The MS File directs the MS to search for the specific analytes on interest (i.e. which masses to look for at which times). The current MS File is “MC\_MRM\_Reduced Transitions\_060214”.
  - The Inlet File determines the LC conditions such as flow rate, gradient schedule, column, column temperature, etc. The current Inlet File is “Waters 2010 Toxicon Inlet AB Channels 052914”.
  - The auto-sampler is currently configured with two, 48-locations trays. The Bottle Locations must include both the tray number and the numeric location in the tray (i.e. 1:16 or 2:1).
  - The MS Tune file sets both the conditions of the ionization (gas flows, temperatures, voltages, etc.) as well as the general tune specifications of the mass spectrometer. The current MS Tune file for this method is “MC\_Unit\_051514\_2kv”.
- Columns of data in the sample list can be automatically filled in by the software using the Fill Down and Fill Series options. To do this, highlight the cells to be filled, click the right mouse button and select the appropriate action. Fill Down will fill the cells with the exact information in the first cell. Fill Series will add one to the last numeral in the field (i.e. 062514-01 to 062514-02), but only the last numeral.



- It is recommended that sample lists begin and end with a blank sample. This gives the system time to settle in the beginning of the run and gives the operator time to add additional samples as necessary before system shutdown at the end of the run. Be sure to include all appropriate QC, including a MRL

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check and sufficient CCV samples. When the table is complete, save the sample list under a new name (Disk Icon or File/Save As).

### 11.4. Starting the Run

- When the LC has stabilized, the sample list has been completed, and at least the first sample (usually a blank) is placed in the auto-sampler tray, a run may be started. Begin a run by highlighting the samples to be analyzed and selecting the “Play Icon” (see large arrow below). The software will then display a prompt confirming the samples to be analyzed. Once confirmed, analysis will begin and a description of the samples to be analyzed will appear in the sample Queue. The Queue can be accessed via the “Queue” icon above the sample list (small arrow below).

File Name	Sample ID	File Text	MS File	Inlet File	Bottle	Inject Volume	Sample Type	Conc A	Quan Reference	MS Tune File
060914-01	Algal Toxins in DI @ 1.0 ug/L-4	400	MC_MRM_Reduced Transitions_060214	Waters 2010 Toxicon Inlet AB Channels 052914	1:19	10.000	Blank			MC_Unit_051514_2kv_400
060914-02	Algal Toxins in DI @ 1.0 ug/L-5	400	MC_MRM_Reduced Transitions_060214	Waters 2010 Toxicon Inlet AB Channels 052914	1:19	10.000	Blank			MC_Unit_051514_2kv_400
060914-03	Blank5		MC_MRM_Reduced Transitions_060214	Waters 2010 Toxicon Inlet AB Channels 052914	1:1	10.000	Blank			MC_Unit_051514_2kv_450
060914-04	Algal Toxins in DI @ 1.0 ug/L-6	450	MC_MRM_Reduced Transitions_060214	Waters 2010 Toxicon Inlet AB Channels 052914	1:19	10.000	Blank			MC_Unit_051514_2kv_450
060914-05	Algal Toxins in DI @ 1.0 ug/L-7	450	MC_MRM_Reduced Transitions_060214	Waters 2010 Toxicon Inlet AB Channels 052914	1:19	10.000	Blank			MC_Unit_051514_2kv_450
060914-06	Blank5		MC_MRM_Reduced Transitions_060214	Waters 2010 Toxicon Inlet AB Channels 052914	1:1	10.000	Blank			MC_Unit_051514_2kv_400
060914-07	Algal Toxins in DI @ 1.0 ug/L-4	400	MC_MRM_Reduced Transitions_060214	Waters 2010 Toxicon Inlet AB Channels 052914	1:19	10.000	Blank			MC_Unit_051514_2kv_400
060914-08	Algal Toxins in DI @ 1.0 ug/L-5	400	MC_MRM_Reduced Transitions_060214	Waters 2010 Toxicon Inlet AB Channels 052914	1:19	10.000	Blank			MC_Unit_051514_2kv_400
060914-09	Blank5		MC_MRM_Reduced Transitions_060214	Waters 2010 Toxicon Inlet AB Channels 052914	1:1	10.000	Blank			MC_Unit_051514_2kv_400
060914-10	Algal Toxins in DI @ 1.0 ug/L-6	450	MC_MRM_Reduced Transitions_060214	Waters 2010 Toxicon Inlet AB Channels 052914	1:19	10.000	Blank			MC_Unit_051514_2kv_450
060914-11	Algal Toxins in DI @ 1.0 ug/L-7	450	MC_MRM_Reduced Transitions_060214	Waters 2010 Toxicon Inlet AB Channels 052914	1:19	10.000	Blank			MC_Unit_051514_2kv_450
060914-12	Blank5		MC_MRM_Reduced Transitions_060214	Waters 2010 Toxicon Inlet AB Channels 052914	1:1	10.000	Blank			MC_Unit_051514_2kv_450
060914-13	Algal Toxins in DI @ 1.0 ug/L-8	500	MC_MRM_Reduced Transitions_060214	Waters 2010 Toxicon Inlet AB Channels 052914	1:19	10.000	Blank			MC_Unit_051514_2kv_450
060914-14	Algal Toxins in DI @ 1.0 ug/L-9	500	MC_MRM_Reduced Transitions_060214	Waters 2010 Toxicon Inlet AB Channels 052914	1:19	10.000	Blank			MC_Unit_051514_2kv_450
060914-15	Blank5		MC_MRM_Reduced Transitions_060214	Waters 2010 Toxicon Inlet AB Channels 052914	1:1	10.000	Blank			MC_Unit_051514_2kv_450
060914-16	Algal Toxins in DI @ 1.0 ug/L-11	550	MC_MRM_Reduced Transitions_060214	Waters 2010 Toxicon Inlet AB Channels 052914	1:19	10.000	Blank			MC_Unit_051514_2kv_550
060914-17	Algal Toxins in DI @ 1.0 ug/L-12	550	MC_MRM_Reduced Transitions_060214	Waters 2010 Toxicon Inlet AB Channels 052914	1:19	10.000	Blank			MC_Unit_051514_2kv_550
060914-18	Blank5		MC_MRM_Reduced Transitions_060214	Waters 2010 Toxicon Inlet AB Channels 052914	1:1	10.000	Blank			MC_Unit_051514_2kv_550
060914-19	Algal Toxins in DI @ 1.0 ug/L-14	600	MC_MRM_Reduced Transitions_060214	Waters 2010 Toxicon Inlet AB Channels 052914	1:19	10.000	Blank			MC_Unit_051514_2kv_600
060914-20	Algal Toxins in DI @ 1.0 ug/L-15	600	MC_MRM_Reduced Transitions_060514	Waters 2010 Toxicon Inlet AB Channels 052914	1:19	10.000	Blank			MC_Unit_051514_2kv_600

- Unlike some other systems in the laboratory, the entire sample list will not be analyzed unless it has been specifically selected for analysis. Also, once a sequence has begun, additional samples may be selected for analysis. The additional samples will appear as a separate item in the Queue and will be analyzed once the first list in the Queue is complete.
- Samples may now be prepared and placed in their appropriate auto-sampler positions.

### 11.5. Sample Preparation Procedure

All environmental and quality control samples are prepared in the same manner.

- Remove the samples and standards from the refrigerator so that they may warm at room temperature. Meanwhile, assemble amber vials, syringes, pipettes, filters and other appropriate equipment.
- When warm, pipette 5mL of each sample into an amber vial. Spike LFM samples as appropriate.

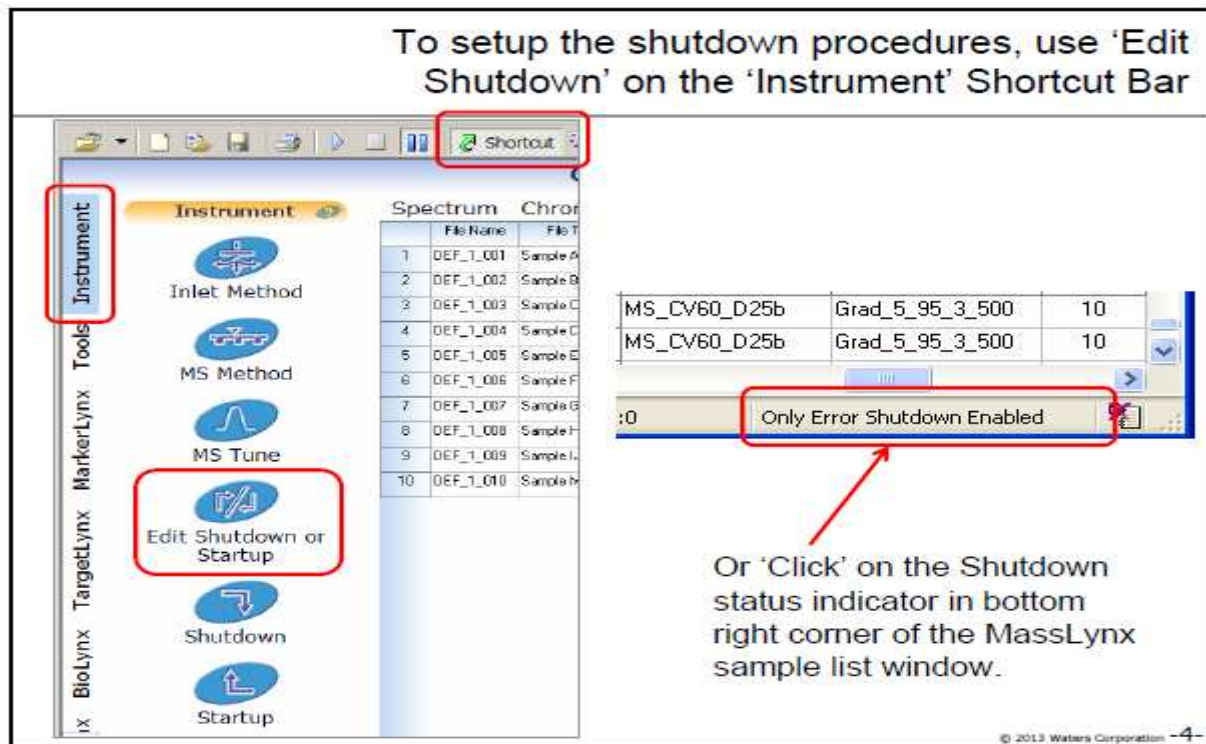
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- Spike 10 $\mu$ L of the 1 $\mu$ g/mL monuron surrogate standard into each vial. Invert to mix.
- Using a 3mL disposable syringe, draw up approximately 3mLs of sample into the syringe.
- Connect a 0.2  $\mu$ m Nylon/PVDF filter onto the syringe tip and depress the syringe plunger to force the sample through the filter. Filter the samples directly into a 2mL auto-sampler vials and cap.
- Place each auto-sampler vial in its appropriately numbered cell of the auto-sampler tray.

### 11.6. System Shutdown

It is very important that the system be shutdown following analysis both to save mobile phases and gases as well as to extend the lifetime of the equipment. MassLynx can be configured to automatically shut down the system after a batch of samples is complete.

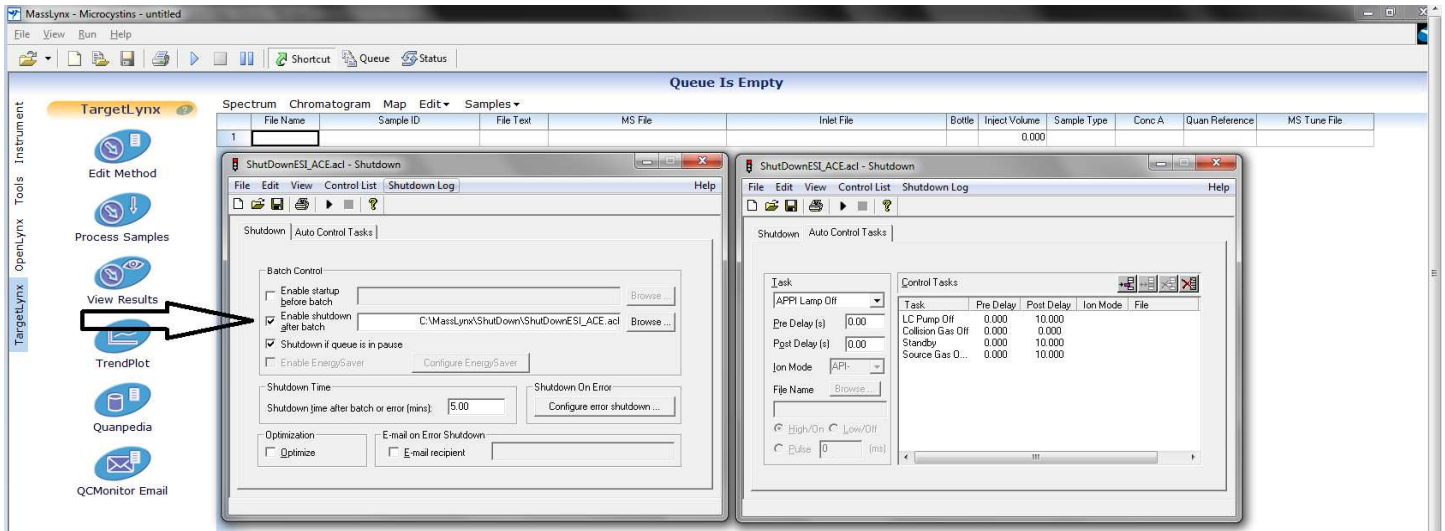


- This brings up the Shutdown Setup window. A default shutdown procedure has already been created by Waters service personnel named "ShutDownESI\_ACE and should load automatically. This file has been



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set to automatically shutdown the system when there is a MS or LC error to prevent any problems that might arise from a partial system failure. These do not need to be edited.



- As highlighted above, if not already checked, the system will be set to automatic shutdown by clicking the “Enable shutdown after batch” cell. Once checked, hit the save button and exit the shutdown window. MassLynx indicates the current shutdown status in the lower right corner of the sample list window.



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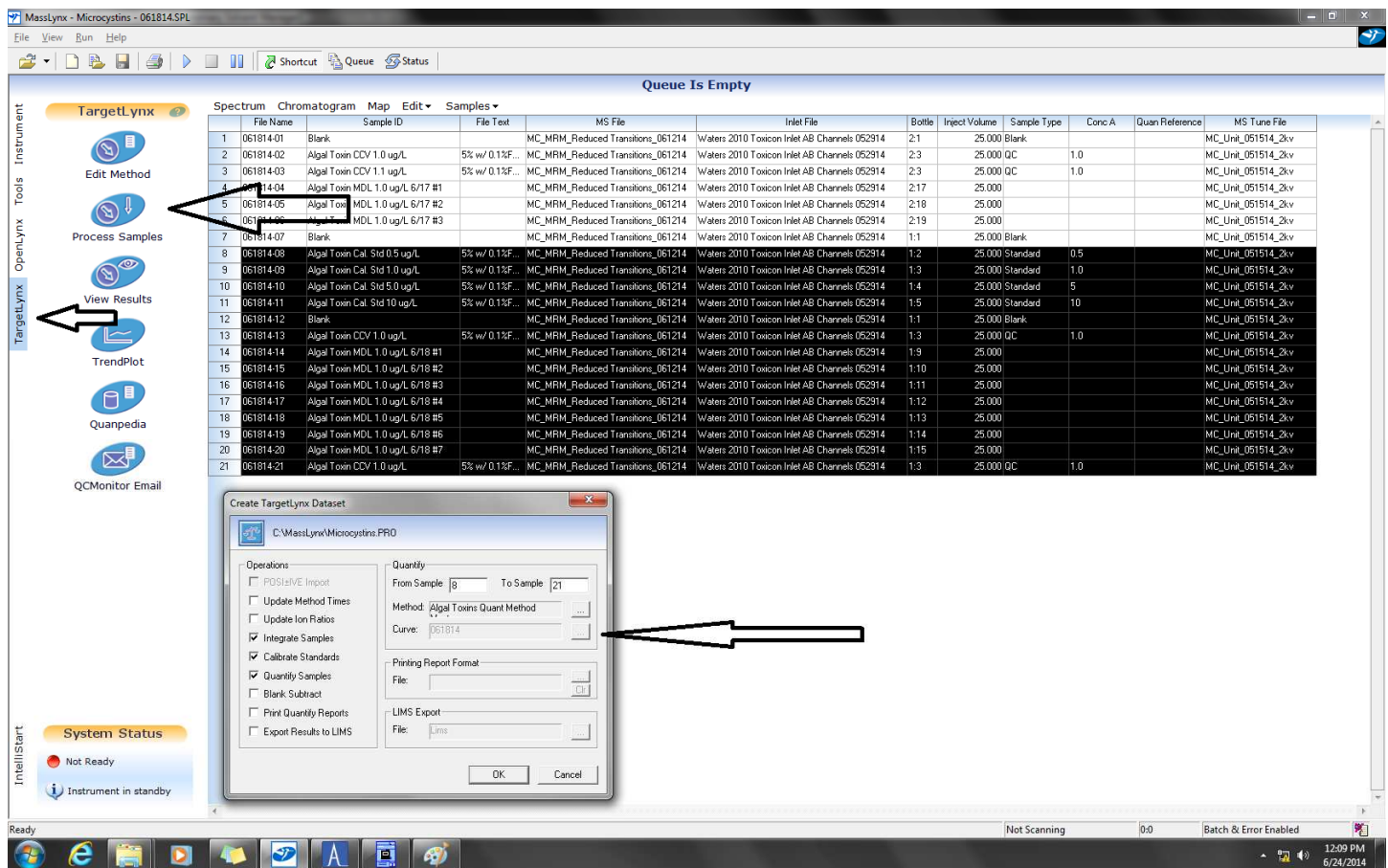
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## 12. Identification of Analytes and Calculations

Identification, integration and quantitation of peaks are done using Waters TargetLynx software. The concentration of each analyte is derived directly from the external calibration curve. The concentration of the calibration standards in the sample list will be automatically used when creating a curve.

If a target analyte is detected in the sample with a concentration that is greater than the highest standard in the calibration, the sample is diluted with DI to a concentration that should fall within the span of the curve.

### 12.1. Sample Quantitation



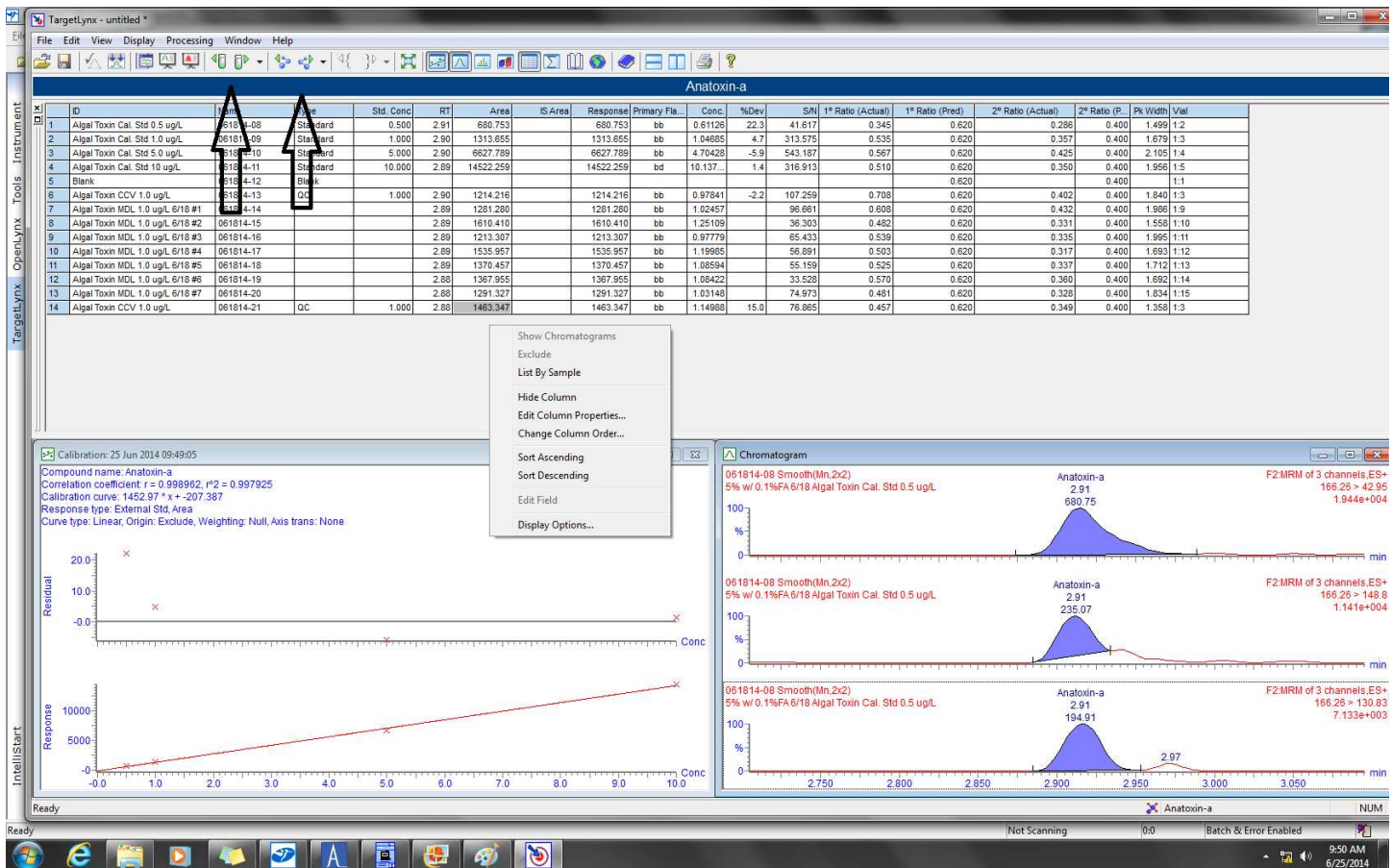
- To quantitate samples, enter the TargetLynx software by selecting the tab on the left of the MassLynx software (small arrow above). Open the appropriate sample list and highlight the samples of interest.

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- Next, click on the “Process Samples” tab (top arrow). This will bring up a prompt asking which actions are required (quantitate vs. calibrate and quantitate). If using a previously generated curve, click on the “Curve” icon (bottom arrow) and select the desired calibration file. The current analysis method for the test is “Algal Toxins Quant Method”. Click OK and the software will process the selected samples.
- TargetLynx will generate an untitled report listing the results of each sample for the first analyte listed in the method. Green arrows on the top of the window (left arrow) toggle between samples, while blue arrows toggle between analytes (right arrow). There are many options for displaying calibration curves, chromatograms, etc that can be selected within the report.



- The report displays some default data fields that can be changed depending on the nature of the data and the report needed. Right clicking within the data fields displays the options “Hide Column” and “Edit Column Parameters”. Selecting “Hide Columns” will open a list of all the various data that can be displayed (ion ratios, calculations, signal to noise, etc.). Check the boxes for the fields desired and these will be added to the report. “Edit Column Properties” is used to change column specific characteristics

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like number of decimals, column width, shading, etc. It should be noted that any changes to the report will only affect this single report. If global changes (changes to all future reports) are required, the report format should be exported and saved (File/Export/Report Format).

- Review the report for possible problems such as poor integrations. Once reviewed, save the report in the data directory and print for review.

### 13. Preventive Maintenance

There are several routine maintenance procedures for the system, including cone cleaning, pump oil checks and pump purging. Specific instructions are listed in the system manual.

Preventive maintenance is performed yearly as part of the service contract kept with Waters for this system. This service is performed by Waters engineers. Their work will be recorded and logged into the maintenance/service manual kept in the Laboratory.