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MassDEP

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| **Massachusetts Department of Environmental Protection**  **Division of Watershed Management** |

STANDARD OPERATING PROCEDURE

**Enumeration of Cyanobacteria in Water Samples**

CN 150.1

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| Prepared by: | **sig_jb.jpg** | Date: |  |
|  | **Joan Beskenis, Environmental Analyst** |  |  |
| Approved by: | **sig_rc4.jpg** | Date: |  |
|  | **Richard Chase, QA/Data & Assessments** |  |  |
| Approved by: | **sig_aj.jpg** | Date: |  |
|  | **Arthur Johnson, Monitoring Coordinator** |  |  |
| Approved by: | sig_kg.jpg | Date: |  |
|  | **Kimberly Groff, Program Supervisor** |  |  |

### *\* see pdf version for valid signatures*

1. **SCOPE AND APPLICATION**

Blooms of cyanobacteria can be toxic to humans and to pets. *Anabaena, Nostoc, Microcystis, Nodularia* may contain the hepatotoxin microcystin, a liver toxin, others like *Aphanizomenon flos-aquae, Anabaena circinalis* and *Cylindrospermopsis raciborskii* may contain the neurotoxin saxitoxin. Counts of the cyanobacteria are performed to determine the level of risk to the public using the waterbody for recreational or drinking water purposes. The World Health Organization (WHO 1999) has found that when cyanobacteria cell counts exceed 100,000 cells/ml the risk of adverse health effects to swimmers is considered moderate. Massachusetts Dept. of Public Health (MDPH) (2007) used the WHO cell count and developed a relationship between cyanobacteria cell counts and associated toxin levels based upon modified average weights and amount of ingestion and determined that a cell count of 70,000 cells/mL would correspond to a toxin level of approximately 14 ppb which is the current Massachusetts guideline for contact recreational waters. A method was developed for collecting a water sample and conducting the counts to determine if this guideline is being met.

**2.0** **SUMMARY**

Counts of the cyanobacteria are used to provide a means of determining if toxins may be present in potentially harmful amounts. Cyanobacteria are often a constituent of the algal community, but where blooms are present ingestion and contact of the water should be avoided.

**3.0 SAFETY CONSIDERATIONS**

###### Care must be taken while collecting the sample, especially in areas where a scum is present, to avoid contact with the skin since the cyanobacteria can cause skin rashes. If samples are to be preserved care should be taken in adding and using Lugol’s solution – such as using gloves and eye protection-since it can be an irritant to the skin and eyes.

**4.0 SAMPLE COLLECTION, PRESERVATION AND HANDLING**

Samples for cyanobacteria counts should be collected at beaches and other recreational areas especially if a visible bloom is present. Grab samples are collected by wading into the water, ~ 1 meter depth and inverting an open wide-mouth sampling bottle (250-500 ml) ~0.25 meters below the surface and then bringing it back up to the surface and re-capping it. Glass bottles are required if microcystin testing (or testing of other toxins) will be done otherwise plastic containers are suitable. If a scum is present on the surface a separate sample can be collected for identifications, but for the sample for counts no effort should be made to swirl the scum into the sample. Individual grab sample results from a geographically defined area like a cove or a beach should be averaged. Do not average water column and scum sample values together.

In order to learn more about the spatial extent of a bloom within a lake additional samples may be collected to help characterize a waterbody.

The sample bottle is put on ice in a cooler to keep it both cool and out of the sunlight. The identifications and counts are done within 24 hours with the only preservative typically being refrigeration. If samples will be examined later than 24 hours Lugol’s solution is typically used. If microcystin measurements will be made samples should be collected in glass containers otherwise plastic wide-mouth jars are suitable. Lugol’s should not be added to samples for microcystin analysis instead they should be frozen if they need to be stored for an extended period.

Water

The temperature of the water as well as the movement of the water are important factors contributing to bloom formation. Although cyanobacteria blooms can occur in any season, with water temperatures increasing (especially above 25o C) the frequency of blooms also increases. Many cyanobacteria species also do well at high light levels. Not all cyanobacteria form surface scums. The development of surface scums requires both still water without wind and waves as well as species present that are positively buoyant because of the presence of gas vacuoles. *Microcystis* sp. often is found in surface blooms. In contrast *Planktothrix* sp. will remain dispersed throughout the water column. Once formed, bloom densities and locations are directly affected by wind-direction and intensity. This affects sampling strategies since a bloom observed in one area of a pond today may not be in the same location tomorrow.

Any toxins present in a bloom may be released when the cells die. The toxins are not easily destroyed by sunlight or other environmental factors. So, to ensure safety to water users after a bloom, people should wait 2-3 weeks after the bloom has disappeared to reestablish contact recreation or allow humans and or dogs back into the water.

Besides the issue of potential toxicity, cyanobacteria blooms can affect water tastes by the release of geosmin or 2-methylisoborneol (MIB) which can cause a musty taste or odor.

Representative Sampling Locations and Frequency

Shore-line samples-collected by wading into an area-are used to determine if a bloom is present or if counts have changed from the previous sampling event. Once a waterbody has been posted by the Local Board of Health because of a cyanobacteria bloom it is necessary that cyanobacterial counts go below 70,000 cells/ml for two consecutive weeks (Mass DPH) in order to re-open it for recreational use. Sampling frequency can also be affected by visible changes in the waterbody.

If possible, samples should be collected between 10:00 and 3:00, the time when the accumulation of the cyanobacteria will be greatest in the upper part of the epilimnion. Cyanobacteria can regulate their buoyancy based on accumulation or reduction of the storage products from photosynthesis i.e. dense carbohydrates or less dense proteins which leads to the development or loss of gas vacuoles. During periods of moderately high irradiances excess carbohydrate is produced that causes the cyanobacteria to sink below the surface layers. After a period in the dark the photosynthate is converted to less dense protein allowing the cells which had sunk over night to rise in the water column (Visser et. al. 2005).

Samples collected as part of a lake survey will have one duplicate and one field blank per day. Field replicates are collected by simultaneously inverting and thrusting two sample bottles into the water and bringing them back up again. Or if a sampling tube is used then water from the same hose sample is used to fill two bottles.

**5.0 APPARATUS, EQUIPMENT, MATERIALS**

* Compound Microscope with long working distance so that a Sedgwick Rafter counting chamber can be used, 20 x objective, typically the Olympus BX51 or Zeiss Axioskop 2 are used for id’s and counts.
* An occular Whipple grid
* Stage micrometer
* Sedgwick Rafter Counting Chamber
* 1 ml disposable pipets
* Taxonomic keys
* Counter

Commonly Used Taxonomic Keys for Cyanobacteria

Cronberg, G. and H. Annadotter. 2006. *Manual on Aquatic Cyanobacteria: A Photo Guide and a Synopsis of Their Toxicology.* Intergovernmental Oceanographic Commission of UNESCO, International Society for the Study of Harmful Algae. 106 pp.

Prescott, G. W. 1982. *Algae of the Western Great Lakes Area*. Otto Koeltz Science Publishers. Koenigstein/West Germany. 977 p.

Smith, G. M. 1950. *The Fresh-water Algae of the United States*. 2nd edition McGraw Hill Publishers. New York. 719 p.

Prescott, G. W. 1982. *How to Know the Freshwater Algae*. Wm C. Brown. New York. 293 p.

VanLandingham, S. L. *Guide to the Identification, Environmental Requirements and Pollution Tolerance of Freshwater Blue-green Algae (Cyanophyta).* Environmental Monitoring and Support Laboratory. U.S. Environmental Protection Agency. Cincinnati.

Wehr, J. D. and R. G. Sheath. 2003. *Freshwater Algae of North America: Ecology and Classification*. J. H. Thorp editor. Academic Press, Inc. 917 pp.

Whitford, L. A. and G. J. Schumacher. 1984. *A Manual of Fresh-Water Algae*. Sparks Press. Raleigh. 337 p.

#### REAGENTS

The primary preservative used is Lugol’s solution which is purchased commercially (Fisher Scientific, Inc. ). The dosage is 1 ml/100 ml of sample. Samples should be stored in the dark. Occasionally, M3 (Meyer’s Magic Mix) is used. It is prepared at the State Laboratory-Wall Experiment Station in Lawrence. The dosage for this is 2 ml/100 ml of sample. These samples too should be stored in the dark

M3 (Meyer’s Magic Mix) for preserving samples

1. recipe-add 5 g KI, 10 g I, 50 ml glacial acetic acid, 250 ml formalin to make 1 liter of dH2O
2. dosage- 2 ml/100 ml of sample
3. comment-store in dark

**7.0 CALIBRATION**

Samples will be analyzed by George Zoto, Ph.D. (MADEP-Southeast Regional Office-Lakeville/Hyannis) or Joan Beskenis, Ph.D (MADEP-Central Regional Office-Worcester). Split samples will be conducted with personnel from other agencies or organizations involved in cyanobacteria counts and ID’s on an occasional basis or when an issue comes up regarding a particular sample. Also, a seasonal employee may be instructed in how to do the counts and will do occasional check samples or parts of samples.

1. **PROCEDURE**

Log-In

Samples are logged into the lab book found with the Zeiss-Axioscope microscope in the Mary Wheeler Memorial Microscopy Lab-room 122 at the MADEP-CERO offices. They are given a chronological number that is written on the sample bottle with indelible marker. Information regarding the sample is recorded in the lab book e.g. location, station number, date sampled, time sample collected, name of collector, any warnings about high bacteria counts or potentially harmful samples.

The samples are returned to the refrigerator after being logged in.

Cyanobacteria counts

Before the actual counts are done identifications need to be made of the dominant genera present. The sample is shaken and a drop is put on a glass slide and examined at 200 or 400 x. An estimate is made of the number of cells contained in a filament or a portion of a colony. This is done by lining up a filament under the Whipple grid and then counting the number of cells in the section of the filament found under the large square within the filament. This is repeated for other filaments ten times.

Re-shake the sample and remove 1 milliliter and add it to the Sedgwick Rafter Counting Chamber. The chamber holds 1 milliliter of water that requires 15 minutes for the cells to settle.

Filling a Sedgwick-Rafter Cell from *Standard Methods for the Examination of Water and Wastewater* (APHA, 1992)

Place the cover slip diagonally across the cell and transfer sample with a 1 ml pipette

The cover slip will slowly rotate and cover the filled portion of the S-R cell

To prevent air bubbles from forming and distorting and ruining the slide, add a drop of DI water to the edge of the cover slip

After 15 minutes the bottom of the counting chamber is examined. A strip of the chamber is counted by moving the Whipple grid along a transect from one end of the slide to the other. All organisms are counted that are within the area defined by the grid, however, if the organism is half out of the bottom of the slide you don’t count it, but if it is half out of the top you do count it.

When you get to the end of the slide you need to refocus on the underside of the coverslip and follow the same procedure used for the bottom of the slide.

As you move along the bottom and the underside of the counting chamber try to count either the individual cells or the large squares that are covered by the colonial cyanobacteria.

Two hundred power is the highest magnification that can be used with a Sedgwick Rafter counting chamber. For a colony like *Microcystis* sp. the large square within the Whipple grid is moved over the colony and the individual cells are counted using best professional judgment while focusing up and down. Repeat this process on different parts of the colony or other colonies ten times and calculate the average number of cells/large square. Then total the number of large squares that represent the area the colony occupies. At the end of the count multiply the total number of large squares by the average number of cells per square.

If filaments e.g. *Anabaena* sp.are present try to count the number of cells that are within the large square on the Whipple grid. Repeat this estimate a total of ten times and calculate the average number of cells/large square. If the filaments are fairly uniform in length then an estimate can be made of the cells per filament. Repeat either count (part of filament within the square or the entire filament) 10 times and calculate the average number of cells per filament. When doing the counts just total the number of large squares needed to cover the length of the filament within the strip. At the end of the count multiply the total number of large squares by the average number of cells per filament. There is a place on the Excel spreadsheet (template is at N\:cyanobacteria cell counts) to calculate average number of cells/square. If you averaged cells/filament then keep a running count of the number of filaments per strip.

Typically, 2-3 strips are counted per cell counting chamber.

After the strips are counted the number of cells of each cyanobacteria are tallied. Cells/large square\*large squares counted or cells/filament\*# filaments.

Use the following formula to calculate # cells/ml=

Strip count (#/ml)=(C\*1000mm2)/(L\*D\*W\*S) where:

C=# organisms counted

L=length of a strip, mm

D=depth of a strip, mm

W=width of a strip (Whipple grid image width), mm

S=number of strips counted

Samples for identification are typically not preserved to avoid some of the cellular changes that occur in preserved samples such as flagella falling off, and the chloroplast constricting. However, iodine in the M3 will color the stored starch in the green algae (Chlorophyceae) to turn black which aids in identification.

Cyanobacterial results are reported to MADPH, particularly if the count is over 70,000 cells/ml. Currently, the results also go to the watershed coordinator at MADEP or other personnel that requested the information. Occasionally, this may mean someone from a Board of Health or Conservation Commission. A more formalized reporting system is under development and will aid in determining who to contact in the event of a cyanobacteria bloom in a recreational waterbody or at a drinking water facility.

The counts and identifications are written in a lab book that is kept in the Microscopy Room-122. Calculations are kept as an Excel spreadsheet on Joan Beskenis’s P: drive and will be added to the cyanobacteria database N\: Cyanobacteria Database.

1. **QUALITY CONTROL**

Pictures of rare or difficult organisms to identify are sent either to individuals or companies for assistance in their identification.

One sample per field day should be collected in duplicate.

Every ten samples should be recounted either by the same biologist or a temporary seasonal employee if they are available.

Typically, the cyanobacteria are dominated by 1 or 2 species, so according to WHO (1999) a precision level of 20 % can be attained by counting 400 individual units (cells, colonies, filaments) per species. For colonial species, breaking the colonies up and then counting them is preferable to counting colonies and estimating colony size (Box, 1981)

We will be trying to reach this level of precision although in the past we, like our colleagues, have just been trying to have two replicate counts be ‘reasonably’ similar. We will determine a precision level by calculating the standard deviation of the transects, dividing it by the mean of the transects and multiplying by 100. This precision level will be reported in the QA/QC report. If the deviation in two transects is <20% no additional transects need to be counted (WHO 1999), unless the unit count is less than 400 then one or two more transects will be counted. If after four transects the 400 unit number is not obtained then a second slide is prepared and re-counted to ensure that a well mixed sample was tested in the first slide.

1. **INTERFERENCES**

It is particularly difficult to get a good estimate of the number of cells in colonial species. The thickness of the colony and the number of cells in colonies of *Microcystis*, for example, make it difficult to count accurately. An effort should be made to count in rows and focus up and down to examine the thickness of the cell. Colonial cyanobacteria like the *Microcystis flos-aquae* are difficult to count accurately because the colonies are spherical and several cell layers thick. Ten different counts of the cells per square should also be obtained and then averaged. A method of boiling the sample for 6 minutes to break up the polysaccharides holding the cells together (Joung 2006) has occasionally been used, but most samples are examined intact.

Filaments can also cause problems in the counts. Some filaments do not have an indentation marking the end of one cell and the beginning of the other. Thus, some genera-particularly the *Planktothrix* sp. and the *Lyngbya* sp., are difficult to count. It is helpful to view the filament at higher magnification than the 200 x just to examine the cells better and then return to the count 200 x for the count. In some difficult cases-where cells are not easily discerned, literature values might be used to estimate cell width and length and those dimensions used to determine the # cells per large square.

**11.0 PREVENTIVE MAINTENANCE**

The Sedgwick-Rafter counting chamber must be rinsed out after use along with the special glass cover slip. The cover slip is fragile and must be carefully handled. The counting chamber and the cover slip should be washed frequently with detergent and rinsed well.

1. **CORRECTIVE ACTIONS**

Samples can not typically be recollected, but when possible photo-micrographs will be taken to document sample appearance or cell/colony structure.

**13.0 WASTE AND POLLUTION PREVENTION**

The majority of samples are typically examined without added preservatives thus reducing the addition of formalin into the waste stream. Samples with preservatives can be collected along with the acetone waste and brought to WES for proper disposal.

**14.0 REFERENCES**

American Public Health Assoc. 1992. *Standard Methods for the Examination of Water and Wastewater*, 18 th edit. American Public Health Assoc. Washington, DC.

Box, J.D. 1981. Enumeration of cell concentrations in suspensions of colonial freshwater microalgae, with particular reference to *Microcystis aeruginosa*. *Brit. Phycol. J*. 16:153-164.

MDPH. 2007 *MDPH Guidelines for Cyanobacteria in Freshwater Recreational Waterbodies in Massachusetts*. Massachusetts Dept. of Public Health. Boston. 14 pp.

Joung, S. H., Kim, C. J., Ahn, C. Y., Jang, K. Y., Boo, S. M. and H. M. Oh. 2006. Simple method for a cell count of the colonial cyanobacterium. *Journal of Microbiology.* 44:562-565.

Visser P.M, Ibelings, B.W., Mur, L.R. and A. E.Walsby. 2005. Ecophysiology of the harmful cyanobacterium *Microcystis* sp.:Features explaining its success and measures for its control. *Harmful Cyanobacteria* edit J. Huisman, Hans, C.P. Matthijsand Petra M. Visser. Springer, Inc. Netherlands-Aquatic Ecology Series 3, 2005 pp 109-142.

WHO. 1999. *Toxic Cyanobacteria in Water: A Guide to Their Public Health Consequences, Monitoring and Management* . I. Chorus and J. Bartram editors. World Health Organization. Spon Press. London. 416 p.