



Cyanobacteria Identification and Enumeration

B-002-OWQ-WAP-XX-23-T-R1

Technical Standard Operating Procedure

Office of Water Quality

Branch Watershed Assessment and Planning

Section All

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Purpose

This technical standard operating procedure (TSOP) describes the method for counting and identifying cyanobacteria, commonly referred to as blue-green algae. Specifically, this TSOP covers the processes of loading the nanoplankton counting chamber, counting and identifying cyanobacteria cells using a microscope with Nomarski differential interference contrast (DIC) and epifluorescence capabilities, and calculating the concentration of cyanobacteria cells present in the sample.

Water samples are collected from designated swimming beaches managed by the Indiana Department of Natural Resources (DNR), as described in “Collecting Surface Water Samples for Cyanobacteria and Cyanotoxin Analysis, S-003-OWQ-WAP-TGM-23-T-R1”. Samples are collected monthly unless cyanobacteria cell counts exceed 100,000 cells/milliliter (mL), at which point sampling occurs biweekly. Samples are taken to the Watershed Assessment and Planning Branch (WAPB) laboratory for identification, quantification, and toxin analyses, as described in “Determination of Cyanobacteria Toxins in Ambient and Drinking Water by ELISA, S-001-OWQ-WAP-TGM-21-T-R4”.

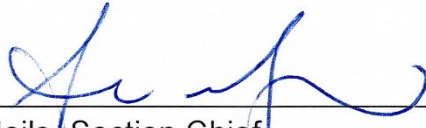
Scope

This TSOP applies to agency staff in the Office of Water Quality (OWQ) Targeted Monitoring Section who are responsible for quantifying and identifying cyanobacteria cells.

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Authorizing Signatures

I approve and authorize this technical standard operating procedure (TSOP):



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3/22/2023
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3-22-2023
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This TSOP is consistent with agency requirements.



Quality Assurance Staff
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3/30/2023
Date

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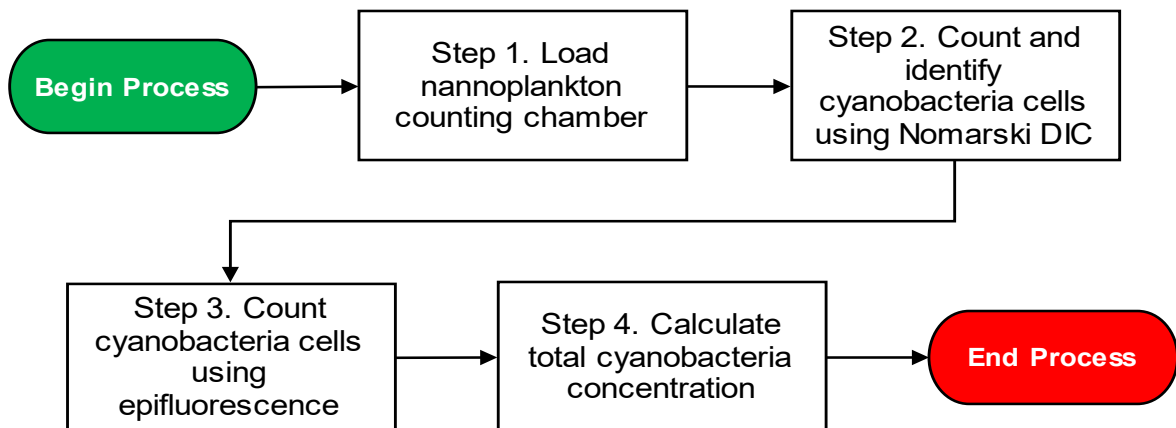
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1.0. Overview Flowchart

Not applicable.

2.0. Procedure

2.1. Procedural Flowchart



2.2. Procedural Steps

- Step 1. Loading the PhycoTech, Inc. nanoplankton counting chamber
- All steps are to be completed by an IDEM biologist. Select a sample from the refrigerator and record all necessary site information, including the sampling date and time on a Cyanobacteria Benchsheet (Appendix 1).
 - Choose a PhycoTech, Inc. nanoplankton chamber to use for counting cells and record the chamber's identification number on the Cyanobacteria Benchsheet. It is important to record which chamber is used because each chamber is individually calibrated and holds a slightly different volume. The volume of the chamber is used in the calculation to determine total cyanobacteria concentration in cells/mL.
 - Place a 22 x 22 mm² glass coverslip on the side of the counting chamber and leave a small opening to add sample. See Appendix 2 for an example of instructions that are included with each nanoplankton chamber.
 - Thoroughly agitate the sample.
 - Using a disposable plastic pipette, collect an aliquot of the sample and deposit it into the open space of the nanoplankton chamber that the coverslip is not covering.

- F. As the sample fills the chamber, it will draw the coverslip over the chamber. Position the coverslip over the entire opening to seal in the sample.
- G. Are air bubbles present in the chamber? If yes, proceed to Step 1.H. If no, proceed to Step 1.I.
- H. Since air bubbles are present in the chamber, the sample will have to be reloaded. Thoroughly rinse the nanoplankton chamber and coverslip with deionized water. This will remove any adherent cyanobacteria from the previous attempt to fill the chamber and ensure that the total cyanobacteria concentration that is calculated accurately represents the aliquot of sample. Dry the chamber and coverslip with a Kim-Wipe, and then proceed to Step 1.C to start the filling process over.
- I. Since air bubbles are not present in the chamber, place the nanoplankton chamber on the microscope stage and let the sample settle for 10 minutes before beginning to count cells.

Step 2. Cyanobacteria identification and enumeration using Nomarski differential interference contrast (DIC) microscopy

- A. Turn on the microscope and the light source. The power switch on the Nikon Eclipse 80i is located on the back left side of the microscope and the light source switch is on the front of the microscope at the bottom. If the microscope is connected to a power supply box, use the controls on the power supply box to turn on the power and adjust the light setting. Make sure the upper turret is in a position other than #1 or #2. Positions #1 and #2 are used during epifluorescent microscopy as explained in Step 3.
- B. Position the 20X objective over the nanoplankton chamber.
- C. Make sure the multiple tally counter is set at "0" before beginning to count. Be sure to keep track of the number of grids that are counted since this is used in the calculation to determine total cyanobacteria concentration.
- D. Choose a transect, or row, on the nanoplankton chamber to count. Start either at the top or bottom of the chamber to allow for counting 100 grids without ever counting a grid more than once. Using a gridded reticle in the microscope eyepiece as a boundary, identify and count the number of cyanobacteria cells that occur within that grid. Count individual cells for colonial species or any solitary cells present in the grid. Count and measure individual trichomes for filamentous species and use

standard cell lengths predetermined by IDEM biologists to calculate the number of cells. Use the multiple tally counter to keep track of the number of grids and cells counted. Record taxa and the number of natural units of each taxon on the Cyanobacteria Benchsheet. In most cases, multiple transects will need to be scanned. Some colonies and filaments are only partially in the grid. The rule for counting cells that fall partially outside of the boundary is to count them if they touch the upper or left boundaries. **They should not be counted if they touch the bottom or right boundaries of the grid.**

- E. Continue the identification and enumeration process until 100 natural units or 100 grids have been counted. For samples that are dense with cyanobacteria cells, it is common to record 100 natural units before 100 grids have been counted. If cyanobacteria cells are sparse, it is more likely to count 100 or more grids and potentially not even find 100 natural units. The latter scenario is common at the beginning of the season in May and June when cyanobacteria are not yet blooming. Counting can end once either of these criteria is met.

Step 3. Cyanobacteria identification and enumeration using epifluorescent microscopy

- A. Turn off the bottom light source of the microscope. If the microscope is connected to a power supply box, dial down the light setting to zero.
- B. Turn on the Nikon Intensilight. The switch is located on the bottom right corner of the Nikon Intensilight power box.
- C. Move the upper turret to Position #2 for phycoerythrin/phycoerythrin. Phycoerythrin will fluoresce bright orange-red and phycocyanin will fluoresce orange-yellow. (Epi filters for phycoerythrin/phycoerythrin – Exciter: D546/10x, 25mm, Dichroic: 560 DCLP, Emitter: 580/30m)
- D. Pull the nosepiece analyzer filter (A) halfway out.
- E. Check that the manual shutter at the nosepiece is set to open “O” (moved to the right).
- F. Turn the light source on using the remote control. You will see a green light emitting from the objective after the light source is turned on. The intensity of the light can also be controlled by the remote control. To return to DIC microscopy, do the opposite of the steps listed above in reverse order from Step 3.F to 3.A.
- G. Position the 20X objective over the nanoplankton chamber.

- H. Make sure the multiple tally counter is set at “0” before counting begins. Be sure to keep track of the number of grids that are counted since this is used in the calculation to determine total cells/mL.
- I. Choose a transect, or row, on the nanoplankton chamber to count. Using the gridded reticle in the microscope eyepiece as a boundary, identify and count the number of solitary coccoid cyanobacteria cells that occur within that grid. Only small, solitary coccoid cells, or picoplankton, that are fluorescing yellow should be counted. These cells are hard to see under DIC. Using epifluorescence causes them to glow, thus making it easier to see and count them. It is best to perform the epifluorescent scan in a darkened room to better see the dimly lit fluorescing cells. Use the multiple tally counter to keep track of the number of grids and cells counted.
- J. Continue the identification and enumeration process until 50 natural units or 50 grids have been counted. Again, the rule for counting cells that fall partially outside of the boundary is to count them if they touch the upper or left boundaries. They should not be counted if they touch the bottom or right boundaries of the grid.
- K. When the sample has been viewed under both DIC and epifluorescent microscopy, thoroughly rinse and dry the nanoplankton chamber and coverslip with deionized water to avoid cross-contamination between samples.

Step 4. Calculating total cyanobacteria concentration (cells/mL)

- A. There are two separate sets of calculations per sample: one to determine the total cyanobacteria concentration of discernable cells that are counted using DIC microscopy and another to determine the total cyanobacteria concentration of picoplankton that were counted using epifluorescent microscopy. The two calculations are added together to determine the total cyanobacteria concentration (cells/mL) of the sample.

The calculations begin with the multiplication of the number of grids counted by the area of the entire grid (0.246 mm² using 20X objective) to determine the area of the chamber that was enumerated. Remember that these steps are done separately for each of the counting methods.

$$\text{Area} = \text{Number of grids counted} \times 0.246$$

- B. Use the number calculated in Step 4.A (total area counted) and multiply it by the depth of the nanoplankton chamber to determine the volume of the sample that was counted. Each chamber has been individually calibrated so the number used in this calculation will be different depending on which chamber is being used. The depth of each nanoplankton chamber is recorded on the chamber and on the instructions sheet that is supplied by PhycoTech, Inc. This calculation gives the volume in mm³.

$$\begin{aligned} \text{Volume} &= \text{Area (from Step 4.A)} \\ &\times 0.278 \text{ (depth of chamber \#713)} \end{aligned}$$

- C. Add up all the cells that were enumerated and identified by taxon to determine the total number of cyanobacteria cells that were counted.
- D. Divide the total number of cyanobacteria cells counted by the volume of sample that was enumerated.

$$\text{Concentration} = \frac{\text{Total number of cells (Step 4.C)}}{\text{Volume enumerated (Step 4.B)}}$$

- E. Multiply the number calculated in Step 4.D by 1000 to get total cyanobacteria concentration in cells/mL (1 mL = 1000 mm³).
- F. On the computer, open the macro-enabled Excel file titled "20XX Cyano Benchsheets..." that corresponds with the analysis set number. The analysis set number, also known as the trip number, is shown in the upper right corner of the Cyanobacteria Benchsheet.
- G. On the benchsheet for the appropriate sample number, fill in the fields (chamber #, grids counted, taxa names, calculated cells, etc.) and the total concentration is automatically calculated in the Excel workbook. Using the Excel file with the built-in calculations reduces errors and allows total cell counts to be reported quicker. Manually calculate the total cyanobacteria concentrations using Steps 4.A to 4.E for a few samples throughout the season to make sure the Excel formulas were not inadvertently altered.

H. Enter the data from the benchsheet into the Assessment Information Management System (AIMS) database in the “Concentration” tab under “Algae”.

2.3. Related Technical Issues

A. Health and Safety Warnings

1. Handle glassware such as coverslips and nanoplankton counting chambers with care. Broken glassware should be disposed of in the “broken glass” container instead of in regular trash.

B. Cautions

1. Handle glassware with care. The nanoplankton chamber should be stored in its protective case when not in use to minimize accidental breakage since they are expensive to replace.
2. Use a new disposable plastic pipette for each sample to eliminate the possibility of cross-contamination between samples.
3. Be diligent in recording the correct sample number and site information on the Cyanobacteria Benchsheet.
4. Be sure to keep track of how many grids are counted during the identification and enumeration process because this number is used in the total concentration calculation. If the number of grids counted is not recorded, the process of enumeration and identification of the sample **must be restarted**.
5. Do not count or identify cells in grids that have air bubbles. Move over to the next grid before continuing the identification and enumeration process. If grids with air bubbles are counted, the total cyanobacteria concentration would be inaccurate.
6. Do not let the sample dry out in the nanoplankton counting chamber. When dry patches form, sample distribution will shift and make identification difficult.
7. When using epifluorescence in Step 3, be sure to keep the Nikon Intensilight on for at least 20 minutes before turning it off, and make sure it stays off for at least 20 minutes before turning it back on. This will prevent causing damage to the Intensilight bulb in the unit.
8. Make sure to thoroughly rinse the nanoplankton chamber and coverslip with deionized water and dry them after each sample to avoid cross-contamination between samples.
9. Be sure to double check calculations. If calculations are done in Excel, it is important to check a few calculations by hand periodically throughout the season to ensure the formulas have not been inadvertently altered.

C. Interferences

1. The multiple tally counter should be set at “0” before counting begins. Also make sure to always record the number of grids that are encountered during the cell counting process. Not having an accurate record of the number of grids used in the counting process will result in an incorrect calculation of total cyanobacteria concentration for the sample.
2. Refer to taxonomic literature, keys, and archived photographic images to resolve any uncertainties regarding taxa present in the sample in order to avoid making taxonomic errors. Duplicate samples are collected at one site per week which serves as a QA/QC measure so the IDEM biologist can compare their identification and enumeration results from the same sample to ensure they are consistent with counts and identifications.

D. Calibration

1. PhycoTech, Inc. nanoplankton chambers are individually calibrated by PhycoTech, Inc. Each chamber comes with its own identification number and the exact volume (in milliliters) that the chamber holds, along with instructions on how to calculate the total number of cells/mL. See Appendix 2 for an example of instructions that are included with each nanoplankton chamber.
2. The gridded reticle in the eyepiece of the Nikon Eclipse 80i microscope was calibrated with a stage micrometer to ensure that measurements are accurate. The calibration was done before the ocular reticle was used for measurements.

E. Troubleshooting

1. To avoid the sample drying out while counting, measure only 20 trichomes of each species and use the average length of those trichomes as the length for all trichomes of that taxon instead of measuring each trichome individually. Measure several cells from different trichomes to come up with a standard cell size to be used for all samples. Counting would take too long if the IDEM biologist measured each trichome and/or counted every cell in each trichome. Using estimations where necessary ensure that samples are counted before they dry out in the chamber.
2. During the identification and enumeration process, the IDEM biologist might come across trichomes and/or colonies that lie on the boundary lines of the grid on the gridded reticle. To be consistent, trichomes and colonies that extend beyond the grid boundary should only be counted if they touch the left or upper

boundary lines. Do not count cells that touch the lower or right boundary lines.

3. For unidentifiable specimens, use the lowest possible classification level (e.g., if the family can be determined but the genus is unknown, record only the family name for the identification). If no classification level can be determined, use “Unknown coccoid” or “Unknown trichome” for the taxonomic identification.
4. The manuals for the Nikon Eclipse 80i microscopes are stored in the Algae Lab at the Shadeland Office. For trouble with the microscope, contact a customer service representative from Nikon.

3.0. Roles

3.1. Responsibilities

A. IDEM biologist

1. Exercises proper laboratory safety
2. Loads the PhycoTech, Inc. nanoplankton chamber
3. Identifies and enumerates cyanobacteria samples using both Nomarski DIC and epifluorescent microscopy
4. Calculates total cyanobacteria concentration (cells/mL)
5. Records taxonomic information in AIMS

3.2. Training requirements

A. Loading the nanoplankton counting chamber

1. IDEM biologist

B. Identification and enumeration of freshwater cyanobacteria

1. IDEM biologist

C. AIMS database management

1. IDEM biologist

4.0. Required Forms, Equipment, or Software List

4.1. Forms

- A. Cyanobacteria benchsheet (Appendix 1)

4.2. Equipment

- A. Nikon Eclipse 80i microscope
- B. Nikon microscope camera (optional)
- C. Epifluorescence capability on microscope

- D. PhycoTech, Inc. nanoplankton counting chamber
- E. Disposable plastic pipettes
- F. Kim-Wipes
- G. 22 x 22 mm² glass coverslips
- H. Multiple tally counter
- I. Gridded reticle
- J. Stage micrometer

4.3. Software

- A. AIMS
- B. Nikon NIS-Elements D (optional)

5.0. Records Management

- 5.1. Original hard copies of Cyanobacteria Benchsheets are stored in a filing cabinet in the Algae Lab at the Shadeland Office. Benchsheets have been scanned and stored as attachments to the project in AIMS. Starting with the 2023 season, benchsheets will be scanned and stored in the Virtual File Cabinet (VFC). Identification and enumeration data are entered and stored in AIMS.

6.0. Definitions

- 6.1. "AIMS sample number (AA/AB/AC)" – A number assigned to each individual watershed sampling event conducted by Indiana Department of Environmental Management (IDEM) field crews. This number is used to identify the sampling event in the Assessment Information Management System database (AIMS database).
- 6.2. "Agency staff" – Any employee or representative of the Indiana Department of Environmental Management including regular employees, temporary employees, contractors, and interns.
- 6.3. "Assessment Information Management System database (AIMS database)" – The database contains information related to water chemistry, aquatic habitat, macroinvertebrate, fish, and algae communities, fish tissue analyses, sediments and *E. coli* bacteria data collected by agency staff from watershed sampling events.
- 6.4. "Benchsheet" – A paper form where data such as cyanobacteria taxa names and cell count information are recorded.

- 6.5. "Chain of custody (COC)" – The records documenting the possession of the samples from the time they are obtained until they are disposed of or shipped off-site.
- 6.6. "Cyanobacteria" – A division of microorganisms that are related to the bacteria but are capable of photosynthesis. They are also referred to as blue-green algae.
- 6.7. "Deionized water" – Water that has had its mineral ions removed.
- 6.8. "Filament" – A trichome including the sheath.
- 6.9. "Gridded reticle" – Clear disc marked with a grid pattern that fits in the eyepiece of a microscope.
- 6.10. "IDEM biologist" – Indiana Department of Environmental Management (IDEM) scientist educated and trained to understand the life of living organisms, including their structure, function, growth, origin, evolution, and distribution. In this particular case, the IDEM biologist needs to have specific knowledge of algae and taxonomy of cyanobacteria.
- 6.11. "Nannoplankton counting chamber" – Microscope slide counting chamber that holds a known volume of sample for quantitative counting of cells with a microscope.
- 6.12. "Natural Units" – Natural grouping of algae (i.e., individual filament, colony, or isolated cell). An individual filament, colony, or isolated cell would all count as one natural unit even though the natural unit might be comprised of several cells.
- 6.13. "Nomarski differential interference contrast (DIC)" – An optical microscopy illumination technique used to enhance the contrast in unstained, transparent samples.
- 6.14. "Picoplankton" – The fraction of plankton composed of cells between 0.2 and 2 μm in size. This technical standard operating procedure (TSOP) is only concerned with cyanobacterial picoplankton that glows yellow under epifluorescence.
- 6.15. "Taxonomist" – A biologist that groups organisms into categories.
- 6.16. "Technical standard operating procedure (TSOP)" – A standard operating procedure that involves environmental data generation, manipulation, or compilation of an analytical process.
- 6.17. "Trichome" – A strand or chain of cells (as in a filamentous colony of bacteria or algae).

- 6.18. “Virtual File Cabinet (VFC)” – The agency’s electronic digital image document repository system, that stores, files, indexes, redacts, reassembles and securely accesses electronic documents of all types both received and created by the various program areas within the agency.

7.0. Quality Assurance and Quality Control

- 7.1. QA/QC will be documented by checks of both field and laboratory data. AIMS will assign QC samples for each sampling trip prior to sample collection. This includes a field blank and a field duplicate.
- 7.2. A field blank, using ASTM D1193-91 Type 1 water, is collected each week following procedures outlined in the Collecting Surface Water Samples for Cyanobacteria and Cyanotoxin Analysis TSOP. The field blank is kept in the cooler with the normal samples and is analyzed under the microscope following the same procedural steps outlined in Section 2.2 as used for the normal samples. This QC check ensures that the sample was not somehow contaminated in the cooler or field. It also serves as a way for the IDEM biologist to assess whether the nanoplankton chamber and coverslip are adequately rinsed between samples. If cyanobacteria cells are ever found in the field blank sample, it can be assumed the nanoplankton counting chamber and/or coverslip were not thoroughly rinsed after the last sample or that the sample was contaminated in the cooler or field.
- 7.3. A field duplicate sample is collected each week following procedures outlined in the Collecting Surface Water Samples for Cyanobacteria and Cyanotoxin Analysis TSOP. The duplicate sample is analyzed under the microscope following the same procedural steps outlined in Section 2.2 as used for the normal samples. This helps the IDEM biologist determine whether there is consistency between samples for both identification and enumeration purposes. The relative percent difference (RPD) between the normal and field duplicate samples should be $\leq 20\%$.
- 7.4. 10% of all samples identified by one IDEM biologist will be re-identified and counted by another IDEM biologist trained in this TSOP. This serves as a QC check of taxonomic data and ensures consistency between taxonomists. The RPD between IDEM biologists should be $\leq 20\%$.

8.0. References

- 8.1. Agency Standard Operating Procedures

- A. [Collecting Surface Water Samples for Cyanobacteria and Cyanotoxin Analysis](#), S-003-OWQ-WAP-TGM-23-T-R1, effective January 23, 2017, last revised February 9, 2023.
- B. [Determination of Cyanobacteria Toxins in Ambient and Drinking Water by ELISA](#), S-001-OWQ-WAP-TGM-21-T-R4, effective June 12, 2013, last revised March 30, 2021.

8.2. Other Guidance

- A. [PhycoTech, Inc. nanoplankton chamber product page](#)

9.0. Appendices

- 9.1. Appendix 1 – Example of a Cyanobacteria Benchsheet
- 9.2. Appendix 2 – Example of instructions that accompany a PhycoTech, Inc. nanoplankton counting chamber
- 9.3. Appendix 3 – List of cyanobacteria taxonomic references (located in the Algae Lab)

Appendix 2 – Example of instructions that accompany a PhycoTech, Inc. nanoplankton counting chamber



Nannoplankton Chamber Information and Instructions

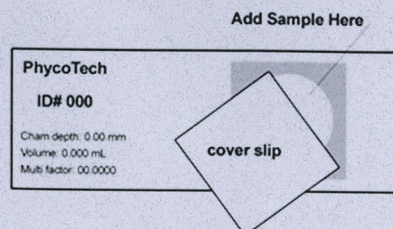
ID# 713

This nanoplankton chamber is fashioned after the Palmer-Maloney counting chamber, and is used to hold a known volume of sample for quantitative counting of cells with a microscope. This nanoplankton chamber has several advantages over other chambers and hemocytometer: 1) It is thinner, which allows examination and counting of microalgae at higher magnification, 2) larger cells will not be crushed by the weight of the coverslip, as this chamber uses a #1, 22 mm² coverslip, 3) it is less expensive and ease to use, and 4) it is individually calibrated. It is suitable for counting organisms up to approximately 300 µm in length. It can be used up to 600x using a high, dry objective.

Each chamber has been individually calibrated and this particular chamber holds exactly 0.052 milliliters (mL). Retain underlined data for future use with this chamber. After counting all of the algae in the entire chamber, simply multiply by 19.098 to calculate the number of cells/mL.

A less tedious counting method involves extrapolating from counts on several individual microscope fields. To do this, measure the diameter of the field at a given power with a stage micrometer. Then calculate the area of the field using the formula $3.14159(\pi)$ times the radius squared (radius = $\frac{1}{2}$ of the diameter): $\text{area} = \pi \times r^2$. Use millimeters for all length measurements. To convert this value into volume, multiply by the depth of the chamber, which is calibrated to 0.278 mm for this chamber. The formula for calculating volume is (area) x (depth). Keep in mind that 1000 mm³ is equal to 1 mL.

To use this chamber, place a #1, 22 mm² glass coverslip on the side and leave a small space to add sample as illustrated below. As the sample fills the chamber, it will draw the coverslip over the chamber. Then position the coverslip over the entire opening to seal in the sample. If air bubbles are present, repeat the filling process. Live samples can be examined immediately and preserved samples after settling.



Appendix 3 – List of cyanobacteria taxonomic references (located in the Algae Lab)

- Clerin, N. 2010. An Identification Guide to the Cyanobacteria of Indiana, Center for Earth and Environmental Science, Indiana University Purdue University Indianapolis.
- John, D.M., Whitton, B.A. & Brook, A.J. 2002. The Freshwater Algal Flora of the British Isles, Cambridge University Press, New York, New York.
- Komarek, J. & Zapomelova, E. 2008: Planktic morphospecies of the cyanobacterial genus *Anabaena* = subg. *Dolichospermum* – 2. part: straight types. *Fottea* 8:1-14.
- Prescott, G.W. 1951. Algae of the Western Great Lakes Area, Cranbrook Institute of Science, Bloomfield Hills, Michigan.
- Prescott, G.W. 1970. How to Know the Freshwater Algae, Third Edition, WCB McGraw-Hill Burr Ridge, Illinois.
- St. Amand, A. & Wagner, K.J. 2012. Collection, Identification, Ecology of Freshwater Algae, PhycoTech, Inc.
- Wacklin, P., Hoffmann, L. & Komarek, J. 2009: Nomenclatural validation of the genetically revised cyanobacterial genus *Dolichospermum* (Ralfs ex Bornet et Flahault) comb. nova. *Fottea* 9:59-64.
- Wehr, J.D. & Sheath, R.G. 2003. Freshwater Algae of North America, Academic Press, Boston, Massachusetts.