Spring 2015

# Toxic Cyanobacteria Aerosols: Tests of Filters for Cells

Sean R. Perry
*University of New Hampshire - Main Campus*, sra8852@wildcats.unh.edu

Alexis Mack
*University of New Hampshire - Main Campus*

James F. Haney
*University of New Hampshire - Main Campus*, jfhaney@gmail.com

Amanda Murby
*University of New Hampshire - Main Campus*, mana.murby@gmail.com

# Toxic Cyanobacteria Aerosols: Tests of Filters for Cells

*S. R. Perry, A. Mack, J. F. Haney, A. J. Murby*
Dept. of Zoology, University of New Hampshire, Durham, NH, 03824

**Abstract**

Aerosolization of toxic cyanobacteria released from the surface of lakes is a new area of study that could uncover a previously unknown route of exposure to toxic cyanobacteria. Since toxic cyanobacteria may be responsible for adverse human health effects, methods and equipment need to be tested and established for monitoring these airborne bacteria. The primary focus of this study was to create controlled laboratory experiments that simulate natural lake aerosol production. I set out to test for the best type of filter to collect and analyze the aerosolized cells as small as 0.2-2.0 µm, known as picoplankton. To collect these aerosols, air was vacuumed from just above a sample of lake water passing through either glass fiber filters (GFF) or 0.22 µm MF-Millipore™ membrane filters (0.22 Millipore™). Filter collections were analyzed through epiflourescence microscopy for determining cell counts. Data analysis revealed that 0.22 Millipore™ filters were the best option for cell enumeration providing better epiflourescence optical quality and higher cell counts.

## Introduction

Many recent studies have investigated the composition of various particles contained in the air released from the surface of aquatic environments (Dueker, 2012). Composition of this air can consist of bacteria and cyanobacteria that are exhausted from aquatic environments due to disruptions of the water surface. These disruptions consisting primarily of bubble-bursting actions can be caused by waves, changes in temperature, or aeration of the water (Dueker, 2012). Dueker et al. observed that bacterial fallout from the air was much greater in areas surrounding a superfund site than areas surrounding other water bodies. The superfund site, Newtown Creek, was located along the Hudson River Estuary (New York, NY, USA). This site was receiving large amounts of untreated sewage along with waste dumping and oil seepage. Additionally, this site was aerated as a way to increase dissolved oxygen concentration. Bacterial fallout around Newtown Creek was significantly higher than in other areas and was representative of the sewage bacteria that were drained into this superfund site. Also, during aeration the concentration of large aerosolized particles, was greater than times when aeration was not performed. This study demonstrated that aerosolized particles are characteristic of the composition of the aquatic environments from which they come and their concentration can be influenced by factors such as bacterial water concentration and disruption of the water surface.

Many cyanobacteria that become aerosolized are as small as 0.2-2.0 µm, known as picocyanobacteria. Cyanobacteria can produce a variety of toxins that are detrimental to human health, such as hepatotoxin microcystins (MCs) and neurotoxic β-methylamino-L-alanine (BMAA). Removal of MCs requires compound actions of physical removal, chemical inactivation, and biological inactivation. This involves cleansing of the water using filters and activated carbon, chlorination, and pH and temperature manipulation to remove and inactivate MCs (Pantelića, 2013).

Cox et al. (2003) discovered a relationship between amyotrophic lateral

sclerosis/parkinsonism-dementia complex (ALS/PDC), and the diet of the Chamorro people of Guam. The Chamorro people had diets composed of multiple food sources containing concentrated BMAA. One of their primary food sources was cycad seeds, which were consumed as a carbohydrate source. Cycad seeds contained high levels of BMAA due to biomagnification from symbiotic relationships between cyanobacteria and their roots. Toxicity of cycad seeds was known to the Chamorro people and therefore was subjected to extensive washing to make it edible when made into a four. Biomagnification of BMAA increased each step up the food chain beginning with cyanobacteria containing 0.3 µg/g of BMAA, which led to 9 µg/g in cycad seeds, and finally, 3556 µg/g of BMAA in flying foxes, a delicacy of the indigenous people's diet. Biomagnification led to a concentration of 7.2 µg/g of BMAA in the frontal cortex of Chamorro people who died of ALS/PDC (Bradley 2009). High intake of BMAA by the Chamorro people was correlated with the high rate of neurodegenerative diseases present in these communities. Rates of ALS/PDC in the Chamorro people reached levels as high as 100 times greater than the expected rates of ALS found worldwide. Chamorro people are the only community in their indigenous area to eat flying foxes which contained the highest BMAA concentration. Rises in consumption of flying foxes, as the Chamorro people acquired larger amount of flying foxes through access to firearms, were correlated with increased rates of ALS/PDC. Following this rise was a delayed decrease in the rates of ALS/PDC as flying foxes became scarce due to over hunting by the Chamorro people (Cox 2003).

In 2013, Stommel and colleagues hypothesized a correlation between aerosolized cyanobacteria and cases of sporadic ALS (sALS). Emphasizing the prevalence of conjugal couples and coworkers who develop sALS in conjuncture implies an environmental trigger. Stommel references the discovery of BMAA in the brains of ALS patients but not in those of patients afflicted with Huntington's disease, a genetic neurodegenerative disease (Pablo, 2009). This eliminates the possibility of BMAA as a byproduct of neurodegeneration. BMAA functions through binding to N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors while also causing protein misfolding, which all result in selective motor neuron loss. Possible exposure routes include ingestion as was seen in the Chamorro people, recreational activities involving water experiencing cyanobacterial blooms, or through inhalation of aerosolized cyanobacteria. All provide the possibility of cyanobacteria exposure, which can then implant in human organs and chronically secrete cyanotoxins.

Caller et al. in 2009 further investigated the relationship between BMAA and ALS. A geographic information system (GIS) map was created documenting all cases of ALS in the town of Enfield, NH. This town surrounds Lake Mascoma, which frequently experiences cyanobacterial blooms. The town of Enfield had 10-25 times the expected ALS prevalence and it was hypothesized that while the residents of the town may have been exposed to the cyanobacteria by ingestion or contact with the water, they may have also been gaining additional exposure as the cells were aerosolized carrying with them the harmful toxins. Ultimately, this study determined that there was a spatial association between ALS and lakes known to experience cyanobacterial blooms, however, this does not imply causation of the high rates of ALS.

Since research into aerosolized cyanobacteria is just beginning the appropriate methods and

materials need to be established, first determining which filters are best suited to collect aerosols.

**Methods**

*Aerosol Collection* - Filters used for collecting the aerosolized cyanobacteria were either 0.22 µm MF-Millipore™ Membrane Filters (0.22 Millipore™) or Whatman glass fiber filters (GFF). The 0.22 Millipore™ were autoclaved and stored in sterile cases before use. GFF were by first cleaned by filtering with 5 mL of Milli-Q water. The filters were then placed in a muffle furnace at 500 $^{o}$C for 30-60 min in a covered petri dish and later stored in a desiccator. Firing the GFF reduces the effective pore sizes from 0.6 µm to approximately 0.3 µm.

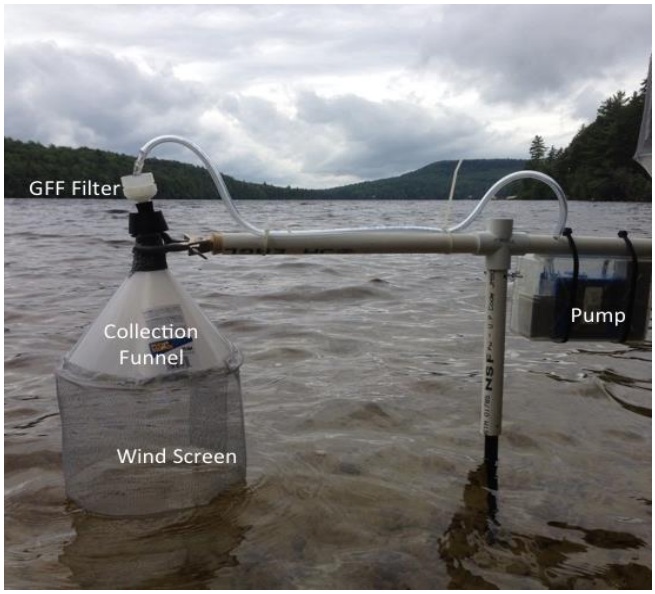

Image 1: Aerosol field collection on Christine Lake, Stark, NH
Photo by J. Haney


Image 2: Aerosol laboratory collection apparatus
Photo by S. Perry

Aerosolized cyanobacteria were collected in both the field and laboratory. The field apparatus consisted of a wire mesh wind screen guided into a funnel (Figure 1). The mesh rested just above the water surface and had tubing connected to a Swinnex® Filter Holder (25 mm diam) containing the filter attached by tubing to a Gilian BDX-II Personal Air Sampling Pump. The aerosol collector was staked into the lake bottom near the shore. To measure aerosolization in the laboratory, lake water samples (500 mL) were placed in 1000 mL pyrex bottles that were sealed by a rubber stopper with inlet and exhaust tubes (Figure 2). The exhaust tube was lowered to 2 cm above the water surface and connected by tubing to a Swinnex® Filter Holder (25 mm diam) and Gilian BDX-II Personal Air Sampling Pump

Table 1: Summarization of aerosol collection experiments with lake samples and corresponding experiment durations and pump flow rates.

| Lake Sample | Run Time (hr) | Air Flow Rate (Liter $min^{-1}$) |
|---|---|---|
| Willand Pond (Oct. 01) | 4.08 | 2 |
| Lake Attitash (Oct. 02) | 4 | 2 |
| Lake Attitash (Oct. 22) | 4 | 2 |
| York Pond (Sept. 18) Day | 4 | 2.5 |
| York Pond (Sept. 18) Night | 4 | 2.5/3 |
| Willand Pond (Sept. 20) Day | 3 | 2.5 |
| Willand Pond (Sept. 20) Night | 4 | 2.5 |

Experiments had collection times from 3-4 h and flow rates from 2-3 LPM (Table 1). The Willand Pond day trial was run for 3 h while the October 1st Willand Pond sample was run for 4.08. The York Pond Night sample had two filters that were run at 2.5 LPM and one filter run at 3 LPM. At termination of each experiment aerosol filters were placed in Lacons™ 1.25 in. diam. snap caps indicating the side of the filter exposed to the aerosols. The snap caps were then labeled and stored upright in a freezer at -40 C.

*Microscopy* – Cells were enumerated at 400X using epifluorescence microscopy with two different excitation wavelengths. The filters were placed on a glass slide on top of 2-3 drops of distilled water with the filter side exposed to the aerosolized cyanobacteria facing upwards. The depth of focus was first determined with standard light. The filter was then exposed to the chlorophyll excitation cube (blue light at 435 nm). This cube was used to identify cells that contained chlorophyll a, i.e. all photosynthetic cells. After each image was taken cells were exposed to a second excitation light (green-yellow light at 572 nm) to cause fluorescence by cyanobacteria that contain phycobilin proteins, phycocyanin. Comparison of images with the two cubes allowed for identification of cyanobacteria (Glazer, 1989). To minimize variations due to patchy distribution of cells approximately 40 images were taken per collection filter, equally divided between the chlorophyll and phycocyanin cubes. Areas containing fluorescing cells were captured with matching chlorophyll and phycocyanin images allowing comparison between the excitation wavelengths. To increase the counting accuracy, cell counts were performed by two people, averaging the two independent counts. Fluorescing cells indicative of cyanobacteria were counted. Areas appearing as clusters were estimated as accurately as possible. For images containing ≥100 cells a grid was laid over the picture so that an accurate count could be made.

*Statistics* - Chlorophyll and phycocyanin counts were performed separately. After all images were counted, the average number of cells per image was found for each filter. The filter area covered by each image was 250 µm x 160 µm or 0.400 $mm^2$. The Swinnex® Filter Holder (25 mm diameter) had a functional filter diameter of 23 mm, or a filtering area of 415.476 $mm^2$, equivalent to 10386.9 images, the factor used to

convert from image counts to cells per filter. Average cells per filter was divided by the corresponding air volume filtered to give cells $m^{-3}$ air. Statistically significant differences ($p < 0.05$) were determined by two-tailed T-tests based off of sample mean.

**Results**

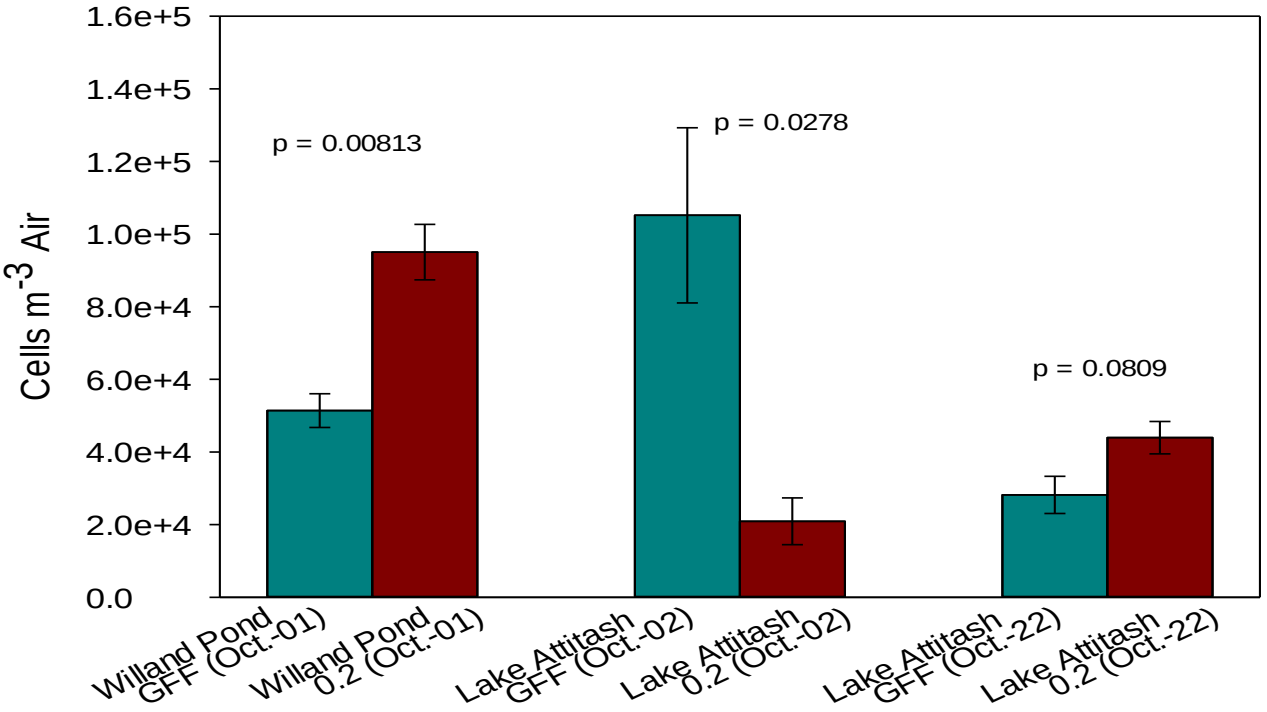


Figure 3: Comparison of GFF vs. 0.22 membrane filter cell counts under chlorophyll cube between various lake samples with std. error.

Table 2: Summarization of filter comparison experiments under chlorophyll cube

| **Lake Sample** | **Cell Count (Cells $m^{-3}$ Air)** | **Std. Error** | **T-Test P Values** |
|---|---|---|---|
| Willand Pond (Oct.-01) GFF | 51390.9 | 4641.5 | 0.00813 |
| Willand Pond (Oct.-01) 0.22 Millipore™ | 95056.0 | 7637.3 | |
| Lake Attitash (Oct.-02) GFF | 105204.5 | 24108.2 | 0.0278 |
| Lake Attitash (Oct.-02) 0.22 Millipore™ | 20924.0 | 6437.6 | |
| Lake Attitash (Oct.-22) GFF | 28176.5 | 5116.8 | 0.0809 |
| Lake Attitash (Oct.-22) 0.22 Millipore™ | 43942.1 | 4461.9 | |

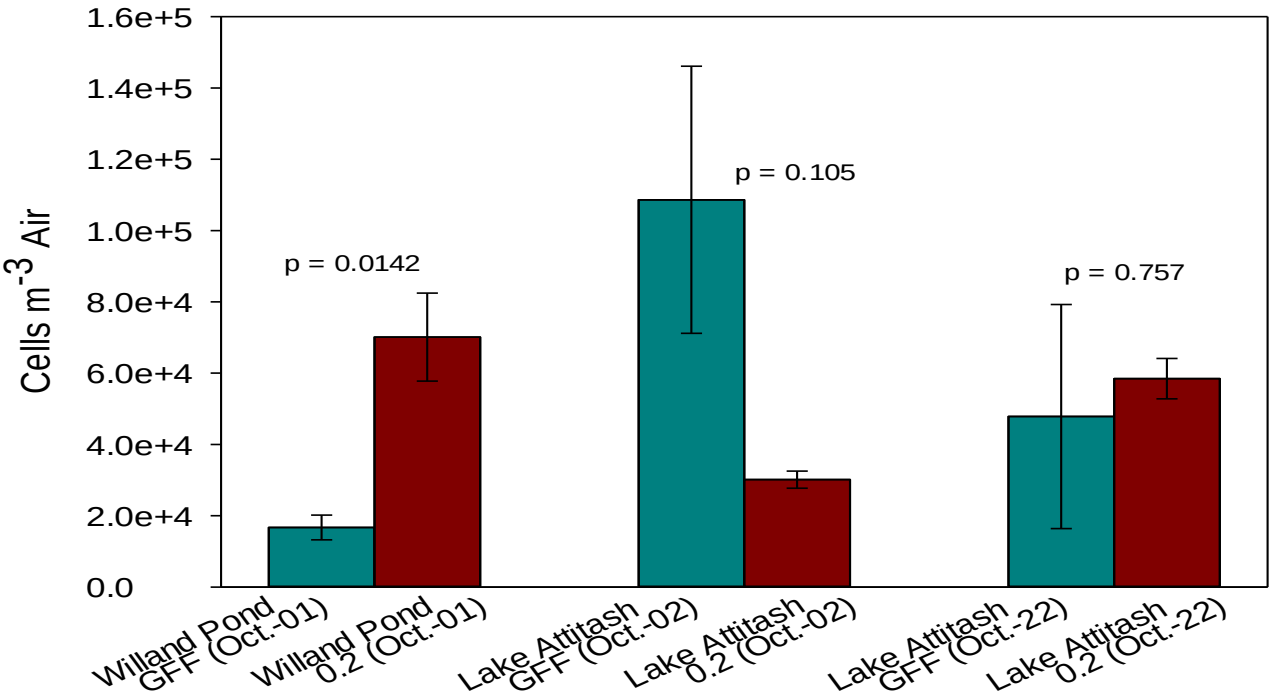


Figure 4: Comparison of GFF vs. 0.22 membrane filter cell counts under phycocyanin cube between various lake samples with std. error.

Table 3: Summarization of filter comparison experiments under phycocyanin cube

| **Lake Sample** | **Cell Count (Cells $m^{-3}$ Air)** | **Std. Error** | **T-Test P Values** |
|---|---|---|---|
| Willand Pond (Oct.-01) GFF | 16706.1 | 3485.7 | 0.0142 |
| Willand Pond (Oct.-01) 0.22 Millipore™ | 70136.9 | 12371.1 | |
| Lake Attitash (Oct.-02) GFF | 108656.6 | 37467.9 | 0.105 |
| Lake Attitash (Oct.-02) 0.22 Millipore™ | 30131.9 | 2394.9 | |
| Lake Attitash (Oct.-22) GFF | 47834.4 | 31435.3 | 0.757 |
| Lake Attitash (Oct.-22) 0.22 Millipore™ | 58439.2 | 5677.4 | |

*GFF vs. 0.22 Millipore*™ - Counts between the two filter types were made using three lake samples under both excitation wavelengths. The 0.22 Millipore™ filters returned higher averages for four counts split between both excitation wavelengths, two of which were significant, while the GFF returned higher averages for two counts split between both excitation wavelengths, one of

which was significant (Figures 3 &4). The 0.22 Millipore™ filter counts were significantly higher for the October 1st Willand Pond sample under both cubes (Tables 2 & 3). The GFF count was significantly higher for the October 2nd Lake Attitash sample under the chlorophyll cube (Table 2) but not under the phycocyanin cube (Table 3). October 22nd Lake Attitash counts were higher for 0.22 Millipore™ filters under both cubes but were not significantly different (Tables 2 & 3). Variation of cell counts between excitation wavelengths was limited as 0.22 Millipore™ filters consistently had higher counts for both the October 1st Willand Pond and October 22nd Lake Attitash samples, while GFF consistently had higher counts for the October 2nd Lake Attitash sample (Figures 3 & 4).

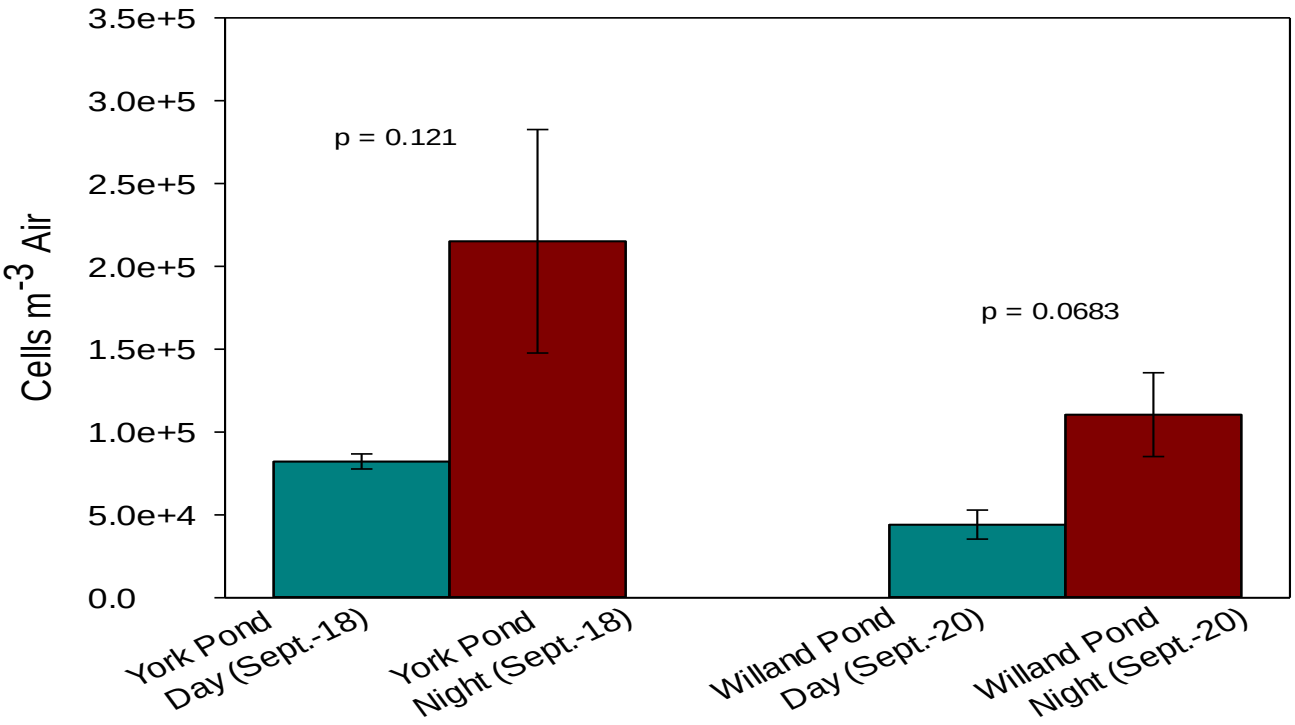


Figure 5: Comparison of day vs. night collection time cell counts under chlorophyll cube between York Pond (18 Sept 2014) and Willand Pond (20 Sept 2014) with std. error.

Table 4: Summarization of diel effects experiments under chlorophyll cube

| **Lake Sample** | **Cell Count (Cells m$^{-3}$ Air)** | **Std. Error** | **T-Test P Values** |
|---|---|---|---|
| York Pond (Sept.-18) Day | 82173.4 | 4560.7 | 0.121 |
| York Pond (Sept.-18) Night | 215185.1 | 67507.5 | |
| Willand Pond (Sept.-20) Day | 44053.4 | 8752.5 | 0.0683 |
| Willand Pond (Sept.-20) Night | 110473.7 | 25323.8 | |

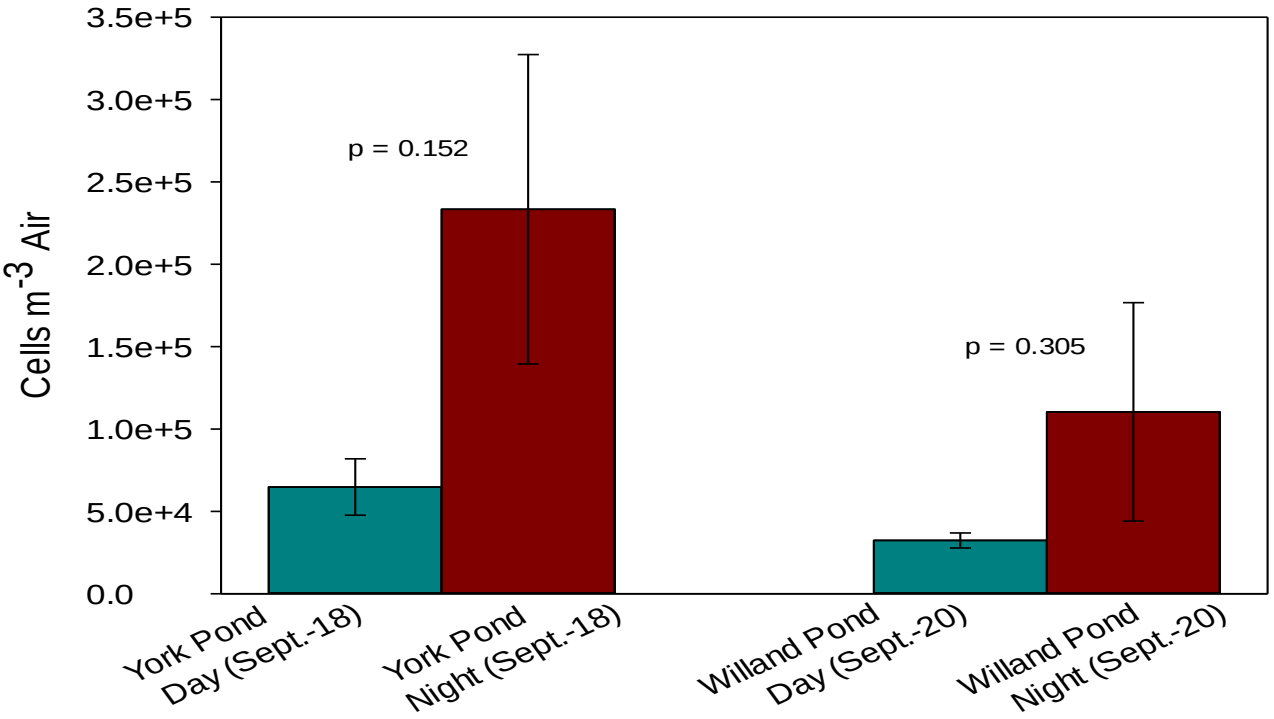


Figure 6: Comparison of day vs. night collection time cell counts under phycocyanin cube between York Pond (18 Sept 2014) and Willand Pond (20 Sept 2014) with std. error.

Table 5: Summarization of diel effects experiments under phycocyanin cube

| **Lake Sample** | **Cell Count (Cells $m^{-3}$ Air)** | **Std. Error** | **T-Test P Values** |
|---|---|---|---|
| York Pond (Sept.-18) Day | 64781.8 | 17089.2 | 0.152 |
| York Pond (Sept.-18) Night | 233414.4 | 93936.6 | |
| Willand Pond (Sept.-20) Day | 32312.8 | 4550.1 | 0.305 |
| Willand Pond (Sept.-20) Night | 110395.4 | 66331.7 | |

*Diel Effects* – Counts for diel effects were made between two lake samples using GFF under both excitation wavelengths. Night sampling times returned counts roughly 2.5 times greater under the chlorophyll excitation wavelength and 3.5 times greater for the phycocyanin excitation wavelengths (Figure 5 & 6). Cell counts did not differ significantly in any of the diel comparisons (Table 4 & 5).

**Discussion**

*Filter Types Analysis* – Comparisons of GFF versus 0.22 Millipore™ contained instances where both filter types had occurrences of

containing a statistically significant ($p<0.05$) larger amount of cells. In the filter comparisons two cases of 0.22 Millipore™ filters contained more cells ($p<0.05$) and one case of GFF had higher cell counts ($p<0.05$). These results suggest neither filter was consistently higher. These inconsistencies may be related to difficulties in cell counts. During epifluorescence enumeration there were difficulties pertaining particularly to the GFF. GFF consist of strands of glass fibers with varying layers of depth. As lake aerosols are vacuumed through the GFF the cyanobacteria are collected in the matrix of interwoven fibers at varying depths within the filter. This creates focusing difficulties when using a single focal plane, potentially missing cells outside the range of focus. The 0.22 Millipore™ filters are produced as a membrane film that is thinner than the GFF filters. Due to the flatter filter the problem of focal depth of 0.22 Millipore™ filters was much reduced. This led to better optical quality and ease of use during epifluorescence for 0.22 Millipore™ filters and presumably more accurate cell counts. Overall, the preferred filter type was the 0.22 MF-Millipore™ Membrane Filters. On the other hand, the thicker GFF filters have an advantage where higher volume samples are collected, especially for toxin analysis.

*Diel Effects Analysis* – Higher cell counts were consistently found during night sampling times in both samples that compared diel effects on cyanobacteria aerosolization. Due to high sample variability, neither of the cell counts differed significantly ($p<0.05$). The higher counts at night could have been due to a variety of environmental factors that influence bubble bursting actions in the lake. Some possible factors that could influence diel effects on cyanobacteria aerosolization are changes in temperature or relative humidity, varying levels of aquatic life activity such as diel vertical migration (Haney, 1988). Temperature and relative humidity impacts the amount of evaporation occurring off of lakes. For example, one possibility is that as lakes warm up evaporation increases stemming from the lakes carrying with it more cyanobacteria, but as lakes cool air could condense around the lake and concentrate the aerosolized cyanobacteria near the surface. Diel changes in the activity of aquatic organisms could also impact lake aerosolization. For example, as both fish and zooplankton become more active by feeding and moving they disrupt the surface more often; more disruption of the lake surface causes greater aerosolization and more cyanobacteria to be suspended in the air. All of these factors vary from day to night and could have impacts on rates of evaporation and aerosolization (Haney 1988).

*Future Studies* – This study provided a preliminary evaluation of differences in filter types when studying aerosolized cyanobacteria. Further studies are needed to determine if 0.22 Millipore™ filters are the best option. Testing should also investigate other filter types, such as 0.45 µm MF-Millipore™ membrane filters. These studies should be performed with a single water sample to minimize sample variability. Also, a greater number of replicate samples would provide smaller variance and higher sensitivity to resolve statistical differences Diel effects also need to be investigated further as preliminary data provided interesting trends of higher night time aerosolized cyanobacteria counts. When performing these studies in the future additional environmental factors that need to be monitored, specifically differences in the day and night sampling times concerning temperature, humidity, wind levels, and tide changes. Finally, toxicity of aerosolized cyanobacteria needs to be investigated using longer collection times to maximize the cellular mass and toxins contained on the filters.

## References

BRADLEY, W. G. AND D. C. MASH. 2009. Beyond Guam: The cyanobacteria/BMAA hypothesis of the cause of ALS and other neurodegenerative diseases. Amyotrophic Lateral Sclerosis **2**: 101-108.

CALLER, T.A., J. DOOLIN, J. F. HANEY, A.J. MURBY, K.G. WEST, H.E. FARRAR, A. BALL, B.R. HARRIS AND E. W. STOMMEL. 2009. A cluster of amyotrohic lateral sclerosis in New Hampshire: A possible role for toxic cyanobacteria blooms. Amyotrophic Lateral Sclerosis **2**: 101-108.

COX, P. A., S. BANACK, & S. MURCH. 2003. Biomagnification of cyanobacterial neurotoxins and neurodegenerative disease among the Chamorro people of Guam. Proceedings of the National Academy of Sciences of the United States of America **100**(23), 13380–13383.

DUEKER, M. E., G. D. O'MULLAN, A. R. JUHL, K. C. WEATHERS, AND M. URIARTE. 2012. Local Environmental Pollution Strongly Influences Culturable Bacterial Aerosols at an Urban Aquatic Superfund Site. Environmental Science & Technology **46**. 10926-10933.

GLAZER, A. N. 1989. Light Guides: Directional energy transfer in a photosynthetic antennae. The Journal of Biological Chemistry **264**(1): 1-4.

HANEY, J. F. 1988. Diel Patterns of Zooplankton Behavior. Bulletin of Marine Science **43**(3): 583-603

PABLO, J., S. BANACK, P. COX, T. JOHNSON, S. PAPAPETROPOULOS, W. BRADLEY, A. BUCK, AND D. MASH. 2009. Cyanobacterial neurotoxin BMAA in ALS and Alzheimer's disease. Acta Neurologica Scandinavica **120**(4): 216-225

PANTELIĆA, D., Z. SYIRČEVA, J. SIMEUNOVIĆA, M. VIDOVIĆB, AND I. TRAJKOVIĆ. 2013. Cyanotoxins: Characteristics, production and degradation routes in drinking water treatment with reference to the situation in Serbia. Chemosphere **91**(4): 421-441

STOMMEL, E.W., N.C. FIELD and T.A. CALLER. 2012. Aerosolization of cyanobacteria as a risk factor for amyotrophic lateral sclerosis. Medical Hypotheses **80**: 142-145.