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The Effects of Staining Toxic Cyanobacteria with SYTOXGreen after Lysis by Freeze-Thaw and Lysis by Ethanol

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Abstract

I attempted to find the most effective way to kill and stain dead cyanobacteria cells from the genera Aphanizomenon and Planktothrix with SYTOXGreen. This was done by culturing *A. flos-aquae* and *P. agardhii* and then killing the cells by freeze-thaw or ethanol. The cells were then stained at various concentrations of SYTOXGreen and then viewed under a fluorescent microscope. It was found that the most effective way of staining both *A. flos-aquae* and *P. agardhii* was lysis by ethanol. A SYTOXGreen stock concentration of 500X at 2 microliters with stain goal of 1 micromolar showed the highest fluorescence in *A. flos-aquae* when using ethanol. A SYTOXGreen stock concentration of 100X at 2.5 microliters with stain goal of 0.25 micromolar showed the highest fluorescence for *P. agardhii*. *A. flos-aquae* proved to be harder to kill and stain due to its heterocytes in the trichrome structure.

Introduction

Cyanobacteria, also known as blue-green algae, are ubiquitous in surface water when conditions are favorable for growth and often form of algal blooms. The overgrowth of cyanobacteria are called algal blooms due to their ability to double size twice per day by harvesting energy from the sun and nutrient in the water (Huisman and Hulot, 2005). Cyanobacteria also release cyanotoxins upon cell death. These toxins can stay present in water for weeks, but as long as multiple months (Carmichael, 1992). Although cyanobacteria have several detrimental effects on the environment, they are also one of the most important bacteria to date. Cyanobacteria have been very important in shaping the course of evolution and ecological change throughout Earth's history. The oxygen atmosphere that the Earth depend on today was generated by numerous cyanobacteria (Whitton and Malcom, 2012). Cyanobacteria

are also the main source of nitrogen for plants such as rice and beans. This paper focuses on two strains of toxic cyanobacteria known as *Aphanizomenon- A. flos-aquae* and *Planktothrix - P. agardhii*.

Planktothrix are a filamentous cyanobacteria that produce toxic heptapeptides microcystins and grows in shallow, eutrophic lakes (Kurmayer and Gumpenberger, 2006). Microcystin are produced via ATP by the microcystin synthetase complex to make clusters of *mcy* genes: *mcy A-C* and *mcy D-J* (de Figueiredo et al., 2004). In particular, Planktothrix agardhii consists of both microcystin genotypes and genotypes without the *mcy* gene as opposed to other strains (Kurmayer et al., 2004). This means that they can produce toxic and non-toxic algal blooms. *P. agardhii*, thrives in lakes that have a low nitrogen and low phosphorus environment (Rücker et al., 1995).

Aphanizomenon are a genus of cyanobacteria that are responsible for producing toxic and non-toxic algal blooms (Lyra, et al. 2001). *Aphanizomenon* are increasingly growing at high altitude levels and thrive particularly in artic conditions. A particular strain, *A. flos-aquae*, have the ability to grow in harsh conditions due to their trichome structure which produce akinetes (Komárek and Komárková, 2006). These akinetes allow the bacteria to sink in the water and survive around 22℃ (Yamamoto and Nakahara, 2009). In spring, *A. flos-aquae* recruit vegetative cells and heterocyst to compose their trichrome. The bacteria grow in size up to several hundred micrometers and in summer/early autumn they produce algal blooms. The blooms degrade in the end of autumn and the akinetes allow the bacteria to survive the winter before the cycle restarts in the spring (Yamamoto and Nakahara, 2009).

Aphanizomenon flos-aquae is often confused for *Anabaena*, another filamentous cyanobacterium genus. To tell the difference between the two, it is important to note that *A. flos-*

aquae fascicles are 2 cm long and fascicles with trichomes are 4.5-6.5 cm. The trichomes are straight but the akinetes are long cylindrical. Finally, their terminal cells are elongated, hyaline with plasm and rounded (Komárek and Komárková, 2006). However, because *Anabaena* and *A. flos-aquae* often grow in clusters together they are simply labeled as *Aphanizomenon* (Komárek and Komárková, 2006).

Methods

There were two different methods used in this experiment. The first was to kill the cyanobacteria with 200 proof ethanol and the second was to kill with a freeze/thaw technique. Both strains of cyanobacteria followed each technique. Fluorescence was rated on a scale from low, medium, and high intensity. A low intensity grade shows background fluorescence on the slide and the cell is not fully stained and therefore not fluorescing in some parts. A high intensity grade shows no background fluorescence and the cell is fully stained and fluorescing. A medium intensity grade would fall in between.

Lysis by Freeze-Thaw

Obtain a culture of cells. Then pipette 0.5mL of culture and 0.5mL of dH2O into a 5mL tube and vortex. Aliquot the diluted solution into seven 1.5mL Eppendorf tubes by 250uL each. Set up a 35-degree water bath and a dry ice station. Place six of the seven tubes on dry ice. The tube not going through the freeze-thaw cycle will be the control. Once the solution has frozen fully, move the three tubes to the water bath. Let the tubes thaw completely. This is a freeze/thaw cycle. Repeat four more times. Add SYTOXGreen to all six tubes for a total concentration of 0.1uM. Store the tubes out of the light and allow to incubate for 10 minutes. Add a sample from

tube-1 to a hemocytometer and count the live cells (red chlorophyll filter) and the dead cells (green filter) under the fluorescent microscope at 10x magnification. Fluorescence was than ranked on a scale of none, low, medium, and high. Repeat for all tubes.

Lysis by Ethanol

Obtain a culture of cells. Add 50mL of culture and 25mL of ethanol into a 50mL falcon tube. Vortex and aliquot 1.5mL into seven 1.5mL Eppendorf tubes. Set aside one tube for the control. Spin tubes at 14000 RPM for five minutes in the centrifuge. Make sure that the cells become pelleted and spill off supernatant. Add another 1mL of culture/ethanol solution. Spin and spill off supernatant again. Resuspend pelleted cells in 50mL of dH2O. Add SYTOXGreen to all four tubes for a total concentration of 0.1uM. Store the tubes out of the light and allow to incubate for 10 minutes. Add a sample from tube-1 to a hemocytometer and count the live cells (red chlorophyll filter) and the dead cells (green filter) under the fluorescent microscope at 10x magnification. Fluorescence was than ranked on a scale of none, low, medium, and high. Repeat for all tubes.

Results

Table 1: *P. agardhii* Freeze-Thaw

Tube	SYTOXGreen Stock (uM)	Total Volume (uL)	Diluted Sample (mL)	Stain Goal (uM)	Green Fluorescence
Control	-	-	1	-	none
1	10	5	1	0.05	Low
2	100	1	1	1	Medium
3	100	2.5	1	0.25	Medium
4	500	1	1	0.5	High
5	500	2	1	1	High
6	500	4	1	2	High

Caption: Tube-4 was the first to show high fluorescence, therefore its SYTOXGreen Stock Concentration and Stain Goal is what should be used to stain all *P. agardhii* when using the Freeze-Thaw technique. Tube-5 and Tube-6 also fluoresced at a high intensity; however, it requires more SYTOXGreen and the amount of stain used should be kept as low as possible.

Table 2: *P. agardhii* Ethanol

Tube	SYTOXGreen Stock (uM)	Total Volume (uL)	Diluted Sample (mL)	Stain Goal (uM)	Green Fluorescence
Control	-	-	1	-	None
1	10	5	1	0.05	Low
2	100	1	1	0.1	Medium
3	100	2.5	1	0.25	High
4	500	1	1	0.5	High
5	500	2	1	1	High
6	500	4	1	2	High

Caption: Tube-3 was the first to show high fluorescence, therefore its SYTOXGreen Stock Concentration and Stain Goal is what should be used to stain all *P. agardhii* when using the Ethanol technique. Tube-4, Tube-5 and Tube-6 also fluoresced at a high intensity; however, it requires more SYTOXGreen and the amount of stain used should be kept as low as possible.

Table 3: *A. flos-aquae* Freeze-Thaw

Tube	SYTOXGreen Stock (uM)	Total Volume (uL)	Diluted Sample (mL)	Stain Goal (uM)	Green Fluorescence
Control	-	-	1	-	None
1	10	5	1	0.05	None
2	100	1	1	0.1	None
3	100	2.5	1	0.25	Low
4	500	1	1	0.5	Low
5	500	2	1	1	Medium
6	500	4	1	2	High

Caption: Tube-6 was the only tube to show high fluorescence, therefore its SYTOXGreen Stock Concentration and Stain Goal is what should be used to stain all *A. flos-aquae* when using the Freeze-Thaw technique.

Table 4: *A. flos-aquae* Ethanol

Tube	SYTOXGreen Stock (uM)	Total Volume (uL)	Diluted Sample (mL)	Stain Goal (uM)	Green Fluorescence
Control	-	-	1	-	None
1	10	5	1	0.05	None
2	100	1	1	0.1	Low
3	100	2.5	1	0.25	Low
4	500	1	1	0.5	Medium
5	500	2	1	1	High
6	500	4	1	2	High

Caption: Tube-5 was the first to show high fluorescence, therefore its SYTOXGreen Stock Concentration and Stain Goal is what should be used to stain all *A. flos-aquae* when using the Ethanol technique. Tube-6 also fluoresced at a high intensity; however, it requires more SYTOXGreen and the amount of stain used should be kept as low as possible.

Discussion

It was found that for *P. agardhii* and *A. flos-aquae*, the most effective way of killing the cells was lysis by ethanol. The data shows that more cells were able to fluoresce at a lower concentration of SYTOXGreen meaning that more cells were dead and able to be dyed. In *Table 1*, Tube-4 was the first to show high fluorescence, therefore its SYTOXGreen stock concentration of 500X at 1 microliter and stain goal of 0.5 micromolar is what should be used to stain all *P. agardhii* when using the freeze-thaw technique. In *Table 2*, Tube-3 was the first to show high fluorescence, therefore its SYTOXGreen stock concentration of 100X at 2.5 microliters and stain goal of 0.25 micromolar is what should be used to stain all *P. agardhii* when using the ethanol technique.

A. flos-aquae was much harder to stain than *P. agardhii*, this is due its ability to survive in colder conditions and heterocytous makeup. In *Table 3*, Tube-6 was the only tube to show high fluorescence, therefore its SYTOXGreen stock concentration of 500X at 4 microliters and stain goal of 2 micromolar is what should be used to stain all *A. flos-aquae* when using the freeze-thaw technique. In *Table 4*, Tube-5 was the first to show high fluorescence, therefore its SYTOXGreen stock concentration of 500X at 2 microliters and stain goal of 1 micromolar is what should be used to stain all *A. flos-aquae* when using the ethanol technique.

Aphanizomenon and Planktothrix are can produce harmful algal blooms in various conditions and threaten water all over the world. By knowing the best ways to kill the cyanobacteria and at what concentration to stain, mores studies can be done so that biologist can properly treat for cyanobacteria. To do so, a confocal microscope can be used to quantify the fluorescent signal. It is important that the value of cyanobacteria and non-toxin algal blooms are understood but also know how to treat and eradicate the toxic strains that threaten ecosystems.

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