

## Why Bother?

The purpose of this Application Note is to make the reader aware of the utility as well as some of the limitations of Beagle's Amiscience fluorometers, especially for the purpose of monitoring phytoplankton populations in water. Each user will have to establish one's own procedures for use, and this Application Note is written to assist in that process.

Phytoplankton are aquatic, microscopic algae that utilize photosynthesis. In addition to light and carbon dioxide, they need other nutrients to grow, such as nitrogen and phosphorus. Changes in levels of these nutrients and overall environmental conditions can thus impact phytoplankton populations, and as such phytoplankton are among the indicators used to assess when a system may become eutrophic (nutrient-rich) or hypereutrophic. Most phytoplankton are harmless, and indeed serve as primary producers (carbon fixers) for a healthy ecosystem. Green algae and diatoms are common examples. Unfortunately, not all phytoplankton are beneficial, especially when they grow into very dense "blooms." Blooms can contribute to anoxia because when the phytoplankton die they are decomposed by microbes that consume oxygen in that process. Additionally, some types of phytoplankton can produce toxins, which can accumulate to dangerous concentrations during a bloom condition. Cyanobacteria, or "blue-green algae," are one type of toxic phytoplankton, and are commonly associated with freshwater Harmful Algal Blooms (HABs). The toxins produced by blue-green algae have implications for human health, and are well known to cause the death of wildlife and pets that might drink or swim in affected waters. HABs and the toxins they produce are therefore a concern of organizations that range from homeowners' associations through city-level reservoir and water treatment plant operators, up to state, national, and international organizations like the Environmental Protection Agency and World Health Organization. All concerned with the potential impacts of HABs must have a means for monitoring them.

What exactly is monitored to follow the progression of a bloom, and assess whether it has the potential to be toxic, can vary widely. Microscopy can be used to identify and enumerate all types of phytoplankton, or even to just generally get a

sense of the dominant types of phytoplankton present (*e.g.*, cyanobacteria vs. green algae). Identification and enumeration of phytoplankton take a great deal of both time and expertise. Monitoring may alternatively involve collection of the algae followed by laboratory-based extractions of specific molecules that are then analyzed. Such molecules can be "markers" for certain types of algae. The photosynthetic pigment chlorophyll is a very common marker used in this manner, and processing of samples to measure chlorophyll typically involves trained technicians and laboratory equipment. There are now easier alternatives that can be performed by most people, though they may sacrifice some of the accuracy or resolution that occurs with laboratory-based methods. These alternative methods may be better suited as preliminary screening tools to not only follow the relative progression of a bloom, but also to guide choices regarding more sophisticated (and more expensive) tests. One such alternative is to measure fluorescence using a handheld fluorometer (pictured left). These portable instruments can be taken directly into the field to perform on-the-spot measurements of general phytoplankton abundance.



## Measuring Fluorescence

Pigment	Chlorophyll	Phycocyanin
<b>Excitation <math>\lambda</math></b>	440 nm	620 nm
<b>Emission <math>\lambda</math></b>	670 nm	650 nm
<b>Found in...</b>	most photosynthetic phytoplankton	only blue-green algae

Fluorescence is a natural phenomenon that occurs when a molecule absorbs light of one wavelength, then emits light of a different wavelength. Fluorometers can illuminate a sample with a specific wavelength of light (measured in nm), and detect the amount of light that the sample emits at a

different wavelength, which will be indicative of the concentration of specific fluorescent molecules in the sample. Thus, fluorescent molecules have specific ranges of light wavelengths for absorption (sometimes called excitation) and emission. The photosynthetic pigments chlorophyll and phycocyanin are such molecules. Phycocyanin absorbs light of wavelength 620 nm, and it emits light of wavelength 650 nm. Chlorophyll absorbs light of wavelength 440 nm, and it emits light at 670 nm. All phytoplankton have chlorophyll to capture light energy for photosynthesis. In addition to chlorophyll, blue-green algae have phycocyanin, which is not present in other green algae.

Beagle's Amiscience fluorometer comes in models that can measure fluorescence from phycocyanin, chlorophyll or both. Fluorometers that measure one pigment's fluorescence are called single-channel fluorometers, while a dual-channel fluorometer can be used to measure both pigments. Dual-channel fluorometers can thus be used to monitor both general phytoplankton and blue-green algae from the same sample, at the same time.

The units on the readout of the Amiscience fluorometer can be either a relative fluorescent unit (RFU) or a calibrated unit such as parts per billion (ppb). An RFU may be thought of as the "raw" measurement taken by the instrument. To understand how RFUs relate to a specific quantity of a fluorescent pigment, every instrument must be calibrated so that the correlation between RFU and pigment concentration (usually in ppb) is known. That relationship is unique to each instrument. The channels can be calibrated by measuring a sample with a known concentration of the pigment of interest, or a known concentration of a "surrogate" pigment if the real pigment is not available for calibration. The instrument will measure RFU in the calibrator, and the user will tell the instrument to fix that RFU for the known concentration of the pigment in the calibrator sample. The fluorometer will then be able to measure concentrations of pigment that are either above or below that calibrated value, provided that the calibrator was prepared to be well within the instrument's dynamic range. The width of the acceptable calibrated range (a percent, an order of magnitude?) is decided by the user and their own comfort with a single-point calibration, as well as their experience and understanding of the instrument. Some of the experiments shown below will aid in gaining that understanding. The instrument will always measure RFU, but once calibrated it will calculate and yield data with the same units as the calibrator. In this manner, the user can receive data regarding the ppb of the pigment in that sample.

Measuring the fluorescence of the calibrators or samples is straight-forward. All that is required is to fill a 0.2 ml tube with sample, place the tube into the fluorometer, and tell the instrument to either calibrate or measure. The measurements can be immediately recorded by the user, or saved to the device's internal memory to be viewed at a later time or to be downloaded as a .csv file. Once an instrument is calibrated, it will hold calibration for days to months. "Drift" of calibration can be recognized by measuring a water blank every time the instrument is used, as described in the user's manual. As the experiments below demonstrate, one must be aware of the useful ranges of the instruments for measuring pigments, as well as some potential interferences when making such measurements. With this understanding, Beagle's Amiscience fluorometers may be a valuable component of a phytoplankton monitoring approach.

## Measuring Pigments in Unialgal Samples

A calibrated dual-channel fluorometer was used to measure both the phycocyanin and chlorophyll fluorescence from cultures of two different species: a cyanobacterium, *Microcystis aeruginosa*, and a green alga, *Scenedesmus dimorphus*. The fluorescence from each culture was measured *in vivo*, that is without extracting either of the pigments, to mimic how they might be measured if they had originated directly from a lake. Once diluted to where turbidity did not affect measurements (more on that in the section below), the fluorescence from each culture correlated well with its dilution, meaning that a twofold dilution, for example, would yield a twofold reduction in fluorescence ( $R^2=0.99$ ). It was found that for this fluorometer's calibration setting, *M. aeruginosa* had about 50 times more phycocyanin fluorescence than chlorophyll fluorescence, as would be expected for a blue-green alga that contains a high level of phycocyanin. *S. dimorphus* had about 8.5 times more chlorophyll fluorescence than *M. aeruginosa* when the cultures had the same turbidity, again as expected. Interestingly, the *S. dimorphus* culture had a small yet measureable fluorescence under the phycocyanin channel (10 times less than a corresponding amount of *M. aeruginosa*), even though biologically it does not contain phycocyanin. This was also observed when *S. dimorphus* was measured with other fluorometers as well. As expected these results show that different species have different amounts of fluorescence, but the results will further become useful to estimate the abundance of each species in a mixed culture.

Before proceeding to the mixed culture, experiments were performed to determine why *S. dimorphus* has fluorescence under the phycocyanin channel. Because *S. dimorphus* is not a cyanobacterium, it should not contain any phycocyanin and therefore should not fluoresce under the phycocyanin channel. To understand why this was occurring, phycocyanin and chlorophyll were extracted and enriched from mixed algal lake samples in order to obtain separate fluorescent signatures. UV absorbance was used to quantify the pigments in the extracts. Then, fluorescence of each extract was measured under both phycocyanin and chlorophyll channels. The phycocyanin extract fluoresced well under its own channel and had less than 0.5% fluorescence under the chlorophyll channel, meaning that 100 ppb of phycocyanin caused a reading of less than 0.5 ppb on the chlorophyll channel. The chlorophyll extract also fluoresced well under its own channel but had greater than 60% fluorescence under the phycocyanin channel – meaning that 100 ppb of chlorophyll registered at least 60 ppb of “phycocyanin.” This complicates data analysis because this positive phycocyanin fluorescence was not due to a low level of phycocyanin (or cyanobacteria), but rather the fluorescence was due to background chlorophyll fluorescence. Fortunately, from the experiments above, it was shown that *M. aeruginosa* cultures contained far more phycocyanin fluorescence (about 10 times more) than *S. dimorphus* cultures. As such, the background fluorescence due to chlorophyll would be so low that a cyanobacteria-rich sample would barely register the phycocyanin background that might be due to chlorophyll. Further, if a sample contains only green algae, the chlorophyll background fluorescence being picked up by the phycocyanin channel would likely be too low to raise concerns about high populations of blue-green algae. Meaningful assessments can be made, then, about relative populations and especially about changes in those populations with time. Below we demonstrate this with mixed cultures.

## Mixed Cultures

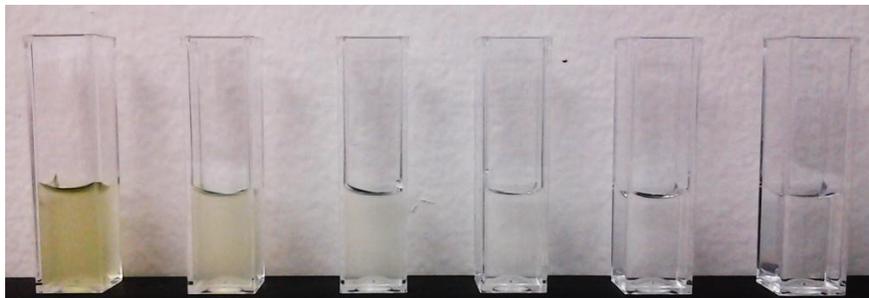
If one's goal is to just monitor phytoplankton then only chlorophyll readings need to be measured. If the relative amount of blue-green algae in that population is also of interest, then phycocyanin readings should also be collected. Given the interference from chlorophyll there are two ways to estimate the cyanobacterial population from a sample: measure the phycocyanin fluorescence and the turbidity of the sample or measure both the phycocyanin and chlorophyll fluorescence.

For practical purposes, if the phycocyanin reading is low then the amount of cyanobacteria is also low. If the phycocyanin reading is moderate or high, though, then this could mean any of three things: 1) the sample could be of a dense bloom

dominated by green algae, with little or no cyanobacteria, so the phycocyanin is actually just chlorophyll interference, 2) the sample is dominated by cyanobacteria and the measurement is due primarily to actual phycocyanin, or 3) a mixture of green algae and cyanobacteria is in the sample and the overwhelming majority of the phycocyanin fluorescence is still due to the cyanobacteria.

*The key to distinguishing these three possibilities is the density of the sample.* Since fluorescence is measured via light that both reaches and is emitted by the sample, light has to be able to pass through the sample relatively unhindered. If the sample is too turbid to see through then it will not give an appropriate reading. In the case of a lake sample, turbidity can be due to a dense algae bloom itself or could be from sediment or other particles suspended in the water. Regardless of the cause, turbid samples must be diluted so light can penetrate and escape the sample to produce an accurate reading.

For a moderate phycocyanin reading, if the sample is as dense a sample as the left two cuvettes in the figure below then it may be dominated by green algae (case #1 above) because if there were cyanobacteria in that sample then the phycocyanin reading would be very high. If it is as clear as the right two samples then it is dominated by cyanobacteria (case #2 above) because only highly fluorescent cyanobacteria can generate a moderate reading at very low concentrations. If the turbidity is somewhere in the middle then it is a mixture (case #3 above). This assumes that the turbidity is only from algae, not from suspended sediment. Also, the numbers that actually represent low, moderate, and high will vary by fluorometer calibration and by case, so historical data and experience become important for drawing conclusions. Thus, turbidity and fluorescence should be monitored regularly and frequently to establish a context for measuring cyanobacterial relative abundance. This further suggests that when dealing with environmental samples, it might be prudent to measure two or more dilutions of the sample, and obtain two or more readings of fluorescence. As will be demonstrated in the following experiments, the wide dynamic range of these instruments should allow one to find a range where a twofold dilution should yield a twofold decrease in fluorescence. When one is operating within that range, the measurements of fluorescence can be trusted.



Dilutions of a *Microcystis aeruginosa* sample. The left-most sample is too turbid to read accurately, but the fluorescence from all other samples can be accurately measured. The right-most sample, though it looks clear, still has a measureable amount of phycocyanin due to the cyanobacteria present.

A more accurate way to determine the amount of cyanobacteria and green algae is to measure both phycocyanin and chlorophyll fluorescence. Since the fluorescence from *M. aeruginosa* and *S. dimorphus* was measured for the individual species in the above experiments, it is therefore possible to back-calculate from total phycocyanin and total chlorophyll fluorescence measurements to the expected ratio of these organisms. This experiment was performed on three cultures with known ratios of *M. aeruginosa* (*Ma*) and *S. dimorphus* (*Sd*). After measuring fluorescence, the calculated ratios were less than 5% different from the expected ratios, thus validating this model (see table below).

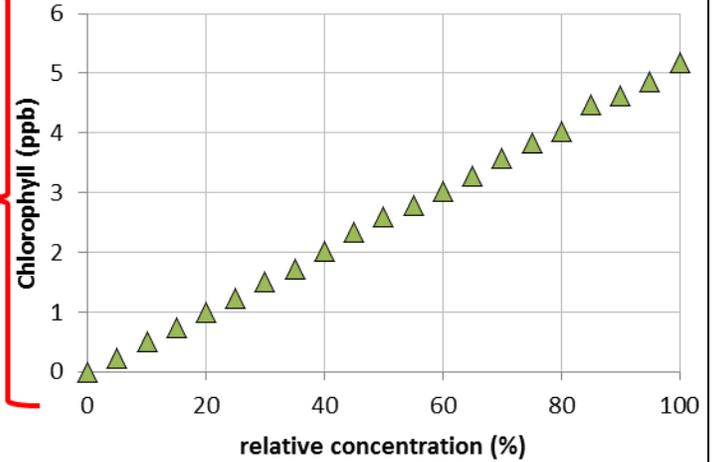
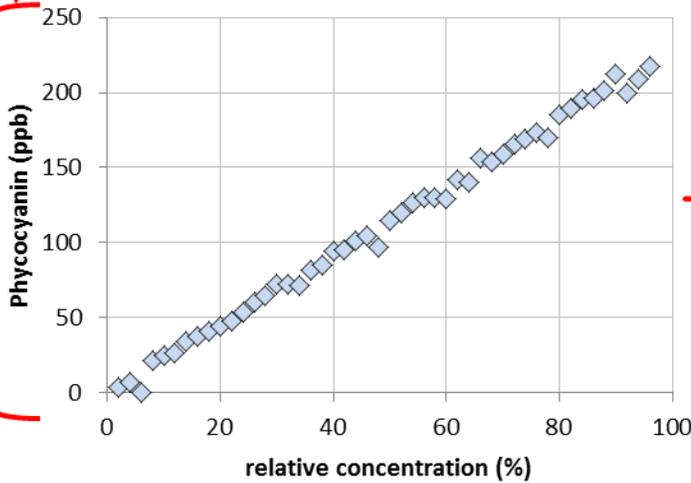
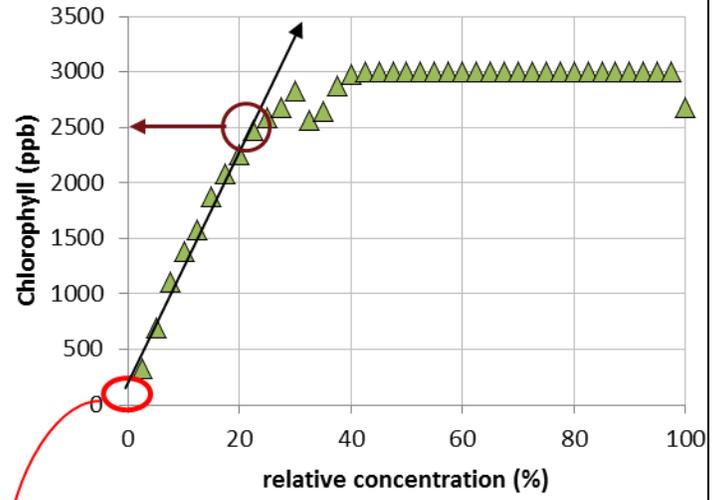
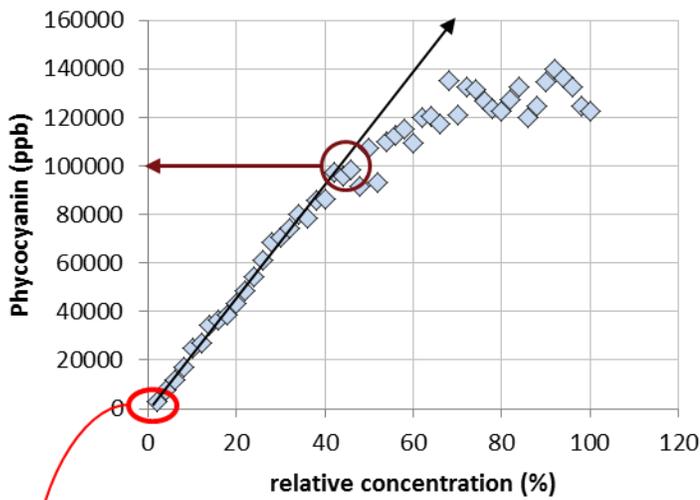
Actual:	Measured:		Calculated:
Ratio of <i>Ma</i> : <i>Sd</i>	PC (ppb)	Chl (ppb)	Ratio of <i>Ma</i> : <i>Sd</i>
Pure <i>Ma</i>	574.10	13.66	-----
2:1	400.00	47.13	2.08 : 1
1:1	318.90	63.73	1.03 : 1
1:2	223.06	79.73	1 : 2.06
Pure <i>Sd</i>	57.84	104.03	-----

This two-species mixture is an idealized situation because in environmental samples there will be a mixture of many species. Such a complex environment will make it impossible to determine an exact ratio of cyanobacteria to green algae, but changes in the ratio of phycocyanin to chlorophyll fluorescence will still indicate changes in the species composition. So as stated above: examining historical data, in this case of phycocyanin and chlorophyll fluorescence, is necessary to guide further action.

### **Technical Specifications**

The precision and range of the Amiscience fluorometer were determined. (The accuracy of the fluorometer will depend on the accuracy of the calibration standard.) For this experiment, extracted pigments were prepared and UV absorbance was used to quantify the concentration of the pigments. Fluorescence of the extracts was then measured using the fluorometer. Both phycocyanin and chlorophyll channels were precise, with a measured relative standard deviation from 25 readings of 5 samples of about 2%. While pigments could be measured at very low concentrations, confidence in the lower limit of quantitation was obtained by adding ten times the error of the y-intercept to the y-intercept of a linear regression derived from a dilution series (the X-axis represents that dilution series, as relative concentrations). The upper limit of quantitation was assessed as the point at which the R<sup>2</sup> values for the linear regression decreased with the addition of further points on the graph. With this approach, the useful range of the phycocyanin channel was found to be from 10 ppb of phycocyanin to about 100,000 ppb, and the linearity was excellent in the low range (see graphs on left). For the chlorophyll channel, the range was from 0.25 ppb of chlorophyll to about 2500 ppb (see graphs on right). The upper limit of the range was determined by assessing the concentration where a dilution series deviated from linearity.

Note that these ranges were determined with extracted phycocyanin or chlorophyll, since extracts have no turbidity to alter readings. When actual cells, which contribute to turbidity, were measured the upper limit of linearity decreased significantly, to about 1000 ppb for phycocyanin and 200 ppb for chlorophyll, because light could not penetrate the sample as well. So again, the user is strongly encouraged to understand the turbidity of samples, and to measure diluted samples as well as the original samples that were collected. With experience, one will develop a strong sense of the turbidity that “matters” for your samples, and may even be able to develop a turbidity measurement range within which the fluorometric measurements can be taken with the greatest confidence.



Low and high dilutions of extracted phycocyanin (blue diamonds) or extracted chlorophyll (green triangles), also showing the approximate concentration of the upper limit deviating from linearity. The bottom graphs show the excellent linearity at very low concentrations of the pigments.

## Conclusions

The technical specifications of both the phycocyanin and chlorophyll channels for Beagle's Amiscience fluorometer are more than sufficient for environmental monitoring and also allow the fluorometer to ably function for laboratory measurements. It was noted that turbidity greatly affects both channels, so the user should be aware of this effect, but it is generally accepted that all fluorometers are prone to errors caused by turbidity. Readings of samples under both phycocyanin and chlorophyll channels resulted in measureable background fluorescence for the phycocyanin channel. This points to yet another reason that turbidity should also be assessed. Therefore, we advise that fluorometer measurements be used for a quick and low resolution analysis of water quality and in conjunction with a larger data set help guide further management of the water body.