

# Application of Fluorescence Spectroscopy to Environmental Studies

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## **Abstract**

CTC is a tetrazolium salt that is reduced to a fluorescent, insoluble formazan (CTF) in the presence of aerobically respiring bacteria. CTF is excited by long-wave UV radiation between 400 and 500nm and fluoresces in the red region of the visible spectrum between 600 and 700nm. In a water sample, CTC is reduced intracellularly by the processes of aerobic respiration. The amount of CTF produced is therefore indicative of the relative number of aerobically respiring bacteria. This type of measurement is typically done using an instrument called a fluorescence microscope. With this method, the fluorescent particles within the bacteria are visually observed and counted. This is a time-consuming process and it is not possible to take the instrumentation out into the field to take measurements. Spectrometric determination of the red fluorescence can be done much faster, allowing more samples to be analyzed in a shorter amount of time. At the same time, the instrumentation can be taken into the field and used in conjunction with fiber optics for true, real-time, *in situ* measurements.

The goal of this project was to develop and optimize a method for applying fluorescence spectroscopy to the measurement of the aerobically respiring bacteria in a water sample. CTF samples were produced by reduction with sodium dithionite. Although CTF is soluble in organic solvents, it was determined that fluorescence is only emitted from the solid state. Samples were, therefore, filtered onto black polycarbonate membrane filters for fluorescence analysis.

The results of this project show a definite linear relationship between fluorescence intensity and CTC concentration. However, the specific line equation varies slightly between different standard sets. This indicates that a new standard curve will have to be determined each time unknown bacteria samples are measured in order to deal with sources of random error inherent to the measurement process.

### **Introduction and Background**

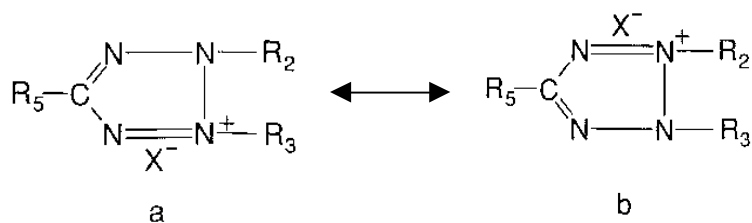
The release of chemicals into the environment is a concern because of their possible ill effects on human health. When these often-toxic compounds are degraded in the environment, the degradation products can be of little or no harm to humans or to the environment. Studies have been conducted in a series of constructed wetlands near Tennessee Technological University (TTU) to determine the fate of two pesticides found in the run-off from a nearby nursery. It has been determined that the two pesticides, metolachlor and simazine, are being naturally degraded to non-harmful products in these wetlands. Dr. Henry Spratt, a biology professor at UTC, is working with three professors at TTU, Dr. K. Stearman, Dr. S. George, and Dr. L. Weathers, to determine the mechanism by which this degradation occurs. Once this mechanism is understood, wetlands can be constructed in other locations to facilitate the natural removal of these pesticides from the environment.

(8)

The wetlands project involves dividing the wetlands into sections based on depth and plant populations, determining in which sections each pesticide is degraded

using  $^{14}\text{C}$  labeled metolachlor and simazine, and constructing mesocosms to model the physical conditions of each section. Dr. Spratt's hypotheses include (1) metolachlor is degraded anaerobically (in an oxygen depleted environment), and (2) simazine is degraded aerobically (in an oxygen rich environment). The relative number of aerobically respiring bacteria in a given environment is indicative of the amount of oxygen present in that environment. Therefore, these two hypotheses can be tested by studying bacterial respiration in each area of the wetlands. (8) The ultimate goal of this project is to develop and optimize a method for applying fluorescence spectroscopy to the measurement of aerobically respiring bacteria in a water sample and to implement this method in the field as part of Dr. Spratt's overall project.

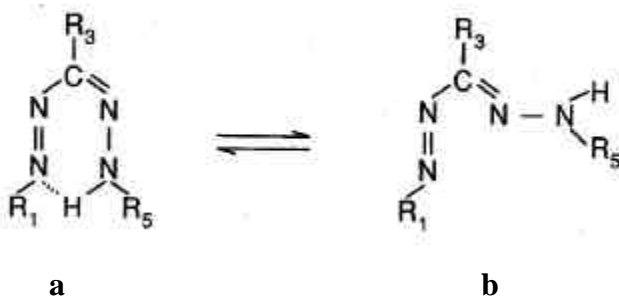
Tetrazolium salts are a group of organic compounds frequently used in biological assays. All are five-membered rings consisting of four nitrogen atoms and one carbon atom with two double bonds. All biologically useful tetrazolium salts belong to the 2-H-group and have the same basic structure. As shown in Figure 1, a 2-H-tetrazolium salt exists as the resonance hybrid of two structural isomers. Although the substituents on  $\text{N}_2$  and  $\text{N}_3$  are usually heterocyclic or aromatic, thousands of these compounds can be synthesized by altering substituents on  $\text{N}_2$ ,  $\text{N}_3$ , and  $\text{C}_5$ . (6)



**Figure 1:** Generic structure of a 2-H Tetrazolium Salt. (6)

Almost all oxidized tetrazolium salts are positively charged and yellow, but mild reduction opens the ring to produce a neutral, highly colored compound called a formazan. Formazans can exist as one of two possible structures. The six-membered ring structure shown in Figure (2a) gives rise to a red color, while the open structure shown in Figure (2b) gives rise to a yellow color. Formazans generally have low melting points, are sensitive to light, and are soluble in organic solvents and lipids.

(6)

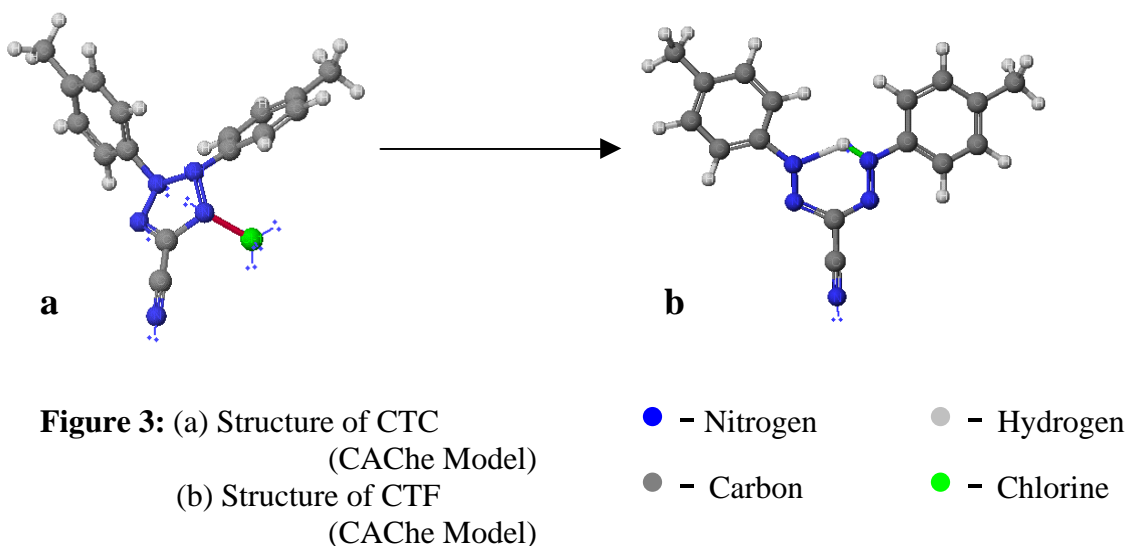


**Figure 2:** Two possible structures of a tetrazolium formazan. (6)

Several spectrometric methods are available for using tetrazolium salts for the enumeration of aerobic bacteria in a water sample. Blenkinsopp and Lock describe the use of 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) as a respiration indicator. INT is a water-soluble tetrazolium salt that is reduced to a water-insoluble formazan deposit within the cell. These deposits can be extracted

using organic solvents and measured spectrophotometrically. Such measurements allow quantification of bacterial respiration in marine and freshwater samples as well as in soil samples. INT formazan is studied quantitatively by measuring absorbance at 480nm. (2)

Roehm, et al. describe the use of another tetrazolium salt, sodium 3'-[1-[phenylamino)-carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate (XTT) as a respiration indicator. Like INT, XTT is reduced to a colored formazan by microbial respiration activity. Unlike INT, however, the XTT-formazan is water soluble, eliminating the need to extract formazan particles with organic solvents. XTT formazan shows maximum absorbance between 440 and 490nm. (4)



5-cyano-2,3-ditolyl tetrazolium chloride (CTC-Figure 3a) is a tetrazolium salt that is reduced to a fluorescent formazan (CTF-Figure 3b) by the byproducts of the metabolic processes involved in the electron transport system of aerobically respiring

bacteria (3, 10). Since oxidized CTC is water soluble, it can easily be applied to a bacterial water sample. Bacteria in the water take molecules of CTC into the electron transport chain inside the cell. CTC molecules are then reduced by electrons in the system as they pass down the chain (1, 9). The five-membered carbon-nitrogen ring is broken and a hydrogen bond forms between N-2 and the hydrogen of N-3, creating a rigid, planar pseudo-six-membered ring capable of producing fluorescence (from CAChe models). These fluorescent, insoluble formazan particles can be detected intracellularly through illumination by long-wave UV radiation between 400 and 500nm, producing fluorescence in the red region of the visible spectrum between 600 and 700nm. This fluorescence range is especially beneficial because many biotic and abiotic components of natural water samples fluoresce in the blue-green region of the visible spectrum. The bright red fluorescence emitted by CTC, thus allows it to be easily distinguished from background fluorescence in the sample (3, 11).

Fluorescence measurement is preferable to the absorbance methods described for INT and XTT. UV light absorbance in a water sample is not necessarily all due to reduced XTT or INT. For example, one method for determining relative numbers of bacteria in water samples is through optical density testing. A SPEC20 instrument is used to measure UV light transmitted through a water sample. Individual cells scatter the light so that less light is transmitted through a sample with a high density of cells. This scattering effect is present in any water sample containing cells or other debris and can artificially amplify absorbance measurements. Since there is no natural component of a water sample that emits light in the red region of the spectrum, all

radiation detected between 500 and 600nm can be attributed to CTC fluorescence.

(3,9) Fluorescence analysis, therefore, allows greater selectivity and accuracy than absorbance measurement.

This paper reports microbial respiration studies currently being performed using CTC with fluorescence microscopy. A water sample is treated with CTC at concentrations ranging from two to five millimolar and incubated for one to four hours at temperatures comparable to the source of the sample. The sample is then filtered through a black polycarbonate 0.2- $\mu\text{m}$  pore-size membrane filter (3). Using immersion oil, the filter is mounted on a microscope slide and studied under a high-power fluorescence microscope. Since the formazan is reduced intracellularly by aerobically respiring bacteria, the number of aerobic bacteria can be determined by counting the number of fluorescent deposits on the slide. Several fields are randomly selected on each slide and the number of bacteria counted are averaged. These cell counts are compared to counts obtained from other water samples to determine the relative number of aerobic bacteria in each sample (9). This number is indicative of the relative amount of oxygen in each sample. It is important to note that this technique is used only to determine the relative number of aerobically respiring bacteria in a sample. The analysis does not provide information about the specific species of bacteria present.

Fluorescence microscopy is a widely used, relatively accurate method for performing this type of analysis. However, there are several sources of error inherent to the process of microscopic analysis that can be reduced by implementing

fluorescence spectroscopy. Fluorescence microscopes are very expensive and are not portable. Therefore, samples collected in the field, must be transported back to the lab for analysis. This transportation increases the risk of sample contamination, and potentially changes the conditions of O<sub>2</sub> solubility in the samples. Small, battery powered fluorescence spectrometers and laptop computers are now widely available. The application of fluorescence spectroscopy to CTC assays could allow for onsite testing of water samples, greatly reducing the risk of sample contamination or other changes in route to the lab. Microscopic assays are also very time-consuming. It takes several hours to make cell counts for each water sample. Fluorescence measurements require only a few minutes, greatly reducing sample processing time. This can provide a great reduction in the systematic error between the first and last sample measured. Fluorescence spectroscopy also reduces the chances for human error, since all measurements are taken and analyzed by a computer. Counting fluorescent cells under a microscope is very subjective, but little to no estimation is involved in analysis of a fluorescence signal.

### **Experimental Section**

#### **Liquid vs. Solid**

The first determination involved in this experiment was whether to test the samples in solution or as a solid. Since the bacteria samples are aqueous, measuring CTF fluorescence as a solid requires extensive handling of the sample in filtering and drying the precipitate, greatly increasing the possibility of error due to sample

contamination. Measuring fluorescence of a solid also involves several inherent complications. In solution, the sample is homogeneous and spectroscopic measurements can be made with relative consistency. A filtered sample will never be completely homogeneous, so fluorescence measurements are not consistent throughout the sample. For these reasons, it is preferable to measure fluorescence of the sample as a liquid.

**Solvent Selection.** CTF is soluble in solutions that are greater than 50% organic. Several organic solvents were tested including acetone, ethanol, 2-propanol, and toluene. Absorbance measurements were taken of solutions made with each of these solvents using a Varian UV-VIS Scanning Spectrophotometer. All solutions showed maximum absorbance in the expected range of 400-500nm. Formazan particles dissolve faster and remain in solution better in the ethanol solvent than in the others, so it was selected as the best solvent.

**Liquid Tests.** A solution of CTF was made by dissolving CTC (purchased from Polysciences, Inc.) in deionized water and treating with ascorbic acid. After a few hours, ethanol was added to the solution to dissolve all of the CTF particles that had formed. Samples of varying CTF concentration were then made by diluting this solution with deionized water, keeping the solution more than 50% ethanol. Absorbance of these samples was measured using the UV-VIS Spectrometer. Maximum absorbance, as expected, was found to be between 400 and 500nm for all the samples. No linear relationship was found between absorbance and concentration, however, and no fluorescence was measured for the liquid samples.

Fluorescence measurements were also made for each of these solutions using an S2000-FI fiber optic spectrometer (Ocean Optics, Inc.) connected to a *DELL Inspiron 7500* laptop computer with *OOIBase32 FL* data analysis software. Not only did data fail to show a linear relationship between fluorescence intensity and CTF concentration, but the expected trend of increased fluorescence with increased concentration was also not observed. It was finally determined that CTF does not fluoresce in solution. This was confirmed in an article by Erhard Severin, Joachim Stellmach, and Hans-Martin Nachtigal (7).

### **Light Source Selection**

Although many light sources are available for spectroscopic studies, it is important to choose a light source that provides high intensity radiation in the range of maximum absorbance for the sample. Some literature sources state that CTF is excited by radiation at 420nm (3,5) and some suggest 450nm (7,9). However, UV-VIS experiments indicate that maximum absorbance occurs over a range of wavelengths between 400 and 500nm. Three different light source combinations were tested for this project.

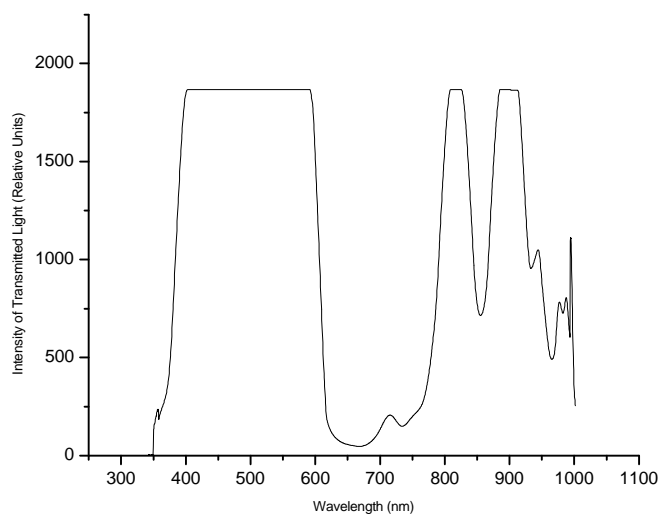
**Xenon Lamp with band-pass filters.** Xenon lamps provide high intensity radiation over a wide range of wavelengths (150nm-850nm). Therefore, this lamp provides radiation in the desired range of excitation, but it also gives radiation in the range of measured fluorescence. This artificially amplifies the fluorescence signal, and decreases the accuracy of measurements taken. For CTF samples with low

concentration, the actual fluorescence signal was indistinguishable from the noise signal created by the light source. A 450nm band-pass filter was used between the light source and the sample to insure that only wavelengths in the excitation range actually illuminated the sample. A 620nm band-pass filter was used between the sample and the detector to reduce signal noise caused by source light reflecting off the filter. This light source produces a high intensity signal, but the double filter combination greatly reduces the intensity of the source light and of the fluorescence signal collected. High signal intensity is necessary to detect small changes in fluorescence produced.

**Dye Laser.** Lasers produce very intense, narrow beams of radiation. For these experiments, a nitrogen dye laser was used with Coumarin 120 dye. Coumarin dye is a yellow/green powder, which dissolves in ethanol to form a slightly blue solution. When exposed to UV light inside the laser, the solution fluoresces in a very bright blue. A very intense beam of light around 450nm is emitted from the laser and focused through a lense into a fiber optic, which focuses the light on the sample. Another fiber optic collects the fluorescence signal at a 90° angle to the source light and connects to the detector. A 620nm band-pass filter was again required to reduce noise in the signal. Using this set-up produced good signals for individual filters, but no linear relationship between concentration and fluorescence intensity could be obtained. For example, filters with very low amounts of CTF showed fluorescence readings lower than blank filters with no CTF. This is partly due to the reduced signal intensity as a consequence of using a collection filter. It was also noted that

the dye laser produces a very narrow band of wavelengths. Although literature sources suggest the use of very narrow wavelength bands around 420 or 450nm, UV-VIS experiments show absorption over a much wider range of wavelengths from 400 to 500nm. If all of these wavelengths can excite CTF to fluoresce, the measured fluorescence intensity will be greater if all of them are available to the sample.

**Xenon lamp with cyan subtractive filter.** As previously discussed, the xenon lamp provides the wide range of excitation wavelengths needed, but also emits unwanted radiation in and near the range of fluorescence measurement. In order to maximize the fluorescence signal, a filter is needed that will cut out unnecessary light without significantly decreasing intensity of light in the desired wavelength range. With the cyan subtractive filter in place, the light source is allowed to saturate the detector between 400 and 500nm, but virtually all light is filtered out between 600 and 700nm(see Figure 4). This combination of light source and filter was chosen as the best for these experiments.



**Figure 4:** Spectrum of source light transmitted through a cyan subtractive filter.

## **Reducing Agent Selection for Standard Preparation**

**Ascorbic Acid.** Since bacterial reduction of CTC is unpredictable, CTF must be produced using a chemical reducing agent for use as a standard. A CTC solution of 10mM concentration was treated with an equal volume of 10mM ascorbic acid. No immediate change was visibly apparent. Over time, however, an orange precipitate began to form. Maximum precipitate formation seems to occur after about four hours at room temperature. Initially, ascorbic acid was used exclusively for CTF synthesis. However, the long incubation time required and unpredictable yield suggested the need for a stronger reducing agent.

**Sodium Dithionite.** Sodium dithionite is a much stronger reducing agent. 10mM solutions of CTC were treated with an equal volume of 10mM sodium dithionite. Bright red formazan particles formed immediately. With the use of sodium dithionite, it is possible to produce very large amounts of CTF almost instantaneously. For this reason, it was decided to use sodium dithionite as the reducing agent to produce all CTF used for the latter portions of this project.

## **Other Materials**

0.2 $\mu$ m pore-size, black, polycarbonate membrane filters were used to filter CTF samples. These were purchased from Fisher Science Inc. A 0.2 $\mu$ m pore-size is necessary when dealing with bacterial samples. Any larger pore-size presents the risk of losing bacterial cells during filtering. The filters need to be black in order to facilitate viewing the red fluorescence of the formazan. Also, white polycarbonate

filters will auto-fluoresce, interfering with the fluorescence measurement. One side of the filter is much darker and shinier than the other. The shiny surface of the filter creates noise in the sample due to reflection of source light. The use of the cyan subtractive filter greatly reduces this problem by cutting out source light in the range of detection. It was also determined that the lighter side of the filter is not as shiny as the darker side, and therefore reflects much less source light.

CTF adheres to glass, causing relatively large amounts of the sample to be lost during the filtering process. In order to reduce this loss, Teflon beakers were used for sample preparation. Because Teflon filtering columns are not available, small amounts of the formazan are still lost during filtering.

### **Sample Preparation**

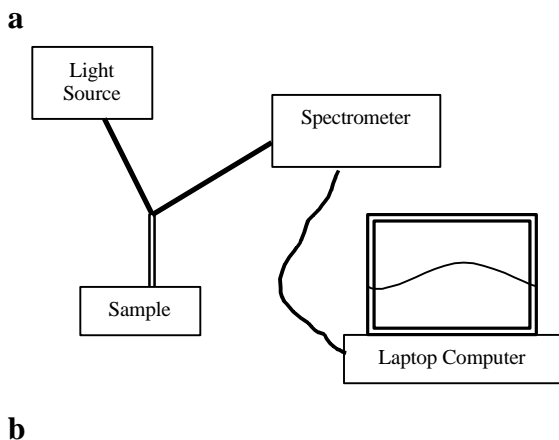
A 10.0mM solution of CTC was made using deionized water. 3mL aliquots of this solution were treated with differing amounts of sodium dithionite. Based on a 1:1 reaction ratio, the amount of CTF produced was calculated. Each sample was filtered and allowed to air-dry before fluorescence measurements were taken for each filter. This procedure was repeated numerous times, but no linear relationship was found between fluorescence and the calculated amount of formazan. The physical appearance of filters with the same calculated amount of formazan was also not consistent. These calculations depend on the assumption of 100% completion of the reaction. It was finally determined that the reaction does not consistently go to

completion. As a result, a more reliable way of producing known amounts of CTF was needed.

A 10.0mM solution of CTC was treated with excess sodium dithionite causing the solution to become bright red and opaque with formazan particles. The solution was filtered to extract the particles and the filtrate was re-treated. It should be noted that if ethanol solutions are not sufficiently diluted with water before filtering, the ethanol seems to partially dissolve the surface of the polycarbonate filter, allowing formazan particles to escape into the filtrate and causing very uneven coverage of the filter surface. This process was repeated until no further formazan could be obtained. The collected formazan particles were allowed to dry. Dry, solid CTF was dissolved in ethanol to produce a stock solution of approximately 2mM CTF. The exact concentration of the stock solution was calculated using the known mass of CTF dissolved. Various  $\mu\text{L}$  volumes were pipetted into Teflon beakers. Since CTF is only soluble in solutions greater than 50% ethanol, approximately 3mL of deionized water was added to cause the particles to precipitate out of solution. These samples were filtered and allowed to dry before fluorescence measurements were made. Using the exact concentration of the stock solution, the number of nmol of formazan on each filter was stoichiometrically calculated.

## Experimental Set Up

After choosing an appropriate light source and sample form, the final experimental set-up for collecting fluorescence measurements was designed to maximize both the intensity of source light focused on the sample and the amount of fluorescence detected. A diagram and photograph of this set-up is shown in Figure 5. Source light from a PX-2 pulsed xenon light source (Ocean Optics, Inc.) travels through a fiber optic to the cyan subtractive filter. Filtered light is focused on the solid sample through a fiber optic probe. This probe consists of six fiber optics arranged in a circle around one other fiber optic. The source light travels through the six outer fiber optics and the fluorescence signal is collected by the single fiber optic in the center. The probe is held perpendicular to the sample by a black, opaque holder that prevents any outside light from entering the system. The fluorescence signal travels through the fiber optic to an S2000-FI fiber optic spectrometer (Ocean Optics, Inc.), which digitizes the signal and transmits it to a *Dell Inspiron 7500* laptop computer. *OOIBase32Fl* software plots fluorescence intensity as a function of wavelength.



**Figure 5a:** Diagram of Experimental Set-up.



**Figure 5b:** Photograph of Experimental Set-up.  
Taken by Dr. Wendy Cory.

### **Fluorescence Measurement and Data Analysis**

Three to six fluorescence spectra were obtained for each filter. Since the CTF layer on the filter is not uniform, the probe was moved to a different area for each measurement to account for intra-filter error. Data was analyzed using *Microcal Origin* software. Since data cannot be converted directly from *OOIBase32 FL* to *Origin*. Data files were opened using *Microsoft Excel* and data points between 600 and 700nm were copied and pasted into data tables in *Origin*. Each spectrum was plotted between 600 and 700nm (x-axis = wavelength; y-axis = fluorescence intensity (relative units)) and integrated. The five measurements for each filter were averaged and the average intensity was graphed versus known amount of formazan on the filter.

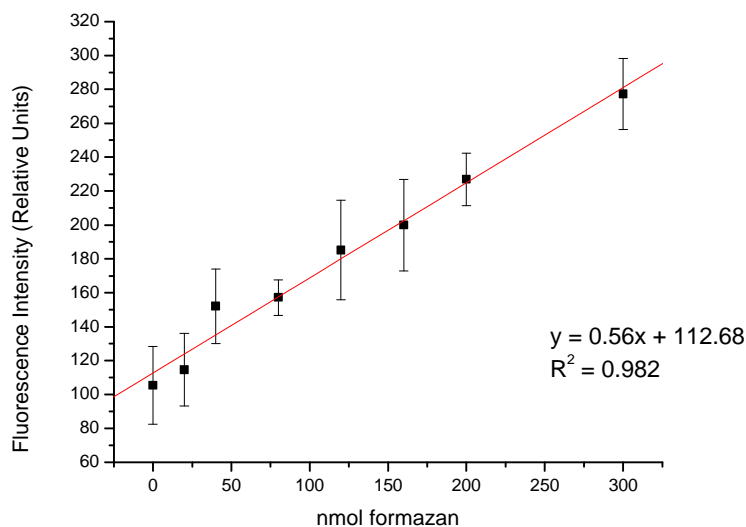
### **Results and Discussion**

Figures 6-8 show three examples of linear calibration curves obtained for fluorescence as a function of nmol of formazan calculated for each filter. Data for each calibration curve were collected in the same manner. A stock solution of approximately 2.0mM CTF was prepared. Various  $\mu\text{L}$  volumes of this stock solution

were pipetted into Teflon beakers and approximately 3mL of deionized water was added to precipitate the formazan particles. Each sample was filtered onto a black 0.2- $\mu\text{m}$  pore-size polycarbonate membrane filter. Each beaker was rinsed with both ethanol and water. The filters were allowed to dry and fluorescence was measured as described in the Data Analysis section.

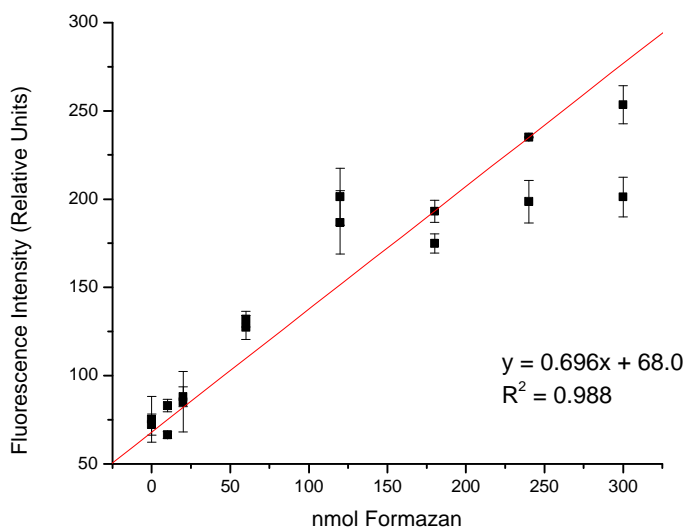
For Curve A, a 2.7mM stock solution of CTF in ethanol was used. One filter was prepared for each of eight volumes of this stock solution. Blank measurements were made using a blank filter prepared by filtering a solution of ethanol and deionized water. Six fluorescence spectra were collected for each filter and the data were averaged using *Microcal Origin*. Error bars indicate the intra-filter error (the standard deviation of measurements from the same filter).

**Figure 6:** Calibration Curve A



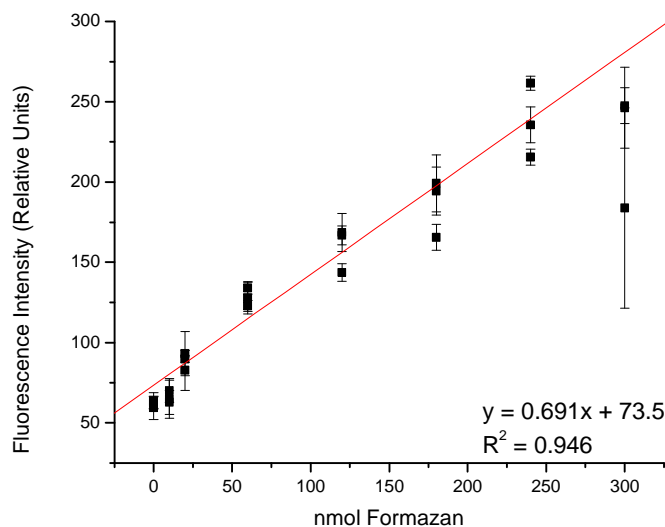
For Curve B, a 2.0mM stock solution of CTF in ethanol was used. Samples were prepared and data collected in the same manner as for Curve A, but two filters were prepared for each volume of stock solution. *Microcal Origin* was used to calculate both the intra-filter error for each filter and the inter-filter error (the standard deviation of average fluorescence measurements for multiple filters with the same CTF concentration)

**Figure 7:** Calibration Curve B



For Curve C, the same stock solution was used as for Curve B, but three filters were made for each volume used. Again, *Microcal Origin* was used to calculate both the inter- and intra-filter errors.

**Figure 8: Calibration Curve C**



All three calibration curves presented show a definite linear relationship between the amount of formazan in a sample and the intensity of measured fluorescence. Correlation coefficients ( $R^2$ ) of 0.982, 0.988, and 0.946 indicate a very close linear fit of the data. This provides convincing evidence that when samples are prepared carefully and correctly, there is a linear correlation that can be used to determine the CTF concentration of an unknown sample. The slope of each calibration curve indicates the relationship between fluorescence and formazan amount. For these three examples, the slopes range from 0.56 to 0.70. These data give an average slope of  $0.65 \pm 0.077$ . The major concern with these data is the large variation in the y-intercept of the three curves, ranging from 68.0 to 112.7. The average y-intercept is  $84.7 \pm 24.4$ . The y-intercept indicates the fluorescence measured when there is no CTF on the filter (i.e.-the fluorescence of the blank).

There are several possible sources of error in this experiment. First of all, there is intra-filter error. This error includes variations in multiple fluorescence

measurements taken from the same filter. In these three experiments, this error ranges from 0.6 to 62.4 standard deviation units. This is a very wide range of error values. Sources of this type of error measurement include the uniformity of the filtered layer and the random error associated with the instrument itself. Visual examination of the filters with the naked eye shows great variance in the thickness of the CTF layer on the filter from one area to another. A measurement taken in an area with a thicker layer of CTF will show higher fluorescence intensity than a measurement taken in an area with a thinner layer. This non-uniformity can be attributed to uneven suction pressure during vacuum filtration. It is also possible that smaller particles may clog the pores on some parts of the filter. This would greatly slow the flow of water through those areas, in turn reducing the CTF deposited there. This error should be accounted for in the final results, because several measurements are taken and averaged. Since each measurement is taken from a different point on the filter, the average should be between the highest measurement and the lowest measurement, which should be indicative of the overall filter. If a fluorescence measurement could be taken for the entire filter at one time, this averaging process would not be necessary. There is also a certain amount of random error associated with any instrumental procedure. This error should also be minimized by averaging several fluorescence measurements, since random fluctuations are just as likely to cause an increase in the measured fluorescence intensity as they are to cause a decrease in the measurement.

For calibration curves B and C above, there is also a certain inter-filter error, which includes variations in the average fluorescence measured for multiple filters of the same CTF concentration. In these experiments, this error ranges from 2.0 to 36.9 standard deviation units. Possible sources of this error include pipetting errors during sample preparation, losses during filtering, and the same random instrumental error discussed above. These sources of error also affect the linearity of the calibration line due to their unequal effects on measurement taken from filters of differing CTF concentrations. In these experiments, micro-liter volumes are being pipetted. When dealing with such small volumes, a single drop of solution can have a major effect on the final results; an extra drop may cling to the outside of the pipette tip from the stock solution being dropped into the sample container, or a single drop can remain in the pipette tip after it is emptied into the sample container. This source of error is greatly reduced through very careful calibration of automatic pipetters as well as careful and consistent pipetting technique. Inter-filter error is also greatly increased by losses during filtering. It was found that solid CTF will stubbornly adhere to anything it comes into contact with. Initially, this was a significant problem because glass beakers were used. CTF clinging to the beaker can be dissolved with ethanol and re-precipitated with water, resulting in the removal of some of the adhering CTF, but never all of it. This problem was greatly improved with the use of Teflon beakers. It is possible to remove all the CTF from the Teflon beaker with one to two ethanol/water rinses. The rinse solution can be added to the filter column to collect the leftover CTF on the filter. Unfortunately, a Teflon filter column was not available

for use. Therefore, quite a bit of CTF was still lost in the glass filter column. While the amount of CTF appears insignificant, this is probably the largest source of error in this experiment. It is again important to note that very small (nano-molar) amounts are being used, so very small losses of material can have a large effect on the final results.

Inter-filter sources of error are most likely much more significant in these experiments because they are not as easily accounted for in averaging and they involve much larger deviations from the mean measurement due to such small amounts being used. It seems that all of these error sources can be satisfactorily dealt with through very careful handling of the sample during sample preparation, filtering, and fluorescence measurement.

The results of this project show that there is indeed a linear relationship between CTF concentration and fluorescence intensity. The equation for this line can be used to determine the amount of CTF produced by unknown bacterial samples from the wetlands being studied. The amounts of CTF produced by each bacterial sample can be compared to estimate the relative number of aerobically respiring bacteria in corresponding areas of the wetland. Because the slope and y-intercept of the calibration line varies between different groups of samples and even between separate measurements of the same samples, it is necessary to make new standard filters and to obtain a new calibration curve each time unknown samples are going to be measured.

## Conclusions

The results of this project show that fluorescence spectroscopy is a reliable method for determining the relative amount of reduced CTC in a water sample. Even though a new set of standards must be prepared to obtain a calibration curve each time unknown samples are measured, the process still requires much less time than microscopic analysis of the same samples. The small standard deviation (0.077) between the slopes of the three sample calibration curves indicate that this method is precise and reproducible. As shown in figure 5b, the experimental set-up designed for this analytical method utilizes several small, battery-powered instruments. It is, therefore, possible to transport the fluorescence set-up into the field to take measurements.

In order to validate this method for use in biological assays, fluorescence measurements will be compared to cell counts from the fluorescence microscope. It has been determined that aerobically cultured *Escherichia coli* samples are capable of reducing CTC in a water sample, but a more detailed method must be developed for preparing samples and treating them with CTC without killing all the cells.

The linear relationship found between CTF concentration and fluorescence intensity can be used to compare relative numbers of aerobically respiring bacteria in water samples from the wetlands. Higher fluorescence intensity indicates a greater number of aerobic bacteria. Dr. Spratt and his colleagues will be able to use this information to better understand the environment in which Metolachlor and Simazine are degraded.

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