# Investigation of the algal toxicity of Triclosan (5-chloro-2-(2,4-dichlorophenoxy)phenol) and Triclocarban (3,4,4'-trichlorcarbanilide).

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**Abstract** Triclosan and Triclocarban are antimicrobial compounds that are present in several disinfectant hand-soaps currently on the market. The compounds have recently been found in surface waters, possibly due to soil runoff or ineffective waste water treatment. The motivation for this study stems from the lack of peer-reviewed published information on aquatic toxicity of these compounds, a rise in the use of these chemicals and that equally effective alternatives exist in the prevention of hand-washing related illnesses. This study investigates the algal toxicity of these compounds over a wide range of concentrations. The target organisms are green freshwater algae that were enriched from a local agricultural drain. Growth inhibition of the alga is measured using in-vivo chlorophyll a fluorescence. Results show that the $IC_{50}$ of Triclosan is 1.1mg/L, 10 orders of magnitude higher than previous studies. This decrease in toxicity is likely due to a difference in the species of algae studied and suggests that local algae species are more resistant than originally thought.

**Introduction**

There has recently been concern over the potential negative impacts of pharmaceuticals on U.S. water resources. Many of these pharmaceutical compounds are reaching concentrations that are hazardous to aquatic life (Kolpin *et al.* 2002, Kümmerer 2004). Of the pharmaceuticals found or thought to be present in aquatic ecosystems, two anti-bacterial chemicals, triclosan (5-chloro-2-(2, 4-dichlorophenoxy) phenol) (TCS) and triclocarban (3, 4, 4'-trichlorcarbanilide) (TCC), are of increasing concern due to their high toxicity to aquatic life, potential for bio-accumulation (Orvos *et al.* 2002, Fraker *et al.* 2004, Wilson *et al.* 2003) and a lack of peer-reviewed published information on the toxicity of Triclocarban. This potential for aquatic ecosystem impact is increasing due to the suspected rise in prevalence and concentration levels of these compounds in surface waters (Halden and Paull 2005).

TCS and TCC are the active ingredients in most of the antibacterial soaps currently on the market. A recent study of the frequency of TCC and TCS in soaps and found that 76% of liquid soaps and 29% of bar soaps surveyed contained these antibacterial agents with an overall frequency of 45% (Harris *et al.* 2001). Estimates of usage in the U.S. are between 2 and $4x10^5$ kg/yr for TCS and 3 and $10x10^5$ kg/yr for TCC (Halden and Paull 2005). Recent studies investigating the effectiveness of these compounds found no increased protection from the transmission of infectious diseases over regular hand-washing. Alcohol based hand sanitizers were found to be the most effective (Luby *et al.* 2005, Larson *et al.* 2004) and these sanitizers do not have a potential adverse impact on aquatic ecosystems.

TCS and TCC enter the environment through incomplete remediation in wastewater treatment plants and also through accumulation in soils (McAvoy *et al.* 2002, Rooklidge 2004). A USGS survey conducted on streams and rivers in the U.S., found the frequency of detection for TCS was 57.6% (Kolpin *et al.* 2002). A recent study set out to quantify the presence of TCC in the environment using the co-occurrence of the published values of TCS concentrations as a co-factor. Based on the models used in the study, the frequency level of detection was estimated to be 60%. The nationwide median was estimated to be 10.9 µg/L with a maximum concentration of 675.0 µg/L (Halden and Paull 2005).

Based on the previous research, this study sets out to answer two main questions. The first question is: are the methods used in growing algae, without the addition of toxicants, yielding data that are reproducible? This is of importance during the follow-up toxicity study because we

want to ensure that control data received are reproducible and that any differences in growth can be explained by concentration of the toxicant and not poor methodology. Our first hypothesis was that chlorophyll fluorescence is an accurate measure of chlorophyll a concentration. Our second hypothesis was that SSC media, because of a larger buffer capacity, will provide more consistent algae growth patterns.

The second question of the study is: are TCS and TCC toxic to green freshwater algae collected from the San Luis Drain? The San Luis Drain is an agricultural drainage system in the San Joaquin Valley. The drainage system was closed in 1985 due to Selenium poisoning of birds in the area. The system was reopened in 1996 with a by-pass system to facilitate drainage of agricultural runoff directly to the San Joaquin River without contamination of surrounding wetland areas (Bekon *et al*. 1997). There are large algal blooms that develop in this area due to fertilizers present in agricultural runoff. We used algae harvested from the drain because it contains many species that are found in local natural waters. Our hypothesis for this portion of the study is that TCC and TCS will inhibit the growth rate and yield of native algae. Our second hypothesis is that the toxicity of the compounds will be similar to previously published values.

## Methods

### Calibration of Shimadzu RF-1501 Spectrofluorophotometer:

This portion of the methods was used to measure chlorophyll a fluorescence of a sample. First we calibrated the Shimadzu RF-1501. This was achieved by preparing serial dilutions of chlorophyll a using spinach. Serial dilutions were made by adding approx. 0.1 grams of spinach and 40 mL of acetone to a 50mL glass centrifuge tube. The tube was shaken on level 8 of the vortex shaker for two minutes. After use, extract was kept in the dark at approx 4 degrees C for no longer than one week. Dilutions of the spinach extract were then performed by adding volumes of the spinach extract to acetone. These standard dilutions were then analyzed using a Perkin Elmer Lambda 20 spectrophotometer. 0.5mL, quartz, two sided, black cuvettes were used. An acetone blank was used during the analysis and to zero the machine. After each measurement the cuvette was rinsed twice with acetone and once with the standard dilution being measured. The absorptions of each dilution were recorded and then concentration of chlorophyll a was calculated using the trichromatic method, eq. 1 (APHA 2005).

Eq 1: Chlorophyll a (mg/L) = $11.85(A_{664}-A_{750})-1.54(A_{647}-A_{750})-0.08(A_{630}-A_{750})$.

Once we knew the concentrations of chlorophyll a in the dilutions, the values were then entered into the RF-1501 to establish a calibration curve. This allowed the machine to calculate a concentration of chlorophyll a based on the fluorescence of the sample. The following parameters were used for the RF-1501. 10 nm excitation bandwidth, 10 nm emission bandwidth, response set to auto, high sensitivity, automatic shutter on, 425nm excitation wavelength, 665nm emission wavelength, at least 30 minutes of warm-up time, and glass 4 sided 10 mm light path cuvettes. The machine was auto-zeroed using acetone and then a water blank was measured. The data from the calibration was then analyzed using linear regression techniques.

**Preparation of Growth Media, Inoculation and Algae Growth**

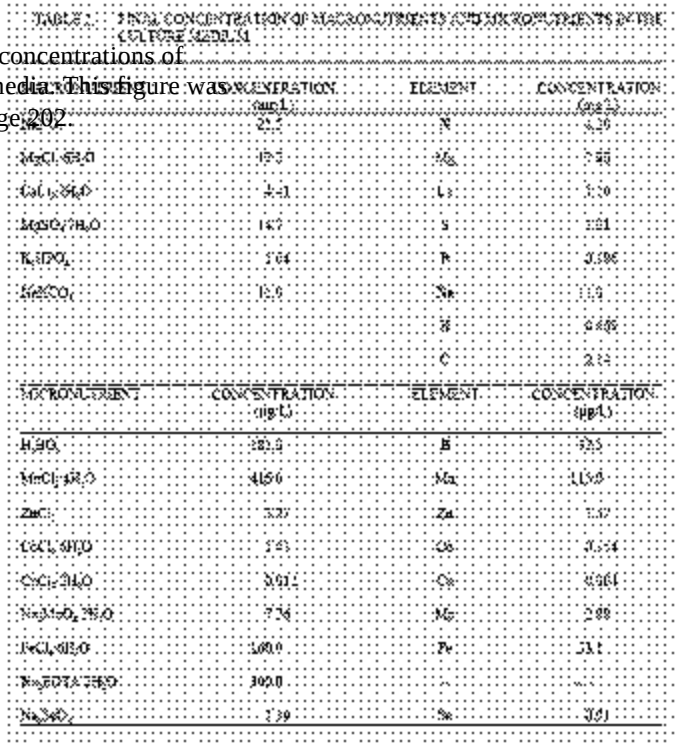

Table 1: This table illustrates the concentrations of nutrients in solution for the EPA media. This figure was taken from EPA-821-R-02-013 page 202.

We analyzed two different media to test the hypothesis that SSC media, because of a larger buffer capacity, will provide more consistent algae growth patterns. The single source carbon (SSC) media was prepared in accordance with Stringfellow & Alvarez-Cohen 1999. Table 2 shows the concentrations of chemicals in the SSC media. The EPA media was prepared in accordance with EPA-821-R-02-013. Table 1 shows the concentrations of the chemicals in the EPA media. A 250 mL volume of media was transferred to a 500 mL Erlenmeyer flask and inoculated with 2mL of previously cultured algae. The flask containing the culture was covered with a 100mL beaker and then placed in a water bath equipped with an orbital shaker. The cultures were incubated at 25° C and shaken at approx 150 rpm. The cultures were illuminated with fluorescent lighting at 105 µmol E/$m^2$/s. In order to monitor the growth of the algae, FI of the algae cultures were measured once following inoculation, twice on days three, four, five and six; once on subsequent days. The pH of the culture was measured on the days that the FI was measured. These cultures were used as starter cultures for the toxicity test.

The statistics we used to analyze the growth of the media was log transforming the data. We then used correlation coefficients to assess the linearity of the model during the log transformed exponential growth phase.

| Macronutrients: | Micronutrients: |
|---|---|
| 2000mL Millipore H20 | 995.8mL Millipore H2O |
| 2.07g KH2PO4 | 3.5mg MnSO4 * H2O |
| 1.72g Na2HPO4 | 6.8mg CuSO4 * 5H2O |
| 2.01g NH4Cl | 7.5mg ZnSO4 * 7H20 |
| 0.25g MgSO4 * 7H2O | 12.8mg Na2MoO4 * 2H2O |
| 0.13g CaCl2 * 2H2O | 66.3mg FeSO4 * 7H2O |
| 2mL of Micronutrients | |

Table 2: Media Recipe for SSC media

### Toxicity Test

EPA media was used for the toxicity test. A 250mL volume of media was measured into a 500mL Erlenmeyer flask. Concentrations of Triclosan are then diluted in acetone and introduced to the media in 1ml portions. The test cultures have one duplicate and contain concentrations of 0.5µg, 5.0µg, 50µg, 500µg, 5.0mg. One control was used with a duplicate each containing 1ml of acetone. Starter cultures, from which the test cultures are to be inoculated from, were first measured for FI to find their relative concentrations. The test cultures were then inoculated with 2mL portions of the starter culture. Test flasks were then measured for FI and pH periodically throughout the growth period. Test cultures were grown under the same conditions as the starter cultures. The endpoint of the test calculates the $IC_{50}$ during the exponential phase of growth and when the growth ends and reaches its final yield (carrying capacity). This $IC_{50}$ estimate will be calculated from the measured data by log transformation and regression techniques.

## Results

### Preliminary Tests

Figure one shows the relationship of fluorescence intensity to chlorophyll a concentration. This relationship is with extracted (in-vitro) measurements. The model shows that the ratio of fluorescence intensity (FI) to chlorophyll a is approx. 3.82. The correlation between FI and concentration using this model is high ($R^2$ >0.99). This leads us to accept the hypothesis that chlorophyll fluorescence is an accurate measure of the chlorophyll a in the sample.

The figures 2-5 represent the natural log of the fluorescence intensity verses time. The results show that the EPA media produces more reproducible data which caused us to reject our original hypothesis that the SSC data would produce more consistent algae growth patterns. Combining the data from cultures "EPA 7" and "EPA 8," this model shows a higher correlation ($R^2$ = 0.989 see figure 3) than the combined "SSC7" and "SSC 8" cultures ($R^2$ = 0.963 see figure 5). The EPA cultures as a whole, are more closely correlated and have similar exponential growth rates independent of the culture they are inoculated from ($R^2$ = 0.986, k = 0.810 $d^{-1}$ see figure 2).

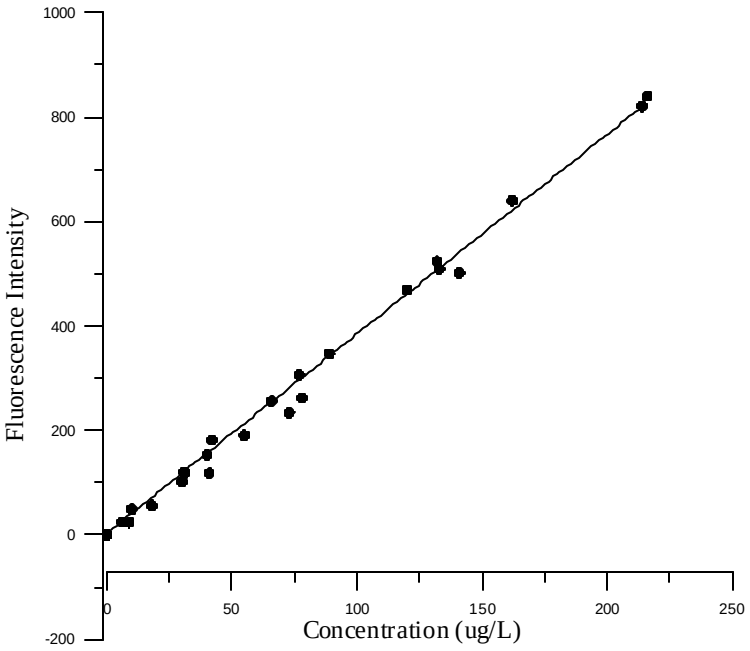


Figure 1: This table illustrates the relationship between fluorescence intensity and concentration of chlorophyll a in vitro. The data conforms to a linear model given by the equation: Y = 3.817* X n = 30. The model is significant $R^2$ value =0.997

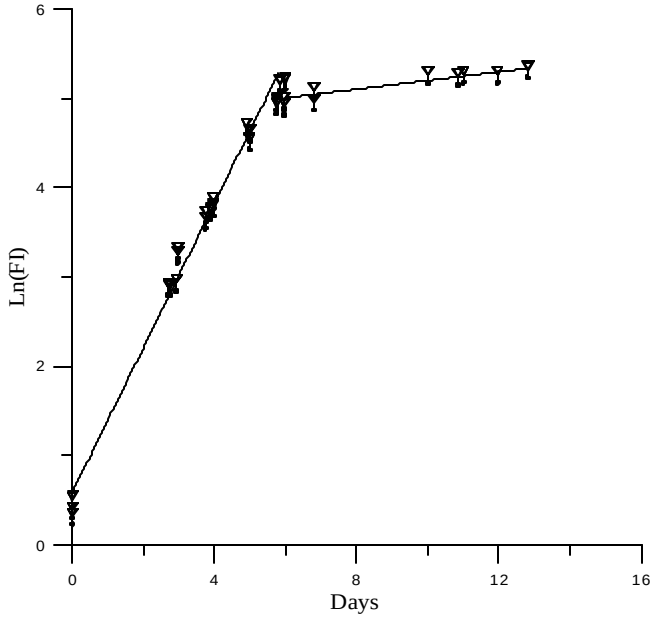


Figure 2: Combined data from EPA5-022806, EPA7-031006 and EPA8-031006. The data conforms to an exponential growth model. This is illustrated above where the natural log of the data is increasing linearly. During this phase the growth rate is 0.810 $days^{-1}$ n = 20 The model has a high correlation, $R^2$ = 0.986. This shows that the method using the EPA media is more reproducible than the SSC media.

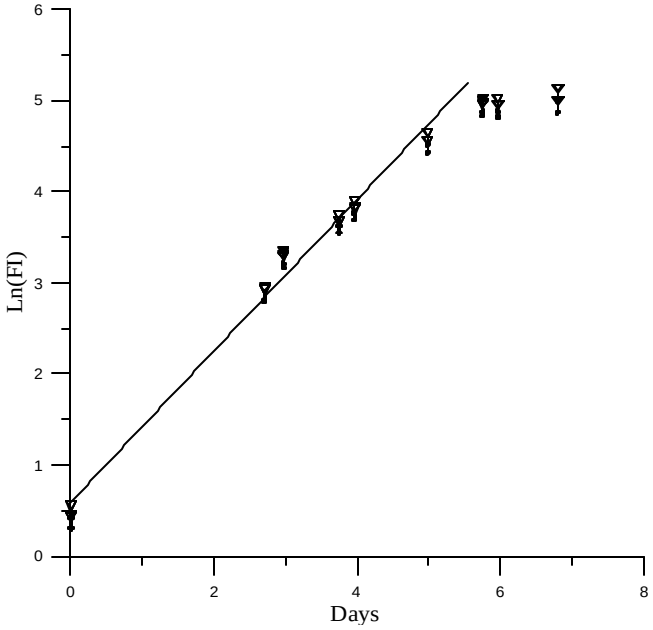


Figure 3: Combined data from EPA7-031006 and EPA8-031006, which were inoculated from the same culture. The data conforms to an exponential growth model. This is illustrated above where the natural log of the data is increasing linearly. During this phase the growth rate is 0.829 $days^{-1}$; n = 12. The $R^2$ = 0.989

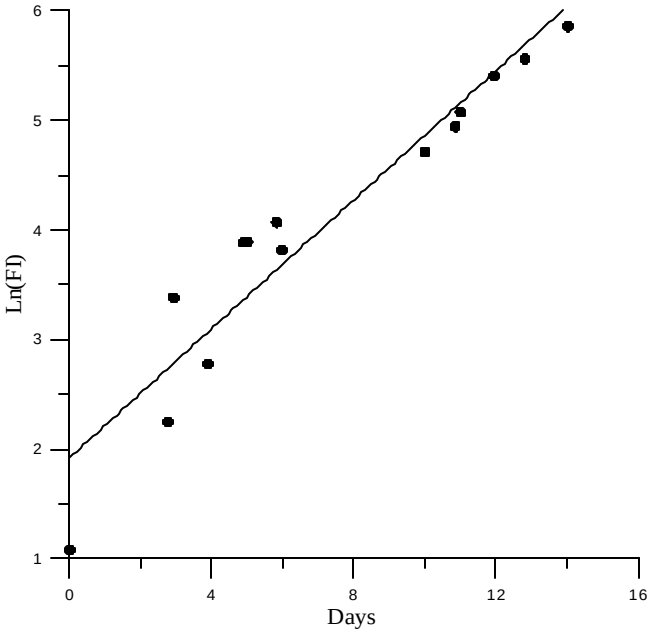


Figure 4: Data from SSC5-022806. During the period measured, the media exhibited no carrying capacity. This and the lack of confidence of the data conforming to the linear model, $R^2$= 0.907, n = 14, is most likely due changes in growth due to competition from zooplankton observed via microscope.

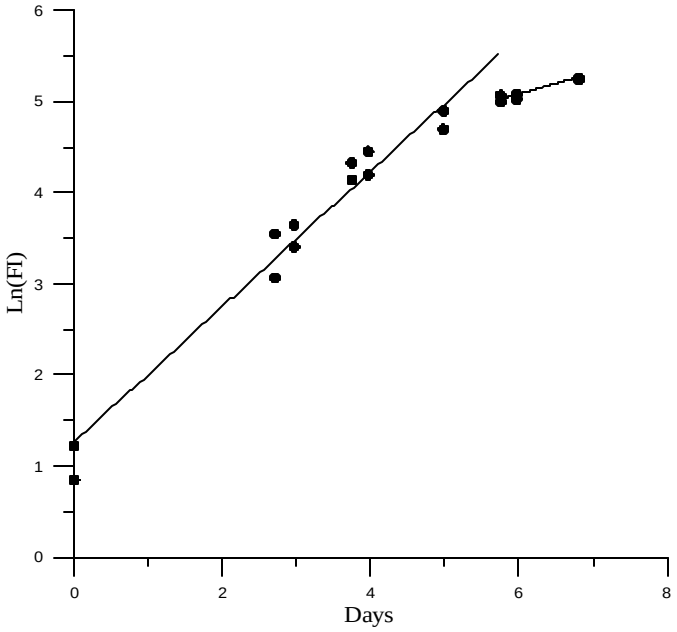


Figure 5: Combined data from SSC7-031006 and SSC8-031006, which were inoculated with the same starter culture. The data conforms to an exponential growth model. This is illustrated above where the natural log of the data is increasing linearly. During this phase the growth rate is 0.744 $days^{-1}$, n = 13. The correlation of the data is high for this model: $R^2$ = 0.9663, but is not as strongly correlated as the EPA model (see figure 2, 3 for $R^2$ values)

**Toxicity Test**

The test cultures and controls were grown for a period of 5 days. The log of the growth for each test concentration and its duplicate were plotted as a function of time (data not shown). This allowed us to calculate the growth rate and yield of the algae using standard regression techniques. The yield of the algae at the end of the test, the yield of the algae at t=67 days and the exponential growth rates were each plotted as a function of triclosan concentration. (See figures 6, 7 & 8). The $IC_{50}$ was calculated for both growth models by calculating the triclosan concentration at which there was a 50% reduction in growth. The values were found to be 1.1 mg/L for t = 67days and 0.9mg/L for t = 5 days. The 50% reduction in growth rate was then calculated from the model of growth rate as a function of triclosan concentration. This value was found to be 3.9 mg/L.

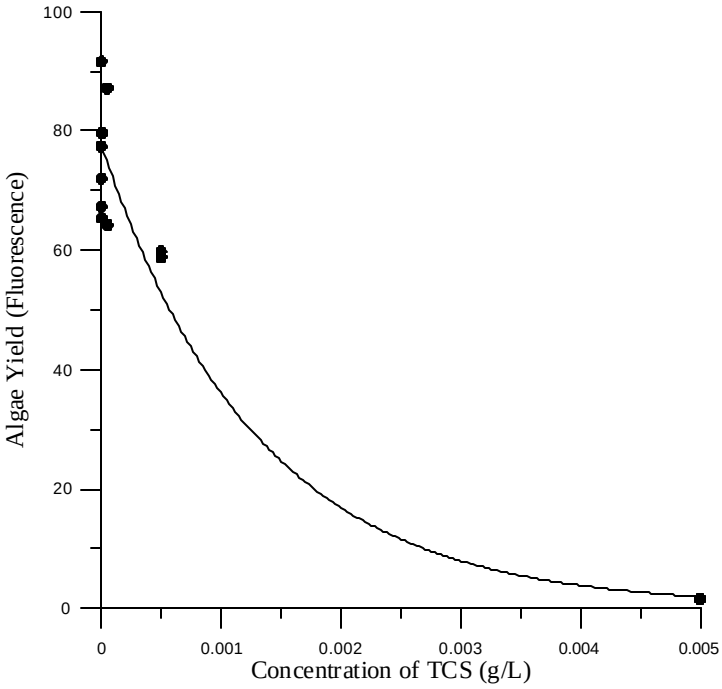


Figure 6: Growth yield at t=96 hours as a function of triclosan concentration. The data fits the model poorly despite a $R^2$=0.99. The estimation of the $IC_{50}$ from this model is 0.9mg/L

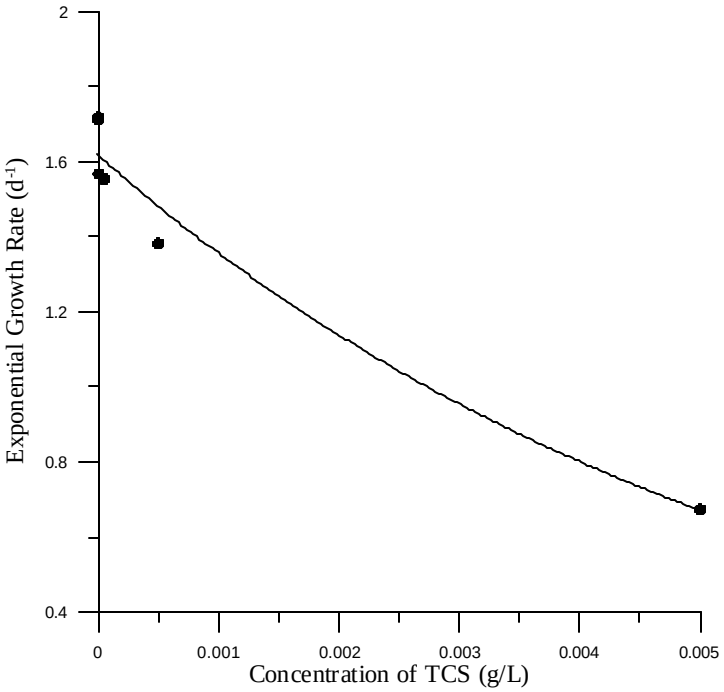


Figure 7: Growth Rate as a function of triclosan concentration. The data fits the model well $R^2$= 0.98. The concentration of TCS that produces a 50% reduction in growth rate is 3.9mg/L

**Discussion**

Our hypothesis that the chlorophyll fluorescence was an accurate measure of chlorophyll fluorescence was supported by the data that we received. The model was linear with a high correlation coefficient. It was also possible to find the upper and lower limits of the RF-1501 by collecting data sets in this method. We found that the machine had an upper limit of 1000 fluorescence units, which equated to approx 250 µg of chlorophyll a in the sample. The measure of biomass or growth is the key outcome variable in these types of toxicity tests. We were able to use chlorophyll a concentration as an indication of growth rate

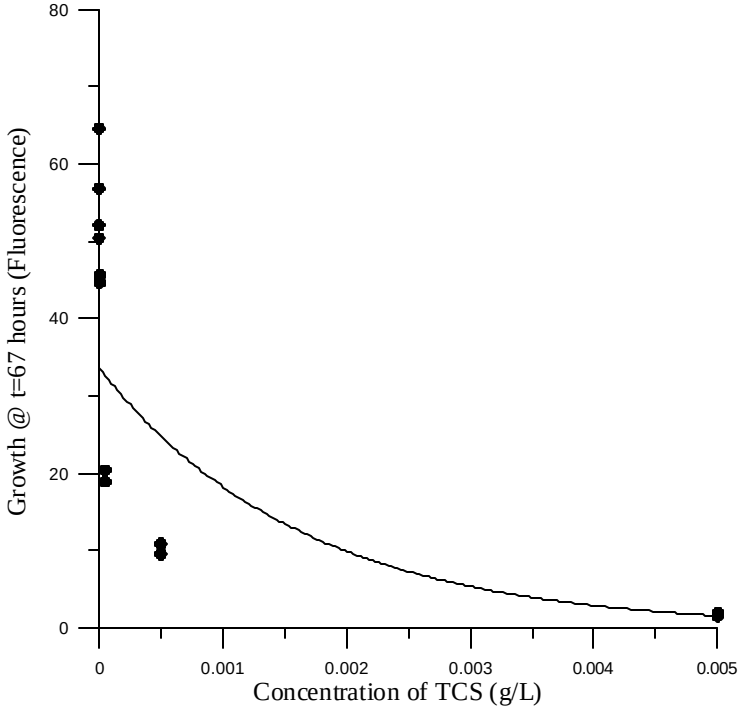


Figure 8: Growth yield at t=67 hours as a function of triclosan concentration. There is variation unexplained by the model, $R^2$ =0.82. The $IC_{50}$ estimated from this model is 1.1mg/L

first, because it has been established that chlorophyll concentration is directly proportional to the biomass of the sample (APHA 2005) and second, that our measurements were periodic, measuring growth relative to the difference in between time periods. This allowed us to calculate reductions in growth without quantifying biomass. To ensure quality with future tests, it is recommended that cell counts be performed on samples of chlorophyll a in an effort to quantify a correlation between biomass and chlorophyll a.

For the investigation of the differences in culture media, the data in the figures 2, 3, 4 and 5 are broken into two periods. The first period is in which the line has as increasing slope. During this period the culture is experiencing an exponential growth phase. We break the data at this point in order to calculate a growth rate and linear correlation coefficient. The period over which the slope is zero represents the time period in which we see no growth and the algae has reached its carrying capacity (yield). One of the main concerns with the use of algae media in toxicity testing is that the media will either increase or decrease the toxicity of a compound by impacting the yield of the algae. EPA-821-R-02-013 is the accepted method at which to test the toxicity of compounds and receiving waters to many different aquatic animals. The media suggested in this protocol did produce more consistent results than the SSC media, despite the fact that pH levels increased rapidly with the EPA media and stayed consistent with the SSC media (data not shown). It is unclear if the pH has an effect on the yield of the algae and therefore an effect on the toxicity values. This was unclear because we found evidence that the yields between EPA and SSC media were similar in some cultures, specifically ones that had been inoculated from the same starter batch. We are currently in the process of using SSC media as the test media to investigate if its produces the same $IC_{50}$ as the EPA media.

None of the models used for calculation of $IC_{50}$'s fit the data well, even though some of them had high $R^2$ coefficients. This was due to the wide range of concentrations that we tested. It is possible, now that the $IC_{50}$ has been estimated using this data, to repeat the test using concentrations that are closer to that estimation. This will allow us to create a model with less variation and we will be able to calculate the $IC_{50}$ with higher confidence. We estimated two different $IC_{50}$ values from the toxicity test. The $IC_{50}$ value which was calculated at the last measured point in which the growth was considered to be exponential (t =67 hours), is what we are choosing to report ($IC_{50}$ = 1.1mg/L). This value is in between two and three orders of magnitude higher than the previously measured value of 4.46µg/L (Orvos *et al*. 2002). We

considered this difference to be large enough to reject the hypothesis that the toxicity of triclosan will be similar between local native algae and *Selenastrum* sp. In order to explain the difference between the published and reported toxicities, we looked at the testing techniques. The differences between the test techniques are first, that we measure the toxicity through out the test. The reason for this is that by measuring during the period in which the algae are growing exponentially, it allows for a better approximation of the growth rate. This does introduce some error due to the lack of sterility during the test. It is unclear whether this error would have a stimulating or inhibiting effect on the toxicity of the compounds and further analysis is planned. The second difference in technique is that the species used are different. Orvos et al. (2002) uses the species suggested by the EPA test method (*Selenastrum capricornutum*). Our test culture incorporates many different algae; we were able to identify *Scenedesmus* sp. and the presence of unknown centric diatoms. It is likely that during the test that the presence of triclosan inhibits the less robust strains of harvested algae and therefore the more resistant strains are allowed to grow. It is also highly likely that algae in the San Luis Drain are subjected to low back ground levels pesticides and other agricultural chemicals and possibly even triclosan. This would therefore favor the growth of robust strains of algae that would be less sensitive to toxicity testing using triclosan. Both of these scenarios would help account for the wide difference between measured data and published data. In order to ensure that the algal strains account for the difference in toxicity, rather than our methodology, we plan on repeating the test using *Selenastrum* sp.

We were unable to test the chemical triclocarban due to time constraints. Previously published NOEC levels using *Selenastrum* sp are reported to be 30µg/L (EPA HPVC 2002). We hypothesize that using strains of algae harvested from the drain will produce a large order of magnitude difference as seen in the triclosan toxicity testing We plan on doing future testing using both algae harvested for the San Luis Drain and pure *Selenastrum* sp. cultures.

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