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The Effect of Azo Textile Dyes on Gross Primary Production and Community Respiration in an Artificial Environment

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A thesis submitted to the faculty of the Environmental Studies Program in partial fulfillment of the graduation requirements for the Degree of Bachelor of Arts with honors in Environmental Studies

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ABSTRACT

Azo dyes are the largest category of textile dyes in production today, with over 100,000 tons of dye produced yearly in the United States. Ten to fifty percent of this dye is not fixed to the fabric during the textile production process, and is therefore discarded as effluent. Because dye waste is difficult to process in wastewater treatment facilities, it is important to understand how this waste affects aquatic systems using a whole-ecosystem approach. This study used an artificial stream to model the effects of four azo dyes on benthic biofilm production and respiration. Dyes were found to have varying effects on primary production, respiration, and net ecosystem production, with Acid Red 1 being the most inhibitory of gross primary production. A pulse release of this dye monitored over time revealed the main factor influencing primary production to be light limitation, rather than acute toxicity. Because legislation surrounding dye effluent is based upon perceivable color, and therefore light limitation, it was concluded that current legislation regulates textile effluent in an appropriate manner.

ACKNOWLEDGEMENTS

I would like first and foremost to acknowledge Denise Bruesewitz, who saved me from countless sleepless nights in the lab with her guidance and expertise, and always had a cup of tea, advice, and perspective waiting for me when I thought everything was falling apart. I would also like to acknowledge Rebecca Forgrave, who was companionably miserable with me on countless late lab nights and solved all of my technological, social, and emotional struggles. I would also like to acknowledge two gentlemen who saved my thesis: Jacob Adamson, who courageously braved the perils of hypothermia with me to collect water from a frozen stream in the forest in February (and didn't laugh too hard when I fell into said stream), and Chuck Jones, who properly grounded the artificial stream that had been previously throwing me against the wall with the strength of its static electric shocks. And lastly, I would like to acknowledge Russ Cole, who asked concerned questions about my lab activities in the most tactful way possible and never failed to compliment my exemplary lab fashion. Without these people, and the support of the rest of the Environmental Studies Program, what you are about to read would not exist, and for that, I am eternally grateful.

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CHAPTER 1: INTRODUCTION

Overview of Textile Industry

Textile production was largely a domestic and small-scale industry up until the Industrial Revolution. In the United States, the first mill with power looms was built in Lowell, Massachusetts in the early 1800s (U.S. EPA 1997). As of 2012, textiles in the US were a $54 billion industry, and employed >200,000 individuals, with 70% of these living in ten key textile-producing states concentrated in the Southeast (Platzer 2013; Figure 1). The history of textile dyeing as we know it today arguably starts with the synthesis of mauveine, a purple dye, in 1856 (Easton 1995). Reactive azo dyes that react directly with the fiber to achieve a color fastness not feasible with water-soluble dyes were developed in the 1950s (Greenberg 2003). In 1986, over 107,000 tons of textile dyes were produced in the US (Greenberg 2003). As of 1996, there were 500 dyeing and finishing plants in the United States, and in May 2013, the textile dye industry employed 11,680 individuals in the United States, with the highest employment in the Southeast and California (U.S. EPA 1997, U.S. Department of Labor 2014, Figure 1.2). Most color in effluent is from plants finishing woven fabrics, as opposed by operations involved in the finishing of wool or knit fabric (Correia et al. 1994).

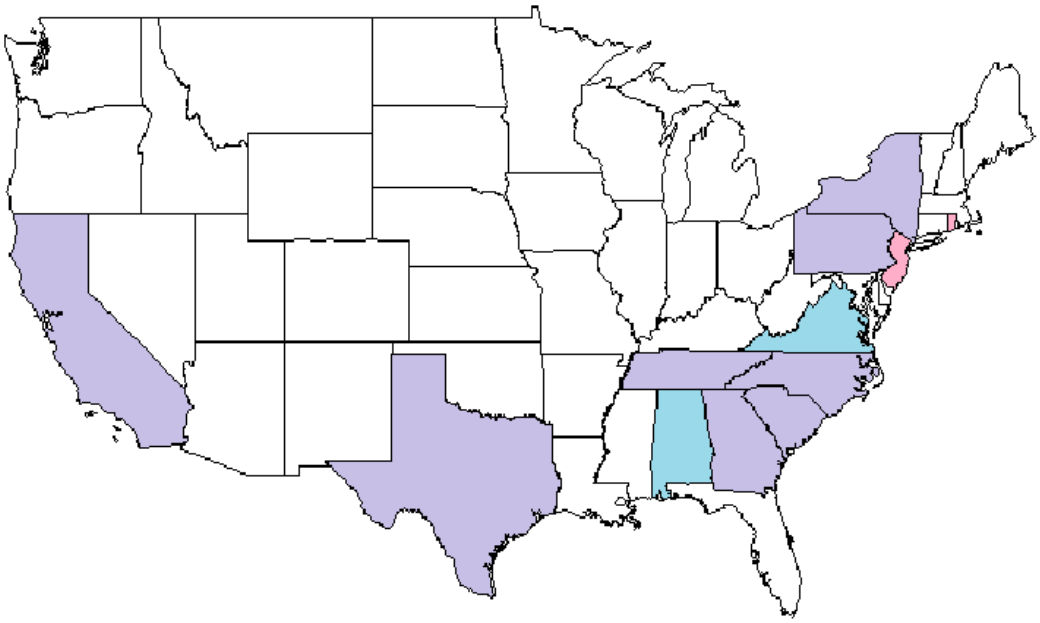


Figure 1.1. Distribution of the textile industry in the United States. Blue shading represents the ten states with the highest employment in the textile industry, pink shading represents the ten states with the largest number of textile dying and finishing facilities, and purple shading indicates that these states are in the top ten of both classifications (Platzer 2013).

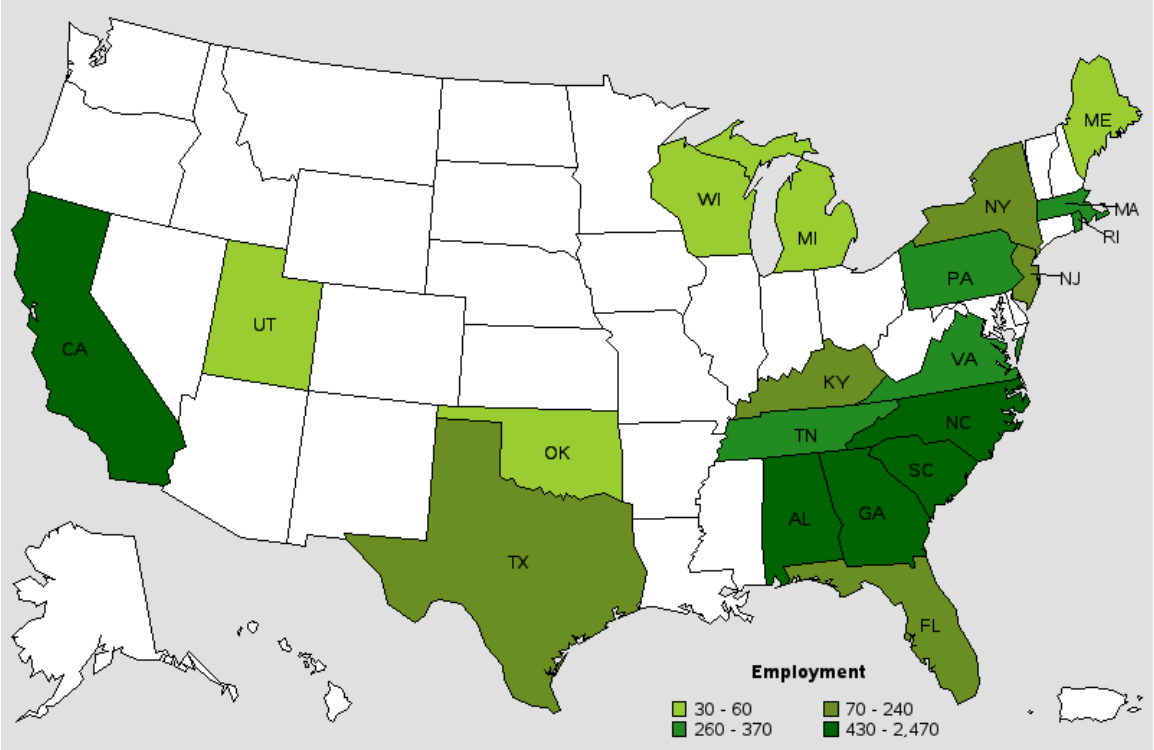


Figure 1.2. Distribution of textile bleaching and dyeing machinery operators as of May, 2013 in the United States. Blank states indicate either that these areas have no employment or that data are unavailable. *Image reproduced from US Department of Labor 2014.*

The process of creating a finished product from raw materials in the textile industry is long, and often requires multiple factories (U.S. EPA 1997). Generally, the raw material is spun or texturized to create yarn, which is then either knit or woven to make fabric. The fabric is then usually transferred to a finishing facility, where it is dyed, printed, and any special coatings such as fireproofing are added. The colored fabric is then transferred to a facility handling the cutting and sewing (U.S. EPA 1997; Figure 1.3).

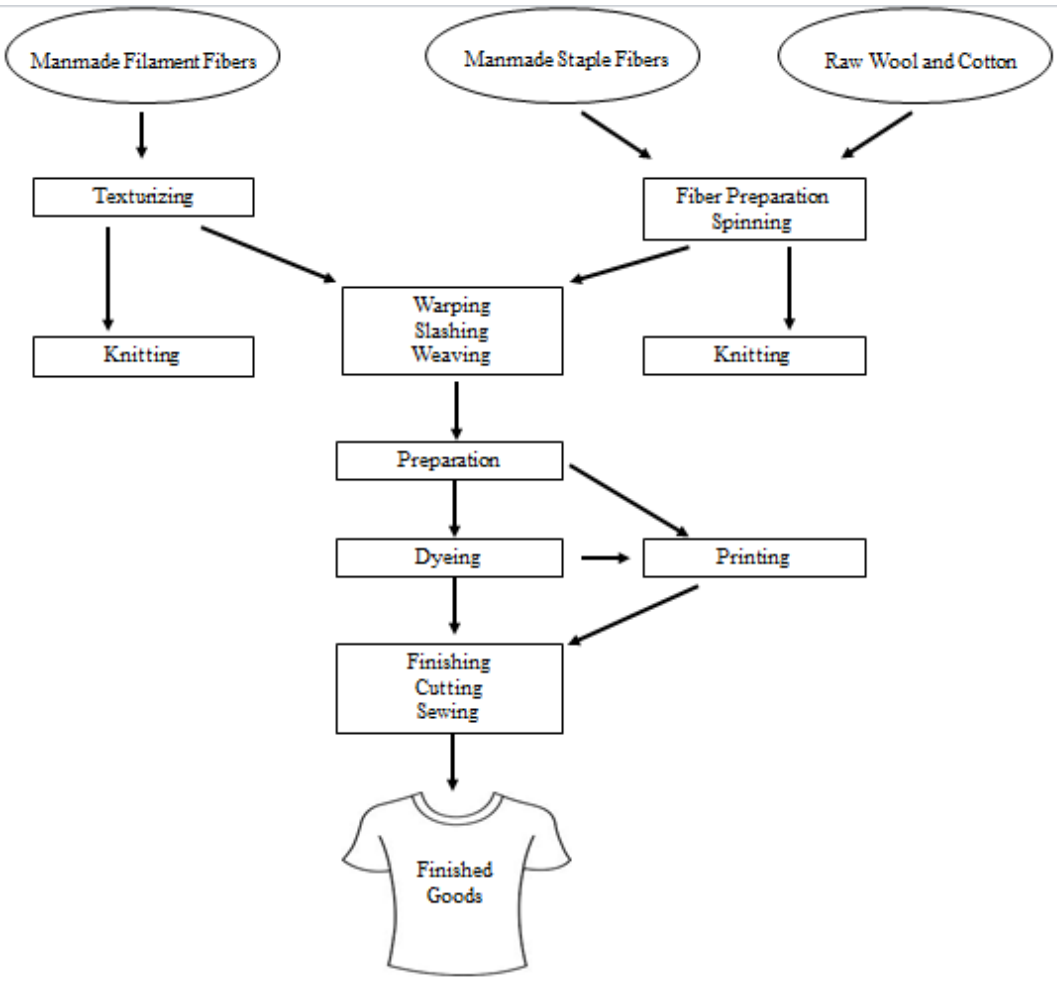


Figure 1.3. Flowchart depicting the production of textiles from raw or artificial fibers to the finished product. *Adapted from U.S. EPA 1997.*

Azo Dyes

General Characteristics and Importance

Textile dyes account for 56% by weight of synthetic colorant production, and of the 360,000 tons textile dye produced, about 43,000 tons are lost in the manufacturing and dyeing processes, and approximately 20% of this reaches aquatic ecosystems (Clarke and Anliker 1980). Thus, about 2.5%, or 8,600 tons, of total textile dyes produced are released to the environment. The most common dyes used in the coloring of textiles are reactive and direct dyes for cotton, and disperse dyes for polyester (U.S. EPA 1997). These dyes have fixation rates of 60-90%, 90-95%, and 80-90%, respectively, with the unfixed dye either recycled through the dye process or released in wastewater, depending

upon the dyeing process employed (U.S. EPA 1997, Table 1.2). In general, as the depth of the shade increases, the degree of fixation decreases (Easton 1995).

Table 1.2. Summary of common azo dye types, including the substrates dyed, and fixation ranges.

Dye Type	Substrate (Correia et al. 1994)	Fixation (Easton 1995)
Acid	Protein, Polyamide, Polyacrylic	80-95
Direct	Cellulose	70-95
Disperse	Cellulose, ester polyamide, polyester, polyacrylic	90-100
Reactive	Cellulose	50-90

Azo dyes make up the majority of synthetic dyes and are characterized by one or more N=N bonds. The class of azo dyes is subdivided into categories based upon the substrate being dyed: disperse dyes for polyesters; acid and basic dyes for ionic substrates like nylon, wool, and silk; direct and reactive dyes for cotton, rayon, and linen (General Introduction to the Chemistry of Dyes 2010). These dyes must bind fast to the fabric and remain bright, impervious to wear, sweat, biodegradation, water, detergent, even bleach – anything that the fabric could come in contact with over its lifetime. However, the same qualities that keep our sweaters bright make textile dyes difficult to remove from factory effluent: 10 to 50% of the dye used in textile production is discharged (Gonçalves et al. 2000), and the dyes are largely unaffected by the usual methods of wastewater treatment plants (WWTP). They do not settle out, they are not degraded by the typical WWTP bacterial community (in some cases, they are actually toxic to these bacteria), and the most effective physio-chemical removal techniques generate large amounts of saturated sludge that must be disposed of safely (Robinson et al. 2001). In order to reduce the color in textile effluent to below detection by the human eye, and thus to meet consent standards, about 99% of the dye concentration must be decolorized across changing concentrations and discharge rates (O'Neill et al. 1999).

Wastewater Treatment

The literature surrounding azo dyes focuses mainly on its decolorization and mineralization, defined respectively as the elimination of color and the breakdown of the molecule into smaller component groups, in wastewater treatment. Methods for achieving these goals vary, and much of the literature focuses on optimizing existing processes. A 2005 comparative study found that wet-air oxidation methods (WAO) were most effective at decolorizing and mineralizing dye, followed by chemical reagent processes utilizing ultraviolet (UV) light as a catalyst, and lastly chemical reagent processes not exposed to UV light (Kusvuran et al.). Several studies have found that the Fenton Reagent (Hydrogen Peroxide (H_2O_2) and Iron II) is especially effective in decolorization, and the pairing of this technique with UV radiation facilitates effective mineralization (Bali et al. 2004, Lucas and Peres 2006). Another well-studied catalyst in decolorization and mineralization is titanium oxide (TiO_2), which is also optimized when combined with UV radiation (Poulios and Tsachpinis 1999, Tang and Chen 2004, Aguedach et al. 2005).

The most common method for dye removal in effluent is adsorption to biomass, which works well on most water-soluble dyes (Waters 1995). An important exception is the class of reactive dyes, of which only about 10% is removed by adsorption, which is especially problematic since fixation rates for reactive dyes are relatively low, about 50-90% (Waters 1995).

Documented Toxic Effects

Because azo dyes are not easily removed by traditional wastewater treatment and successful removal techniques are expensive, dyes in effluent have a significant possibility of being released to the environment (Zaharia and Suteu 2012). A study carried out by the American Dye Manufacturers Institute (ADMI) investigated the effects of 56 dyes of differing classifications on algal growth. They found that 15 of the tested dyes were inhibitory at a concentration of 1 mg L^{-1}, and 13 of those dyes were of the Basic dye class, indicating that there are similarities in toxicological effects within dye classes (Laing 1991). A study by Wang et al. (2002) used a bioluminescence method to determine the bacterial toxicity of effluent in a dye plant in Turkey at various stages of

production within the plant. The study found mid- to high toxicity in about half of the samples, with the highest toxicity in dye baths and the lowest in rinse waters, and toxicity varied among the dye colors studied (Wang et al. 2002).

Azo dyes also tend to be mutagenic and/or carcinogenic, since they metabolize into aromatic amines and other potentially harmful byproducts (de Lima et al. 2007, Oh et al. 1997). A study of a textile effluent containing Disperse Blue 373, Disperse Violet 93, and Disperse Orange 37 found that ingestion of effluent water significantly increased the odds of developing colon cancer in rats, and the study notes that this could be due to nitro-aromatics, aromatic amines, and benzidine byproducts in the effluent (de Lima et al. 2007). Human studies show that azo dyes can be reduced to aromatic amines by intestinal flora, which may be a cause for the connection between intestinal cancer and economic development, as developed countries typically consume more processed food, which may be colored with azo dyes (Chung 1983). Thus, even if the dye itself is not toxic, it may break down into harmful products.

Laws and Regulating Institutions Concerning Textile Wastes

The problem of dye pollution is often perceived by both the public and by regulators as more of an aesthetic problem than an ecotoxic one, meaning that red and purple rivers cause more concern than blue, green, or brown ones, regardless of the toxicity of the dyes involved (Zaharia and Suteu 2012). Despite this perspective on textile effluent management, numerous studies examine linkages between azo dyes, dosage, and toxicological response. Clarke and Anliker (1980), in a review of about 3000 colorants, found that only 2% were lethal to 50% (LC_{50}) of fish when present in concentrations under 1.0 mg L^{-1} and 98% of commercial dyes had an acute oral toxicity in rats of greater than 2000 mg kg^{-1}. Algal studies show a similarly low level of acute toxicity (Clarke and Anliker 1980). Because the human eye can detect dye concentrations as low as 0.005 mg/L in water, regulation by aesthetic concerns also promotes safety from a toxicology standpoint (Clarke and Anliker 1980, O'Neill et al. 1999). Also, dyes do not appear to bioaccumulate, or concentrate as they are transferred to higher trophic levels, minimizing the future toxicological damage of current inputs (Easton 1995)

In 1978, the EPA published the most recent available regulations governing release of textile effluent into aquatic systems. These regulations cite the main effects of textile effluent as containing solids that may hinder oxygen transfer and reduce light penetration, containing dyes that increase demand for dissolved oxygen (DO) in the receiving water, and containing a variety of other materials that may upset the ecosystem in various ways (U.S. EPA 1978). For example, dye baths may also contain acids, bases, and salts that are not retained on the textile product and are rinsed into aquatic systems, where they upset the natural pH and conductivity balances (U.S. EPA 1978). Organics from the process, such as starches and detergents, consume DO as they are broken down (U.S. EPA 1978). Nitrogen and phosphorus in the dyes and dye bath materials can contribute to stream eutrophication, and the heavy metals present in some dyes are harmful to aquatic life if released (U.S. EPA 1978). The EPA states that limits should be based upon pounds of pollutant discharged relative to pounds of product produced, but state and local requirements should be followed, as these institutions have jurisdiction over waterways under the Clean Water Act (U.S. EPA 1978). In the state of Maine, the color pollution laws are based upon the paper industry, but similar legislation would likely apply to a textile factory in Maine. The guidelines are 150 pounds or less of color per ton unbleached pulp, and no discharge should increased the color in the receiving water body by more than 20 color pollution units (State of Maine 1997). Regulating by color is standard practice, as the color produced by a given concentration varies between dyes, and the concentration itself is dependent upon daily dye usage, fixation, effluent treatment, and dilution in the receiving waters (Zaharia and Suteu 2012).

Treatment of wastewater is difficult due to the mixing of different dye types in the dying process as well as the waste stream, so processes tailored to specific dyes or concentrations would not be able to handle the waste stream (Correia et al. 1994). Additionally, the types of dyes used change daily, as the plant changes color or substrate, and seasonally, as fashions change and new textiles become popular, so it is economically difficult for textile manufacturers to effectively manage their waste streams (Correia et al. 1994). Since many textile processes are batch processes, concentrations may vary over time (U.S. EPA 1978). However, generalized processes such as adsorption of dye to sludge can work well for many disperse and direct dyes (Easton

1995). Clarke and Anliker (1980) found that 40-80% of dyes are adsorbed to sludge in wastewater treatment, and suggest a combination of physical, chemical, and biological processes to most effectively handle a constantly changing waste stream.

CHAPTER 2: EFFECT OF AZO DYES ON GROSS PRIMARY PRODUCTION AND RESPIRATION IN AN ARTIFICIAL ENVIRONMENT

Introduction

Azo Dyes

Azo dyes are the largest class of dyes used in the textile industry. They are organic molecules characterized by at least one N=N bond. Azo dyes are classified by the substrate type on which they are most effective at coloring. Approximately 20% of textile dyes produced are released into the environment (Clarke and Anliker 1980), meaning that textile effluent is a significant problem and its effects on the aquatic ecosystems into which it is released should be explored. As the world population grows and countries develop economically, the demand for textiles, and therefore textile dyes, can be expected to increase.

Previous studies conducted to determine the toxic effects of azo dyes have found high variability based upon the dyes tested, the test subject, and the dosage. A study carried out by the American Dye Manufacturers Institute (ADMI) found that 15 of the 56 tested dyes inhibited algal growth at a concentration of 1 mg L^{-1} (Laing 1991). However, a study by Greene and Baughman (1996) found that dye type was a significant factor in algal toxicity, as no reactive dyes were toxic to the algae studied. A study by Wang et al. (2002) found mid- to high bacterial toxicity, defined as the dilution level at which under 20% inhibition occurs is greater than 10 and 100 respectively, in about half of the dye samples, while Hu and Wu (2001) found that cyanobacteria were susceptible to dye concentrations under 5 mg L^{-1}. Azo dyes also tend to be mutagenic and/or carcinogenic, since they metabolize into aromatic amines and other potentially harmful byproducts (Oh et al. 1997, de Lima et al. 2007). Studies have shown that azo dyes generally have minimal toxicity to fish and mammals, but it should also be noted that there is variability at the dye level, and that few long-term chronic exposure studies have been conducted (Clarke and Anliker 1980, Chung and Stevens 1993). Thus, the literature suggests that dyes do affect aquatic system, but the affect varies by dye type and organism studied, and whole-ecosystem approaches are not well explored.

Importance of Benthic Communities

Algae and bacteria make up the base of the food web in aquatic systems, and changes affecting these groups of taxa affect the system as a whole (Hall et al. 2000). Algae are important to the stream ecosystem as primary producers of organic carbon and oxygen. Bacteria are important in making allochthonous nutrients and organic carbon available to upper level consumers (Simon et al. 2003, Hall and Meyer 1998).

Research Questions and Hypotheses

The goal of this research was to determine if a suite of dyes inhibits primary production by benthic algal communities or aerobic respiration of bacteria. The main questions asked by this study are:

- Does exposure to textile dyes negatively affect benthic bacterial and algal communities, and is this negative effect the same across dye families?
- Is there a relationship between dye concentration and toxicity?
- How do the effects of a prolonged exposure differ from those of a highly concentrated pulse?

I hypothesize that algae may be more detrimentally affected than bacteria, because they use pigments to photosynthesize (Novotný et al. 2006). I also expect that all dyes will have detrimental effects on the local benthic communities, but that the level of response to each dye may vary due to variances in dye chemistry in the different dye classes (Laing 1991). I also expect that very low levels of dye may not be toxic to benthic biofilms, but that toxicity may increase with increasing concentration of the dye.

Methods

Conceptual Framework

This work follows the model outlined by Rosi-Marshall and Royer (2012), who proposed that ecologists should recognize the difference between a substance's toxicology as measured in laboratory studies and its potential effects on ecosystem structure and function. They proposed that the tools ecologists already use to measure structure and function, including chamber and core experiments, solute diffusing substrata, and mesocosm and whole-system manipulations, could be adapted to answer

questions about how anthropogenic pollutants (in this case specifically pharmaceutical and personal care products) affect these measurements. Rosi-Marshall et al. (2013) followed the procedure outlined, using solute diffusing substrata to detect production and respiration suppression by common pharmaceuticals, indicating that such an approach is successful.

Like pharmaceuticals and personal care products (PPCPs), the effect of textile dyes on aquatic ecology and ecosystem structure are largely unknown (Rosi-Marshall and Royer 2012). The main pathways for PPCPs to enter aquatic ecosystems include discharge from wastewater treatment plants and discharge from pharmaceutical manufacturers, similar to the pathways for textile dyes (Rosi-Marshall and Royer 2012). The conceptual framework outlined by Rosi-Marshall et al. (2012, 2013) above therefore translates well to the study of textile dyes, which can be easily dissolved into the substrata and their effects measured in the environment without exposing the whole system to an influx of color from dyes.

The experiment took place in a greenhouse in an artificial stream tank inoculated with natural biofilm from a nearby stream. Dye diffusing substrates were used to measure community response to low exposure to dye over time, while time series experiments were used to measure algal response to a concentrated discharge. Bae and Freeman (2007) draw a distinction in aquatic toxicology testing between acute and chronic toxicity. While acute toxicity is measured after a short-term exposure and displays a direct relationship with the toxicant, chronic toxicity is measurable after long-term exposure at low doses, and the effects tend to be indirect. For example, death following one day of exposure indicates acute toxicity, while lower fecundity later in the organism's lifecycle indicates chronic toxicity. This study attempts, within the confines of the laboratory, to explore both of these potential toxic effects of azo dye families on benthic microbial biofilms, using dye diffusing substrates over a timespan of three weeks to capture chronic toxicity and acute exposure experiments to capture acute toxicity.

Differences between control and treated groups were determined using t-tests and ANOVAs at $\alpha=0.05$ in Stata 12.1 (StataCorp). Assumptions for normality were tested using Shapiro-Wilk normality test and assumptions for equal variance were tested using

the Robust Variance test. If necessary, data were log transformed to meet these assumptions.

Artificial Stream Tank

An artificial stream was prepared by inoculating tap water with approximately 7 L of stream water collected in March 2014 from the Arboretum Stream where it crosses Mayflower Hill Drive on the Coly College campus in Waterville, ME. Rocks and sediment found in the streambed were also added to inoculate the stream with benthic microbiota. Nitrogen (N) and phosphorus (P) were added in a 16:1 ratio to bring the N concentration in the tank to 1 mg L^{-1}. Figure 2.1 shows a schematic of the artificial stream. Two propellers (A) create a circulation current in the tank. On the shelf, the ceramic tiles (B) and dye diffusing substrates (C) are colonized by biofilm algae and bacteria introduced from a local stream (D).

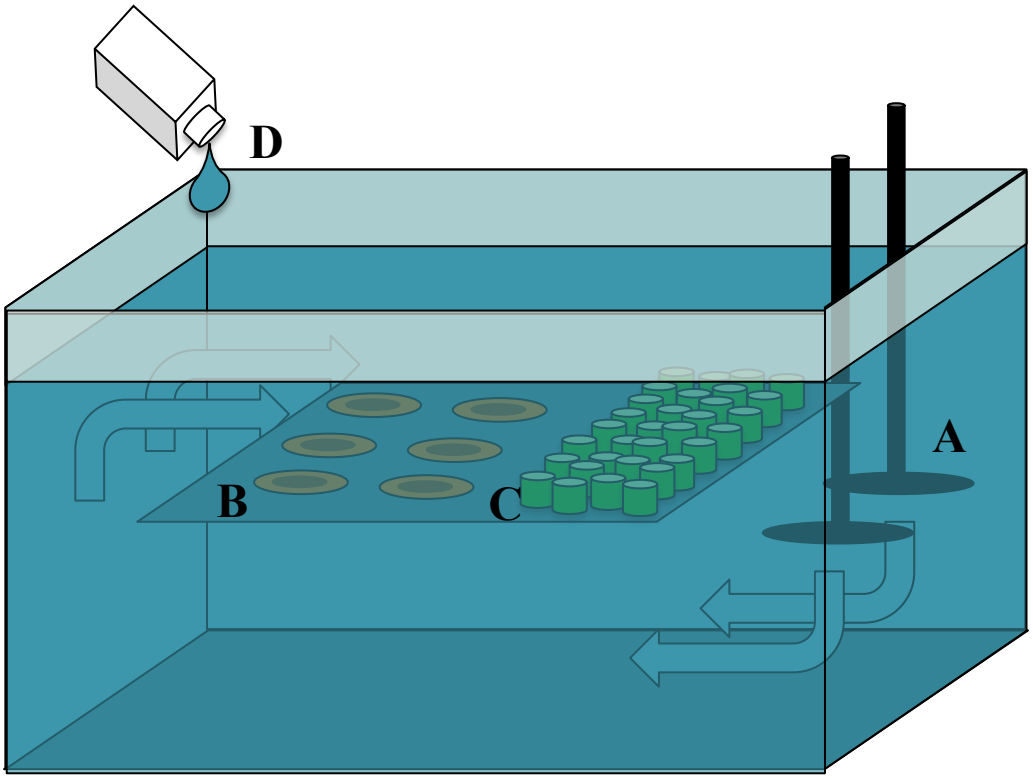
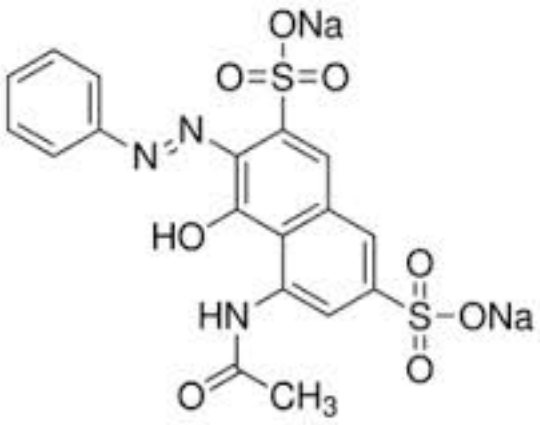


Figure 2.1. Illustration of artificial stream used in the dye experiments. The stream was placed in a temperature- and humidity-controlled greenhouse for the duration of the

experiment to allow for a natural light:dark cycle, and inoculated with water collected from a local stream.

Dye Selection

Azo dyes were chosen because they make up the majority of colorants commercially used, and the test dyes were chosen based on availability from the Acid, Direct, Disperse, and Reactive azo dye families. The dyes chosen were Acid Red 1, Direct Red 81, Disperse Orange 1, and Reactive Black 5, and the structures of each of these are shown in Figure 2. Azo dyes are organic molecules characterized by one or more N=N bonds, which can be clearly seen in these structures. None of the Material Safety Datasheets (MSDS) for these compounds indicate significant health risks to humans, and none of the MSDS indicate a significant environmental concern.



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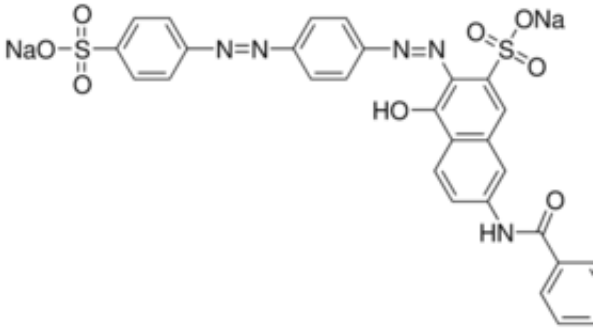

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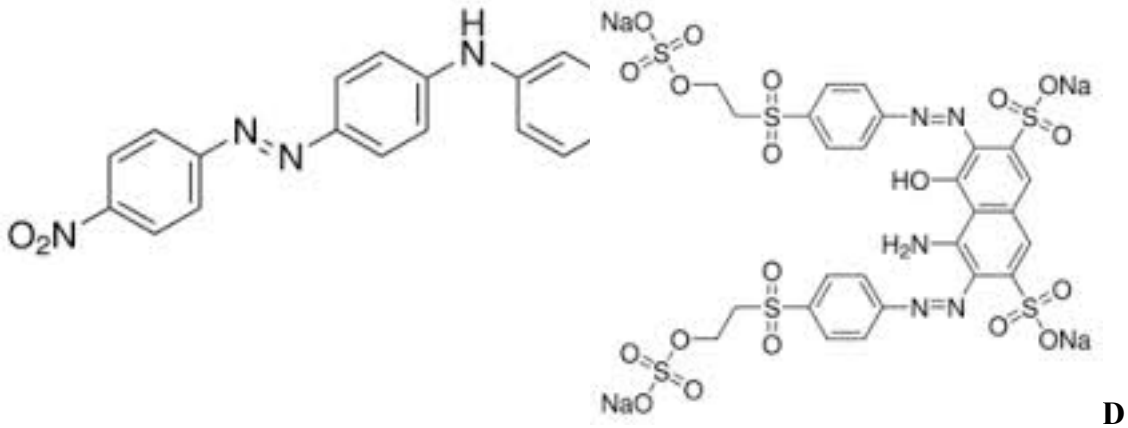

D

C

Figure 2.2. Chemical structures of each of the dyes studied. A: Acid Red 1, B: Direct Red 81, C: Disperse Orange 1, D: Reactive Black 5

Dye Diffusing Substrates

Dye diffusing substrates were prepared in 30 mL cups with a 2% agar (by weight) solution amended with approximately 30 mg L^{-1}, or not amended as a control. The target dye concentration was chosen based on estimates that dye concentrations in effluent range from 10 to 50 mg L^{-1} (Laing 1991). Seven reps of each amended agar solution were covered with fritted glass to select for autotrophs, and seven were covered in cellulose to select for heterotrophs. The cups were then placed in the stream tank for 21 days.

Following incubation, the glass frits and cellulose discs were removed from the agar and placed in 50 mL centrifuge tubes. The dissolved oxygen (DO) was measured in ambient water from the stream, and this water was then used to fill the centrifuge tubes. The tubes were overfilled slightly and then capped tightly to minimize air in the sample, and the time of capping was recorded. The tubes were incubated under fluorescent light for 2 hours, and the DO following incubation was recorded, along with the time the measurement was taken. The tubes were then refilled with fresh stream water and the procedure was repeated, but incubation took place in the dark. Glass frits were then frozen for chlorophyll *a* analysis and the cellulose discs were discarded.

The oxygen data was used to calculate gross primary production (GPP), community respiration (CR) and net ecosystem production (NEP). Each of these values was expressed as a change in oxygen per unit surface area per unit time. The oxygen concentration in each tube was converted from mg L^{-1} O_2 to μg O_2. Change in time was converted to decimal hours, and the final value was obtained by dividing μg O_2 by (decimal hours x surface area). NEP was obtained by adding together GPP and CR. Treatments with an NEP greater than 0 were classified as autotrophic, and those with an NEP less than 0 were classified as heterotrophic.

Concentrated Exposures

Ceramic tiles were placed in the artificial stream for approximately five weeks to colonize a community of biofilm. Dye solutions of Acid Red 1 were prepared in concentrations of 30 mg L^{-1} and 100 mg L^{-1}. Four tiles were placed in each of these baths and stored under fluorescent light for the duration of the study to simulate a pulse exposure to a high concentration of dye. Samples of algae were collected at specific time intervals (t=0, t=5, t=15, t=30, t=60, t=3h, t=6h, t=12h, t=24h), concentrating the majority of the sampling soon after submersion and continuing to sample up to 24 hours, by scraping the algae from an area of 1 cm^2 of the tile into a centrifuge tube, diluting with deionized water, and then filtering the solution through a 0.45 μm glass fiber filter. These solutions were then stored in the freezer until analysis for chlorophyll *a*.

Chlorophyll a *Analysis*

Chlorophyll *a* concentrations were measured as a proxy for phytoplankton activity. These measurements differ from GPP calculations, because GPP calculations measure the change in dissolved oxygen in the community as a whole and therefore are a more robust measurement, while chlorophyll *a* data are a proxy for this activity using the assumption that more primary production is the result of more chlorophyll. Chlorophyll *a* concentrations were determined using a protocol modified from EPA Method 445.0 (Arar and Collins 1997). The chlorophyll *a* pigment was extracted using 90% acetone and a fluorometer (Trilogy, Turner Designs) was used to determine the concentration of

chlorophyll *a* (μg L^{-1}). The samples were then acidified and rerun to determine the concentration of phaeophytin *a* (μg L^{-1}), a chlorophyll degradation product.

Results

Effect of Textile Dyes on Community Production and Respiration

Gross Primary Production (GPP) on glass substrates across all dye treatments was 2.87 ± 0.27 μg L^{-1} O_2, while on cellulose substrates GPP was 3.33 ± 0.25 μg L^{-1} O_2. Community Respiration (CR) on glass substrates was -1.21 ± 0.23 μg L^{-1} O_2), while CR on cellulose substrates was -2.34 ± 0.20 μg L^{-1} O_2). Net Ecosystem Production (NEP) on glass substrates was 1.66 ± 0.31 μg L^{-1} O_2, while NEP on cellulose substrates 0.974 ± 0.267 μg L^{-1} O_2. Direct Orange 1 exhibited a significant difference from the control in CR and NEP in algal samples (glass substrate), and Reactive Black 5 showed a significance difference in NEP in bacterial samples (cellulose substrate, Figure 2.3). Although the trends are not significant, the suppression of GPP and/or CR, and the maintenance of ecosystem balance in NEP is interesting to note. In the cellulose substrates, we can see that the addition of dyes tended to make the community more autotrophic, indicating that the dyes may have modified the environment in a way that made autotrophy a more competitively favorable condition, either by toxically affecting the bacteria or by rendering the cellulose substrate unusable.

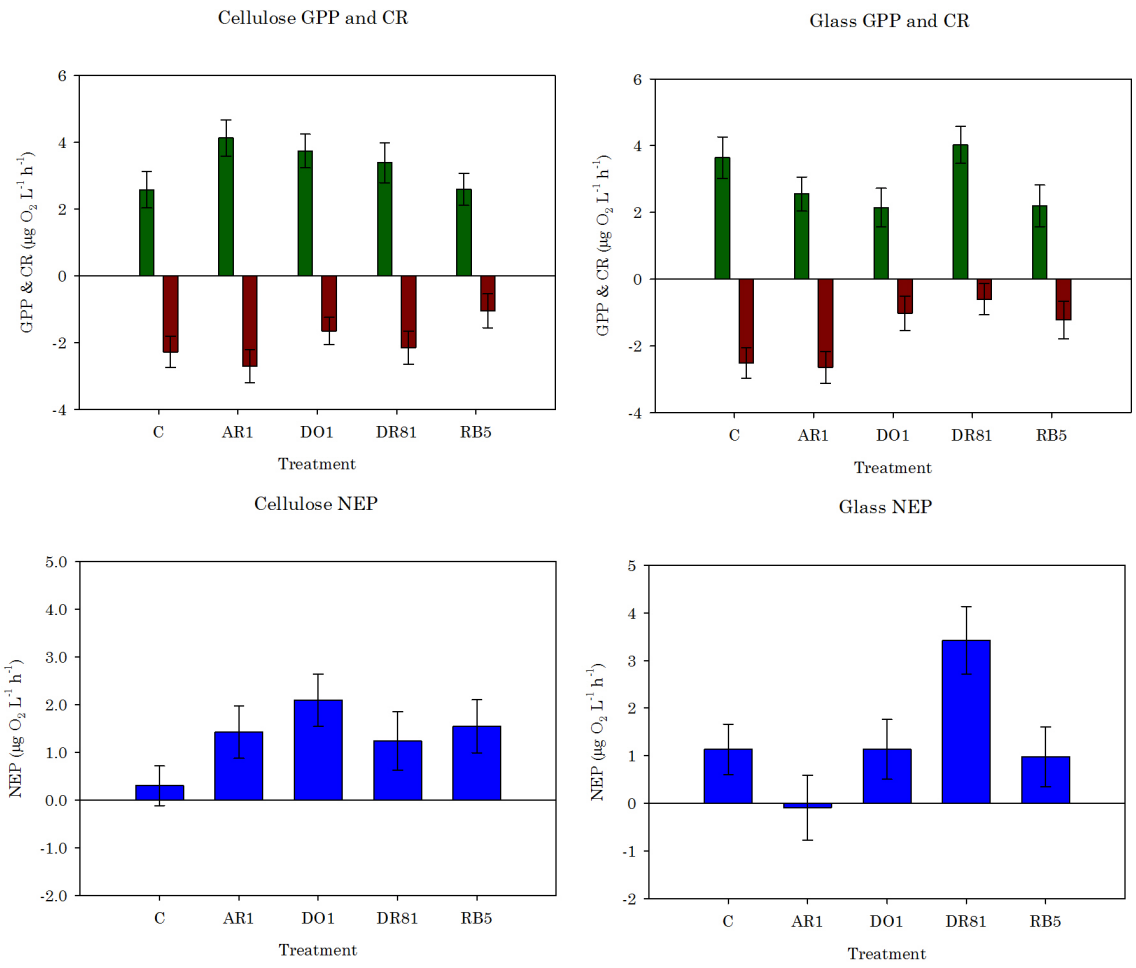

Figure 2.3. Gross Primary Production and Community Respiration (top) and Net Ecosystem Production (bottom) on cellulose (left) and glass (right) substrates, after 23 days exposure in the stream. C: Control, AR1: Acid Red 1, DO1: Disperse Orange 1, DR81: Direct Red 81, RB5: Reactive Black 5. DO1 glass CR, RB5 cellulose NEP, and DO1 glass NEP differ significantly from the control ($p < 0.05$).

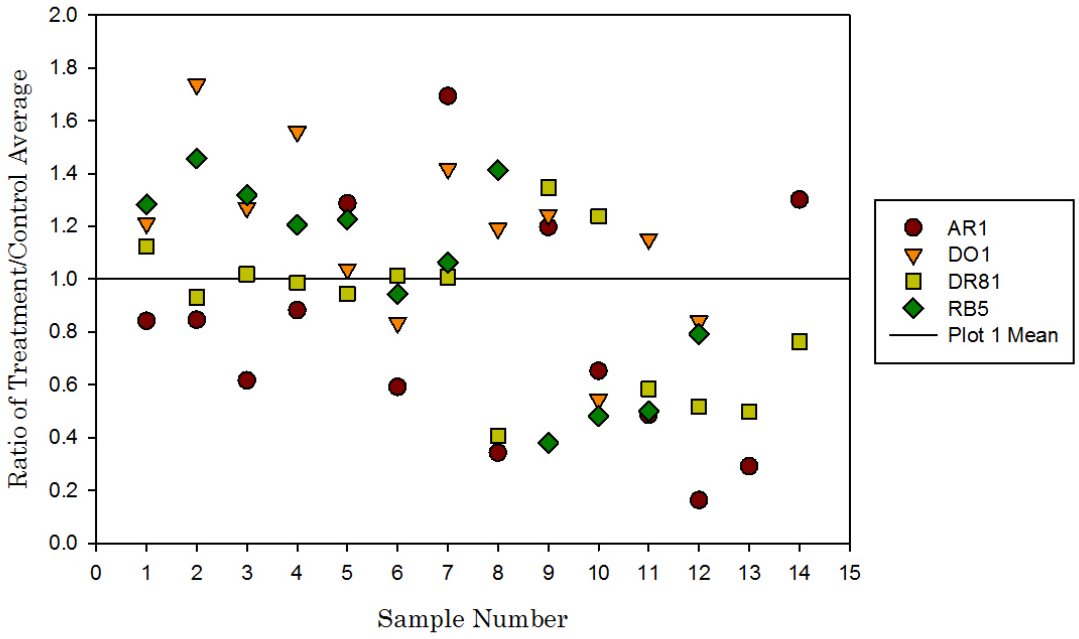

Figure 2.4. Ratios of treatment to control gross primary production for each dye type, measurements by chlorophyll *a* analysis and change in oxygen concentration shown, as both of these methods were used to estimate GPP. The reference line at y=1 indicates a lack of deviation from the control, and values <1 show suppression of chlorophyll *a* or GPP, values >1 show increased chlorophyll *a* or GPP.

The ratios of treatment: control for each dye type can be used to examine the influence of each dye on primary production, with points closest to the line y=1 showing the least change in GPP and the points furthest away showing the greatest change. 9 of 12 Disperse Orange 1 trials (orange triangles) are located above the line y=1, indicating increased GPP in those treatments, while 10 of 14 Acid Red 1 trials (red circles) are located below the line y=1, indicating suppression of GPP. Reactive Black 5 trials are dispersed evenly above (7/12) and below (5/12) y=1 and trials of Direct Red 81 are clustered near the line, and therefore do not indicate a deviation from the control.

Effect of Acid Red 1 on Primary Production Over Time

At 30 mg L^{-1}, there may be a significant change in primary production over time (ANOVA, n=35, p=0.0539) (Figure 2.5). There is a significant difference between the

control and chlorophyll at t=30, but no further significance between any binary combination of sample times. However, there is a significant difference between unexposed (t=0) and exposed (all other t) samples (t=2.06, df=36, p=0.0466). The chlorophyll *a* concentration before exposure was 4848 ± 751 µg L^{-1}, while the chlorophyll *a* concentration following exposure was 3276 ± 299 µg L^{-1}. This indicates that while dyes may suppress primary production, this effect was not detectable between each time fraction. We can see in Figure 2.5 that primary production has a general decreasing trend in the first 60 minutes of dye exposure.

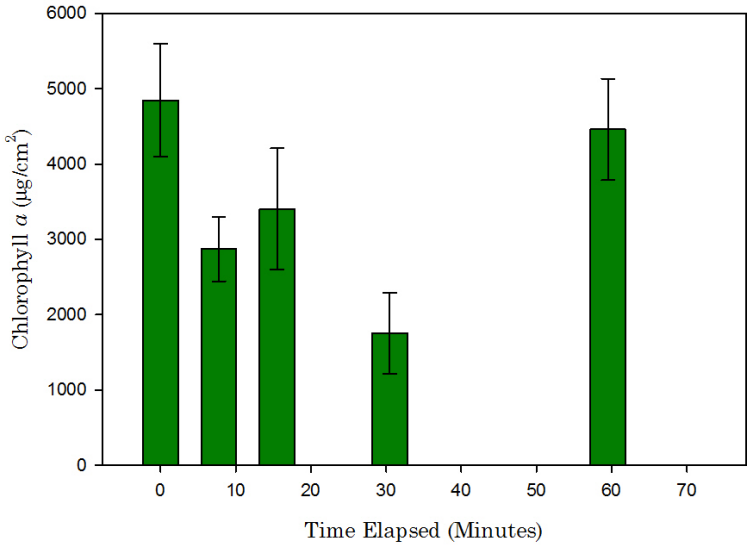

Figure 2.5. Chlorophyll *a* response over one hour to a pulse concentration of 30 mg L^{-1} of Acid Red 1. The measurement at t=30 differs significantly from the initial ($p < 0.05$), and a generally decreasing trend can be seen in the first 60 minutes of exposure. The time chart has been cut at 60 minutes as the rest of the data was relatively stable out to 24 hours.

There was no significant difference in primary production over time at an Acid Red 1 concentration of 100 mg L^{-1} (ANOVA, n=34, p=0.4725), but we can see a general decreasing trend in the first hour (Figure 2.6). The measurement at t=60 differs significantly from the control ($p<0.05$), but there is no other significance between any binary combinations of sampling times. Although we see a general decline through the first hour, high variability makes it difficult to determine if this is a statistically-

significant trend. There was a significant difference in exposed (t=0) and unexposed (all other t) samples (t=2.57, df=35, p=0.0147). The chlorophyll *a* concentration before exposure was 4848 ± 751 µg L^{-1}, while the concentration after exposure was 2951 ± 292 µg L^{-1}. This indicates that although significant decreases in algal activity cannot be statistically detected between time fractions, the dyes have a general effect of suppressing algal activity.

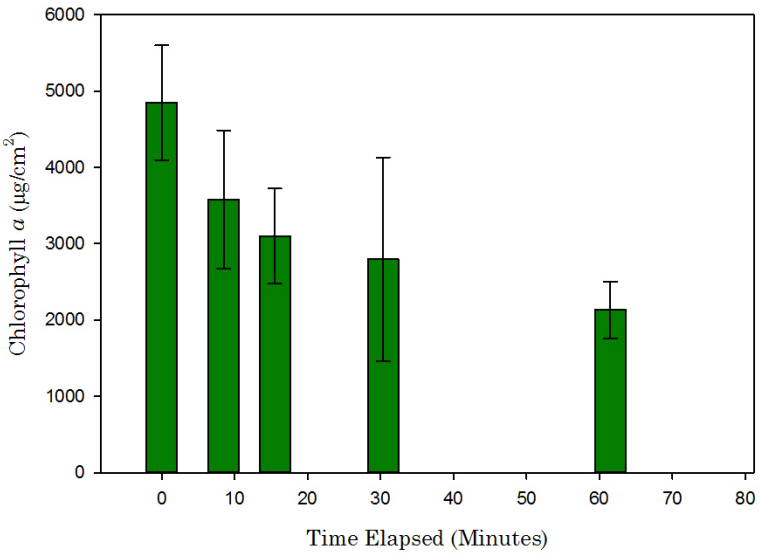

Figure 2.6. Chlorophyll *a* response over one hour to a pulse concentration of 100 mg L^{-1} of Acid Red 1. The measurement at t=60 differs significantly from the initial ($p<0.05$), and a generally decreasing trend can be seen in the first 60 minutes of exposure. After 60 minutes, the chlorophyll *a* concentration remained roughly stable (data not shown).

Discussion

Effects of Dyes on NEP and GPP

There were some interesting trends in the effects of treatment with dye at prolonged low concentration on Net Ecosystem Production (NEP; Figure 2.3). In the glass substrates, we see that Disperse Orange 1 and Reactive Black 5 have very similar NEP in comparison to the control, but decreased rates of primary production and community respiration from the control. Thus, it appears that these dyes suppress both carbon production and carbon consumption in equal amounts, maintaining a balance between the two but at a reduced production capacity. A wastewater treatment study on

the effects of azo dyes on activated sludge found that azo dyes inhibit microbial respiration (Ogawa et al. 1978).

Acid Red 1 also had decreased NEP relative to the control, and Direct Red 81 increased NEP relative to the control (Figure 2.3). It appears that this was achieved in the case of Acid Red 1 by suppression of production, and in the case of Direct Red 81 by suppression of respiration. This highlights again the individuality of the dyes effect, as both of these dyes result in red color but have varying effects. All of the dyes, except for Acid Red 1, show average NEPs that are greater than 0, indicating that these dyes do not upset the net autotrophy of the stream. This is important, because preserving ecosystem balance, particularly a net carbon sink such as an autotrophic system, has relevance in both local and global warming concerns.

Figure 2.4 illustrates further the differing responses of GPP to the different dyes. Acid Red 1 was found to generally suppress production, while Disperse Orange 1 was found to augment production and Direct Red 81 and Reactive Black 5 were found to have no effect. This illustrates that detrimental ecosystem effects are dye-specific, and can't be generalized across the category of "azo dyes". Further study would be necessary to determine whether these effects are generalizable across subsets of azo dyes, as I sampled only one dye from each category.

Inhibition of Photosynthesis

In the first hour of the concentrated exposure data, we see a definite trend toward decreasing chlorophyll *a* concentrations over time, after which there is high variability. This indicates that inhibitory effects occur within the first hour of exposure, meaning that although effluent pulses may travel downstream quickly, the time it is in contact with the benthos may be sufficient to affect photosynthetic rates and therefore the energy available to the ecosystem, especially if the pulse occurs in the middle of the day, when photosynthesis rates are highest (Odum 1956). Additionally, samples exposed to concentrated solutions of Acid Red 1 had significantly lower levels of gross primary production than control samples, but there was no significant difference between responses at 30 mg L^{-1} and at 100 mg L^{-1}. This indicates either that dosage may not be a controlling factor in the suppression of primary production by Acid Red 1.

In other studies, toxic effects in bacteria have been documented at very low concentrations, and there is high variability in both testing methods and response. In a study of the effects of azo dye RP_2B on chlorophyll *a* in *Anabaena*, a cyanobacteria, increasing doses showed increasing toxic effects, and the toxic effects were seen at concentrations as low as 5 mg L^{-1} (Hu and Wu 2001). In a study of 46 dyes, including basic, metal-complex, and reactive dyes, none of the reactive dyes were found to be toxic to the sensitive alga *S. capricornutum* (Greene and Baughman 1996), indicating that dye type can be an important variable in assessing toxicity. Because I was only able to complete the pulse assay with one dye, it is impossible to determine whether my results are similar.

Because there was no detected effect of Acid Red 1 on gross primary production in the dye diffusing substrata, but an inhibitory effect was detected in the pulse exposure experiment, it appears that a potential mechanism for primary production inhibition by Acid Red 1 may be the limitation of available light for photosynthesis. The physical presence of dyes in the water may affect the transmittance of light, therefore affecting photosynthesis of aquatic producers (Hu and Wu 2001). This could be especially detrimental if high concentration pulses occurred in the natural environment during periods of high primary production, such as midday (Odum 1956). Because of the nature of the assay, it is impossible to determine whether the inhibitory effect of the dye is acute toxicity to algal cells or reduction of photosynthetic activity in existing algal cells.

Another explanation for reduced photosynthetic activity in live cells is that the dyes interfere with the mechanism of photosynthesis. Aniline, an azo dye byproduct, has been linked to methemoglobinemia, in which the affinity of the hemoglobin for oxygen is reduced (Mieyal and Blumer 1976). Because aniline affects hemoglobin with an oxidation/reduction process, it or other azo dye byproducts may play a similar role in disrupting the oxidation and electron transfer that is integral to photosynthesis. Such an explanation would explain the slight increase in Community Respiration of Acid Red 1 seen in Figure 2.1, as algae that are unable to photosynthesize efficiently would have to respire additional carbon reserves to make up the energy deficit. Similarly, because aniline blocks transport of oxygen, it may inhibit the respiratory process of algae and bacterial cells, a pattern seen in Figure 2.1 in all dyes except Acid Red 1.

The dyes or any of their breakdown products may be toxic to specific species of algae using biochemical pathways that disrupt fundamental cell processes not related to photosynthesis. Assuming that each alga is as productive after dye exposure as it was before, this would explain the reduction in primary production that does not completely collapse to zero. Because of the documented mutagenic and carcinogenic effects of azo dyes and their byproducts, it seems possible that these mechanisms could also act on algae and bacteria cells to induce cell death (Chung 1983, de Lima et al. 2007).

Study Limitations

Because this study took place in a controlled laboratory environment, and the benthic community was cultivated under artificial conditions in the greenhouse with adequate nutrients, sunlight, and warm temperatures, it is an idealized system in comparison to natural systems. Factors such as variation in source water and diurnal changes in the community were not a part of the artificial design, as the water in the stream was recirculated throughout the experiment and represents the benthic community at a specific location at a snapshot in time. Additionally, a winter community was transplanted into summer conditions to facilitate colonization, so the artificial stream was representative of neither winter nor summer conditions, as some organisms that normally have low activity in the winter may have been stimulated by the heat and light. However, because it is not feasible to release potentially toxic textile dyes into the natural environment due to their unknown effects, the best method is to study them in the controlled, but artificial, environment first.

The study is also limited because it looks only at each dye as its whole entity, without taking into account transformations that occur throughout its lifetime in the ecosystem. For example, many azo dyes can be biotransformed or photodegraded into toxic benzidine or aromatic amine byproducts, which have mutagenic and carcinogenic properties (Chung and Stevens 1993, Pinheiro et al. 2004). Wastewater treatment procedures that remove color may also result in hydrolyzed byproducts that can be damaging to downstream ecosystems (Gottlieb et al. 2003). Metabolism and photodegradation of dyes occurs very slowly in the natural environment (Clarke and Anliker 1980). Therefore, although the dyes themselves were found to have limited

effects on ecosystem production and respiration in this study, and have been found to have low toxicity to fish and mammals in other studies, byproducts of transformations as these chemicals move through the ecosystem may have a significant impact (Clarke and Anliker 1980).

Conclusions and Future Research

In the future, the time series study could be repeated with each of the dyes to determine the effects seen are particular to Acid Red 1 or generalizable across dye families. Additionally, the Dye Diffusing Substrates could be incubated outdoors in a natural stream system, as the dye leaching out is in a sufficiently low concentration as to not disrupt the general community, but rather only the communities growing on the surface of the substrates.

If the primary effect of azo dyes is the limitation of light penetration and therefore the energy available at the base of the food chain, then current approaches to policy that focus upon aesthetic standards are likely sufficient for protecting stream biofilms, as any color concentrated enough to significantly affect benthic production and respiration would be easily detectable by the human eye and therefore not be allowed under current legislation (U.S. EPA 1978, State of Maine 1997, O'Neill et al. 1999). However, if a biochemical toxicological effect is the main mechanism, such legislation may need to be revisited to determine a safe level (if any) of contaminant. Therefore, because benthic algae and bacteria are crucial elements in the stream energy cycle, it is important for further research to isolate these mechanisms and inform policy changes if necessary.

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