Decolorization of an azo and anthraquinone textile dye by a mixture of living and non-living Trametes versicolor fungus

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DECOLORIZATION OF AN AZO AND ANTHRAQUINONE TEXTILE DYE BY A MIXTURE OF LIVING AND NON-LIVING TRAMETES VERSICOLOR FUNGUS

by

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ABSTRACT

Wastewater from the textile industry is difficult to treat effectively due to the prevalent use and wide variety of synthetic dyes that are resistant to conventional treatment methods. White-rot fungi, such as Trametes versicolor, have been found to be effective in decolorizing many of these synthetic dyes and current research is focusing on their application to wastewater treatment. Although numerous studies have been conducted on the ability of both living and nonliving Trametes versicolor to separately decolorize textile dyes, no studies were found to have investigated the use of a mixture of live and dead fungus for decolorization. This study explored potential interactions between live and dead, autoclaved Trametes versicolor biomass in a mixed system by utilizing a series of batch tests with two structurally different synthetic textile dyes. Samples were analyzed by spectrophotometer and compared with controls to determine the effect of any interactions on decolorization. The results of this study indicate that an interaction between living and nonliving biomass occurred that affected the specific dye removal for both Reactive Blue 19, an anthraquinone textile dye, and Reactive Orange 16, an azo textile dye. This interaction was seen to improve the specific dye removal during the first 10-46 hours of experimentation but then diminish the specific dye removal after this period. This effect could be due to hydrophobins, which are surface-active proteins excreted by live fungi that may alter hydrophobicity. Additionally, the presence of adsorptive dead biomass could affect dye contact with degrading enzymes released from the live fungus. By expanding current knowledge of the interactions that take place in a fungal bioreactor and their effect on textile dye decolorization, this research aims to inspire more effective and less costly bioreactor designs for the treatment of textile wastewater.

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CHAPTER ONE: INTRODUCTION

Textile Industry Overview

Textiles can be found almost everywhere in modern-day life and are utilized in a wide variety of products such as clothing, carpeting and upholstery. These textiles begin their life in the shape of yarn or fabric that is formed from raw materials. They are then passed through a water-intensive process called "wet processing", which consists of fabric preparation, dyeing, printing and finishing. Following wet processing, the yarn or fabric then enters the final fabrication stage where finished textiles are assembled into their final product (EPA 1997).

Of the processes involved in producing textiles, the wet processing stage has the largest environmental impact due to its significant wastewater generation. This water-intensive operation consumes 4,200 to 22,400 liters of water for each kilogram of synthetic textile goods produced and 6,050 to 44,700 liters of water per kilogram of cotton textiles (Ayaz Shaikh 2009). The scouring, dyeing, printing and finishing conducted during this process generates the majority of the textile industry's wastewater (Lens 2002).

The textile industry uses approximately two-thirds of the world's dyestuffs (Pinheiro, Touraud et al. 2004). Synthetic dyes represent the majority of dyes used in textile processing (EPA 1997). The industry continues to shift in favor of synthetic dyes over natural dyes because synthetic dyes are cost-effective, produced in a wider variety of colors and are able to maintain their color longer. Synthetic dyes are more colorfast than natural dyes because they can resist degradation from exposure to light, detergents and bacteria (Jarosz-Wilkolazka, Kochmanska-

Rdest et al. 2002; Couto 2009). These qualities, while certainly desirable to consumers, also make synthetic dyes highly recalcitrant and difficult to treat in wastewater.

The global textile industry has enjoyed a history of steady, moderate growth. Although the unfavorable economic climate in 2009 and 2010 saw a reduced demand for textiles, the current textile dye market is on track to recover its pre-recession level by the end of 2011 ("Global textile exports" 2011). Future growth in the textile industry is predicted to be the greatest in China and India, while the U.S. and Europe are expected to have declining market shares (Nordas 2004). China, Taiwan and Korea make up the largest segment of the market for disperse dyes, while India is the most prominent country in the reactive dye market ("Global textile exports" 2011). The growth in these regions is due in part to their access to raw materials and inexpensive labor. India, the world's second largest textile producer (behind China), grows about 15% of the world's cotton, making it the third largest global cotton producer. In addition, India's hourly labor costs in the textile industry are less than five percent of the comparable U.S. labor costs (Shetty 2001). The availability of raw textile materials and low labor costs makes countries such as India highly competitive in the global textile market.

Many of the regions experiencing growth in the textile industry have historically maintained less stringent environmental regulation and enforcement than the U.S. (Moore and Ausley 2004; Salomaa and Watkins 2009). This is a concern because when textile effluent is not properly treated, the potential for harmful environmental effects is significant. Recently, however, China and India have experienced significant economic growth and along with it has come an increased pressure to address environmental concerns. The textile industry itself currently faces pressure to address environmental concerns from both regulatory bodies and

consumers. Multinational corporations have pushed suppliers and textile manufacturers to adopt more environmentally-friendly practices in response to consumer demand (U.N. 2005). The increasingly stringent environmental regulations and outside pressure for better practices renews the call for more efficient and cost-effective means of treating textile industry wastewater.

Wastewater from the Textile Industry

Most of the water used in the wet processing stage leaves as effluent after use, resulting in the release of large quantities of wastewater. Dyeing and rinsing operations using disperse dyes produce an average of 12 to 17 gallons of wastewater for each pound of product produced. Even more wastewater is generated with reactive and direct dyes, which generate approximately 15 to 20 gallons per pound of product. The average dyeing facility produces one to two million gallons of wastewater per day (EPA 1997).

The wastewater generated from the dyeing and rinsing process contains a significant level of dye and associated chemicals (EPA 1997). Dye color, which can be seen at concentrations as low as 1 mg/L, is often released in concentrations of 300 mg/L (Couto 2009). After a reactive dye process, effluent may even contain concentrations as high as 800 mg/L (Lens 2002). The wastewater released from the textile industry also has a high alkalinity, Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD) and Total Dissolved Solids (TDS) (Kaushik and Malik 2009).

Textile dye poses a number of problems when it is released in wastewater discharge. Some dyes are carcinogenic or may release toxic aromatic compounds when metabolized by bacteria (Martins, Lima et al. 2003; Couto 2009; Osma, Toca-Herrera et al. 2010). However, regardless of their toxicity, dyes are problematic because of the color they impart to the water.

In natural waters, even very small amounts of dye can be visible and prevent sunlight from filtering down to the aquatic plants and other organisms that rely on photosynthesis. By blocking the sunlight and reducing photosynthetic activity in water, colored dyes indirectly reduce the dissolved oxygen concentration. This means there is less oxygen available to support fish, plants and other aquatic life (Sukumar, Sivasamy et al. 2009). Because of these problems, it is necessary to treat textile effluent by decolorizing and degrading the various dyes it contains.

Current methods of treating textile dye in effluent are expensive, inefficient and have several challenges (Couto 2009). Treatment with bacteria in conventional methods such as activated sludge or biofilm systems is not effective (Yang, Li et al. 2009). Although the activated sludge process effectively removes COD, the biodegradation of dyes is insignificant (Lens 2002). Bacteria are not effective at treating the complex mixtures of dyes released from the textile industry because they tend to specialize in breaking down very specific compounds (Yang, Li et al. 2009). Additionally, bacteria often need to be preconditioned and are susceptible to high levels of toxicity (Barr and Aust 1994). Because of the unfavorable conditions and large variation of dyes present in textile effluent, bacteria are not able to effectively treat it.

Other methods of removing textile dyes from effluent have previously been explored. Coagulation and flocculation can achieve significant removal of sulfur and disperse dyes; however, it is not effective in removing acid, direct, reactive or vat dyes. This is significant because reactive dyes alone make up 20-30% of the total dye market. Oxidative processes, which are the most widely-used method of decolorizing textile dyes, have their own disadvantages. They require large amounts of chemicals such as hydrogen peroxide and also require chemical additions to adjust the pH (Lens 2002). Even with these tools, the

effectiveness of both chemical and biological methods in the treatment of textile manufacturing wastewater is only around 20-30% (Yang, Li et al. 2009).

Treatment of Textile Dyes with White-Rot Fungi

Fungi have been increasingly studied in their application to the problem of treating textile effluent. Studies have determined that "by far the single class of micro-organisms most efficient in breaking down synthetic dyes is the white-rot fungi" (Couto 2009). Unlike bacteria, these fungi can decolorize a wide range of dyes without the need for preconditioning. They can also withstand higher toxicity levels than bacteria due to their extracellular enzymes. White-rot fungi are also capable of adjusting the pH of their environment (Barr and Aust 1994).

White-rot fungi remove dyes through dye molecule adsorption onto the fungal cells and through enzymatic degradation. The process of adsorption occurs when fungi take up dye molecules, removing them from the water. Although the underlying mechanisms of adsorption are not entirely understood, it is believed that the surface of the fungal biomass acts as an ion exchanger and binds the dye molecules to it (Anastasi, Prigione et al. 2009).

Enzymatic degradation occurs when fungi produce extracellular enzymes that break down the dye molecules in the water. The main enzymes produced by white-rot fungi are lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase, which are designed to break down lignin in wood and other materials. These ligninolytic enzymes also have the ability to degrade a wide array of pollutants including mixtures of dyes in wastewater (Couto 2009).

The combined ability of white-rot fungi to adsorb and degrade dyes makes it promising for use in textile wastewater treatment. The development of an efficient bioremediation method

to remove textile dyes from wastewater could result in a more cost-effective and environmentally-friendly treatment approach (Sukumar, Sivasamy et al. 2009).

CHAPTER TWO: LITERATURE REVIEW

Fungi Suitable for Textile Dye Removal

Most research into fungal remediation of textile dye focuses on white-rot fungi. This type of fungus gets its name because it is able to degrade the lignin in wood, leaving behind the light-colored cellulose. This leaves the wood substrate with a bleached appearance, which is what gives the lignin-degrading fungi their name. Since the term "white-rot fungi" is based on a physiological characteristic, it does not denote a taxonomic classificiation. Most white-rot fungi are basidiomycetes, although white-rot fungi belonging to other classifications such as ascomycetes have been found (Pointing 2001).

The research on textile dye decolorization has focused on white-rot fungi due to their unique characteristics that make them well-suited to this application. White-rot fungi utilize nonspecific mechanisms that allow them to degrade a wide variety of recalcitrant compounds. When these types of fungi are subjected to limited carbon, nitrogen or sulfur nutrients, ligninolytic enzymes are produced. The purpose of these enzymes is to degrade lignin molecules, which are difficult to break down due to their complex and irregular polymer structure (Barr and Aust 1994). In fact, white-rot fungi are the only known microorganism that is able to significantly degrade lignin (Pointing 2001). In addition to degrading a wide range of pollutants, the extracellular enzymes produced by white-rot fungi also allow the fungi to withstand high concentrations of toxic materials that would inhibit the growth of other microorganisms (Barr and Aust 1994).

Another advantage of using white-rot fungi to degrade dyes is that they do not require preconditioning. Bacteria produce degrading enzymes in response to their exposure to pollutants and therefore normally require preconditioning. Fungi, however, produce degrading enzymes when nutrients such as nitrogen are limited. Because the production of enzymes in fungi is independent of exposure, low concentrations of dye do not decrease the production of enzymes as is seen with bacteria. This means that white-rot fungi are able to achieve nearly complete removal of dyes without diminishing enzyme production (Barr and Aust 1994).

Of the papers reviewed, the majority focused on the strains Trametes versicolor, Phanerochaete chrysosporium, Pleurotus ostreatus and Bjerkandera adusta, although several studies attempted to isolate new strains or screened other types of white-rot fungi (Ali, Hameed et al. 2008; Hernandez-Luna, Gutierrez-Soto et al. 2008; Anastasi, Prigione et al. 2009; Yang, Li et al. 2009). Aspergillus niger has also been studied for its ability to remove textile dye as a biosorbent (Fu and Viraraghavan 2000; Fu and Viraraghavan 2002; Aksu and Karabayur 2008; Ali, Hameed et al. 2008).

Textile Dyes

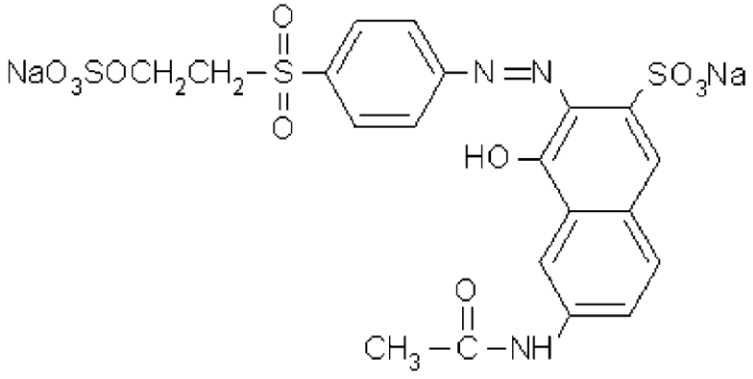
The recent research focuses on azo dyes and anthraquinone dyes because of their common use in the textile industry and their recalcitrant qualities (Osma, Toca-Herrera et al. 2010). Of the sources surveyed, researchers used the dyes Drimarene Blue, Acid Red, Remazol Brilliant Blue Reactive (RBBR, Reactive Blue 19 or RB-19) and Reactive Orange 16 (RO-16) the most.

Reactive dyes form strong, covalent bonds with textile fibers, producing textiles that are colorfast and do not fade or run. This property, along with their ease of application, make

reactive dyes very popular in the textile industry. Because these dyes are so resistant to degradation, they are also very recalcitrant in wastewater. Additionally, they have a low fixation efficiency, which means that a large percentage of the dye does not affix to the textile and passes through in the wastewater (Rezaee, Ghaneian et al. 2008). This results in the presence of significant quantities of textile dye in the effluent, which is problematic for treatment.

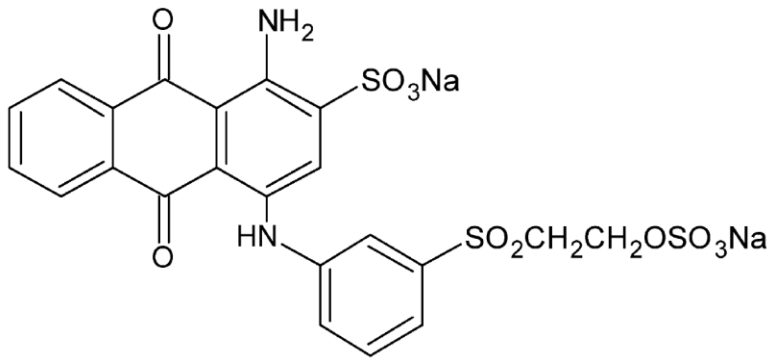
Reactive Orange 16 is an example of a monoazo dye, which is a class of dye that contains one nitrogen double bond, or azo bond (Pinheiro, Touraud et al. 2004). The molecular structure of Reactive Orange 16 is shown in Figure 1 (Pointing 2001). The molecule's azo bond has a tendency to withdraw electrons, shielding the dye molecule from oxidative reactions (Maddhinni, Vurimindi et al. 2006). Azo dyes are targeted for decolorization research studies because they make up an estimated 60-70% of all textile dyes used. Additionally, their azo bonds and sulfonate groups make them highly resistant to degradation (Pinheiro, Touraud et al. 2004), presenting a significant problem in textile wastewater treatment.

Figure 1. *Reactive Orange 16 Molecular Structure*



Anthraquinone dyes are the second most important class of textile dyes, behind azo dyes. Reactive Blue 19, shown in Figure 2 (Song, Yao et al. 2008), is an example of an anthraquinone dye, which is a class of dyes derived from anthracene with a general formula containing a quinoid ring (Osma, Toca-Herrera et al. 2010). It is this highly-stable aromatic anthraquinone structure that makes this dye very resistant to chemical oxidation (Rezaee, Ghaneian et al. 2008). Reactive Blue 19 is one of the most common anthraquinone dyes in the textile industry because it is used as the foundation for the creation of polymeric dyes (Osma, Toca-Herrera et al. 2010).

Figure 2. *Reactive Blue 19 Molecular Structure*



Although the structural properties of azo and anthraquinone dyes make these synthetic dyes difficult to treat with conventional methods, several types of white-rot fungi have been studied for their ability to degrade these dyes. In particular, Trametes versicolor has been shown to successfully treat these types of dyes in prior studies (Swamy and Ramsay 1999; Erkurt, Unyayar et al. 2007; Srinivasan and Murthy 2009).

Fungal Application Methods

Living Fungal Biomass

Current research studies on fungal remediation of textile dyes may be classified by the type of decolorizing material they use: living cells, non-living cells or enzyme extracts. Living fungal biomass has been studied for its ability to employ both biosorption and enzymatic degradation of dye molecules. Biosorption is a dye removal mechanism in which fungal biomass uptakes or accumulates a contaminant. This process occurs because of an active metabolism-driven transport mechanism and a passive binding of dye molecules to the mycelium (Aksu and Tezer 2000). Enzymatic degradation of dyes is the breakdown of chemical compounds by biological enzymes released from fungi (Kaushik and Malik 2009).

One advantage of using live fungal cells is that they can use both the active and passive biosorption mechanisms as well as direct enzymatic degradation to remove dyes from water (Kaushik and Malik 2009). Enzymatic degradation, which only occurs with live biomass, removes the largest share of dye. In one study, Benito et al. (1997) found that adsorption only represented 10% of color removal by Trametes versicolor while the remaining 90% was removed biologically.

Although highly effective, the use of live fungi does present a few challenges as well. One limitation is that the appropriate conditions must be maintained in a live cell reactor in order to sustain fungal growth (Kaushik and Malik 2009). Some live fungi may also be susceptible to disruption from bacteria, but Hai et al. (2009) were able to achieve 93% removal of an azo dye using white-rot fungi in a non-sterile environment. Yang et al. (2009) successfully developed a

biofilm reactor that used a mixture of bacteria and fungi to effectively treat real textile wastewater; the fungi were found to be the predominant microorganism present.

Live white-rot fungi produce three main extracellular enzymes that are capable of degrading dye molecules: laccase, lignin peroxidase (LiP) and manganese peroxidase (MnP). These ligninolytic enzymes are designed to break down lignin in wood but also have the ability to degrade a wide array of pollutants including mixtures of dyes in wastewater (Couto 2009). As these enzymes break down dye molecules, intermediate products are formed. These intermediary byproducts are currently the focus of studies to determine if they are hazardous. When azo dyes are broken down by bacteria, it is possible for toxic anilines to form. However, Martins et al. (2003) examined the metabolites from the degradation of an azo dye by Trametes versicolor and found no toxic anilines were present. Osma and Toca-Herrera et al. (2010) identified two intermediate products and two final products in the degradation of RBBR dye by Trametes pubescens and found they were less phytotoxic than the original dye. A number of studies have also proposed metabolic pathways based on the observed metabolites during enzymatic degradation of dye molecules (Martins, Lima et al. 2003; Vanhulle, Enaud et al. 2008; Osma, Toca-Herrera et al. 2010). Additionally, some research papers have discussed immobilizing fungi in their live state to improve bioreactor effectiveness (Couto 2009; Enayatzamir, Alikhani et al. 2009; Hai, Yamamoto et al. 2009).

Non-living Fungal Biomass

Non-living fungal biomass is able to remove textile dyes through the process of adsorption. Because the biomass has no metabolism, it is unable to produce extracellular

enzymes or actively uptake dye molecules, although passive adsorption still occurs (Aksu and Tezer 2000). Dead fungal biomass has been studied as a potentially useful adsorbent to remove textile dyes because it may be cheaply obtained as a byproduct from industrial fermentations (Kaushik and Malik 2009).

One advantage of using adsorption as a dye removal process is that it is relatively quick and is usually complete within a matter of hours (Mou, Lim et al. 1991). Additional factors may play a part in the kinetics of adsorption. In one study, Binupriya et al. (2008) determined that the rate of adsorption onto Trametes versicolor biomass was dependent upon pH and achieved a better dye removal at a lower pH value. This study also found that the adsorptive properties of Trametes versicolor pellets increased when the biomass was autoclaved. Fungal biomass adsorbent can be applied in a wider range of conditions because it is not affected by any contaminants and the biomass may even be reused (Aksu and Karabayur 2008).

Enzyme Extracts

There are also a number of studies on the use of fungal enzyme extracts to decolorize textile dyes. These enzymes degrade dye in the same way that live fungal biomass does, although extracts allow for greater control over the available enzyme concentration in bioreactors. Higher enzyme concentrations may be useful in remediating pollutants that can be directly broken down by the enzyme, but in some instances a greater concentration of enzyme may actually hinder removal. Like lignin, some pollutants are not directly broken down by enzymes but are oxidized by the intermediate free radicals produced. In these cases, excess enzyme could oxidize these radicals and reduce the dye degradation capability. Indeed, studies

have shown that the addition of an excess of enzyme beyond a certain point actually decreased lignin degradation (Barr and Aust 1994). In order to overcome problems such as this, a better understanding of the pathways of dye degradation needs to be achieved.

Enzyme extracts are an attractive treatment option because enzymes are easier to use than live fungi. Enzymes may be applied like a chemical treatment and can be used in cases where the effluent contains chemicals toxic to fungi (Kaushik and Malik 2009). The main disadvantage of using enzyme extracts is that large quantities must be used because the enzymes are washed out of the system with the effluent (Hai, Yamamoto et al. 2009). To alleviate the problem of enzyme wash-out, Bayramoglu et al. (2010) used a reversible process to adsorb laccase onto magnetic beads that would remain within the system. This immobilized laccase enzyme from Trametes versicolor was able to successfully degrade three different reactive textile dyes.

Conclusions

Many of the research studies surveyed aimed to optimize the process of fungal decolorization of textile dyes. Some focused on improving the type of reactor to be used. In the research studies surveyed, most experimental setups used batch bioreactors, although a few studies had success with other types such as a membrane bioreactor (Hai, Yamamoto et al. 2009) and a fixed-bed bioreactor (Enayatzamir, Alikhani et al. 2009).

Other studies examined the role of parameters such as growth media, pH, temperature and agitation on dye removal. Interestingly, some of the papers came to opposite conclusions. Ali et al. (2008) reported no major differences in the dye removal effectiveness of fungal strains grown on different culture media. In contrast, Anastasi et al. (2009) found that the type of culture media greatly affected the adsorption capability in their experimentation. Also, Ali et al.

(2008) determined that maximum decolorization occurred when experiments were agitated, however Sukumar et al. (2009) reported maximum decolorization when experiments were static. While these conflicting results could possibly be attributed to different conditions in each experiment, they highlight the need for further study to optimize the decolorization process.

Currently, there are a few different research areas that are being explored. One area is the collection and isolation of new strains of fungi that may be able to outperform the more commonly studied strains such as Trametes versicolor (Hernandez-Luna, Gutierrez-Soto et al. 2008; Yang, Li et al. 2009). Another area being explored is the optimization of bioreactor parameters like pH, temperature and incubation conditions for specific strains (Aksu and Karabayur 2008). Additionally, researchers are looking at utilizing fungi in different types of bioreactors in order to reduce the time required for treatment, reduce enzyme washout and protect against bacterial interference (Enayatzamir, Alikhani et al. 2009; Hai, Yamamoto et al. 2009). These studies will continue to improve our knowledge of fungal decolorization of textile dyes and allow for the future development of optimized processes.

CHAPTER THREE: THEORY AND HYPOTHESIS

Overview

Of the literature reviewed, a number of studies examined live fungi and dead fungi separately, but none were found that addressed the effectiveness of a mixture of live and dead biomass. Mixtures have some potential advantages if they are able to maintain an acceptable dye removal efficiency. This is because less live cells would be present, which would reduce the need for any necessary nutritional additions. Additionally, spent live biomass could be collected and reused as dead adsorbent biomass, reducing the amount of sludge disposal required.

However, the effectiveness of a mixture of live and dead fungal biomass may be affected by interactions between the active and non-active biomass. Surface-active proteins secreted by fungi can alter hydrophobic and hydrophilic surfaces (Wösten and de Vocht 2000; Linder 2009), which may alter the dye-removal of dead biomass or change an absorptive quality of dye molecules. The physical presence of dead biomass could also impact the contact between degrading enzymes and dye molecules. Alternately, the presence of the enzymes may interfere with the adsorption of dye molecules onto the dead biomass.

The effect on dye decolorization of any potential interactions between living and non-living fungal biomass shall be examined in this paper. If mixtures are found to be effective at dye removal, the development of mixed systems to reduce the cost of textile effluent treatment and sludge disposal should be explored. If mixtures inhibit decolorization, then a goal of fungal

bioreactors with live cultures should be to reduce the amount of dead or inactive biomass present in the system.

Pretreatment of the Biomass Adsorbent

Like other white-rot fungi, Trametes versicolor is capable of removing dye molecules from water through the process of biosorption. This process may involve an active uptake of dye molecules as well as a passive adsorption of dye onto the biomass. When inactivated fungi are used for dye removal, they do not produce enzymes and therefore decolorization is due to adsorption (Bayramoglu, Bektas et al. 2003). Although enzymatic degradation does not occur, non-living fungal biomass can be an effective adsorbent. In some cases, non-living biomass may even achieve higher dye removals than live fungi (Fu and Viraraghavan 2001). Additionally, it is an attractive adsorbent because biomass may be generated in mass quantities and is inexpensive to produce (Bayramoglu, Bektas et al. 2003).

The ability of dead fungal biomass to remove dye may be enhanced through the use of certain pretreatments. Arica and Bayramoglu (2007) tested the dye biosorption of fungal biomass that had been subjected to heat, acid and base pretreatments. They found that the heat-treated biomass exhibited the highest biosorption, followed by the acid-treated biomass, the live fungi and then the base-treated biomass. Binupriya et al. (2008) tested live, autoclaved, acid-treated and alkali-treated Trametes versicolor and found that the autoclaved biomass achieved a higher percent removal than the live biomass; both the acid-treated and alkali-treated biomass achieved a lower percent removal than the live biomass. These studies suggest that adsorptive properties of the biomass may be improved with the application of heat and pressure.

These pre-treatments may be effective in enhancing adsorption because of their ability to alter physical and chemical characteristics of the biomass. When fungal biomass is autoclaved, its physical surface properties are altered, which may affect its adsorption capability by increasing adsorption sites or facilitating dye contact. Images taken from a scanning electron microscope show there are marked differences between the surface textures of native and autoclaved biomass (Binupriya, Sathishkumar et al. 2008). Alternately, Bayramoglu and Arica (2007) suggested that heat-treating the biomass "may lead to an increase of the charge on the cell surface or open the available sites for the adsorption and enhance ion-exchange". Although the mechanisms are not entirely clear, the ability to enhance dye adsorption by autoclaving fungal biomass has been documented in several studies (Arica and Bayramoglu 2007; Bayramoglu and Arica 2007; Binupriya, Sathishkumar et al. 2008).

Reuse of Biomass

In addition to its effectiveness, the use of non-living fungal biomass for an adsorbent is an attractive option for textile dye removal because biomass may be reused several times. Chemicals such as methanol, ethanol and sodium hydroxide may be used to desorb dye molecules from fungal biomass and regenerate it for future cycles (Fu and Viraraghavan 2001). Using ethanol, Binupriya et al. (2008) achieved 99.9% desorption of Congo red dye from Trametes versicolor biomass in 30 minutes. Ethanol desorbs dye by dissolving the lipid components of the cell wall, thereby releasing dye molecules that have adsorbed onto the surface of the cells. The ethanol used to desorb the dyes may then be distilled and reused, while at the same time recovering dyes for reuse in industries such as plastic, glass and ceramic manufacturing that utilize low-grade dyes (Binupriya, Sathishkumar et al. 2008). Other studies

have also shown that regenerated fungal biomass may be used effectively as an adsorbent over several cycles (Fu and Viraraghavan 2001). Because of its potential for regeneration and reuse, fungal biomass is an economical alternative to more costly adsorbent materials such as activated carbon.

Potential Interactions Affecting Dye Decolorization

Although non-living fungal biomass is an effective adsorbent, living biomass is capable of removing dye through enzymatic degradation as well. The dye removal of live and dead biomass varies depending on the fungal species, culture conditions, type of dye and initial dye concentration. Fu and Viraraghavan (2001) highlighted this variability by describing three studies of different dyes that came to separate conclusions. In one study the dead biomass attained the same dye removal as the living biomass. In another, the live biomass outperformed the dead biomass, and in the third study the dead biomass outperformed the live biomass. Since both the dye and the state of the biomass can affect dye removal, mixtures of living and non-living biomass may be useful for decolorizing mixtures of dyes. This could be useful for the treatment of textile wastewater containing a mixture of dyes that are best removed by live biomass and dyes that are best removed by dead biomass.

Live and dead fungal biomass have the potential to interact within a bioreactor and this could affect the ability of the biomass to remove dye molecules. The presence of hydrophobins is one mechanism by which the two types of biomass have the potential to interact. Hydrophobins, which are surface active proteins secreted by live fungi, "play a role as a coating/protective agent, in adhesion, surface modification, or other types of functions that require surfactant-like properties" (Linder 2009). These proteins have the ability to coat

surrounding objects (Linder 2009), and self-assemble into membranes at an air-water or solid-liquid interface. Hydrophobins have both a hydrophilic end and a hydrophobic end, which allows them to change a hydrophobic surface to a hydrophilic surface, and the reverse. For example, when hydrophobins assemble on a hydrophilic surface, the hydrophilic ends are attracted to the hydrophilic surface. This causes the hydrophobic ends to face outward, effectively making the surface hydrophobic. In the same way, hydrophobins can also make a hydrophobic surface hydrophilic (Wösten and de Vocht 2000). Since dye adsorption may be due in part to hydrophobic/hydrophilic interactions (Rezaee, Ghaneian et al. 2008), it is possible that in a batch reactor undergoing agitation, hydrophobins from the live fungus will coat the dead biomass in the solution and alter the adsorption capability of the dead fungi. Alternately, hydrophobins could coat the dye molecules and alter their ability to adsorb to the biomass.

Physical interactions of the biomass could also potentially affect the dye decolorization of a mixture of living and non-living fungal biomass. If the dead biomass adsorbs or becomes coated in the degrading enzymes, the enzyme molecules could occupy available sites on the dead biomass where dye molecules could have adsorbed. It is also possible that it may affect the rate of degradation by altering the interaction between enzymes and dye molecules. Additionally, the physical presence of the dead biomass could affect the growth of the live fungi or their release of degrading enzymes, thereby impacting dye removal.

Hypothesis

Mixing live and dead fungal biomass will create an interaction that will enhance dye decolorization by the fungus Trametes versicolor. This hypothesis will be tested by determining the effectiveness of using mixed live and dead Trametes versicolor fungus to decolorize the common textile dyes Reactive Blue 19 (anthraquinone dye) and Reactive Orange 16 (azo dye). Batch experiments containing a fixed amount of live biomass and varying amounts of dead biomass will be conducted to determine the percent decolorization and the specific dye removal, which is defined as milligrams of dye removed per gram of biomass (dry weight). These results will be compared with a live fungus control and a dead fungus control to determine whether combining live and dead biomass has a beneficial effect on dye decolorization.

CHAPTER FOUR: MATERIALS AND METHODS

Microorganism and Culture Conditions

A pure culture of the white-rot fungus Trametes versicolor, strain ATCC 11235, was obtained from the American Type Culture Collection. This culture was transferred to Petri dishes containing a malt agar medium and was maintained at 30 ºC until full colonization of the plate. After colonization, the agar plates were transferred to a refrigeration unit and maintained at 4 ºC to slow growth of the fungus and preserve the live culture. Subculturing was performed every month to maintain active Trametes versicolor cultures.

The malt agar growth medium was prepared using 15.0 g of malt extract, 7.5 g of Bacto agar and 500 mL of distilled water. While stirring, the growth medium was heated on a hot plate until the agar dissolved into solution. It was then sterilized by autoclaving for 15 minutes at 121 ºC and allowed to cool in a biosafety cabinet. When the growth medium was cool enough to be handled, twelve agar plates were poured and allowed to dry inside a biosafety cabinet.

After drying and cooling, the agar plates were inoculated with the fungus. The initial fungal culture arrived in a frozen ampoule and was thawed by placing it in a 25-30ºC water bath for approximately five minutes. Using a sterile pipette, 1 mL of sterile water was added to the ampoule and then drawn back into the pipette along with the fungus. This was transferred to a test tube with 5 mL of sterile water. The fungus was allowed to rehydrate for two hours and then was transferred to the agar plates by inoculating each dish with 0.5 mL of the rehydrated fungus solution. When subculturing, new agar plates were inoculated using a previously colonized plate. With a sterile pipette, 0.5 mL of sterile water was introduced onto a plate containing an

active culture. This water was then drawn back into the pipette and transferred to a new agar plate.

Each plate was incubated for approximately 7 days at 30ºC to allow the fungus to colonize the growth medium. After colonization, the plates were refrigerated for approximately three weeks before being used for subculturing.

Dyes

An azo dye and an anthraquinone dye were chosen as representative textile dyes. Reactive Blue 19 (Remazol Brilliant Blue R) and Reactive Orange 16 (Remazol Brilliant Orange 3R) were obtained from Acros Organics and Sigma-Aldrich, respectively, for use in this study. These dyes were chosen because they are textile dyes that are highly recalcitrant in wastewater and are commonly used in the textile industry. These two dyes also have significantly different molecular structures to explore the ability of Trametes versicolor to decolorize both types.

In addition to different molecular structures, Reactive Blue 19 and Reactive Orange 16 have different chemical properties that may affect their removal from water. In the absence of experimental data, a software program was used to estimate the octanol-water partition coefficient and the solubility of each dye. The software used for this analysis was the Estimation Programs Interface (EPI) Suite, Version 4.10, which uses the chemical structure to estimate the physico-chemical properties of a substance (EPA 2011). The octanol-water partition, k_{ow}, represents the ratio of the dye concentration in octanol to the dye concentration in water. This value is an indicator of the hydrophobicity of the dye, with higher values indicating a more hydrophobic substance. This property may impact dye decolorization because hydrophobic substances are often more easily removed from water than hydrophilic substances. The k_{ow} of

Reactive Blue 19 was estimated to be $10^{-1.85}$ and the k_{ow} of Reactive Orange 16 was estimated to be $10^{-3.31}$. Additionally, the solubility of Reactive Blue 19 and Reactive Orange 16 were estimated to be 314.4 mg/L and 5,668 mg/L respectively. This suggests that Reactive Blue 19 is more hydrophobic and less soluble than Reactive Orange 16, indicating a possibility that Reactive Blue 19 may be removed more easily. In addition to providing k_{ow} and solubility estimates, the EPI software estimated only a 1.85% total removal of either dye in conventional wastewater treatment, with the majority (1.75%) due to sludge adsorption.

To determine dye concentrations during this experiment, a standard curve was created for both Reactive Blue 19 and Reactive Orange 16. Each dye was diluted to concentrations of 25, 50, 75, 100, 200 and 300 mg/L. Liquid growth medium was used to dilute the dye samples in order to better approximate the absorbance characteristics of the experimental samples. The prepared dye concentrations were analyzed by a spectrophotometer at the maximum absorption wavelength for the respective dye (590nm for Reactive Blue 19 and 494nm for Reactive Orange 16). This data was then used to create a dye concentration curve for determining the concentration of a sample based on the spectrophotometer reading.

Preparation of Live Cultures

For the dye decolorization experiments, Trametes versicolor was grown in a liquid growth medium. This medium was composed of 10 g glucose, 2.6 g KH_2PO_4, 1 g yeast extract, 0.5 g $MgSO_4*7H_2O$, 0.12 g NH_4Cl, 0.10 g $CaCl_2*2H_2O$, 0.001 g thiamine and 1 L of deionized water. 250 mL of growth medium was poured into each of twelve 500 mL Erlenmeyer flasks, covered loosely with foil and autoclaved for 15 minutes at 121 ºC. The sterilized flasks of growth medium were then allowed to cool within a biosafety cabinet.

Once the flasks were cooled to room temperature, plugs of actively growing fungus were taken from colonized agar plates and used to inoculate them. This was done by pressing a sterile metal coupling into colonized agar, cutting out a 3/8-inch diameter plug. A sterile spatula was used to remove the plug and transfer it to an Erlenmeyer flask containing the growth medium. Sterile cotton balls were used to plug the mouth of the flasks so that gas exchange could take place while reducing the risk of contamination.

The innoculated flasks were placed on a shaker table and incubated for 5 days to allow for colonization of the growth medium. The use of the shaker table provided mixing and aeration of the culture to improve fungal growth. Once completely colonized with mycelial balls, the cultures were ready to be used in the dye decolorization experiments.

Preparation of Dead Biomass Adsorbent

The dead biomass used in the dye decolorization experiment was prepared by first culturing live biomass as described in the Preparation of Live Cultures section. Once fully colonized flasks were achieved, the live biomass was filtered out of the growth medium. The filtered biomass was rinsed three times with deionized water to remove the growth medium and then autoclaved to enhance its adsorption capability as described by Binupriya et al. (2008). This biomass was then dried and stored in a desiccation chamber until used in the later experiments.

Dye Decolorization Experiments

Two dye decolorization experiments were performed. The objective of the first experiment was to determine the optimal initial dye concentration to use in later testing. In this

dye concentration experiment, dye was added in varying amounts to flasks of liquid growth medium colonized with Trametes versicolor. Sampling was used to determine the dye concentration with the greatest overall removal rate.

The objective of the second dye decolorization experiment was to determine if any interaction existed between live and dead biomass that would affect the dye decolorization. This was tested by measuring the dye decolorization rate of mixtures of live and dead biomass. The ratio of live to dead biomass was varied for each flask and the initial dye concentration was held constant at the pre-determined optimal concentration. To determine if any interaction occurred, the specific dye removal of each mixture was compared to two controls containing only live or only dead biomass.

Initial Dye Concentration Test

The optimum initial dye concentration for use in later experimentation was determined separately for each dye. Six flasks of live cultures were prepared and five were dosed with Reactive Blue 19 dye to achieve concentrations of 50, 100, 150, 200 and 300 mg/L; the remaining flask was designated a control and was not dosed with dye. Reactive Orange 16 was tested at concentrations of 0, 50, 100, 200, 300 and 400 mg/L due to its higher solubility. Once the dye was thoroughly mixed into solution, an initial sample was obtained from each flask. Samples were taken from the flasks every day and the dye decolorization was tracked. The optimal initial dye concentration was then determined by calculating which flask produced the greatest dye decolorization per gram of biomass.

Measurements were taken from duplicate 4 mL samples that were withdrawn from the cultures with a sterile pipette. These samples were centrifuged for 10 minutes at 7,000 rpm and then 3.5 mL of the sample was placed in a 10-mm square cuvette. Using a Hach DR 5000 spectrophotometer, the samples with Reactive Orange 16 dye were analyzed at 494 nm and the samples with Reactive Blue 19 dye were analyzed at 590 nm. The measured absorbances were compared with the standard curve to determine the dye concentration present in each sample.

Once the dye decolorization had been tracked for several days, the biomass in each flask was filtered out, dried and weighed. The specific dye removal per gram of biomiass was computed and the initial dye concentration in the flask with the maximum dye removal was chosen as the optimal concentration. This dye concentration was used in the experimentation with mixtures of living and nonliving biomass.

Mixed Living and Nonliving Fungus Test

While preparing the dead adsorbent biomass, the amount of biomass recovered from each flask was measured by filtering, drying and weighing it. From the measured amounts of biomass recovered, an average mass was determined. This was estimated to represent the average amount of living biomass present in the liquid cultures.

Live cultures in liquid growth medium were prepared as described in the Preparation of Live Cultures section. Once fully colonized, four flasks were dosed with varying amounts of the prepared dead biomass so that the flasks contained a ratio of live to dead biomass of 2:1, 1:1, 1:1½ and 1:2. Two flasks were controls; one contained only live biomass and the other contained only dead, autoclaved biomass in approximately the same amount. These flasks were

then dosed with dye to achieve the optimal initial dye concentration determined previously in the dye concentration test.

Samples were taken periodically to determine the extent of dye decolorization achieved by each flask. Enzymatic degradation is a relatively slow process but adsorption usually takes place within a matter of hours (Kaushik and Malik 2009). From reviewing prior studies, the adsorption was expected to be complete within the first eight hours and the majority of the enzymatic degradation complete by the eighth day. In order to better describe the contribution of adsorption to the process of decolorizing the dye, duplicate 4 mL samples were taken initially, at 2, 4, 6, 8, 10 and 12 hours, and then at 24 hours. After that time, samples were taken every 24 hours through the fifth day. The samples were centrifuged at 7,000 rpm for 10 minutes to remove the mycelium and then were analyzed in the spectrophotometer to determine dye concentration.

The theoretical specific dye removal of each mixed fungus experiment was determined using the two controls. The actual specific dye removal of the live fungus and the dead fungus was determined from the control experiments and then used in the following equation to determine the theoretical specific dye removal of the mixed cultures:

$$\mathbf{D_{mix} = (D_{live} * M_{live}) + (D_{dead} * M_{dead})} \qquad \textbf{(mg dye removed/g biomass)}$$

D_{live} = observed specific dye removal of the live control (mg/g)

D_{dead} = observed specific dye removal of the dead control (mg/g)

M_{live} = mass percent of live fungus in the biomass

M_{dead} = mass percent of dead fungus in the biomass

The calculated theoretical specific dye removal was then compared with the observed specific dye removal. If the two values were not approximately equal, this indicated that an interaction between the live and dead biomass affected the dye decolorization. If the two values were approximately equal, then this indicated that no interaction took place.

CHAPTER FIVE: ANALYSES AND FINDINGS

Overview

The two textile dyes that were tested were removed to varying degrees by the living and non-living, autoclaved biomass. Based on the solubility and octanol-water partition coefficients of the dyes, the Reactive Blue 19 dye was expected to be more readily removed. This is because Reactive Blue 19 has a larger octanol-water partition coefficient, indicating greater hydrophobicity, as well as a lower solubility than Reactive Orange 16. Indeed, the experimental results showed that Reactive Blue 19 was removed to a greater extent than Reactive Orange 16. Live biomass was able to achieve the greatest removal of the Reactive Blue 19 dye with 94% removal. Dead biomass was able to achieve the greatest removal of the Reactive Orange 16 dye with 64% removal over the course of the experiment. Additionally, the removal of Reactive Blue 19 was significantly quicker than the removal of Reactive Orange 16. While the live culture achieved 93.7% removal of Reactive Blue 19 within 46 hours, only 48.3% of the Reactive Orange 16 had been removed by the dead biomass at this time.

The results show that adsorption and enzymatic degradation are both effective mechanisms for the removal of Reactive Blue 19, although degradation is the most effective. The dead biomass control and the live biomass control both removed approximately the same amount of dye over the course of the experiment (93% and 94%, respectively). Because the biomass in the live control had a lower mass, the live biomass was able to remove more dye per gram of biomass. However, the results indicate that Reactive Blue 19 is readily removed through both adsorption and enzymatic degradation.

Unlike Reactive Blue 19, adsorption was found to be the more effective mechanism for removal of Reactive Orange 16. The dead biomass achieved 64% removal by the end of the experiment but the live biomass only removed 42%. The dead biomass also achieved a higher rate of dye removal.

To screen for potential interactions that affect dye removal, living and nonliving biomass was mixed to various approximate ratios and dosed with textile dye. The results show that the mixing of live and dead biomass did not affect the overall dye removal for Reactive Blue 19. The trajectory of the Reactive Orange 16 dye concentration curves suggests that this would also be the case with this dye if the systems were allowed to reach equilibrium. Although no interactions were detected that affected the overall dye removal, significant interactions were found that affected the dye removal kinetics.

Standard Curves

To determine the concentration of dye present in a sample using absorbance measurements, standard curves were created for each dye. Samples containing known concentrations of dye were analyzed with a spectrophotometer and the absorbance measurements were plotted. Using linear regression, a curve equation for each dye was determined. This equation was used in the later testing to determine the concentration of dye present in a sample. The standard curve and linear equation for Reactive Blue 19 dye is shown in Figure 3. The standard curve and linear equation for Reactive Orange 16 dye is shown in Figure 4.

Figure 3. *Reactive Blue 19 Dye Standard Curve*

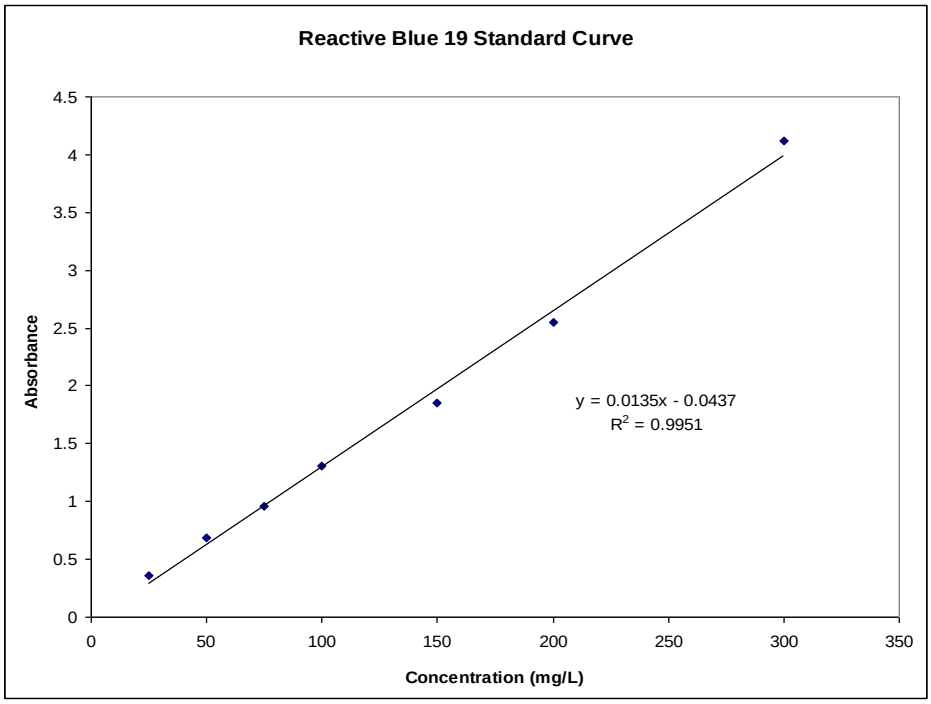
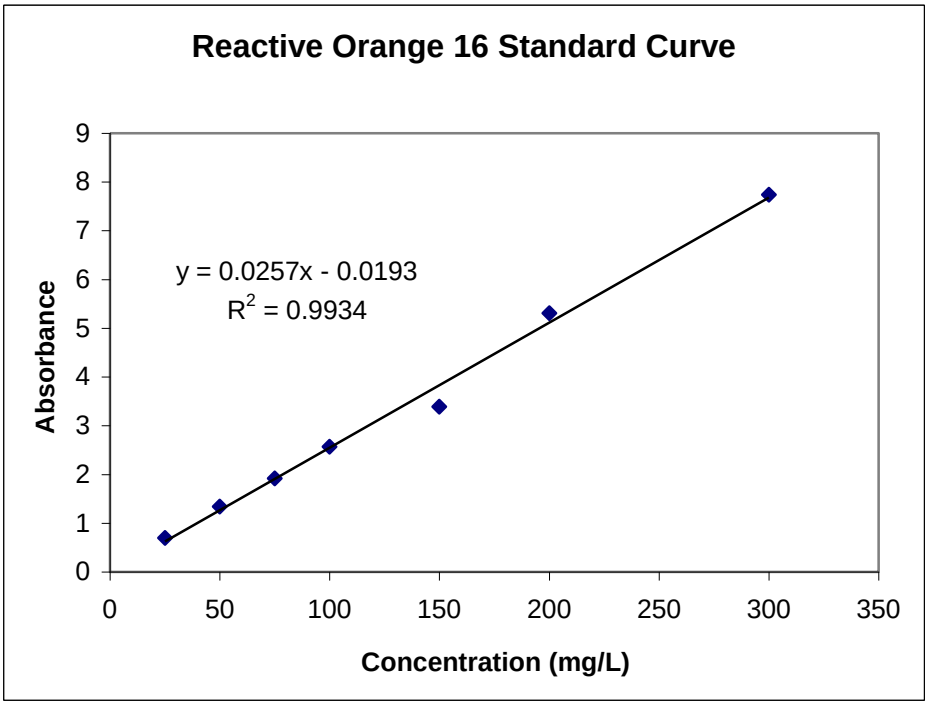


Figure 4. *Reactive Orange 16 Dye Standard Curve*



Initial Dye Concentration

Varying concentrations of dye were added to flasks containing live cultures to determine the effect on dye removal. The initial dye concentration was found to affect both the overall amount of dye removed and the rate at which it was removed. The remaining Reactive Blue 19 and Reactive Orange 16 dye concentrations are shown in Figures 5 and 6, respectively.

Figure 5. *Reactive Blue 19 Initial Dye Concentration Tests*

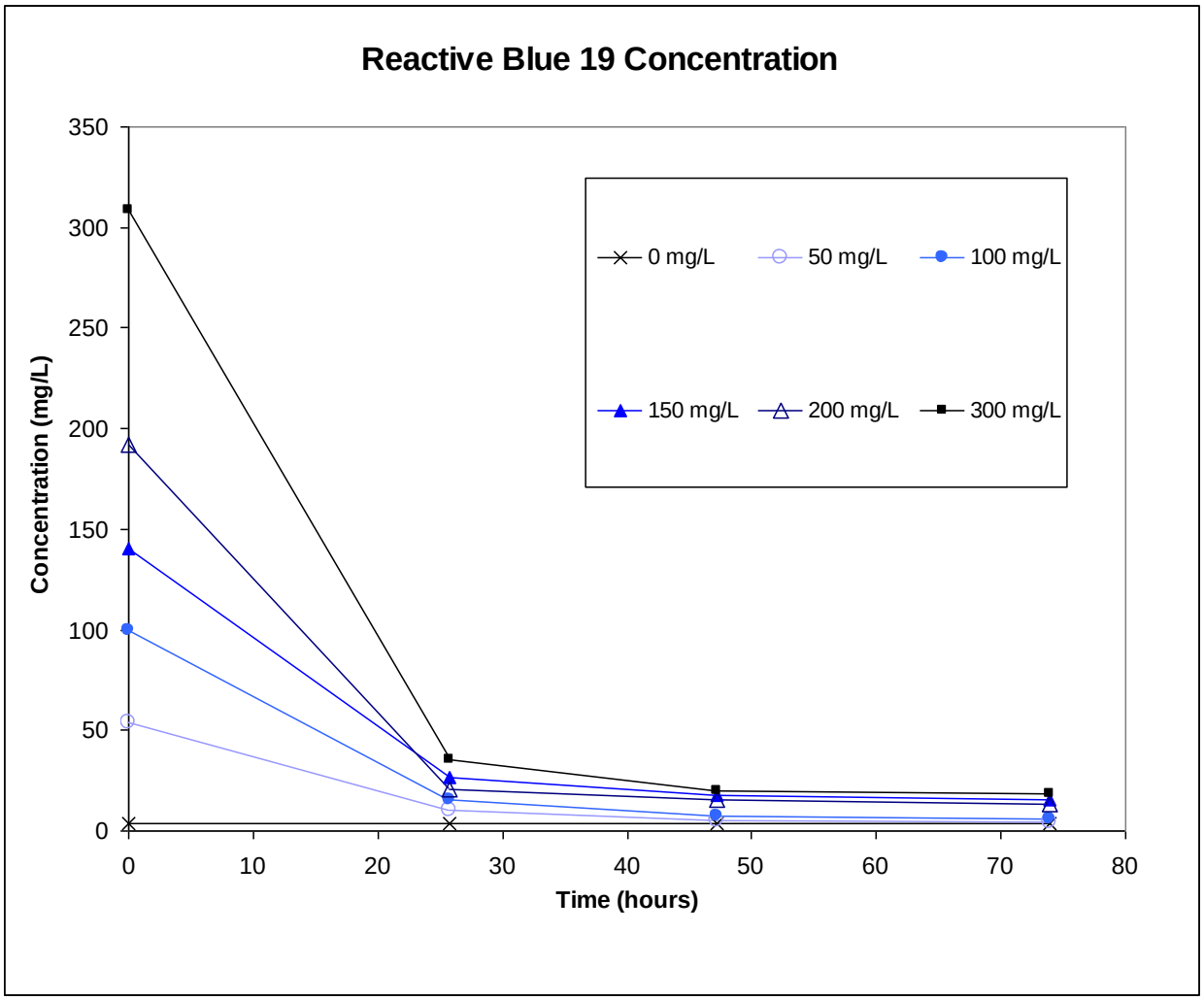
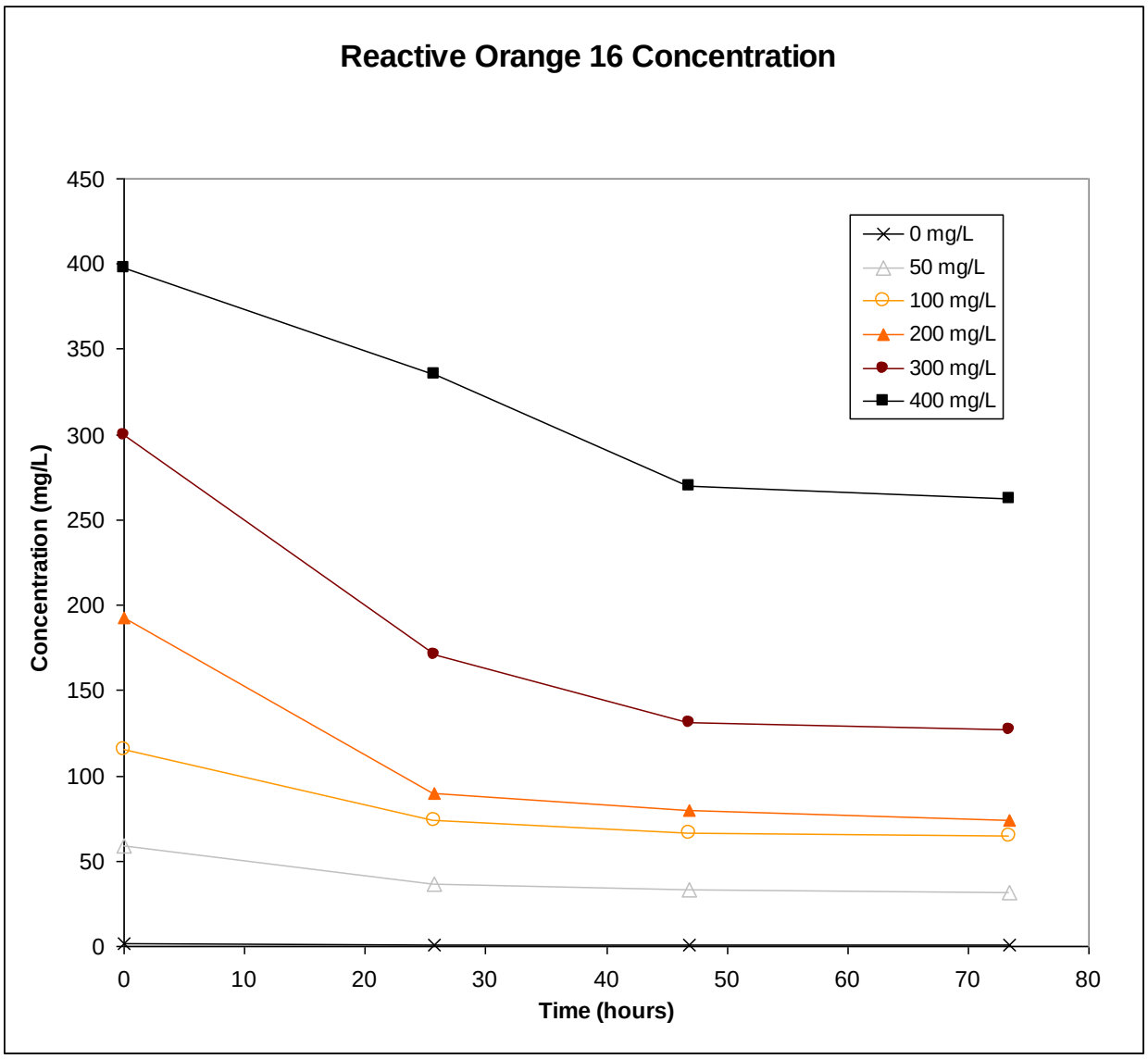


Figure 6. *Reactive Orange 16 Initial Dye Concentration Tests*



The rate of removal of Reactive Blue 19 increased with higher initial dye concentrations. The final concentration of Reactive Blue 19 was slightly higher for flasks with a larger initial dye concentration, with the exception of the flask containing 200 mg/L of dye initially. This flask attained a lower final dye concentration than the flask dosed with 150 mg/L. Although the 200

mg/L flask had a lower concentration by 6.0 mg/L at 25 hours, by the end of the experiment the difference had decreased to 2.2 mg/L. Nevertheless, based on the results of this experiment, an initial dye concentration of 200 mg/L of Reactive Blue 19 dye was selected for subsequent experimentation.

The initial dye concentration of the Reactive Orange 16 also affected the rate of removal and the final dye concentration. Flasks with higher initial dye concentrations also exhibited quicker rates of removal except for the flask with the highest initial dye concentration. This could be due to a toxic effect on the live culture at such a high concentration. The final Reactive Orange 16 dye concentration was more significantly affected by the initial dye concentration than was seen with Reactive Blue 19. The flasks with initial concentrations of 200 mg/L and 300 mg/L removed 63% and 69% of the dye, respectively. The other flasks achieved only 35-36% removal. Because of the higher percent removals and the quicker initial rate of removal, an initial dye concentration of 250 mg/L of Reactive Orange 16 dye was selected for subsequent experimentation.

Dye Removal of Mixed Systems

Reactive Blue 19 Dye

The actual dye removal per gram of biomass of the live biomass control, dead biomass control and the four tested mixtures are reported in Table 1. The live biomass was found to have the highest specific dye removal of 131.9 mg/g biomass. The dead biomass exhibited a specific dye removal of 96.04 mg/g biomass, which was the second highest value of the systems tested. Therefore, in this experiment, living Trametes versicolor biomass was found to be more effective

in the ultimate amount of dye removed per gram of biomass present. The live and dead biomass controls were able to remove 94.1% and 92.7% of the dye, respectively. Because the dead biomass control removed the majority of the dye and removed approximately the same amount of dye as the other systems, it was not likely that the maximum adsorbent loading of the dead biomass was exceeded. Therefore, the amount of dye removed by the dead biomass could potentially have been greater if more available dye was present.

Table 1. *Reactive Blue 19 Actual Dye Removal of Separate and Mixed Biomass*

Flask #	Description	Flask Volume (L)	Initial Dye Concentration (mg/L)	Final Dye Concentration (mg/L)	Mass of Dye Removed by Biomass (mg)	Mass of Biomass (g)	Actual Specific Dye Removal (mg/g biomass)
1	Control #1 - Dead Biomass	0.250	200	14.5	46.37	0.4828	96.04
2	Control #2 - Live Biomass	0.250	200	11.8	47.06	0.3568	131.90
3	Live to Dead Ratio 1:2	0.250	200	13.76	46.56	1.2932	36.00
4	Live to Dead Ratio 1:1	0.250	200	29.35	42.66	0.8306	51.36
5	Live to Dead Ratio 1:(1/2)	0.250	200	14.31	46.42	0.6719	69.09
6	Live to Dead Ratio 1:(1/4)	0.250	200	15.31	46.17	0.5666	81.49

Visual observations of the live biomass and dead biomass controls revealed significant color differences in the flasks over the course of the experiment. The dye solution containing the live culture changed color from dark blue to purple to a light iced-tea color. This observation suggested that enzymatic degradation was occurring in the live culture and causing the dye molecules to change color as they were broken down. Additionally, when the live biomass was separated from samples by centrifuging, the removed biomass did not have a noticeable color. This suggests that either adsorbed dye molecules are decolorized by the live biomass or that adsorption onto live biomass does not occur.

Unlike the color change observed with the live control, the color of the dye solution containing only the dead biomass took on a cloudy, dark blue color as the biomass dispersed throughout the flask. After this, the apparent color remained unchanged until the biomass was removed by centrifuging. After separating the biomass, the supernatant was a light blue hue while the removed biomass retained a dark blue color from the adsorbed dye molecules. Because no noticeable color change was observed in the solution containing dead biomass, visual observation supports adsorption as the primary dye removal mechanism in this case. The significant visual difference between the two controls was also seen in the mixed cultures. When the experiment was stopped and the biomass was filtered out, the live biomass and the dead biomass were visually distinct. The live fungus had a whitish color with a hint of light gray; the dead fungus was a dark blue.

The dye removal endpoint was nearly the same for each of the mixtures and controls, with the exception of the mixture containing a 1:1 ratio of live to dead biomass. However, this mixture displayed a continuing decrease in dye concentration, suggesting that the endpoint might have been reached with additional time. This result shows that the presence of dead biomass in live cultures does not affect the final dye concentration or the ultimate amount of dye removed.

Reactive Orange 16 Dye

The actual dye removal per gram of biomass of the live biomass control, dead biomass control and the four tested mixtures are reported in Table 2. The greatest overall specific dye removal was observed in the dead biomass control, with 82.3 mg of dye removed per gram of biomass. The live biomass control, which had a removal of 60.6 mg dye per gram of biomass,

performed nearly the same as the mixture with the least amount of dead biomass, which had the lowest specific dye removal of 59.4 mg/g biomass. The mixture with the greatest specific dye removal had a live to dead ratio of 1:2 (69.6 mg/g biomass), followed closely by the mixture with a ratio of 1:1 (69.0 mg/g biomass). Therefore, the mixed systems with the greatest amount of dead biomass exhibited the greatest specific dye removals. However, this result may be misleading because of the arbitrary endpoint in the experiment. As was seen in the Reactive Blue 19 experimentation, the dead biomass may not actually affect the ultimate specific dye removal but only the kinetics of the dye removal. Therefore, if the experiment length was not long enough to allow the remaining dye concentration to converge as observed in the Reactive Blue 19 experiment, then the difference in the final dye concentrations may be due more to altered kinetics than to an interaction affecting the ability of the biomass to remove dye. Thus, an examination of the effect of dead biomass on the kinetics of the dye decolorization may be more meaningful than an analysis of the specific dye removal seen at the arbitrary endpoint of the experiment.

Table 2. *Reactive Orange 16 Actual Dye Removal of Separate and Mixed Biomass*

Flask #	Description	Flask Volume (L)	Initial Dye Concentration (mg/L)	Final Dye Concentration (mg/L)	Mass of Dye Removed by Biomass (mg)	Mass of Biomass (g)	Actual Specific Dye Removal (mg/g biomass)
1	Control #1 - Dead Biomass	0.250	250	91.0	39.75	0.4828	82.32
2	Control #2 - Live Biomass	0.250	250	146.0	26.00	0.4287	60.64
3	Live to Dead Ratio 1:2	0.250	250	110.42	34.89	0.5013	69.61
4	Live to Dead Ratio 1:1	0.250	250	138.13	27.97	0.4052	69.02
5	Live to Dead Ratio 1:(1/2)	0.250	250	153.20	24.20	0.4074	59.40

Unlike the experiments with the Reactive Blue 19 dye, the live cultures did not cause a significant change in visible hue of the Reactive Orange 16 dye. Although no apparent color changes were noted, a visible reduction in color intensity did occur as the concentration decreased. However, this reduction was less apparent in the Reactive Orange 16 than in the Reactive Blue 19. This could be because the difference between Reactive Orange 16 concentrations is more difficult to visually distinguish and because less dye was removed.

Observations of the biomass highlighted the mechanisms of adsorption and enzymatic degradation. The live biomass that was removed by centrifuge was lightly colored, which indicated that few unaltered dye molecules were adsorbed onto the surface. It is likely that this is because the adsorbed dye molecules were decolorized by the extracellular enzymes. The dead biomass control took on a cloudy orange color as the dried biomass rehydrated and adsorbed dye. When the dead biomass was separated from the solution by centrifuging, it had a bright orange color that indicated dye adsorption had occurred. As with the Reactive Blue 19 dye, the live and dead biomass were visually distinct when filtered out of the mixed solutions. The live biomass had a whitish color with a hint of light pink; the dead biomass had a dark red-orange color.

Kinetics of Dye Removal

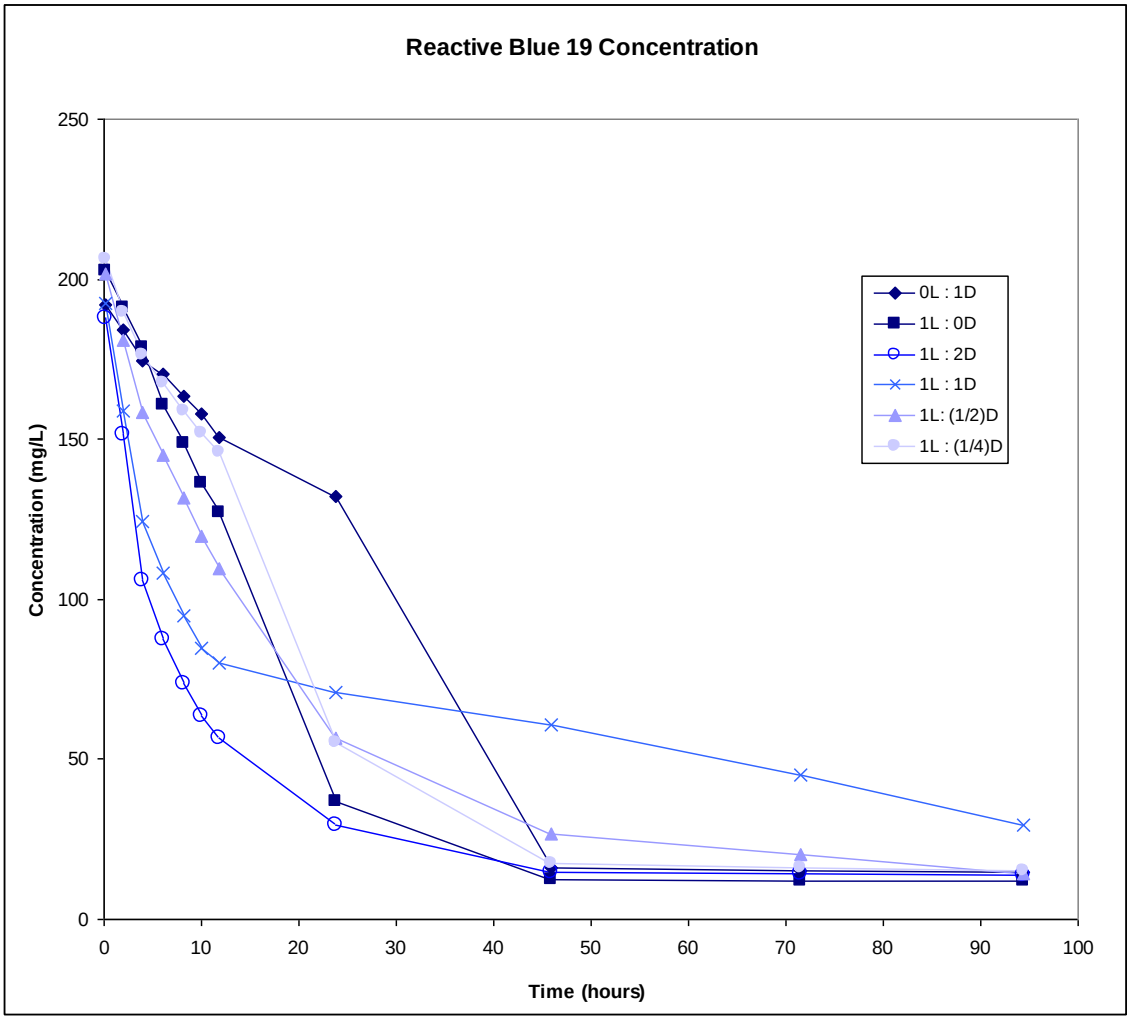
Although the results do not indicate that the presence of dead biomass impacts the final amount of dye removed, they do show that it affects the dye removal kinetics. In general, mixtures with a greater percentage of dead biomass had a higher rate of removal but a lower specific dye removal. For each dye, the remaining concentrations in the flasks were graphed and then zero-order, first-order and second-order kinetic models were fitted to each. For each flask,

the suitability of the kinetic models was judged by comparing the correlation coefficients, R^2, and visually examining the data correlation. These models showed significant kinetic differences among the mixtures, which indicated that the addition of dead biomass had altered the dye removal rates.

Reactive Blue 19 Dye

Duplicate samples were taken at two hour intervals for the first twelve hours and then once every twenty-four hours following this initial period. The remaining dye concentration at these sampling times is shown in Figure 7. The mixture with a live to dead biomass ratio of 1:2 was seen to have the quickest initial rate of dye removal, although this mixture also contained the most biomass. The flasks with the next quickest dye removal rates were the mixtures with live to dead biomass ratios of 1:1, 1:½, 1:0, 1:¼, and then 0:1. These results show that the addition of dead biomass improved the initial rate of dye removal. However, after the first 48 hours, the rates of dye removal for every mixture and control were nearly equal.

Figure 7. *Remaining Reactive Blue 19 Concentration Over Time*



Zero-order, first-order and second-order kinetic models were fitted to the data and the correlation coefficient, R^2, was calculated for each model. The models and their best-fit lines are shown in Figures 13-30 in Appendix A. A summary of these results is displayed in Table 3. Highlighted values indicate the models with the highest R^2 value, or model correlation. Based on the R^2 values, the dead biomass control was best represented by a zero-order model, the live bioimass control was best represented by a first-order model and the mixtures were best

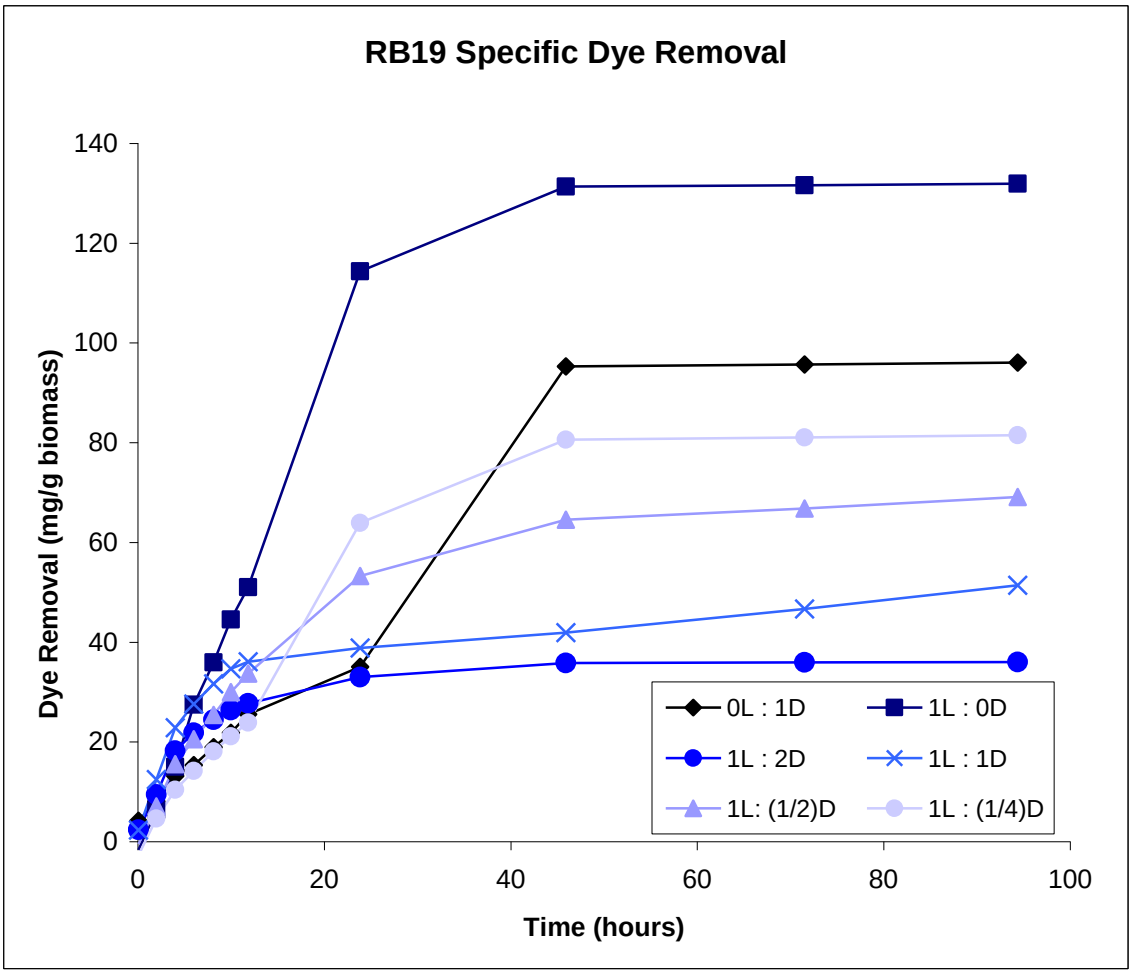
represented by a second-order model. Although the zero-order model of the dead biomass control had the highest R^2, visual inspection of the best-fit line (Figure 14, Appendix) suggested that a first-order model was a better fit.

Table 3. *Comparison of Reaction Constants and Correlation Coefficients of Kinetic Models for RB19 Dye Removal*

		Zero Order		First Order		Second Order	
		k	R^2	k	R^2	k	R^2
Flask 1	0L : 1D	2.1374	0.8835	0.0331	0.8810	0.0015	0.8810
Flask 2	1L : 0D	2.7232	0.6412	0.0354	0.8586	0.0014	0.7891
Flask 3	1L : 2D	1.3220	0.5293	0.0268	0.7957	0.0008	0.9023
Flask 4	1L : 1D	1.1914	0.6092	0.0158	0.8461	0.0003	0.9387
Flask 5	1L: (1/2)D	1.8475	0.7745	0.0290	0.9397	0.0007	0.9918
Flask 6	1L : (1/4)D	2.1371	0.7998	0.0322	0.8828	0.0008	0.9141

The concentration measurements shown in Figure 7 do not account for the differing amounts of biomass present in each flask. A flask with more biomass may be capable of removing dye more quickly than a flask with less biomass. To account for this, the specific dye removal at each sample point was calculated and graphed as shown in Figure 8. This graph shows that the mixture with a live to dead biomass ratio of 1:1 initially had the highest specific dye removal until the live control surpassed it at around six hours. Initially, the specific dye removal of the dead control was greater than the live control, but the live control exhibited a higher specific dye removal after two hours. After a lag, the dead biomass achieved a higher specific dye removal than the mixtures. This lag could be due in part to the rehydration of the dried fungus; as the biomass expanded, more surface area was available for adsorption.

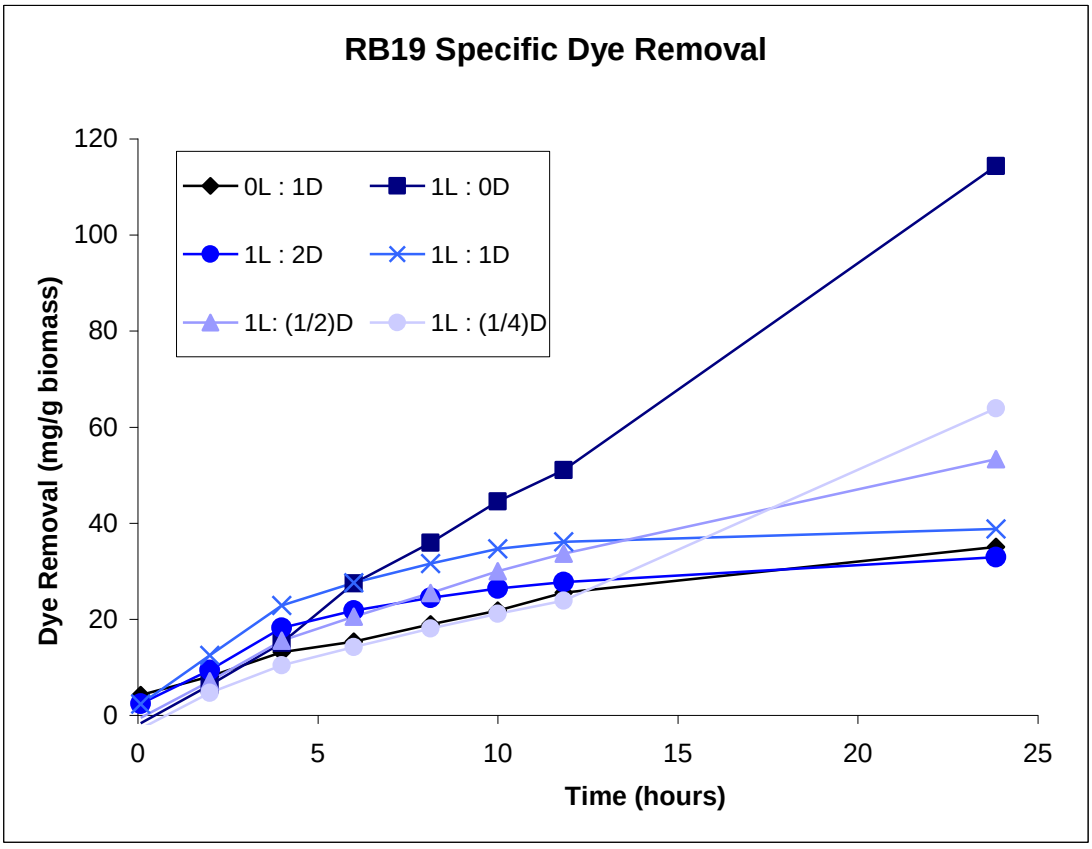
Figure 8. *Specific Dye Removal of Reactive Blue 19 Dye*



The final specific dye removals are less meaningful than the specific dye removals during the first 24 hours. This is because each flask removed approximately the same amount of dye over the course of the experiment and therefore the final specific dye removals were simply a reflection of the amount of biomass present in each flask. However, the specific dye removals during the initial 24-hour period were more useful because these measurements were taken before most of the available dye had been used up. A plot of the specific dye removal during the

first 24 hours is shown in Figure 9. This graph shows that during the first four hours, the specific dye removals of the mixtures containing live to dead biomass ratios of 1:1 and 1:2 were greater than either the live control or the dead control alone. This suggests that a mixture of both live and dead biomass may have a higher initial specific dye removal than either types of biomass alone. However, after six hours, the live biomass had the greatest specific dye removal and the mixtures' specific dye removals began to level off. At the end of the 24-hour period, the live biomass had the greatest specific dye removal, followed by the 1:¼, 1:½, 1:1 mixtures. The dead control and 1:2 mixture had the lowest specific dye removal. This indicates that the presence of dead biomass decreases the specific dye removal with Reactive Blue 19.

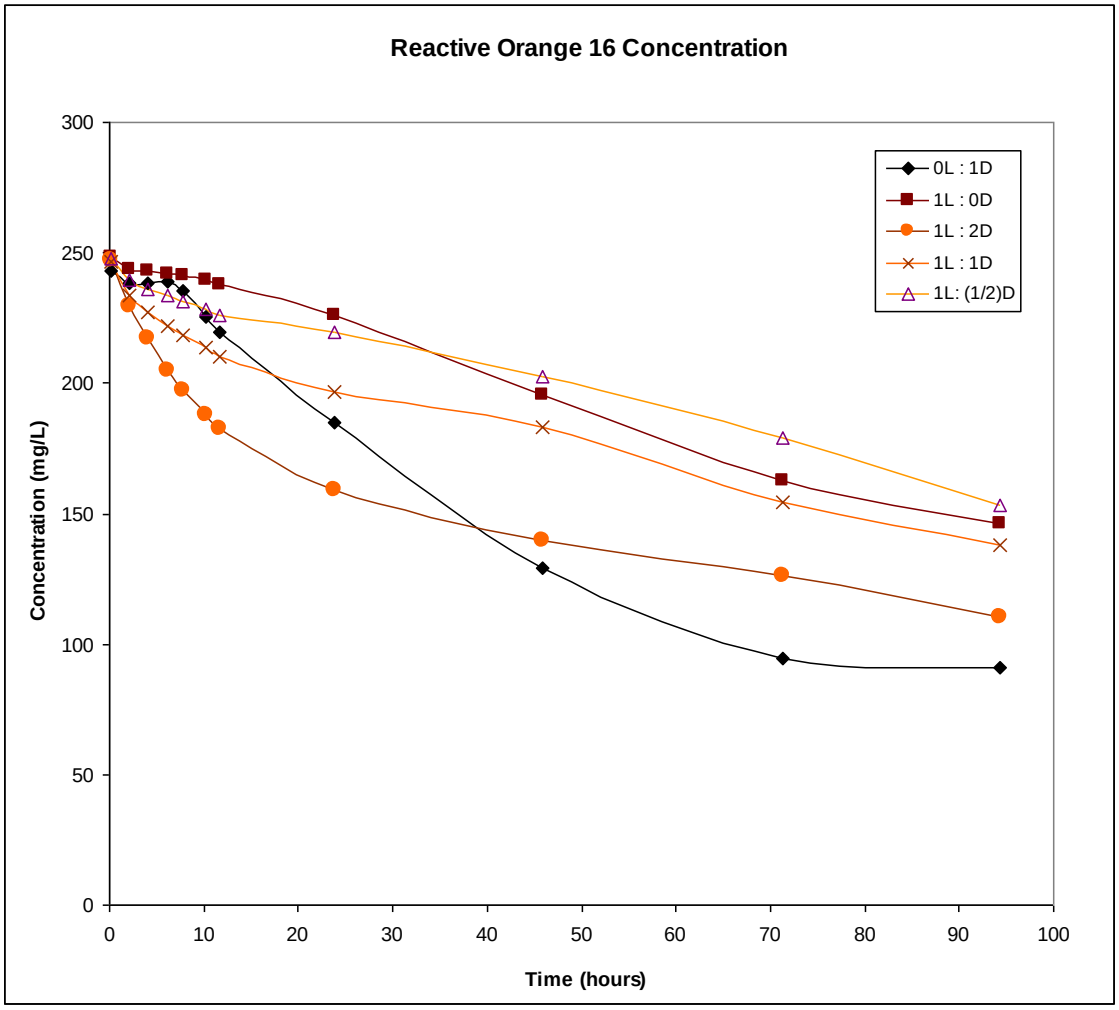
Figure 9. *Specific Dye Removal of Reactive Blue 19 Dye Within 24 Hours*



Reactive Orange 16 Dye

As with the Reactive Blue 19 experiments, duplicate samples were taken at two hour intervals for the first twelve hours and then once every twenty-four hours. The remaining dye concentration at these sampling times is shown in Figure 10. The mixture with a live to dead biomass ratio of 1:2 was seen to have the quickest initial rate of dye removal, although it also contained the most total biomass. Within the first twenty hours, the mixtures with the next quickest dye removal rates had live to dead biomass ratios of 1:1, 1:½, and then 1:0. The dead biomass control had an initial lag before the concentration began to fall, likely because of the rehydration and dispersion of the biomass. However, after the first six hours, the dye concentration in this flask had the sharpest rate of decrease and, after 40 hours, it had the lowest dye concentration of all the flasks. The lag in dye concentration reduction could potentially be reduced if the dead biomass adsorbent was added in a powder form and/or rehydrated prior to addition. In this situation, it is possible that the dead biomass control could exhibit a more rapid reduction in dye concentration than the other flasks over the initial six hour period.

Figure 10. *Remaining Reactive Orange 16 Concentration Over Time*



The live biomass control and the mixture with the least amount of dead biomass exhibited the slowest decreases in dye concentration and the highest final dye concentrations at the end of the experiment. Compared with the dead biomass control, the live biomass control removed less dye over the course of the experiment and exhibited a slower rate of removal. This suggests that Reactive Orange 16 dye is more readily removed through adsorption than enzymatic degradation. At the end of the experiment, however, the flasks containing live cultures exhibited

continuing dye removal, whereas the dye removal in the dead biomass control had leveled off. This means that the live cultures could potentially remove an equal or greater amount of dye as the dead biomass control, if given a long enough period of time.

Zero-order, first-order and second-order kinetic models were explored as a tool to describe the rate of dye removal. The data and best-fit lines for each flask and kinetic model are included as Figures 31-45 in Appendix B. A summary of these results is displayed in Table 4. Highlighted values indicate the models with the highest R^2 value. Based on the R^2 values, the flask with the live to dead biomass ratio of 1:½ was best represented by a zero-order model, the live biomass control was best represented by a first-order model and the dead control and mixtures with live to dead biomass ratios of 1:2 and 1:1 were best represented by a second-order model.

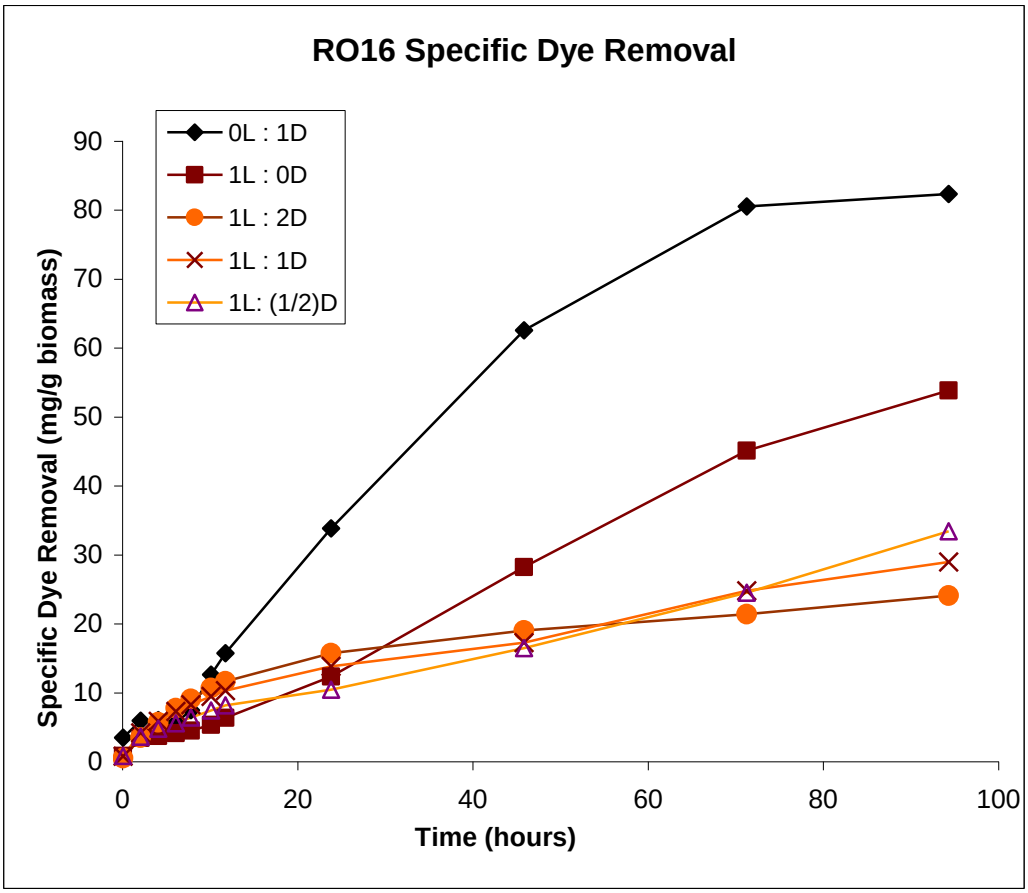
Table 4. *Comparison of Reaction Constants and Correlation Coefficients of Kinetic Models for RO16 Dye Removal*

		Zero Order		First Order		Second Order	
		k	R^2	k	R^2	k	R^2
Flask 1	0L : 1D	1.8614	0.9479	0.0120	0.9702	8.E-05	0.9771
Flask 2	1L : 0D	1.5543	0.9553	0.0058	0.9921	4.E-05	0.9803
Flask 3	1L : 2D	1.2614	0.8362	0.0077	0.9085	5.E-05	0.9595
Flask 4	1L : 1D	1.0307	0.9509	0.0056	0.9748	3.E-05	0.9848
Flask 5	1L: (1/2)D	0.9004	0.9877	0.0046	0.9843	2.E-05	0.9722

A comparison of the amount of dye removed from each flask does not take into account the differing amounts of biomass present in each. Therefore, the specific dye removal is calculated to determine the amount of dye removed per gram of biomass. A plot of this data is shown in Figure 11. After a six hour lag, the specific dye removal of the dead control rose more quickly than any of the other systems. After 35 hours, the dead biomass took on the greatest

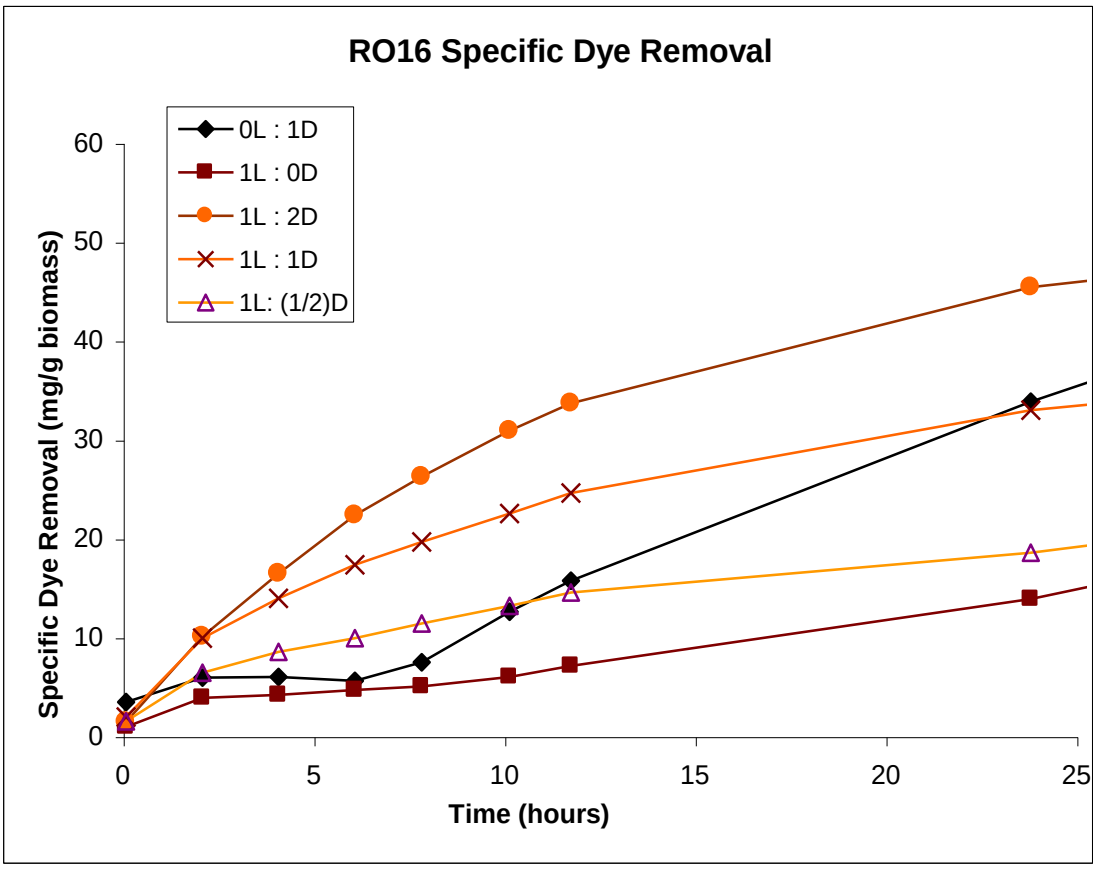
specific dye removal. The live biomass and the mixture with a live to dead biomass ratio of 1:½ had the lowest specific dye removals. In general, the mixtures with more dead biomass had larger specific dye removals. This means that the dead biomass was able to remove more dye on a per mass basis than the live biomass. Therefore, the results imply that Reactive Orange 16 dye is more readily removed through adsorption than through enzymatic degradation.

Figure 11. *Specific Dye Removal of Reactive Orange 16 Dye*



A plot of the specific dye removals during the first twenty-four hours is shown in Figure 12. This graph details the differences in the specific dye removals during the initial phase when an abundant amount of dye is available and the remaining dye concentration is not yet nearing an equilibrium concentration. This figure clearly shows a lag in the dye removal by the dead biomass control, which is most likely due to time required to rehydrate and disperse the biomass in the solution. The mixtures exhibited increasing specific dye removals but the rate of increase gradually declined over time. Mixtures with more dead biomass experienced a slower decline in the rate of increase in specific dye removal. Both the live and dead controls experienced an initial lag phase before exhibiting a rise in their specific dye removals. After the lag phase, the dead biomass control experienced the greatest increase in specific dye removal.

Figure 12. *Specific Dye Removal of Reactive Orange 16 Dye Within 24 Hours*



Interactions Affecting Kinetics

To determine if any interaction occurred between the live and dead biomass that affected the kinetics of the dye removal, the actual and theoretical specific dye removals for each mixture were compared. If a significant difference existed between the two values, this was an indication of an interaction affecting the rate of dye removal. To find the difference, the theoretical specific dye removal was subtracted from the actual specific dye removal. Therefore, a positive result indicated a beneficial effect that improved the specific dye removal of the mixture, while a

negative result indicated that the interaction reduced the specific dye removal. This method of comparison was used in lieu of comparing the reaction rate constants because the dye removal did not follow one single kinetic model and therefore comparison of the rate constants would not yield meaningful results.

The difference between actual and theoretical specific dye removal for the mixtures containing Reactive Blue 19 are shown in Table 5. In the flasks containing an approximate ratio of 1:2, 1:1 and 1:½ of live to dead biomass, a beneficial effect was seen in the early part of the experiment. The flask with a ratio of live to dead of 1:1 experienced the greatest increase in specific dye removal during the first 10 hours. However, after this initial period, the theoretical specific dye removal was greater than the actual specific dye removal, indicating a negative impact on dye removal.

Table 5. *Difference Between Theoretical and Actual Specific Dye Removal for Reactive Blue 19*

Specific Dye Removal (mg/g biomass) Difference Actual - Theoretical				
Time (hours)	**Flask 3 1L : 2D**	**Flask 4 1L : 1D**	**Flask 5 1L: (1/2)D**	**Flask 6 1L : (1/4)D**
0.1	-0.33	0.58	-1.00	-2.36
2.0	1.77	5.10	0.14	-2.03
4.0	4.56	8.90	1.18	-4.22
6.0	3.40	7.18	-2.60	-10.68
8.1	1.25	5.58	-4.39	-14.25
10.0	-1.18	3.34	-6.41	-18.6
11.8	-4.28	-0.11	-8.21	-21.8
23.8	-22.2	-29.4	-32.6	-33.6
45.9	-68.6	-68.5	-53.9	-43.1
71.5	-68.9	-64.1	-51.9	-42.9
94.4	-69.1	-59.7	-49.9	-42.8

The difference between the actual and theoretical specific dye removal of Reactive Orange 16 is shown in Table 6. The positive numbers between hours two and 46 indicate that a beneficial interaction took place at this time. These numbers are significantly greater than those seen in the Reactive Blue 19 experiment, so it appears that the beneficial interaction is more vigorous with this dye. The flask with an approximate live to dead biomass ratio of 1:2 generated the greatest beneficial interaction. After the first 46 hours, the beneficial interaction disappeared and a negative impact on the specific dye removal was observed. These results indicate that an interaction between the live and dead biomass occurred that improved the specific dye removal initially but decreased it after a period of 12 to 46 hours.

Table 6. *Difference Between Theoretical and Actual Specific Dye Removal for Reactive Orange 16*

Specific Dye Removal (mg/g biomass) Difference Actual - Theoretical			
Time (hours)	**Flask 3 1L : 2D**	**Flask 4 1L : 1D**	**Flask 5 1L: (1/2)D**
0.1	-1.16	-0.72	-0.38
2.1	4.86	4.66	1.78
4.1	11.06	8.53	3.67
6.1	17.09	12.05	4.90
7.8	19.60	12.95	5.47
10.1	20.54	12.06	4.74
11.7	20.86	11.65	4.22
23.8	18.4	5.6	-2.73
45.8	2.9	-11.4	-14.0
71.3	-8.6	-11.8	-18.3
94.3	-5.3	-6.3	-9.3

One reason this beneficial interaction is only seen in the initial phase of the experiment may be that the interaction between live and dead biomass affects adsorption and enzymatic degradation differently. Adsorption is a process that takes place more quickly than enzymatic degradation and therefore adsorption may be the primary driver of dye removal during the initial phase. If this is the case, then the improved specific dye removal during this period may be due to an interaction that improves adsorption. If adsorption is improved and it is the dominant dye removal mechanism during the first several hours, an increase in specific dye removal may be expected. Additionally, a negative impact on the enzymatic degradation may decrease the specific dye removal later in the experiment when the adsorbent biomass nears saturation and enzymatic degradation becomes the dominant dye removal mechanism.

It is possible that the hydrophobins present from the live fungi improve adsorption by either coating the dead biomass and changing its adsorptive properties or by coating the dye molecules and improving their hydrophobicity. This could give an initial boost to the specific

dye removal by allowing dye molecules to adsorb more readily. On the other hand, it may interfere with enzymatic degradation later on because the adsorption may withdraw dye molecules from the solution and decrease the concentration present. As was seen in the initial dye concentration tests, solutions with a lower concentration of dye exhibited a slower rate of removal. Therefore, by increasing the initial adsorption and decreasing the available concentration of dye in solution, the enzymatic degradation may occur more slowly, resulting in a decrease in specific dye removal in the later stages of the experiment.

CHAPTER SIX: CONCLUSIONS AND FUTURE RESEARCH

Conclusions

This study shows that Trametes versicolor is effective in decolorizing textile dyes in water and has significant potential for use in the treatment of textile wastewaters. The initial dye concentration was shown to have a substantial effect on the rate of dye removal by live cultures. As the initial dye concentration was increased, the dye removal rate also increased, except in the case of a 400 mg/L concentration of Reactive Orange 16 dye. This could be because of a toxic effect at this high concentration. In the case of the Reactive Blue 19 dye, an increase in the initial dye concentration did not significantly affect the final dye concentration. However, in the case of Reactive Orange 16, a higher initial dye concentration led to a higher final dye concentration. Reactive Blue 19 was more readily and more completely removed by the live cultures than Reactive Orange 16. This was anticipated due to the higher octanol-water partition coefficient and lower solubility of Reactive Blue 19.

The live biomass and dead biomass removed approximately the same amount of Reactive Blue 19 dye over the course of the experiment. However, the live culture was found to have less mass than the dead biomass control and therefore, the live biomass had a higher specific dye removal. Although the live fungus was more effective at removing Reactive Blue 19 from water, the dead biomass was also effective and had a higher final specific dye removal than any of the mixed cultures. In contrast, Reactive Orange 19 was most effectively removed by the dead biomass. This suggests that Reactive Blue 19 is more effectively removed through enzymatic degradation but Reactive Orange 16 is more readily removed through adsorption. In the mixed

systems, the addition of dead biomass to live cultures increased the initial rate of removal but decreased the overall specific dye removal. This could be due to an interaction between the live and dead biomass that improved adsorption but diminished enzymatic degradation. Although the results of this study are not able to confirm what type of interactions took place, they do indicate that a beneficial interaction between live and dead biomass occurred within the initial period up to 46 hours that increases the specific dye removal. After this, a negative effect on the specific dye removal was seen to occur.

The results of this study show that the extent and rate of dye removal in a fungal bioreactor is heavily dependent upon the type of dyes present. For a wastewater containing Reactive Orange 16, the use of dead biomass as an adsorbent may be more beneficial than the use of a live system. This study showed that dead biomass was able to accomplish a greater amount of Reactive Orange 16 dye removal than live cultures. The use of dead biomass as an adsorbent is advantageous because it does not require any supplemental nutrients or hospitable growth conditions to effectively remove dye.

However, these results may not be applicable to all textile dyes, so some dyes may be removed more effectively through enzymatic degradation. In a wastewater with dyes that are more readily removed through enzymatic degradation, a live fungal bioreactor may be more effective. When live systems are used, the rate of dye removal may be improved by adding dead biomass for adsorbent. This could be beneficial because spent live biomass could be recycled for use as adsorbent, producing an adsorbent material on site. Additionally, textile wastewater typically carries a mixture of many dyes, some of which may be more readily removed through

either adsorption or degradation. Therefore, in some cases it may be beneficial to use a mixed system containing both live and dead biomass.

Future Research

This study provides the basis for future research into the interactions that occur between live and dead biomass in a mixed system. Further research is needed to determine the nature and cause of the interactions between the live and dead biomass. It is recommended that hydrophobins be investigated as the cause by directly testing their effect on the octanol-water partition coefficient and solubility of the dyes. If an addition of hydrophobins increased the k_{ow} and/or decreased the solubility, this would indicate that the dye had become more hydrophobic, which may allow it to be more readily removed.

It is also recommended that future research examine the ability of mixed live/dead systems to remove a mixture of dyes, as well as actual textile wastewater. This would more closely simulate the conditions of an actual fungal bioreactor in removing textile dyes and provide a better idea of its performance on a larger scale.

APPENDIX A: REACTIVE BLUE 19 KINETIC MODELS

Appendix A: Reactive Blue 19 Kinetic Models

Figure 13. *Reactive Blue 19 Flask 1, Zero-Order Kinetic Model*

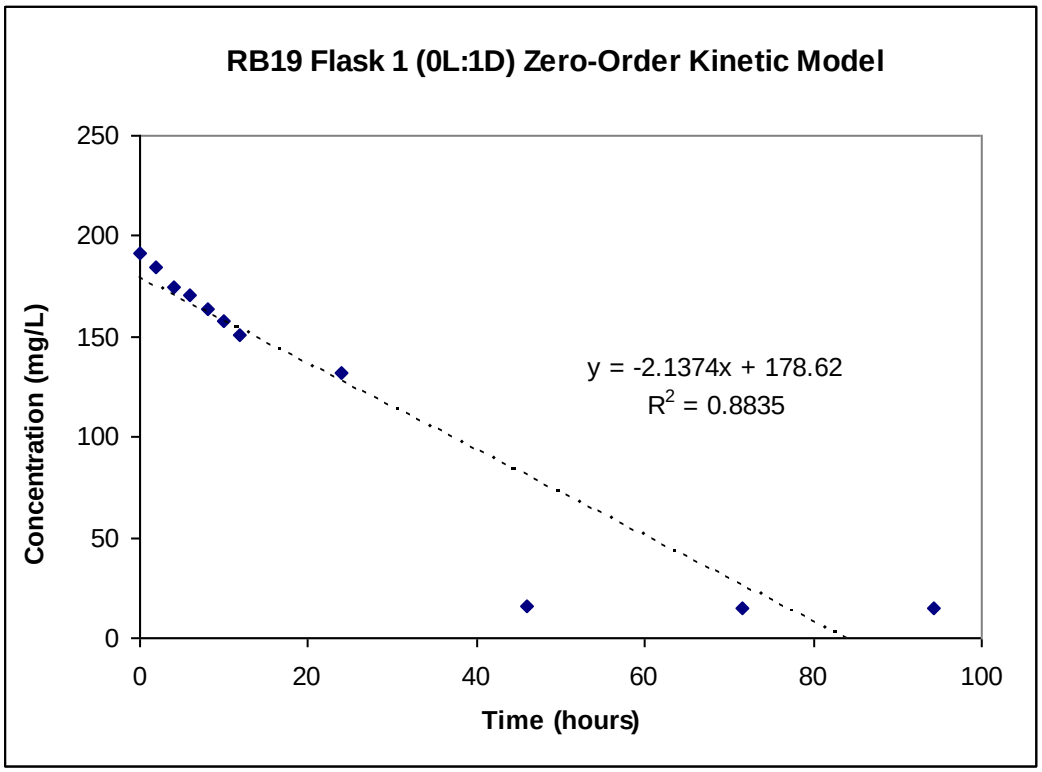


Figure 14. *Reactive Blue 19 Flask 1, First-Order Kinetic Model*

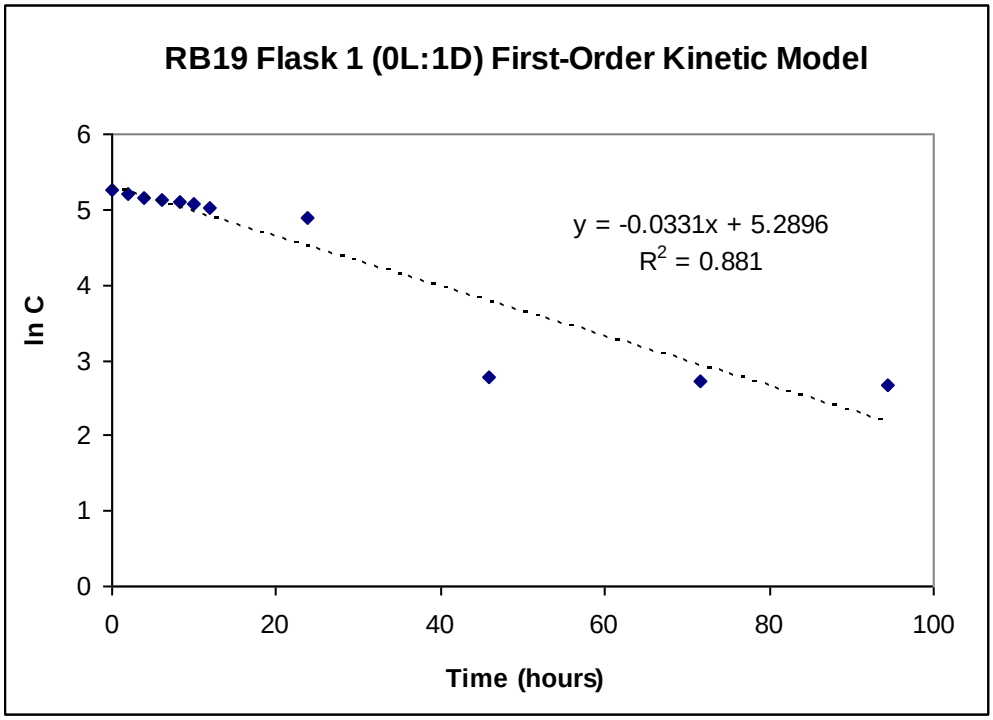


Figure 15. *Reactive Blue 19 Flask 1, Second-Order Kinetic Model*

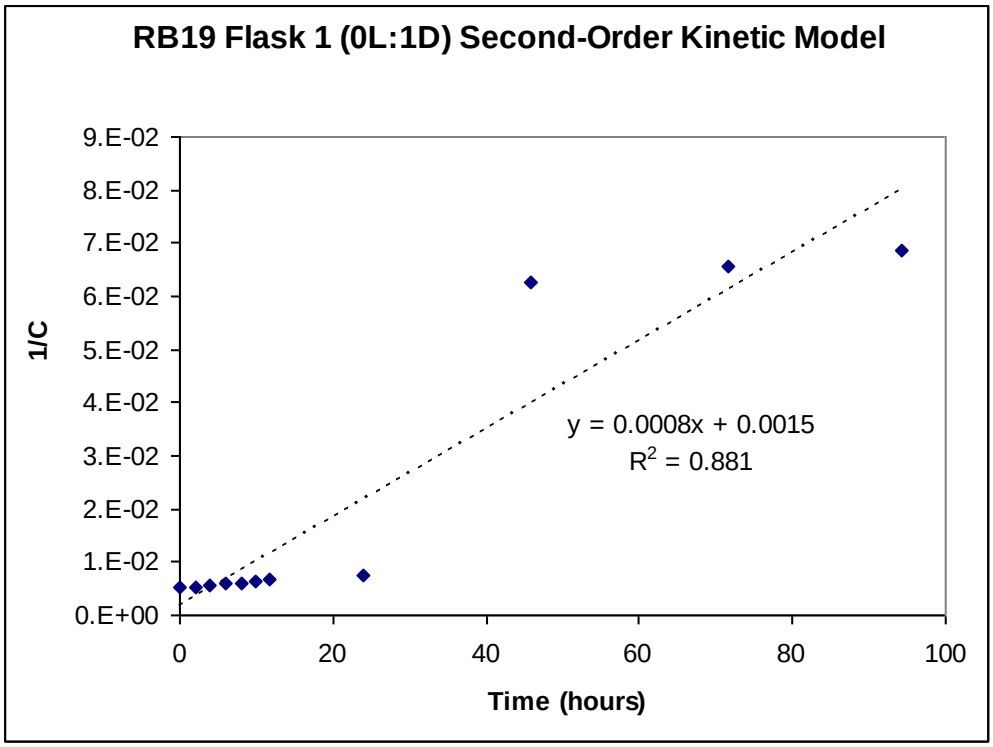


Figure 16. *Reactive Blue 19 Flask 2, Zero-Order Kinetic Model*

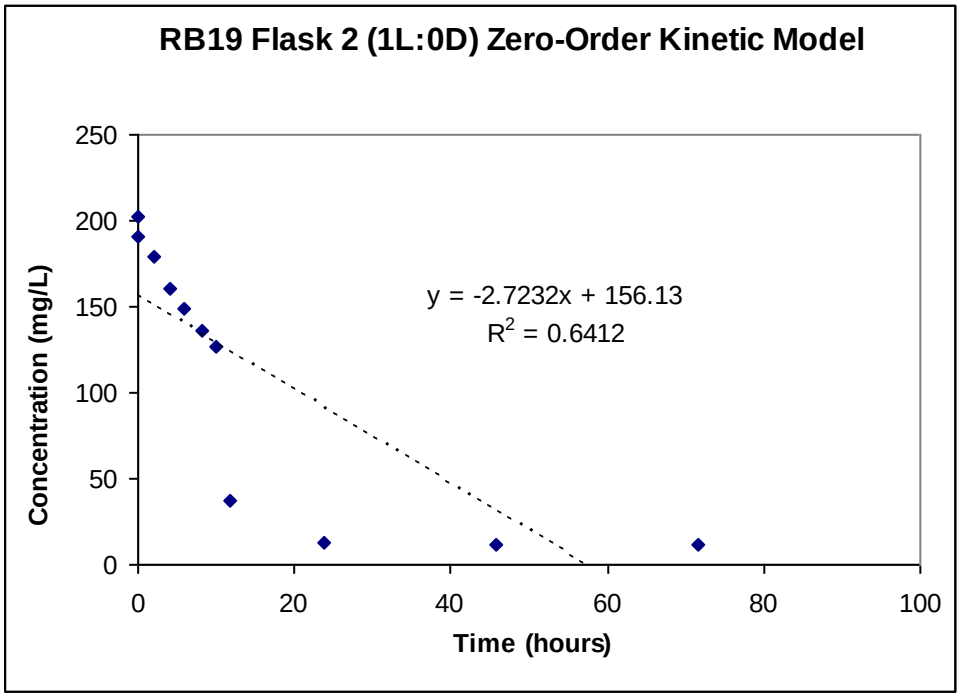


Figure 17. *Reactive Blue 19 Flask 2, First-Order Kinetic Model*

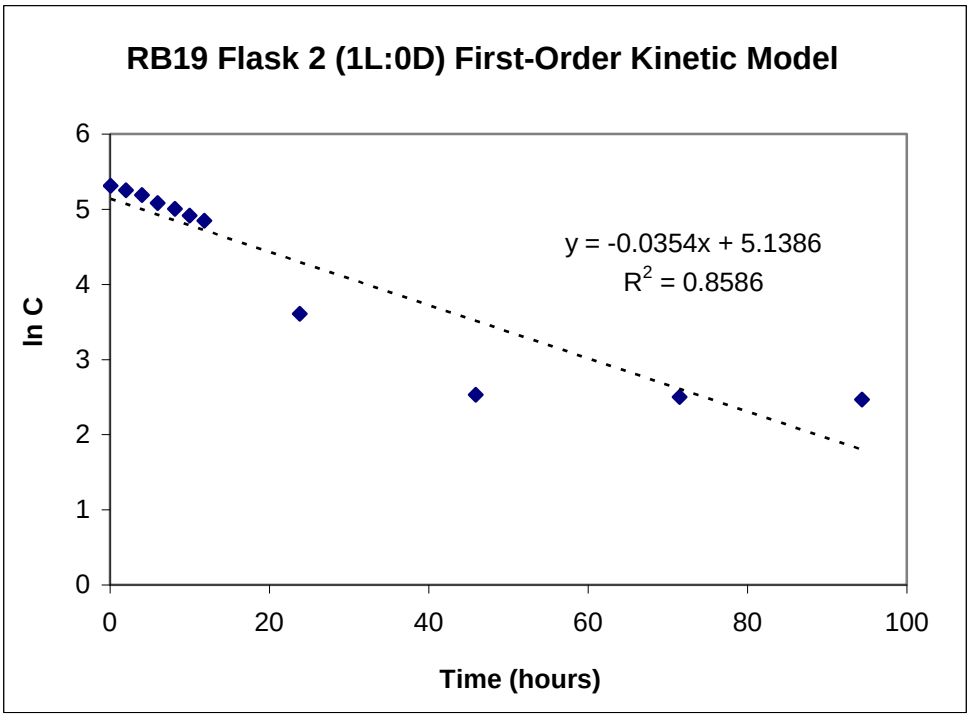


Figure 18. *Reactive Blue 19 Flask 2, Second-Order Kinetic Model*

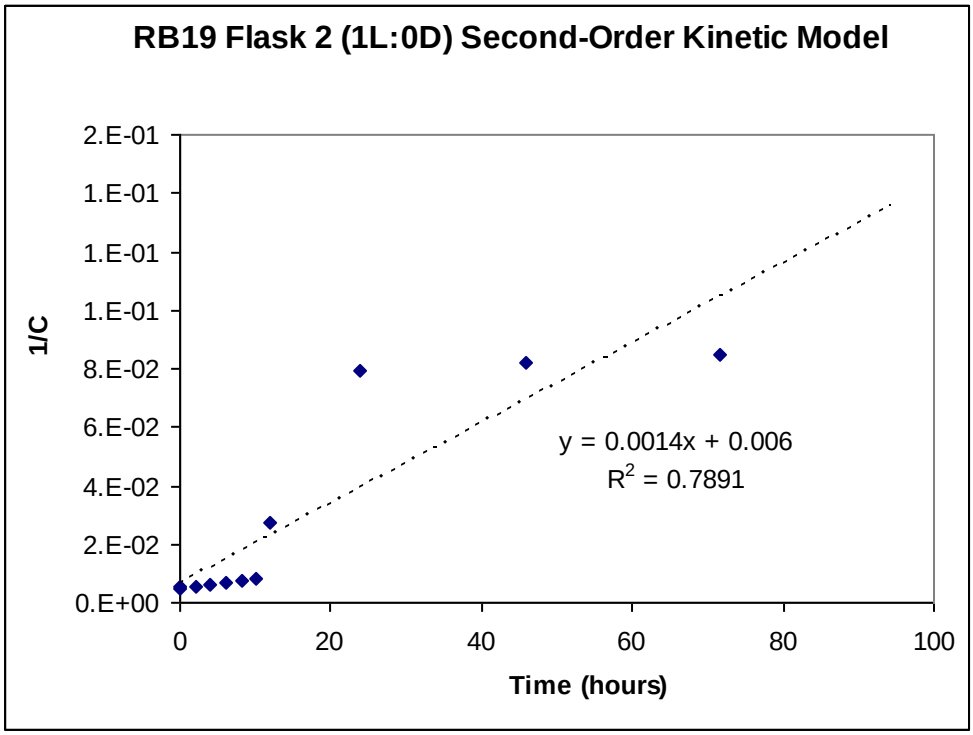


Figure 19. *Reactive Blue 19 Flask 3, Zero-Order Kinetic Model*

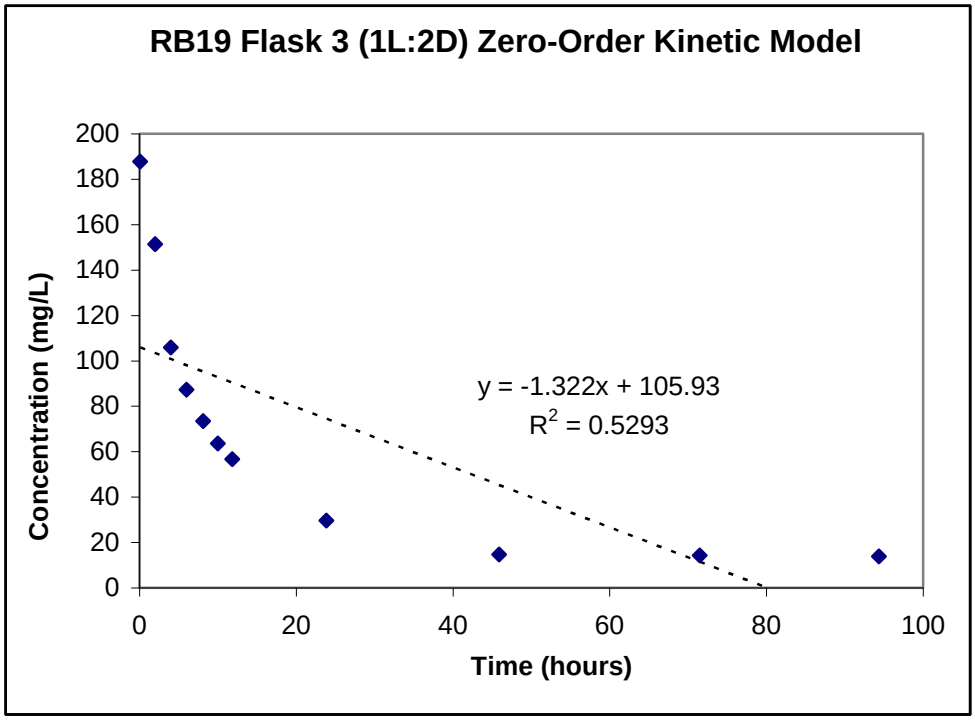


Figure 20. *Reactive Blue 19 Flask 3, First-Order Kinetic Model*

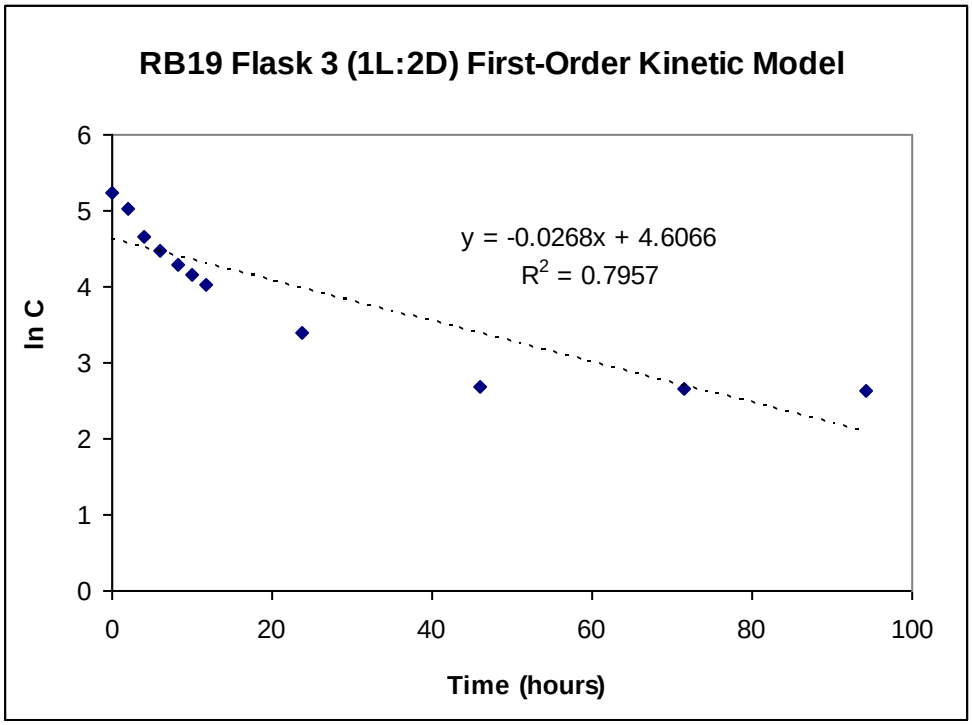


Figure 21. Reactive *Blue 19 Flask 3, Second-Order Kinetic Model*

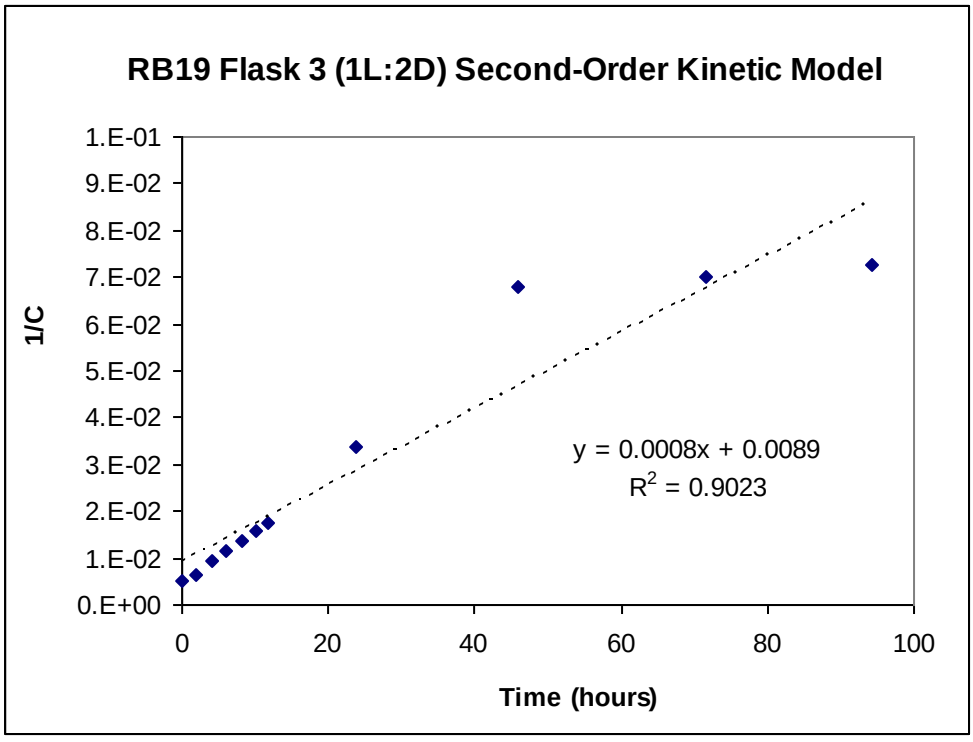


Figure 22. *Reactive Blue 19 Flask 4, Zero-Order Kinetic Model*

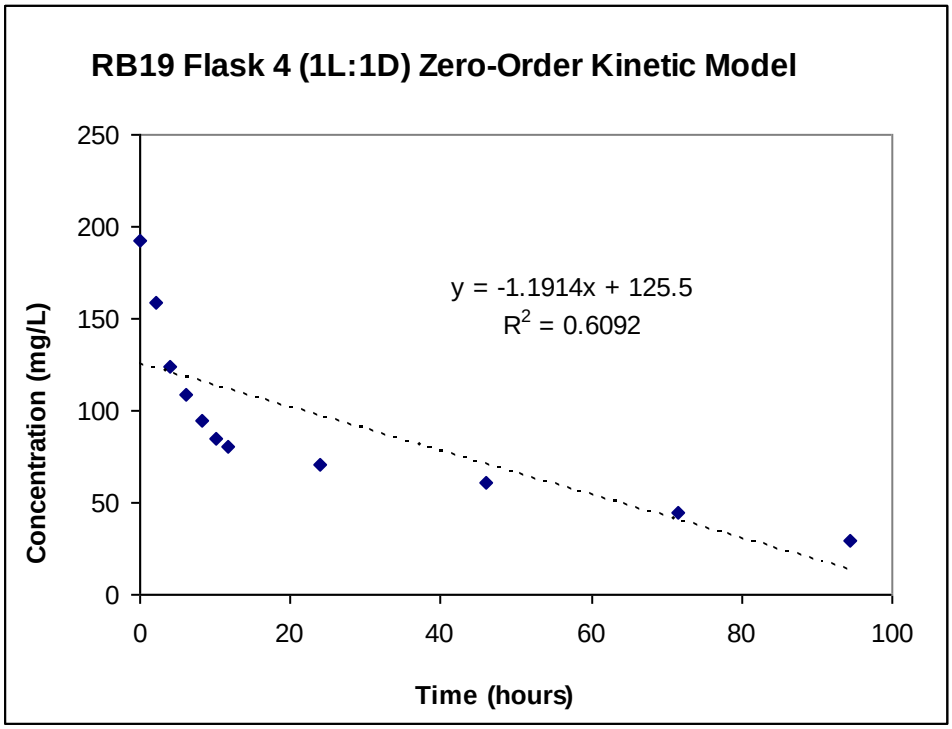


Figure 23. *Reactive Blue 19 Flask 4, First-Order Kinetic Model*

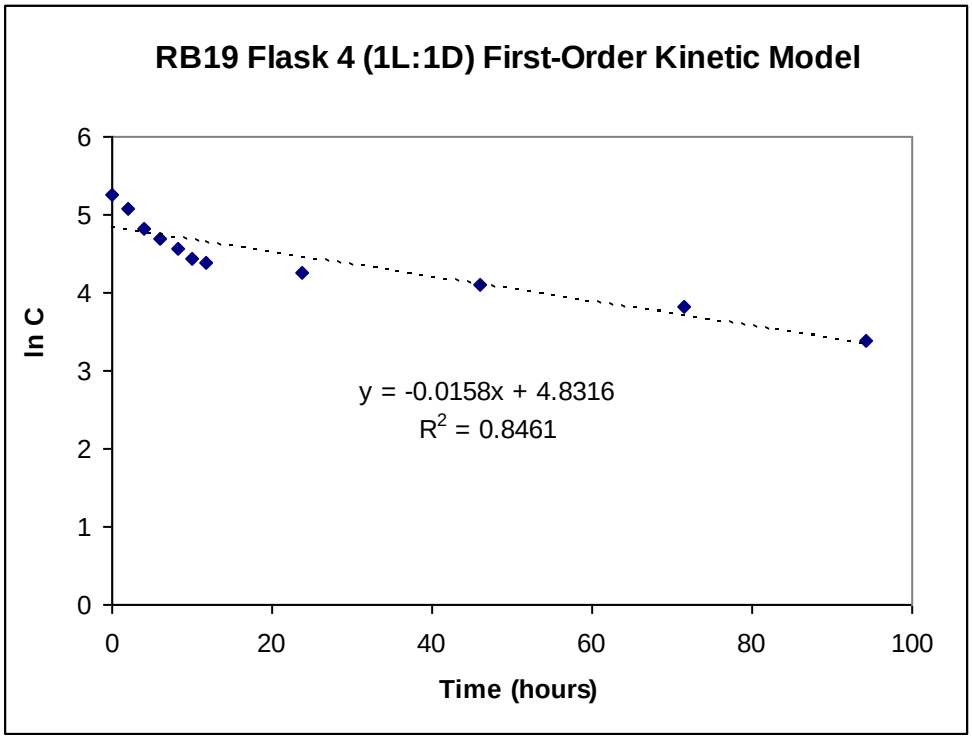


Figure 24. *Reactive Blue 19 Flask 4, Second-Order Kinetic Model*

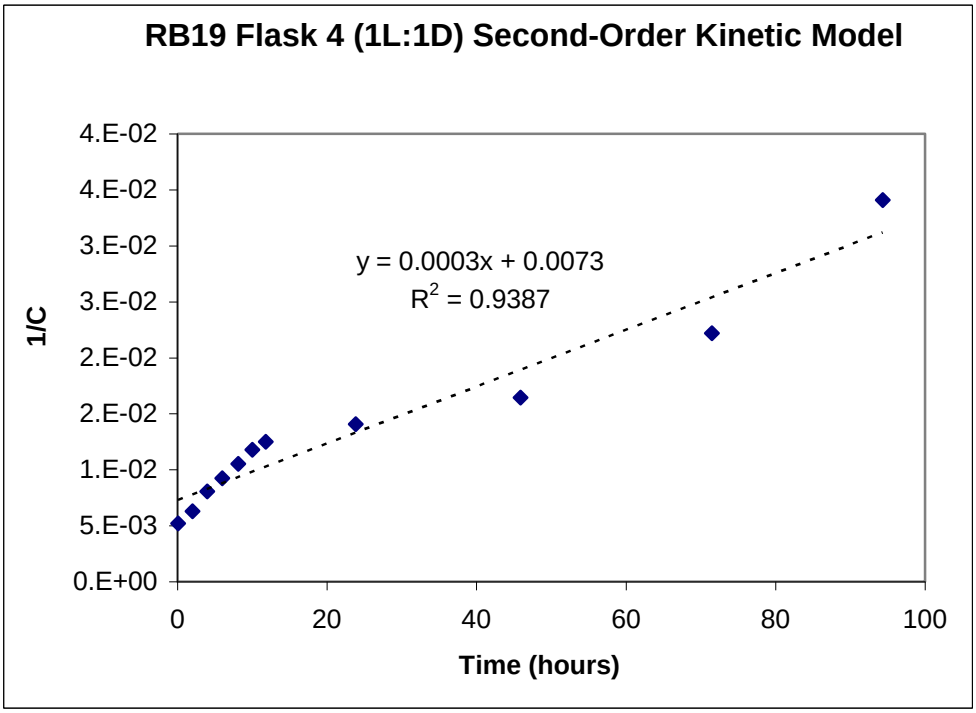


Figure 25. *Reactive Blue 19 Flask 5, Zero-Order Kinetic Model*

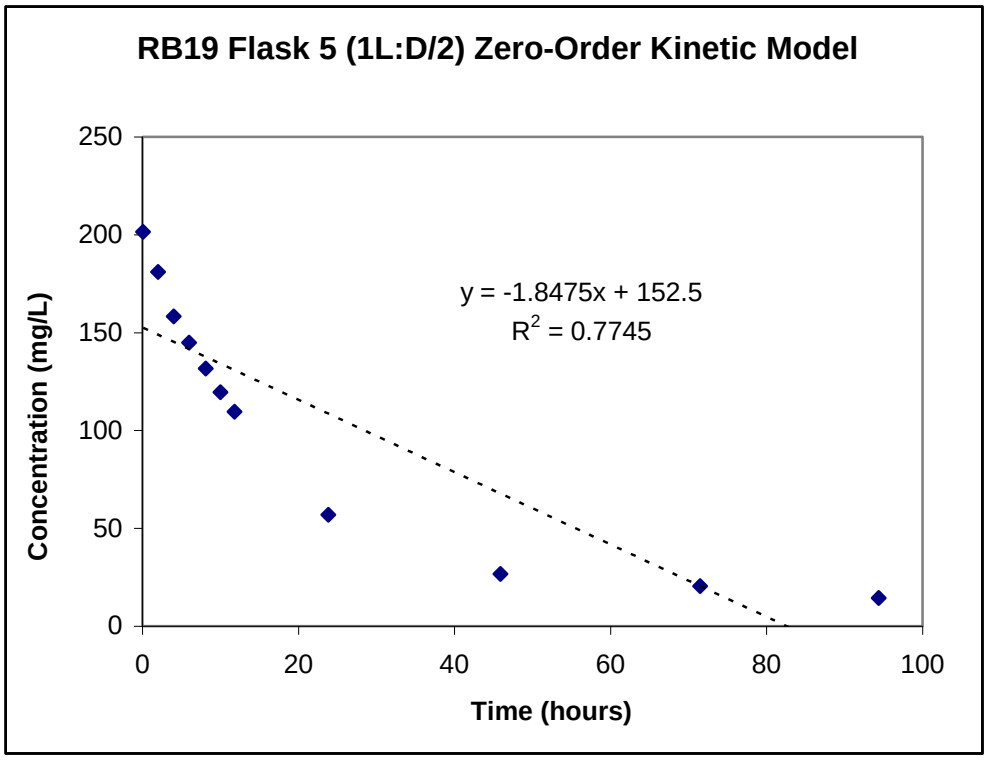


Figure 26. *Reactive Blue 19 Flask 5, First-Order Kinetic Model*

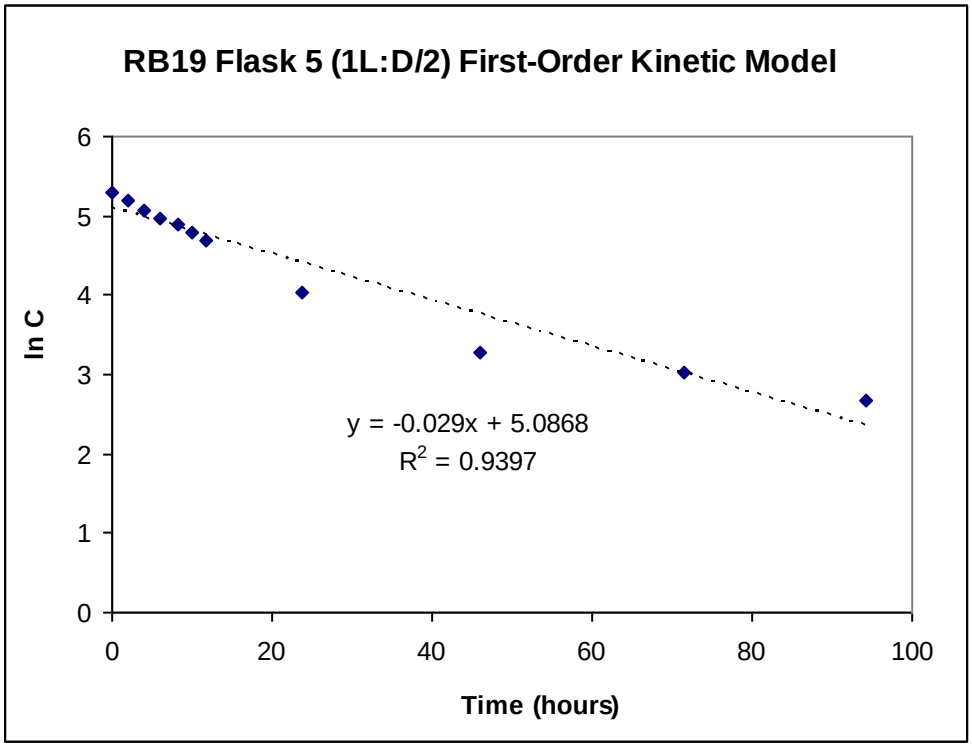


Figure 27. *Reactive Blue 19 Flask 5, Second-Order Kinetic Model*

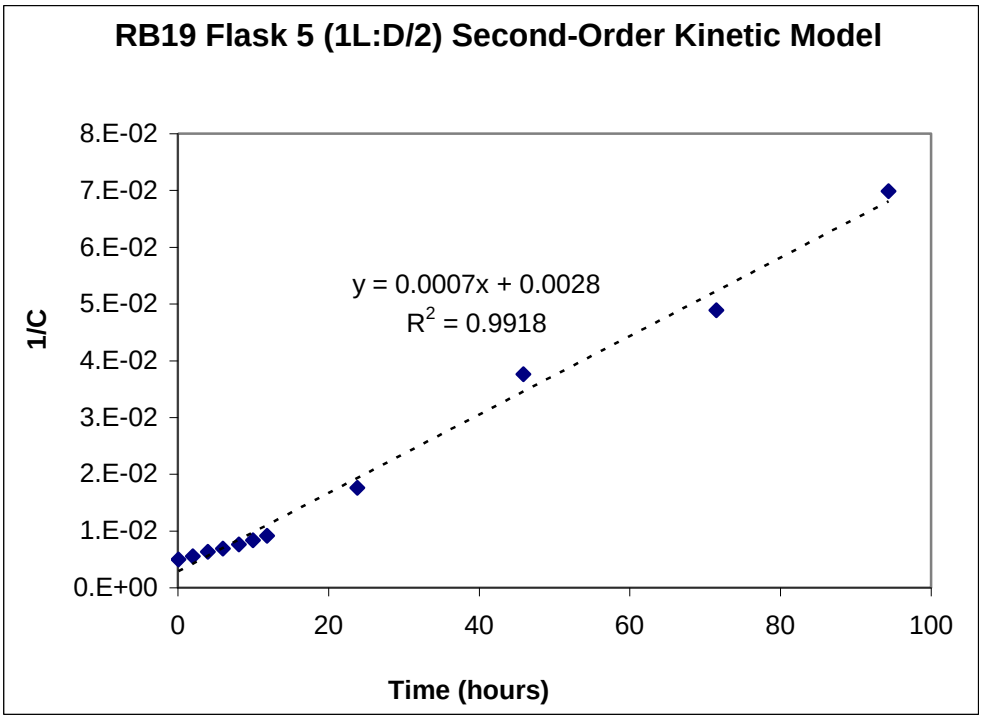


Figure 28. *Reactive Blue 19 Flask 6, Zero-Order Kinetic Model*

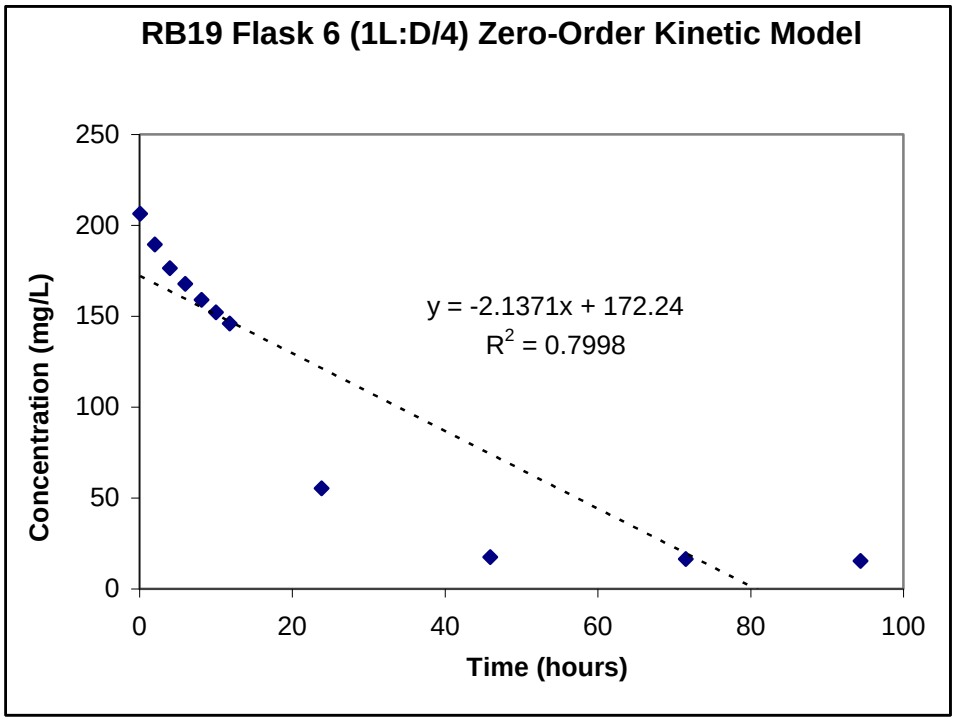


Figure 29. *Reactive Blue 19 Flask 6, First-Order Kinetic Model*

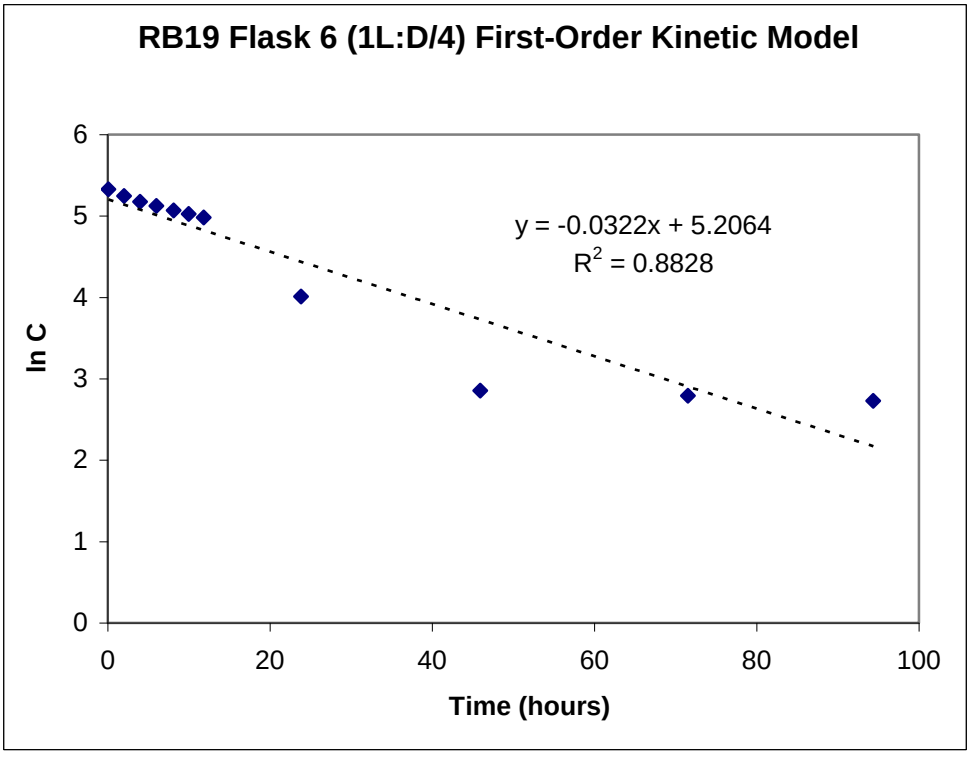
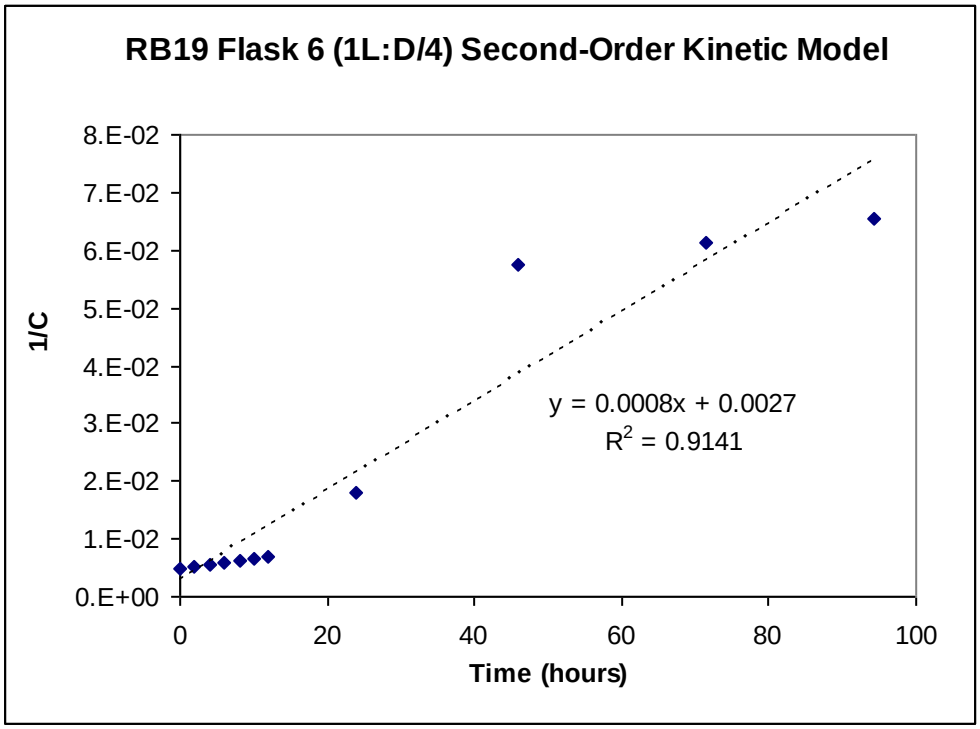


Figure 30. *Reactive Blue 19 Flask 6, Second-Order Kinetic Model*



APPENDIX B: REACTIVE ORANGE 16 KINETIC MODELS

Appendix B: Reactive Orange 16 Kinetic Models

Figure 31. *Reactive Orange 16 Flask 1, Zero-Order Kinetic Model*

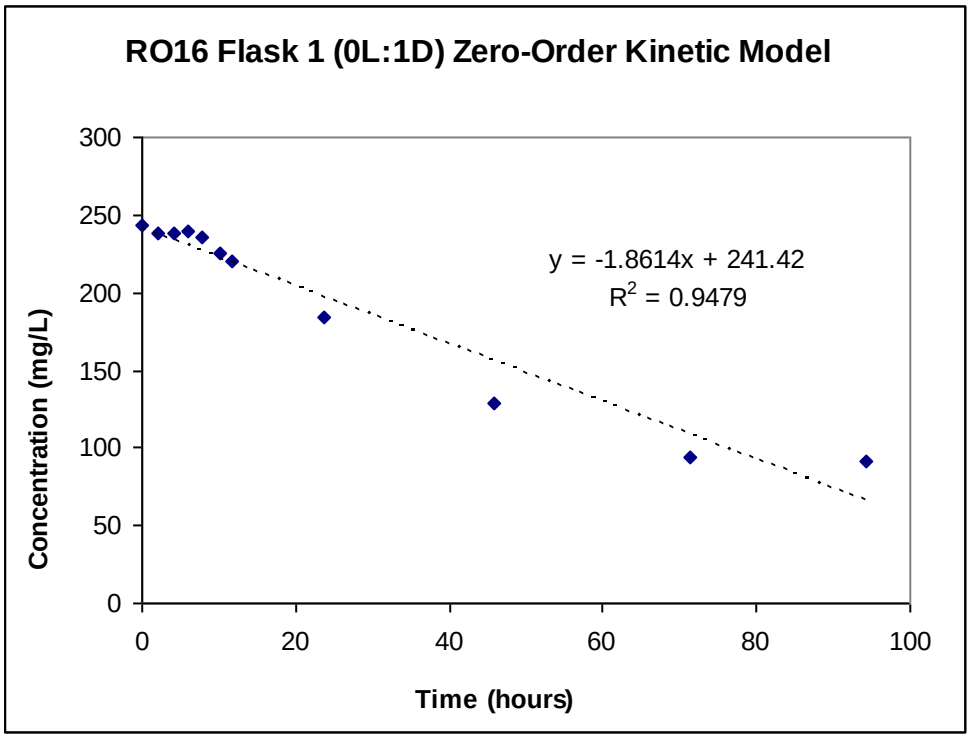


Figure 32. *Reactive Orange 16 Flask 1, First-Order Kinetic Model*

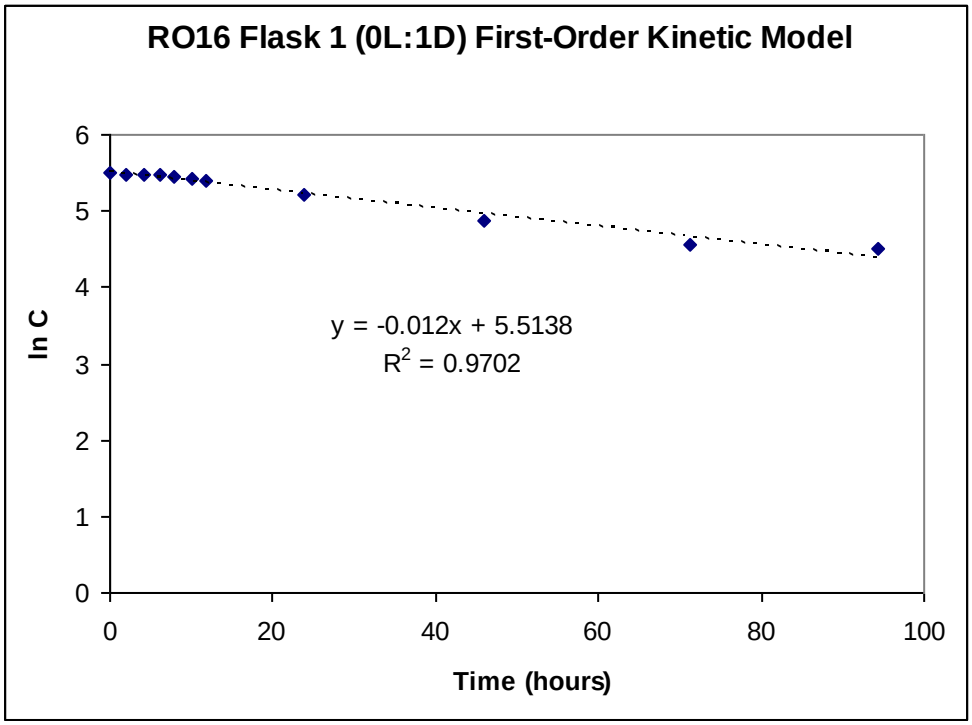


Figure 33. *Reactive Orange 16 Flask 1, Second-Order Kinetic Model*

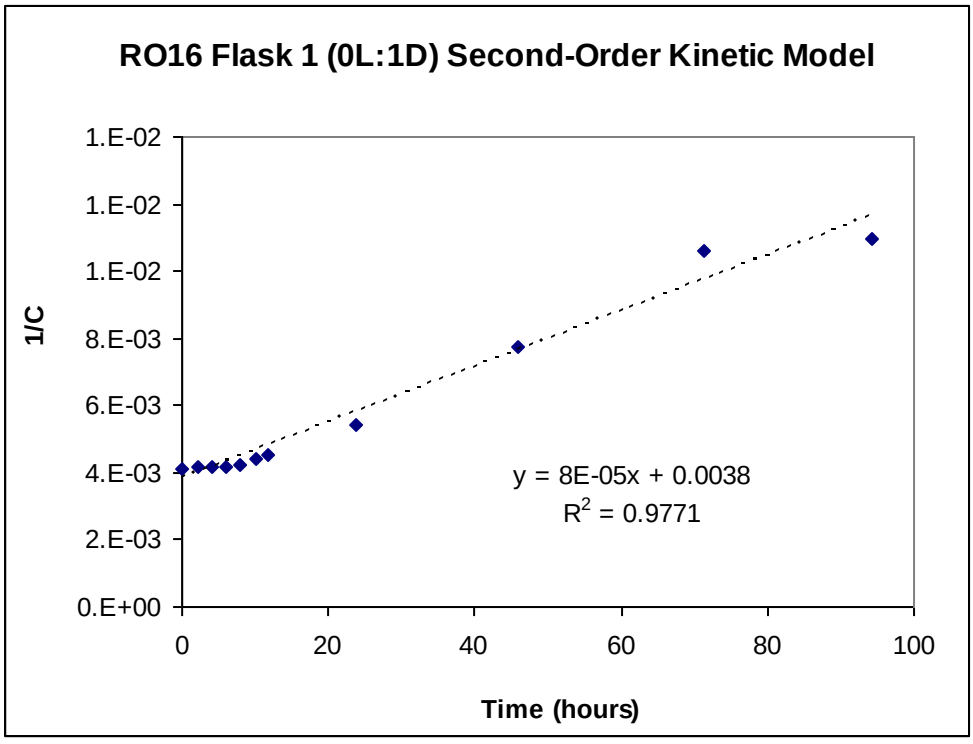


Figure 34. *Reactive Orange 16 Flask 2, Zero-Order Kinetic Model*

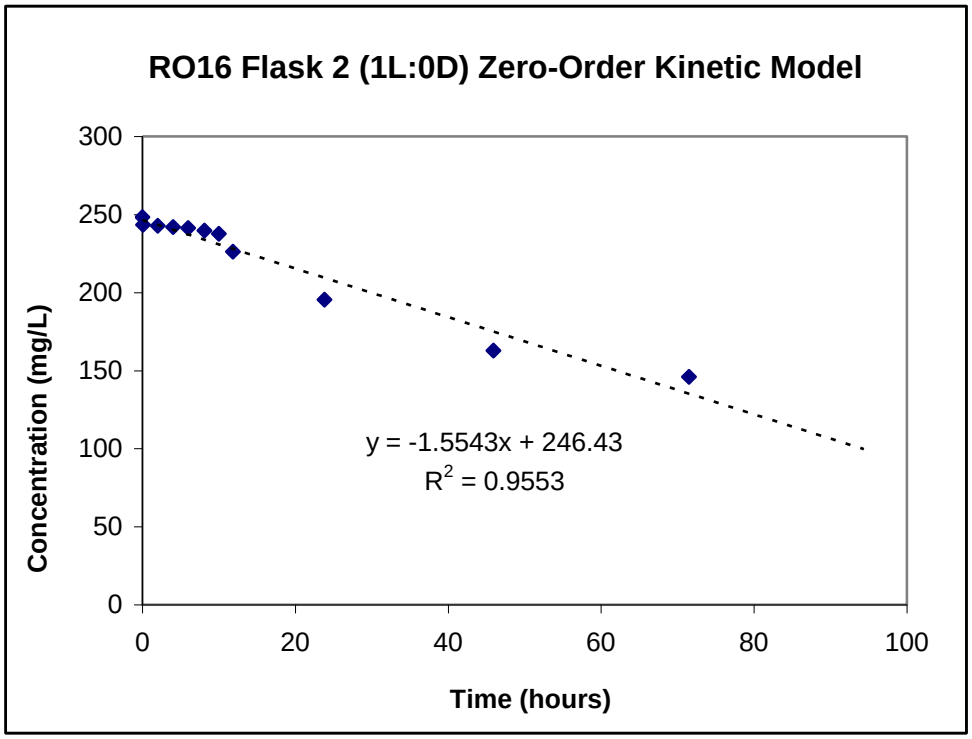


Figure 35. *Reactive Orange 16 Flask 2, First-Order Kinetic Model*

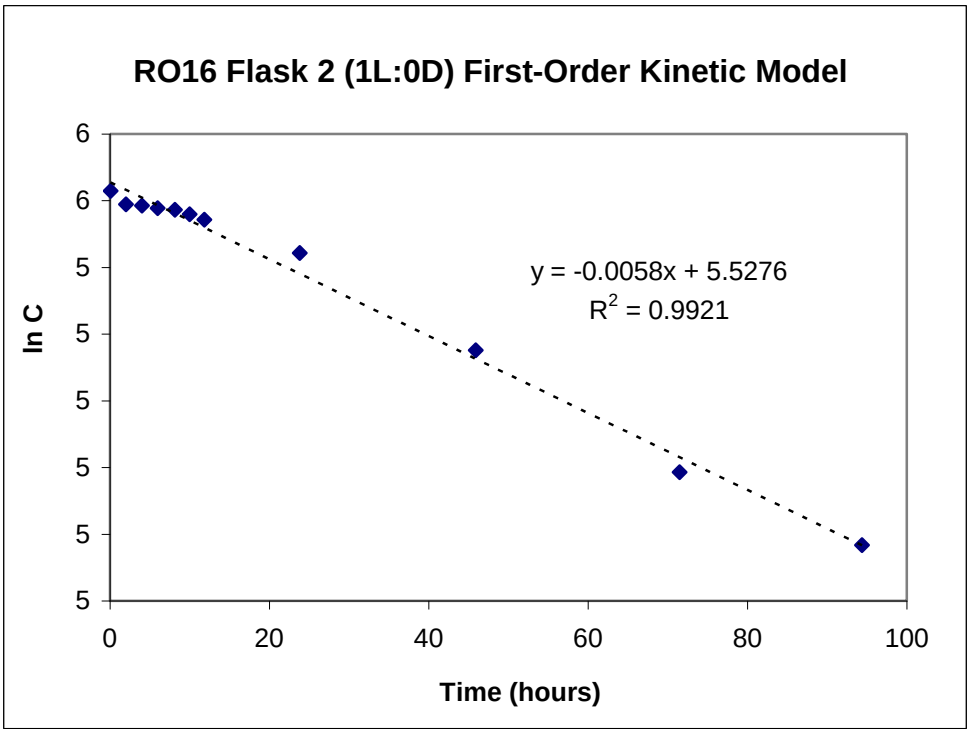


Figure 36. *Reactive Orange 16 Flask 2, Second-Order Kinetic Model*

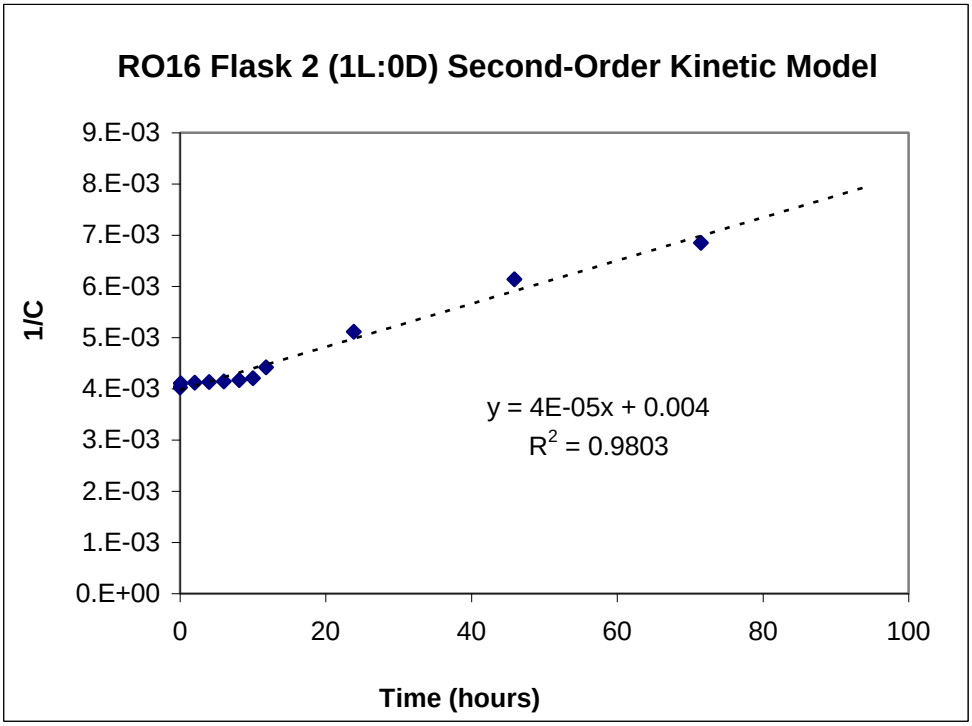


Figure 37. *Reactive Orange 16 Flask 3, Zero-Order Kinetic Model*

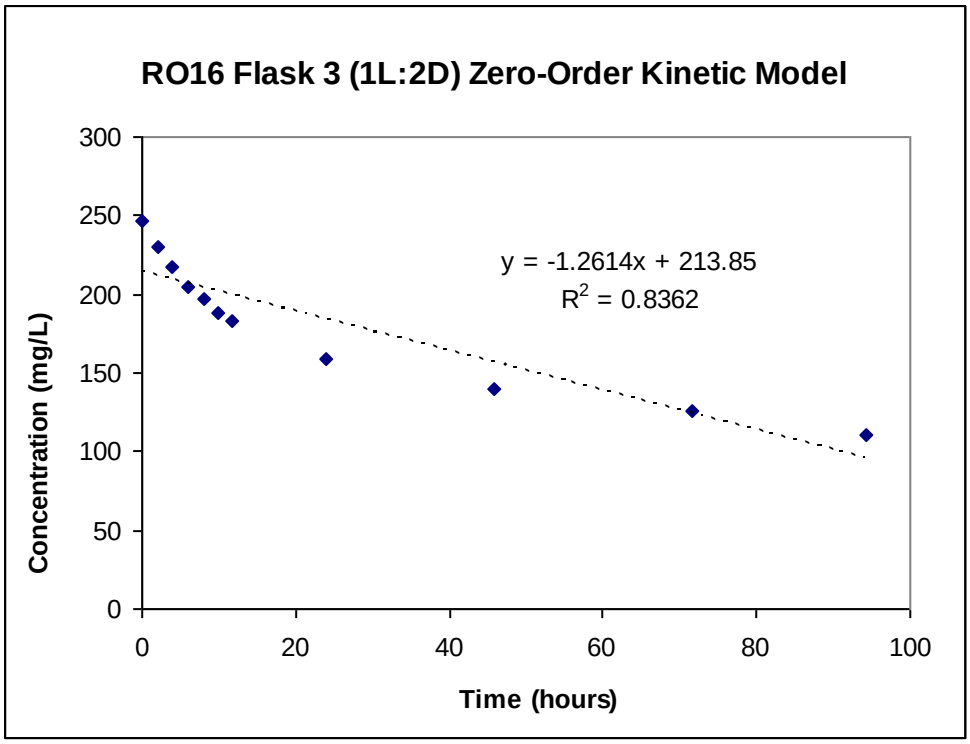


Figure 38. *Reactive Orange 16 Flask 3, First-Order Kinetic Model*

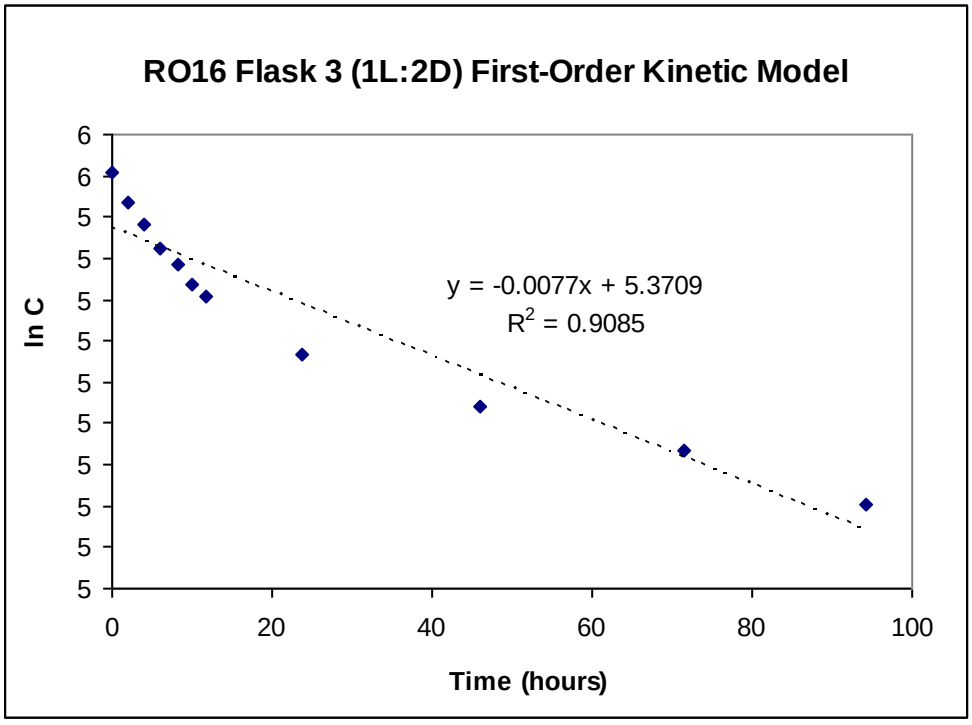


Figure 39. *Reactive Orange 16 Flask 3, Second-Order Kinetic Model*

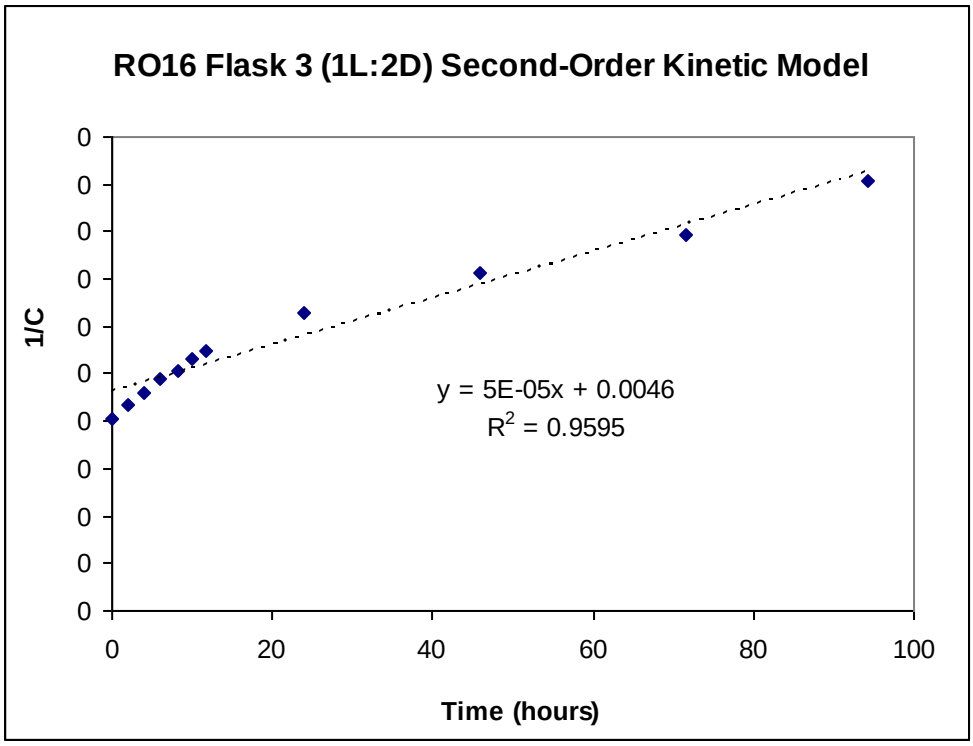


Figure 40. *Reactive Orange 16 Flask 4, Zero-Order Kinetic Model*

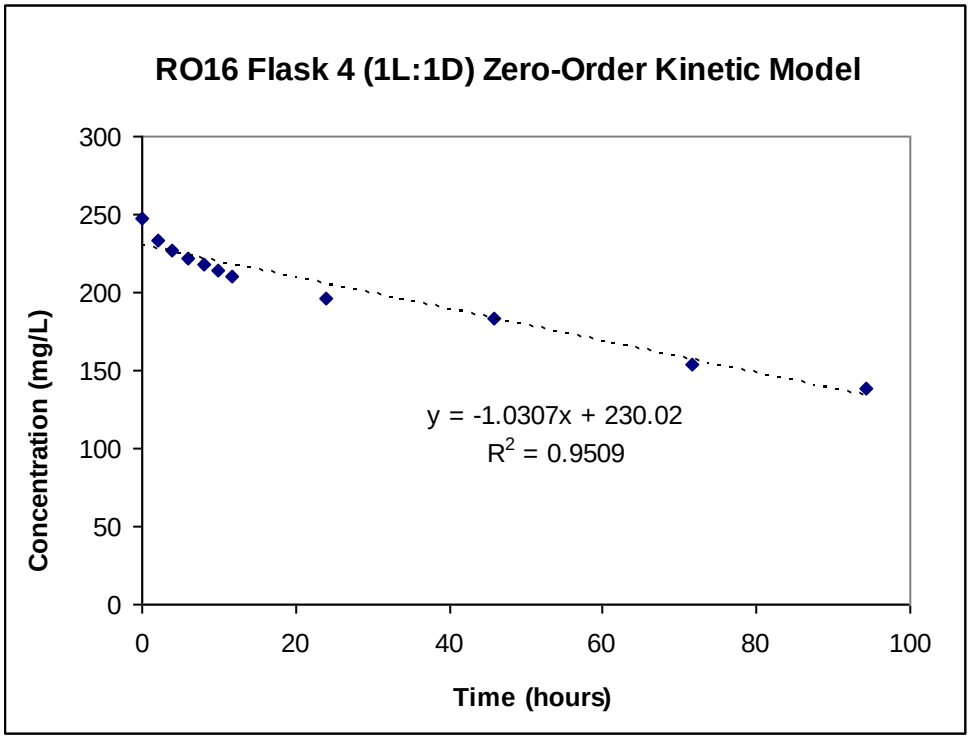


Figure 41. *Reactive Orange 16 Flask 4, First-Order Kinetic Model*

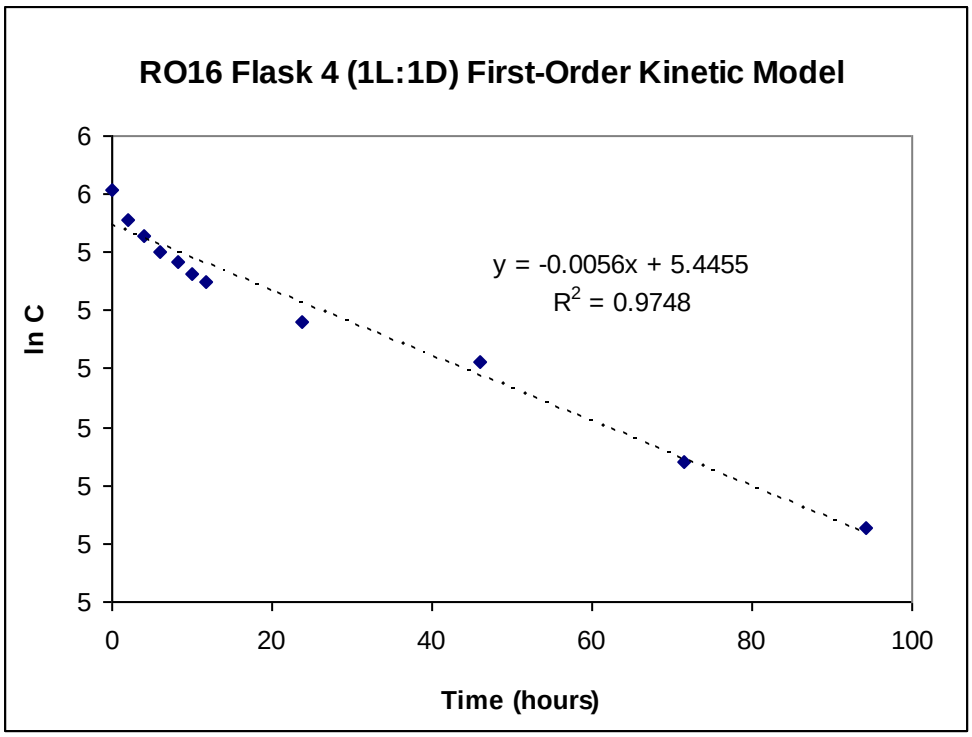


Figure 42. *Reactive Orange 16 Flask 4, Second-Order Kinetic Model*

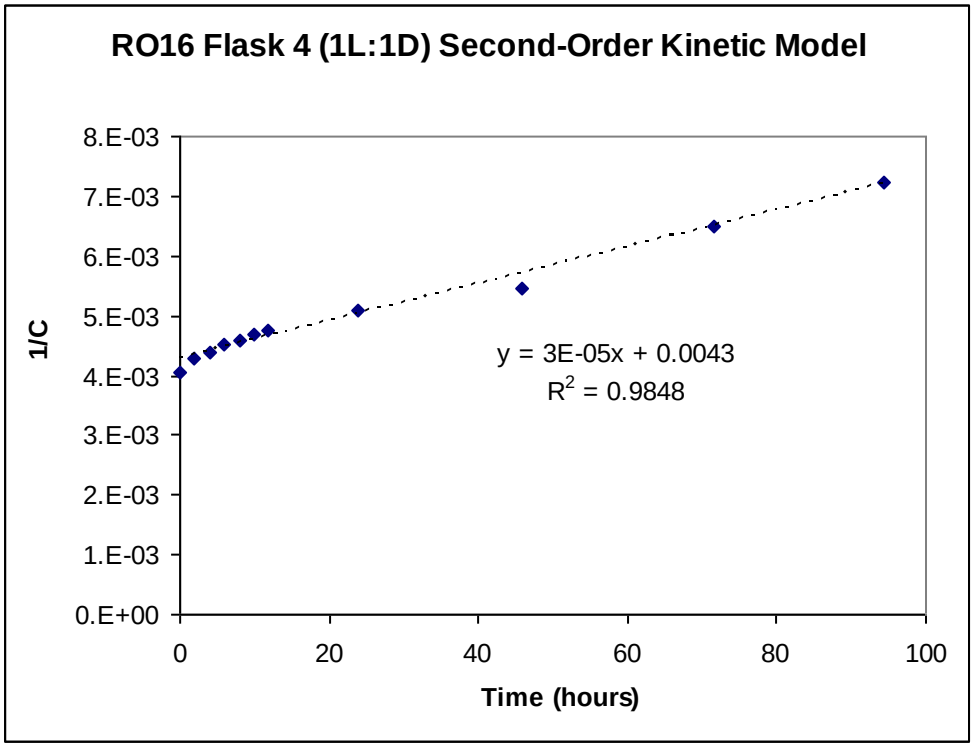


Figure 43. *Reactive Orange 16 Flask 5, Zero-Order Kinetic Model*

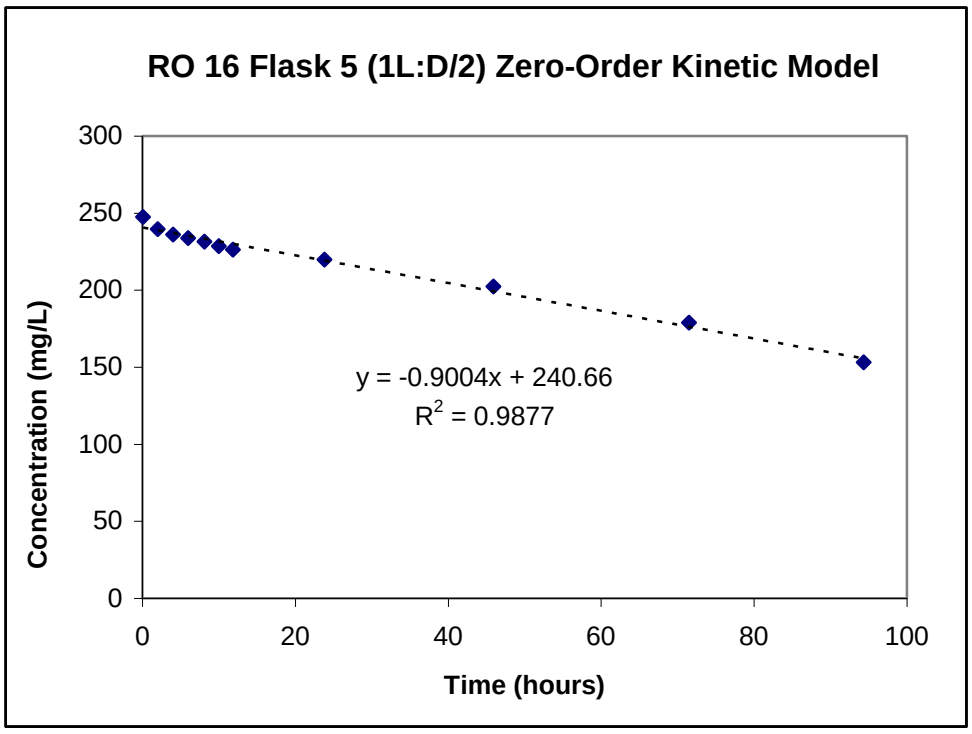


Figure 44. *Reactive Orange 16 Flask 5, First-Order Kinetic Model*

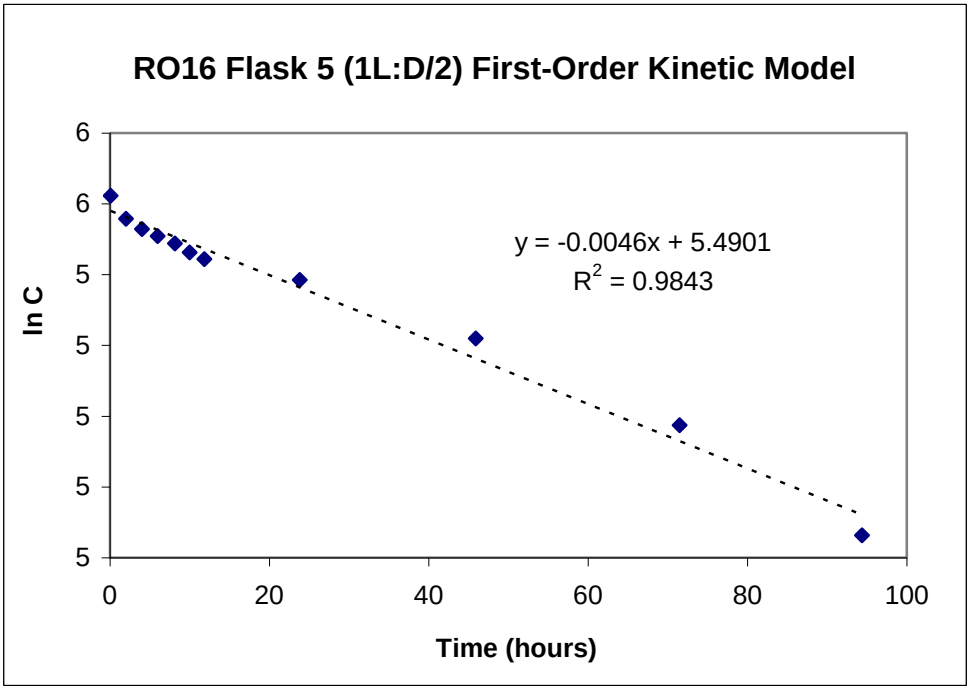
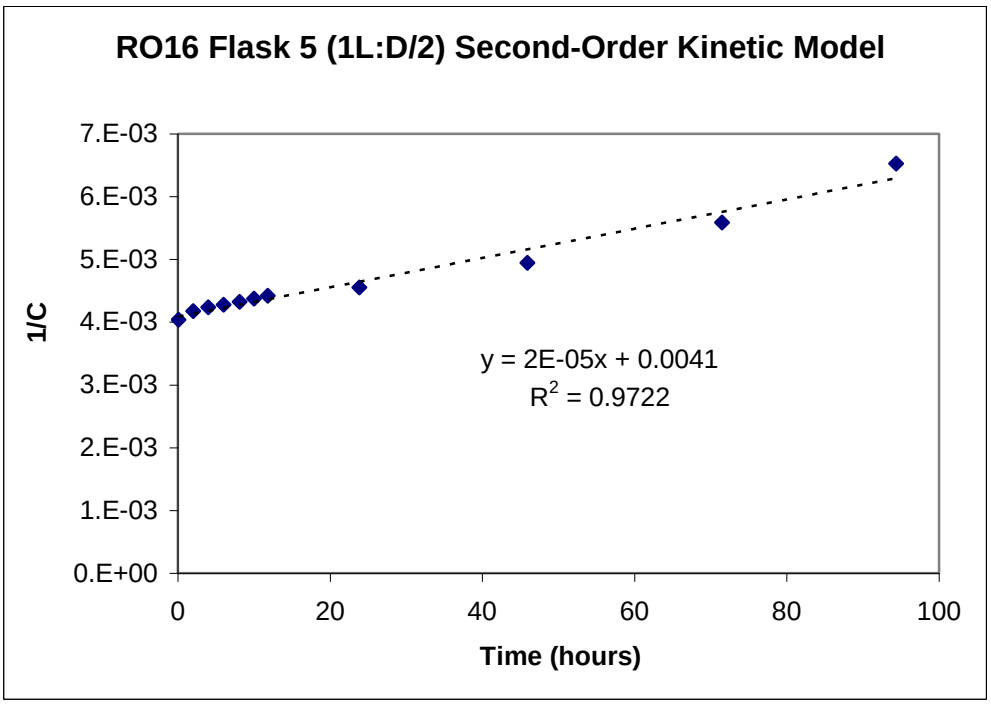


Figure 45. *Reactive Orange 16 Flask 5, Second-Order Kinetic Model*



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